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Steffen Backert *Editors*

Molecular Pathogenesis and Signal Transduction by *Helicobacter pylori*

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Foreword

I was honoured to be asked by Nicole Tegtmeyer and Steffen Backert to provide a foreword for "Molecular Pathogenesis and Signal Transduction by *Helicobacter pylori*" which they have edited. I was fascinated by the topic list and impressed with the large group of international experts they had gathered together to contribute. Writing the foreword gave me the perfect excuse to read the book, and I was honoured and excited to do so.

And I was glad I did. This book comprehensively covers the current state of research concerning *H. pylori*–human interactions and the broader infection biology of *H. pylori*. The chapter authors discuss the strategies *H. pylori* uses to persist lifelong within its human partner, largely through expressing a number of host interaction (or virulence) factors. This has occurred throughout human evolutionary history, such that *H. pylori* and humans are closely co-adapted. The authors describe in detail how the effects of *H. pylori* on epithelial cell biology, while allowing *H. pylori* to persist, also result in high-impact human diseases, notably gastric cancer and peptic ulcer. They also describe how humans have adapted over millennia to *H. pylori* and how *H. pylori*'s absence for the first time in human evolutionary history has led to changes in human immunology and physiology. In the final chapter, they speculate how these may have contributed to increases in some of the diseases of modern life, in particular allergic and auto-immune diseases.

In short, this book updated me and gave me cause for thought and reflection. I saw previous findings in different ways and learnt a lot which was new to me. Most of all, the book showed me where the leaders in the field have got to with their experimentation and thinking, how much more we all have to learn, and importantly what directions we might take to progress the field further. I recommend it very highly indeed and am sure you will enjoy reading it as much as I have.

To whet your appetite further, I thought it might be useful to very briefly preview the contributions. Karen Robinson, Darren Letley, and Kazuyo Kaneko open by elegantly discussing how *H. pylori* adapts to the gastric microenvironment and in particular how it survives periods of acid exposure and both hides from and

manipulates the host immune response. They summarise how it does this mainly through the expression of host interaction or virulence factors and they provide a concise review of these.

Alain Gobert and Keith Wilson describe how *H. pylori's* host interaction factors stimulate the expression of innate immune response effectors. The best known effects are indirect, through stimulating expression of the chemokine interleukin-8, but they also describe stimulated expression of two direct anti-microbial effectors—reactive oxygen species and nitric oxide—which of course also have tissue damaging effects. They finish with interesting observations on the extent to which human colonisation by *H. pylori* strains which are less co-adapted with their host (due to a different geographic origin) are associated with increased damage and inflammation. In Chap. 3, Racquel Mejias-Luque and Markus Gerhard discuss some of the ways in which *H. pylori* evade and manipulates human immune responses. In particular, they describe how it minimizes innate immune recognition and downregulates host release of anti-microbial peptides. Moving to the acquired immune response, they then describe how it induces tolerogenic dendritic cells and how this in turn increases the regulatory T cell response. Both this and specific *H. pylori* factors then downregulate the effector T cell response.

In a change of gear, Anna Pawlik-Zawilak and Jolanta Zakrzewska-Czerwinska summarize what is known about *H. pylori* replication. Among other interesting observations, they point out that *comH* is close to the *H. pylori* genome's origin of replication and so will be among the first genes to double its copy number during replication. This underlines the importance of DNA uptake to *H. pylori,* perhaps in order to repair its genome during genotoxic stress. Rebecca Gorrell and Terry Kwok then describe the *H. pylori* methylome, and speculate that, like other bacteria, methylation may have a role in virulence as well as its well-understood role in protection against foreign endonucleases.

In Chap. 6, Celia Berge and Laurence Terradot return to *H. pylori's* best known host interaction system, Cag, and describe recent insights from structural analyses. The structure and assembly of the Cag apparatus for translocating CagA into epithelial cells is still poorly understood. However, the interactions of CagA with host cell proteins, particularly signalling proteins, is further clarifying some of the multiple effects CagA induces in host cells.

Malvika Pompaiah and Sina Bartfeld describe the progress made in producing gastric organoids from stem cells to use as an experimental model. They speculate that as well as their use to study carcinogenesis they may be useful in *H. pylori* coinfection studies. They will certainly allow a way of checking that mechanisms uncovered in transformed cancer cell lines are also pertinent in non-transformed cells. More interestingly, they should provide a way of studying *H. pylori* interactions with cell types which cannot be cultured, for example, parietal cells—loss of which is a crucial step in progression to gastric atrophy and thence carcinoma.

Returning to the innate immune system, Matthew Varga and Rick Peek elegantly describe the evasion by *H. pylori* of recognition by toll-like receptors (TLRs), and also how *H. pylori* manipulates signalling through TLRs and through the intracellular innate immune receptor Nod-1. Most interestingly though, they describe

their recent discovery of *cag*-dependent DNA translocation into epithelial cells and subsequent TLR-9 activation. This is ripe for further exploration and could well provide new models of pathogenesis. Steffen Backert and co-authors then describe *H. pylori's* complex manipulation of intercellular junctions and provide an elegant sequential model of how its host interaction factors induce these effects. Presumably this allows *H. pylori* to gain nutrients from the host, but of course loss of cell polarity may also be a crucial step in carcinogenesis.

In Chap. 10, Adam Smolka and Mitchell Schubert discuss the two main gastric colonisation patterns of *H. pylori*, and how this leads to either an increased acid state and risk of duodenal ulcer disease, or a reduced acid state and risk of gastric carcinogenesis. They also speculate that changes in the gastric acid barrier may affect the microbiome lower down the gastrointestinal tract and that this is turn may modulate other human infectious and other diseases. Back in the stomach, Teresa Alarcon, Laura Lorca, and Guillermo Perez-Perez give a fascinating description of the non-Helicobacter gastric microbiota and how this is affected by gastric site, inflammation, disease, and other factors. They speculate that this may be important in carcinogenesis and provide some data to support this. It will be an exciting area for further study, particularly as it provides a tractable target which could potentially be manipulated to reduce cancer risk.

Ceu Figueiredo and co-authors give a detailed description of the work from the Cancer Genome Atlas project on the molecular phenotyping of sporadic gastric cancer. The description of four main phenotypes: chromosomal instability, microsatellite instability, genomically stable, and EBV positive will likely not only help in understanding the mechanisms of carcinogenesis, but also have implications for stratified treatment. Takahiro Shimizu, Tsutomu Chiba, and Hiroyiki Marusawa then discuss how *H. pylori* likely induces genetic instability through inducing expression of reactive oxygen and nitrogen species from inflammatory and gastric epithelial cells and also through inducing epithelial cell expression of the genome editing enzyme, cytidine deaminase. Which cells are the origin of most cancers, though, remain unanswered.

In the final chapter, Andreas Kyburz and Anne Müller review the evidence around lack of *H. pylori* increasing the risk of a variety of diseases, including allergic diseases, asthma, and inflammatory bowel disease. As well as describing the epidemiological evidence, they review the evidence from animal models and discuss the likely mechanism. Both animal and human studies show that *H. pylori* induces a strong regulatory T cell response and that this is diminished in the absence of *H. pylori*. This brings us full circle to how *H. pylori* coadapts with the human host to ensure its lifelong survival, and how its absence changes the human state and may increase the risk of various diseases.

Read this book! It is fascinating!

December 2016

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Preface

Helicobacter pylori colonizes the gastric mucus layer of about half of the world's population. The bacterium was discovered in 1983 by the Australian scientists Robin Warren and Barry Marshall and designated as a gastric pathogen, causing peptic ulcer disease. In 2005, they received the Nobel Prize in Physiology or Medicine for their discoveries, primarily because the application of antibiotics to treat ulcers has changed radically the practice of medicine since that time. Colonization by *H. pylori* results in superficial gastritis without clinical symptoms in most individuals, but can progress to gastric or duodenal ulcers, gastric adenocarcinomas, and mucosa-associated lymphoid tissue-lymphomas. Disease outcome is highly complex and depends on the interplay between host, bacterial, and environmental parameters. However, irrespective of disease outcome, the majority of *H. pylori*-infected people remain colonized for their entire lifetime. Today, *H. pylori* is established as a prime example of a persistent pathogen and cancer-inducing bacterium. *H. pylori* exhibits very unique characteristics in its metabolism and survival strategies, and has been the subject of intensive research to discover the mysteries of its genetics and cellular biology. In contrast to this high importance of *H. pylori* for human health, it appears that a comprehensive volume on molecular infection mechanisms and intracellular signal transduction pathways during colonization is not fully developed.

The number of publications in the *H. pylori* field has increased substantially in recent years, making it very difficult for even the most diligent readers to stay abreast of research progress. With the breathtaking expansion of studies on *H. pylori*, this is an opportune time to review the present knowledge about this exciting research topic. Accordingly, a comprehensive collection of reviews on the multiple facets of *H. pylori* pathogenesis and signal transduction mechanisms seems both timely and appropriate for a book series. The present volume on "Molecular Pathogenesis and Signal Transduction by *H. pylori*" summarizes our current scientific understanding of *H. pylori* biology in 14 chapters by internationally recognized experts in this research field. It is designed to provide important cutting-edge findings on this fascinating microbe and molecular pathogenic processes for advanced undergraduates, graduate students, medical students,

postdoctoral fellows, clinicians, and (bio)medical investigators, who are interested in infectious diseases and host cell signaling. We discuss the most recent insights into the major signal transduction pathways and highlight their mechanism of action, in particular in response to infection with *H. pylori* and the corresponding disease pathologies.

The first chapter was designed to provide the necessary background and a general overview for understanding the topics covered in the following chapters. This introduction includes advances in the general strategies of *H. pylori* infection and specialized metabolism at the molecular level. In the subsequent chapters, we specifically discuss the current state of research concerning the regulation and action of bacterial virulence factors, genetics, and infection biology of *H. pylori*. The chapters include frontline findings and discuss the overall strategies of *H. pylori* infection, replication and persistence, cross talk with the microbiota, innovative and novel model systems and signaling mechanisms, risk factors and genetics of gastric disease and stomach cancer, as well as the impact of *H. pylori* infection on non-gastric diseases.

As will become evident from these detailed review articles, there is much more complexity in the triggered pathways than was originally anticipated, adding greatly to the overall interest in these signaling factor cascades. Within the individual chapters, readers will find not only consensus and paradigm, but also differing perspectives on the regulation and functions of the multitude of *H. pylori* factors. Importantly, all of the reviews point out specific areas, where the lack of sufficient knowledge and understanding raises intriguing new questions for further experimentation in the future. These outstanding questions often pertain to the increasingly complex biological functions of the infection and diverse mechanisms of regulation in a variety of applied systems, ranging from mouse models via gastroids to humans. Recurring themes are: (i) How the pathogen can dampen the host immune system in order to establish long-term chronic infection, (ii) what is the evolutionary benefit for *H. pylori* by hijacking distinct host signaling pathways, (iii) how many and which signal cascades are most crucial for developing gastric malignancy, (iv) what exact molecular mechanism(s) decide whether a patient remains asymptomatic or develops a given type of gastric disease and not another, and (v) can we define biomarkers for the different gastric diseases. In the future, better characterization of the cellular and molecular biology of the *H. pylori* infections will pinpoint important new therapeutic targets for the treatment and prevention of multiple infectious gastric diseases. If this comprehensive collection of reviews on *H. pylori* pathogenesis and disease-associated mechanisms stimulates fresh new thinking and research on the involved signaling pathways, this book will have accomplished its goal.

The above-discussed advances in the field have helped to shape the core of this volume. We are very grateful to all the scientific contributors from around the globe, who have participated in the preparation of these outstanding chapters

covering our growing knowledge of *H. pylori* pathogenesis and signaling. We hope that this volume will become an invaluable resource to readers new to the field and expand the resources for those professionals already working in the *H. pylori* area. We would like to thank all participants for their support and help in making this book a tremendous success.

Erlangen, Germany
November 2016

Nicole Tegtmeyer
Steffen Backert

Contents

Abbreviations

~P	Phosphate group
8-OHdG	8-hydroxy-2-deoxyguanosine
Abl	Abelson kinase
ACRG	Asian cancer research group
ADP	Adenosine diphosphate
ADR	Alternate DNaseI resistant
AID	Activation-induced cytidine deaminase
AJ	Adherens junction
AKT/PKB	Protein kinase B
Am-1	Amerindian-I
AmiE	Amidase
AmiF	Formidase
AP	Apurinic/apyrimidinic
AP-1	Activator protein-1
APC	Antigen presenting cell
APE1	Apurinic/apyrimidinic endodeoxyribonuclease 1
APOBEC	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like
ArcA	Response regulator of a two-component system ArcA-ArcB
ArcB	Sensor histidine kinase of two-component system ArcA-ArcB
Arg2	Arginase II
ARHGAP26	Gene name for: Rho GTPase-activating protein 26
ARHGAP6	Gene name for: Rho GTPase-activating protein 6
ARID1A	AT-rich interactive domain-containing protein 1A
ASC	Adult stem cell
ASPP2	Apoptosis-stimulating protein of p53 2
ATF-2	Activating transcription factor 2
ATM	Ataxia telangiectasia mutated kinase
ATP	Adenosine triphosphate
B2M	Beta-2-microglobulin

BabA	Blood group antigen-binding adhesin
BAC	Bacterial artificial chromosome
Barx1	BarH-like homeobox 1
BE	Barrett's esophagus
BER	Base excision repair
BMDC	Bone marrow-derived cell
BMP	Bone morphogenic protein
BrdU	Bromodeoxyuridine
cag	Cytotoxin-associated gene
cagA	Cytotoxin-associated gene A
*cag*PAI	*cag* pathogenicity island
CAMP	Cationic antimicrobial peptides
cAMP	Cyclic adenosine monophosphate
Cas9	CRISPR-associated protein 9
c-boxes	Classic DnaA boxes
CCKBR	Cholecystokinin B receptor (also called CCK_2)
CCND1	Gene name for: Cyclin D1
CCNE1	Gene name for: Cyclin E1
CcrM	*Caulobacter crescentus* DNA methyltransferase
CD	Cluster of differentiation
Cdc6	Cell division control protein 6
CDH1	Gene name for: E-cadherin
CDKN	Cyclin-dependent kinase inhibitor gene
Cdx2	Caudal-type homeobox 2
CFU	Colony-forming unit
CGD	Chronic granulomatous disease
CGRP	Calcitonin gene-related peptide
CHK2	Checkpoint kinase 2
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CK1	Casein kinase 1
CLD18	Gene name for: Claudin 18
CLR	C-type lectin receptor
CM	CagA multimerization sequence
c-Met	Tyrosine kinase (also called hepatocyte growth factor receptor)
comB	*H. pylori* competence gene B
comH	*H. pylori* competence gene H
Cox2	Cyclooxygenase 2
CpG	Cytosine-guanine repeats
CREB	cAMP responsive element-binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CRPIA	Conserved repeat responsible for phosphorylation independent activity
Csk	Carboxy-terminal Src kinase
CTNNA1	Gene name for: α-1-Catenin

CTNNB1	Gene name for: β-1-Catenin
CTNND1	Gene name for: δ-1-Catenin
CXCL8	C-X-C motif chemokine ligand (also called IL-8)
Dam	Deoxyadenosine methylase
DARS	DnaA-reactivating sequence
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific HIV-1 receptor (also called CD209)
DGC	Diffuse gastric cancer
DiaA	DnaA initiator-associating factor
dif	Sequence bound by Xer complex
DKK	Dickkopf 1
DLBCL	Diffuse large B cell lymphoma
DNA	Deoxyribonucleic acid
DNA Pol I	DNA Polymerase I
DNA Pol II	DNA Polymerase II
DNA Pol III	DNA Polymerase III
DNA Pol IV	DNA Polymerase IV
DNA Pol V	DNA Polymerase V
DnaA	Initiator protein of chromosomal replication
DnaB	Replicative DNA helicase
DnaC	Helicase loader
DnaE	α-subunit of DNA Pol III
DnaG	Bacterial primase
DnaN	β-sliding clamp of DNA Pol III
DnaQ	ε-subunit of DNA Pol III
DnaX	γ/τ-subunit of DNA Pol III
DoMo	Domain movement
DOT1L	Disruptor of telomeric silencing 1-like histone H3K79 methyltransferase
Dps	DNA-binding protein from starved cells (also called NapA)
DSB	Double-strand DNA breaks
dsDNA	Double-stranded DNA
dTMP	2′-deoxythymidine-5′-monophosphate
DUE	DNA unwinding element
DupA	Duodenal ulcer promoting gene A
DUS4L-BCAP29	Dihydrouridine synthase 4-like and B-cell receptor-associated protein 29
EAC	Esophageal adenocarcinoma
EBER	EBV-encoded small RNA
EBNA	EBV nuclear antigens
EBNA-LP	EBNA-leader protein
EBV	Epstein–Barr virus
ECL	Enterochromaffin-like
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

EHEC	Enterohemorrhagic *Escherichia coli*
EMT	Epithelial mesenchymal transition
EPIYA	Glu-Pro-Ile-Tyr-Ala sequence motif
ERBB2(HER2)	Erb-b2 receptor tyrosine kinase 2
ERK1/2	Extracellular signal–regulated kinase 1/2
ESC	Embryonic stem cell
FAK	Focal adhesion kinase
FAP	Familial adenomatous polyposis
FasL	Fas ligand
FBXO24	F-box protein 24
FGC	Familial gastric cancer
FGF-10	Fibroblast growth factor-10
FGFR2	Fibroblast growth factor receptor 2
Fic	Filamentation-induced by cAMP
FIGC	Familial intestinal gastric cancer
Fis	Factor for inversion stimulation
FlaA	Flagellin A
FoxP3	Forkhead box P3
FtsK	Filamentus temperature-sensitive cell division protein K
GAPPS	Gastric adenocarcinoma and proximal polyposis of the stomach
GAP	GTPase-activating protein
gB	EBV glycoprotein B
GC	Gastric cancer
G-DIF	Diffuse subtype of GC
GERD	Gastro-esophageal reflux disease
GGT	Gamma-glutamyl transpeptidase
gH	EBV glycoprotein H
G-INT	Intestinal subtype of GC
gL	EBV glycoprotein L
gp350	EBV glycoprotein 350
gp42	EBV glycoprotein 42
Grb2	Growth factor receptor-bound protein 2
GS	Genomically stable
GSH	Glutathione (reduced form)
GSK-3β	Glycogen synthase kinase 3 beta
GyrA	Subunit A of DNA gyrase
GyrB	Subunit B of DNA gyrase
H2AX	Histone H2A variant X
H_2RA	H_2-receptor antagonist
hBD	Human beta-defensin
HBV	Hepatitis B virus
Hda	Homologous to DnaA protein
HDGC	Hereditary diffuse gastric cancer
HGF	Hepatocyte growth factor

HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen class II
HLA-B	Histocompatibility complex class I
HNF4α	Hepatocyte nuclear factor 4α
HNPCC	Hereditary non-polyposis colorectal cancer
HobA	*Helicobacter* orisome binding protein A
HolA	δ-subunit of DNA Pol III
HolB	δ'-subunit of DNA Pol III
HP1021	Orphan response regulator of *H. pylori*
HR	Homologous recombination
HU	Histone-like protein
Hupki	Human TP53 knock-in
IARC	International agency for research on cancer
IBD	Inflammatory bowel disease
IFN	Interferon
IGHV	Immunoglobulin heavy chain variable region
IHF	Integration host factor
IKK	IκB kinase
IL	Interleukin
IM	Intestinal metaplasia
IMC	Inner membrane complex
iNOS	Inducible nitric oxide synthase
INSR	Insulin receptor
IP_3	Inositol triphosphate
IPD	Interpulse duration
iPSC	Induced pluripotent stem cells
IRAK	IL-1 receptor-associated kinase
IRF	Interferon-regulatory factor
JAK	Janus kinase
JUP	Junction plakoglobin
KCNQ1	Potassium voltage-gated channel subfamily Q member 1
KLF5	Krueppel-like factor 5
Kras	Kirsten rat sarcoma oncogene
LabA	lacdiNAc-specific adhesin
LES	Lower esophageal sphincter
LFA-1	Lymphocyte function-associated antigen 1
LFS	Li-Fraumeni Syndrome
Lgr5	Leucin-rich-repeat-containing G-protein-coupled receptor 5
Lig	DNA ligase
LL37	37-residue amphipathic α-helical cathelicidin
LMP	Latent membrane protein
LOH	Loss of heterozygosity
LP	Leader peptide
LPS	Lipopolysaccharide
LR	Low risk

Lrp	Global regulatory protein
LRR	Leucin-rich repeat
m4C	N4-methylcytosine
m5C	5-methylcytosine
m6A	N6-methyladenine
MAGI-1	Membrane-associated guanylate kinase with inverted orientation 1
MALT	Mucosa-associated lymphoid tissue
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MARK	Microtubule affinity regulating kinase
MDCK	Madin-Darby canine kidney
MHC	Major histocompatibility complex
Mincle	Macrophage inducible C-type lectin
Mist1	Basic helix-loop-helix family member a15
MKI	MARK kinase inhibitor
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLH	Human homolog of MMR from *Escherichia coli*
MLN	Mesenteric lymph nodes
MMP	Matrix metalloprotease
MMR	DNA mismatch repair
mob	Mobility genes
MPF	Mating pair formation
MRN	MRE11-RAD50-NBS1 complex
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSI	Microsatellite instability
MSI-H	High microsatellite instability
MSI-L	Low microsatellite instability
MSS	Microsatellite stable
Mtase	Methyltransferase
mTOR	Mechanistic target of rapamycin
MtrA	Response regulator of a *M. tuberculosis* two-component signal transduction system MtrAB
MtrB	Histidine kinase of a *M. tuberculosis* two-component signal transduction system MtrAB
MUC6	Mucin-6
MukB	SMC homolog
MUPP	Multi-PDZ domain protein
MyD88	Myeloid differentiation primary response gene 88
MZB	Marginal zone B
NADPH	Nicotinamide adenine dinucleotide phosphate
NAP	Nucleoid associated protein
NapA	Neutrophil-activating protein A (also called HP-NAP or Dps)

NER	Nucleotide excision repair
NF-κB	Nuclear factor-kappa B
NFAT	Nuclear factor of activated T cells
NGS	Next-Generation Sequencing
NHEJ	Non-homologous end joining
NK	Natural killer
NLR	Nucleotide-binding domain and leucine-rich-repeat-containing-proteins
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOG	Noggin
NOS2	Nitric oxide synthase 2
NOX	NAPDH oxidase
NOXA1	NADPH oxidase activator 1
OCT1	Octamer transcription factor 1
ODC	Ornithine decarboxylase
ODN	Oligodeoxynucleotide
OipA	Outer inflammatory protein A
OMV	Outer membrane vesicle
$ONOO^-$	Peroxinitrite
ORC1	Origin recognition complex subunit 1
ORF	Open reading frame
oriC	Origin of chromosome replication
oriT	Origin of transfer
oriV	Origin of vegetative replication
PAI	Pathogenicity island
PAMP	Pathogen-associated molecular pattern
Pap	Pyelonephritis-associated pili
PAR1	Protease-activated receptor 1
Par1b	Partitioning-defective kinase 1b
ParA	Chromosome partitioning protein ParA
ParB	Chromosome partitioning protein ParB
parS	Centromere-like sequence
pDC	Plasmacytoid dendritic cell
PDCD1LG2	Programmed cell death 1 ligand 2
PD-L1	Programmed cell death ligand 1
Pdx1	Pancreatic and duodenal homeobox 1
PG	Peptidoglycan
PGC	Pepsinogen C
phasevarion	Phase variable regulon
PhoA-PhoB	Two-component system of the Pho (phosphate) regulon
PhoB	Response regulator of two-component regulatory system PhoA-PhoB
PhoR	Histidine kinase of two-component regulatory system PhoA-PhoB

PI3K	Phosphatidylinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PJS	Peutz-Jeghers syndrome
PKC	Protein kinase C
PMN	Polymorphonuclear neutrophil
PolA	DNA Polymerase I
POLE	Polymerase ε
PPI	Proton pump inhibitor
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PSC	Pluripotent stem cells
pSLT	*Salmonella* virulence plasmid
PTEN	Phosphatase and tensin homolog
PUD	Peptic ulcer disease
punB	Purine nucleoside phosphorylase
PW	Pulse width
RA	Retinoic acid
Rac1	Ras-related C3 botulinum toxin substrate 1
RC	Rolling circle
RecG	DNA recombinase
REL	Member of the NF-κB family of transcription factors
Rep	Replication initiator protein
RGD	Arg-Gly-Asp sequence motif
RGDLXXL	Arg-Gly-Asp-Leu/Met-X-X-Leu/Ile sequence motif
RhoA	Ras homolog gene family A
RHS	RGD helper sequence
RIDA	Regulatory inactivation of DnaA activity
R-M systems	Restriction-modification systems
RNA	Ribonucleic acid
RNF43	Ring finger protein 43
RnhA	Ribonuclease H
RNI	Reactive nitrogen intermediate
RNS	Reactive nitrogen species
RocF	Urea-producing arginase
ROCK	Rho kinase
ROS	Reactive oxygen species
RSPO	R-spondin1
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
RUNX3	Runt-related transcription factor 3
SabA	Sialic acid binding adhesin
SeqA	Sequestration protein A
SFK	Src family kinase
SH3	Src homology 3

SHH	Sonic hedgehog
SHP-1/2	Src homology region 2 domain-containing phosphatase-1/2
SLB	Single layer antiparallel β-sheet
SLC1A2	Solute carrier family 1 member 2
SLT	Soluble lytic transglycosylase
SMC	Structure maintenance of chromosomes
SMOX	Spermine oxidase
SMRT	Single molecule real-time
SNP	Single-nucleotide polymorphism
Soj	Sporulation initiation inhibitor Soj
Sox9	Sex determining region Y (SRY)-box 2
SPEM	Spasmolytic polypeptide-expressing metaplasia
SPI-1	*Salmonella enterica* pathogenicity island 1
Spo0J	Chromosome partitioning protein
SPR	Surface plasmon resonance
Src	Sarcoma virus kinase
SSB	Single-strand binding
ssDNA	Single-stranded DNA
STAT3	Signal transducer and activator of transcription factor-3
T4CP	Type IV coupling protein
T4SS	Type IV secretion system
TBK-1	Serine/Threonine protein kinase-1
TCF/LEF	T-cell-specific transcription factor/Lymphoid enhancer binding factor
TCGA	The Cancer Genome Atlas
TER	Transepithelial electrical resistance
terC	Replication terminus
TGF-β	Transforming growth factor beta
T_H	Helper T cell
ThyA	Thymidylate synthase A
ThyX	Thymidylate synthase X
TIMP3	Metalloproteinase inhibitor 3
TIR	Toll/IL-1 receptor domain
TJ	Tight junction
TLR	Toll-like receptor
Tnfrsf19	Tumor necrosis factor receptor super family 19
TNF-α	Tumor necrosis factor alpha
TopA	DNA topoisomerase I
Topo IV	Topoisomerase IV
TP53	Tumor protein p53
tra	Transfer genes
TRD	Target recognition domain
TRD1	Target recognition domain 1
TRD2	Target recognition domain 2
Treg	Regulatory T cell

ts-boxes	Topology-sensitive DnaA boxes
Tus	Terminus utilization substance
UC	Ulcerative colitis
UNG	Uracil-DNA glycosylase
UPEC	Uropathogenic *Escherichia coli*
Ure	Urease
UV	Ultraviolet
VacA	Vacuolating cytotoxin A
Vav	Rac-specific nucleotide exchange factor
VEGFA	Vascular endothelial growth factor A
Vil1	Villin-1
WHO	World Health Organization
XerC	Tyrosine recombinase C
XerD	Tyrosine recombinase D
XerH	Tyrosine recombinase H
Y2H	Yeast two-hybrid
YY1	Ying Yang-binding motif

The Human Stomach in Health and Disease: Infection Strategies by *Helicobacter pylori*

Karen Robinson, Darren P. Letley and Kazuyo Kaneko

Abstract *Helicobacter pylori* is a bacterial pathogen which commonly colonizes the human gastric mucosa from early childhood and persists throughout life. In the vast majority of cases, the infection is asymptomatic. *H. pylori* is the leading cause of peptic ulcer disease and gastric cancer, however, and these outcomes occur in 10–15% of those infected. Gastric adenocarcinoma is the third most common cause of cancer-associated death, and peptic ulcer disease is a significant cause of morbidity. Disease risk is related to the interplay of numerous bacterial host and environmental factors, many of which influence chronic inflammation and damage to the gastric mucosa. This chapter summarizes what is known about health and disease in *H. pylori* infection, and highlights the need for additional research in this area.

Contents

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1 Introduction

The bacterial pathogen *Helicobacter pylori* was discovered by Barry Marshall and Robin Warren in the early 1980s, who first reported its presence on mucosal tissue sections from the stomach of patients with gastritis and peptic ulcers (Marshall and Warren 1984). It is a fastidious and microaerophilic Gram-negative bacterium characterized by a curved rod morphology and positive reactions for catalase, oxidase, and urease, the latter reaction being characteristically rapid and most frequently used for initial identification. In the vast majority of cases, the infection is completely asymptomatic; however, peptic ulcer disease or gastric cancer occurs in 10–15% of those infected. The type and severity of disease depends on several factors including characteristics of the colonizing strain, the host immune response, as well as environmental factors such as smoking, a high-salt diet, and the presence of other concurrent infections (Amieva and Peek 2016). It is important to characterize the effects of these factors in order to understand why disease occurs, and who may be at risk of developing disease in the future.

2 *H. pylori* Infection and Strategies of Persistence

H. pylori usually establishes colonization of the gastric mucosa during early childhood, and it persists life long in the absence of an effective eradication treatment. The organism has co-evolved with humans over at least 60,000 years since their migration from Africa (Atherton and Blaser 2009), and is currently estimated to colonize almost 50% of the world's population. The prevalence of *H. pylori* differs greatly around the world, with the highest infection rates in developing countries. The prevalence of *H. pylori* is declining, with increased urbanization and common use of antibiotics during childhood. *H. pylori* infection rates in young children from developed countries are now very low and, since it is unusual to acquire the infection as an adult, this creates a birth cohort effect. Of those below 20 years of age residing in the USA, for example, only approximately 10% are *H. pylori*-positive, compared with 40% amongst the over 60s (Nagy et al. 2016).

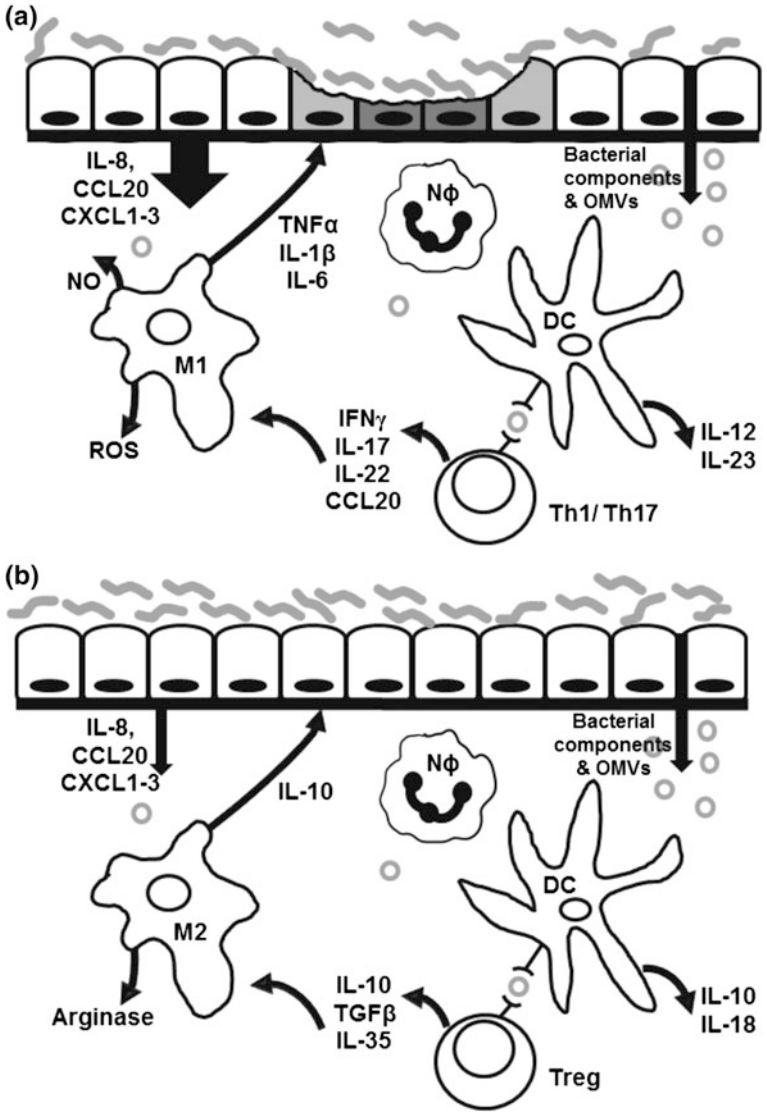
H. pylori is almost exclusively found in humans. The presence of other *Helicobacter* species in the human stomach is comparatively rare, and these are thought to be acquired from domestic pets. The precise routes of transmission of *H. pylori* are unclear; however, close person-to-person contact is required, and the

faecal–oral and oral–oral routes are most likely. Strains of *H. pylori* are usually isolated from gastric biopsy tissue specimens; however, it is also possible for the bacterium to be recovered from saliva, gastric reflux fluid, diarrhoea, and vomitus. The organism does not typically survive passage through the intestine into normal faeces; however, its isolation and transmission from contaminated water supplies and farm animals has also been reported (Breckan et al. 2016).

H. pylori inhabits the harsh, highly acidic environment of the stomach, and has evolved several mechanisms in order to survive. This bacterium is not an acidophile and can only survive for a short period at pH 2, quickly losing motility in a pH- and pepsin-dependent manner. Whilst the basal acidity in the human stomach lumen is usually below pH 2, this can increase to pH 5.5 following a meal in adults and as high as pH 7 in breast-fed children. Furthermore, pepsin activity is exponentially reduced at higher pH, thus allowing *H. pylori* a longer survival time in the gastric lumen if infection occurs postprandial and in younger children compared to adults (Schreiber et al. 2005). *H. pylori* possesses several important enzymes to enable survival under acid shock conditions. 15% of *H. pylori*'s expressed protein is made up of cytoplasmic urease, made up of two subunits, UreA and UreB. In an environment below pH 6.5, a channel in the cytoplasmic membrane, encoded by the *ureI* gene, opens to enable entry of host urea. This becomes hydrolysed by urease to produce ammonia and carbon dioxide, buffering the cytoplasm (Weeks et al. 2000). Another important enzyme is α-carbonic anhydrase. This is located within the periplasm and catalyses the reversible hydration of carbon dioxide, diffusing into the periplasm from the action of cytoplasmic urease, to bicarbonate, buffering the periplasm. Both of these enzymes are regulated by the two component acid-responsive signalling system, ArsRS (Pflock et al. 2005; Wen et al. 2007), which also regulates other non-essential enzymes which aid acid tolerance: the amidase AmiE; the formamidase AmiF; and the urea-producing arginase RocF (Pflock et al. 2006).

As well as surviving the acidic environment of the gastric lumen, *H. pylori* must avoid clearance through frequent gastric emptying. The organism has unipolar flagella, is highly motile, and is able to sense pH and bicarbonate ions. This allows the bacteria to quickly migrate from the highly acidic gastric lumen into the mucus layer overlying the epithelium. The secretion of bicarbonate ions by gastric epithelial cells establishes a pH gradient through the mucus layer, from a near neutral pH close to the epithelial cell surface to the highly acidic stomach lumen. The viscosity of the mucus layer is pH-dependent and ranges from a thick, protective gel near the acidic lumen to a viscous solution near the epithelium. Upon entering the mucus gel from the lumen, *H. pylori* is able to reduce the viscosity of the mucus gel to aid its motility by increasing the local pH through the urease-catalysed hydrolysis of urea to ammonia (Celli et al. 2009). The bacteria use the pH gradient to orientate themselves in the gastric mucus, moving from the acidic lumen to a more neutral environment deeper in the mucus layer. Histological analysis has shown most of the bacteria to be free-swimming in the mucus close to the epithelium, with some adhering to the surface of the epithelial cells or within the gastric glands. This close proximity allows it to deliver bacterial products to the

cells, either in the form of secreted or surface proteins, or in outer membrane vesicles, for its own benefit. One example of this is the loosening of tight junctions and release of interstitial fluid for access to nutrients and urea. *H. pylori* can sense other important signals such as the essential amino acid arginine, urea, and the quorum-sensing molecule autoinducer 2, and is repelled from conditions which reduce electron transport (Keilberg and Ottemann 2016). Host urea appears to be the main attractant that *H. pylori* uses to sense the epithelium (Huang et al. 2015). Urea binds strongly to the chemoreceptor, TlpB, and coupled with the hydrolysis of



◂**Fig. 1** Pro- and anti-inflammatory responses to *H. pylori* in the gastric mucosa. **a** Interactions between gastric epithelial cells and *H. pylori* result in activation of inflammatory signalling, resulting in the expression of high concentrations of cytokines and chemokines including interleukin-8 (IL-8), CCL20, and CXCL1–3. These chemokines stimulate the migration of immune cells, including dendritic cells (DCs), neutrophils (Nϕ), macrophages, and B and T lymphocytes into the mucosa from capillary blood. Upon arrival, the cells respond to the local cytokine milieu, becoming pro-inflammatory in nature. Macrophages and neutrophils phagocytose bacteria that penetrate the mucosa. Bacterial components and outer membrane vesicles (OMVs) pass through the epithelial barrier and further activate immune and inflammatory cells. DCs secrete IL-12 and IL-23, which stimulate the differentiation of naïve T cells into T-helper 1 (Th1) and Th17 cells, respectively. The cytokines secreted by these Th subsets act on macrophages to stimulate their development into an M1 type, being highly bactericidal, secreting reactive oxygen and nitrogen species (ROS, NO) and expressing inflammatory cytokines including IL-1β, IL-6, and tumour necrosis factor alpha (TNFα). This results in damage to the epithelial barrier and the induction of epithelial cell apoptosis. The presence of inflammatory cytokines also exacerbates the chemokine response of gastric epithelial cells. **b** Less pathogenic (*cag*PAI-negative) strains of *H. pylori* stimulate lower-level expression of cytokines and chemokines from gastric epithelial cells, resulting in reduced recruitment of immune and inflammatory cells into the mucosa. Upon penetration of the mucosal barrier (e.g. via OMVs), some bacterial components such as VacA and GGT have a tolerizing effect on DCs. These DCs promote the differentiation of regulatory T cells (Tregs), which express anti-inflammatory cytokines including IL-10, IL-35, and transforming growth factor beta (TGFβ). Macrophages in this environment express markers of an M2 type, express IL-10 and perform wound-healing rather than inflammatory or bactericidal functions. The presence of IL-10 suppresses chemokine expression from gastric epithelial cells

urea within the immediate environment of the bacteria, and *H. pylori* is able to sense levels of the molecule below the nanomolar range (Huang et al. 2015). Interestingly, *H. pylori* has been shown to preferentially colonize sites of gastric injury in mice, and this response is both rapid and requires a functional chemotactic system (Aihara et al. 2014). Such areas of damage may be a good source of nutrients for the bacteria. Whilst close interactions with the epithelium protect *H. pylori* from the harsh environment of the stomach lumen and provide access to nutrients, it comes at a cost: the activation of the immune response and increased induction of bactericidal factors. In order to persist, a careful balance is struck between the bacteria and the host.

H. pylori interacts with the gastric epithelium, stimulating the expression of pro-inflammatory cytokines and chemokines in addition to antimicrobial peptides such as beta-defensin 2 (Fig. 1a). The chemokine response recruits the migration of immune and inflammatory cells. This includes neutrophils, which phagocytose the bacteria. Macrophages also secrete reactive oxygen and nitrogen species to kill bacteria, but also express pro-inflammatory cytokines which can induce epithelial cell apoptosis. Macrophage-derived cytokines also have an important influence on the development and balance of the adaptive immune response (Munari et al. 2014). Dendritic cells (DCs) in a pro-inflammatory cytokine environment stimulate the differentiation of T-helper 1 (Th1), Th17 and Th22 cells, which in turn secrete cytokines to enhance the inflammatory response (Atherton and Blaser 2009).

Despite this potent inflammatory and bactericidal activity, *H. pylori* has multiple mechanisms for evading immunity and directing the immune system towards a suppressive response. It thereby minimizes damage and enables it to maintain persistent, lifelong colonization of the host (Fig. 1b). Many components of *H. pylori* are modified so that they do not potently activate recognition receptors such as the toll-like receptors, and its fucosylated ligands for the C-type lectin receptor DC-SIGN stimulate anti-inflammatory cytokine responses including interleukin-10 (IL-10) (Salama et al. 2013). Some components have the ability to induce regulatory immune responses (White et al. 2015) (reviewed in Chapters "Immune Evasion Strategies and Persistence of *Helicobacter pylori*" and "*Helicobacter pylori* and Extragastric Diseases"), for having a tolerizing effect on DCs and causing them to promote the differentiation of naïve T cells into regulatory T cells (Tregs) (Oertli et al. 2012; Luther et al. 2011). These suppressive cells modulate inflammation by secreting cytokines such as IL-10 and transforming growth factor beta (TGFβ), or they may act via a number of contact-mediated mechanisms (Ai et al. 2014). Such effects result in reduced chemokine expression by gastric epithelial cells, and therefore reduced migration of inflammatory cells into the mucosa and less tissue damage. Macrophages responding to the cytokine milieu of the mucosa may express markers of the M2 type, whose chief role is in wound-healing rather than having a potent bactericidal and inflammatory function, which are attributes of M1 macrophages (Quiding-Jarbrink et al. 2010). *H. pylori* expresses enzymes such as catalase and arginase to protect against the damaging effects of reactive oxygen and nitrogen species (Ramarao et al. 2000; Gobert et al. 2001), and in addition, the bacterium is known to downregulate the expression of some antimicrobial peptides by gastric epithelial cells, including beta-defensins 1 and 3 (Patel et al. 2013; Bauer et al. 2012). These mechanisms are also thought to contribute to the persistence of the infection.

3 *H. pylori* Infection in Health and Disease

Chronic *H. pylori* colonization almost always leads to local inflammation of the gastric mucosa (gastritis), but this does not usually result in any symptoms. The most common disease outcomes of *H. pylori* infection include gastric and duodenal ulcer disease, distal gastric adenocarcinoma, and primary gastric mucosal-associated lymphoid tissue (MALT) lymphoma. Other conditions associated with *H. pylori* infection include dyspepsia, atrophic gastritis, iron deficiency anaemia, and idiopathic thrombocytopenia purpura. Pathogenesis is linked with the level of gastric inflammation, and interestingly the pattern of gastritis within the stomach is an important determinant of disease outcome (see Chapters "Human and *Helicobacter pylori* Interactions Determine the Outcome of Gastric Diseases" and "Pathogenesis of Gastric Cancer: Genetics and Molecular Classification"). What dictates the distribution of gastritis remains unknown; however, antral-predominant gastritis is associated with duodenal ulceration, whereas corpus-predominant or pan-gastritis is associated with the development of gastric ulcers and gastric cancer (Atherton 2006).

It has been proposed that the time of development of atrophic gastritis may an important factor in deciding disease outcome. If atrophic gastritis is not acquired early, high gastric acid output may result, with increased risk of duodenal ulcer later in life. An earlier appearance of atrophic gastritis would cause low gastric acid production, and consequently gastric cancer may occur (Ubukata et al. 2011).

Peptic ulcer disease is a fairly modern outcome of *H. pylori* infection, and the reasons for its emergence are unknown. In the USA, gastric ulcers were described from 1838 and duodenal ulcer disease reported a little later in 1931. Peak prevalence was in the early 1900s (Baron and Sonnenberg 2001). Possible theories for the emergence of peptic ulcer disease include increased strain virulence, and reduced transmission making infection with a single strain more common. Changes in the immune response, due to environmental factors such as cigarette smoking, diet, or exposure to other infections, are also important (Atherton and Blaser 2009). The ancestry and co-evolution of the *H. pylori* strain and its host population has also been shown to influence gastric cancer risk (Kodaman et al. 2014). Within populations in Africa, Asia, and India, some anomalies in the prevalence of disease have been reported, and these "enigmas" may provide clues about the circumstances of disease development. Much controversy surrounds whether these actually exist however (Graham et al. 2009), and the factors influencing the risk of disease are extremely complex (Table 1).

On the other hand, a protective association between *H. pylori* infection and disorders such as gastroesophageal reflux disease (GERD), oesophageal adenocarcinoma, inflammatory bowel disease, multiple sclerosis, and asthma has been variously described (Robinson 2015) (described in Chapter "*Helicobacter pylori* and Extragastric Diseases").

Table 1 Factors influencing the risk of gastric carcinogenesis amongst *H. pylori*-infected individuals

Factor	Evidence	References
Age of *H. pylori* acquisition and birth order	Earlier acquisition of *H. pylori* (most likely from older siblings) increases the risk of gastric cancer	Blaser et al. (2007)
Gender	Gastric cancer prevalence rates in women are half of that in men. This effect may be mediated by sex hormones since gastric cancer rates increase more slowly in women up to menopausal age, and thereafter at the same rate as in men	Chen et al. (2016), Global Burden of Disease Cancer et al. (2015)
Life expectancy	Gastric cancer is most commonly diagnosed in those over 60 years of age, and this may explain reduced gastric cancer prevalence in some countries where there is a low life expectancy	Global Burden of Disease Cancer et al. (2015), Graham et al. (2009)
Pattern of gastritis	Those with pan-gastritis (rather than antral-predominant gastritis) are at risk of developing gastric cancer	Ubukata et al. (2011)

(continued)

Table 1 (continued)

Factor	Evidence	References
H. pylori eradication	Reduced incidence of gastric cancer following eradication therapy, particularly if administered prior to the development of pre-malignant pathology	den Hoed and Kuipers (2016)
H. pylori virulence type	More virulent strains (expressing CagA, the s1/i1/m1 type of VacA, and others including BabA, SabA, OipA) are associated with an increased gastric cancer risk	Amieva and Peek (2016)
Matching of host and strain ancestry	Those with a genetic mismatch between their ancestry and that of their colonizing strain (e.g. Amerindian ancestry, but carrying a strain with a high African genetic content) are more likely to have severe pre-malignant pathology	Kodaman et al. (2014)
Host gene polymorphisms	The risk of gastric cancer is higher in those with gene polymorphisms leading to increased inflammation (e.g. IL-1β, TNFα, IL-10, TLRs 1, 2, 4, 5, and 9)	El-Omar et al. (2008)
Diet	A diet low in fruits and vegetables, high in salt, and with high alcohol consumption is associated with an increased gastric cancer risk	Fang et al. (2015)
Smoking	Data are not consistent; however, some studies show that cigarette smoking increases the risk of gastric cancer, and quitting smoking delays the risk of gastric cancer development and death	Ordonez-Mena et al. (2016)
NSAID use	Regular NSAID use is an independent protective factor for gastric cancer development	Wu et al. (2010)

3.1 Gastritis

The surface of the gastric mucosa is covered by layers of protective mucus, which *H. pylori* swims through to avoid damage from gastric acid and digestive enzymes. *H. pylori* is able to interact with the mucins via major adhesins the blood group antigen-binding adhesin (BabA), sialic acid-binding adhesin (SabA), and the lacdiNAc-specific adhesin, LabA (Ilver et al. 1998; Mahdavi et al. 2002; Rossez et al. 2014). Once in proximity to gastric epithelial cells, *H. pylori* activates inflammatory gene expression via interactions with pattern recognition receptors, for example Toll-like receptor 2 and NOD1 (Viala et al. 2004), and inflammasomes (Kim et al. 2013; Vanaja et al. 2015). Components of the *H. pylori* cytotoxin-associated gene

pathogenicity island (*cag*PAI) encoded type IV secretion system (T4SS) activate inflammatory signalling in gastric epithelial cells via a number of different mechanisms (described briefly in Sect. 3.1, and in detail in Chapter "Structural Insights into *Helicobacter pylori* Cag Protein Interactions with Host Cell Factors"), resulting in the secretion of cytokines and chemokines, including interleukin-8 (IL-8), IL-1β, tumour necrosis factor alpha (TNFα), IL-6, IL-12, CCL2-5, CCL20, and CXCL1-3 (Cook et al. 2014). The presence of chemokines leads to the recruitment of neutrophils, macrophages, mast cells, dendritic cells (DCs), innate lymphoid cells, and lymphocytes, a feature termed gastritis (Atherton and Blaser 2009).

The level and nature of gastritis varies, affecting the risk of disease development (Macarthur et al. 2004). Innate cells including neutrophils, macrophages, and NK cells contribute to gastritis via the secretion of inflammatory and tissue-damaging factors including reactive oxygen and nitrogen species (ROS and RNS) (Italiani and Boraschi 2014), perforin and granzymes (Yun et al. 2005).

It has recently been shown that human gastric epithelial cells and DCs produce retinoic acid (RA), which regulates the level of inflammation. During *H. pylori* infection, RA production is reduced, which leads to more intense inflammation and mucosal damage (Bimczok et al. 2015). DCs also play an important role in regulating the development of the adaptive immune response. They promote the development of pro-inflammatory Th1 and Th17 cells, the numbers of which correlate with the severity of gastritis (Hitzler et al. 2012). In the *H. pylori*-infected gastric mucosa however, DCs are thought to be semi-mature and tolerogenic (Rizzuti et al. 2015), stimulating the development of regulatory T cells (Tregs) which suppress inflammation.

Molecular mimicry by *H. pylori* frequently induces autoreactive antibodies against molecules such as the parietal cell H^+, K^+-ATPase. It is thought that these antibodies may enhance inflammation and damage in the stomach (Smyk et al. 2014). In addition, Th1 cells secrete the cytokines interferon-gamma (IFNγ) and TNFα, which stimulate macrophages to secrete further pro-inflammatory factors. Th17 cells secrete IL-17A, IL-17F, IL-21, and IL-22, which also stimulate the expression of ROS, RNS, and chemokines, leading to further inflammation and neutrophil recruitment (Hitzler et al. 2012).

3.2 Peptic Ulcer Disease

H. pylori is the leading cause of peptic ulceration, with 95% of duodenal ulcers and 70% gastric ulcers being attributable to the infection (Ford et al. 2016). The presence of these breaks in the lining of the duodenal or gastric mucosa is associated with significant mortality, for example from haemorrhage and perforation.

Chronic inflammation of the antrum resulting from *H. pylori* infection leads to destruction of delta cells, and subsequent reduction in the level of somatostatin they secrete. As somatostatin inhibits gastrin production from neighbouring G cells, reduced levels of this hormone lead to hypergastrinemia. In the case of antral-predominant

gastritis, elevated gastrin levels overstimulate the acid-producing parietal cells of the undamaged corpus resulting in hyperchlorhydria. In some people with antral-predominant gastritis, this increased gastric acid output results in gastric metaplasia of the duodenal epithelium. This permits *H. pylori* to colonize and cause inflammation, possibly leading to duodenal ulceration. Gastric ulcers may develop in those with corpus-predominant or pan-gastritis, where the acid output is normal or reduced. Low acid output can arise owing to loss of parietal cells resulting from atrophic changes to the corpus mucosa induced by chronic inflammation of this part of the stomach. Despite increased gastrin production from the *H. pylori*-infected antrum, a state of hypochlorhydria is established. This prevents the development of duodenal ulcers; however, inflammation and damage to the gastric mucosa may result in the development of gastric ulcers. Pre-malignant lesions and gastric adenocarcinoma may also arise as a result of this gastritis pattern (Atherton and Blaser 2009; Malfertheiner 2011).

Peptic ulceration is more frequently found in those with reduced numbers of Tregs in their gastric mucosa, and thus impaired capacity to control inflammation (Cook et al. 2014; Robinson et al. 2008). Gastric Th1 and Th17 cells from the antrum of patients with peptic ulcer disease induce epithelial cells to express higher levels of MHC class II, and exposure to their secreted cytokines leads to activation of mitogen-activated protein (MAP) kinases and transcription factors AP-1 and NF-κB, thereby enhancing the inflammation and damage (Zhou et al. 2007).

3.3 Gastric Adenocarcinoma

Gastric cancer is the fifth most common malignancy worldwide, and there are approximately 100,000 new cases each year (Colquhoun et al. 2015). Most cases are found in Asia, with over two-thirds of those reported in China (Ferlay et al. 2015). It is the third most common cause of cancer-related death, since initial diagnosis is usually at a late stage (Herrero et al. 2014). It can be divided into two subtypes depending on the location: cardia (arising from epithelial cells at the gastroesophageal junction) and non-cardia (arising from the distal stomach). Cardia gastric cancers have similar risk factors to those for oesophageal adenocarcinoma and Barrett's oesophagus, and are thought to be mostly unrelated to *H. pylori* infection (Colquhoun et al. 2015). In contrast, non-cardia gastric cancer is strongly associated with *H. pylori,* with up to 89% of cases attributed to the infection. *H. pylori* has been classified as a carcinogen, and the risk of gastric cancer development for an infected individual is 1–2% (Plummer et al. 2015).

The two histological types of gastric cancer are classed as intestinal and diffuse. The intestinal type develops along a stepwise progression driven by inflammation. *H. pylori* infection leads to chronic gastritis which, after several decades, leads to gastric gland atrophy, intestinal metaplasia, dysplasia, and finally adenocarcinoma. *H. pylori* eradication therapy reduces the incidence of atrophic gastritis, but does not reduce the risk of gastric cancer development unless the eradication is administered prior to pre-malignant changes (Malfertheiner et al. 2006).

Gastric carcinogenesis is associated with ROS/RNS-mediated DNA damage, the silencing of tumour suppressor genes via DNA methylation, histone epigenetic modifications, and epithelial–mesenchymal transition (Na and Woo 2014) (see Chapters "Exploiting the Gastric Epithelial Barrier: *Helicobacter pylori*'s Attack on Tight and Adherens Junctions" and "*Helicobacter pylori*-Mediated Genetic Instability and Gastric Carcinogenesis"). Gene polymorphisms associated with a higher risk of gastric adenocarcinoma tend to result in more severe inflammation, mediated by genetically determined high expression of pro-inflammatory cytokines (IL-6, IL-8, TNFα, IL-1β), low expression of anti-inflammatory cytokines (IL-10, TGFβ), or enhanced responsiveness to bacterial components (Toll-like receptors 1, 2, 4, 5, and 9) (Ramis et al. 2015; El-Omar et al. 2008). High-level Th17 and Th22 responses are associated with increased gastric cancer progression and poor survival, probably because the cytokines that they secrete favour angiogenesis and increased tumour invasiveness (Liu et al. 2012). The Th1 response also contributes to carcinogenesis, but there is evidence that this includes stronger anti-tumour immunity, and thus a better prognosis for gastric cancer patients. Treg responses are important for controlling inflammation; however, as they inhibit anti-tumour immunity, having high levels of these cells is an indication of poor prognosis (Hou et al. 2014).

3.4 MALT Lymphoma

H. pylori infection is present in virtually all patients with gastric MALT lymphoma, but this outcome occurs rarely (approximately 0.8 per 100,000 per year). Around 10% of cases are thought to be independent of *H. pylori,* but may be due to an undiagnosed *H. pylori* infection, or perhaps gastric non-*pylori* Helicobacters. *H. pylori*-mediated inflammation induces the formation of lymphoid follicles in the gastric mucosa, which are not present in the uninfected stomach (Genta et al. 1993). Uncontrolled expansion of marginal zone B cells in these lymphoid follicles occurs as a consequence of chronic inflammation and continuous antigenic stimulation (Du and Atherton 2006). The tumour cells are commonly disseminated throughout the gastric mucosa and often remain localized to this site. Spread to regional lymph nodes and more distant mucosal sites, however, occurs in approximately 40% of cases. Low-grade MALT lymphomas may transform into more aggressive diffuse large B cell lymphomas (DLBCL). This occurs in around half of gastric lymphoma cases, which have a considerably worse prognosis (Du and Atherton 2006).

Low-grade B cell MALT lymphomas usually regress following *H. pylori* eradication treatment. Chromosomal translocation t(11; 18), the most common genetic aberration in gastric MALT lymphoma, is found in one-quarter of cases, and more frequently when advanced to stage II or above. This chromosomal breakage and translocation results in fusion between the activator protein-12 (AP-12) and MALT-1 genes, and the product of this stimulates activation of the transcription factor NF-κB, which regulates the expression of anti-apoptotic genes and cell survival (Du and Atherton 2006). The presence of t(11; 18) also predicts

the non-responsiveness of gastric MALT lymphoma to *H. pylori* eradication therapy (Fischbach 2014). Mutations in immunoglobulin heavy chain variable region (IGHV) genes are also frequently present, and there is a bias in the usage of certain IGHV gene families, suggesting that tumour cells have undergone antigen selection in germinal centres (Zucca and Bertoni 2016). Bacterial virulence factors do not seem to greatly influence the risk of developing gastric MALT lymphoma; however, there is growing evidence that host genetic factors play an important role.

4 *H. pylori* Virulence Factors and their Association with Disease

H. pylori expresses several factors that are strongly associated with increased risk of disease development. These include toxins, adhesins, and chemoattractants. Many are highly polymorphic, phase variable, and have diverse functions. For further information, see Chapters "Human and *Helicobacter pylori* Interactions Determine the Outcome of Gastric Diseases", "Immune Evasion Strategies and Persistence of *Helicobacter pylori*" and "Structural Insights into *Helicobacter pylori* Cag Protein Interactions with Host Cell Factors".

4.1 The cag *Pathogenicity Island*

As described in detail in Chapter "Structural Insights into *Helicobacter pylori* Cag Protein Interactions with Host Cell Factors", the *cag*PAI is a 40 kB horizontally transmitted segment of DNA. It encodes a type IV secretion system (T4SS), with many of the expressed subunits forming a pilus through which CagA, peptidoglycan peptides, and other components are transferred into the host cell cytoplasm. CagL is found at the tip of the T4SS pilus structure and binds α5β1 integrin on epithelial cells (Kwok et al. 2007). Upon interaction of CagL with the integrin, and transfer of CagA and peptidoglycan peptides into cells via the pilus, the transcription factor NF-κB is activated, enters the nucleus, and induces the expression of pro-inflammatory cytokines and chemokines, most notably IL-8 and CCL20 (Cook et al. 2014; Gorrell et al. 2013). CagA rapidly interacts with signalling molecules in the cell cytoplasm. It is tyrosine-phosphorylated at its EPIYA (Glu-Pro-Ile-Tyr-Ala) motifs by Src kinases and interacts with SHP-2 phosphatase, leading to cytoskeletal changes, NF-κB activation and further pro-inflammatory gene expression (Tegtmeyer et al. 2011; Mueller et al. 2012). Unphosphorylated CagA interacts with the tight junction protein ZO-1, causing disruption of tight junctions between epithelial cells, and with E-cadherin, promoting the α-catenin-mediated upregulation of genes with oncogenic potential (Amieva et al. 2003; Franco et al. 2005).

The *cag*PAI may be fully complete and functional, or it may be partially present or absent. Strains with a functional *cag* T4SS are strongly associated with increased gastric cancer risk. The *cagA* gene is polymorphic, with EPIYA motifs categorized

as EPIYA-A, B, C, or D depending on their flanking sequences (Backert et al. 2010). EPIYA-A, EPIYA-B, and EPIYA-C motifs are usually present in Western CagA strain types whilst EPIYA-A, EPIYA-B, and EPIYA-D motifs are commonly found in East Asian CagA (Lind et al. 2014, 2016). A larger number of EPIYA-C motifs or the presence of an EPIYA-D increases the strength of SHP-2 interactions, and is associated with a higher risk of intestinal metaplasia and gastric cancer (Amieva and Peek 2016).

4.2 Vacuolating Cytotoxin (VacA)

VacA is a pore-forming toxin, originally named for its ability to induce vacuolation in gastric epithelial cells in vitro [recently reviewed by Foegeding et al. (2016)]. A wide range of functions have now been identified, including the induction of epithelial cell apoptosis, autophagy, and inhibition of T cell activation, and many of these effects depend on its formation of anion-selective channels (Kim and Blanke 2012; Terebiznik et al. 2009; Gebert et al. 2003). The *vacA* gene is present in almost all strains, and is highly polymorphic, with two alternative allelic variants for the signal (s1/s2), intermediate (i1/i2), and mid (m1/m2)-regions. The mid-region type is important for binding to epithelial cells, with m1 forms binding to a wider range of cell types than m2 forms of the toxin. s2 and i2 types of VacA also have reduced vacuolating activity compared to the s1 and i1 variants (Atherton et al. 1995; Rhead et al. 2007). All forms of VacA appear to be important in the tolerization of dendritic cells, promoting the differentiation of Treg cells and protecting against asthma in a mouse model (Oertli et al. 2013).

The *vacA* s1 and i1 alleles are associated with increased risk of peptic ulceration, atrophy, and gastric adenocarcinoma; however, the amount that is expressed is also important. A genetic determinant of increased *vacA* expression has recently been reported, consisting of a putative stem-loop structure in the 5′ untranslated region of the transcript (Amilon et al. 2015). The presence of this stem-loop region in colonizing strains is associated with more severe inflammation and pre-malignant pathology in the human gastric mucosa (Sinnett et al. 2016).

Genotypic analysis of *H. pylori* clinical isolates from around the world has shown that the majority of *cag*PAI-positive strains are *vacA* s1/i1 type whilst *cag*PAI-negative strains are usually *vacA* s2/i2 type. This makes it difficult to definitively determine the contribution of each individual factor to gastric disease. This association is not due to clonality or genetic linkage, and a simple functional dependence is not responsible either, as *cagA* knockouts still retain vacuolating activity (Tummuru et al. 1994), and a *vacA* null mutant strain still delivers CagA into AGS cells inducing a hummingbird phenotype and IL-8 secretion (Argent et al. 2008). However, mutagenesis studies suggest that CagA and VacA may downregulate each other's effect on epithelial cells presumably to avoid excessive damage to host gastric cells during infection (Argent et al., 2008). Both CagA and VacA localize to lipid rafts (Asahi et al.2003; Nakayama et al. 2006), where CagA associates with tyrosine-phosphorylated GIT1/Cat-1 (G protein-coupled receptor kinase-interactor

1/Cool-associated, tyrosine-phosphorylated 1). GIT1 is an important scaffold protein involved in signalling mechanisms that regulate cytoskeletal dynamics and membrane trafficking, processes central to hummingbird formation and vacuolation. GIT1 is phosphorylated by Src and FAK (focal adhesion kinase) kinases, the latter inactivated by phosphorylated CagA (Tsutsumi et al. 2006), and is dephosphorylated by receptor-like protein–tyrosine phosphatase-β (RPTPβ), the receptor for VacA. Studies have shown that CagA decreases the tyrosine phosphorylation of GIT1 (Asahi et al. 2003) whilst VacA binding to RPTPβ increases it (Fujikawa et al. 2003). Thus, functional interaction between CagA and VacA may be acting through GIT1-regulated signalling. There may also be interaction between these two virulence factors acting through NFAT (nuclear factor of activated T cells) signalling. CagA upregulates the expression of NFAT-regulated genes through stimulation of calcineurin (Yokoyama et al. 2005). However, the anion channel properties of VacA interfere with the calcium ion influx required for calcineurin activity, reducing NFAT activation (Boncristiano et al. 2003; Gebert et al. 2003). In addition to differential effects on signalling pathways, CagA inhibits the endocytosis of VacA, reducing the toxin's ability to induce vacuolation and apoptosis (Akada et al. 2010; Oldani et al. 2009). If VacA channels in the epithelial cell plasma membrane benefit the bacteria by releasing nutrients or urea, there may be a selective advantage in inhibiting its endocytosis. CagA can also increase the uptake of transferrin across the basolateral surface of polarized epithelial cells, whilst VacA redirects the internalized transferrin/transferrin receptor complex to the apical surface of the cell (Tan et al. 2011). In this way, CagA and VacA may work together to modify iron trafficking to support the growth of *H. pylori* microcolonies on the epithelial surface.

4.3 *DupA and* tfs*4*

Duodenal ulcer-promoting gene A, *dupA,* is present in the *tfs4* gene cluster along with other *vir* gene homologues which are thought to encode a type IV secretion system. As the name indicates, this was initially described as associated with duodenal ulcer disease, and *dupA*+ strains were reported to induce increased IL-8 expression in the antral gastric mucosa. *dupA*− clinical isolates and null mutants were also impaired in their ability to stimulate IL-12 production from peripheral blood mononuclear cells and monocytes, compared to *dupA*+ isolates and wild-type strains (Lu et al. 2005; Hussein et al. 2010). Not all studies have been able to demonstrate an association with duodenal ulcer disease, however. This is probably because an intact *tfs4* gene cluster is required for virulence rather than *dupA* alone.

4.4 *NapA*

H. pylori neutrophil activating protein (NapA, sometimes also called HP-NAP) is a highly conserved dodecameric 150-kDa protein, which is able to stimulate the

activation and production of oxygen radicals by neutrophils. The protein is a neutrophil chemoattractant, stimulating these cells to produce pro-inflammatory cytokines and chemokines, and it has been shown to induce a strong Th1 response. NapA may therefore play a central role in *H. pylori* pathogenesis (de Bernard and D'Elios 2010).

4.5 Adhesins

H. pylori expresses several major adhesins including BabA, SabA, LabA, OipA, and AlpAB. It is thought that adhesins mediate a closer association of the bacteria with the epithelium, and thus enhances exposure to other virulence factors, resulting in increased inflammation and damage to the gastric mucosa. BabA (blood group antigen-binding adhesin) binds to difucosylated Le^b blood group antigens on host epithelial cells and mucins, and is the major adhesin involved in the colonization process (Ilver et al. 1998). The *babA* genes exist in two allelic forms: *babA1*, lacking the translational start site and signal peptide, and *babA2*, encoding the full-length, active protein. Expression of functional BabA protein can be modulated by recombination between *babA* alleles and a closely related locus *babB*. The *babA2* allele has been associated with increased *H. pylori* colonization density, higher inflammation, and more severe gastric disease, particularly in combination with other virulence determinants such as *cagA* and the *vacA* s1 allele (Yu et al. 2002). The increased adherence of *babA2*-type *H. pylori* may enhance the virulent actions of CagA- and VacA s1-type toxin. SabA (sialic acid-binding adhesin), which becomes the predominant adhesin in the chronically inflamed stomach, binds to sialylated Lewis antigens (Mahdavi et al. 2002). The expression of functional SabA adhesin is subject to phase variation owing to the presence of dinucleotide repeats near the start of the gene (Mahdavi et al. 2002). Colonization with SabA-expressing strains is associated with an increased risk of gastric cancer (Yamaoka et al. 2006). LabA (lacdiNAc-binding adhesin) is a recently discovered adhesin, which binds a N,N′-diacetyllactosediamine (lacdiNAc) motif on MUC5AC mucin (Rossez et al. 2014). The expression of OipA (outer membrane inflammatory protein) is also controlled by phase variation. OipA-expressing strains are associated with increased risk of duodenal ulcer and gastric cancer, but these are also likely to be *cag*PAI-positive (Yamaoka and Graham 2014). *oipA* was originally identified as a gene required for the induction of IL-8 production by epithelial cells. An *oipA* null mutant strain failed to induce full phosphorylation and activation of FAK in gastric epithelial cells, preventing *H. pylori*-induced actin stress fibre formation and changes in cell morphology (Tabassam et al. 2008). Interestingly, the effects of *oipA* mutagenesis on FAK phosphorylation were greater than those observed for a *cag*PAI deletion mutant. Two further adhesins encoded by adjacent genes *alpA* and *alpB* have been shown to be involved in binding to human gastric tissue, and are required for colonization of animal models. AlpAB adhesins from East Asian and Western *H. pylori* strains have been found to differ in their ability to

modulate pro-inflammatory signalling cascades, which may relate to geographical differences in disease outcome (Lu et al. 2007; Odenbreit et al. 2002).

5 Environmental Factors and their Association with Disease

In epidemiological studies, factors including diet, alcohol consumption, and cigarette smoking have been found to influence the risk of developing peptic ulcer disease and gastric cancer (Fang et al. 2015).

Smoking is well known to increase the risk of morbidity from many diseases and cancers outside the respiratory tract (Ordonez-Mena et al. 2016). Cigarette smoke contains hundreds of toxic and carcinogenic compounds, and within the mucosa of the gastrointestinal tract, these are reported to decrease blood flow, increase gastric acid output, skew immune responses, cause cell death, and inhibit cell turnover (Li et al. 2014). Smokers are more likely to be *H. pylori*-positive, and to develop peptic ulcer disease with ulcers that are frequently more difficult to heal. There is a strong association between smoking and the development of gastric cardia adenocarcinoma, but the association between smoking and non-cardia adenocarcinoma is less convincing as unfortunately most studies have not investigated *H. pylori* status (Malfertheiner et al. 2012). Investigations of the mechanisms in vitro have shown that nicotine enhances the proliferation and epithelial–mesenchymal transition of gastric epithelial cells; however, cigarette smoke extract and nicotine exposure is reported to have conflicting pro- or anti-inflammatory effects (Goncalves et al. 2011).

The composition of the human diet has an important influence on disease risk. High-salt diets predispose to gastric cancer (Fang et al. 2015), probably because of an exacerbated inflammatory response, and this has also been demonstrated in *H. pylori*-infected Mongolian gerbils (Loh et al. 2015). The increased risk of disease may be due to the effects on the bacteria as well as the host, since *H. pylori* expresses increased levels of CagA and VacA when cultured in the presence of high sodium chloride concentrations (Amilon et al. 2015). On the other hand, diets that are high in fruits and vegetables tend to be inversely associated with gastric cancer risk (Fang et al. 2015; Amieva and Peek 2016). A number of clinical trials have demonstrated some significant protective effects from antioxidants, vitamins, and high dietary fibre [reviewed by den Hoed and Kuipers (2016)].

The human gastric mucosa is colonized by bacterial phyla, predominantly including Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (including *Helicobacter*). The composition of the gastric microbiota differs markedly according to the presence or absence of *H. pylori* and gastric pathology, particularly as these changes result in alterations of acid production and mucosal mucin expression [reviewed by He et al. (2016)]. Mouse studies have demonstrated that *H. pylori* infection influences the microbiome of the intestinal tract as well as the

stomach, and that the composition of the gastric microbiota influences disease development (He et al. 2016). The main focus of studies on human gastroduodenal disease has thus far centred on the interaction of *H. pylori* with host cells and mucosal tissue; however, the contributions of the other components of the gastric microbiota are only just beginning to come to light (see Chapter "Impact of the Microbiota and Gastric Disease Development by *Helicobacter pylori*").

6 Treatment of *H. pylori* and Prospects for a Vaccine

Patients with persistent dyspeptic symptoms are normally referred for endoscopy, and gastric biopsies routinely taken for *H. pylori* infection diagnosis by a rapid, commercial CLO (Campylobacter-like organism) test based on urease activity, and/or culture of the organism. Breath tests using ^{13}C-labelled urea, serology, and stool antigen testing are also alternative, non-invasive diagnostic methods employed for determining *H. pylori* status. *H. pylori* is sensitive to a variety of antibiotics in vitro, but the efficacy of single-drug therapy is limited by the localization of the bacterium within the low pH, viscous environment of the gastric mucus layer. The standard treatment for *H. pylori* consists of a triple therapy, with two antibiotics (typically clarithromycin and amoxicillin or metronidazole) and a proton pump inhibitor to suppress gastric acid output. This regimen has been proved effective in healing existing ulcers and preventing their recurrence, and it is recommended in various consensus reports and clinical guidelines (Sugano et al. 2015; Malfertheiner et al. 2012). Unfortunately, resistance to clarithromycin and metronidazole is a growing problem, resulting in more numerous treatment failures. The prevalence of antibiotic resistance varies widely around the world and is linked to the consumption of these drugs in each region. Metronidazole resistance is the most common problem, being present in approximately 75% of strains in Africa, 46% in Asia, and around 30% in Europe and the USA. For treatment regimens to be effective, it is therefore recommended that they should be chosen based on susceptibility testing in that region (Sugano et al. 2015).

With such antibiotic resistance issues, vaccination appears the logical approach to control *H. pylori;* however, progress in this area has not been straightforward despite using a wide range of approaches over the past 30 years. These include formulations of whole cell lysates, outer membrane vesicles, single or multiple purified antigens (e.g. urease, catalase, NapA, VacA, CagA, and heat–shock proteins) and DNA vaccines, with mucosal or parenteral delivery systems and adjuvants (Blanchard and Nedrud 2010). The search for new vaccine antigens is continuing using genomic and proteomic approaches (Walduck et al. 2015).

Since *H. pylori* infections are usually acquired in early childhood, it would be difficult to ensure that prophylactic vaccines are administered at an early enough stage. The most practical alternative is therefore to administer a therapeutic vaccine to eradicate the infection in people who are already colonized. In *Helicobacter*-infected animal models, therapeutic immunization has been shown to reduce the

bacterial load as well as protecting against a subsequent reinfection (Ikewaki et al. 2000; Rossi et al. 2004). A commonly identified problem with therapeutic vaccines in particular, however, is the phenomenon of post-immunization gastritis. This has been reported in mice (Garhart et al. 2002; Sutton et al. 2001), rhesus monkeys (Solnick et al. 2000), and gnotobiotic piglets (Krakowka et al. 1991), and seems to result from the inflammatory response to residual colonizing bacteria. In humans, phase 1 trials have been conducted and the vaccine formulations tested appear safe and immunogenic (Michetti et al. 1999; Kotloff et al. 2001). An avirulent *H. pylori* strain has been developed so that immunization-challenge studies can be carried out in humans (Graham et al. 2004).

Trials in *H. pylori*-infected adult humans have been rather disappointing [reviewed in Anderl and Gerhard (2014)]. Recently, however, a phase 3 clinical trial of a *H. pylori* vaccine based on recombinant urease B subunit fused to heat-labile enterotoxin B subunit showed an efficacy of 71.8% in preventing *H. pylori* infection in *H. pylori*-naïve children aged 6–15 years within 1 year post-immunization (Zeng et al. 2015). This study demonstrates that continued development of effective vaccines coupled with early immunization of young children could be an effective approach to prevent *H. pylori*-related disease in future generations.

7 Concluding Remarks

The most common outcome of *H. pylori* infection is chronic asymptomatic gastritis, and this is probably because the bacteria have adapted to evade and suppress the immune response. Disease outcome is determined by the complex interplay of multiple host, bacterial and environmental factors. The chronic inflammatory response is undoubtedly of extreme importance in disease pathogenesis; however, the mechanisms are not yet completely understood. As research progresses, it may become possible to develop prognostic tests, based on the important risk factors, to predict who might develop gastric cancer in the future. It will be important to ensure that effective antibiotic treatments or vaccines are available for these individuals in particular.

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Human and *Helicobacter pylori* Interactions Determine the Outcome of Gastric Diseases

Alain P. Gobert and Keith T. Wilson

Abstract The innate immune response is a critical hallmark of *Helicobacter pylori* infection. Epithelial and myeloid cells produce effectors, including the chemokine CXCL8, reactive oxygen species (ROS), and nitric oxide (NO), in response to bacterial components. Mechanistic and epidemiologic studies have emphasized that dysregulated and persistent release of these products leads to the development of chronic inflammation and to the molecular and cellular events related to carcinogenesis. Moreover, investigations in *H. pylori*-infected patients about polymorphisms of the genes encoding CXCL8 and inducible NO synthase, and epigenetic control of the ROS-producing enzyme spermine oxidase, have further proven that overproduction of these molecules impacts the severity of gastric diseases. Lastly, the critical effect of the crosstalk between the human host and the infecting bacterium in determining the severity of *H. pylori*-related diseases has been supported by phylogenetic analysis of the human population and their *H. pylori* isolates in geographic areas with varying clinical and pathologic outcomes of the infection.

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Contents

1 *Helicobacter pylori*: From Infection to Diseases

Helicobacter pylori is a Gram-negative microaerophilic bacterium that specifically colonizes the human stomach and exhibits extraordinary genetic diversity. Long-term infection causes diseases including chronic active gastritis, peptic ulcers, B cell lymphoma of mucosa-associated lymphoid tissue, and adenocarcinoma. The bacterium is adapted to its hostile ecologic niche through the activity of its bacterial urease that neutralizes gastric acidity by generating ammonium from urea. Moreover, the common trait of *H. pylori* strains that have increased risk of inducing gastric adenocarcinoma is the expression of specific virulence genes. First, the cytotoxin-associated gene A (CagA) is a bacterial factor that belongs to the *cag* pathogenicity island (*cag*PAI) and is injected in human epithelial cells through a type IV secretion system (T4SS). CagA is then sequentially tyrosine-phosphorylated by the host SRC proto-oncogene, non-receptor tyrosine kinase (SRC, c-Src) and ABL proto-oncogene 1, non-receptor tyrosine kinase (ABL1) kinases (Mueller et al. 2012) and dysregulates the homeostatic signal transduction of gastric epithelial cells (Higashi et al. 2002). This results in a persistent inflammation through the induction of CXCL8 (also known as IL-8) synthesis, and malignancy by loss of cell polarity, resistance to apoptosis, and chromosomal instability (Umeda et al. 2009). Second, the vacuolating cytotoxin A (VacA) contributes to *H. pylori* pathogenesis by regulating the inflammatory process (Supajatura et al. 2002) and by damping cell death by autophagy, thus favoring gastric colonization and oxidative damage (Raju et al. 2012). Although the contribution of VacA to gastric metaplasia has not been directly demonstrated using animal models, epidemiological studies have emphasized a correlation between the

vacA gene structure and severity of *H. pylori*-related diseases; the signal region s1 and the middle region m1 of the *vacA* gene are associated with strains at increased risk for inducing peptic ulcers and/or gastric cancer, compared to s2 or m2 strains (Atherton et al. 1997).

However, the sole expression of virulence factors is not sufficient to explain *H. pylori* pathogenesis and the development of gastric cancer. Environmental components, including iron deficiency (Noto et al. 2013) or high-salt diet (Fox et al. 2003), have been implicated in the outcome of *H. pylori* infection. Moreover, the involvement of host factors in gastric cancer risk is reflected in polymorphisms in genes that govern inflammation, stomach homeostasis (Vinall et al. 2002), and apoptosis/proliferation (Menheniott et al. 2010), as well as epigenetic factors resulting in altered levels of DNA methylation of specific genes (Schneider et al. 2015).

The backbone of *H. pylori*-associated inflammation is the non-specific activation of gastric epithelial cells and the presence of polymorphonuclear neutrophils (PMN), antigen presenting cells, i.e., dendritic cells and macrophages, and the adaptive immune response associated with CD4+ cells of the Th1 and Th17 subtypes in the gastric mucosa (Wilson and Crabtree 2007). The ultimate goal of the innate immune response of stromal and myeloid cells in response to *H. pylori* is to limit colonization by the pathogen; this occurs either indirectly by recruiting immune cells through the synthesis of chemokines, such as CXCL8, or directly by releasing antimicrobial effectors including reactive oxygen species (ROS) and nitric oxide (NO). However, these inducible host factors may also have deleterious effects by favoring persistent inflammation, which can be critical for the progression of gastric diseases according to the Correa cascade model (Correa 1988), and/or by affecting carcinogenesis through the regulation of cellular events (apoptosis/proliferation) or the induction of genetic changes (oxidative damage; mutations).

Here, we review the induction and the role of three major mediators of the innate immune response, namely the chemokine CXCL8, ROS, and NO during *H. pylori* infection.

2 The Chemokine CXCL8

The production of chemokines, and notably CXCL8, by gastric epithelial cells is a recurrent feature of *H. pylori* infection and represents a striking example of how the crosstalk between the host and the bacterium may affect the severity of *H. pylori*-associated diseases. Indeed, CXCL8 is a member of the CXC chemokine family produced by the innate response of myeloid and epithelial cells and is a major mediator of inflammation, acting as a chemoattractant for neutrophils and T cells (Fig. 1). Moreover, CXCL8 acts as a potent angiogenic factor in endothelial cells by stimulating vascular endothelial growth factor expression and enhancing cell

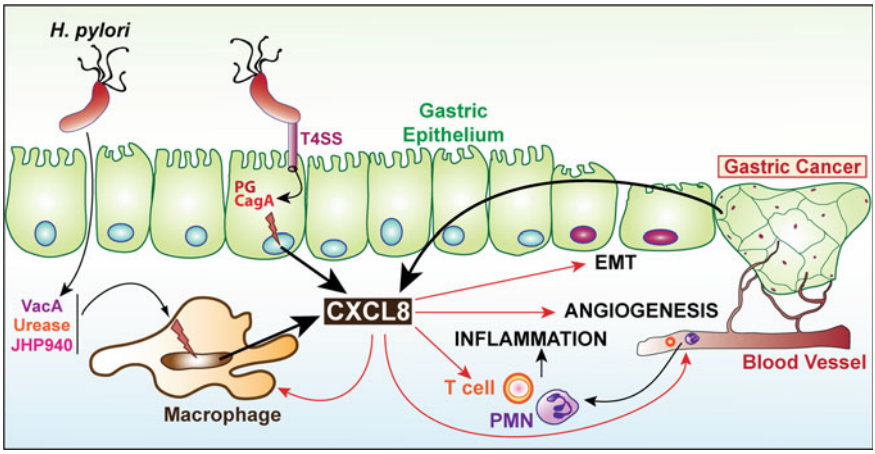

Fig. 1 Hypothetical model for CXCL8-mediated gastric carcinogenesis. Gastric epithelial cells and macrophages produce CXCL8 (also referred to as IL-8) in response to different *H. pylori* factors; VacA, urease, and JHP940 have been shown to stimulate macrophages, whereas the T4SS-dependent injection of CagA or peptidoglycan (PG) has been involved in epithelial cell activation. CXCL8 chemoattracts PMNs and T cells to infiltrate to the site of infection and activates immune cells, thus favoring a persistent inflammatory state. Moreover, CXCL8 stimulates epithelial–mesenchymal transition (EMT) and angiogenesis. These events may contribute to *H. pylori* carcinogenesis. Lastly, tumor cells also produce CXCL8, further potentiating gastric cancer development

proliferation and survival, potentiating epithelial–mesenchymal transition, and activating immune cells at the tumor site (Yuan et al. 2005) (Fig. 1).

The expression of *CXCL8* mRNA and the production of CXCL8 by antral biopsies are increased in *H. pylori*-infected patients compared to *H. pylori*-negative cases, and interestingly, CXCL8 has been immunodetected in both epithelial cells and macrophages (Ando et al. 1996). Further, increased CXCL8 in gastric biopsies is associated with infection by *cagA*-positive *H. pylori* (Li et al. 2001), and the serum concentration of CXCL8 in *H. pylori*-positive patients with gastric cancer is more elevated than those without carcinoma (Konturek et al. 2002) (Fig. 1). Consequently, it has been described that CXCL8 is a critical factor for the development, severity, and spread of gastric carcinoma (Kitadai et al. 1999; Lee et al. 2004; Asfaha et al. 2013).

2.1 Induction of CXCL8 by H. pylori

Numerous investigations have analyzed the mechanisms by which *H. pylori* interacts with epithelial cells to stimulate CXCL8 production. Studies have highlighted the critical role of the T4SS in this process (Fischer et al. 2001; Belogolova

et al. 2013; Varga et al. 2016) (Fig. 1). Although several bacterial components, such as peptidoglycan (Viala et al. 2004) or DNA (Varga et al. 2016), can be translocated into host cells through the T4SS and induce an innate immune response, the role of CagA has been evidenced by the following discoveries: (i) *H. pylori cagA*-positive strains induce more CXCL8 than *cagA*-negative strains (Crabtree et al. 1994; Salih et al. 2014); (ii) *cagA* mutant strains have reduced ability to stimulate CXCL8 (Brandt et al. 2005; Gobert et al. 2013); (iii) ectopic expression of CagA in gastric epithelial cells stimulates CXCL8 expression (Kim et al. 2006); and (iv) inhibition of CagA phosphorylation decreases *CXCL8* mRNA expression (Gobert et al. 2013). Nevertheless, the effect of CagA on CXCL8 induction has not been observed in some studies (Sharma et al. 1995), and the various results could be explained by differences in the type of host cells, in the *H. pylori* strains that have been used, and in the time of infection. Remarkably, it has been reported that *H. pylori*-stimulated CXCL8 production by AGS cells requires phosphorylation of CagA in the early stage of the infection, and then both phospho-CagA and a conserved motif, termed conserved repeat responsible for phosphorylation-independent activity (CRPIA), are implicated in late cell activation (Suzuki et al. 2009).

Again, according to the design of the in vitro experiments, different results have been observed regarding the signal transduction leading to *H. pylori*-induced CXCL8 induction in epithelial cells. Either the pro-inflammatory pathway involving the mitogen-activated protein kinase (MAPK) p38 and the transcription factor nuclear factor-kappa B (NF-κB) (Yamaoka et al. 2004; Gobert et al. 2013) or the extracellular signal-regulated kinases 1/2 (ERK1/2, MAPK1/3)-related signals (Keates et al. 1999; Brandt et al. 2005) have been described to play a role in inducible *CXCL8* transcription. Of note, p38 and NF-κB activation by *H. pylori* is partially mediated by CagA (Allison et al. 2009; Lamb et al. 2009; Gobert et al. 2013), but other *H. pylori* factors, including the outer inflammatory protein OipA (Lu et al. 2005a) and peptidoglycan (Allison et al. 2009), are possibly involved in the stimulation of p38 phosphorylation/activation. Finally, it is evident that multiple ways of signaling can lead to CXCL8 induction in *H. pylori*-infected epithelial cells, as underlined by work showing that the transcription factors activator protein-1 (AP-1) and NF-κB are required for maximal induction of *CXCL8* mRNA expression (Chu et al. 2003).

Myeloid cells are also a major source of CXCL8 during pathological conditions (Fig. 1). Thus, RNA in situ hybridization has revealed that *CXCL8* mRNA is expressed in macrophages and neutrophils in the gastric lamina propria of patients with chronic active *H. pylori* gastritis (Eck et al. 2000). Human peripheral blood mononuclear leukocytes or the human monocyte cell lines THP-1 or U937 stimulated in vitro with *H. pylori* urease (Harris et al. 1996), an *H. pylori* water extract (Bhattacharyya et al. 2002), the *H. pylori* protein JHP940 (Rizwan et al. 2008), or VacA (Hisatsune et al. 2008) express *CXCL8* mRNA and produce CXCL8. Moreover, *H. pylori*-induced CXCL8 production may occur independently of the T4SS because a *cagE* mutant was shown to retain its inducing activity (Maeda et al.

2001). CXCL8 induction in response to *H. pylori* is inhibited in THP-1 cells by ERK1/2, p38, and NF-κB inhibitors, demonstrating the involvement of the MAPK/NF-κB pathway in this process. However, VacA directly increases CXCL8 production in pro-monocytic U937 cells by activation of the p38 MAPK leading to the activation of two transcription factors, namely the heterodimer activating transcription factor 2/c-AMP response element-binding protein-1 (CREB1) that binds to the AP-1 site of the CXCL8 promoter and NF-κB (Hisatsune et al. 2008).

2.2 *CXCL8* Gene Polymorphisms

The *CXCL8*–251 A/A genotype is associated with a higher risk of atrophic gastritis and gastric cancer compared with the A/T or T/T genotype in Japanese (Ohyauchi et al. 2005; Taguchi et al. 2005), Chinese (Lu et al. 2005b; Zhang et al. 2010), and Korean (Kang et al. 2009) populations. Importantly, the CXCL8 promoter activity is enhanced, and CXCL8 is produced in greater amounts in the gastric mucosa of *H. pylori*-infected Asian patients harboring the A/A genotype than those with the A/T or T/T alleles (Ohyauchi et al. 2005; Taguchi et al. 2005; Ye et al. 2009; Song et al. 2010), demonstrating a direct link between the gene polymorphism and phenotypic identity. In the same way, it has been evidenced that the *CXCL8*–251 A allele is correlated with the degree of neutrophil infiltration, atrophy, and intestinal metaplasia (Taguchi et al. 2005; Ye et al. 2009). Interestingly, it has been reported that in Korean patients, the *IL-10*–592 A/A genotype (low promoter activity of the *IL-10* gene) and the *CXCL8*–251 A/A genotype (high promoter activity) each are associated with a greater relative risk of developing gastric adenocarcinoma, and the combination yielded a synergistic increase in risk (Kang et al. 2009). Such findings suggest that a combination of multiple host immune factors is involved in carcinogenesis.

Inversely, the *CXCL8*–251T/T genotype is associated with gastric carcinoma exhibiting a high frequency of microsatellite instability, antral location, and greater depth of invasion in Japanese patients (Shirai et al. 2006) and with an increased risk of adenocarcinomas in a population of Chinese Veterans infected with *H. pylori* (Lee et al. 2005). It should be noted that in the latter study, the T allele is correlated with increased transcriptional activity in comparison with the –251A counterpart (Lee et al. 2005), which is in contrast with previous findings (Ohyauchi et al. 2005; Taguchi et al. 2005; Ye et al. 2009; Song et al. 2010). Similarly, the frequency of the A/A genotype at the position –251 in the *CXCL8* promoter in European Caucasian patients is significantly increased in patients with gastritis, but it is not correlated with gastric cancer (Szoke et al. 2008). Interestingly, a study performed in Brazil showed that the *CXCL8*–845 T/C polymorphism, but not the *CXCL8*–251 A/T, is correlated with increased risk of developing chronic gastritis and gastric cancer in *H. pylori*-infected individuals (de Oliveira et al. 2015). The authors also show that the C variant in position –845 is responsible for the presence of the

binding sites for the transcription factors NF-κB and CREB1 (de Oliveira et al. 2015), which are involved in increased *CXCL8* gene expression. Of importance, no association between the *CXCL8*–251A/T polymorphism and the risk of *H. pylori* infection has been found (Lee et al. 2005), demonstrating that the modulation of chemokine production does not affect the level of *H. pylori* infection.

Together, these data indicate that CXCL8 plays a major function in *H. pylori* pathogenesis. Although the *IL8*–251A/T polymorphism might be a relevant host susceptibility factor for development of gastric cancer, this association is likely to be ethnicity-specific, as previously suggested by a meta-analysis (Canedo et al. 2008). More studies relating human genetic backgrounds, specific gene polymorphisms, and disease outcomes are needed.

3 Induction and Role of ROS

The ultimate goal of the inducible generation of ROS by stromal or myeloid cells in response to pathogenic bacteria is to limit the development of intruders. Nonetheless, persistent production of ROS affects cell signaling and DNA damage that may ultimately lead to carcinogenesis. Two main biochemical pathways leading to ROS production, namely NADPH oxidase and spermine oxidase (SMOX), have been identified in *H. pylori* infection. The mammalian NADPH oxidases are enzymes sharing the capacity to transport electrons across the plasma membrane and to generate superoxide. Seven homologs have been identified (NOX1, NOX2/gp91^{phox}, NOX3, NOX4, NOX5, DUOX1, and DUOX2), but NOX1 and NOX2 are of particular interest for *H. pylori* pathogenesis because NOX1 is highly expressed in the gastrointestinal tract and NOX2, the prototype NADPH oxidase, is abundant in immune cells (Fig. 2). SMOX is expressed in epithelial cells and macrophages and synthesizes hydrogen peroxide through the oxidation of the biogenic polyamine, spermine (Fig. 2).

3.1 ROS During H. pylori *Infection*

Several lines of evidence suggest that *H. pylori* infection is associated with increased ROS production in the stomach. A chemiluminescent method has been used to demonstrate that ROS generation is increased in gastric biopsies of *H. pylori*-positive patients compared to those from uninfected subjects (Papa et al. 2002). Moreover, H_2O_2 generation, assessed by an electrochemical microsensor positioned close to the gastric mucosa, was shown to be increased in Mongolian gerbils infected with *H. pylori* SS1 (Elfvin et al. 2007).

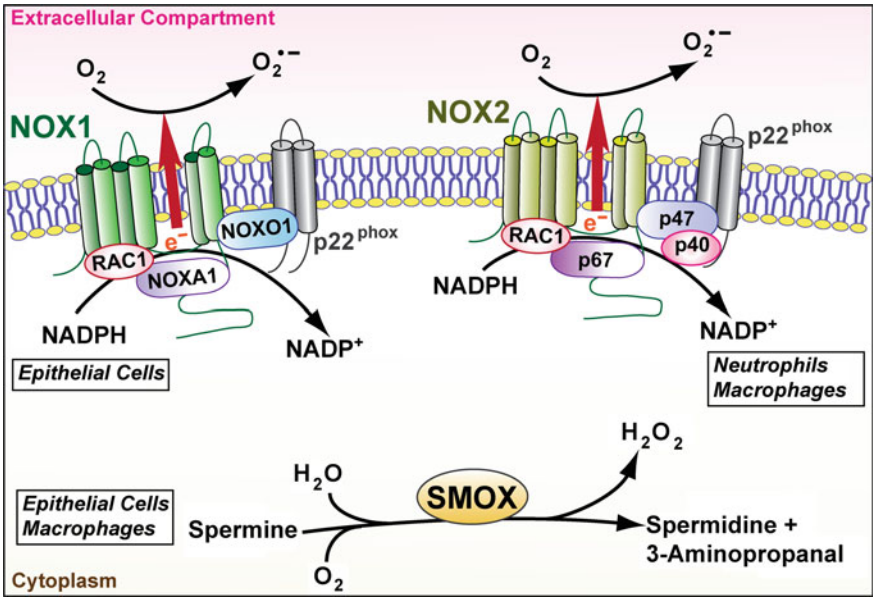

Fig. 2 Structure, function, and cellular localization of the two NADPH complexes, NOX1 and NOX2, and SMOX. The type of gastric cells in which these enzymes have been detected during *H. pylori* infection is indicated in the *boxes*

A significant correlation between the production of ROS in the gastric tissues of *H. pylori*-infected dyspeptic patients and the level of 8-hydroxy-2-deoxyguanosine (8-OHdG), a biomarker of DNA oxidative damage, has been evidenced in European patients (Papa et al. 2002). However, this was not observed in uninfected individuals and in the few patients infected with *cagA*-negative *H. pylori* (Papa et al. 2002). Of note, Pignatelli et al. (2001) questioned whether eradication of *H. pylori* could be associated with reduced oxidative damage to gastric tissues. They showed by immunohistochemistry in gastritis tissues of infected subjects that NOS2 was primarily detected in inflammatory cells, while nitrotyrosine and 8-OHdG localized mainly to foveolar epithelial cells (Pignatelli et al. 2001). While NOS2 and nitrotyrosine levels decreased with *H. pylori* eradication, the authors did not find a significant reduction of 8-OHdG levels (Pignatelli et al. 2001).

3.2 *Expression and Role of NOX1*

Although NOX1 expression has never been documented in the normal human stomach (Salles et al. 2005), in vivo and in vitro data suggest that *H. pylori* can induce this enzyme. It has been shown that the genes encoding NOX1 and one of its partner proteins, NOXO1, are upregulated in tissues from *H. pylori*-infected

patients with intestinal-type or diffuse-type gastric adenocarcinomas and were absent in normal stomachs (Tominaga et al. 2007). These authors also showed by immunofluorescence and confocal microscopy that the proteins NOX1 and the partners NOXO1, NADPH oxidase activator 1 (NOXA1), and $p22^{phox}$ co-localize with the Golgi apparatus in gastric cancer cells, and that their expression levels are increased in gastric cancers compared to the surrounding tissue and compared to areas of chronic atrophic gastritis or adenomas in patients without carcinoma (Tominaga et al. 2007). This provides a rationale for the use of NOX1 as a marker of neoplastic transformation and for the role of NOX-1-derived ROS in *H. pylori*-mediated carcinogenesis in the human stomach. In the same way, non-transformed antral-derived primary epithelial cells and the gastric epithelial cell line AGS infected with *H. pylori* 26695 express NOX1 and produce ROS rapidly (within 30 min) and transiently (den Hartog et al. 2016). The transcription factor(s) involved in *H. pylori*-mediated *NOX1* transcription in gastric epithelial cells have not been characterized yet, but one study implicated the pleiotropic signaling molecule Ras-related C3 botulinum toxin substrate 1 (Rac1) (den Hartog et al. 2016). Further work is warranted to delineate the molecular mechanisms leading to NOX1 expression, since these findings could provide critical insight into *H. pylori*-associated inflammation.

Several articles have analyzed the effect of NOX1-derived ROS on cellular and molecular events that may explain *H. pylori* pathogenesis. Ngo et al. (2016) have demonstrated that the oxidative burst induced by *H. pylori* in human gastric epithelial cells is responsible for the phosphoinositide 3-kinase (PI3-K)/AKT serine/threonine kinase 1 (AKT1)/glycogen synthase kinase 3 beta (GSK3β)-dependent expression of the transcription factor Snail (SNAI1) (Ngo et al. 2016), which is essential for epithelial–mesenchymal transition and therefore in gastric cancer progression and metastasis. Additionally, it has been shown that the transcription factor signal transducer and activator of transcription 3 (STAT3), which plays a major function in inflammation and angiogenesis, is also induced in gastric epithelial cells via upregulated autocrine IL-6 signaling, partially mediated by endogenous ROS (Piao et al. 2016). Lastly, α-lipoic acid, a naturally occurring thiol compound that exhibits antioxidant properties, inhibits NADPH oxidase-derived ROS production in AGS cells and concomitantly dampens NF-κB and AP-1 activation and expression of the oncogenes β-catenin (CTNNB1) and c-Myc (MYC) (Byun et al. 2014). Interestingly, the endonuclease apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1, also known as APE1), a multifunctional enzyme that plays a central role in the cellular response to oxidative stress, including DNA repair and redox regulation of transcriptional factors, is induced by endogenous ROS in AGS cells (den Hartog et al. 2016). APEX1 inhibits RAC1 activation and consequently NOX1-dependent ROS production, and blocking APEX1 leads to sustained NOX1 activation and increased O_2^- generation (den Hartog et al. 2016). This could represent a mechanism by which cells limit NOX1-dependent DNA damage and/or a strategy developed by the bacterium, to promote its own survival by inhibiting ROS production.

3.3 Expression and Role of NOX2

Myeloid cells are also a major source of ROS during pathological process. Live *H. pylori*, a bacterial sonicate, or *H. pylori* lipopolysaccharide (LPS) can each induce a rapid oxidative burst in human primary blood-derived polymorphonuclear cells (PMNs) (Nielsen and Andersen 1992; Nielsen et al. 1994; Allen et al. 2005). However, this effect was not observed in neutrophils primed with a water extract of *H. pylori* (Shimoyama et al. 2003). In studies using a Clark oxygen electrode, it has been demonstrated that *H. pylori* triggers a faster and stronger oxidative burst in PMNs than that induced by other bacteria, including *Staphylococcus aureus* and *Salmonella*, or by phorbol myristate acetate (Allen et al. 2005). Of particular interest, *H. pylori* does not efficiently recruit the NADPH oxidase domain $p47^{phox}$ or $p67^{phox}$ to the phagosome; consequently, NADPH oxidase accumulate in patches at the cell surface and the infection leads to the release of large amounts of superoxide into the extracellular milieu (Allen et al. 2005). This unique ability to prevent the oxidase assembly at the phagosome allows *H. pylori* to evade the oxidative killing and may result in neutrophil-derived oxidative damage to the surrounding cells, mainly from the epithelium.

The gene *napA* of *H. pylori* (Evans et al. 1995) encodes the 17 kDa virulence factor neutrophil-activating protein A (NapA) that forms hexagonal rings, binds up to 40 atoms of iron per monomer, and oligomerizes into dodecamers with a central hole capable of binding up to 500 iron atoms per oligomer (Tonello et al. 1999). It has been shown that NapA is a chemoattractant for leukocytes and induces NADPH oxidase in neutrophils through a SRC/PI3K signaling pathway (Satin et al. 2000). In addition to its effect on ROS production by neutrophils, NapA also displays a role in the bacterium itself. Thus, the *napA* mutant is more sensitive to oxygen (Olczak et al. 2002) and oxidative stress induced by organic peroxides, cumene hydroperoxide, or paraquat (Olczak et al. 2002; Wang et al. 2006), than the wild-type strain and exhibits more bacterial DNA damage (Wang et al. 2006). However, no alteration in gastric colonization was observed in animals infected for 3 weeks with the *napA* mutant compared to the wild-type strain (Wang et al. 2006). Chronic infection of mice or the use of animal models of *H. pylori*-mediated gastric dysplasia, e.g., transgenic FVB/N insulin-gastrin (INS-GAS) mice that over-express pancreatic gastrin or gerbils, would be useful to determine the role of NapA and the associated induction of NOX2 in carcinogenesis.

Similarly, the stimulation of blood-derived monocytes or differentiated THP-1 cells with *H. pylori* or its LPS yields a rapid and sustained production of O_2^- (Mai et al. 1991). More particularly, it has been reported that *H. pylori* cecropin-like peptide Hp(2-20) is a chemoattractant and activator for monocytes through the *N*-formyl peptide receptors 1 and 2, and that monocytes stimulated with Hp(2-20) release ROS, which inhibit the function of antineoplastic lymphocytes (Betten et al. 2001).

Chronic granulomatous disease (CGD) mice are animals with a targeted disruption of the NOX2/gp91^{phox} subunit of the NADPH oxidase. At 12- and 30-week post-infection *with H. pylori*, CGD mice showed no differences in colonization, but more glandular atrophy, proliferation of gastric epithelial cells, and neutrophil infiltration (Keenan et al. 2005). But overall, there were no significant changes in gastritis score between wild-type and CGD mice (Keenan et al. 2005). In contrast, a second study reported that *gp91*$^{phox-/-}$ mice exhibit more inflammation and increased mononuclear cell infiltration in the mucosa during infection with *H. felis* or *H. pylori* (Blanchard et al. 2003). Although this investigation was performed at 3-weeks post-infection, which is not sufficient time to observe strong gastric inflammation, the results are consistent with in vitro data showing that the partial scavenging of ROS by *N*-acetylcysteine led to a decrease of *H. pylori*-induced inflammasome formation, and IL-1β and IL-18 production by *H. pylori*-infected THP-1 cells (Li et al. 2015).

The NOX1 and NOX2 partner p22^{phox} is essential for NADPH oxidase activity. It has been reported that the C242T polymorphism of the *p22*^{phox} gene, which leads to reduced production of ROS, is associated with reduced risk of developing functional dyspepsia and intestinal metaplasia in *H. pylori*-infected patients (Tahara et al. 2009a, b).

In summary, NADPH-derived ROS may be both a consequence of and contributor to the severity of *H. pylori* gastritis and may contribute to the progression to precancerous lesions. However, additional studies are needed to further assess the role of this source of ROS in carcinogenesis.

3.4 Expression and Role of SMOX

An important characteristic of the innate immune response of the host during *H. pylori* infection is the induction of the enzyme arginase 2, but not arginase 1, through an NF-κB-dependent pathway (Gobert et al. 2002). Arginases are enzymes that catabolize L-arginine into urea and L-ornithine; this last product is then converted by ornithine decarboxylase (ODC) into the first polyamine putrescine, which is then sequentially catabolized to spermidine and spermine. Although ODC is mainly regulated at the post-transcriptional level in many cell types, *Odc* mRNA expression, and consequently, ODC protein is increased in murine macrophages infected in vitro with *H. pylori* (Gobert et al. 2002) and in gastric macrophages of infected mice and humans (Chaturvedi et al. 2010), and this favors the synthesis of the three polyamines (Gobert et al. 2002; Chaturvedi et al. 2010). Importantly, the back-conversion of spermine to spermidine occurs through the enzyme SMOX, which oxidizes spermine into spermidine, 3-aminopropanal, and H_2O_2 (Wang et al. 2001). We have reported that SMOX is induced at the transcriptional level by *H. pylori* in macrophages (Chaturvedi et al. 2004) and in gastric epithelial cells (Xu et al. 2004), thus favoring apoptosis and DNA damage by an H_2O_2-dependent mechanism (Chaturvedi et al. 2004; Xu et al. 2004).

Two *cagA*-deficient strains of *H. pylori* and a *cagE* mutant each induced less *SMOX* mRNA expression in conditionally immortalized murine gastric epithelial cells and in human epithelial cells than the wild-type strains (Chaturvedi et al. 2011), suggesting that CagA and the T4SS are involved in SMOX expression. These in vitro observations were confirmed by the analysis of gastric tissues from patients with *H. pylori* infection: individuals infected with *cagA*-positive *H. pylori* showed a significant increase of *SMOX* mRNA expression, and SMOX protein level in the gastric epithelium compared to patients harboring *cagA*-negative strains (Chaturvedi et al. 2011). Similar data were obtained in mice infected with *cagA*-positive *H. pylori* versus a *cagE* mutant, and in gerbils infected with *cagA*-positive versus *cagA*-negative strains (Chaturvedi et al. 2011, 2015).

SMOX expression in isolated epithelial cells from the gastric tissues of *H. pylori*-infected gerbils correlates with 8-oxoguanosine formation (Chaturvedi et al. 2011); there is a subpopulation of SMOX-expressing cells with oxidative DNA damage that were resistant to apoptosis (Chaturvedi et al. 2011), thus increasing the likelihood to favor carcinogenesis. In vitro, inhibition of SMOX with MDL 72527 (N^1,N^4-Di(buta-2,3-dien-1-yl)butane-1,4-diamine dihydrochloride) reduced *H. pylori*-induced 8-oxoguanosine levels, emphasizing the critical role of this enzyme in carcinogenesis. This was further evidenced by in vivo experiments: treatment with either α-difluoromethylornithine, an inhibitor of ODC, or MDL 72527, reduced gastric dysplasia and carcinoma in *H. pylori*-infected gerbils (Chaturvedi et al. 2015). Moreover, the subpopulation of 8-oxoguanosine+ cells that were resistant to apoptosis (8-oxoguanosine^{high}, active caspase-3^{low}) has been significantly reduced in the gastric mucosa of infected animals treated with the ODC or SMOX inhibitor (Chaturvedi et al. 2015).

Excitingly, reduced levels of SMOX, DNA damage, and DNA damage^{high}, apoptosis^{low} cells has been also observed in gastric epithelial cells grown from mice lacking the gene encoding epidermal growth factor receptor (EGFR) or in mice with disruption of EGFR signaling (*Egfr*^{wa5} mice) (Chaturvedi et al. 2014), implicating this receptor in *H. pylori*-mediated SMOX expression. Additionally, the kinase erb-b2 receptor tyrosine kinase 2 (ERBB2) was shown to be critical for the cellular events associated with EGFR signaling in gastric epithelial cells (Chaturvedi et al. 2014). Further identification of the molecular events leading to SMOX transcription is warranted to further understand mechanisms of *H. pylori*-associated carcinogenesis.

3.5 Epigenetic Regulation of SMOX

Recently, the epigenetic control of SMOX has been evidenced. It has been described that overexpression of the microRNA miR-124 blocks *SMOX* mRNA expression, SMOX protein induction, and SMOX activity in *H. pylori*-infected AGS cells by targeting the 3′-UTR of the human *SMOX* gene (Murray-Stewart et al. 2016). The promoter region of the tumor suppressor *miR-124* is hypermethylated in

a variety of cancers, including gastric cancer (Ando et al. 2009). Consistent with this, it was demonstrated that increased methylation of the three *miR-124* loci is associated with increased SMOX protein expression in patients with *H. pylori* infection (Murray-Stewart et al. 2016). Together, the data suggest that in non-transformed cells, *miR-124* is normally expressed and downregulates SMOX, whereas SMOX induction should be maximal during carcinogenesis.

4 Nitrosative Stress

The non-specific defense program of gastric epithelial cells and macrophages against *H. pylori* leads to the production of NO, a ubiquitous free radical synthesized by the enzyme inducible NO synthase 2 (NOS2) through the 5-electron oxidation of the terminal guanidino-N2 of the amino acid L-arginine in the biological milieu. Since the discovery of the immunological properties of the macrophage-derived L-arginine-NO pathway, notably by the work of J.B. Hibbs, Jr in the 1990s (Lancaster and Hibbs 1990), more than 23,000 referenced publications have addressed the thematic field of NOS expression during the immune response and the role of NO in the pathophysiological process of infectious diseases.

4.1 H. pylori *Infection and NOS2*

An increase of *NOS2* mRNA expression has been reported in the gastric tissues of *H. pylori*-infected patients (Tatemichi et al. 1998; Fu et al. 1999; Li et al. 2001), independently of the *cagA* status of the bacteria (Son et al. 2001). Nonetheless, *NOS2* expression is directly related to the presence of the bacteria because NOS2 is less expressed in *H. pylori*-negative gastritis than in infected patients (Fu et al. 1999; Goto et al. 1999), and in the gastric mucosa after the eradication of *H. pylori* (Mannick et al. 1996; Hahm et al. 1997; Antos et al. 2001; Felley et al. 2002).

In *H. pylori* gastritis, NOS2 protein has been localized to the epithelium, endothelium, and lamina propria of the stomach (Fu et al. 1999; Sakaguchi et al. 1999) or suggested to be only in inflammatory cells, including PMNs and mononuclear cells (Mannick et al. 1996; Goto et al. 1999; Sakaguchi et al. 1999; Felley et al. 2002).

Similarly, increased *Nos2* mRNA and protein has been observed in isolated gastric macrophages of *H. pylori* SS1-infected C57BL/6 mice after 4 months (Touati et al. 2003; Chaturvedi et al. 2010; Lewis et al. 2011). Multiple regulators of macrophage NOS2 expression during *H. pylori* infection have been elucidated. Deletion of arginase 2 (Lewis et al. 2011; Hardbower et al. 2016a) and inhibition of ODC (Chaturvedi et al. 2010) or heme oxygenase-1 (Gobert et al. 2014) results in increased NOS2 levels and NO production in vitro and in gastric macrophages. Further, we recently reported that EGFR is phosphorylated in macrophages in response to

H. pylori in vitro and in gastric macrophages of mice and humans, and this is essential for M1-type macrophage activation and NOS2 expression (Hardbower et al. 2016b). The expression of *Nos2* mRNA has also been evidenced in Mongolian gerbils infected with *H. pylori* for 2 weeks (Matsubara et al. 2004) or 3 months (Elfvin et al. 2006). Lastly, like in humans, a reduction of *Nos2* mRNA is observed with the eradication of *H. pylori* in infected INS-GAS mice (Lee et al. 2008).

Interestingly, nitrotyrosine, which indicates the nitration of tyrosine by peroxynitrite ($ONOO^-$) generated from NO and O_2^-, is immunodetected in epithelial cells and macrophages, even in studies in which NOS2 is found only in inflammatory cells (Mannick et al. 1996; Goto et al. 1999; Sakaguchi et al. 1999). This suggests that NO is effectively synthesized from NOS2 and that reactive nitrogen intermediates (RNI) target the cells surrounding NOS2-expressing macrophages, thus providing a rationale for a potential biological effect in the infected tissues.

4.2 *NO-Mediated* H. pylori *Carcinogenesis*

Although the level of *H. pylori* gastritis is similar in wild-type and *Nos2*$^{-/-}$mice (Miyazawa et al. 2003; Obonyo et al. 2003; Hardbower et al. 2016a), several reports suggest that NO may have an effect on *H. pylori*-mediated carcinogenesis. First, NO and certain RNI are considered to be potent mutagens. Hence, *H. pylori* gastritis is associated with enhancement of epithelial cell content of 8-nitroguanine (Ma et al. 2004; Katsurahara et al. 2009), one of the major products formed by the reaction of guanine with $ONOO^-$ (Yermilov et al. 1995), which enables G:C → A:T transversions, one of the most common mutations in the p53 tumor suppressor gene in gastric carcinogenesis (Tsuji et al. 1997). Thus, increased NOS2 protein level, nitrotyrosine immunostaining, and oxidative DNA damage have been detected in patients with gastric cancer compared to individuals with *H. pylori* gastritis (Goto et al. 1999; Hirahashi et al. 2014). In addition, it has been observed that *Nos2* expression in gastric tissues parallels the gene mutation frequency in gastric epithelial cells (Touati et al. 2003). Accordingly, in mice infected with *H. pylori* SS1, DNA fragmentation is observed in wild-type mice, but not in *Nos2*$^{-/-}$ mice, despite the same level of acute inflammation (Miyazawa et al. 2003).

4.3 *NOS2 Polymorphisms*

Different polymorphisms have been described in the promoter region of the *NOS2* gene. A long (CCTTT) repeat (>13) in the 5′ region or the –954G/C and –1173C/T single nucleotide polymorphisms have been associated with increased mRNA expression. The long (CCTTT) repeat and the –954G/C, but not –1173C/T, have been linked with an increased risk of gastric cancer in a Japanese (Tatemichi et al.

2005; Kaise et al. 2007; Sawa et al. 2008) and Brazilian population (Jorge et al. 2010), respectively, providing a rationale for the involvement of NOS2-derived NO in *H. pylori*-associated carcinogenesis.

5 The Host and Bacteria Coevolution: The Crystal Ball of *H. pylori* Pathogenesis?

Hundreds of thousands of years of coevolution have likely shaped the crosstalk between *H. pylori* and the human innate response. Several localized regions of the world with separate outcomes of *H. pylori* infection may help in the understanding of how these interactions may affect bacterial pathogenesis.

Amerindians living in the Peruvian Amazon and infected with *H. pylori* exhibited active gastritis and intestinal metaplasia, but none had peptic ulcer or gastric cancer (Suzuki et al. 2011). The *H. pylori* isolates harbored by these individuals belong to Amerindian strains (hspAmerind); however, the authors have highlighted that these strains possess two types of CagA proteins, namely Amerindian-I (AM-I) and Amerindian-II (AM-II), with particular features (Suzuki et al. 2011). First, AM-I and AM-II have altered Glu-Pro-Ile-Tyr-Ala (EPIYA)-B motifs, which are ESIYT and GSIYD, respectively. Second, the CRPIA, which is responsible for the phosphorylation-independent signaling of CagA, is different from those of Western or East Asian strains; further, AM-I CagA shows two AM-I CRPIA motifs, whereas AM-II CagA have either one AM-II motif or an AM-I plus an AM-II motif. Third, the N-terminal region of the AM-II CagA, involved in plasma membrane anchoring, lacks two large internal segments. The authors next questioned whether these particular CagA motifs could be associated with lower gastric cancer risk. The level of CagA phosphorylation in gastric epithelial cells associated with these particular Amerindian strains does not differ from that associated with Western or East Asian CagA, suggesting that the modified EPIYA motifs in AM-I and AM-II CagA do not have a functional effect (Suzuki et al. 2011). Nonetheless, the authors demonstrated that CXCL8 and cancer-associated Mucin 2 were produced in lower amounts by cultured epithelial cells or the gastric mucosa of gerbils during infection with *H. pylori* with AM-I or AM-II CagA compared to strains harboring Western or East Asian CagA (Suzuki et al. 2011). This effect was attributed to the modified CRPIA motifs that caused decreased interactions between CagA and MET proto-oncogene, receptor tyrosine kinase (MET, c-Met), and less epithelial cell responses to *H. pylori* (Suzuki et al. 2011). The discovery that this particular AM CagA of the native Amerindian plays a major role in the attenuation of the inflammation and consequently to less carcinogenesis is reinforced by the fact that other native Peruvians with high risk to develop gastric cancer are infected with *H. pylori* that possess a Western-type *cag* pathogenicity island (Devi et al. 2006).

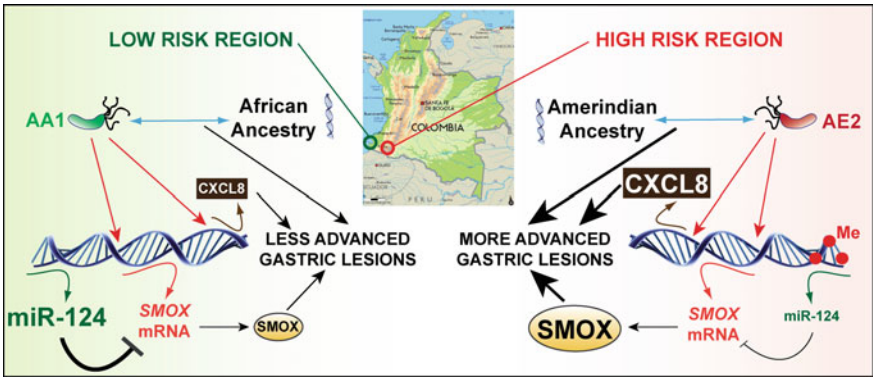

Fig. 3 A hypothesis to resolve the so-called Colombian enigma. The African strains (AA1 phylogenetic group) belonging to the low-risk (LR) region induce less CXCL8 than European strains (AE2) of the high-risk (HR) region. The methylation (Me, methyl) of the *miR-124* gene is greater in gastric tissues from the HR versus LR region; therefore, miR-124 is less expressed and SMOX protein more translated in subjects from the HR region. Moreover, individuals with an African ancestry infected with AA1 isolates have less risk for developing more advanced gastric lesions than Amerindians infected with AE2 *H. pylori* strains

In Colombia, the incidence of gastric cancer is 25 times higher in the towns of Tuquerres and Pasto, in the Andes Mountains [high-risk (HR) region] than in the town of Tumaco located on the coast [low-risk (LR) region] (Correa et al. 1976). HR *H. pylori* isolates were found to all be of European phylogeographic origin, whereas one third of the LR strains were of European origin, and two-thirds had an African origin (de Sablet et al. 2011). Furthermore in the cases from the LR region, the strains associated with higher levels of gastric epithelial DNA damage and premalignant lesions in the source patients were of European phylogeographic origin (de Sablet et al. 2011). Gastric tissues from subjects from the HR region exhibit greater levels of SMOX protein and oxidative DNA damage than those from LR region (Chaturvedi et al. 2015). Also, the HR European isolates induced more CXCL8 and SMOX expression in AGS cells than the clinical strains with an African phylogeographic origin (Sheh et al. 2013; Chaturvedi et al. 2015) (Fig. 3). Although European *H. pylori* express more *cagA*, *vacA*, and *babB* (Sheh et al. 2013) than LR strains, the differences in the induction of gene expression in AGS cells by HR and LR isolates were not attributable to differences in CagA expression and phosphorylation (Chaturvedi et al. 2015). Inversely, the African strains, but not the European ones, significantly induced apoptosis in gastric epithelial cells (Sheh et al. 2013; Chaturvedi et al. 2015).

Furthermore, the hypermethylation, and thus the silencing, of the genes encoding miR-124, the microRNA regulating *SMOX* expression (Murray-Stewart et al. 2016), has been observed in patients with *H. pylori*-associated gastric cancer (Ando et al. 2009). Increased DNA methylation of three *mir-124* gene loci has recently been evidenced in the HR Colombian Andean subjects compared to the LR coastal inhabitants (Murray-Stewart et al. 2016), demonstrating that the epigenetic control

of this miRNA, which is known as a tumor suppressor, could also have an important effect on the innate response of epithelial cells and consequently on *H. pylori*-mediated carcinogenesis (Fig. 3). It would be of interest to determine whether *miR-124* methylation is only associated with the type of *H. pylori* infection or is also dependent on the ethnic origin and genetic features of the human population.

Studies from our group have described that both the host and *H. pylori* phylogeographic variations, rather than the phylogenetic categorization of the bacteria per se, are essential for the development of gastric disease progression along the Correa cascade (Kodaman et al. 2014). In the LR region, humans exhibit a predominant African ancestral cluster and have less chance to develop severe gastric lesions, because they are mainly infected with African *H. pylori* strains, indicative of ancestral coevolution of the bacterium and the host (Fig. 3). In contrast, the risk of more advanced lesions in the histologic cascade toward cancer is increased in individuals showing a predominant Amerindian ancestry, but infected with strains exhibiting a substantial African component (Kodaman et al. 2014). In the HR region, the mountain population is principally Amerindian, but are infected mainly with *H. pylori* strains belonging to a predominant European origin suggesting a loss of coevolution. Further, in the HR mountain region, strains with a significant African component ($\sim$20%) were particularly associated with the development of more severe gastric lesions, especially intestinal metaplasia and dysplasia (Kodaman et al. 2014) (Fig. 3). This host/pathogen crosstalk has been found to be more predictable for the risk of *H. pylori*-mediated diseases than the effect of *cagA* (Kodaman et al. 2014).

Therefore, human and bacterial phylogenetic parameters should be taken into consideration for an accurate determination of the effect of the host response in the development of gastric diseases associated with *H. pylori* infection. More studies in additional human populations are needed to further evaluate the interactions of the bacterium and the host that predict disease outcome.

6 Concluding Remarks

The host innate immune response is certainly one of the major events involved in *H. pylori* pathogenesis. We focused our review on CXCL8, ROS, and NO, since these effectors have been shown to exhibit function in infectious inflammation and carcinogenesis. However, numerous other products of the innate response, including cytokines and other chemokines, prostaglandins, and their metabolites, may be involved in the etiology of *H. pylori*-related diseases. While several correlations suggest that CXCL8 has an important role in the development of gastric malignant lesions, more studies are warranted to prove that it is a key oncogenic molecule during *H. pylori* infection. In contrast, the reduction of carcinoma in *H. pylori*-infected gerbils treated with inhibitors of SMOX or the upstream enzyme, ODC, provide a strong rationale for envisioning the ODC-SMOX pathway as a potential

target to limit the development of the more advanced gastric lesions. Because NOS2 has been linked to *H. pylori* carcinogenesis and NO reacts with ROS to form RNI, the study of their synergistic effect may also be a promising area of investigation. But importantly, the evidence that the phylogenetic features of the human population and/or *H. pylori* are essential for host/pathogen interaction and the outcome of the infection demonstrates the necessity to bring human ancestry into the analysis of innate responses. Our ongoing studies investigating the human and *H. pylori* genetics, utilizing whole genome sequencing in cases from LR and HR in Latin America, are expected to provide new insights into gastric cancer development.

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Immune Evasion Strategies and Persistence of *Helicobacter pylori*

Raquel Mejías-Luque and Markus Gerhard

Abstract *Helicobacter pylori* infection is commonly acquired during childhood, can persist lifelong if not treated, and can cause different gastric pathologies, including chronic gastritis, peptic ulcer disease, and eventually gastric cancer. *H. pylori* has developed a number of strategies in order to cope with the hostile conditions found in the human stomach as well as successful mechanisms to evade the strong innate and adaptive immune responses elicited upon infection. Thus, by manipulating innate immune receptors and related signaling pathways, inducing tolerogenic dendritic cells and inhibiting effector T cell responses, *H. pylori* ensures low recognition by the host immune system as well as its persistence in the gastric epithelium. Bacterial virulence factors such as cytotoxin-associated gene A, vacuolating cytotoxin A, or gamma-glutamyltranspeptidase have been extensively studied in the context of bacterial immune escape and persistence. Further, the bacterium possesses other factors that contribute to immune evasion. In this chapter, we discuss in detail the main evasion and persistence strategies evolved by the bacterium as well as the specific bacterial virulence factors involved.

Contents

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1 Introduction

Despite of eliciting a strong immune response, *H. pylori* can persist lifelong in the gastric mucosa of infected individuals. *H. pylori* infection leads to gastric inflammation, which in most of the cases is asymptomatic, but can progress to a more severe pathology and even gastric cancer (Wroblewski et al. 2010). Therefore, *H. pylori* is considered as class I carcinogen by the World Health Organization (IARC 1994). To survive in the hostile conditions found in the stomach and escape the host's immune response, *H. pylori* has developed different sophisticated strategies, which depend on the presence of certain bacterial virulence factors. Thus, to establish colonization, urease activity as well as flagella are important, while once established in its niche, other virulence factors encoded by the cytotoxin-associated gene A (CagA), vacuolating cytotoxin A (VacA), or gamma-glutamyltranspeptidase (GGT) become decisive to shape the host's immune response in order to favor bacterial persistence. These virulence factors can directly alter cellular processes and signaling cascades of host cells or induce changes in the cellular milieu, which also culminate in altered immune cell responses.

Different cell types are involved in the complex immune response toward *H. pylori*, which the bacterium is able to manipulate in order to escape and persist. Thus, epithelial cells and innate immune cells, which constitute the first line of defense against *H. pylori*, are profoundly altered by the bacterium, leading to an ineffective clearance of the pathogen. These changes are decisive to shape the subsequent adaptive immune response, mainly mediated by effector T cells, whose function is also compromised, and contribute to bacterial persistence.

In this chapter, we discuss in detail different strategies employed by *H. pylori* to escape from the host's immune response and persist in the stomach, paying special attention to the bacterial virulence factors involved.

2 Innate Immune Evasion Strategies of *H. pylori*

In order to ensure its persistence, *H. pylori* manipulates the host's innate immune response (Fig. 1), which is mainly driven by gastric epithelial cells as well as gastric-resident innate immune cells or innate immune cells recruited upon infection, including dendritic cells (DCs), monocytes/macrophages, and neutrophils.

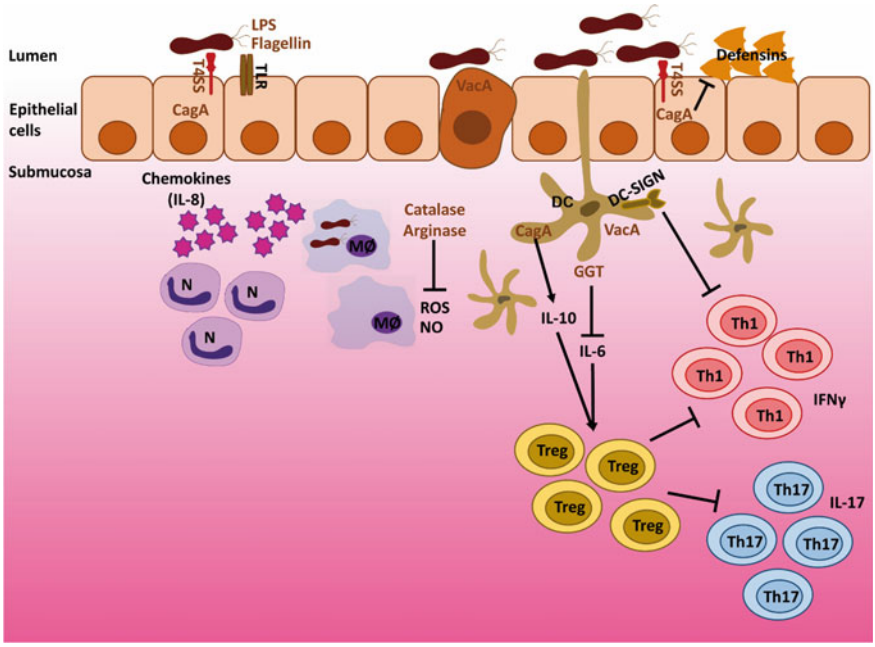

Fig. 1 *H. pylori* evades and manipulates host innate immune responses. Upon *H. pylori* infection, secretion of different chemokines by epithelial cells induces the recruitment of several types of immune cells to the gastric mucosa. However, the bacterium has developed a number of strategies to escape recognition and to dampen immune responses. *H. pylori* LPS and flagellin are weakly recognized by TLRs due to structural modifications, while the secretion of antimicrobial peptides such as defensins by epithelial cells is actively impaired by the virulence factor CagA. The type IV secretion system is important to inhibit phagocytic uptake of the bacterium, which can resist intracellular killing after enfulfement. Thus, the expression of bacterial enzymes as catalase or arginase contributes to the inhibition of phagocytic killing by producing reactive oxygen species (ROS) and nitric oxide (NO). Activation of DC-SIGN signaling in dendritic cells suppresses pro-inflammatory responses, while, by altering the maturation and function of DCs through virulence factors such as CagA, GGT, or VacA, regulatory T cell responses are favored. Abbreviations: N, neutrophil; Mϕ, macrophage; DC, dendritic cell

2.1 *Evasion and Manipulation of Pattern Recognition Receptors*

Epithelial cells and innate immune cells recognize pathogen-associated molecular patterns (PAMPs) by different immune receptors or pattern recognition receptors (PRRs) expressed at specific subcellular compartments. Upon their activation, PRRs induce diverse downstream signaling pathways important for the clearance of pathogens. *H. pylori* has developed a number of strategies to avoid detection by Toll-like receptors (TLRs) and to manipulate and suppress TLR—as well as C-type

lectin receptor (CLR)-mediated signaling. By this, clearance of the bacterium is impaired and persistent colonization of the human stomach is consolidated.

2.1.1 Evasion of TLR Recognition and Manipulation of TLR-Mediated Signaling

TLRs on gastric epithelial cells and immune cells recognize diverse *H. pylori* PAMPs such as lipopolysaccharide (LPS) (TLR2 and TLR4), flagellin (TLR5), and bacterial nucleic acids (TLR8 and TLR9). However, by modulating the expression and structure of surface molecules such as LPS or flagellin, *H. pylori* successfully avoids or dampens recognition by these TLRs (Pachathundikandi et al. 2011, 2015).

LPS is a glycolipid commonly found on the outer membrane of Gram-negative bacteria and composes of three well-defined units: (1) a hydrophobic moiety, lipid A, responsible for the toxic effects; (2) a core oligosaccharide, which contributes to the outer membrane integrity together with lipid A; and (3) the O-antigen, which is a polymer composed of repeating oligosaccharides connected to the core and in direct contact with the extracellular milieu (Whitfield and Trent 2014). By structural modifications in the lipid A and expression and variation of Lewis (Le) antigens terminally exposed on the O-antigen, *H. pylori* has achieved reduced detection by the immune system. *H. pylori* lipid A shows lower biological activity than lipid A of other Gram-negative bacteria (Muotiala et al. 1992; Moran and Aspinall 1998). Uncommon phosphorylation and acylation of lipid A are not only responsible for the reduced endotoxicity of *H. pylori* LPS (Ljungh et al. 1996), but also for its reduced immunogenicity (Moran 2001). Exchange of the 1-phosphate group for a phosphorylethanolamine and removal of the 4′-phosphate group in the *H. pylori* lipid A backbone increase resistance to antimicrobial peptides and reduce recognition by TLRs (Tran et al. 2006; Cullen et al. 2011). These modifications are extremely important for the successful colonization of the gastric mucosa, since *H. pylori* mutants deficient for the phosphatases involved in dephosphorylation of lipid A are not able to colonize mice (Cullen et al. 2011).

The O-antigen of *H. pylori* LPS also contributes to the evasion of bacteria recognition by innate immune cells. The common backbone of this polysaccharide is modified by fucosyltransferases that generate structures mimicking human Lewis antigens and related blood group antigens (Rubin and Trent 2013). Thus, the O-antigen can evade TLR detection since it is not recognized as a foreign molecule but rather as a "self"-antigen. *H. pylori* can adapt to the host by phase variation of its LPS, in which Le antigens of the bacterium evolve to resemble the gastric Le phenotype of the host (Appelmelk and Vandenbroucke-Grauls 2001). Due to the diversity of Lewis antigens expressed in *H. pylori* LPS, the exact role of these molecules in infection and disease is not completely clear. Le^x and Le^y antigens are expressed in almost 80–90% of *H. pylori* strains (Moran 2008). They have been related to bacterial adhesion (Fowler et al. 2006; Heneghan et al. 2000), and more importantly, they contribute to bacterial molecular mimicry to evade immune

responses (Appelmelk and Vandenbroucke-Grauls 2001). It is still under discussion which TLR(s) is/are involved in the recognition of *H. pylori* LPS, since some studies suggest TLR4 as the main sensor (Ishihara et al. 2004; Kawahara et al. 2005), while others support TLR2 as its receptor (Yokota et al. 2007; Smith et al. 2011). Nevertheless, it is clear that the reduced ability of *H. pylori* LPS to bind and activate its receptors contributes to bacterial escape from innate immune responses.

H. pylori flagellin, which is an important bacterial factor for motility and colonization, eludes recognition by TLR5, avoiding pro-inflammatory transcription factor and nuclear factor-kappa B (NF-κB) activation (Pachathundikandi et al. 2016). This is due to a modification in residues 89–96 of the N-terminal D1 domain of *H. pylori* flagellin, important for TLR5 recognition (Andersen-Nissen et al. 2005). In addition, FlaA, the primary flagellar structural component, is not released and is much less pro-inflammatory compared to other bacterial flagellins (Gewirtz et al. 2004).

Apart of having developed mechanisms to escape immune recognition, *H. pylori* has also acquired strategies to actively manipulate TLR-mediated signaling in order to dampen pro-inflammatory responses. For example, activation of TLR2 signaling induces the expression of many anti-inflammatory cytokines via myeloid differentiation primary response gene 88 (MyD88), particularly IL-10 (Rad et al. 2009). In line, infection of *Tlr2*$^{-/-}$ mice indicated an important role for TLR2 signaling in *H. pylori*-induced immune tolerance. *Tlr2*$^{-/-}$ mice were colonized at lower levels than wild-type animals and showed more severe inflammation of the gastric mucosa, reflected by higher levels of interferon (IFN)-γ and lower expression of forkhead box P3 (Foxp3), a transcription factor marking regulatory T cells, IL-10, and IL-17 (Sun et al. 2013).

Another mechanism by which *H. pylori* suppresses gastric inflammation is the activation of TLR9, which was identified as the main intracellular TLR signaling pathway recognizing *H. pylori* DNA in DCs (Rad et al. 2009). Activation of TLR9 signaling is suggested to have anti-inflammatory effects at early phases of *H. pylori* infection. Thus, although *Tlr9*$^{-/-}$ mice showed similar bacterial colonization, they presented a more severe gastric inflammation, which was characterized by increased neutrophil infiltration and upregulation of the expression of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interferon-γ (IFN-γ) (Otani et al. 2012).

2.1.2 Inhibition of CLR-Mediated Signaling

H. pylori can modulate immune responses by interacting with another type of PRR, the C-type lectin receptors (CLRs). CLRs expressed on DCs recognize and bind carbohydrates such as mannose, fucose, or glucan, commonly expressed on bacterial surfaces. Of those, Dendritic cell-Specific Intercellular adhesion *molecule-3*-Grabbing Non-integrin (DC-SIGN) can bind *H. pylori* ligands and is involved in the innate immune response to the bacterium. Specifically, *H. pylori* Le^x and Le^y antigens can interact with C-type lectin DC-SIGN on dendritic cells to block the

development of Th1 cells (Bergman et al. 2004). Further studies confirmed *H. pylori* LPS binding to recombinant human DC-SIGN and showed that addition of fucose or incubation with monoclonal antibodies against Le antigens abolished this binding (Miszczyk et al. 2012). Notably, the fucose residues of the DC-SIGN ligands of *H. pylori* interfere with the signaling complex downstream of DC-SIGN, suppressing pro-inflammatory responses, in contrast to other pathogens expressing mannosylated DC-SIGN that activate pro-inflammatory signaling pathways (Gringhuis et al. 2009). More recently, the expression of macrophage inducible C-type lectin (Mincle) was found to be upregulated in *H. pylori*-infected macrophages. This CLR interacts with Lewis antigens of *H. pylori* and induces an anti-inflammatory immune response, which would also contribute to bacterial immune escape and persistence in the host (Devi et al. 2015).

2.2 *Inhibition of Phagocytosis and Killing by Reactive Oxygen Species and Nitric Oxide*

Bacterial phagocytosis is a central host defense mechanism to eliminate invading bacteria. Upon *H. pylori* infection, different phagocytes such as neutrophils, polymorphonuclear (PMN) lymphocytes, and monocytes are recruited to the gastric mucosa. However, the bacterium successfully inhibits its own uptake by PMNs and monocytes. This antiphagocytic activity depends on different virulence genes, such as *virB7* and *virB11*, and core components of the type IV secretion system (T4SS) (Ramarao et al. 2000a; Ramarao and Meyer 2001) and contributes to bacterial immune escape. Another strategy used by the bacterium to escape phagocytosis involves intrinsic α-glycosylation of cholesterol. Thus, bacteria lacking cholesterol-α-glycosyltransferase HP0421 (Lebrun et al. 2006) were more susceptible to phagocytosis by macrophages and induced a more potent MHC-restricted T cell activation. In addition, HP0421-deficient bacteria were efficiently cleared from the gastric tissue of infected mice (Wunder et al. 2006), indicating that α-glycosylation of cholesterol by *H. pylori* represents a prerequisite for the bacterium to escape phagocytosis, T cell activation, as well as bacterial clearance in vivo.

On the other hand, once engulfed by macrophages, different strategies allow *H. pylori* to survive phagocytosis. *Cag* pathogenicity island (*cag*PAI)-positive and VacA-positive *H. pylori* strains were found to delay actin polymerization and phagosome formation in human and murine macrophages. Once formed, phagosomes underwent clustering and fusion-forming megasomes, wherein *H. pylori* resisted intracellular killing. (Allen et al. 2000). Other strategies to avoid macrophage-mediated bacterial killing include VacA-related arrest of phagosome maturation in association with the retention of tryptophan aspartate-containing protein (Zheng and Jones 2003) and delayed entry and arrest of phosphatidylinositide 3-kinase (PI3 K)-dependent phagosome maturation via synthesis of cholesteryl glucosides (Du et al. 2014).

Survival of *H. pylori* within polymorphonuclear cells (PMNs) is also achieved by disrupting the NADPH oxidase system, which produces reactive oxygen species (ROS) in response to the bacterium. NADPH oxidase assembly is inefficient in the phagosome, and although PMNs containing *H. pylori* produce ROS, they do not accumulate inside the phagosomes but are released to the extracellular space (Allen et al. 2005). *H. pylori* produces catalase and superoxide dismutase, which detoxify ROS and protect the bacterium from the toxic effects of ROS in vitro and in vivo (Spiegelhalder et al. 1993; Odenbreit et al. 1996; Ramarao et al. 2000b; Seyler et al. 2001; Harris et al. 2002, 2003).

Production of nitric oxide by macrophages represents another mechanism to kill bacteria. *H. pylori* urease induces the activation of inducible nitric oxide synthase (iNOS) (Gobert et al. 2002b); however, at the same time, *H. pylori* arginase protects the bacterium against NO-mediated killing by competing with host cells iNOS for the common substrate L-arginine. Thus, macrophages infected with *H. pylori* deficient for arginase efficiently killed the bacterium, whereas *H. pylori* wild type did not show loss of survival under the same experimental conditions (Gobert et al. 2001). The induction of arginase II (Arg2) represents a further mechanism by which *H. pylori* escapes the host innate immune response. Upregulation of Arg2 was detected in *H. pylori*-induced gastritis in humans and mice (Gobert et al. 2002a), and Arg2 activity attenuated *H. pylori*-stimulated NO production by limiting iNOS protein expression in vitro and in vivo (Lewis et al. 2010). Notably, treatment with an arginase inhibitor led to increased iNOS protein expression and NO production in gastric macrophages of *H. pylori*-infected mice (Lewis et al. 2010), confirming that restriction of NO production contributes to *H. pylori* escape from macrophage-mediated killing.

2.3 Inhibition of Antimicrobial Peptides

As part of the innate immune response, epithelial cells generate a number of antimicrobial peptides to protect the gastrointestinal epithelium from invading bacteria. *H. pylori* was reported to induce the expression of human beta-defensin 2 (hBD2) (Hamanaka et al. 2001; Bajaj-Elliott et al. 2002; Wehkamp et al. 2003), hBD3 (Kawauchi et al. 2006) and the amphipathic α-helical cathelicidin LL37 (Hase et al. 2003), which also exhibits a wide spectrum of antimicrobial activity. However, in more recent research, different studies have demonstrated that *H. pylori* is able to manipulate the expression of these antimicrobial substances in order to escape and persist in the gastric mucosa. Thus, the expression of hBD3 is rapidly induced via epidermal growth factor receptor (EGFR)-dependent activation of mitogen-activated protein (MAP) kinase and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling at early stages of *H. pylori* infection. However, during chronic infection, hBD3 is downregulated by *H. pylori* CagA-mediated activation of tyrosine phosphatase Src homology 2 domain-containing phosphatase 2 (SHP-2), which terminates EGFR activation and

downstream signaling, supporting bacterial viability (Bauer et al. 2012). In addition, the *H. pylori* T4SS downregulates the expression of hBD1 through NF-κB signaling (Patel et al. 2013). Interestingly, when analyzing biopsies from infected patients, the same authors observed that patients showing higher bacterial colonization and inflammation showed lower hBD1 expression, whereas they did not detect differences in hBD2 (Patel et al. 2013). Notably, *H. pylori* showed resistance to hDB1, while minimal susceptibility to hBD2 was observed for some strains. On the other hand, hBD3 and LL37 efficiently killed *H. pylori*; however, these two antimicrobial peptides were only marginally detected in the human stomach (Nuding et al. 2013). Therefore, by manipulating defensin expression and developing resistance against these antimicrobial peptides, *H. pylori* can more efficiently colonize the gastric mucosa.

3 Immune Tolerance Driven by *H. pylori* Interaction with Dendritic Cells

DCs are highly specialized antigen-presenting cells, key mediators of the innate and adaptive immune response due to their ability to capture and transfer antigens and regulate T cell responses. DCs can enter the gastric epithelium, where they take up *H. pylori* and its virulence products (Necchi et al. 2009) and as thus shape adaptive immune responses. *H. pylori* has evolved a number of strategies to manipulate DC maturation and cytokine production, skewing them toward a tolerogenic phenotype and therefore instructing a regulatory T cell response that contributes to bacterial persistence (Fig. 2).

In first reports, *H. pylori* undefined secreted factors were reported to inhibit the secretion of IL-12 (Kao et al. 2006), while chronic exposure to the bacterium resulted in increased expression of PD-L1, a member of the B7 family implicated in the inhibition of T cell function, and impaired DC function inhibiting Th1 responses (Mitchell et al. 2007).

Further investigations have implicated the virulence factors CagA, VacA, and GGT in *H. pylori*-induced tolerogenic effects on DCs. In bone marrow-derived dendritic cells (BMDCs), CagA plays an important role in regulating DCs to inhibit $CD4^+$ T cell differentiation toward a Th1 phenotype. Hence, phosphorylation of CagA inside the cells led to the activation of SHP-2, suppressing the activation of serine/threonine protein kinase-1 (TBK-1), the phosphorylation, and nuclear translocation of interferon regulatory factor 3 (IRF-3) and inducing a reduced interferons production by the DCs (Tanaka et al. 2010). In human DCs, once translocated into the cell, CagA induces a DC semi-mature phenotype characterized by low expression of the costimulatory molecule cluster of differentiation (CD86) and the maturation marker CD83 as well as by low expression of the pro-inflammatory cytokine IL-12p70 and increased expression of IL-10, which favors a regulatory T cell response. These changes are orchestrated by the

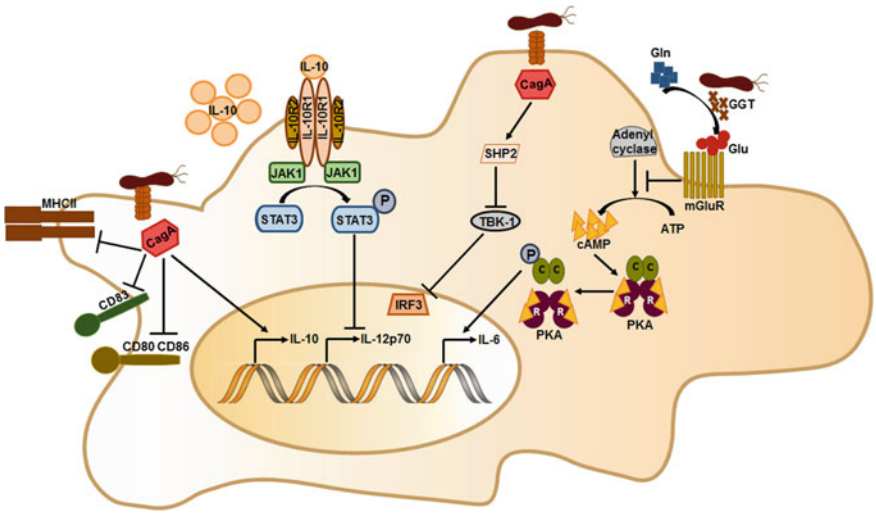

Fig. 2 *H. pylori* induces tolerogenic dendritic cells (DCs). *H. pylori* influences DC maturation and function leading to a tolerogenic phenotype. Translocation of *H. pylori* CagA into DCs dampens the maturation of DCs, which express low levels of MHCII, the maturation marker CD83, as well as costimulatory molecules CD80 and CD86. In addition, it induces high expression levels of the anti-inflammatory cytokine IL-10, which activates STAT3 impairing the expression of pro-inflammatory cytokines as IL-12p70. Moreover, CagA activates SHP-2 leading to the inhibition of IRF3 translocation and IRF-3 interferon-mediated expression. *H. pylori* GGT activity has also a major impact on DC function. Due to *H. pylori* GGT enzymatic activity, glutamine is converted into glutamate that can activate glutamate receptors expressed on the DCs. This leads to the inhibition of cAMP signaling and mediated expression of IL-6. Abbreviations: C, catalytic; Gln, glutamine; Glu, glutamate; JAK, Janus kinase; mGluR, metabotropic glutamate receptor; PKA, protein kinase A; R, regulatory; SHP, src homology phosphatase; STAT, signal transducer and activator of transcription; TBK, TANK-binding kinase

IL-10-dependent activation of signal transducer and activator of transcription factor 3 (STAT3) (Kaebisch et al. 2014).

The *H. pylori* virulence factors VacA and GGT are also important to skew the DC-mediated T cell response toward the bacterium, favoring the development of a predominant regulatory phenotype. In mice, VacA and GGT are required for gastric colonization and DC tolerization in vivo and contribute to neonatally acquired immune tolerance (Oertli et al. 2013). VacA was shown to inhibit DC maturation via restoration of the critical suppressor of DC maturation E2 promoter-binding factor-1 (E2F1) expression (Kim et al. 2011). In human DCs, *H. pylori* GGT also induces a tolerogenic phenotype mainly by repressing the expression of IL-6. This effect is due to the enzymatic activity of GGT, which converts glutamine into glutamate. Glutamate activates metabotropic glutamate receptors expressed on DCs, inhibiting cAMP-mediated regulation of IL-6 expression and thus favoring the expansion of regulatory T cells (Kabisch et al. 2016).

Together, DC tolerization induced by different *H. pylori* virulence determinants has a major impact on the subsequent adaptive immune response to the bacterium, favoring the expansion of regulatory T cells. Indeed, regulatory T cells are massively recruited to the gastric mucosa of *H. pylori*-infected subjects (Lundgren et al. 2003; Cheng et al. 2012) and contribute to bacterial persistence and chronic infection by suppressing effector immune responses (Lundgren et al. 2003; Arnold et al. 2011).

4 Manipulation and Inhibition of Effector T Cell Responses

The adaptive immune response toward *H. pylori* is characterized by the recruitment of $CD4^+$ effector T cells, particularly Th1 and Th17 subsets, which are crucial for the control of the infection and at the same time are implicated in the immunopathological changes resulting from the chronic infection (D'Elios et al. 1997; Bamford et al. 1998; Sayi et al. 2009; Shi et al. 2010; Kabir 2011; Serelli-Lee et al. 2012). As mentioned before, DCs are important regulators and play a key role in defining T cell responses to *H. pylori*. However, in order to persist in the stomach, the bacterium has also developed mechanisms to directly manipulate and inhibit T cells, rendering them hyporesponsive (Fan et al. 1994). In this context, VacA and GGT are the main bacterial virulence determinants impairing effective T cell responses upon infection, although other bacterial factors such as arginase and the *cag*PAI also contribute to dampen T cell proliferation and function (Fig. 3).

VacA can interact with T cells in the lamina propria and enter activated human T cells by binding to β2 integrin (CD18), which associates with CD11a, forming the heterodimeric transmembrane receptor lymphocyte function-associated antigen 1 (LFA-1) (Sewald et al. 2008). VacA uptake is facilitated as *H. pylori* exploits the recycling of LFA-1, and this effect depends on serine/threonine phosphorylation of the β2 integrin cytoplasmic tail by protein kinase C (PKC) (Sewald et al. 2011). Once in the cytoplasm, VacA impairs T cell activation as well as proliferation by different mechanisms. VacA interferes with T cell receptor–IL-2 signaling pathway at the level of calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase. By this, VacA inhibits nuclear translocation of the transcription factor NFAT and prevents transactivation of NFAT-regulated genes specific for T cell immune responses (Gebert et al. 2003). Importantly, VacA suppresses IL-2-mediated cell cycle progression and T cell proliferation through its N-terminal hydrophobic region without affecting IL-2-dependent survival. The N-terminal region of VacA is necessary for the formation of anion-selective membrane channels inhibiting clonal expansion of activated T lymphocytes (Sundrud et al. 2004). Proliferation of T cells is also impaired by the reduction of the mitochondrial membrane potential through *H. pylori* VacA (Boncristiano et al. 2003), while actin rearrangements, as a result of channel-independent activation of intracellular signaling via the MAP kinases

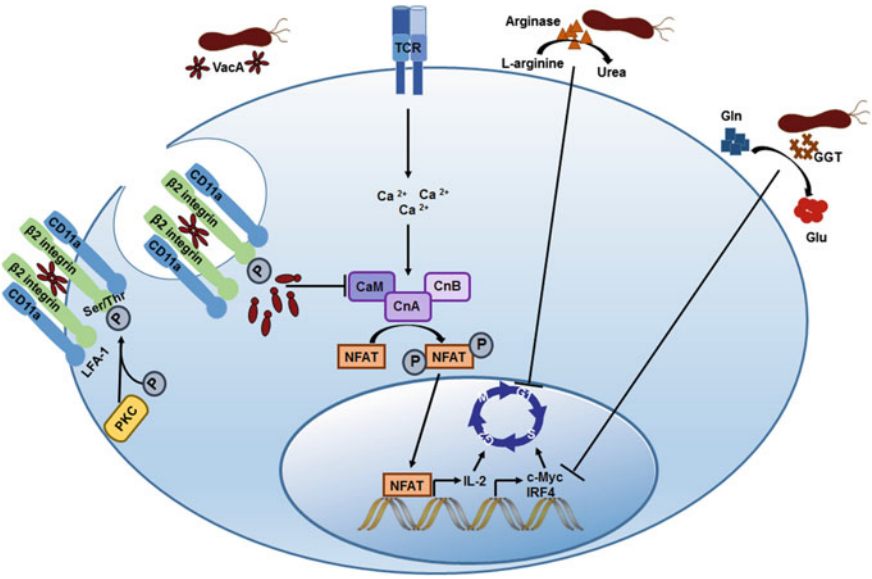

Fig. 3 *H. pylori* inhibits effective effector T cell responses. Effector T cell functionality is altered by *H. pylori* virulence factors. Hexameric VacA binds to the β2 integrin subunit of the LFA-1 receptor, which is internalized after serine/threonine phosphorylation of the β2 integrin cytoplasmic tail by PKC facilitating the uptake of bound VacA. Once in the cytoplasm, VacA prevents nuclear translocation of NFAT by interfering with calcineurin. This leads to impaired IL-2 production and subsequent T cell activation. *H. pylori* GGT also blocks T cell proliferation by depriving cells of glutamine and thereby blocking the expression of transcription factors important for metabolic reprogramming of T cells such as c-Myc and IRF4. Finally, *H. pylori* arginase also contributes to T cell proliferation arrest by depleting L-arginine, which is required for T cell activation and function. Abbreviations: c-Myc, avian myelocytomatosis virus oncogene cellular homolog; CaM, calmodulin; Cn, calcineurin; Gln, glutamine; Glu, glutamate; IRF4, interferon regulatory factor 4; LFA-1, lymphocyte function-associated antigen 1; NFAT, nuclear factor of activated T cell; PKC, protein kinase C; Ser; serine; Thr, threonine

MKK3/6 and p38 as well as the Ras-related C3 botulinum toxin substrate *Rac-specific* nucleotide exchange factor Vav, dampen T cell activation (Ganten et al. 2007).

Another important virulence factor interfering with T cell proliferation and function is GGT. This secreted low molecular weight protein can directly block T cell proliferation by inducing a G1 cell cycle arrest through disruption of rat sarcoma (Ras) signaling pathway (Gerhard et al. 2005; Schmees et al. 2007). More recently, it was shown that *H. pylori* GGT compromises metabolic reprogramming of T lymphocytes by depriving them from glutamine (Wüstner et al. 2015). The expression of IL-2, CD25, and effector cytokines IFN-γ and IL-17 was reduced in the presence of *H. pylori* GGT. Moreover, the expression of the transcription factors c-Myc and IRF4 and signaling cascades as mechanistic target of rapamycin (mTOR) important for T cell metabolic reprogramming were altered by GGT,

indicating that the enzymatic activity of GGT is sufficient to hinder effector T cell responses toward the bacterium and thus contribute to *H. pylori* immune evasion (Wüstner et al. 2015).

Depletion of L-arginine availability by *H. pylori* arginase is another mechanism affecting T cell proliferation. L-arginine is required for T cell activation and function and is depleted by *H. pylori* arginase to produce urea. This induces decreased proliferation of T cells and reduced expression of the chief signal transduction CD3ζ-chain of the T cell receptor, which is necessary for T cell activation (Zabaleta et al. 2004).

*H. pylori cag*PAI-positive strains were found to induce apoptosis of T cells through the induction of the Fas ligand (FasL), limiting host immunity (Wang et al. 2001).

In summary, the inhibition of effector T cells in combination with the development of a regulatory-biased adaptive immune response represents a major mechanism by which *H. pylori* evades the host immune system and persists even though eliciting strong inflammatory responses.

5 Bacterial Plasticity and Immune Evasion

H. pylori is one of the most variable bacterial genus, exhibiting extensive genetic diversity among different strains (Suerbaum 2000). This high level of diversity not only supports adaptation to its host, but at the same time enables *Helicobacter* to avoid several aspects of the immune response. Adhesion molecules of *H. pylori* are not only found in different allelic forms (Pride et al. 2001; Oleastro et al. 2010; Nell et al. 2014), but can also be regulated through on/off mechanisms and were shown to be turned off and replaced by other adhesions after successful colonization (Solnick et al. 2004). These adhesins are also regulated through recombination between various genomic loci. Through this regulation, *H. pylori* avoids immune recognition of important adhesins.

Further, such genomic variability is also observed within a single host (Kraft et al. 2006) and probably reflects adaptation of *H. pylori* to the individual host, including adaptation to specific immune responses. This is reflected by the high variability observed in immune responses among infected individuals. At the humoral level, hardly any antigen is recognized in all infected subjects. Rather, most antibody responses toward a single antigen are detected in a minority of patients. Few antigens, such as CagA, chaperonin GroEL, or flagellar hook-associated protein FliD, are recognized in over 80% of infected individuals (Cover et al. 1995; Nomura et al. 2002; Khalifeh Gholi et al. 2013; Shiota et al. 2014; Pan et al. 2014; Michel et al. 2014). Although far less data are available regarding antigen-specific cellular responses, the same seems to be true here. This could be interpreted as an early adaptation mechanism of *H. pylori*, where protective immune responses lead to rapid epitope changes or selection of subspecies not exhibiting the specific epitope. Over the time, substrains are selected which are weakly recognized.

6 Concluding Remarks

Despite of eliciting strong immune responses, *H. pylori* has, during coevolution with humans, developed a number of strategies to evade the host immune system and persist in the hostile niche that represents the human stomach. Immune evasion and persistence depend on several bacterial virulence factors, which, on the one hand, induce cellular damage and recruitment of immune cells at the site of infection, but, on the other hand, subvert the host's responses in order to create a tolerogenic environment allowing the bacterium to persist. Understanding how *H. pylori* manipulates host immune responses is of foremost importance in order to develop novel and successful therapeutic interventions for the treatment of human populations at high risk of gastric cancer development associated with the infection. This knowledge is also of special relevance in the context of vaccine development against *H. pylori*, since finding adequate antigens to generate vaccine-mediated protection has been extremely challenging so far. Thus, the identification of conserved bacterial proteins well recognized by the host's immune system and important to establish strong immune responses able to clear the bacterium seems imperative for the development of successful vaccine formulations. On the other side, further knowledge on the intricate relations between host and pathogen will be useful to develop strategies to mimic and induce some of the bacterial beneficial effects found in asymptomatic carriers (e.g., asthma protection) while avoiding the deleterious consequences of the infection.

In summary, *H. pylori* is one of the most successful human pathogens able to manipulate the cellular milieu and subvert immune responses in order to ensure its persistence. Although several of the strategies used by *H. pylori* have been identified in the recent years, there are still many lessons to learn from this exceptional bacterium.

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Recent Advances in *Helicobacter pylori* Replication: Possible Implications in Adaptation to a Pathogenic Lifestyle and Perspectives for Drug Design

Anna Zawilak-Pawlik and Jolanta Zakrzewska-Czerwińska

The dream of a bacterium is to become two bacteria.
François Jacob, 1965.

Abstract DNA replication is an important step in the life cycle of every cell that ensures the continuous flow of genetic information from one generation to the next. In all organisms, chromosome replication must be coordinated with overall cell growth. *Helicobacter pylori* growth strongly depends on its interaction with the host, particularly with the gastric epithelium. Moreover, *H. pylori* actively searches for an optimal microniche within a stomach, and it has been shown that not every microniche equally supports growth of this bacterium. We postulate that besides nutrients, *H. pylori* senses different, unknown signals, which presumably also affect chromosome replication to maintain *H. pylori* propagation at optimal ratio allowing *H. pylori* to establish a chronic, lifelong infection. Thus, *H. pylori* chromosome replication and particularly the regulation of this process might be considered important for bacterial pathogenesis. Here, we summarize our current knowledge of chromosome and plasmid replication in *H. pylori* and discuss the mechanisms responsible for regulating this key cellular process. The results of extensive studies conducted thus far allow us to propose common and unique traits in *H. pylori* chromosome replication. Interestingly, the repertoire of proteins involved in replication in *H. pylori* is significantly different to that in *E. coli*, strongly suggesting that novel factors are engaged in *H. pylori* chromosome replication and could represent attractive drug targets.

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Contents

1 Introduction

Helicobacter pylori (and other bacteria) must precisely coordinate chromosome replication with cell growth and division to produce viable offspring. Since *H. pylori* is able to naturally propagate only in the human stomach, the growth of this bacterium strongly depends on the host environment (Amieva and Peek 2016). Indeed, it has been shown that *H. pylori* actively moves towards the human gastric epithelium and subsequently usurps it as a replicative niche (Huang et al. 2015; Schreiber et al. 2004; Tan et al. 2009). In fact, much progress has been recently made in understanding of molecular processes related to colonization of a particular niche by *H. pylori* and the pathogenesis triggered by this bacterium (Amieva and Peek 2016; Backert et al. 2016). In contrast, there are limited data concerning benefits that are achieved by *H. pylori* from choosing particular sub-niche over the other.

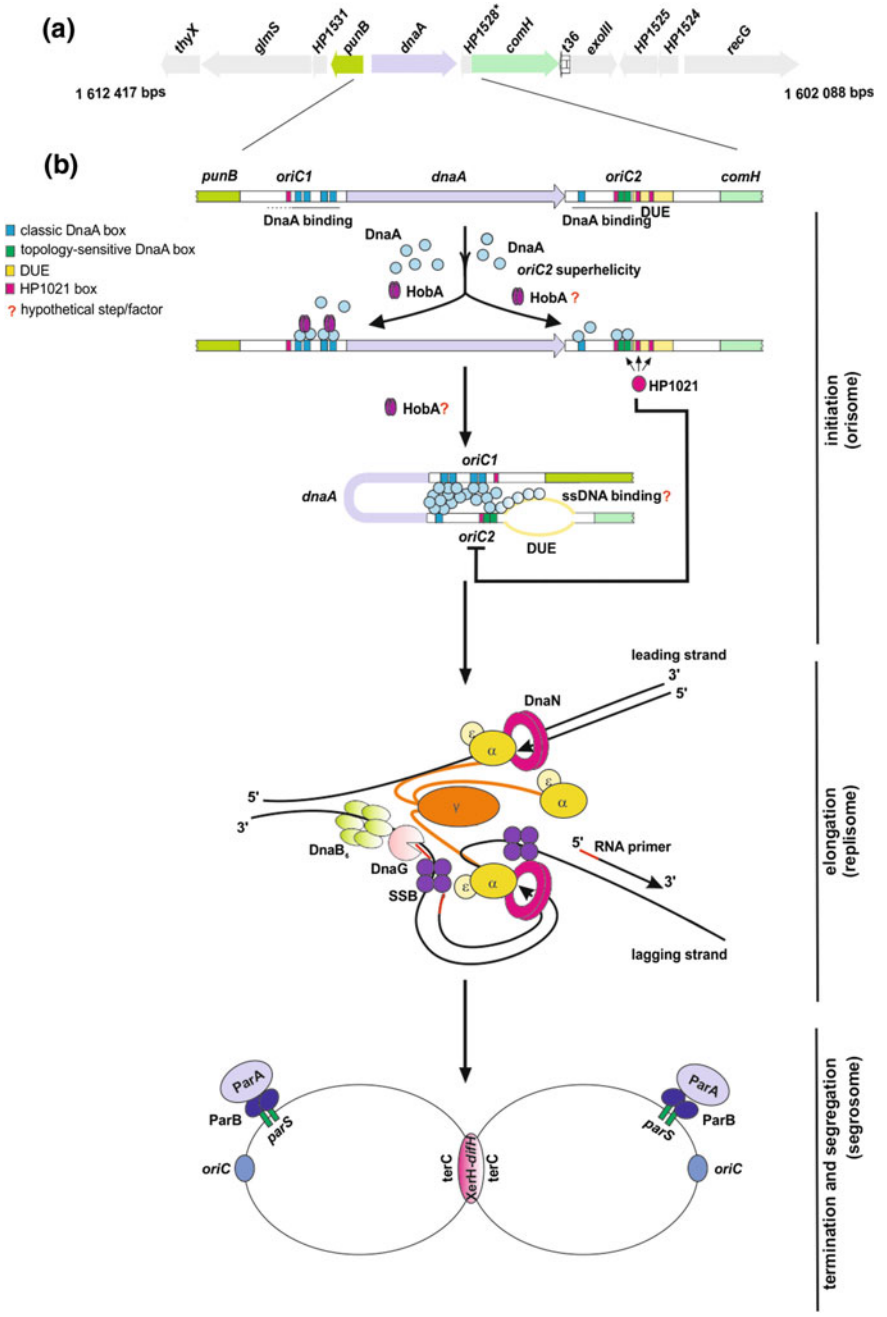
H. pylori uses motility and chemotaxis to move towards to and to reside within the epithelial mucus layer, where it finds optimal conditions for growth, e.g. neutral pH (*H. pylori* is not an acidophile) and nutrients (Lertsethtakarn et al. 2011; Tan et al. 2011). There are two major chemotactic signals sensed by *H. pylori*: pH and urea. In general, low pH acts as a repellent—*H. pylori* moves away from highly acidic gastric lumen towards neutral epithelium; the pH gradient between the lumen and the mucus as well as within the mucus is required for successful colonization (Croxen et al. 2006; Schreiber et al. 2004). Urea acts as an attractant—*H. pylori* senses urea, which is one of the main waste products of human metabolism, and swims towards epithelium (Huang et al. 2015). It should be pointed out that possibly there are many other metabolites emanating from the human gastric epithelium, which are sensed by *H. pylori* as markers of the environmental microniche optimal for growth (Keilberg and Ottemann 2016). Indeed, *H. pylori* actively and specifically chooses gastric micronisches for colonization and/or persistence such as particular stomach region (antrum or corpus), injured tissue or gastric glands; the

latter ones in each stomach region are composed of different cells (Aihara et al. 2014; Howitt et al. 2011; Rolig et al. 2012; Sigal et al. 2015). Most importantly, it has been shown that *H. pylori* does not achieve similar fitness in the inhabited micronisches. For example, *H. pylori* doubles approximately every 6.5 h in the gastric corpus while every 3 h in the antrum (Rolig et al. 2012); *H. pylori* doubling time under laboratory conditions varies from 50 min up to several hours (in average 2.5–6 h in rich media) but depends on the strain and medium (Andersen et al. 1997; Jiang and Doyle 2000). In vitro growth studies and histochemical analyses of the gastric epithelium indicate that even within a single gastric gland, there are subregions more suitable for *H. pylori* growth than others (Hidaka et al. 2001; Kawakubo et al. 2004; Sigal et al. 2015). Taking into account minute differences in the inhabited environment encountered within a single gland, it becomes more obvious that, besides simple availability of nutrients, *H. pylori* possibly receives or exchanges signals that indicate for the preferred niche at which bacteria multiply and establish complex, usually lifelong interplay with the host (Falk et al. 2000). It has to be noted that, unlike many other pathogens, *H. pylori* does not take the control over its host by massive growth and high bacterial load. In contrary, this bacterium somehow limits itself (or is limited by the host) in order not to do too much harm to its host. However, the signals received by *H. pylori* that regulate the growth of this bacterium are unknown. Nonetheless they have to, presumably indirectly, control the frequency and timing of chromosome replication, because in bacteria cell division usually overlaps with ongoing DNA synthesis, i.e. the decision about multiplication is made at the DNA replication step. Since chromosome replication ensures the continuous flow of genetic information from one generation to the next, it might be considered important for bacterial pathogenesis.

Here, we summarize our current knowledge of chromosome replication in *H. pylori* and discuss the mechanisms responsible for the regulation of this key cellular process. Despite the fact that there remains much information missing for a thorough understanding of this process, the mechanisms, factors and interactions identified thus far allow us to propose common and unique traits in *H. pylori* chromosome replication and discuss them below in the context of pathogenesis and putative targets for anti-*H. pylori* therapy.

2 Chromosome Replication in Bacteria

Chromosome replication occurs once per cell cycle. In bacteria, in contrast to eukaryotes, DNA replication overlaps with segregation of nascent DNA, i.e. segregation starts before replication is completed. The accurate copying and transmission of DNA ensures the inheritance of all the parental genetic information by the daughter cells. Thus, chromosome replication is strictly controlled and coordinated with cell cycle progression. DNA replication is divided into three steps: initiation, elongation and termination, with initiation being the main control step in synthesis of bacterial chromosomes.


(a)
thyX
glmS
HP1531
punB
dnaA
HP1528*
comH
136
exoIII
HP1525
HP1524
recG
1 612 417 bps
1 602 088 bps
(b)
punB
oriC1
dnaA
oriC2
comH
DnaA binding
DnaA binding
DUE
classic DnaA box
topology-sensitive DnaA box
DUE
HP1021 box
? hypothetical step/factor
DnaA
DnaA
oriC2 superhelicity
HobA
HobA ?
HP1021
HobA?
oriC1
dnaA
ssDNA binding?
oriC2
DUE
initiation (orisome)
leading strand
3'
5'
DnaN
5'
3'
DnaB6
DnaG
SSB
5'
RNA primer
3'
lagging strand
elongation (replisome)
ParA
ParB
parS
oriC
terC
XerH-dif
terC
ParA
ParB
parS
oriC
termination and segregation (segrosome)

◂**Fig. 1** Model of chromosome replication in *H. pylori*. **a** Conservation of gene organization surrounding the *H. pylori oriC* region (gene numbers according to *H. pylori* 26695 (Tomb et al. 1997)). *The HP1528 ORF is not annotated in every strain; however, the sequence of this region is highly conserved between *H. pylori* species. **b** The basic outline of the replication process. The schemes for initiation and elongation are based on (Donczew et al. 2014a) and (Beattie and Reyes-Lamothe 2015), respectively. Please note that the schemes presenting elongation, termination and segregation steps are speculative

In principle, replication of the bacterial chromosome is initiated by the binding of the initiator DnaA protein to a unique site—an origin of chromosome replication (called *oriC)*. At *oriC*, DnaA–ATP molecules (see Sect. 4) interact with numerous adjacent binding sites (DnaA boxes), which leads to DnaA oligomerization and formation of the active nucleoprotein complex (orisome) that is able to open the DNA double helix at the specific DNA sequence called "DNA unwinding element" (DUE). Subsequently, the DnaB helicase is recruited to the unwound region, followed by other proteins necessary to form a replication machinery (replisome), that is able to synthesize the entire chromosome (Fig. 1 and Table 1). The bacterial chromosome is replicated bidirectionally. Thus, the two replisomes continue replication at both forks until they reach the termination site. Soon after replication has been initiated, the newly replicated regions are bound by the segregation protein (s) that form specialized complexes (segrosomes) capable to drive the correct segregation of chromosomes (see Sect. 3.4). Once chromosome replication and segregation are completed the cell is ready to divide.

Orisome assembly is assisted and controlled by other factors, which help to organize the initiation complex in such a way that opening of the double helix occurs at a precisely determined time point in the cell cycle (Kaguni 2011; Katayama et al. 2010; Leonard and Grimwade 2011). These regulatory factors respond to either intracellular or environmental stimuli. The latter factors raise special interest in pathogenic bacteria because they might be assumed to be virulence-related, as is the case for the *Mycobacterium tuberculosis* MtrB–MtrA two-component system. MtrA–MtrB system functions during macrophage infection, and, via control of *oriC* and *dnaA* promoter activities, it is directly involved in the regulation of the *M. tuberculosis* cell cycle (i.e. replication and cell division) (Fol et al. 2006; Rajagopalan et al. 2010) (see Sect. 4).

Although the replication of bacterial chromosomes is universal and many of the replication factors share a significant level of homology to each other, there is relatively high diversity in the composition and/or interactions between components of the orisomes, replisomes and segrosomes in bacterial species, especially between distantly related bacteria (Heinrich et al. 2015). Therefore, components of the bacterial replication machinery are more often considered to be useful as targets for drug discovery and rational drug design (Dallmann et al. 2010; Georgescu et al. 2008; Johansson and Dixon 2013; Robinson et al. 2012; Sanyal and Doig 2012; Wijffels et al. 2011; Yamaichi et al. 2009). Interestingly, the repertoire of proteins

Table 1 List of proteins involved in *H. pylori* chromosome replication

Protein, gene number	Function	Quaternary structure[a]/ Binding partners[b]	Features
Initiation of chromosome replication (orisome)			
DnaA, HP1529	Initiates replication	A monomer and oligomer/DnaA boxes, HobA	Essential protein; DnaA box consensus sequence: TCATTCACN
HobA, HP1230	Helps DnaA to bind *oriC*	A tetramer/DnaA	Essential protein; functional homologue of *E. coli* DiaA
HP1021	Orphan response regulator, inhibits initiation	ND/HP1021 boxes	Binds *oriC* and inhibits DUE unwinding
Elongation (replisome)			
DnaB, HP1362	Helicase, unwinds dsDNA	A hexamer and a dodecamer/DnaG, SSB, HP0897	Dodecameric structure is unique in bacteria; possibly requires no loader
DnaG, HP0012	Primase, synthesizes RNA primers	A monomer in *E. coli*/DnaB	DnaG stimulates DnaB helicase activity and dissociates the helicase dodecamer into hexamers
DnaN, HP0500	β-sliding clamp of DNA Pol III, ensures DNA Pol III stability	A dimer/ligase	Essential gene; proteins that interact with DnaN are characterized by a conservative clamp-binding motif QL(S/D)LF and QxxL(x)F, promising target of antibacterial drugs
DnaX, HP0717	γ/τ-subunit of DNA Pol III	Bacteria form a clamp loader γ complex: $\tau_3\delta\delta'$/conserved interaction between τ and the catalytic α subunit	*holA* and *holB* are essential genes*; H. pylori* may lack the ψ and χ subunits
HolA, HP1247	δ-subunit of DNA Pol III		
HolB, HP1231	δ′-subunit of DNA Pol III		
DnaE, HP1460	α-subunit of DNA Pol III, catalytic subunit	Core polymerase, in *E. coli* a heterotrimer αεΘ	*H. pylori* may lack a homologue of *E. coli* Θ subunit
DnaQ, HP1387	ε-subunit of DNA Pol III, proofreading with $3' \rightarrow 5'$ exonuclease activity		
Lig, HP0615	DNA ligase, joins Okazaki fragments	A monomer in *E. coli*/DnaN	
PolA, HP1470	DNA Pol I, fills in the nucleotides between the Okazaki fragments	A monomer in *E. coli*/ND	Exhibits less proofreading activity than *E. coli* counterpart

(continued)

Table 1 (continued)

Protein, gene number	Function	Quaternary structure[a]/ Binding partners[b]	Features
SSB, HP1245	Binds and stabilizes ssDNA	A tetramer in *E. coli*/DnaB	An essential gene; stimulates DnaB unwinding activity
Termination of chromosome replication			
XerH, HP0675	Recombinase, decatenates chromosomes	ND/*difH* sequences	*difH* marks the *terC* region
FtsK, HP1090	Translocase, translocates DNA termini	A hexamer in *E. coli*/ND	
Chromosome segregation and topology			
Soj (ParA), HP1139	Segregation proteins, segregate chromosomes to the daughter cells	A monomer in the absence of ATP or ADP/ParB	*parA* is an essential gene
Spo0J (ParB), HP1138		A dimer or a monomer/*parS* sequences, ParA	
TopA, HP0116, HP0440	DNA topoisomerase I, relaxes DNA	A monomer in *E. coli*/ND	HP0440 is an essential gene
GyrA, HP0701 GyrB, HP0501	Subunits of gyrase; introduce negative supercoiling in the DNA	In *E. coli*, a tetramer consisting of two A and two B subunits	Two amino acid residues (87 and 91) are responsible for quinolone resistance (Matsuzaki et al. 2010)
HU, HP0835	Histone-like protein, compacts DNA	A dimer/DNA	An essential gene; protects DNA from stress damage; in *E. coli,* it is involved in initiation of chromosome replication
Dps, HP0243	Protects DNA from oxidizing radicals generated by the Fenton reaction	A dodecamer/DNA, Fe(II)	In *E. coli*, Dps (HP-NAP, neutrophil-activated protein) interacts with DnaA and inhibits replication

The list includes only those proteins that were experimentally confirmed to participate in replication or were identified on the basis of high sequence homology

[a]Unless otherwise stated, the quaternary structure refers to a state of the *H. pylori* protein

[b]Only specific interactions are presented; ND, not determined. The essentiality of proteins was shown in functional studies of particular proteins, while genes are assumed to be essential based on global transposon mutagenesis (Salama et al. 2004)

involved in replication of *H. pylori* is different to that of *E. coli*, opening the possibility for the identification of factors specifically crucial for the control of *H. pylori* growth, including those responding to host–pathogen interactions and/or attractive drug targets.

3 Chromosome Replication in *Helicobacter pylori*

The genome size of *H. pylori* ranges from 1.5 to 1.7 Mbp, and the GC content is 38–39%. The *H. pylori* chromosome is a covalently closed molecule of double-stranded DNA (ds DNA), and approximate half of the analyzed strains carry one or more plasmid(s) (Ali et al. 2015). Since 1997, when the first genome of *H. pylori* 26695 was sequenced (Tomb et al. 1997), the complete genome sequences of 84 different *H. pylori* strains have been determined until today and deposited in NCBI Genome database (https://www.ncbi.nlm.nih.gov/genome); nearly 500 additional *H. pylori* genomes are available as scaffolds or contigs. Comparative genomic analyses of strains from different sources revealed a striking degree of genetic diversity and plasticity in the *H. pylori* genome, including nucleotide polymorphisms, gene mosaicism and distinct gene content. However, despite such overall genome plasticity, the repertoire of genes encoding for chromosome replication and segregation machineries is highly conserved (Table 1) and belongs to the core genome.

3.1 Initiation

The initiation of *H. pylori* chromosome replication follows a general scheme, in which chromosome replication is initiated by the binding of DnaA to *oriC*. The *H. pylori* origin of chromosome replication is located within *punB-dnaA-comH* locus as shown in Fig. 1 (Donczew et al. 2012; Zawilak et al. 2001; see also Sect. 5). It is bipartite, i.e. it is composed of the two subregions, *oriC*1 and *oriC*2, which are located upstream and downstream of *dnaA*, respectively; the DNA unwinding element is located at *oriC*2 (Fig. 1). The *H. pylori oriC* region is the first bipartite origin identified in Gram-negative bacteria and one of very few examples of the divided origins identified in all bacteria so far (Wolański et al. 2014). In vitro, the two DnaA–*oriC*1 and DnaA–*oriC*2 nucleoprotein subcomplexes cross-interact and loop out the region between *oriC*1 and *oriC*2. The role of the loop formation phenomenon in the initiation of chromosome replication is not known but might be connected to the regulation of orisome activity and consequently in replication control (Cournac and Plumbridge 2013; Krause et al. 1997). There are 7 DnaA boxes at *oriC* (Fig. 1), which are classified into two categories: classic boxes (c-boxes) and topology-sensitive DnaA boxes (ts-boxes) (Donczew et al. 2014a). Classic boxes are recognized regardless of DNA topology, while ts-boxes are

recognized only in superhelical form. Consequently, *oriC*1, which contains exclusively c-boxes, is bound by the DnaA protein regardless of DNA topology, while *oriC*2, which contains both the c-box and ts-boxes, is bound and unwound by DnaA only when *oriC*2 assumes a superhelical topology. In fact, *H. pylori* DnaA is the only eubacterial DnaA for which binding to DNA is dependent not only on DNA sequence, but also on topology—a phenomenon reported previously only for Orc1/Cdc6-*ori* interactions in archaea and metazoa (Comoglio et al. 2015; Dueber et al. 2011). In addition to topology-sensitive DNA recognition, the interactions between *H. pylori* DnaA and nucleotide residues of DnaA boxes significantly differ from those of DnaAs from other phyla including *E. coli* and *M. tuberculosis* (Jaworski et al. 2016), which further suggest the distinct molecular mechanism of DnaA–DNA interaction in *H. pylori*. The structural motifs or particular amino acid residues of *H. pylori* DnaA responsible for these unique features remain unknown, but if these unique interactions are driven by a highly distinct protein motif, *H. pylori* DnaA might become an attractive drug target. The topology-sensitive DnaA–DNA interactions are also interesting because of the possible distinct mode of regulation of the initiation of chromosome replication by DNA topology (see Sect. 4).

3.2 Elongation

Once DNA is unwound, replisome assembly starts (the known *H. pylori* replisome components are listed in Table 1, see also Fig. 1). The first three steps, namely DnaB helicase, DnaG primase and DnaN beta-clamp loading, are often considered to be part of the initiation step because in *E. coli* they lead to DnaA inactivation via the DnaN–Hda complex (see Sect. 4). Bacterial DnaBs are enzymes, which act at the replication forks and unwind dsDNA in an ATP-dependent manner. In *E. coli*, the DnaB hexamer, in a complex with its partner protein DnaC, is loaded onto ssDNA. Via direct interactions between DnaA and DnaB, the helicase is positioned specifically in the replication eye. *H. pylori* helicase is characterized by the following two distinct features: the loading factor is still unknown, and the helicase forms a double hexamer (Table 2, Stelter et al. 2012), which is atypical for bacteria but similar to helicases found in some eukaryotes, archaea and viruses. It has to be noted that *H. pylori* DnaB is the only bacterial dodecameric helicase identified so far, which, together with topology-sensitive initiator–*ori* interactions, makes the initiation of *H. pylori* chromosome replication somewhat similar to initiation in eukaryotic species. It has been shown that *H. pylori* DnaB can replace *E. coli* DnaB and bypass DnaC (Soni et al. 2003, 2005), which suggests that in *H. pylori* DnaB is self-loaded into the replication eye. There are numerous species in which DnaB loading does not require a specified loader (Soultanas 2012), but in these species, other factors, such as the DnaA protein located at the replication eye, often recruit DnaB. However, in *H. pylori*, an interaction between DnaA and DnaB has not been shown thus far. Alternatively, self-loading helicases are bifunctional proteins

Table 2 A list of *H. pylori* replication proteins or protein complexes for which the tertiary structures were resolved

Protein or complex	PDB ID	Reference
HobA	2UVP	Natrajan et al. (2007)
N-terminus DnaA–HobA	2WP0	Natrajan et al. (2009)
DnaB	4ZC0	Bazin et al. (2015)
DnaB	Electron microscopy density map	Stelter et al. (2012)
C-terminus of DnaB	4A1F	Stelter et al. (2012)
N-terminus of DnaB	GXV	Kashav et al. (2009)
C-terminus of DnaG	4EHS	Abdul Rehman et al. (2013)
DnaN	4RKI, 4S3I	Pandey et al. (2016)
DnaN–DNA ligase peptide	5FRQ	Pandey et al. (2016)
SSB	2VW9	Chan et al. (2009)

composed of a loader (e.g. a primase or DNA-binding domain) and a helicase. However, the ability of DnaB to bind dsDNA, which is required for self-loading of DnaB onto DNA, is still discussed (Bazin et al. 2015; Nitharwal et al. 2012). It has been recently shown that the DnaB enzymatic activity is stimulated by HP0897 (Verma et al. 2016). *HP0897* is annotated as an unknown ORF in *H. pylori* genomic database. The HP0897 protein, along with *H. pylori* DnaB, colocalizes with the replisome at the initiation step but does not move with the replisome during DNA elongation, which suggests a possible role of HP0897 in loading of *H. pylori* DnaB at *oriC* (Verma et al. 2016). On the other hand, it has been postulated that the DnaB double-hexamer architecture supports an alternative strategy for loading bacterial helicases onto forks in the absence of helicase loaders (Stelter et al. 2012). However, the loading of DnaB into an open complex, either as a hexamer or a dodecamer, has not been shown in vitro thus far. Thus, the mechanism of DnaB recruitment, which allows *H. pylori* DnaB to find its way selectively into a replication bubble and encircle ssDNA, is still not known.

It has been shown that the *H. pylori* DnaB dodecamer dissociates into two hexamers upon interaction with the primase DnaG (Bazin et al. 2015), which in addition stimulates DnaB helicase activity (Bazin et al. 2015; Kashav et al. 2009). Similar DnaB–DnaG interactions and the reciprocal stimulation have also been shown for *Bacillus stearothermophilus* (Thirlway and Soultanas 2006) and *E. coli* (Makowska-Grzyska and Kaguni 2010). In the latter, DnaG directs the release of DnaC from the helicase and stimulates helicase translocation during the transition from the initiation phase to the elongation phase (Makowska-Grzyska and Kaguni 2010). This may also be the case in *H. pylori*. It is postulated that, in vivo DnaB–DnaG interactions during the elongation phase may ensure that primer synthesis occurs only in the replication forks or may regulate primase activity on the lagging strand (Soultanas and Bolt 2014).

Bacterial helicases are also stimulated by the interaction with single-strand binding (SSB) proteins. SSB consists of an N-terminal ssDNA-binding/oligomerization domain and a flexible C-terminal protein–protein interaction

domain. During DNA replication, SSB plays an important role because it binds and stabilizes ssDNA generated initially within *oriC* upon DUE unwinding and later during replisome movement as a result of helicase activity. Such stabilization is especially important on the lagging strand, which is longer exposed to DNA-damaging factors than the leading strand. Moreover, SSB was shown to directly modulate activity of proteins at replication forks, including DnaB (Biswas et al. 2002; Costes et al. 2010). Interestingly, SSB confers species specificity, both in protein–DNA interactions (Huang and Huang 2014) and in protein–protein interactions, including SSB–DnaB interplay. In *E. coli*, noncognate SSB proteins are able to stimulate DnaB helicase activity, while the stimulation of ATPase activity requires a cognate *E. coli* SSB (Biswas et al. 2002). Moreover, *E. coli* SSB, via interaction with the χ-subunit of DNA polymerase III, stimulates DNA synthesis on the leading strand in a species-specific manner (Georgescu et al. 2014). In *H. pylori*, DnaB and SSB interact, and SSB modulates helicase and ATPase activity of DnaB in vitro (Sharma et al. 2009). *H. pylori* SSB can replace *E. coli* SSB in vivo; however, it has not been studied whether *H. pylori* SSB confers any species specificity in protein–DNA and protein–protein interactions in *H. pylori*. Interestingly, in addition to DnaB, the putative *H. pylori* SSB interactome identified by a yeast two-hybrid screen consists of 7 additional proteins and includes the following proteins involved in DNA or RNA metabolism: ribonuclease H (RnhA), the γ/τ subunit of DNA polymerase (DnaX) and DNA recombinase (RecG) (Rain et al. 2001). Taking into account that *H. pylori* SSB is an essential protein (Salama et al. 2004; Sharma et al. 2009) that probably plays a role far beyond DNA replication and presents some degree of species specificity, targeting its activity might be an interesting antimicrobial strategy.

The replisome assembly is completed by the recruitment of the DNA polymerase III holoenzyme (DNA Pol III) that consists of the β-clamp, the clamp loading γ-complex and the enzymatic core (Table 1 and Fig. 1) (Beattie and Reyes-Lamothe 2015). The β-clamp (DnaN) is a ring-shaped protein complex positioned on DNA by the clamp loader γ-complex in an ATP-dependent manner. DnaN slides along the DNA and serves as an anchor for many replication and repair enzymes, modulating their activities and increasing processivity (Beattie and Reyes-Lamothe 2015; Georgescu et al. 2014). DnaN interacts with its partners via a conserved hydrophobic pocket in the C-terminal region of the clamp (Wolff et al. 2014). It should be noted that the ability of the β-clamp to interact with many proteins at the same surface makes it an important drug target, a finding that was clearly confirmed in *M. tuberculosis* (Kling et al. 2015). All of the known clamp-binding proteins contain a conserved peptide sequence motif through which they interact with the clamp (Dalrymple et al. 2001). In *E. coli*, the conserved motifs QL(S/D)LF and QxxL(x)F were experimentally determined (Dalrymple et al. 2001; Lopez de Saro 2003). It has recently been shown that *H. pylori* DnaN interacts with DNA ligase that contains the QEFIRSLF clamp-binding motif (Table 2); in addition, the putative DnaN binding motif QGGNSLF was also located in the catalytic α subunit of DNA Pol III (Pandey et al. 2016). These interactions are important for replication: in *E. coli,* the α subunit

of DNA Pol III replaces the clamp loader γ-complex during replisome formation or at the elongation step, while ligase joins nicks after polymerase I removes the RNA template and fills the gap between Okazaki fragments on the lagging strand. DnaN–ligase interactions were previously reported for *E. coli* (Lopez de Saro 2009, 2003); however, these might not be conserved across species, as there is no interaction between DnaN and ligase in *M. tuberculosis* (Kukshal et al. 2012).

In addition to these sparse data on *H. pylori* DnaN, there is nothing known about the assembly, loading and activity of the *H. pylori* DNA Pol III holoenzyme, except that in comparison to *E. coli* it lacks three subunits: θ, χ and ψ. Although these subunits are not conserved in some other species (for example all three subunits are missing from the Gram-positive bacterium *B. subtilis*), χ and ψ are generally conserved in Proteobacteria, while θ is conserved in Enterobacteria (Beattie and Reyes-Lamothe 2015; Dietrich et al. 2014). The *E. coli* ψ and χ subunits interact with both τ and SSB and stabilize the clamp loading γ-complex and the replisome. In addition, the χψ subassembly may play a role in promoting the access of DNA Pol III to newly synthesized primers. The θ subunit increases the fidelity of replication (Taft-Benz and Schaaper 2004). The lack of these subunits might either impair the *H. pylori* replisome or may be compensated by other interactions within the replisome. Alternatively these subunits may be substituted by yet undiscovered functional homologues that do not exhibit any significant sequence homology to the known ψ, χ and θ subunits. It should be noted that such functional homology has been previously identified in *Pseudomonas aeruginosa* ψ subunit (Jarvis et al. 2005), and also in *H. pylori* HobA (see Sect. 4). If there are indeed substantial differences between the architecture of *H. pylori* DNA Pol III and that of *E. coli* and other Proteobacteria, one can consider designing an *H. pylori*-specific inhibitor of the major replicative polymerase. However, reconstitution experiments with purified DNA Pol III subunits are needed to characterize the *H. pylori* replication machinery.

In addition to DNA Pol III, *H. pylori* encodes a DNA polymerase I (DNA Pol I), but it lacks genes encoding the accessory DNA polymerases: DNA Pol II, DNA Pol IV and DNA Pol V; the three latter polymerases belong to a larger family of translesion polymerases whose members specialize in repairing DNA damage (Andersson et al. 2010; Fijalkowska et al. 2012). *E. coli* Pol I, one of two *bona fide* DNA polymerases, complements Pol III in replication, i.e. Pol II fills in the necessary nucleotides between the Okazaki fragments, proofreading for mistakes as it goes. *E. coli* Pol II is proposed to act as a proofreader for Pol III-produced misinsertion errors and to protect mismatched 3′ termini against the mutagenic action of polymerase IV. DNA Pol IV is involved in repair of DNA lesions caused by DNA damaging agents, while *E. coli* DNA Pol V functions as part of the SOS response to DNA damage. Interestingly, it has been recently found that, in contrast to other bacteria, *H. pylori* Pol I lacks proofreading activity (García-Ortíz et al. 2011). Thus, in addition to having a primary role in DNA replication and repair,

Pol I unexpectedly promotes mutagenesis and thus increases genome plasticity. The lack of other polymerases, especially Pol IV and Pol V, and the lack of θ subunit, in addition to the absence of the DNA mismatch repair (MMR) pathway (Bjorkholm et al. 2001; Dorer et al. 2013; Pinto et al. 2005), may partially explain the fact that the mutation rate is approximately two orders of magnitude higher in *H. pylori* than in *E. coli*. Altogether it may contribute to the plasticity of the *H. pylori* genome, which enables this bacterium to continuously adapt to various host modulation strategies and different human populations (Baltrus et al. 2008, 2009; de Reuse and Bereswill 2007; Kawai et al. 2011).

3.3 Termination

There is little information available about the termination of *H. pylori* chromosome replication. In *E. coli*, the replication forks are arrested at a specific chromosomal locus called *terC* by a Tus–*ter* complex; specifically, by the interaction between Tus and DnaB (Bastia and Zaman 2014). Tus is a protein that interacts with *ter* sequences that are approximately 20 bp long and positioned in inverse orientations on each side of the *terC* locus. Thus, the two Tus–*ter* complexes formed at each side of *terC* are oppositely oriented and block the replisome in a strictly directional manner; i.e. only replisomes that have passed *terC* are blocked. This ensures that the entire chromosome is replicated before the replisome stops. After replisome disassembly, the two chromosomes are decatenated by topoisomerase IV (Topo IV) and resolved by a XerC–XerD recombinase (Bussiere and Bastia 1999). Heterodimeric XerC–XerD targets a 28-bp *dif* site located at *terC*. The *dif* site is presented to the Xer complex by the FtsK DNA translocase protein (Thanbichler 2010). FtsK is anchored at incipient cell division septa and simultaneously and specifically interacts with DNA, the XerD component of the XerCD complex and several other proteins, including topoisomerase IV. After separation of the two chromosomes by the Topo IV/Xer system, FtsK translocates chromosomes into daughter cells. In *H. pylori*, *ter* sequences and Tus protein have not been identified. In *H. pylori* and *Campylobacter jejuni*, no homologues of the *parE* and *parC* genes, that encode subunits of Topo IV, have been found; thus, there is possibly no topoisomerase IV. A single *xerH* gene and *difH* recombination site, which are the counterparts of *xerC*/*xerD* genes and *dif* site, were identified and shown to be required for proper chromosome segregation into nascent cells (Carnoy and Roten 2009; Debowski et al. 2012). Intact FtsK was needed for *difH* recombination, presumably for effective *difH* site presentation to the *H. pylori* XerH recombination complex at the end of each DNA replication cycle. Interestingly, despite a moderate effect on cell viability and growth in vitro, the *xerH* deletion mutant is unable to infect mice (Debowski et al. 2012).

3.4 Segregation

Unlike segregation in eukaryotes, bacterial chromosome segregation begins soon after the initiation of chromosome replication, e.g. in rod-shape bacteria, the newly replicated *oriCs* move towards the cell poles while the synthesis of the chromosome is still ongoing. The active segregation of chromosomes requires ParA and ParB proteins and *parS* centromere-like sequences in most bacteria, with the exception of *E. coli* and other gamma-proteobacteria, which do not have *parABS* homologues (Mierzejewska and Jagura-Burdzy 2012). ParB binds *oriC*-proximal *parS* sequences, organizing the origin of replication region into a compact nucleoprotein complex (named the segrosome), while ParA (a cytoskeletal Walker A type ATPase) provides the force for segregation. Interestingly, in some bacteria such as *Caulobacter crescentus*, the deletion of *parAB* is lethal, while deletion in other bacterial species causes different effects but mainly an increased number of anucleate cells i.e. cells without chromosomes. In *H. pylori*, the *parABS* system also exists; all elements were identified, including two *parS* sequences that are located within the origin-proximal 20–30% of the chromosome (Lee et al. 2006). In vitro studies showed that ParB binds specifically to the two *parS* sequences (Lee et al. 2006) and forms a high-order nucleoprotein complex (Chen et al. 2015), suggesting that ParB might form segrosomes in *H. pylori* cells. Indeed, immunolocalization of ParB protein revealed the presence of discrete foci (Lee et al. 2006) similar to those observed in other bacteria, including mycobacteria (Trojanowski et al. 2015). However, the biological role of the *parABS* system in *H. pylori* is still poorly understood and requires further investigation.

4 Regulation of *H. pylori* Chromosome Replication

As mentioned above, chromosome replication is mainly regulated at the initiation step. In *E. coli*, there are numerous proteins that regulate orisome formation. They either interact with DNA (e.g. SeqA, ArcA) or with the initiator protein (e.g. DiaA, HU, Hda) (Chodavarapu and Kaguni 2016; Wolański et al. 2014). DnaA activity is also regulated by cofactor ATP/ADP binding (Katayama et al. 2010) or by post-translational modifications such as acetylation (Zhang et al. 2016), while *oriC* activity is additionally controlled by topology or methylation (Donczew et al. 2014b; Marczynski et al. 2015; Wolański et al. 2014). In *E. coli*, only ATP-bound DnaA assumes the conformation that enables it to initiate replication (Katayama et al. 2010). Upon replisome formation, the DnaN–Hda complex interacts with DnaA–ATP, stimulates the weak ATPase activity of DnaA and inactivates DnaA in a mechanism called replication inactivation of DnaA activity (RIDA) (Katayama et al. 2010; Katayama and Sekimizu 1999; Wargachuk and Marczynski 2015). *E. coli* DnaA–ADP requires reactivation to exchange ADP for ATP. This process, called DnaA rejuvenation, is stimulated by the interaction of DnaA–ADP with

specific DNA sequences called DARS (Fujimitsu et al. 2009) and acidic phospholipids (Saxena et al. 2013). There is no information about the possible regulation of DnaA activity by ATP/ADP binding in *H. pylori*. It has not been shown whether any system similar to RIDA or DARS exists in *H. pylori*. Moreover, neither homologues of Hda nor DARS sequences have been identified. However, it has been shown that *H. pylori* DnaA interacts with an inner membrane of the cell wall, which could suggest that a process similar to rejuvenation does exist in *H. pylori* (Zawilak et al. 2003). In *H. pylori*, the following two proteins are known to affect orisome formation: HobA, which interacts with DnaA, and HP1021, which binds to *oriC* (Fig. 1). HobA, a protein essential for *H. pylori* growth (Zawilak-Pawlik et al. 2007), is a structural and functional homologue of the nonessential *E. coli* DiaA, despite low sequence homology (15% identity and 31% similarity) (Table 2, Natrajan et al. 2007; Zawilak-Pawlik et al. 2011). Both proteins interact with the N-terminus of a cognate DnaA and promote DnaA oligomerization at *oriC* (Keyamura et al. 2007, 2009; Natrajan et al. 2009; Zawilak-Pawlik et al. 2011). The role of HobA in orisome formation is not fully understood, especially in the context of *H. pylori* physiology and cell cycle. However, this protein is of special interest because of the high specificity of DnaA–HobA interactions and due to the fact that HobA is essential for *H. pylori* viability. DiaA and HobA cannot substitute for each other in vitro and in vivo. Indeed, DiaA–*E. coli* DnaA and HobA–*H. pylori* DnaA interaction surfaces coevolved with each other (Zawilak-Pawlik et al. 2011). Moreover, HobA cannot be exchanged in vivo even for its close sequence homologue Cj0545 from *C. jejuni* (27% identity and 52% similarity) (Zawilak-Pawlik et al. 2011). Therefore, the species specificity of the DnaA–HobA/DiaA interaction might be especially interesting as a potential target for antimicrobial drug design. In bacteria in which DiaA/HobA proteins are essential, as in *H. pylori*, such compounds could specifically affect orisome formation and hence prevent chromosome replication and bacterial growth.

HP1021, a member of the atypical, orphan response regulator family (Schär et al. 2005) and one of very few regulatory proteins in *H. pylori*, specifically interacts with *H. pylori oriC* at HP1021 boxes (5′-TGTT[TA]C[TA]-3′). These binding sites overlap with DnaA topology-sensitive DnaA boxes and the DUE (Fig. 1, see also Sect. 3.1). Consequently, HP1021 binding to *oriC* precludes DnaA–*oriC* interactions and inhibits DNA unwinding at the DUE. Thus, HP1021 constitutes a negative regulator of *H. pylori* orisome assembly in vitro (Donczew et al. 2015). HP1021 is involved in the regulation of dozens of *H. pylori* genes (Pflock et al. 2007) and might represent an important factor in a signalling pathway regulating *H. pylori* growth during host infection and/or persistent colonization. The exact mode of HP1021 activity in *H. pylori* cells, both in the regulation of gene transcription and chromosome replication, is still unclear. It has been shown that HP1021 is not regulated by differential expression or phosphorylation (Müller et al. 2007; Schär et al. 2005), but no other possible mechanisms that could modulate HP1021 activity are known. However, it is plausible to assume that HP1021 may sense, directly or indirectly, environmental stimuli and regulate *H. pylori* chromosome replication in a host-dependent manner. Indeed, classical two-component

systems regulate the initiation of chromosome replication in other bacteria, including pathogens. These include for example ArcB–ArcA and PhoR–PhoB in *E. coli*, which respond to anaerobic conditions and reduced availability of phosphate, respectively (Han et al. 1999; Lee et al. 2001). The activated, phosphorylated response regulator controls bacterial replication either directly by binding to *oriC* (e.g. ArcA ~ P) or indirectly by regulating the transcription of genes encoding proteins involved in the initiation of chromosome replication (e.g. PhoB, which induces expression of *oriC*-binding IciA). The particularly interesting in the context of bacterial growth within the host is *Mycobacterium tuberculosis* MtrB–MtrA, which functions during macrophage infection (Fol et al. 2006; Rajagopalan et al. 2010). MtrA is the essential response regulator of the MtrB–MtrA two-component system of *M. tuberculosis* (Bretl et al. 2011; Zahrt and Deretic 2000). The proliferation of *M. tuberculosis* upon infection depends in part on the optimal ratio of phosphorylated to nonphosphorylated MtrA (Fol et al. 2006). Phosphorylated MtrA (MtrA ~ P) binds *oriC* and functions as a regulator of *oriC* activity; MtrA also binds the *dnaA* promoter and interacts with DnaA (Fol et al. 2006; Purushotham et al. 2015). Thus, MtrA is directly involved in the regulation of the *M. tuberculosis* cell cycle (i.e. replication and cell division). Interestingly, MtrA does not bind the *oriC* of the closely related *M. smegmatis*, which indicates the high species specificity of MtrA in the regulation of *oriC* activity and, in consequence, chromosome replication (Purushotham et al. 2015). This is an example of how a pathogenic bacterium has specified its regulatory pathways to adapt to its host during coevolution with humans.

OriC activity in *E. coli* is modulated by DNA superhelicity. It is known that DNA unwinding is favoured at negatively supercoiled regions, because it is energetically less costly to separate strands in negatively supercoiled than in relaxed DNA. In bacteria, chromosome supercoiling depends on environmental conditions such as temperature, osmolarity, anaerobic shock and nutrient availability (Drlica 1992). On the other hand, local superhelicity is affected by the transcription of adjacent genes. Supercoiling has been shown to modulate the expression of virulence genes in pathogenic bacteria at different phases of the host–pathogen interactions (Dorman et al. 2016; Rosario and Tan 2016). In *E. coli*, only negatively supercoiled *oriC* is unwound in vitro by DnaA at the DUE. However, it should be noted that *E. coli* DUE unwinding is facilitated by the torsional tension that diminishes the DNA helicity and thus aids strand separation rather than by the direct influence of the DNA topology on the binding of the initiator protein DnaA, because no such interplay has been reported for *E. coli* DnaA and *oriC* thus far. In contrast to *E. coli*, in *H. pylori*, DNA topology directly controls DnaA–DNA interactions. *H. pylori oriC* contains DnaA boxes that are recognized only when supercoiled (Donczew et al. 2012, 2014a, see also Sect. 3.1). Consequently, upon DNA relaxation, such as in the stationary phase, DnaA is probably not able to bind to *oriC* and to unwind DUE, i.e. is unable to initiate replication. Moreover, DNA topology affects the expression of many *H. pylori* genes (Ye et al. 2007). Thus, it cannot be excluded that supercoiling also influences the expression of replication genes in a growth phase and possibly host-infection dependent manner.

DNA methylation is a common but species-specific mechanism of regulation of cellular processes, mainly those involving DNA–protein interactions. It is important to note that adenine methylation in bacteria is becoming increasingly recognized as a crucial epigenetic signal that may be transmitted to daughter cells (Adhikari and Curtis 2016). An epigenetic role has been suggested for Dam methylase in *E. coli* and CcrM methylase in *C. crescentus* (Adhikari and Curtis 2016). *E. coli oriC* contains 11 GATC sites, overrepresented within this region, that are subject to methylation by Dam methylase. Newly replicated sequences are hemimethylated and as such are bound by a SeqA protein that in turn prevents DnaA binding to *oriC* and premature initiation until the cell is ready for the next round of chromosome replication. Dam methylation was also shown to regulate replication in other species such as *Vibrio cholerae* or *Salmonella typhimurium*. Many species belonging to the class of α-proteobacteria possess another methylase called CcrM that is also implicated in DNA replication. In *C. crescentus*, CcrM is an essential cell cycle-regulated protein that affects the synchrony of replication, possibly via controlling DnaA assembly on *oriC* and influencing *dnaA* and *ctrA* expression. CtrA is an essential pleiotropic response regulator that, when phosphorylated, directly binds to *C. crescentus oriC* and displaces bound DnaA, thus inhibits DNA replication (Collier et al. 2007; Gonzalez et al. 2014; Stephens et al. 1996; Taylor et al. 2011). *H. pylori* is especially rich in methylases and restriction-modification systems that affect gene expression, including genes involved in DNA replication, such as *topA*, *ssb*, *ruvC* and *dnaQ* (Beaulaurier et al. 2015). It has been shown that most DNA is methylated, which may affect the binding of proteins to DNA. However, whether methylation affects DNA replication in *H. pylori* remains to be elucidated.

5 Plasmid Replication in Bacteria

Plasmids are extrachromosomal, usually circular, mobile DNA fragments that replicate in an autonomous and self-controlled way. They are composed of the two major functional parts: 1/genes or loci required for plasmid replication, maintenance and spread, and 2/genes or loci, which, though nonessential, give some benefits to their bacterial host in respect of such processes as detoxication, virulence, ecological interactions or antibiotic resistance. In addition, plasmids can carry sequences or genes used in inter- and intra-species recombination or transposition, by which they facilitate genetic changes in bacterial populations.

Circular bacterial plasmids replicate via three basic mechanisms: theta (Θ) replication, rolling circle replication (RC) and strand displacement. It should be noted that initiation, synthesis and control of plasmid replication greatly depends on the type of the plasmid and the mechanism of its replication (del Solar et al. 1998; Lilly and Camps 2015). Plasmid replication is usually initiated by a specific, plasmid-encoded initiator Rep protein at a single origin or multiple origins of replication (*ori*, *oriV*). There are plasmids, such as ColE1, which do not possess its own initiator protein but requires DNA Pol I to initiate replication. Plasmid *ori*

usually contain a Rep binding site—a sequence or repeated sequences (iterons) interacting with the Rep protein; *ori*s of plasmids, in which initial DNA melting and recruitment of replisome machinery depends on the bacterial DnaA initiator protein, also contain DnaA boxes (del Solar et al. 1998; Lilly and Camps 2015; Wegrzyn et al. 2016). Once DNA duplex is destabilised upon Rep–*ori* interactions, a replisome is formed that is able to synthesize a nascent strand. The replisome is assembled from bacterial proteins; however, in some plasmids and to some extent, plasmid-encoded proteins participate in DNA synthesis (del Solar et al. 1998; Lilly and Camps 2015; Wegrzyn et al. 2016). Replicated plasmids are maintained within a cell at a relatively stable copy number, and they are transmitted to daughter cells upon cell division. The low copy plasmids are actively segregated by proteins and loci encoded on a plasmid, while high copy plasmids are randomly passed between generations (Million-Weaver and Camps 2014). Plasmids can be transmitted horizontally between bacterial cells via natural transformation (nonmobilizable plasmids) or via conjugation (conjugative and mobilizable plasmids) (Smillie et al. 2010). Conjugative plasmids encode genes (*mob*) and loci (origin of transfer, *oriT*) required for plasmid mobilization as well as all the genes required for the assembly of a mating complex (membrane-associated mating pair formation (MPF) complex). Mobilizable plasmids encode only *mob* genes and *oriT*, while MPF genes are encoded on another genetic element. MPF is basically a protein secretion channel, which transports protein-bound DNA similarly as type IV secretion systems (Alvarez-Martinez and Christie 2009; Waksman and Orlova 2014).

6 Plasmid Replication in *Helicobacter pylori*

Plasmids are found in nearly 50% of *H. pylori* strains (Höfler et al. 2004; Penfold et al. 1988; Smith et al. 2002); some strains such as *H. pylori* Aklavik117 or *H. pylori* AL236 harbour two or three plasmids, respectively (Kersulyte et al. 2015; Shintani et al. 2015). In spite of the fact that the number of available plasmid sequences increases, our knowledge not only about replication, maintenance and transfer of *H. pylori* plasmids, but also about the role of plasmids in *H. pylori* physiology and pathogenicity is merely revealed. There are 43 plasmids currently annotated in Genbank database, out of which the smallest plasmid (pAL236-5; 1216 bps) encodes only one ORF, a homologue of the RepH plasmid initiator protein, while the biggest sequenced plasmid (unnamed plasmid from strain South Africa7; 25916 bps) encodes 28 ORFs, which include homologues of plasmid replication and transmission proteins (RepA, MobA and MobC) and 12 ORFs of unknown function. Only about 20 *H. pylori* plasmids were experimentally analyzed for their genetic content, transcription profile, replication or possible mobilization capacity (Fernandez-Gonzalez and Backert 2014; Höfler et al. 2004; and references herein). Based on homology analyses, these plasmids were grouped into two replication types: Θ (~75%) or RC (~15%). All of the plasmids contain genes encoding Rep proteins. The *ori* region was determined in some of the plasmids by the identification of repeated iteron Rep

binding sites (e.g. De Ungria et al. 1999; Hofreuter and Haas 2002; Hosaka et al. 2002; Joo et al. 2012; Quiñones et al. 2001). However, there are no data available about molecular mechanism of *H. pylori* plasmids replication. Three out of ca 20 plasmids analysed thus far were experimentally shown to be mobilizable. However, *mob* genes were identified in many *H. pylori* plasmids, including 8 out of ca 20 experimentally analyzed plasmids, which suggest that the number of mobilizable plasmids exceeds 40% (Fernandez-Gonzalez and Backert 2014). Other plasmids, which do not encode *mob* gens, are probably transmitted by natural transformation. Interestingly, in contrast to molecular mechanism of plasmid replication and maintenance in *H. pylori* cell, the transmission mechanisms of *H. pylori* plasmids were studied in greater details. There are four type IV secretion systems in *H. pylori* which could be involved in plasmid transmission: *cag*PAI, *comB*, *tfs3* and *tfs4* (Backert et al. 2005). ComB system was shown to be absolutely required by a recipient bacterium for DNA uptake via natural transformation and conjugation (Hofreuter et al. 1998, 2001; Rohrer et al. 2012). Other three systems are supposed not to be involved in plasmid transfer since deletion of each of them or all three systems does not change the efficiency of plasmid transfer (Rohrer et al. 2012). Interestingly, it has been shown that plasmid transfer between *H. pylori* can be mediated by a novel, T4SS-independent, but DNaseI-resistant pathway (DNaseI-resistant mechanism indicates that there is a direct contact between donor and recipient bacteria which supports DNA transfer). This pathway was designated as alternate DNaseI-resistant (ADR) pathway and two possible ADR mechanisms were suggested that could mediate plasmid transfer: outer membrane vesicles (OMVs) or nanotubes (Rohrer et al. 2012).

As noted above, the role of plasmids in *H. pylori* is merely known, and therefore, they are described as cryptic plasmids (Fernandez-Gonzalez and Backert 2014; Höfler et al. 2004). Many of the plasmid-encoded ORFs are of unknown function. There are a number of very small plasmids (6 out of 43 sequenced plasmids) that possess only a single ORF. That single ORFs are homologues of the Rep initiator proteins that possibly initiate autonomous replication of these plasmids in *H. pylori* cells. Many other plasmids are relatively small and encode only few ORFs (2–4), which are also homologues of genes ensuring plasmid propagation (*rep*, *mob*). Therefore, the question arises why *H. pylori* maintain these plasmids since *H. pylori* is supposed to have no benefits from them. One possibility is that these plasmids serve as DNA transfer molecules and increase population diversity of *H. pylori*. Interestingly, some plasmids, both small and larger ones, encode an ORF similar to filamentation induced by cAMP (Fic) protein. Fic proteins and cAMP may be involved in a regulatory mechanism of bacterial cell division (Utsumi et al. 1982) and thus could affect *H. pylori* cell cycle. In addition, the Fic protein is widespread in bacteria, and various pathogenic species secrete Fic as toxins that mediate post-translational modifications of host cell proteins to interfere with cytoskeletal, trafficking, signalling or translation pathways in the host cell (Roy and Cherfils 2015). Thus, Fic-encoding plasmids could be beneficial to *H. pylori* in the context of adaptation to its human host. Further analyses of mechanisms responsible for plasmid replication and spread will help us to better understand the diversity of *H. pylori* and its ability to adapt to its human host.

7 Beyond the Replication Flowchart

In contrast to eukaryotes, chromosomal replication in *H. pylori* as in other bacteria begins at a single site on the chromosome and proceeds bidirectionally to the terminus. Recently, Slager and Veening showed that the regulation of many bacterial processes depends on the chromosomal location of genes and that the location of *oriC*-proximal genes is evolutionary conserved and has implications for bacterial fitness (Slager and Veening 2016). Interestingly, the *H. pylori oriC* region is located in an atypical gene context (Fig. 1a), distinct from that in many other bacteria (Briggs et al. 2012; Wolański et al. 2014). Genomic analysis of different *H. pylori* strains revealed that the gene arrangement around the *oriC* region is conserved within these species and that some *oriC*-proximal genes (e.g. *comH* and *thyX*) might be especially important for *H. pylori* physiology and/or pathogenicity (Fig. 1). The *comH* gene, which is located immediately downstream of the *oriC*, encodes a protein which appears to be unique to *H. pylori* and is involved in natural DNA uptake from the environment (natural competence); however, its exact role in this process remains unknown (Smeets and Kusters 2002; Smeets et al. 2000). It is noteworthy that natural competence might be essential for the protection of *H. pylori*'s own DNA against oxidative stress encountered by *H. pylori* upon the contact with the gastric epithelium and immune system of the host (Krüger et al. 2016). Consequently, it has been shown that natural competence also promotes *H. pylori* chronic infection (Dorer et al. 2013). Thus, doubling of *comH* copy number, which takes place right after initiation of chromosome replication, might be important for protection of DNA during replication—a process especially sensitive to DNA damage. Interestingly, in *Streptococcus pneumoniae*, the genes responsible for DNA uptake (*com*) are also located in the vicinity of *oriC*. The antibiotic-induced replication stress triggered in this pathogen compensates by increasing *com* gene copy number, facilitating the more efficient uptake of genes associated with antibiotic resistance (Slager et al. 2014). A second important gene located in the vicinity of *H. pylori oriC* is *thyX* that encodes thymidylate synthase X, which produces the essential DNA precursor 2′-deoxythymidine-5′-monophosphate (dTMP). ThyX, in contrast to thymidylate synthase A (ThyA), is a low-activity enzyme that is supposed to limit the DNA synthesis rate. Indeed, in *H. pylori*, the mean DNA replication speed (120 bp/s) is approximately one order of magnitude lower that corresponding value for *E. coli*, which harbours *thyA* (Escartin et al. 2008), but it is similar to that of other slow-growing, *thyX*-encoding bacteria, such as *M. smegmatis* (Trojanowski et al. 2015) or *M. tuberculosis* (Hiriyanna and Ramakrishnan 1986). The presence of *thyX* in the proximity of *oriC* may allow *H. pylori* to increase the ThyX dosage shortly after the initiation of replication without the need for complex gene-regulatory pathways. Altogether, this mode of regulation of *comH* and *thyX* seems to be a smart strategy for pathogens such as *H. pylori* that contain only a few regulatory proteins.

In fact, the *H. pylori* chromosome encodes not only fewer regulatory genes, but also many genes involved in cellular processes, including DNA metabolism, are

missing from *H. pylori*. Analysis of the gene content in *H. pylori* genomes revealed a reduced number of genes responsible for the structural organization and replication of the chromosome compared to *E. coli*. The *E. coli* chromosome encodes at least 12 nucleoid-associated proteins (NAPs) that help to maintain its chromosome structure (Dorman 2014). Amongst others, *H. pylori* lacks Fis and IHF, which in *E. coli* also regulate chromosome replication at the initiation step (Kasho et al. 2014; Ryan et al. 2004). It must be noted that the corresponding genes are also missing from other pathogens (e.g. *C. jejuni* or *Neisseria gonorrhea*) (Ambur et al. 2009). *H. pylori* encodes few NAP proteins, including the histone-like HU and Dps (DNA-binding proteins from starved cells) proteins, which in *E. coli* play a role in regulation of chromosome replication. *E. coli* HU is indispensable for *oriC* unwinding in vitro (Hwang and Kornberg 1992). In addition, both the HU and Dps *E. coli* proteins, the primary role of which is to bind DNA, interact with DnaA to regulate the frequency of DNA replication: HU stimulates replication while Dps inhibits this process, probably to reduce initiations, providing an opportunity for mechanisms to repair oxidative DNA damage (Chodavarapu et al. 2008a, b). *H. pylori* HU is dispensable for *oriC* unwinding in vitro (Donczew et al. 2012), but we cannot exclude that in vivo HU and also Dps participate in the regulation of *H. pylori* chromosome replication. Interestingly, in addition to HU and Dps roles in maintaining DNA integrity and/or structure (Ceci et al. 2007; Chen et al. 2004), both proteins were reported to be involved in *H. pylori* pathogenesis (Satin et al. 2000; Sehrawat et al. 2015; Wang et al. 2012). *H. pylori* presumably also lacks SMC (structure maintenance of chromosomes) proteins or its functional homologue—*E. coli* MukB. SMC/MukB proteins play an important role in bacterial chromosome organization and compaction and additionally are involved in chromosome segregation and cell cycle progression. Moreover, the genes encoding topoisomerases III and IV, which are essential for maintaining the DNA topology in many bacteria, are also absent from the *H. pylori* genome. However, it has yet to be determined whether *H. pylori* possesses functional homologues of SMC/MukB and topoisomerases III and IV, which due to low sequence homology were not identified upon genome annotation. Interestingly, *H. pylori* J99 and 26695 contain two paralogs of the DNA topoisomerase TopA, but their role is not known.

8 Concluding Remarks

Understanding the *H. pylori* cell cycle, including chromosome replication, is crucial to decipher the mechanisms leading to *H. pylori* growth during host infection or persistent colonization. As illustrated in this chapter, recent research significantly enhanced our knowledge about chromosome replication and regulation of this process in *H. pylori.* The mechanisms, factors and interactions identified thus far allow us to propose common and unique traits in *H. pylori* chromosome replication. Because there is sufficient variation between the structures of replication proteins and complexes from *H. pylori* and other bacteria (Tables 1 and 2), it is tempting to

speculate that in the near future some of the proteins involved in *H. pylori* replication may become targets of new antibacterial therapeutics. However, it has to be noted that most of the studies regarding DNA replication and regulation of chromosome replication in *H. pylori* have been performed in vitro or in the laboratory conditions while *H. pylori* proliferation is strictly dependent on host environment. Therefore, investigation of the chromosome replication during the colonization of its host seems to be a major direction of future research.

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The *Helicobacter pylori* Methylome: Roles in Gene Regulation and Virulence

Rebecca Gorrell and Terry Kwok

Abstract The methylome is defined as a map of DNA methylation patterns at single-base resolution. DNA methylation in bacteria was first discovered as a function of restriction–modification (R-M) systems. R-M systems in *Helicobacter pylori*, like those in other bacteria, are important host-specificity determinants that provide protection against foreign DNA. Moreover, the gene regulatory role of the methyltransferase (Mtase) unit of various *Helicobacter pylori* R-M systems is being increasingly recognized. Recent advances in the application of single-molecule real-time (SMRT) DNA sequencing to analyse DNA methylation have revealed for the first time comprehensive pictures of the genome-wide distribution of methylation sites in various strains of *H. pylori.* The methylomic data published so far have not only confirmed the significant inter-strain diversity of *H. pylori* Mtases and their DNA methylation profiles, but also identified numerous novel Mtase target recognition sites. The precise knowledge of the nucleotide sequence of Mtase recognition sites and their distribution within the *H. pylori* genome will in turn enable researchers to more readily test hypotheses on how *H. pylori* Mtases function to orchestrate gene regulation and/or modulate virulence. Methylomic studies hold promise for providing a deeper understanding into the roles of *H. pylori* Mtase and R-M systems in the physiology, epigenetics and possibly also pathogenesis of this important human pathogen. Consequently, the knowledge gained will provide crucial insights into the potential application of *H. pylori* methylomes as novel biomarkers for the prediction of disease outcome and/or antibiotic susceptibility.

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Contents

1 Introduction

The methylome is defined as the set of nucleotide methylation modifications in a particular genome. Alternatively, it can be viewed as a map of DNA methylation patterns at single-base resolution. DNA methylation in bacteria was first discovered as a function of restriction–modification (R-M) systems (Arber and Dussoix 1962). R-M systems are present in most bacteria and are important host-specificity determinants that provide protection against bacteriophage attack and contribute to cell cycle and gene regulation (Vasu and Nagaraja 2013). These systems are multicomponent, with methyltransferase (Mtase) activity for modifying self-DNA at a specific nucleotide sequence and endonuclease activity for digesting foreign DNA at, or adjacent to, the same target sequence. Depending on the substrate specificity, the enzymatic activities of Mtases result in modified bases including 5-methylcytosine (m5C), N4-methylcytosine (m4C) and N6-methyladenine (m6A) in the target DNA. Since the genomic DNA in a given bacterium is typically fully or close to being fully methylated by its own Mtases at target sites that are otherwise cleaved by the cognate restriction endonucleases, it is protected against degradation by the bacterium's own R-M systems. In contrast, foreign DNA most often lacks the appropriate methylation profile and is therefore cleaved by the restriction endonuclease activities of the bacterium's R-M systems. Thus, R-M systems are classically viewed as a defence mechanism (bacterial immune system) to confer protection against foreign DNA. However, in the past few decades, there has been accumulating evidence for a role of bacterial Mtases in gene regulation and cell cycle regulation (Vasu and Nagaraja 2013; Casadesus and Low 2006; Marinus and Casadesus 2009). Bacterial R-M systems are therefore being increasingly recognized as complex, multifunctional machineries that are associated with diverse biological functions.

There are four groups of R-M systems in prokaryotes, namely Types I, II and III, which target unmethylated DNA, and Type IV, which restricts modified DNA (Roberts et al. 2003). The different R-M systems are classified based on their subunit composition, target sequence features, cleavage site characteristics and requirements for the cofactor and methyl donor *S*-adenosylmethionine, and Type I and Type II systems are further classified into subtypes mostly based on their target specificities (Roberts et al. 2003). Of particular interest with respect to prokaryotic gene regulation are the Type III R-M systems; these systems consist of a Mtase which carries a target recognition domain (TRD) and can function alone (Bachi et al. 1979), and a restriction endonuclease which can only bind DNA in complex with the Mtase subunit (Meisel et al. 1995).

Compared to other bacteria, genes encoding R-M systems are unusually abundant in *H. pylori*, comprising approximately 2% of the total number of genes in the genome (Alm et al. 1999; Tomb et al. 1997). Types I, II and III R-M systems have been identified in *H. pylori*, and a significant number of *H. pylori* Mtases and their corresponding R-M systems have been studied. However, it was not until the recent advances in the application of single-molecule real-time (SMRT) DNA sequencing to analyse DNA methylation that the complete methylomes of an organism could be readily determined. This has provided for the first time comprehensive pictures of the genome-wide distribution of methylation sites in various different *H. pylori* strains. The SMRT data published so far also provide convincing support for the great diversity of Mtases and their DNA recognition motifs in *H. pylo*ri and their extensive variations among different strains. Such analyses have also enabled previously unknown Mtases to be identified and studied in detail; some of the findings obtained provide further evidence for the abundant presence of phase-variable Mtases in *H. pylori* and the ability of this genetically plastic organism to generate new Mtases via homologous recombination between different Mtases-coding genes. These various aspects of *H. pylori* methylomes and their implications in host adaptation and *H. pylori* evolution will be dealt with in the first half of this chapter. While the SMRT data have provided further insights into the diversity of Mtases in *H. pylori*, recent findings from gene expression studies and functional analyses have suggested that some *H. pylori* Mtases may play important roles in gene regulation including the regulation of virulence genes. This exciting aspect of the *H. pylori* methylomes will be discussed in detail in the second part of this chapter.

While the R-M systems of *H. pylori* have long been a topic of considerable interest, the specific aim of this book chapter is to present an overview of the latest developments in the analyses of complete *H. pylori* methylomes and the recent findings that have shed light on the role(s) of *H. pylori* Mtases in gene regulation.

2 The Restriction–Modification (R-M) Systems and Their Diversity in *H. pylori*

Among the various types of R-M systems, Type II systems are the most abundant in *H. pylori* for reasons that remain to be understood. Moreover, *H. pylori* has an unusually large number of R-M genes, which represent approximately 2% of the total gene number (Alm et al. 1999; Oh et al. 2006; Tomb et al. 1997). This is a high proportion compared to other bacteria (Roberts et al. 2007). Analysis of the sequenced *H. pylori* genomes shows that on average a given *H. pylori* genome can encode 30 R-M genes (Roberts et al. 2007).

Another striking feature of the R-M systems in *H. pylori* is that they exhibit a high degree of inter-strain diversity, with each strain possessing a unique set of Mtases genes (Tomb et al. 1997; Nobusato et al. 2000; Alm et al. 1999). Such diversity has been proposed to be important for host adaptation. In addition, given that *H. pylori* is naturally competent (Hofreuter et al. 2000) and capable of inter-strain competition (Dorer et al. 2011), the diversity in R-M systems is believed to be important for regulation of genetic transfer between different *H. pylori* strains (Ando et al. 2000; Humbert et al. 2011). A recent study based on the analysis of 221 *H. pylori* strains from Africa, America, Asia and Europe suggests that diversity in R-M systems correlates with the geographic origins of the corresponding *H. pylori* strains (Vale et al. 2009). This leads to the proposition that R-M systems of *H. pylori* might play a role in host adaptation during the long history of *H. pylori* co-evolution with humans. Several other studies examined the important question of how such diversity is generated. There is evidence suggesting that horizontal gene transfer has mediated the recent acquisition of the genes encoding R-M systems in *H. pylori* and that old systems are constantly inactivated and eliminated while new systems are being acquired (Lin et al. 2001). Thus, the geographic association of some *H. pylori* R-M systems observed by Vale et al. (2009) suggests that they might have been acquired early during human migration out of Africa, whereas others acquired later by geographically isolated populations. An additional mechanism for generating the extensive methylome diversity of *H. pylori* has been proposed (Furuta et al. 2011). This mechanism, termed domain movement (DoMo), occurs in the specificity (S) genes and the *mod* genes of *H. pylori* Type I and Type III R-M systems, respectively, and involves the shuffling of TRD DNA sequences between two homologous systems by recombination at their adjacent repeat sequences (Fig. 1). This process of interchanging TRDs within a given system or between different strains has been proposed to contribute to the diversity of Mtase DNA recognition specificity. Furthermore, allelic recombination, point mutations and changes in the copy number of the tandem repeats between the multiple TRDs present in Type I R-M systems are likely to further shape the diverse methylomic landscapes among different *H. pylori* populations (Furuta et al. 2011; Furuta and Kobayashi 2012).

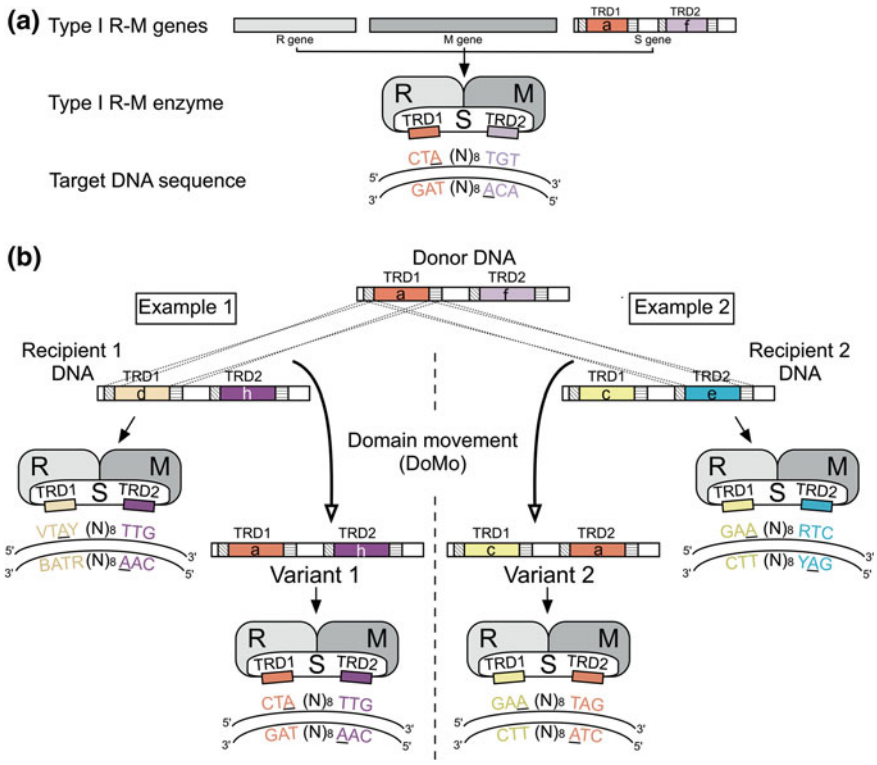


Fig. 1 Domain movement (DoMo) within the TRD of Type I S genes mediates extensive diversity within Type I R-M systems of *H. pylori*. **a** *H. pylori* strains typically carry 3 Type I R-M systems, designated groups 1, 2 and 3. Each Type I R-M group contains genes encoding restriction (R), modification (M) and one or more specificity (S) subunits, the products of which combine to produce a functional Type I R-M complex. The S subunit is responsible for DNA binding and typically contains 2 target recognition domains (TRD1 and TRD2), each of which possesses its own DNA sequence specificity that combines to form a bipartite TRD. The target sequence illustrated is that recognized by the S subunit encoded by *H. pylori* strain 26695 gene HP0790 (Krebes et al. 2013). Adenine residues within the target sequence are methylated (*underlined*) by the R-M. **b** The S gene TRD cassettes switch freely via DoMo to generate new variants. Each TRD is flanked by identical sequences that are conserved within each group, thereby facilitating TRD DoMo, i.e. the TRD cassettes reassort freely within and between individual S genes via homologous recombination at the conserved flanking sequences. *TRD colours and letters* denote previously described homology groups within the S genes of Type I R-M Group 2 (Furuta et al. 2011). Example 1—reassortment of the donor TRD1 with TRD1 of recipient 1 switches the specificity of the target sequence from 5′-VTAY$(N)_8$TTG-3′ to 5′-CTA$(N)_8$TTG-3′, and underlined nucleotides on each DNA strand are thereby targeted for ^{m6}A modification. Example 2—reassortment of the donor TRD1 with the TRD2 of recipient 2 not only changes the recipient target sequence, but also switches the target of the newly acquired TRD to the opposite orientation, such that the target specificity switches from 5′-GAA$(N)_8$RTC-3′ to 5′-GAA$(N)_8$TAG-3′. Such target re-orientation typical of reassortment of donor TRD1 with recipient TRD2 is in accordance with the rotational symmetry observed for the two TRDs in the tertiary structure of the S subunit (Kim et al. 2005)

3 The *H. pylori* Methylomes

To characterize the patterns of DNA methylation in a cell, various methods have been used to determine the type of DNA modification associated with a particular Mtase, including identification of methylated nucleosides by chromatography (Janulaitis et al. 1983; Dunn and Smith 1958) and the use of polyclonal antisera with specificities for m6A or m4C (Kong et al. 2000). In addition, the target DNA sequences of the Mtase(s) of interest, i.e. the DNA sequence contexts of the modified nucleotides, were often determined by one of the following approaches: (i) identification of m5C by bisulphite sequencing, (ii) identification of m6A by radiolabeling of DNA with [^{3}H] S-adenosylmethionine followed by mapping and sequencing of individual sites (Bitinaite et al. 1992; Bachi et al. 1979) and (iii) a method based on Sanger sequencing that directly detects methylated bases (Bart et al. 2005). These approaches are, however, labour-intensive and have limited capacity for genome-wide analyses. Recently, a new method known as single-molecule real-time (SMRT) DNA sequencing capable of directly detecting m6A, m4C or m5C modifications has been described (Clark et al. 2012; Flusberg et al. 2010). This method measures the rate at which each dNTP is incorporated by the DNA polymerase, which is characterized by the two kinetic parameters: the pulse width (PW) and the interpulse duration (IPD). When DNA polymerase encounters the modified bases m6A, m5C or 5-hydroxymethylcytosine on the template strand of DNA, the rate of dNTP incorporation changes, which is then reflected in an altered magnitude of PW and IPD. This technique generates kinetic data that enable the type and position of the base modification in a given DNA template to be determined. One of the major advantages of this technique is its ability to generate m6A, m5C or 5-hydroxymethylcytosine signatures for the entire genome. Indeed, with the advent of SMRT, a number of *H. pylori* methylomes have been determined in recent years and the data obtained have dramatically enhanced our knowledge regarding the DNA methylation repertoires, the diversity of R-M systems and even the potential roles of Mtases in *H. pylori*, as described below.

The first two methylomes of *H. pylori* were published by Krebes et al. in 2013. Scrutiny of the methylomes of the *H. pylori* strains 26695 and a derivative of J99 (J99-R3) showed that the genomes of these two strains were densely methylated throughout. However, the distribution of methylation sites was not uniform throughout the genome. A relatively lower methylation density was observed in a previously identified plasticity zone (Alm et al. 1999), whereas a slightly higher density was associated with the *cag*PAI region compared with the rest of the genomes of 26695 and J99-R3. This uneven distribution of methylation sites across the entire genome seems to be typical of *H. pylori* methylomes, as confirmed by a recent study that examined the methylomes of seven different *H. pylori* strains including P12, the P12 derivatives HPYF1 and HPYF2, F16, F30, F32 and F57 (Furuta et al. 2014). The latter study has shown that a region within the *rpoB* gene which encodes the RNA polymerase β subunit and a region within the *groEL* gene which encodes a chaperonin are hypermethylated in the majority of the strains

examined and are associated with a high density of 5′-CATG sites. Other hypermethylated regions are found within *fusA* which encodes the translation elongation factor Ef-G, *mreB* gene which encodes a protein important for determining cell shape, *flgE*, *ureC* and *cagY*, but the degree of methylation in these genes varies between strains. Apart from an uneven distribution of methylation sites in the genome, another important feature of the *H. pylori* methylomes is that the methylation patterns vary significantly between strains (Furuta et al. 2014; Krebes et al. 2013; Lee et al. 2015). The latter is in accordance with previous observations showing that *H. pylori* MTases exhibit significant inter-strain variations in DNA recognition sequence specificities (Furuta et al. 2011; Lin et al. 2001).

With the aid of the SMRT sequencing technology, methylome analysis has become a powerful means to deduce the recognition sites of known Mtases whose target sites were previously unknown. For example, the study by Krebes et al. (2013) revealed numerous novel Mtase recognition sites in *H. pylori* 26695 and J99-R3. To this end, open reading frames in the *H. pylori* genomes that potentially encode the candidate Mtase genes were selected based on the data in the REBASE database. To then identify the corresponding target sites, SMRT sequencing was performed to determine the methylome of wild-type bacteria and that of the isogenic mutant in which the Mtase gene of interest was either inactivated by insertional mutagenesis or overexpressed in *E. coli* (Krebes et al. 2013). This approach revealed a number of novel features regarding *H. pylori* MTases. First, several *H. pylori* Type I Mtases show multiple specificities (JHP1422/JHP1423; JHP0785/JHP0786 and JHP0726). For example, JHP1422, which encodes the S subunit of a Type I Mtase with three TRDs, can recognize both a palindromic and a non-palindromic recognition site, whereas it was previously believed that an S subunit can only recognize either a palindromic or a non-palindromic site. Second, there is evidence of a new mechanism for varying the specificity of a given MTase. For example, Krebes et al. (2013) observed that the specificity of the Type IIG Mtases, JHP1272 and HP1353-HP1354, varied depending on the absence or presence of an extra C-terminal domain.

The methylome study by Krebes et al. (2013) also enabled further identification of a number of Mtase genes with homopolymeric nucleotide repeats, the analysis of which provided additional insights into the diversity and variability of *H. pylori* R-M systems. Such homopolymeric nucleotide repeats have been found in *H. pylori* to be hotspots for slipped-strand mispairing which can result in phase variation of the expression of a gene. Two homologous Type IIG systems, namely JHP1272 in J99 and HP1353-HP1354 in 26695, were found in the Krebes et al. (2013) study to contain Mtase genes that harbour homopolymeric repeat lengths that result in frameshifts. These frameshifts were then corrected and the methylomes of the resultant mutants compared with those of the wild type (Krebes et al. 2013). The findings obtained indicate that these genes are switched on and off through phase variation at their first homopolymeric tract, while their specificity can be altered through the phase variation at their second homopolymeric tract. In particular, the activity of JHP1411 (alternatively designated as ModH1) in J99-R3, a Type III Mtase, was restored after adjustment of the repeat length (Krebes et al. 2013), suggesting that it

might be phase variable. However, such modification did not restore the methylase activity of the homologue in 26695, HP1522 (alternatively designated as ModH2). Consistent with the role of the homopolymeric tract in the phase variation of these ModH homologues, our previous study has shown that the ModH5 homologue (HPP12_1497) of these proteins in *H. pylori* P12 is active and is even capable of influencing the expression of multiple genes (Srikhanta et al. 2011) (see Sect. 4.3 for details). The aforementioned mechanism of phase variation may play important roles in *H. pylori* evolution and host adaptation as the rapid switching on and off of Mtase genes may result in rapid variation of methylation patterns, hence providing subpopulations with altered protection against phage infection, altered susceptibility to genetic exchange between strains and/or altered gene expression in response to environmental stimuli or host responses.

Complementing the Krebes et al. (2013) study, which focussed more on the Types II and III R-M systems of *H. pylori*, another study interrogated the methylomes of a number of hpEurope strains (P12 and its two derivatives HPYF1 and HPYF2) and hspEAsian strains (F16, F30, F32 and F57) and yielded novel information on the sequence specificity of primarily the Type I R-M systems in *H. pylori* (Furuta et al. 2014). First, the methylome data of Furuta et al. (2014) confirm the fact that *H. pylori* genomes have the highest methylation density compared to all other bacterial genomes analysed so far by SMRT. Second, close examination of the relationship between the TRD groups (mainly for groups 2 and 3) of the Type I S gene products and the different repertoires of methylation motifs among these various strains allowed deduction and assignment of recognition sequences to each TRD group. The findings of Furuta et al. (2014) suggest that TRDs in the Type I S genes of *H. pylori* can be classified into homology groups based on their amino acid sequences. Members of the same homology group usually have identical or highly similar TRD sequences. Different *H. pylori* strains harbour Type I S gene products that are associated with different TRD homology groups. Adding to the complexity of this variability, each Type I S gene encodes two TRDs in tandem, each recognizing half of a bipartite DNA target sequence (Fig. 1a). The combination of these two tandem TRDs also varies between strains. These results confirm the diversity in the combination of TRD-encoding DNA in multiple Type I S genes and in a Type IIG S gene (Furuta et al. 2011). Thus, DoMo may represent a mechanism for generating diversity in the recognition sequences of Type I (Fig. 1b) and possibly also some Type IIG S gene products. Additionally, the results of methylome analyses by Furuta et al. (2014) also indicate that some *H. pylori* Type I S genes exhibit variations in the number of the central tandem repeats that are flanked by the two TRDs. It is plausible that this variation could further enhance the diversity of sequence specificity of Type I R-M systems and ultimately the diversity of overall methylation patterns in *H. pylori* genomes. These findings, once thoroughly verified experimentally (e.g. by comparing the methylome of wild type with that of an appropriate isogenic mutant of the S gene), would enable future prediction of the target recognition sequence of any given S gene product based on the amino acid sequences of its TRDs (Fig. 2).

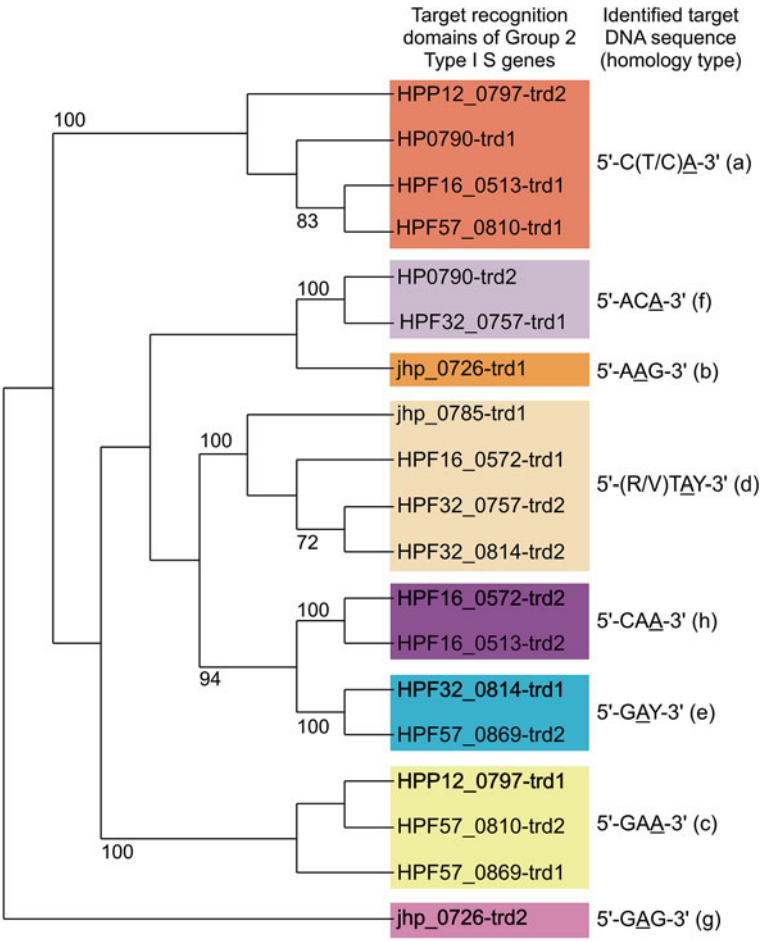


Fig. 2 Phylogenetic analysis of Type I R-M Group 2 S gene TRD sequences correlates with the specificity of the cognate Type I methyltransferase. Individual TRD1 (gene name—trd1) and TRD2 (gene name—trd2) amino acid sequences from the Type I R-M Group 2 S subunits from *H. pylori* strains, for which the methylation specificity has been confirmed by SMRT sequencing, were compared using SeaView (Gouy et al. 2010) to perform multiple sequence alignment and parsimony analysis; 1000 bootstrap replicates and 5 randomizations of sequence order; *numbers* indicate the % of bootstrap replicates each node appeared in (values <70% not shown); *coloured boxes* correlate with homology groups reported by Furuta et al. (2011). The corresponding target DNA sequences (modified ^{m6}A *underlined*) identified experimentally by SMRT sequencing are listed against each node, together with the homology *group letters in brackets*. Each TRD sequence includes the flanking N-term LL(Q/H)LAPKGV(E/G)F and C-term PIPPLEIQQEIV conserved sequences; jhp_0785 only contains a single TRD. The analysis demonstrates that the evolutionary relatedness of the Type I R-M Group 2 S gene TRD sequences indeed correlates with the homology of their identified target sequence specificities

The methylome of the *H. pylori* strain UM032, which was isolated from a gastric ulcer patient in Malaysia, has also been determined recently using SMRT DNA sequencing (Lee et al. 2015). Seventeen methylated motifs were identified. Only 3 of these 17 methylated motifs identified are also present in other *H. pylori* strains with known methylomes, further confirming the strain-to-strain diversity of Mtases and methylation patterns suggested by previous studies. Moreover, all except one motif were shown to be recognized by Type II Mtases, consistent with previous observations that Type II R-M systems are the most predominant R-M system in *H. pylori*. Two of these motifs, namely GAA**A**G and CY**A**NNNNNNNTRG, are found to be novel recognition sites of Type II Mtases.

4 Roles of *H. pylori* Methyltransferases (Mtases) in Gene Regulation

Some prokaryotic R-M systems, including those of *H. pylori*, consist of only active Mtases but no functional restriction endonucleases. This is most likely because the corresponding endonuclease-encoding genes are either not expressed or affected by mutations that result in a non-functional gene product. These Mtases are termed 'orphan' Mtases. A number of bacterial orphan Mtases have been found to play important roles in gene regulation, with the *E. coli* deoxyadenosine methyltransferase (Dam) that methylates the adenine residue in the DNA sequence 5′-GATC-3′ being the best characterized (Casadesus and Low 2006). Dam homologues have been found in many Gram-negative bacteria, including *E. coli*, *Salmonella* spp., *Neisseria meningitides*, *Haemophilus influenza* and *Caulobacter crescentus*. Indeed, bacteria tend to use DNA adenine methylation as an epigenetic signal in contrast to the use of cytosine methylation in eukaryotes. Although a gene homologous to *E. coli dam* has not been identified in *H. pylori*, gene regulation by Dam and Dam homologues is one of the most important paradigms of bacterial epigenetic regulation. Moreover, a role in regulation of flagella gene expression has been attributed to a Dam homologue in *Salmonella enterica* as well as non-homologous Mtases in *Campylobacter jejuni* and *H. pylori* alike, suggesting that common mechanisms may exist among these different Mtases. Thus, before we focus on the role of *H. pylori* Mtases in gene regulation, it is pertinent to review the current state of knowledge regarding how Dam modulates gene regulation.

4.1 Gene Regulation by E. coli *Deoxyadenosine Methyltransferase (Dam) and Its Homologues*

Gene regulation through DNA methylation by Dam occurs via more than one mechanism, whereby the methylation can either (i) affect interaction of regulatory

proteins with their target by causing steric hindrance, (ii) alter DNA structure and (iii) enhance binding of regulatory proteins to the methylation site, which, if the methylation site overlaps with the promoter, could result in competition between the regulatory proteins and RNA polymerase (Casadesus and Low 2006). Thus, depending on whether the regulatory protein involved is a repressor or enhancer and where the methylation site is with respect to the promoter, the differential binding of a regulatory protein to an unmethylated or methylated DNA target site can result in an overall repression or activation of transcription. In fact, DNA methylation can influence gene expression at both transcriptional and post-transcriptional levels. The molecular basis of such complexity and intricacy of epigenetic regulation by Dam are best illustrated by the various examples discussed in the following sections.

A typical example of how DNA methylation can impact on gene regulation at the transcriptional level by complex mechanisms is the regulation of expression of pyelonephritis-associated (Pap) pili by Dam in uropathogenic *Escherichia coli* (UPEC). Pap pili are crucial for mediating the attachment of UPEC to the host cells, and Pap pili expression is regulated in a phase-variable fashion. UPEC Dam controls the phase-variable expression of Pap by methylation of two GATC target sites: one located proximally to the Pap pilin promoter and the other at 102 bp upstream. Both GATC sites are found within the DNA binding sites of the global regulatory protein Lrp. Both the methylation status of these *pap* GATC sites and the configuration of methylated GATC sites modulate the binding affinities of Lrp and PapI, another regulatory protein, for the regulatory region of the *pap* operon in a highly complex manner; the end result is a highly intricate mechanism for switching the expression of Pap pili on and off (Hernday et al. 2004; Casadesus and Low 2006).

Dam methylation in *S. enterica* has been found to repress the transcription of *traJ*, which encodes a transcriptional activator of the transfer (*tra*) operon of the *Salmonella* virulence plasmid (pSLT) (Torreblanca et al. 1999). In this example, Dam methylation also functions together with the global transcriptional regulatory Lrp in controlling *traJ* expression. The latter is an activator of *traJ* transcription. A GATC site, target site of Dam, is present in one of the two Lrp binding sites in the upstream activation sequence of *traJ*. Dam methylation of the GATC site reduces binding affinity of Lrp to the upstream activation sequence of *traJ*, contributing to repression of *traJ* transcription. In addition, Dam methylation activates the transcription of *finP*, which encodes a small RNA that also contributes to repression of *traJ* expression. Evidence exists to suggest that this latter pathway may occur via a global effect of Dam methylation on nucleoid organization which then affects the binding of the nucleoid protein H-NS to the regulatory region of *finP*, resulting in repression of *finP* transcription (Camacho et al. 2005).

The potential role of *S. enterica* Dam in global gene regulation has been examined in a transcriptomic study (Balbontin et al. 2006). Comparison of the transcriptomes of wild-type *S. enterica* and an isogenic Dam$^-$ mutant showed that the genes encoded by the pathogenicity island SPI-I that are involved in invasion were activated by Dam methylation, whereas certain flagella genes, the fimbrial operon *std* and genes of the SOS regulon, were repressed. In particular, the

expression of two flagella structural genes, namely *fliC* and *fliD*, was increased in the Dam$^-$ mutant. Specific repression of *fliC* expression by Dam methylation was further confirmed using a *fliC::lac* transcriptional fusion construct. Furthermore, the Dam$^-$ mutant showed significantly reduced motility compared to the wild type, which is likely due to the observed dysregulated expression of flagella genes in the absence of Dam methylation. Despite these observations, the molecular mechanism by which *S. enterica* Dam regulates the transcription of *fliC* and *fliD* remain to be understood. Interestingly, mutation of the *C. jejuni* Mtase gene cj1461, which encodes a putative adenine Mtase (but not a Dam orthologue), has been shown to also influence motility. Compared to the wild type, the Δcj1461 mutant exhibits aberrant flagella morphology and a significant reduction in motility. However, the effect of the Δcj1461 mutation on the transcriptional level or protein level of flagella genes has not been examined. The mechanism by which Cj1461 functions in flagella regulation thus remains elusive. These various findings, together with the fact that the *H. pylori* phase-variable Type III Mtase ModH is also involved in regulation of flagella gene expression (see Sect. 4.3), suggest that the role of Mtases in flagella gene regulation could be a recurrent theme among different bacteria.

Meanwhile, there are data suggesting that DNA methylation by Dam can act at the post-transcriptional level, such as in the scenario of Dam-mediated expression of *vsr*, a gene that encodes the endonuclease Vsr that initiates very short-patch repair that corrects G-T mismatches to GC. The precise mechanism involved is poorly understood but may occur via an indirect pathway, whereby Dam methylation may control the transcription of one or more cell functions involved in post-transcriptional control. Another line of evidence for post-transcriptional regulation by Dam methylation was provided by Campellone et al. (2007). It was observed that Dam$^-$ mutants of enterohemorrhagic *E. coli* (EHEC), compared to the wild type, exhibited increased abundance of the virulence proteins EspFU, Tir and intimin, but the corresponding mRNA levels of these proteins remained unchanged (Campellone et al. 2007), suggesting that the Dam-dependent regulation of the expression of these proteins occurs at the post-transcriptional level. More recent data show that Dam methylation post-transcriptionally regulates the expression of *hilD*, which encodes a transcriptional regulator that controls the expression of genes located in the *S. enterica* pathogenicity island 1 (SPI-1). Multiple lines of evidence suggest that Dam methylation modulates *hilD* expression by influencing the stability of *hilD* mRNA via mechanisms that remain to be understood (Lopez-Garrido et al. 2010).

It has also been speculated that DNA methylation is able to regulate the expression of small noncoding RNAs (sRNA) and antisense RNA, which could then impact on the translation of the corresponding target gene product(s). According to this model, sRNA could bind to the target mRNA to prevent translation in the case of negative regulation, whereas in the case of positive regulation, sRNA might relieve some structural constraints of the target mRNA that prevent translation, allowing translation to occur (Marinus and Casadesus 2009). However, experimental evidence supporting this hypothesis is yet to be reported.

Finally, there are examples in the literature suggesting that DNA methylation could exert indirect and pleiotropic effects on gene regulation through its housekeeping role in maintaining DNA integrity and preventing double-strand breaks in DNA (Robbins-Manke et al. 2005). Thus, in studying the role of DNA methylation with the use of methylase-deficient mutants, one must exercise caution in the interpretation of gene expression data as the effects observed could be an indirect consequence of cellular stress that results from DNA damage due to a deficiency in global methylase activity. Conversely, the use of methyltransferase-overexpressing strains to study Dam-mediated gene regulation can cause indirect effects on global gene expression possibly due to an increase in the overall amount of fully methylated DNA and subsequent aberrant changes in DNA structure (Lobner-Olesen et al. 2003).

In conclusion, the role of DNA methylation in the regulation of virulence-related genes is widespread, arguing that this prokaryotic epigenetic mechanism plays a fundamental role in regulating bacterial gene regulation and the regulation of microbial virulence. Increasing evidence suggests that gene regulation by DNA methylation could operate at the transcriptional and/or post-transcriptional level and in most cases involves complex molecular mechanisms that remain to be fully understood.

4.2 *Regulation of Gene Expression by* H. pylori *Type I and Type II Mtases*

To date, a handful of examples of *H. pylori* Type I, Type II and Type III Mtases being directly or indirectly involved in gene regulation have been described. One of the earliest reports providing evidence for a role of a *H. pylori* Mtase in gene regulation was published in 2002 (Donahue et al. 2002). This study showed that inactivation of the gene *hpyIM*, which encodes a Type II Mtase, increased expression of the stress-responsive *dnaK* operon. This adenine DNA Mtase is highly conserved among *H. pylori* strains. It methylates the sequence 5′-CATG-3′ and a 5′ flanking gene, named *iceA*. The genetic locus *iceA* itself has attracted considerable interest because its transcription in an ulcer-derived *H. pylori* strain was found to be activated upon adherence to cultured gastric epithelial cells (Peek et al. 1998). Moreover, the presence of the *iceA1* allele of *iceA* associates with carriage of duodenal ulcer disease (van Doorn et al. 1998; Peek et al. 1998), and the *iceA* locus contains a promoter that drives the transcription of *hpyIM* (Xu and Blaser 2001; Donahue et al. 2000). To further understand the biological function of *hypIM*, the gene expression profiles of wild-type *H. pylori* and an isogenic *hpyIM*-inactivated mutant were compared using gene array hybridization (Donahue et al. 2002). The steady-state mRNA level of the *dnaK* operon, which consists of *hrcA*, *grpE* and *dnaK*, is significantly higher in a stationary phase culture of the wild-type strain compared to that of the *hpyIM* mutant. Moreover, transcripts of the *dnaK*

operon are more strongly induced in the *hpyIM* mutant compared to the wild type, both during stationary phase growth and upon adherence to gastric epithelial cells. Complementation of *hpyIM* restored wild-type *dnaK* operon transcript levels. Thus, M.*Hpy*I is capable of repressing the transcription of the stress-response operon *dnaK* operon although it remains to be ascertained whether and how such repressive effect contributes to the association of *iceA* with duodenal ulcer pathology. Notably, the promoter region of the *dnaK* operon does not contain the target sequence CATG of M.*Hpy*I, suggesting that the mechanism by which this Mtase influences *dnaK* transcription could be indirect.

M.HpyAIV, which recognizes the DNA sequence GANTC, is another *H. pylori* Type II Mtase shown to be involved in gene regulation (Skoglund et al. 2007). The presence of homopolymeric repeats within the *M.hpyAIV* gene suggests that it may be subject to phase variation by slipped-strand mispairing. Insertional mutagenesis of *M.hpyAIV* resulted in a significant decrease in the mRNA level of *katA* which encodes the *H. pylori* catalase, suggesting that this phase-variable Type II Mtase plays a role in regulating gene expression. Bioinformatic analysis of *H. pylori* strains 26695 and J99 showed that although GANTC sites are found in both intra- and intergenic regions of the genome, their occurrence in the latter seems to be under-represented (Skoglund et al. 2007). Moreover, GANTC sites within intergenic regions are located close to the translation start codon more often than would be expected by chance. In particular, regions upstream of the start codons of a large number of genes were found to be enriched in GANTC sites. These observations suggest that M.HpyAIV, together with its GANTC target sites, may influence gene expression. However, the molecular mechanism involved remains to be investigated.

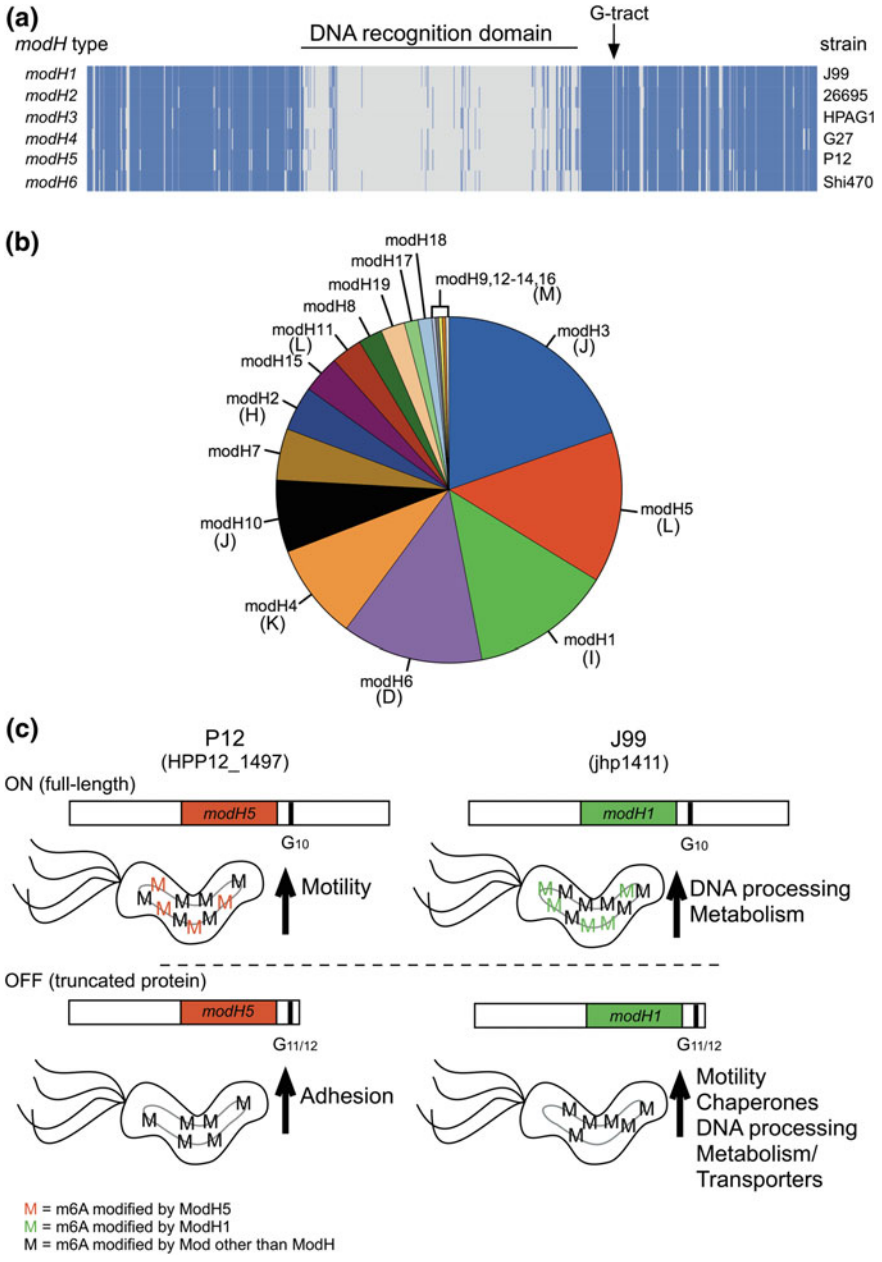
Apart from the two aforementioned Type II Mtases, a Type I Mtase of *H. pylori* has also been implicated to play a role in gene regulation. Furuta et al. (2014) provided evidence in a methylome study that a Type I S gene product, HPP12_0797, is involved in regulating gene expression in *H. pylori* P12. Inactivation of HPP12_0797 by insertion of a kanamycin-resistance gene cassette led to significantly increased mRNA levels of an operon of 4 genes (HPP12_0959, HPP12_0960, HPP12_0961 and HPP12_0962) compared to those in the wild type. Two of these genes code for ATP/GTP binding proteins, whereas the exact functions of the other two genes are unknown. Comparison of the methylome of the wild-type strain with that of the HPP12_0797-knockout strain identified 5′-GAA $(N)_8$TAG-3' to be the target sequence of this Type I Mtase. Indeed, this sequence is present in all of the 4 genes whose expression was affected and the corresponding sites are within the coding regions. The underlying mechanisms by which DNA methylation at these sites within the open reading frame of these genes results in alteration of gene expression levels remain to be elucidated. These observations nevertheless provide yet another example of a potentially important role of *H. pylori* Mtases in gene regulation.

4.3 *The* H. pylori *Mtase-Based Phasevarion, ModH, Plays Key Roles in the Regulation of Virulence Genes*

The complexity of methylation-mediated gene regulation in prokaryotes is further augmented by the concept of the 'phasevarion' (phase-variable regulon). This regulatory system is controlled by random ON/OFF switching of a phase-variable restriction–modification system, resulting in the coordinated and reversible switching of expression in multiple genes via randomly altered genome methylation (Srikhanta et al. 2010). Phasevarions are virulence gene associated in a variety of pathogenic bacteria, including *H. influenza* (Brockman et al. 2016; Srikhanta et al. 2005; VanWagoner et al. 2016), *Neisseria mengititidis* (Seib et al. 2011; Srikhanta et al. 2009), *Neisseria gonorrhoeae* (Srikhanta et al. 2009), *Moraxella catarrhalis* (Blakeway et al. 2014) and *C. jejuni* (Zautner et al. 2015).

Within the large number of Mtase genes carried by each *H. pylori* strain, there are a subset that are phase variably regulated via a homopolymeric tandem repeat, typically a G or C tract (Salaun et al. 2004). Slipped-strand mispairing randomly alters the length of this polynucleotide tract resulting in either ON (full-length open reading frame) or OFF (premature stop codon) status of the gene. One of these phase-variable Mtase genes, which is homologous to 26695 gene HP1522 and termed *modH*, contains a polyG tract and has been shown to possess immense inter-strain diversity in the DNA recognition domain (Fig. 3a) (Srikhanta et al. 2011). Early analysis of the prevalence of *modH* in *H. pylori* strains did not account for this substantial diversity and reported *modH* carriage in only 6 of 23 strains examined (Salaun et al. 2004). However, this was a significant underestimation with a later study identifying *modH* in all 57 *H. pylori* isolates examined at that time (Srikhanta et al. 2011). That same study examined ModH diversity and identified at least 17 distinct types, as defined by >90% amino acid identity in the DNA recognition domain, with *modH3* and *modH5* being equally most prevalent. This extensive ModH diversity has been shown to be DoMo-mediated, with reassortment of TRD cassettes occurring both within each of the Type III *mod* loci via homologous recombination in the conserved flanking sequences and occasionally between the different Type III *mod* loci 1, 2 (*modH*), 3 and 4 at very short sequences flanking the TRD with sufficient similarity between different loci to allow infrequent recombination (Furuta and Kobayashi 2012). With the recent public availability of several hundred *H. pylori* genome sequences, a larger-scale analysis indicates that *modH3* is the most prevalent type followed by *modH5* and *modH1* and that there are at least 19 *modH* types (*modH1—modH19*) in circulation (Fig. 3b). These *modH* types mostly correlate with the DoMo analysis of the Type III *mod* locus genes, but include additional types attributable to further recombination events between some of the TRD cassettes giving rise to new types (e.g. *modH3* and *modH10* both identified as TRD type J) (Furuta and Kobayashi 2012; Srikhanta et al. 2011).

The first evidence of a phasevarion within *H. pylori* was obtained following comparative transcriptional profiling of *H. pylori* strain P12, which carries a type 5



modH gene (*modH5*, HPP12_1497) (Srikhanta et al. 2011). Microarray analysis of P12 isogenic variants bearing ON (wild type) versus OFF (deletion or fixed OFF polyG tract) *modH* genes showed differential gene expression in motility- and adhesion-associated genes dictated by *modH5* ON/OFF status (Fig. 3c). Motility

◂**Fig. 3** Sequence analysis, prevalence and transcriptional analysis of *H. pylori modH* types. **a** Multiple sequence alignment of ModH amino acid sequences from *H. pylori* strains representing types *modH1-modH6*. Open reading frames have been restored at the polyG tract for strains J99, 26695 and G27. *Blue bars* denote >50% amino acid identity between the sequences; refer to Srikhanta et al. (2011) for comparison of further *modH* types. **b** Prevalence of *modH* types in 311 *H. pylori* genome sequences sourced from GenBank; reference strains for *modH18* and *modH19* are GAM103Bi (accession NZ_KB636899.1) and SA156C (CBPV010000003), respectively; all other types are as defined previously (Srikhanta et al. 2011). Homology types described by Furuta and Kobayashi (2012) are in *parentheses* (*capital letters*) beneath the corresponding type(s). **c** Summary of the mechanism and transcriptional profiling results of the ModH5- and ModH1-regulated phasevarions from *H. pylori* strains P12 (Srikhanta et al. 2011) and J99 (Beaulaurier et al. 2015), respectively. In the ON variants, the full-length ModH protein is expressed and is able to methylate the genome along with a variety of other Mod proteins within each strain; in the OFF variants, the ModH protein is terminated prematurely and the truncated ModH is unable to methylate the genome, whereas other Mod proteins are unaffected unless they are regulated as a member of the ModH phasevarion. Functional families of genes upregulated in the ON or OFF variants of each strain are listed next to the *arrow*

and adhesion are both essential colonization determinants of *H. pylori*. A more recent RNAseq analysis of *H. pylori* strain J99 variants containing ON versus OFF *modH* (type *modH1*, jhp1411) further indicated the presence of ModH-regulated phasevarions in *H. pylori* (Beaulaurier et al. 2015). This analysis identified motility-, chaperone-, DNA processing-, metabolism- and transport-associated genes as being differentially expressed in a *modH1* ON/OFF-dependent manner (Fig. 3c). While the phasevarions regulated by ModH5- and ModH1-mediated methylation contained discrete gene cohorts, there was a commonality of motility-associated genes and, more specifically, shared regulation of *flgE-1*. However, the two phasevarions showed opposite effects of ModH-mediated methylation on motility gene regulation, i.e. *modH5* ON increased flagellin and hook protein expression, whereas *modH1* ON decreased flagella hook and rod proteins, and *flaG* expression. The marked overall differences between the phasevarions controlled by *modH5* and *modH1* suggest it is likely that the 19 different *modH* type-encoded methyltransferases possess differing target specificities, thereby coordinating discrete phasevarions based on where these target motifs reside in the genome. The target motif of J99 ModH1 has been identified as GWC^{m6}AY (Krebes et al. 2013). It is noteworthy that this site is not methylated in wild-type P12 (Furuta et al. 2014), in which *modH* has an unusually short and stable G-tract that consistently produces functional ModH, as it further suggests disparate target specificities for the different *modH* types.

The contribution of phasevarions to bacterial pathogenesis has been examined in *H. influenzae* animal models of disease (Atack et al. 2015; Brockman et al. 2016; VanWagoner et al. 2016). These studies have indicated that loss or gain of specific genome methylation can significantly alter disease severity in the host (Atack et al. 2015; VanWagoner et al. 2016) and that the ability of a bacterial population to freely switch between *mod* ON and OFF state can itself be important for in vivo bacterial pathogenesis (Brockman et al. 2016). A comprehensive examination of the contribution of phasevarions to *H. pylori* pathogenesis in vivo remains to be

conducted. However, a study comparing colonization efficiency of ON and OFF variants of several phase-variable *mod* genes of *H. pylori* strain OND79 indicated that the fixed ON state of the HP1522 homologue carried by this strain was detrimental to efficient gastric colonization (Gauntlett et al. 2014). Based on the ModH5-regulated phasevarion, the authors proposed this attenuation was due to downregulation of the outer membrane protein-encoding gene *hopG*. However, given the diversity of genes within the phasevarions regulated by ModH5 versus ModH1, it is not possible to attribute the observed phenotype to genes within the ModH5-regulated phasevarion without first determining the *modH* type of the infecting strain. Nevertheless, this is an important first line of evidence that phase-variable methyltransferases may play an important role in *H. pylori* pathogenesis in vivo, most likely via coordinated phasevarion regulation.

Whether any of the phase-variable methyltransferase genes carried by *H. pylori* strains in addition to the ModH HP1522-homologue also encode phasevarion regulators remains to be examined. However, the accumulating evidence suggests that methyltransferases provide an important and varied mechanism of gene regulation in *H. pylori* that is highly suitable for the rapid host adaptation and immune evasion that is essential for maintenance of chronic colonization of the human stomach.

5 Concluding Remarks

The recent explosion in the availability of *H. pylori* methylomic data has revealed that the *H. pylori* R-M systems are more complex than previously envisaged. It is becoming clear that most of the *H. pylori* Mtases exhibit significant diversity and variability, a notion that has important implications for their roles in host adaption and *H. pylori* evolution. Moreover, there is also increasing evidence pointing to a role of DNA methylation in gene regulation and hence the notion of Mtases functioning as key epigenetic regulators. Some bacterial Mtases, including Mtases in *H. pylori*, have been shown to regulate the expression of even multiple virulence genes. In fact, transcriptomic studies such as those performed on the *H. pylori* ModH phasevarion support the view that Mtases may function as ‘master switches’ of gene expression, although the mechanisms involved are likely to be intricate and highly complex. Previous studies on the gene regulatory functions of *E. coli* Dam and Dam homologues have indicated that DNA methylation patterns can be modulated at the transcriptional and/or post-transcriptional level(s) by various environmental conditions via alterations in regulatory protein binding and/or phase variation of the Mtases themselves. Whether these aspects also apply to gene regulation by *H. pylori* Mtases and their methylomes remains to be investigated. However, regardless of the precise mechanisms involved, if Mtases indeed play a key role in the regulation of bacterial virulence, it would be tempting to speculate their role as novel anti-infective targets.

How Mtases orchestrate epigenetic regulation at the molecular level is a critically important question to address. In this context, many specific questions remain open. For example, how does DNA methylation within the coding region of a gene affect transcription or translation? Do any *H. pylori* Mtases regulate gene expression by mechanisms analogous to those of *E. coli* Dam and Dam homologues? What environmental cues influence the switching on and off of phase-variable Mtases such as *H. pylori* ModH? Which other regulatory proteins do *H. pylori* Mtases interact with during regulation of transcription/translation? How do Mtases that mediate hemimethylation of DNA (methylation of only one of the two strands of DNA) orchestrate epigenetic regulation and how does the underlying mechanism differ between methylation on coding strand and methylation on template strand?

With the advent of the SMRT sequencing technology, extensive methylomic data can be generated at an unprecedentedly rapid pace, allowing global analyses of DNA methylation patterns in a large range and number of bacteria/strains. Future development of a high-throughput workflow for this technology would be desirable as it would further enhance the ease and speed of conducting comparative methylomic studies and hence the capability to examine a large variety of biological conditions and experimental time points that could potentially influence Mtase diversity, the methylomic footprints or the gene expression profiles associated with specific Mtases. The data yielded are anticipated to be instrumental for elucidating how the Mtases and methylomes of *H. pylori* vary during the course of infection, thereby facilitating a better understanding of the role of Mtases in *H. pylori* pathogenesis. Last but not least, given the significant inter-strain diversity of the *H. pylori* methylome, high-throughput methylomics could be a useful tool for genotyping *H. pylori* strains. Future investigations into the relationship between such 'epigenotypes' of *H. pylori* strains and disease outcomes would provide crucial insights into the potential use of *H. pylori* methylomes as biomarkers for the prediction of disease severity (e.g. the prediction of gastric cancer risk of *H. pylori*-infected patients). It would also be of interest to examine whether the *H. pylori* methylomes could be useful tools for antibiotic resistance profiling. Taken together, it is anticipated that further research on the *H. pylori* methylome and its functions will unveil many secrets behind *H. pylori* pathogenesis, while paving ways for the exciting prospect of using methylomes as novel biomarkers for disease outcome prediction and drug susceptibility profiling.

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Structural Insights into *Helicobacter pylori* Cag Protein Interactions with Host Cell Factors

Célia Bergé and Laurent Terradot

Abstract The most virulent strains of *Helicobacter pylori* carry a genomic island (*cag*PAI) containing a set of 27–31 genes. The encoded proteins assemble a syringe-like apparatus to inject the cytotoxin-associated gene A (CagA) protein into gastric cells. This molecular device belongs to the type IV secretion system (T4SS) family albeit with unique characteristics. The *cag*PAI-encoded T4SS and its effector protein CagA have an intricate relationship with the host cell, with multiple interactions that only start to be deciphered from a structural point of view. On the one hand, the major roles of the interactions between CagL and CagA (and perhaps CagI and CagY) and host cell factors are to facilitate *H. pylori* adhesion and to mediate the injection of the CagA oncoprotein. On the other hand, CagA interactions with host cell partners interfere with cellular pathways to subvert cell defences and to promote *H. pylori* infection. Although a clear mechanism for CagA translocation is still lacking, the structural definition of CagA and CagL domains involved in interactions with signalling proteins are progressively coming to light. In this chapter, we will focus on the structural aspects of Cag protein interactions with host cell molecules, critical molecular events precluding *H. pylori*-mediated gastric cancer development.

Contents

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1 The *Helicobacter pylori* Cytotoxin-Associated Gene Pathogenicity Island (*Cag*PAI)

The discovery of the *cag* pathogenicity island (*cag*PAI) was a decisive step towards the understanding of key factors associated with the virulence of *Helicobacter pylori* (Censini et al. 1996). The *cag*PAI is a 37-kb DNA fragment present in the genomes of the so-called type 1 (*cag*-positive) and absent in those of type 2 strains (*cag*-negative). The *cag*PAI contains 27–31 genes, but always includes the cytotoxin-associated gene A (*cagA*) (which gave its name to the genomic island). CagA was initially identified as an immuno-dominant antigen (Covacci et al. 1993), and a large number of studies have now established that *H. pylori* type 1 strains are associated with the more severe forms of gastric diseases as recently reviewed (Cover 2016; Yong et al. 2015; Hatakeyama 2014). Compelling evidence for this was the finding that mice expressing CagA showed gastric epithelial hyperplasia, gastric polyps and adenocarcinomas of the stomach and small intestine (Ohnishi et al. 2008). It has been estimated that people infected with type 1 strains are at 5.8-fold higher risk of developing cancer than uninfected people, while type 2 strains induce only a 2.2-fold higher risk (Parsonnet et al. 1997). The genetic diversity of the *cag*PAI strains isolated from around the globe indicated that the genomic island was probably acquired prior to around 60,000 years ago, the time when *H. pylori* associated with humans' migration "out of Africa" (Moodley et al. 2012; Linz et al. 2007). A strong selective pressure operates on key *cag*PAI components, suggesting that the presence of a functional *cag*T4SS provides a selective advantage to the type 1 over the type 2 strains (Olbermann et al. 2010).

The function of the *cag*PAI is to produce a molecular device called a type IV secretion system (T4SS) (reviewed by Backert et al. 2015). These systems are widespread in bacteria and used for diverse functions including exchange of genetic material (conjugation or transformation) or delivery of effector molecules (Chandran et al. 2015; Alvarez-Martinez and Christie 2009). The latter category is found in pathogens such as *Legionella*, *Bordetella*, *Brucella* or *Bartonella* species for which T4SSs are key virulence factors to inject protein effectors into the host cell (Chandran et al. 2015). In the case of *H. pylori*, the *cag*T4SS injects CagA into gastric epithelial cells but might also be used for other purposes. The *cag*T4SS is involved in the delivery of bacterial peptidoglycan, which triggers nucleotide-binding oligomerization domain-containing protein 1 (NOD1) response

(Viala et al. 2004). Very recently, the *cag*T4SS was also found to mediate DNA injection into gastric cells and trigger a TLR9 response (Varga et al. 2016). The *cag*T4SS is composed of 28 proteins based on the *cag*PAI of the strain 26695. By sequence comparison with the prototypical VirB/D proteins from the *Agrobacterium tumefaciens* T4SS, some putative homologues have been identified, with most having only very limited sequence similarity, and the remaining 17 proteins being unique to *H. pylori*. The composition, architecture and functions of the *cag*T4SS have recently been reviewed in Backert et al. (2015). The *cag*T4SS can be separated into three structural entities based on its resemblance to *Escherichia coli* or *A. tumefaciens* T4SSs: a cytoplasmic complex where processing of the substrate (CagA) is taking place, a core complex in the periplasmic space that might act as a channel and is prolonged by an external appendage named pilus (Terradot and Waksman 2011).

Biochemical and bioinformatics studies have facilitated the localization of these proteins in *H. pylori* cells (Fischer 2011). A recent attempt to determine the architecture of the *cag*T4SS core complex has led to isolation of a multimeric assembly containing CagT, CagX, CagY, CagM and Cag3 (Frick-Cheng et al. 2016). As shown in Fig. 1, CagF might act as a chaperone to anchor CagA to the *cag*T4SS and to facilitate its delivery to the core complex (Couturier et al. 2006; Pattis et al. 2007). Because of its extracellular position, the pilus is the main instrument for the *cag*T4SS to attach the apparatus to the host cell and to mediate CagA injection. The exact composition of the *cag*T4SS pilus is still not clear. Because of its homology to the *A. tumefaciens* VirB2 protein, CagC was proposed as the main pilin (Fischer 2011). Despite its localization at the surface of *H. pylori* (Andrzejewska et al. 2006), the protein has not been detected on the pilus and it is not essential for pilus formation (Johnson et al. 2014). Evidence from immuno-localization studies suggested that CagL, CagY, CagT, CagX and CagA are components of the pilus (Jimenez-Soto et al. 2009; Kwok et al. 2007; Tanaka et al. 2003; Rohde et al. 2003). Some discrepancies exist in the literature regarding CagY involvement in the appendage. The protein was detected at the surface of the pilus (Rohde et al. 2003) and is part of the core complex (Frick-Cheng et al. 2016). However, different studies showed that CagY was not present on the pili (Barrozo et al. 2013) and is not necessary for pilus formation (Johnson et al. 2014). CagY is a large modular protein, which contains two N-terminal distinct repeat regions and also a C-terminal portion homologous to VirB10 proteins (Delahay et al. 2008; Liu et al. 1999). The *cagY* gene might in fact undergo rearrangements in the repeat regions of the protein to generate diversity in *H. pylori*, thereby facilitating escape from the immune system (Barrozo et al. 2013). Evidence for CagI, CagL and CagH association with the pilus is probably more convincing. The proteins can form a ternary complex, and each of them is required for CagA translocation (Fischer et al. 2001; Backert et al. 2011; Shaffer et al. 2011). Moreover, *H. pylori* strains deleted of *cagI* or *cagL* are unable to produce any pilus but *cagH*-deleted *H. pylori* mutants are “hyperpiliated” and produce longer and thicker pili, suggesting that CagH plays an important role in the correct assembly of the appendage and acts as a “molecular ruler” (Shaffer et al. 2011).

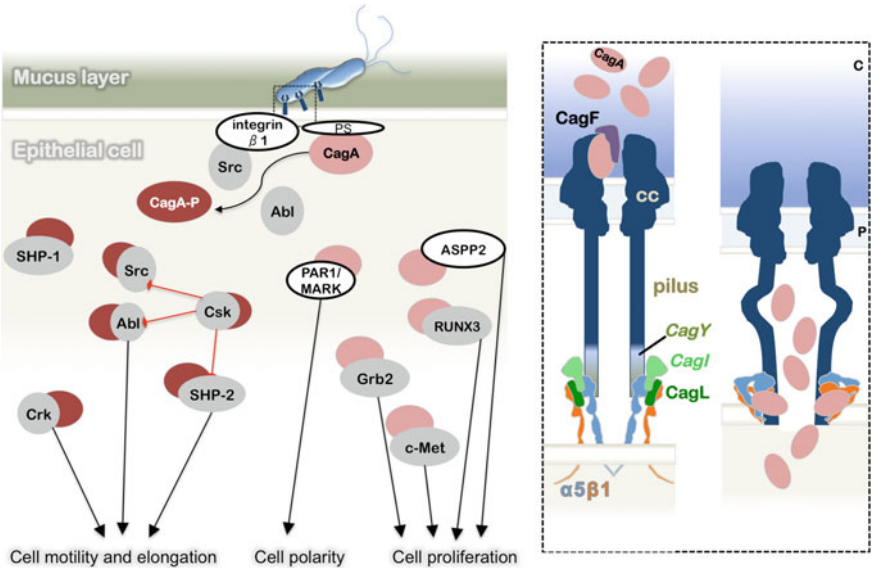

Fig. 1 Overview of CagA injection and selected interacting partners. The cell pathways affected by a given interaction are indicated. CagA partners in a *white* background indicate that some structural information has been provided for the interaction. Phosphorylated and non-phosphorylated CagA are represented by *dark red* and *pink circles*, respectively. *Red arrows* indicate inhibition of effect. PS: phosphatidylserine. The *inset* represents a schematic representation of the injection of CagA by the *cag*T4SS. The cagT4SS core complex (cc), pilus and some proteins present in the pilus, the cytoplasm (c) or the periplasm (p) are represented. This process involves interactions of CagF with CagA, integrin α5β1 with CagL and CagA (and perhaps CagI and CagY in *italic*). A conformational change of α5β1 might be required for CagA translocation

2 Integrins as Receptors of the *Cag*T4SS

Several lines of evidence point towards interactions of the *cag*T4SS apparatus with the host cell. The presence of a functional *cag*PAI, but not CagA, is essential to trigger the secretion of interleukin-8 (IL-8) (Fischer et al. 2001). The best-characterized interaction between *cag*T4SS apparatus and host cell is the one involving integrins. These proteins form a family of heterodimeric transmembrane receptors consisting of α and β subunits. Integrins are essential for cell–cell adhesion and interaction of cells with the extracellular matrix (Luo et al. 2007). The two subunits form a flexible receptor consisting of an extracellular part composed of the "head" and "leg" pieces, a short transmembrane region and a cytoplasmic tail. Many integrins have the property to recognize the Arg-Gly-Asp (RGD) motif in their different ligands, such as fibronectin (Takagi 2007). The RGD motif of the ligand binds to the extracellular headpiece of integrin, and the binding depends on

the activated state of the protein and on multiple factors (Takagi 2007). When they are inactive, integrins display a bent conformation, but once activated, the two subunits undergo spectacular conformational changes that result in an extended conformation. These different conformations are associated with inside-out or outside-in signalling events transmitted via transmembrane domains in a tightly controlled manner (Luo et al. 2007). Because of their ubiquitous distribution integrins are targeted by many pathogens that utilize them as receptors to promote adhesion, host cell invasion or injection of virulence effectors (Stewart and Nemerow 2007; Hauck et al. 2012). In the case of *H. pylori*, four Cag proteins have been shown to interact with integrins: CagI, CagY, CagA and CagL (Kwok et al. 2007; Jimenez-Soto et al. 2009). So far, only the extracellular part of integrin has been found to play a role in the injection of CagA, suggesting that it is the interacting partner of the *cag*T4SS (Jimenez-Soto et al. 2009). The role of CagY and CagI interactions with integrins is not known, and no structural information is available for these proteins. It is noteworthy that CagY and CagI have been detected at the surface of the bacterium and are thus in principle available to interact with integrin. The interactions of CagL, and to a lesser extent CagA, have been more documented and are detailed below.

3 CagL, a *Cag*T4SS-Associated Pilus Adhesin

The importance of integrins in the injection of CagA was first unveiled via the adhesion of the CagL protein to α5β1 (Kwok et al. 2007). CagL is an essential component of the *cag*T4SS because it is necessary for IL-8 induction, CagA translocation and pilus biogenesis (Shaffer et al. 2011; Jimenez-Soto et al. 2009; Kwok et al. 2007; Fischer et al. 2001). CagL has been detected at the surface of the pilus (Kwok et al. 2007) where it is able to bind to several integrins including α5β1, αVβ6, αVβ5, αVβ3, αVβ8 (Barden and Niemann 2015; Wiedemann et al. 2012; Conradi et al. 2012; Jimenez-Soto et al. 2009; Kwok et al. 2007). CagL possesses a RGD motif at residues 76–78 (strain 26695). It has been shown by surface plasmon resonance (SPR) that CagL has a higher affinity (Kd = 0.09 μM) for the integrin than its RGD mutant $CagL^{RGA}$ (Kd = 0.36 μM), suggesting that the RGD motif is important but not absolutely required for the interaction between the two proteins (Kwok et al. 2007). Discrepancies exist regarding the requirement of the RGD motif of CagL in the attachment of the *cag*T4SS in the in vivo context. Indeed, in one study a *H. pylori* strain deleted of *cagL* and complemented with $cagL^{RGA}$ was not able to translocate CagA (Kwok et al. 2007). Yet, another study showed that the RGD motif was not necessary for CagA injection and IL-8 induction (Jimenez-Soto et al. 2009). CagL and $CagL^{RGA}$ mutant are also able to bind the αVβ5 with a similar affinity (CagL Kd = 0.20 μM, $CagL^{RGA}$ Kd = 0.35 μM). So CagL can

interact with αVβ5 in an RGD-independent manner, and the interaction causes an upregulation of gastrin expression (Wiedemann et al. 2012). CagL was also found to interact with integrins αVβ6, αVβ3 and αVβ8, but in an RGD-dependent manner (Barden and Niemann 2015; Wiedemann et al. 2012; Conradi et al. 2012). Altogether these studies demonstrated that CagL is able to interact with different integrins. Although it is now clear that the RGD motif can play a role to interact with some integrins, it is not necessarily the case for all of them.

4 Structures and Interaction Motifs of CagL

Five crystal structures of CagL have been solved so far and show some significant structural differences (Barden et al. 2013, 2014; Bonsor et al. 2015; Choi et al. 2015) (Fig. 2a). In three CagL structures (from strain 26695 and strain K74) solved at neutral pH (named here $CagL^{KKQEK}$, $CagL^{meth}$ and $CagL^{K74}$), the protein forms an elongated four-helix bundle (α1, α2, α5 and α6) flanked by two short perpendicular helices (α3 and α4). The key difference between these three structures is the conformational flexibility of the N-terminal portion of α2, which contains the RGD motif (Fig. 2a). Artificial reduction of this flexibility abolishes the cell adhesion properties of CagL (Barden et al. 2013). The helical conformation of the CagL RGD motif is unusual since this motif is generally located in an extended or flexible loop (Luo et al. 2007). The remaining two crystal structures (from strain 26695) have been solved at acidic pH (named $CagL^{4x5u}$ and $CagL^{C\text{-}His}$). At this pH CagL adopts a three-dimensional domain-swapped dimer arrangement where the α5 and α6 helices form a single extended helix (Barden et al. 2014; Bonsor et al. 2015). As in the structure solved at neutral pH, $CagL^{4x5u}$ and $CagL^{C\text{-}His}$ structures present important flexibility of the N-terminal portion of α2. As mentioned, a comparison reveals that in the $CagL^{KKQEK}$ and $CagL^{meth}$ structures, the RGD motif is surface-exposed and does not interact with other residues. In contrast the RGD motif of $CagL^{4x5u}$ and $CagL^{C\text{-}His}$ interacts with nearby side chains (Arg^{76} with Asp^{46}, Asp^{78} with Thr^{170} and Asp^{78} with Asn^{166}, respectively; Fig. 2b) (Bonsor et al. 2015). As a consequence, the Arg^{76} is buried in the structure of $CagL^{4x5u}$ (Bonsor et al. 2015). Based on these observations, the RGD of the domain-swapped dimer does not appear accessible to the integrins. Accordingly, it has been show that when CagL is a dimer in solution, the protein is not able to attach to cells in vitro (Bonsor et al. 2015). These differences point towards a highly flexible region of CagL. Based on CagL structural variability and in vivo experimental results, an activation mechanism of CagL was proposed in which the α2 helix may partly unfold during the interaction with the integrins and only at permissive pH (Barden et al. 2014).

Additional CagL patterns might also be involved in the interaction with integrins. The RGD of CagL is followed by a LXXL motif ((leucine/methionine)-X-X-

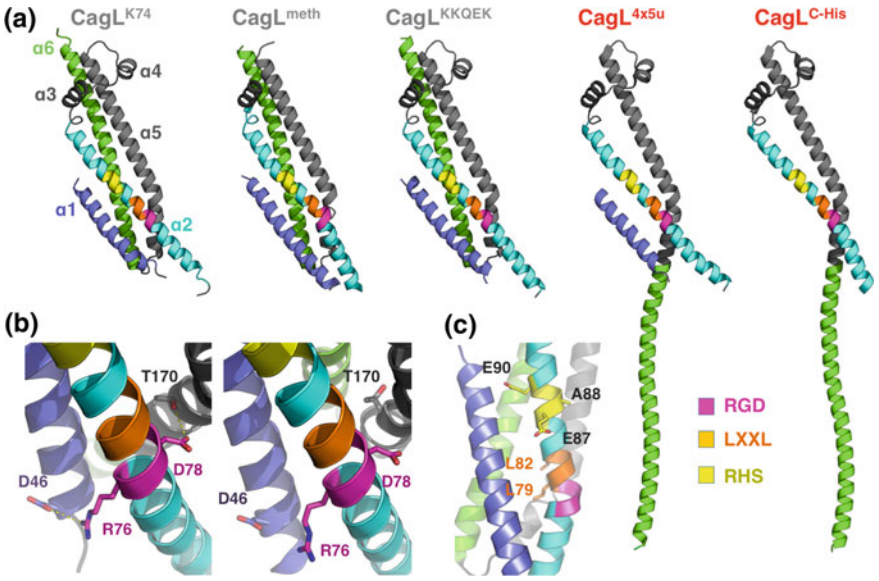

Fig. 2 Structural properties of CagL. **a** Crystal structures of CagL^{K74}, CagL^{meth}, CagL^{KKQEK} solved at neutral pH (pdb codes 4YVM, 3ZCJ, 3ZCI, respectively) of CagL^{KKQEK} and CagL^{C-His} solved at acidic pH (pdb codes 4X5U, 4CII, respectively). All structures are displayed in the same orientation to illustrate the flexibility of the N-terminal portion of α2. **b** Comparison of the RGD motif position in CagL^{meth} and CagL^{4x5u}. **c** L79 and L82 of the LXXL motif are buried in the CagL structure (3ZCJ) in contrast to E87, A88 and E90 of the RHS motif, which are surface-exposed

(leucine/isoleucine)). RGDLXXL is present in a picornavirus, and it is recognized by integrin αVβ6 (Burman et al. 2006). In the CagL structures, the two leucine residues are buried in the hydrophobic core of the protein and consequently unlikely to interact with integrins (Fig. 2c). However, mutation of these leucines to threonines (CagL^{L79T}, CagL^{L82T}) reduced the cell adhesion to CagL (Barden and Niemann 2015). Thus again, some flexibility might exist in this part of the α2 helix and the RGDLXXL motif might be able to interact with integrins (Barden et al. 2013). An early analysis of the CagL sequence identified a third motif in proximity of RGD consisting of Phe^{86}-Glu-Ala-Asn-Glu^{90} (Backert et al. 2008). This "FEANE" motif has been reported to enhance the interaction of CagL with integrins (Conradi et al. 2012). The sequence was also termed RGD helper sequence (RHS) since it is also located in the α2 helix. Residues Glu^{87}, Ala^{88} and Glu^{90} are surface-exposed in CagL^{meth} and seem particularly important to enhance the interaction between CagL and integrin and also to allow CagA phosphorylation (Fig. 2c).

5 CagA Interaction with Host Cell Components

5.1 *Structural Properties of CagA*

Despite 25 years of study, it has been only recently that the structural properties of the CagA oncoprotein came to light. CagA is indeed a challenging protein to work with in vitro since it is prone to heavy degradation when expressed in *E. coli* (Angelini et al. 2009). Even in the host, the protein might not be very stable (Moese et al. 2001), and its half-life is limited to a couple of hours (Ishikawa et al. 2009). CagA is a modular protein, which can be described as composed of two major regions based on biochemical studies on the protein from strain 26695: a N-terminal folded region ($CagA^{NTD}$, residues 1–822) and a C-terminal disordered region ($CagA^{CTD}$, 823–1186) (Hayashi et al. 2012). The structure of $CagA^{NTD}$ was solved by X-ray crystallography (Kaplan-Turkoz et al. 2012; Hayashi et al. 2012) and revealed three domains D1 to D3 (following the nomenclature in (Hayashi et al. 2012)) (Fig. 3a). D1 is a helical bundle of 220 residues separated from the "core"

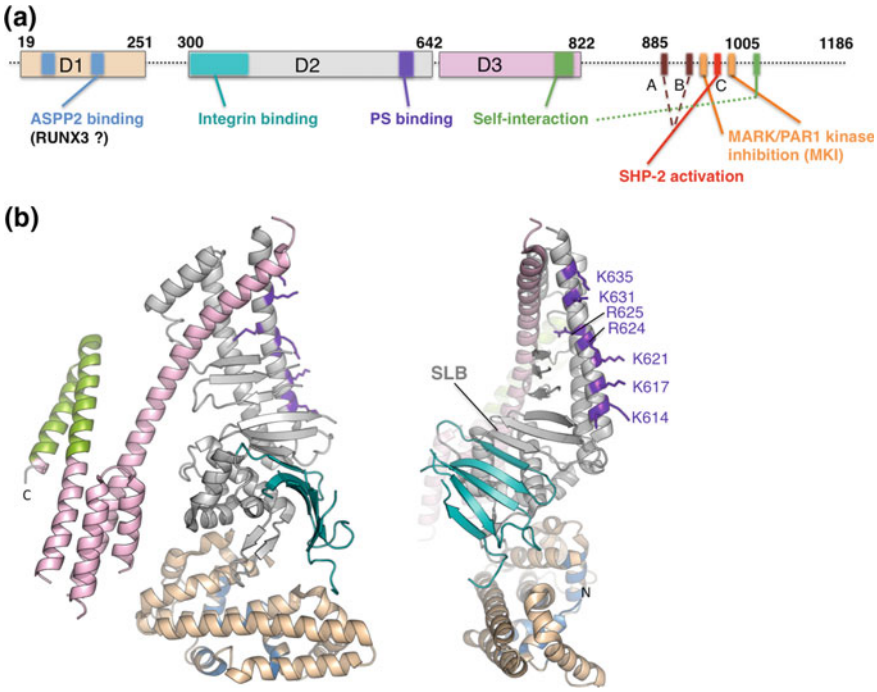

Fig. 3 Structure of CagA. **a** Schematic representation of the structural domains of CagA. Boxes delineate the three structural domains D1, D2 and D3 described in Hayashi et al. (2012) (pdb code 4DVY). The regions of CagA known to interact with host cell components are annotated. PS: Phosphatidyl serine. **b** Structure of CagA domain D1, D2 and D3 in two different views showing the regions or residues involved in host cell interaction coloured as in **a**. The part of SLB (single-layer beta sheet) involved in integrin binding (Kaplan-Turkoz et al. 2012) is coloured in *teal*

D2 domain by a flexible linker of around 60 residues. The D2 domain is an intricate assembly that can be divided into three subdomains. Eleven antiparallel β-strands assemble a single-layer antiparallel β-sheet (SLB), which forms an extended and hydrophobic surface on one side of the protein. A small globular α-helical domain (D2′) supports the lower part of the SLB, while its upper part is stabilized by two long α-helices (D2″). The D3 domain contains a 60-residue-long helix protruding away from the core. At the end of this helix, a small helical bundle followed by two short antiparallel helices complete the D3 domain (Fig. 3b).

While $CagA^{NTD}$ has now defined domains, limited structural information is available for $CagA^{CTD}$. When expressed isolated, this fragment is predominantly disordered (Hayashi et al. 2012). However, $CagA^{CTD}$ is able to interact with the $CagA^{NTD}$ when co-expressed in *E. coli* (Hayashi et al. 2012). $CagA^{CTD}$ and $CagA^{NTD}$ share a similar sequence (named NBS and CBS for N- and C-terminal binding sequence, respectively) identified in the last helices of D3 in $CagA^{NTD}$ (Hayashi et al. 2012). Theses sequences are able to interact with each other, and as a consequence, the CBS undergoes a disordered to helical structural transition. This conformational change might be linked to CagA activation since the sequence is located nearby other functional motifs (Hayashi et al. 2012). Indeed, $CagA^{CTD}$ contains multiple EPIYA (for amino acids Glu-Pro-Ile-Tyr-Ala) sequences that are tyrosine-phosphorylated by the human Src and c-Abl kinases once CagA penetrates the gastric cells (Mueller et al. 2012). EPIYA motifs were later found present in the human protein pragmin, suggesting an evolutionary or functional resemblance between the two proteins (Safari et al. 2011). Based on the sequences surrounding the EPIYA residues, four different motifs have been identified: A, B, C and D—types, with A and B motifs generally present in all CagA sequences (Lind et al. 2014, 2016). Isolates carrying EPIYA-C motifs are more frequent in European and American strains (Western-CagA), while the EPIYA-D sequence is more frequent in Asia (Eastern-CagA). Strains carrying multiple EPIYA-C motifs are more frequently associated with cancer (reviewed in Yamaoka 2010; Polk and Peek 2010; Hatakeyama 2004). The EPIYA phosphorylation is the key determinant for interaction of CagA with the SH2-domain-containing host proteins such as growth factor receptor-bound protein 2 (Grb2), phosphatase SHP-2, C-terminal Src kinase (Csk), which perturb multiple cellular pathways leading to cell transformation (Fig. 1) (Selbach et al. 2009). Finally, very little is known about the remaining part of $CagA^{CTD}$, although the sequence contains important information for translocation (Hohlfeld et al. 2006).

5.2 CagA Interaction with Membrane Components

Despite a large amount of CagA being synthesized by *H. pylori,* less than 20% of the protein is injected into host cells in cell cultures (Jimenez-Soto and Haas 2016), suggesting that the protein might have other functions for *H. pylori*. One intriguing aspect of CagA is that it localizes at the tip of the *cag*T4SS pilus (Fig. 1). It was

found that $CagA^{NTD}$ interacts with integrin α5β1 and αVβ3 with very high affinities (Jimenez-Soto et al. 2009). The interacting region was then narrowed down by yeast two-hybrid (Y2H) mapping, where CagA residues 303–404 were identified as the minimal region for interaction with β1 (Kaplan-Turkoz et al. 2012). Based on the structure and a competition assay, the lower part of the SLB domain was proposed to be involved in the interaction (Fig. 3b), since a fragment containing SLB-D2′ but not the one containing D2′ alone could inhibit CagA injection in vitro (Kaplan-Turkoz et al. 2012). Interestingly, the SLB structure is reminiscent of the structure of the protein OspA from *Burkholderia burgdorferi*. This bacterium is a human pathogen, transmitted by ticks and is the causative agent of Lyme disease. OspA plays a critical role in the infection by interacting with integrin αMβ2 in dendritic cells (Garcia et al. 2005). Although CagA and OspA do not share any sequence similarity, the SLB could represent a common feature mediating adhesion to integrins (Kaplan-Turkoz et al. 2012). The mechanism by which CagA exploits integrin might require structural rearrangements of the receptor. Mn^{2+}-activated integrin was more efficient as a receptor, and a monoclonal antibody which stabilizes the open active conformation of β1 integrin heterodimers was able to block CagA translocation. It was thus proposed that the transition from extended to bent conformation of the extracellular domain of integrin was required for CagA translocation (Jimenez-Soto et al. 2009).

After injection, CagA is tethered at the inner leaflet of the host cell membrane and this localization is essential to its function. There, CagA interacts with proteins associated with tight and adherens junction proteins including E-cadherin, β-catenin and the Src kinase (Murata-Kamiya et al. 2007). Ectopic expression of $CagA^{NTD}$ was sufficient to target the protein to the inner leaflet of the membrane and to the cell junctions (Bagnoli et al. 2005). Interestingly, part of the $CagA^{NTD}$ domain D3 resembles the eukaryotic F-actin binding domain and is highly conserved, although no direct interaction has yet been identified between this domain and actin (Kaplan-Turkoz et al. 2012). In addition, CagA binds directly to some lipid bilayer components. Phosphatidylserine (PS) is a component of the eukaryotic membrane bilayer and is found at the inner leaflet. However, during *H. pylori* colonization, membrane destabilization results in the transient externalization of PS at the site of *H. pylori* adhesion (Murata-Kamiya et al. 2010). CagA was found to interact specifically with PS, and it was proposed that the interaction of CagA with PS could play a role in the protein internalization (Murata-Kamiya et al. 2010). Anchoring of CagA to PS is based on charge complementation. The two helices forming D2″ expose arginine and lysine residues which form a dense positive patch at the surface of $CagA^{NTD}$ which can thus interact with the negatively charged PS. Point mutations of some of these residues showed that localization of CagA at the membrane was affected (Hayashi et al. 2012; Murata-Kamiya et al. 2010). A comparison with other membrane-associated proteins pointed out a resemblance of the combination of D2″ and D3 with the eukaryotic Fes/CIP4 homology-Bin-Amphiphysin-Rvs (F-BAR) domains that are present in many proteins involved in membrane trafficking and frequently also linked to cytoskeletal dynamics (Roujeinikova 2014). The attachment of CagA to host cell membrane PS might also have additional

consequences. *H. pylori* infection associates with diseases outside the stomach, including cardiovascular diseases, haematologic diseases, diabetes mellitus, idiopathic parkinsonism. A recent study showed that cells-derived exosomes containing the CagA protein could be released from gastric epithelial cells (Shimoda et al. 2016). Exosomes are extracellular vesicles that are produced by several different cell types via exocytosis and that are able to diffuse in the blood. After isolation, these CagA-containing exosomes are able to trigger morphological changes when incubated with cells in vitro, suggesting that CagA can be transported by this means in other parts of the human body (Shimoda et al. 2016).

5.3 *CagA Interactions with Oncosuppressors RUNX3 and ASPP2*

CagA association with cancer was reinforced by two studies that found that the protein directly modulates the activities of the tumour suppressor proteins runt-related transcription factor 3 (RUNX3) (Tsang et al. 2010) and the apoptosis-stimulating protein of p53 2 (ASPP2) (Buti et al. 2011). ASPP2 is a pro-apoptotic protein that associates with and activates p53 upon DNA damage or oncogenic stimuli, thereby inducing apoptosis. CagA prevents ASPP2-driven P53 degradation although the latter two proteins can still interact with each other (Buti et al. 2011). CagA forms a complex with ASPP2 at the membrane, thereby preventing the human protein to play its role in the nucleus (Buti et al. 2011). The crystal structure of the CagA domain D1 in complex with the ASPP2 revealed that a deep hydrophobic groove in D1 accommodates a short fragment of ASPP2 (residues 746–765) that forms an α-helix. Binding of ASPP2 does not trigger any significant structural changes in CagA structure since the structure of D1 in the complex is very similar to the structure of D1 in the context of the large fragment (Hayashi et al. 2012). The interaction with a short segment of ASPP2 appears to rely on CagA-specific and conserved structural features. Predictably from the structure of the complex, the interaction is very strong and only multiple amino acid substitutions in the protein CagA were capable of disrupting the interaction. In the absence of interaction with CagA, no change in ASPP2 localization could be observed. The CagA-interacting region of ASPP2 also interacts with the Src Homology 3 (SH3) domain of ASPP2 ankyrin. This self-interaction is proposed to diminish the availability of the ankyrin-SH3 domain to p53 and possibly other ASPP2 targets. By binding ASPP2, the N-terminal domain of CagA might thus release the autoinhibitory activity of ASPP2 and increase association with p53, hereby provoking the decrease in apoptosis (Nesic et al. 2010). This high-affinity binding site is completed by additional interactions between the two proteins. An extensive molecular dissection of the binding mechanism identified a disordered region of ASPP2 that also interacts with CagA (Reingewertz et al. 2015). Although the mechanisms used by CagA to subvert the p53 pathway are still not understood,

the mode of interaction of CagA with ASPP2 might explain how the latter protein can simultaneously interact with p53, via its Ank-SH3 domain and with CagA via the disordered region (Reingewertz et al. 2015).

Interestingly, the D1 domain of CagA is also involved in the interaction with the tumour suppressor RUNX3. The interaction stimulates RUNX3 degradation in the cell and thus promotes carcinogenesis (Tsang et al. 2010). Two CagA motifs, named WW1 and WW2, encompassing residues 120–152 and 204–236, respectively, were proposed to play a role in the binding (Tsang et al. 2010). Interestingly, many of these residues are part of the hydrophobic groove that binds ASPP2, thus raising the interesting possibility that ASPP2 and RUNX3 are targeted by the same CagA interface (Fig. 4a). However, the CagA-interacting region of RUNX3 was delineated to a proline tyrosine motif (PY). Given that this segment is unlikely to adopt a helical structure, as does ASPP2, it is yet unclear whether the binding mechanism of CagA to the two proteins is the same.

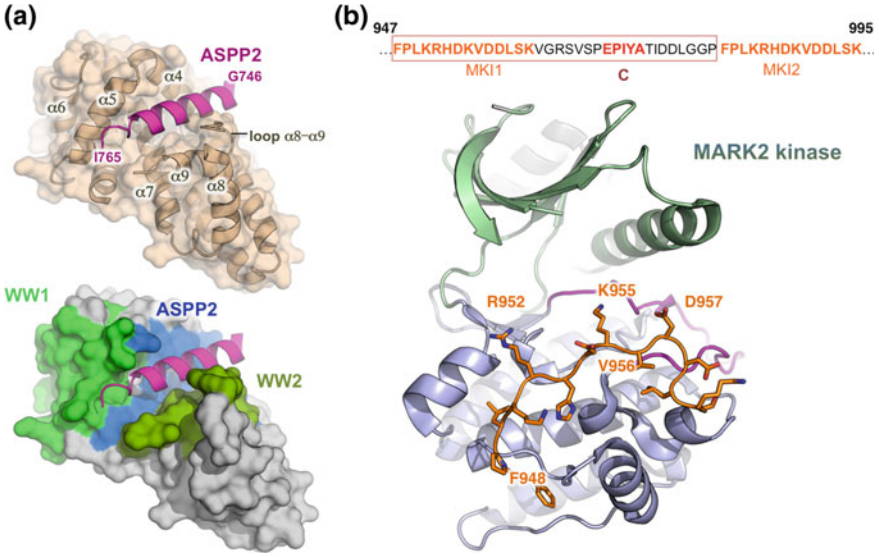

Fig. 4 Structures of CagA in complex with cellular targets. **a** *Upper panel* crystal structure of ASPP2 peptide (magenta) bound to CagA D1 domain (pdb code 4IRV). *Lower panel* surface representation of CagA D1 with residues forming the binding site of ASPP2 is coloured in *blue*. WW1 and WW2 sequences involved in RUNX3 binding are coloured in two shades of *green*. **b** Sequence of CagA (strain 26695) with the MARK kinase inhibitor (MKI) (*orange*) and EPIYA-C motifs (*red box*) indicated. Diagram representation of the crystal structure of MARK2/CagA MKI complex (pdb code 3IEC). MARK2 kinase regions are coloured as follows: N-lobe in *green*, C-lobe in *blue* and the kinase activation loop in *magenta*

5.4 CagA Interaction with Host Cell Signalling Proteins

CagA can also modulate the activity of key signalling enzymes, including kinases and phosphatases (Pachathundikandi et al. 2013). The $CagA^{CTD}$ region contains several short motifs involved in these interactions. C-terminal sequences (termed MKI for MARK Kinase Inhibitor or CM for CagA Multimerization) of CagA bind to the PAR1/MARK kinase (Nesic et al. 2010). Interestingly, a natural variant of CagA lacking MKI isolated in the Amazon rainforest was less potent for pro-oncogenic activities (Hashi et al. 2014; Suzuki et al. 2011). The interaction of MKI inhibits the kinase activity or PAR1/MARK, which perturbs atypical protein kinase C (PKC) signalling, and provokes disruption of tight junctions and loss of cell polarity (Nesic et al. 2010; Saadat et al. 2007). In the CagA sequence from strain 26695, one MKI motif is found between the motifs EPIYA-B and EPIYA-C and one is after the EPIYA-C motifs (Figs. 3a and 4b). The structure of the MARK2 kinase in complex with MKI-peptide (Fig. 4) has provided essential information on CagA mode of action on this essential signalling molecule (Nesic et al. 2010). MKI consists of short stretches of 14 residues that insert into the MARK2 kinase active site and interact with residues located between the N- and C-lobes of the enzyme. In the structure of the complex, MARK2 is locked in an inactivated state by direct interaction of its activation loop with CagA MKI (Fig. 4b). By comparison of MARK2 in activated and inactivated states, it was clearly established that CagA mimics PKI, a natural inhibitor of MARK2 (Nesic et al. 2010).

Another potential oncogenic effect of CagA in the host cell is its interactions with SH2 domain-containing proteins. CagA binds selectively to many signalling proteins with SH2 domains, acting as a "master key" (Backert et al. 2010). Among these proteins, phosphatases are key targets for CagA. SHP-1 and SHP-2 are cytoplasmic protein tyrosine phosphatases involved in the signalling pathways of a variety of growth factors and cytokines (Qu 2000). These enzymes are highly homologous and contain two tandem Src Homology 2 (SH2) domains at the N terminus and one phosphatase domain at the C terminus. Phosphorylated CagA (P-CagA) is able to interact with both SHP-1 and SHP-2 (Selbach et al. 2009; Higashi et al. 2002). P-CagA activates SHP-2 phosphatase via its SH2 domains, leading to dephosphorylation and inactivation of Src family kinases (SFKs), resulting in morphological transformation and dramatic cytoskeletal rearrangements in later stages of infection (Hatakeyama 2004). Inactivation of SFKs results in dephosphorylation of vinculin, disrupting vinculin interaction with Arp2/3, which leads to decreased lamellipodia formation and decreased number of focal adhesions (Moese et al. 2007). The sequence and number of the EPIYA motif are essential in this process, and duplication of EPIYA-C motif is associated with increased risk of cancer (Hatakeyama 2011). Recently, a SPR study demonstrated that duplication of EPIYA-C from one to two increases SHP-2 binding of CagA by more than 100-fold (Nagase et al. 2015) explaining CagA sustained SHP-2 activation reported earlier (Naito et al. 2006). In addition, a common variant of the *cagA* gene with A/T

polymorphism, encoding an EPIYT motif, has an altered function by profound binding and activating phosphoinositol 3-kinase (PI3-kinase), thus also influencing cancer risk (Zhang et al. 2015).

6 Concluding Remarks

The recent years have seen considerable progress in our understanding of the structural biology of Cag proteins. CagA and CagL structures together with biochemical and functional analysis have revealed that the flexibility and the modular architecture of these proteins might be key parameters to enable complex binding events. There are still many fundamental questions regarding the injection of the CagA oncoprotein into the host cell that remain to be addressed. The structure, composition and dynamics of the *cag*T4SS are still poorly understood. The discrepancies observed in several studies regarding the attachment of the *cag*-pilus to integrins may illustrate diverse strategies employed by the bacterium to ensure CagA delivery. With structural information progressively being available, some of these mechanisms might be deciphered soon. Indeed, structures of complexes between CagA and cellular partners have proven extremely informative. One remarkable outcome of the discoveries made during the past years is that while $CagA^{NTD}$ and $CagA^{CTD}$ have obviously different structural properties, they both contain important, dedicated interaction modules. In the future, structural information on the *cag*T4SS and full-length CagA and of the numerous complexes they form with host cell proteins should provide more detailed and mechanistic insights. In turn, this information might be translated into novel strategies to inhibit this sophisticated bacterial deadly machinery.

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Gastric Organoids: An Emerging Model System to Study *Helicobacter pylori* Pathogenesis

Malvika Pompaiah and Sina Bartfeld

Abstract *Helicobacter* research classically uses fixed human tissue, animal models or cancer cell lines. Each of these study objects has its advantages and has brought central insights into the infection process. Nevertheless, in model systems for basic and medical research, there is a gap between two-dimensional and most often transformed cell cultures and three-dimensional, highly organized tissues. In recent years, stem cell research has provided the means to fill this gap. The identification of the niche factors that support growth, expansion and differentiation of stem cells in vitro has allowed the development of three-dimensional culture systems called organoids. Gastric organoids are grown from gastric stem cells and are organized epithelial structures that comprise all the differentiated cell types of the stomach. They can be expanded without apparent limitation and are amenable to a wide range of standard laboratory techniques. Here, we review different stem cell-derived organoid model systems useful for *Helicobacter pylori* research and outline their advantages for infection studies.

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1 Introduction

1.1 Current Models for H. pylori *Research*

In 1983, Barry Marshall and Robin Warren described the staining, isolation and culture of curved bacilli from the stomach, now known as *Helicobacter pylori* (Warren and Marshall 1983). Their causative role in disease was highly questioned at the time. To demonstrate that this bacterium causes gastritis, they tried to infect healthy animals, such as pigs, rats and mice, but initially failed. So to fulfill Koch's postulates, Marshall himself drank a suspension of 10^9 colony-forming units of the bacteria, previously isolated from a gastritis patient (Marshall et al. 1985). He fell ill after about 1 week, with "feeling of fullness," vomiting and headache. Endoscopy confirmed the colonization of his stomach as well as mononuclear infiltrates (Marshall et al. 1985). This self-experiment did not only prove that *H. pylori* indeed causes acute gastritis, but also demonstrated the need for a study subject that reflects the natural host for infection research. Since then, several models have been established and we now have a variety of tools to analyze pathogenicity mechanisms.

Perhaps the most commonly used tools in *H. pylori* research are gastric epithelial cell lines that were originally derived from gastric tumors. They have advantages, since they are immortalized, cost-effective, accessible, easily maintained and a wide variety of experiments are well established. However, many molecular characteristics are modified in cancer cell lines, including genetic and epigenetic changes in tumor suppressor genes, oncogenes, cell cycle regulator genes, growth factors and their receptors. Additionally, cell lines have accumulated mutations during decades of culture in vitro and some are infected with viruses. The AGS cell line, for example, the workhorse of *Helicobacter* research, is commonly infected with parainfluenza virus type 5, influencing central pathways involved in immunity, proliferation and oncogenesis, such as signal transducer and activator of transcription (STAT) signaling as well as the interferon response (Young et al. 2007).

Primary cell culture models are generally a very good alternative to tumor cell lines, as they are not transformed. Culture models of human antral epithelial cells and normal gastric epithelial cells from biopsies have been developed (Boxberger et al. 1997; Richter-Dahlfors et al. 1998; Ootani et al. 2000), and some of these were already used to study bacterial infection (Richter-Dahlfors et al. 1998). While these studies laid important groundwork, the cells cannot be expanded and cultured long term as they lack the ability to renew themselves. Thus, a need to generate freshly isolated primary cells for each experiment makes this model less practical than others.

Conventional primary and tumor cell culture are two-dimensional (2D) and lack higher 3D organization. There have been efforts to grow cancer cell lines in gels composed of extracellular matrix proteins to support 3D organization. Indeed, in these cultures cancer cell lines can form 3D spheres and studies have shown that whether a cancer cell line was grown in 2 or 3D can influence the efficiency of applied drugs (Tung et al. 2011). This indicates that the 3D organization is an important feature of tissue reactions. The 3D tumor cell spheres, however, lack the combination of different cell types that are typical for tissue and organs. In the end, the stomach is—just as any other organ—much more complex than a monolayer of a single type of cells.

To analyze the impact of infection on any whole organ, such as the stomach, research currently has to turn to whole organisms. Many central descriptive studies, such as Warrens initial staining, are performed on human fixed tissue. This gives invaluable information allowing correlations to be made between human disease, marker expression and infection status. Experimental infection of humans has also been used for vaccine studies (Aebischer et al. 2008). Animal models include mice and gerbils, as well as pigs with a defined microbiota ("gnotobiotic" pigs) and even cats and dogs. All of them have their advantages and limitations. Because there are excellent recent reviews on animal models for *H. pylori* research (Lee 1998; Noto et al. 2016; Solnick et al. 2016), we will not discuss this here. Despite their usefulness, there is room between 2D monotypic cancer cell cultures and full organisms for new models that ideally combine the practicability, technical and ethical advantages of cancer cell lines and yet are closer to the human in vivo situation.

In the past decade, advances in stem cell research have allowed the development of new 3D stem cell-derived primary cell models called "organoids" for their striking resemblance to the in vivo counterpart tissue. In this review, we aim to provide an overview of gastric organoids for *H. pylori* infection. For this, we will introduce the development of organoids and their general advantages before discussing in detail the different types of gastric organoids, and their usefulness for *H. pylori* research.

2 Understanding of Adult Stem Cells and Their Niche Led to the Development of Organoid Culture

2.1 The Gastric Epithelium

Anatomically, the stomach is divided into three different regions: the proximal cardia (human) or forestomach (mouse) followed by the corpus (also termed fundus or body), which is the largest part of the stomach. Distally, the antrum leads to the pyloric sphincter that controls the entrance into the intestinal duodenum. Only the murine forestomach is lined by a keratinized squamous epithelium—all other parts of murine and human stomach are lined by a simple columnar epithelium that is organized into many regular, flask-like invaginations called gastric pits. Each pit feeds into a gastric unit, which is divided into the regions isthmus, neck and base. Neck and base together are named gland (Fig. 1a). The epithelium constantly renews itself and parts of it turn over every three days. Thus, the stomach epithelium harbors a tremendous regenerative capacity and the stem cells that fuel this constant need for new cells have long been thought to reside in the tissue itself (Lee et al. 1982).

The isthmus is a site of massive proliferation from where the cells migrate toward either the pit or the gland. Pit cells are characterized by dense apical mucus and a short life span of about 3 days (Karam and Leblond 1993b). Cells migrating to the base of the gland differentiate into either mucus neck cells or pepsinogen-secreting chief cells, which can have life spans of up to 6 months

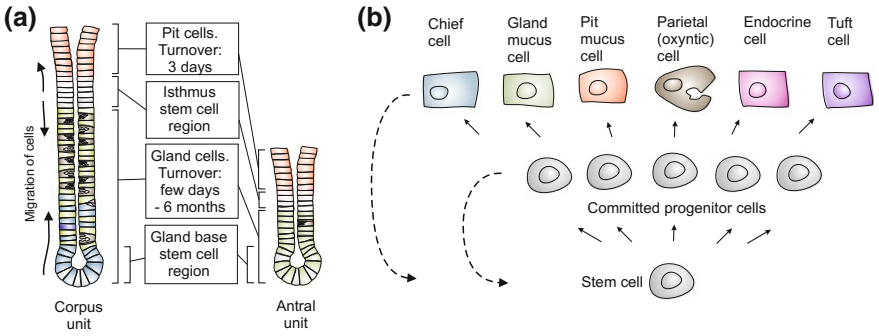

Fig. 1 Organization of gastric glands. *Left side*: Compared to antral units, corpus units are longer and characterized by high abundance of acid-producing parietal cells (*brown*). Each gastric unit is divided into the regions pit, isthmus and gland. The gland is further divided into the regions neck and base. Stem cells reside in the isthmus and at the base. The pit has very high turnover compared to the gland, where individual cells can have life spans of several months. The isthmus is the site of highest proliferation. *Right side*: Gastric glands harbor four main cell types: chief cells, gland mucus cells, pit mucus cells and parietal cells. Additional rare cell types are endocrine cells and tuft cells. Stem cells proliferate to give rise to generate committed progenitor cells, which can further proliferate and differentiate into the cell types of the stomach. Differentiated cells (i.e., chief cells) can gain stem cell capacity, indicating high levels of plasticity (*dashed arrow*)

(Karam and Leblond 1993a). Acid-secreting parietal cells migrate bidirectionally and are found in all regions of the gastric glands with highest numbers in the neck region (Karam 1993). The rare enteroendocrine cells, which account for <2% of the epithelium, are interspersed throughout the glands and secrete regulatory hormones such as gastrin, ghrelin, somatostatin and serotonin. The cellular composition of a gland defines its function, and glands in the corpus region are specialized to secrete acid and pepsinogen, while glands in the antrum are mainly mucus-producing. Mouse and human gastric glands have similar but not identical cellular compositions. Specifically, human antral glands are reported to contain higher numbers of chief and parietal cells than murine antral glands, which are virtually devoid of chief cells and contain only parietal cells when located at the intermediate zone toward the corpus (Lee et al. 1982; Lee and Leblond 1985a; Choi et al. 2014)

2.2 Gastric Stem Cells

It was long hypothesized that several tissues including stomach and intestine harbor resident adult tissue stem cells that constantly regenerate the organ during homeostasis (Fig. 1b). Early studies identified undifferentiated cells in the isthmus region of the glands (Lee and Leblond 1985b). The development of genetic lineage tracing empowered scientists to map the fate of the progeny from specific cells (reviewed in Kretzschmar and Watt 2012). Genetic marking of cells expressing the leucine-rich-repeat-containing G-protein-coupled receptor 5 (*Lgr5*) gene, which was originally described as a WNT response gene, showed that *Lgr5* identified very peculiar, slender cells wedged between the Paneth cells in the intestine, as well as cells at the base of gastric glands. Lineage tracing using *Lgr5-Egfp-IRES-CreERT2* mice, in which administration of tamoxifen induces irreversible genetic labeling in all *Lgr5*-expressing cells and their offspring, revealed that they can generate all cell lineages of the intestine as well as the stomach. Lineage tracing further showed that cells expressing the actin-binding protein villin (*Vil1*) (Qiao et al. 2007), sex-determining region Y (SRY)-box 2 (*Sox9*) (Arnold et al. 2011), gastrin receptor cholecystokinin B receptor (*Cckbr*) (Hayakawa et al. 2015b), transcription factor basic helix-loop-helix family member a15 (*Bhlha15* or *Mist1*) (Hayakawa et al. 2015a), or tumor necrosis factor receptor super family 19 (*Tnfrsf19*), encoding the protein TROY (Stange et al. 2013), have stem cell capacity in the stomach. Currently, the (possibly hierarchical) relations between the different stem cell populations are unclear (reviewed in Bartfeld and Koo 2016).

Homeostasis and proliferation of tissue-resident stem cells is maintained by the stem cell niche. Niche factors for gastric stem cells include Wnt family members (WNT), Notch, epidermal growth factor (EGF), fibroblast growth factor 10 (FGF-10) and gastrin (GAST) as well as an absence of bone morphogenetic protein (BMP) (Goodlad et al. 1987; Wang et al. 1996; Nyeng et al. 2007; Bredemeyer et al. 2009; Barker et al. 2010; Radulescu et al. 2013; Demitrack et al. 2015; reviewed in Bartfeld and Koo, 2016).

2.3 Organoids: Self-organizing Three-Dimensional Systems of Stem Cells and Differentiated Cells

The identification of the *Lgr5*$^+$ stem cells and their niche factors laid the groundwork for their maintenance in culture. In 2009, Toshiro Sato and colleagues in the laboratory of Hans Clevers published a landmark study, in which single sorted murine intestinal *Lgr5*$^+$ stem cells were seeded into an extracellular matrix and embedded in a medium containing the essential niche factors R-spondin1 (RSPO, a WNT agonist), EGF and noggin (NOG, a BMP antagonist). These four components (matrix, RSPO, EGF and NOG) allowed the stem cells to proliferate and expand. Initially, the cells form small cysts, but after few days, buds appear and grow into the matrix. The cells become polarized, with the apical side facing the lumen of the cystic structure. Individual cells differentiate into the specific cell types of the intestine, and a single organoid contains stem cells, progenitor cells, Paneth cells, enterocytes, enteroendocrine cells and goblet cells. Moreover, the cells even self-organize into domains that resemble the intestinal crypts, harboring mostly stem cells interspersed between Paneth cells and progenitor cells, and villus structures harboring enterocytes, goblet cells and enteroendocrine cells (Sato et al. 2009). Probably, the most important feature that makes these organoids so attractive for the laboratory is that they are long-lived: They can be split and re-seeded, frozen and thawed, and appear to have unlimited expansion capacity. Further, they are amenable to a wide range of standard laboratory techniques, including microscopy, RNA analysis, protein analysis, genetic manipulation using lentiviruses, bacterial artificial chromosomes (BACs) or genome editing based on clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) (Koo et al. 2012; Li et al. 2012; Schwank et al. 2013a; Andersson-Rolf et al. 2014; Bartfeld et al. 2015; Drost et al. 2015). Organoids can be grown from different vertebrates and different tissues, such as small intestine, colon, stomach, liver, pancreas, prostate, fallopian tube, gallbladder and others (Sato et al. 2009; Barker et al. 2010; Huch et al. 2013; Karthaus et al. 2014; Boj et al. 2015; Kessler et al. 2015; Scanu et al. 2015). Since the development of conditions for the growth of human organoids (Sato et al. 2011), it became clear that organoids can be grown from apparently every patient, from biopsies as well as from resection material (VanDussen et al. 2015; Fujii et al. 2016). Established living biobanks of intestinal organoids have also enabled drug testing (Van de Wetering et al. 2015). After the establishment of an organoid line, it can be used in a similar way to standard cell lines, with the important difference that organoids are untransformed, primary cells that faithfully mimic the epithelial organization in 3D. For further excellent general overviews about organoid cultures, we would like to direct the reader to other reviews (Sato and Clevers 2013; Lancaster and Knoblich 2014; Huch and Koo 2015; Fatehullah et al. 2016; Werner et al. 2016; Dedhia et al. 2016). Here, we will focus on gastric organoids and their use as an infection model for *H. pylori*.

3 Gastric Organoids

3.1 Organoids Grown from Adult or Pluripotent Stem Cells

Gastric organoids have been generated from tissue-resident adult stem cells (ASCs) (Barker et al. 2010) as well as from two types of pluripotent stem cells (PSCs): induced PSC (iPSC) and embryonic stem cells (ESCs) (McCracken et al. 2014) (Fig. 2). A distinctive difference between ASC- and PSC-derived organoids is the presence of mesenchymal cells. Initial seeding of ASC-derived organoids as well as PSC-derived organoids contains epithelial as well as mesenchymal cells. During further expansion by splitting and re-seeding, ASC-derived organoids become purely epithelial. Thus, this model allows the initial observation of mesenchymal–epithelial interactions and long-term study of pure epithelium. PSC-derived organoids are usually limited in their expansion and remain a combination of epithelium and mesenchyme.

The main technical differences between the systems regard the source and the handling: On the one hand, ASC-derived organoids are generated directly from tissue and mature within 1–2 weeks (depending on the tissue type). Once established, it appears that they can be expanded for an unlimited period of time. Lines can also be shared between laboratories, so for research on organoids expertise in their establishment is not required. On the other hand, PSC-derived organoids are generated from ESCs or iPSCs and each experiment requires their differentiation

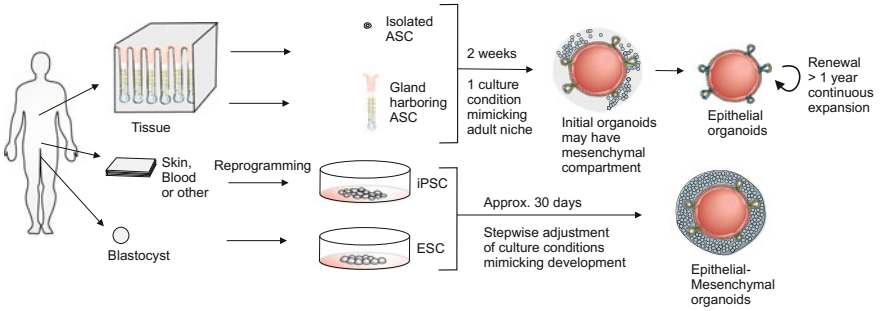

Fig. 2 Generation of organoids. Organoids can be generated either from tissue-resident adult stem cells (ASCs) or from pluripotent stem cells (PSCs). PSCs can be obtained either from blastocysts (embryonic stem cells, ESCs) or by reprogramming of differentiated cells from skin, blood or other tissues (induced PSCs, iPSCs). To generate organoids from ASCs, single ASCs can be isolated, for example based on marker gene expression. Alternatively, whole glands can be isolated from the tissue. Placed in optimal culture conditions, isolated glands round up, stem cells proliferate, and daughter cells differentiate to generate 3D organoids that harbor stem cells next to differentiated cells. After seeding, the culture may contain a mesenchymal compartment. By expansion (simple splitting and re-seeding), pure epithelial cultures can be maintained long term. To generate organoids from PSCs, lines of iPSCs or ESCs are cultured under stepwise changes of conditions to mimic the exposure to different developmental cues during organ development. Duration and steps vary between protocols. Resulting organoids have epithelial and mesenchymal compartments

from PSCs toward the (adult) epithelium, which usually takes about 30–60 days and a fine-tuned sequence of growth factors and inhibitors to lead the cells through the different stages of embryonic development and tissue-specific patterning. PSC-derived organoids have a limited life span; thus, each experiment starts with pluripotent cells and the purity of gastric cells (and the presence of non-wanted phenotypes) in the final culture needs to be monitored (Fig. 2) (reviewed in Wells and Spence 2014).

3.2 *Long-Term Self-renewing Purely Epithelial Organoids*

The first gastric organoid system to be developed was a direct advancement of the above-mentioned original intestinal culture system from the Clevers group (Sato et al. 2009). Meritxell Huch isolated murine antral gastric glands, embedded them into extracellular matrix and identified WNT, FGF-10 and GAST as stomach-specific niche factors that had to be added to the culture in addition to the intestinal factors RSPO, EGF and NOG to allow growth of the glands into organoids (Barker et al. 2010). Murine antral organoids grow into more cystic structures than their intestinal counterparts, but also have small buds that harbor the *Lgr5*$^{+}$ stem cells. Cells in the organoids differentiate into mucin 6 (MUC6)+ neck cells, MUC5AC+ pit cells, pepsinogen C (PGC)+ chief cells and enteroendocrine cells. After seeding, they expand within about 1 week to full size and can be split at a ratio of 1:5 every week, and cultures can be expanded long term (9 months at initial publication) (Barker et al. 2010). The same conditions also allow culture of organoids from the murine corpus (Stange et al. 2013; Schumacher et al. 2015a) and from isolated single *Tnfrsf19*/TROY+ chief cells (Stange et al. 2013). Corpus organoids also harbor neck, pit, chief and enteroendocrine cells (Stange et al. 2013). In addition, parietal cells are present in the initial seeding, but numbers decrease over time (Schumacher et al. 2015a) (Fig. 2).

To enable research on human stem cells, the culture system was then adapted for human intestine, which in addition to WNT, EGF, NOG, and RSPO requires inhibition of transforming growth factor beta (TGF-β) signaling, inhibition of p38 signaling as well as addition of nicotinamide to inhibit sirtuin activity. A Rho-kinase (ROCK) inhibitor is also added after seeding to inhibit anoikis (Sato et al. 2011). The conditions used for growing mouse gastric organoids plus the addition of the nicotinamide allow initial growth of human organoids (Bertaux-Skeirik et al. 2015) but not the long-term expansion (Bartfeld et al. 2015). The culture conditions used for growing human intestinal organoids also support growth of gastric glands into spheres (Schlaermann et al. 2016). The minimal and optimized culture for human gastric stem cells (including only EGF, NOG, RSPO, WNT, FGF10, GAST and TGF-β inhibition) allows the growth of gastric glands or single cells into complex organoids with a cystic body comprised mostly of pit cells and glandular buddings harboring neck cells, chief cells and rare enteroendocrine cells (Bartfeld et al. 2015). By manipulating the culture conditions, researchers

were able to differentiate organoids into three different types distinguished by differences in the mucus-producing cells. The first one consists of all the four gastric lineages, termed "complete type," the second has only the gland domain and is termed "gland type," and the third contains mainly pit cells and hence is termed "pit type." Although these cultures lack parietal cells, they contain the other four different cell types and can be maintained for more than a year without loss of expansion or differentiation capacity (Bartfeld et al. 2015).

ASC-derived organoids are probably the most practical, because they have the ability to self-renew and thus can be cultured long term (Fig. 3, right column). After establishment, they have a short maturation time of only one (for mouse) or two (for human) weeks. An interesting feature of this system is further that it allows the expansion of healthy tissue stem cells, as well as cells from metaplasia, such as Barrett's esophagus (Sato et al. 2011), or gastric cancer cells (Bartfeld et al. 2015). Thus, both cancer and healthy organoids can be established from the same patient, rendering this an ideal model for drug testing (Bartfeld et al. 2015; Van de Wetering et al. 2015). While establishment of cell lines or patient-derived xenograft models is only successful from a comparatively small number of patients, organoids can be grown from apparently every patient. Thus, uniquely, organoids allow the establishment of living biobanks and the genotypes in the biobanks cover the range known from large-scale genetic screening of patients, indicating that the generation

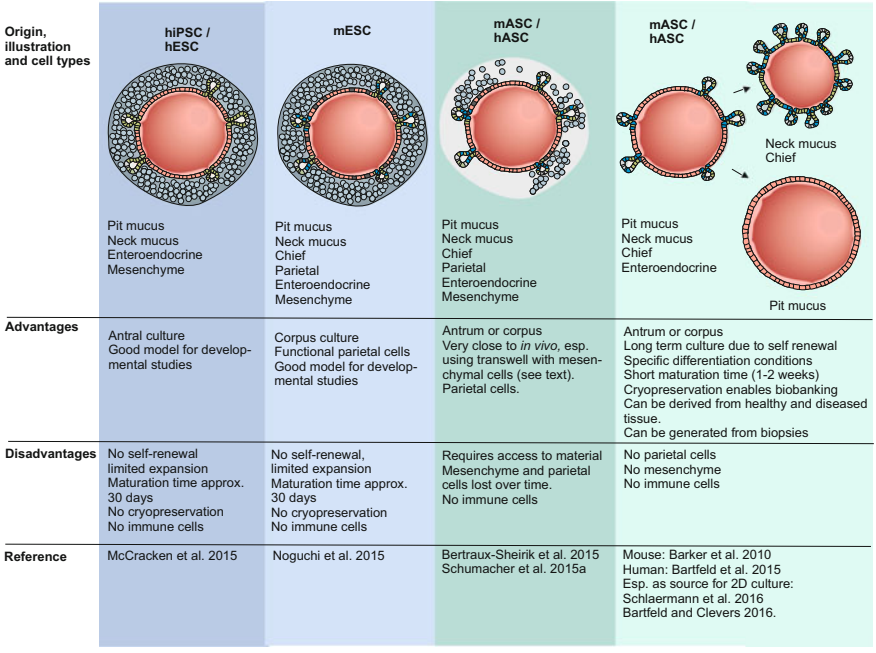

Fig. 3 Overview of different gastric organoid systems, their cellular composition, advantages and disadvantages. See text for more details on each system

of organoids is non-selective and thus represents the entire spectrum of patient genotypes (Van de Wetering et al. 2015; VanDussen et al. 2015; Fujii et al. 2016). This is a unique feature of organoids and enables research approaches targeting patient specificity: (i) experimental analysis of a specific parameter in a wide range of patients to identify patient-specific risk factors for disease development and (ii) the use of a specific subset of organoids, for example based on the genotype, to determine a response, for example to test the efficiency of a drug for a subset of patients. While all other methods (discussed below) also allow establishment of organoids from individual patients, this specific system is the only one that can be expanded long term and allows the generation of frozen stocks that can be stored in a living biobank to be thawed and expanded again for later studies.

Because of their purely epithelial nature, ASC-derived organoids allow the study of isolated epithelium without potentially confounding factors from mesenchyme or the immune system. Because the growth conditions maintain stem cells and progenitors, these organoids also allow the study of a possible direct effect of infection on stem cells. This may also have high impact for the clinic, as cancer can originate from stem cells (reviewed in Mills and Sansom 2015).

3.3 Epithelial/Mesenchymal Organoids Derived from Adult Stem Cells

In the initial seeding, ASC-derived gastric organoids cultured by the above-described or similar, adapted methods (Schumacher et al. 2015a; Bertaux-Skeirik et al. 2015) may also contain a mesenchymal layer (Feng et al. 2014). Interestingly, this coincides with the presence of parietal cells in initial culture of corpus organoids (Schumacher et al. 2015a), indicating that the development of parietal cells may depend on interactions with the mesenchyme, a hypothesis that is supported by ESC-derived organoids (Noguchi et al. 2015). In ASC-derived organoids, markers of mesenchymal cells and parietal cells decline with time and especially after passage (Feng et al. 2014; Schumacher et al. 2015a) (Fig. 2). Addition of immortalized stomach mesenchymal cells in the lower compartment of a transwell, with the matrix-supported organoids in the upper compartment, increased expression of differentiation markers including expression of the H^+/K^+ATPase of parietal cells (Schumacher et al. 2015a). Thus, short-term culture of mouse or human ASC-derived gastric organoids allows the study of mesenchymal–epithelial interactions and also contains parietal cells. These features are, however, not supported by long-term expansion of the cultures.

In parallel developments, Ootani and colleagues established a system for primary cell cultures that contain epithelial as well as mesenchymal cells. Their early work had shown that gastric primary cells build short-lived but well-differentiated monolayers of columnar MUC5AC+ pit cells when embedded in a collagen matrix

and exposed to an air–liquid interface (Ootani et al. 2003). In 2009, they published an intestinal culture system, in which neonatal tissue from murine intestine was seeded into collagen matrix. The tissue pieces built spheres that consisted of an inner polarized layer of epithelial cells surrounded by mesenchymal myofibroblasts. Supported by additional RSPO, the intestinal air–liquid interface cultures survived for one year with high levels of proliferation in the first month, which later decreased. Adult tissue-derived cultures clearly had shorter in vitro viability (Ootani et al. 2009). Murine neonatal gastric tissue also formed 3D epithelial/mesenchymal structures in the collagen-based air–liquid interface system. They could be maintained for an average of one month with a maximum of 80 days and mostly differentiated into MUC5AC+ pit cells and to a lesser extent into MUC6-positive neck cells and few parietal cells (Katano et al. 2013; Li et al. 2014). When these cultures were generated from mice harboring mutations predisposing for cancer development [such as in the genes Kirsten rat sarcoma viral oncogene homolog (*Kras*) or tumor protein p53 (*p53*)], gastric cultures could be passaged and expanded at least six passages (Li et al. 2014).

This organoid system is extremely close to the in vivo situation (Fig. 3, third column). Because it maintains parietal cells, the acidic environment of the stomach can be mimicked and the effect of the infection on parietal cells can also be studied. This has high clinical impact, because loss of parietal cells and accompanied antralization of the tissue is a pathologic feature of chronic *H. pylori* infection in patients. This system, however, requires access to human material, as it has a limited life span.

3.4 Epithelial/Mesenchymal Organoids Derived from PSC

Understanding and in vitro recapitulation of the adult stem cell niche provided the last building block for the generation of human gastrointestinal cells from PSCs. Contrary to ASCs, which are programmed to generate all cells of one particular tissue (such as the stomach), PSCs can become all cells of any tissue (such as stomach, nervous system or heart muscle) (Fig. 2). In two studies, McCracken and colleagues described the precise choreography of stepwise differentiation, mimicking the stages of embryonic development necessary to differentiate human PSCs into intestinal (McCracken et al. 2011) or gastric cells (McCracken et al. 2014). Generally, in this type of experiment, the stepwise differentiation is indicated by markers characteristic of specific developmental stages. For gastric development, human ESCs or iPSCs were first differentiated into definitive endoderm by adding activin A and BMP4. Next, the addition of WNT3a and FGF4 induced caudal-type homeobox 2 (*Cdx2*) expression, a marker for mid-hindgut. Simultaneous inhibition of BMP signaling by NOG resulted in the generation of foregut, as indicated by expression of the foregut endoderm marker sex-determining region Y (SRY)-box 2 (*Sox2*). Transferring these cultures to 3D extracellular matrix and treating them with EGF and retinoic acid (RA) induced antral development, characterized by

expression of pancreatic and duodenal homeobox 1 (*Pdx1*) and *Sox2*. After 34 days of stepwise differentiation, human gastric organoids expressed markers *Muc5AC*, *Muc6* of mucus pit and neck lineages as well as markers for several types of enteroendocrine lineages. Numbers of enteroendocrine cells were increased at low levels of EGF, indicating a previously unanticipated role for EGF in enteroendocrine cell development (McCracken et al. 2014).

Using a similar stepwise path through the developmental stages of the stomach, Noguchi and colleagues generated corpus organoids from mouse ESCs (Noguchi et al. 2015). In the first step, they cultured ESCs in suspension. In this condition, they grow into 3D aggregates called embryoid bodies, which consist of cells from all three germ layers. Transferred to adherent culture conditions and exposed to sonic hedgehog (SHH) and the Wnt antagonist Dickkopf 1 (DKK1), the embryoid bodies formed primordium spheroids expressing *Sox2* and the mesoderm marker BarH-like homeobox 1 (*Barx1*). The spheroids were embedded in extracellular matrix and supplemented with FGF10, WNT, NOG and RSPO. After a total of 60 days of differentiation, this led to formation of corpus organoids containing not only pit cells, neck cells and enteroendocrine cells, but also fully mature chief cells and functional parietal cells. Chief cells were able to secrete pepsinogen and parietal cells secreted acid upon stimulation with histamine (Noguchi et al. 2015).

Organoids derived from PSCs have been extremely useful for the study of developmental processes (Fig. 3, two left columns). They also provide the possibility to study the impact of infections on functional parietal cells, with high medical importance as outlined above. Demonstration of parietal cell development from human PSCs will be an important development in the future.

3.5 2D Monolayers Derived from Epithelial Organoids

While 3D cultures have their clear advantages, long-term organoid cultures from adult stem cells can also be used as a source for primary cells seeded in conventional 2D settings, either adherent on plastic, grown on a layer of extracellular matrix, or supported by a transwell (VanDussen et al. 2015; Bartfeld and Clevers 2015; Schlaermann et al. 2016). The ease and simplicity by which cells of various origins can be cultured long term makes it a practical alternative for primary cultures directly from tissue, freeing the researcher from the dependence on surgical specimens.

In summary, gastric organoids represent a useful bridge between traditional cell culture and in vivo models. Given the diversity of organoids grown from human and mouse tissue in vitro, it will be important to consider the experimental strengths and limitations of each system when designing experiments. For example, ASC-derived stomach organoids can be generated in less than two weeks, whereas PSC-derived stomach organoids require more than five weeks. The fine-tuned steps undertaken for the development of PSCs into gastric organoids may pose an initial barrier for inexperienced researchers. On the other hand, PSC-derived organoids

will be the best source to answer developmental questions. Interactions with the mesenchyme or parietal cells can best be studied in unpassaged ASC- or PSC-derived cultures.

4 The Use of Stem Cell-Derived Organoids for *H. pylori* Research

Organoids hold great promise as new tools for infection research. Future studies will show whether they can recapitulate phenotypes that are only present in 3D, deliver relevant results for infection-induced carcinogenesis that were impossible to obtain in mutated cancer cell lines or identify patient-specific responses and risk factors for disease development. However, one of the many exciting possibilities of organoids that have already been published is to study cell types that could previously not be cultured, for example mucus gland cells or parietal cells.

When Marshall infected himself with *H. pylori*, he developed acute gastritis characterized by polymorphonuclear neutrophil leukocyte infiltrates (Marshall et al. 1985). Today, it is known that inflammation is driven by the potent neutrophil attractor interleukin 8 (IL-8), which is a target gene of the central pathway of innate immunity, nuclear factor kappa B (NF-κB) (reviewed in Shimoyama and Crabtree 1998). Despite its importance, it is still not well understood how *H. pylori* activates NF-κB. In human gastric cancer cell lines, NF-κB activation depends on the cytotoxicity-associated gene pathogenicity island (*cag*PAI) of the bacterium, but not on the gene *cagA* (Bartfeld et al. 2010; Sokolova et al. 2013). In human gastric organoids, *H. pylori* also strongly induces NF-κB and expression of its target gene *IL-8*. However, contrary to what has been shown in cancer cell lines, *IL-8* expression does not depend on the presence of a functional *cag*PAI in *H. pylori* (Bartfeld et al. 2015). While the bacterial factor inducing NF-κB remains elusive (Backert and Naumann 2010), the study indicated an influence of the differentiation of the host cells: Organoids that contain mainly cell lineages of the gland produce higher levels of IL-8 than organoids that contain mainly cell lineages of the pit, leaving room for speculation whether this may be due to expression of a putative receptor or a possible barrier function of the pit mucus (Bartfeld et al. 2015). This demonstrates the unique potential of organoids: No in vitro system before has allowed the parallel infection of pit or gland cells. Similarly, another study used the unique possibility of organoids to study the response of previously non-culturable parietal cells (Schumacher et al. 2015b). A central pathologic feature of *H. pylori*-induced chronic gastritis is the loss of parietal cells, which leads to normal chief cell differentiation and antralization of corpus tissue (reviewed in Mills and Sansom 2015). Parietal cells are the main providers of SHH (Van Den Brink et al. 2001), which induces BMP in the mesenchyme and is necessary for differentiation (Shyer et al. 2015). Schumacher and colleagues examined *Shh* expression in gastric organoids in response to *H. pylori* infection. Importantly, these ASC-derived

organoids were used without passaging; thus, they contained parietal cells. Two days after infection, *H. pylori* induced *Shh* in organoids and the induction was abrogated by pretreatment with NF-κB inhibitor IV, a form of resveratrol (Schumacher et al. 2015b). It will be most interesting to follow up on the interplay between *H. pylori*, NF-κB, *Shh* induction and parietal cell survival in this unique model.

While the new possibilities of organoids are promising, it is also necessary to sound out which previously known hallmarks of infection are recapitulated in the new model. So how well can *H. pylori* infect gastric organoids? Several studies laid the groundwork and addressed colonization, adherence to epithelial cells, bacterial replication, CagA translocation and induction of host cell responses such as apoptosis and proliferative response (Posselt et al. 2013; Backert et al. 2015).

In the human stomach, the majority of *H. pylori* is found motile in the mucus layer and only a part of the bacterial population (between 0 and 41%) is found adherent to the epithelial cells (Hessey et al. 1990). Adherence of *H. pylori* to epithelial cells contributes to transient or persistent colonization of the host gastric mucosa and to efficient delivery of virulence factors such as CagA (Blaser et al. 1995; Blaser and Kirschner 1999). Naturally, the bacterium first interacts with the apical side of the epithelium. As the apical side normally faces the lumen of the organoids [although exceptions have been reported (Huang et al. 2015)], bacteria are commonly microinjected into the luminal space. Here, bacteria face a thick mucus barrier shielding the epithelial cells. Cancer cell lines that were classically used for *H. pylori* research, such as AGS cells, lack this mucus expression—thus, this is one of the factors that render organoids much closer to the in vivo situation, especially because the bacteria establish their replicative niche in this mucus layer in vivo (Schreiber et al. 2004). Indeed, studies in organoids demonstrated localization of bacteria close to or within the mucus layer after injection (Wroblewski et al. 2014; Bartfeld et al. 2015; Schumacher et al. 2015b; Bertaux-Skeirik et al. 2015). Bacteria are viable inside the organoids, as shown through re-culturing of previously injected bacteria (Bartfeld et al. 2015; Bertaux-Skeirik et al. 2015), and can also replicate there (Bertaux-Skeirik et al. 2015). In vivo, *H. pylori* uses taxis toward urea and away from gastric acid to reach the mucus (Schreiber et al. 2004; Huang et al. 2015) and this chemotaxis may in part also be functional in organoids, as they secrete urea, and this indeed attracts *H. pylori* (Huang et al. 2015). The contribution of a possible pH gradient to chemotaxis in organoids is currently unclear: There are some systems that have parietal cells and therefore should generally be able to establish a pH gradient (see above), but this has not been demonstrated. It will be interesting in the future to study a possible effect of a pH gradient on *H. pylori* localization and replication.

In contrast to classical cancer cell culture studies, in which often 100 bacteria are added per host cell, many of which also attach due to the lack of a physical barrier, in organoids attachment to epithelial cells is a comparatively rare event, but it can be observed with electron microscopy (Bartfeld et al. 2015). This opens up the possibility to study attachment, its prerequisites and local consequences under more physiological conditions, which is of medical interest because attachment rates of

bacteria in human gastritis patients vary with disease state: High attachment rates coincide with high pathology (Hessey et al. 1990).

After attachment, *H. pylori* translocates at least one bacterial protein into the host cell, CagA (Covacci et al. 1993; Backert et al. 2000). There, CagA is phosphorylated by Src (Selbach et al. 2002) and Abl family kinases (Mueller et al. 2012). While CagA phosphorylation in organoids remains to be shown, it can be detected in monolayers of cells derived from human organoids (Schlaermann et al. 2016), indicating that *H. pylori* is generally able to translocate CagA into cells derived from organoids. Bacterial CagA interacts with the tyrosine kinase receptor c-MET, leading to increased cell proliferation and morphological changes such as cell elongation. The interaction depends on the type IV secretion system (T4SS), encoded by the *cag*PAI, suggesting that c-MET interacts only with translocated CagA (Churin et al. 2003). In human gastric organoids, this interaction can also be observed, giving an indication that CagA may also be efficiently translocated into primary cells (McCracken et al. 2014; Bertaux-Skeirik et al. 2015). Also, CagA-dependent doubling in proliferation has been observed, based on quantification of newly incorporated labeled nucleoside analogs (5-ethynyl-2'-deoxyuridine, EdU) in organoids (Wroblewski et al. 2014; McCracken et al. 2014; Bertaux-Skeirik et al. 2015). This may be due to c-MET activation and not compensation for *H. pylori*-induced cell loss in the organoids, as levels of apoptosis were unchanged (Wroblewski et al. 2014). In infected organoids, c-MET co-immunoprecipitated with cell surface antigen cluster of differentiation 44 (CD44) and hepatocyte growth factor (HGF) and this interaction depended on CagA. Proliferation was dependent on this interaction, as organoids derived from a CD44-deficient knockout mouse model showed neither complex formation nor proliferation (Bertaux-Skeirik et al. 2015).

In summary, many characteristic hallmarks of *H. pylori* infection such as attachment, colonization, NF-κB activation, CagA-c-MET interaction and increased host cell proliferation have been recapitulated in organoids, indicating that they are a useful new model for *H. pylori* research. Further, several studies highlighted previously unknown aspects of host response to infection, using the unique features of organoids, for example the effects of host cell differentiation on the innate immune response.

5 Concluding Remarks

In addition to their invaluable utility for better understanding development, organoids open up new avenues for a wide range of applications. They have been used to model cancer development using stepwise addition of cancer mutations using CRISPR/Cas9 (Matano et al. 2015; Drost et al. 2015) or knockout mice (Li et al. 2014). CRISPR/Cas9 has also been used to repair a genetic mutation in the CFTR gene in organoids generated from cystic fibrosis patients (Schwank et al. 2013b). Organoids have been transplanted into the mouse colon (Yui et al. 2012). Biobanks

of colon cancer organoids (Van de Wetering et al. 2015; Fujii et al. 2016) represent the range of mutations known from previous genetic analysis and can be used for drug screening (Van de Wetering et al. 2015). Human gastric organoids can also be grown from tumors (Bartfeld et al. 2015), and it will be interesting to see whether this technique can be expanded to tissue from gastric precancerous lesions in the future.

For infection biology, and especially for *H. pylori* research, organoids promise not only a unique resource for primary cells, but also the potential to study the different cell lineages in the stomach in 3D organization. As the presence of parietal cells seems to coincide with the presence of mesenchymal cells, the interplay between infection, mesenchyme and parietal cells may unravel functional insights into the early steps of metaplastic changes in gastric tissue that are characterized by depletion of parietal cells. Biobanks of organoids may identify interindividual factors that play a role in infection and could have a high clinical relevance as putative risk factors for cancer development.

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DNA Transfer and Toll-like Receptor Modulation by *Helicobacter pylori*

Matthew Gordon Varga and Richard M. Peek

Abstract *Helicobacter pylori* is the most common bacterial infection worldwide, and virtually all infected persons develop co-existing gastritis. *H. pylori* is able to send and receive signals from the gastric mucosa, which enables both host and microbe to engage in a dynamic equilibrium. In order to persist within the human host, *H. pylori* has adopted dichotomous strategies to both induce inflammation as a means of liberating nutrients while simultaneously tempering the immune response to augment its survival. Toll-like receptors (TLRs) and Nod proteins are innate immune receptors that are present in epithelial cells and represent the first line of defense against pathogens. To ensure persistence, *H. pylori* manipulates TLR-mediated defenses using strategies that include rendering its LPS and flagellin to be non-stimulatory to TLR4 and TLR5, respectively; translocating peptidoglycan into host cells to induce NOD1-mediated anti-inflammatory responses; and translocating DNA into host cells to induce TLR9 activation.

Contents

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1 Introduction

Helicobacter pylori is a Gram-negative pathogen that infects the stomachs of human hosts; greater than fifty percent of the world's population is colonized by this pathogen, thus making it the most common bacterial infection worldwide. Although most persons infected with *H. pylori* remain asymptomatic, a minority of patients can develop gastric or duodenal ulcers (10–15%) or gastric adenocarcinoma (1–3%) (Peek and Blaser 2002; Amieva and Peek 2016). However, a mere one percent of half the world's population represents a prodigious number of individuals that are plagued by cancer of the stomach, which is why gastric cancer is currently the third leading cause of cancer-related death worldwide and why the World Health Organization has classified *H. pylori* as a class I carcinogen (Carneiro 2014; Ferlay et al. 2015).

With only 1% of infected individuals progressing toward gastric cancer, *H. pylori*-induced disease is the exception rather than the rule. Carcinogenic capabilities of *H. pylori* are mediated by a triumvirate of factors including bacterial virulence constituents, host genetics, and environmental influences, and there is increasing evidence that a dis-symbiosis of these factors mediates disease progression. In order to survive, *H. pylori* must initiate colonization as well as liberate nutrients from the host; however, a consequence of this is a phenotype that is pro-inflammatory and thus not sustainable for prolonged infection. Therefore, to be successful, *H. pylori* must develop and maintain strategies to both liberate nutrients and also avoid the host immune response.

2 Toll-like Receptor Modulation by *H. pylori*

2.1 Toll-like Receptors

Toll-like receptors (TLRs) belong to a class of transmembrane receptors known as pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) (Peek et al. 2010; Castano-Rodriguez et al. 2014;

Pachathundikandi et al. 2015). PAMPs can originate from a wide array of molecules such as lipids, nucleic acids, and specific proteins that can be derived from organisms of bacterial, viral, or fungal origin. So far there are 10 identified human TLRs, however, not all are manipulated by *H. pylori*; TLR2 as a homo- or hetero-dimer with TLR1 or TLR6 (lipoteichoic acid, NapA, Hsp60), TLR4 (lipopolysaccharide), TLR5 (flagella), TLR9 (hypo-methylated DNA), and TLR10 hetero-dimerized with TLR2 (hypothesized to detect lipopolysaccharides) have been shown to interact with *H. pylori* (Peek et al. 2010; Kim et al. 2013; Nagashima et al. 2015; Koch et al. 2015; Pachathundikandi and Backert 2016). TLRs are an essential component of the innate immune system and are expressed both on cell surfaces and intracellularly. They are localized to a wide array of cell types including macrophages and dendritic cells, as well as non-immune cells including gastric epithelial cells. Upon microbial ligand binding to a leucine-rich repeat (LRR) ectodomain, TLRs dimerize and adaptor molecules such as MyD88 or TRIF complex with the intra-cytoplasmic Toll/IL-1 receptor (TIR) domain (Motshwene et al. 2009). Activation of TLRs induces signaling cascades that eventually lead to the transcription of both pro- and anti-inflammatory cytokines, as well as type I interferons. Despite extensive research into the mechanisms of TLR activation, their exact role in *H. pylori* infection remains controversial.

2.2 *TLR4*/H. pylori *Lipopolysaccharide Interactions*

H. pylori lipopolysaccharide (LPS) is a highly specialized structure that is uniquely adapted to maintain persistence within the gastric niche. This is primarily accomplished through decoration of the *H. pylori* LPS O-antigen with Lewis antigen, which is the outermost domain of the LPS molecule. Lewis antigens mimic host Lewis antigens that are expressed on the apical surface epithelium and within the glands of both the antrum and corpus (Kobayashi et al. 1993; Nogueira et al. 2004). Through this molecular mimicry, *H. pylori* can evade immune detection, but this heightens the risk of eliciting autoimmune responses (Monteiro 2001). Additionally, *H. pylori* harbors unique modifications to the lipid A core domain (Perez-Perez et al. 1995; Hajjar et al. 2002; Cullen et al. 2011). The lipid A core is the inner most domain of LPS, referred to as endotoxin, and is the ligand for the TLR4-MD2 immune complex. Compared to other bacterial LPS moieties such as *Escherichia coli*, *H. pylori* LPS has ~1000 fold less endotoxicity (King et al. 2009; Li et al. 2016), and this reduction is attributed to 3 major modifications to the lipid A core. The first modification is a hypoacylation pattern where *H. pylori* is tetra-acylated compared to hexa- or penta-acylated chains. Secondly, hypoacylated fatty acids have longer carbon chain lengths (2 18-carbon and 2 16-carbon chains) compared to the optimal chain lengths required for robust TLR4 activation. Lastly, *H. pylori* LPS is hypo-phosphorylated, an adaptation that renders it less susceptible to destruction by cationic antimicrobial peptides (CAMPs) (Cullen et al. 2011; Li et al. 2016). The role of TLR4 in immune activation is controversial.

Monoclonal anti-TLR4 antibodies in the presence of *H. pylori*-epithelial cell co-cultures failed to block IL-8 secretion (Su et al. 2003) and *H. pylori* infected HEK293 cells transfected with TLR4 failed to induce NF-κB activation (Smith et al. 2003b). However, *H. pylori* can up-regulate TLR4 expression in gastric epithelial cell lines, and it has been shown that *H. pylori* may up-regulate TLR4 for adherence to the epithelial cell surface (Su et al. 2003). In contrast, immune recognition of *H. pylori* has also been shown to be TLR4 independent (Backhed et al. 2003; Ishihara et al. 2004), and *H. pylori* LPS may actually antagonize TLR4 (Lepper et al. 2005).

2.3 *TLR5*/H. pylori *Flagellin Interactions*

The natural ligand of TLR5 is flagellin, specifically the highly conserved N-terminus of the D1 domain (Smith et al. 2003a). Since *H. pylori* is a flagellated bacterium, and TLR5 is expressed in the gastric epithelium, this pathogen should be capable of inducing TLR5-mediated pro-inflammatory signaling cascades. However, *H. pylori* flagellin is not recognized by TLR5 (Lee et al. 2003; Gewirtz et al. 2004; Andersen-Nissen et al. 2005), due to a mutation in the conserved domain of FlaA (Andersen-Nissen et al. 2005). This mutation, which occurs in the D0-D1 domain between amino acids 89–96, renders *H. pylori* flagella inert for TLR5 activation, and when these amino acids are substituted into the corresponding region of the *Salmonella enterica* serovar Typhimurium FliC, its ability to activate TLR5 is similarly lost (Andersen-Nissen et al. 2005). Taken together, these data suggest an important role for *H. pylori* FlaA in maintaining persistence within the gastric niche by dampening the innate immune response.

3 TLR9 Functions in the Innate Immune Response

DNA is the fundamental molecule of nearly all living organisms and consequently is normally sequestered within eukaryotic nuclear envelopes, bacterial cell walls, or viral capsids. During the course of infection, DNA can be actively released or released as a result of degradation from invading microbes or damaged host cells. TLR9 is an endosome-bound, transmembrane receptor that detects these aberrant DNAs and orchestrates a cognate immune response (Hemmi et al. 2000). TLR9 expression is most abundant in dendritic cells (DCs), B cells, macrophages, and other antigen presenting cells (APCs); however, it is also expressed in epithelial cells. In plasmacytoid dendritic cells (pDCs) and B cells, TLR9 activation classically leads to the release of pro-inflammatory cytokines and type I interferons, while epithelial responses are less well defined. TLR9 was originally thought to discern pathogenic DNAs based upon the presence of hypo-methylated CpG DNA motifs (which are rare in eukaryotic genomes); however, accumulating evidence

suggests that TLR9 can also recognize DNA in a sequence-independent manner via structural components such as the saccharide backbone (Haas et al. 2008; Wagner 2008; Ohto et al. 2015). Therefore, in an attempt to prevent the recognition of "self" DNA, evolutionary pressure has relegated TLR9 to endosomal sequestration (Barton et al. 2006). The consequences of TLR9 surface expression were characterized in a study in which TLR9 transmembrane expressing mutant mice were generated by exchanging the localization signal of TLR9 with that of TLR4. TLR9 transmembrane mutant mice died from systemic inflammation and anemia within 4 weeks (Mouchess et al. 2011). Since TLR9 is constrained to the endosome, cells of the immune system must internalize pathogens or pathogenic DNA before it can be detected by TLR9. Most immune cells accomplish this task through either receptor-mediated endocytosis in response to scavenger receptor binding, phagocytosis of complement-mediated opsonized material, or Fc receptor-mediated uptake of antibody opsonized material or a combination of these processes (Brencicova and Diebold 2013). In the case of "self" DNA detected from apoptotic cells, select marginal zone B (MZB) and B1a B cells can internalize chromatin complexes and induce a TLR9-mediated immunoregulatory response through IL-10 to counteract the pro-inflammatory signaling induced by DNA-antibody complexes internalized by rheumatoid factor expressing B cells (Miles et al. 2012). The TLR9-mediated immune response, therefore, is a highly complex response that is not only dependent on cellular localization both between cell types and within cells, but is also dependent upon the ligand and its origin.

3.1 TLR9 Regulation and Signaling

Takeshita et al. have extensively studied *tlr9* gene regulation in both humans and mice. The authors found that *tlr9* is regulated by 4 *cis* promoter elements: CRE, 5′-PU Box, 3′-PU box, and C/EBP. The transcription factors that interact with these *cis* promoter elements include CREB1 (CRE site), Ets2, Elf1, Elk1 (5′PU site), and C/EBPα (CEBP site); the authors could not identify the protein that bound the 3′-PU box. Additionally, this group found that CREB and members of the C/EBP family directly *trans*activate the *tlr9* promoter while the Ets family members Ets2, Elk1, and Elf1 enhance *trans* activity. Furthermore, the authors posit that CREB1, Ets2, Elf1, Elk1, and C/EBPα physically interact with each other and with CBP/p300 to attain maximal transcription. To garner a deeper comprehension of *tlr9* gene regulation in response to stimuli such as CpG DNA, the authors challenged cells transfected with a luciferase reporter linked to the entire *tlr9* promoter. Challenge with CpG DNA significantly reduced *tlr9* promoter activity which resulted from an interaction with the pro-inflammatory transcription factors NF-κB, c-Jun, and AP-1. The authors also found that the Ets family member Spi1 acted as a *tlr9* gene repressor but could not identify if this protein acted directly or indirectly to inhibit transcription (Takeshita et al. 2004). Taken together, these data can

provide valuable insight into cell type-specific *tlr9* gene regulation and could lead to more targeted strategies for the use of CpG DNAs as therapeutics.

Posttranscriptional regulation of TLR9 begins in the endoplasmic reticulum (ER) where it is synthesized as a full-length TLR9 (FL-TLR9) protein under the supervision of chaperone proteins GP96 and PRAT4A, which ensure proper protein folding and function. Inside the ER, FL-TLR9 homo-dimerizes and binds the chaperone protein UNC93B1 (Kim et al. 2008). UNC93B1 is a multipass transmembrane protein that is integral in TLR9 trafficking. UNC93B1 facilitates the transport of FL-TLR9 to the Golgi via a COP-II vesicle (Lee et al. 2013). Once in the Golgi, FL-TLR9 bound UNC93B1 complexes with adaptor-protein 2 and is either secreted in a $VAMP3^+$ endosome or may be transiently cycled through the plasma membrane before being compartmentalized into an endosome (Lee et al. 2013). Inside the acidified endosome, FL-TLR9 ($\sim$150 kDa) is cleaved by cathepsin and asparagine endopetidases to form a cleaved, untrimmed TLR9 protein ($\sim$90 kDa). This version of TLR9 is cleaved once more by cathepsin to form the mature, cleaved form of TLR9 that is capable of binding DNA (Ewald et al. 2008; Park et al. 2008; Ewald et al. 2011). Granulin, HMGB1, and LL-37 have all been implicated as a DNA binding proteins that may facilitate DNA delivery to TLR9 (Lande et al. 2007; Tian et al. 2007; Yanai et al. 2009; Park et al. 2011). Although DNA can bind un-cleaved TLR9, downstream signaling cannot occur until after proteolysis (Ewald et al. 2008). CpG DNA binding to mature, cleaved TLR9 within the endo-lysosome induces a conformational change that enables the interaction between the cytoplasmic TIR domains of the homodimer (Owyang et al. 2012). These dimerized TIR domains recruit the adaptor molecule MyD88 to form the myddosome (Latz et al. 2004). Once localized to an endosome, TLR9 trafficking dictates the type of downstream response (Honda et al. 2005; Guiducci et al. 2006). For example, ligand binding within the $VAMP3^+$ endosome can yield pro-inflammatory responses mediated by transcription factors such as NF-κB, AP-1, or CREB (Latz et al. 2004; Leifer et al. 2004; Kim et al. 2008). However, adaptor-protein 3 can bind the myddosome and facilitate endosomal fusion with a $LAMP2^+$ lysosome-related organelle (LRO), and together with TRAF-3, facilitate an IRF-7-mediated, type I interferon response to CpG DNA (Sasai et al. 2010) (Fig. 1).

3.2 Duality of TLR9-Mediated Responses

Not all TLR activation promotes pro-inflammatory signaling cascades, and there have been numerous investigations into how TLR9 may promote both pro- and anti-inflammatory responses in the gut. These studies were particularly focused on how dysregulation of TLR9 signaling could lead to the development of inflammatory bowel disease or colitis (Rachmilewitz et al. 2004; Katakura et al. 2005; Lee et al. 2006; Luther et al. 2011; Hotte et al. 2012; Jounai et al. 2012; O'Hara et al. 2012; Owyang et al. 2012).

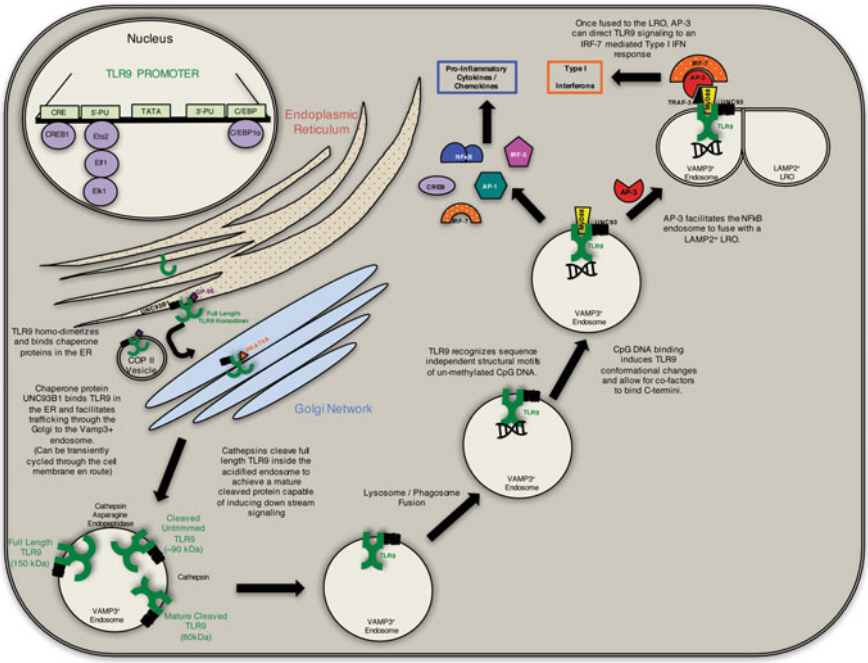

Fig. 1 TLR9 regulation and signaling: TLR9 expression is regulated by *cis* and *trans* elements including the CRE site, 5′-PU box, 3′-PU box, and a C/EBP site. These sites bind the transcription factors CREB1, Ets2, Elf1, Elk1, and C/EBP1α. Full-length TLR9 (FL-TLR9) is then synthesized in the ER as a homodimer with the aid of chaperone proteins UNC93B1 and GP96. The full-length homodimer is then shuttled to the Golgi via a COPII vesicle where it binds the chaperone protein PRAT4a to facilitate trafficking. The fully synthesized FL-TLR9 is then incorporated into a $VAMP3^+$ endosome where cathepsin and asparagine endopeptidases cleave the receptor to form the mature, cleaved form. This mature form of TLR9 is then free to induce downstream signaling cascades upon ligand binding. Cellular localization and interactions with adaptor proteins can further differentiate the type of signaling response upon binding its cognate ligand

pDCs perpetually monitor the microbiota and present microbial antigens to immune cells within the lamina propria. However, healthy individuals do not develop overt inflammatory responses to commensals while they can develop inflammatory responses to pathogens. In a study designed to elucidate the mechanism of how TLR9 may contribute to the maintenance of intestinal homeostasis, the authors found that TLR9 deficiency alone was not sufficient to alter the general composition and signaling capacity of the intestinal immune system (Hofmann et al. 2014). However, when the authors investigated specific sub-populations of immune cells from wild-type and TLR9 knockout mice, they found significant functional changes. These were related to differences in the regulation of the transcription factors NF-κB and CREB from both sets of mice. While both transcription factors compete for binding with CBP, the NF-κB-CBP complex predominantly regulates pro-inflammatory gene transcription while the CREB-CBP

complex is critical for the regulation of the anti-inflammatory cytokine IL-10. In TLR9 deficient mice, myeloid cells in the lamina propria displayed a significant bias toward NF-κB activity, while intestinal T cell populations displayed a lack of counter-regulatory mechanisms to control the inflammatory response (perhaps due to lack of APC derived IL-10). Furthermore, when $CD4^+$ T cells from *Tlr9*$^{-/-}$ mice were transplanted into wild-type mice, recipient mice developed severe colitis similar to mice that received naive splenic T cells (Hofmann et al. 2014). These findings were corroborated by another study that revealed that the colitogenic potential of transplanted splenic T cells from germ-free mice could be reversed by exclusively exposing them to synthetic CpG oligodeoxynucleotides (ODNs) treatment (Bleich et al. 2009) The anti-inflammatory responses induced by TLR9 in the gut may be due to the ability of this receptor to regulate the secretion of type I interferon (Katakura et al. 2005; Mancuso et al. 2007; Otani et al. 2012; Steinhagen et al. 2012). TLR9 is localized in many different cell types; however, it is the most abundant in pDCs, a class of immune cell that readily secrete type I interferons (IFNs). Studies have shown that TLR9 signaling activated by probiotic DNA binding can inhibit colitis through the induction of type I IFNs (Katakura et al. 2005). The same study also suggested that the probiotic DNAs were absorbed in the small intestine and could have a systemic anti-inflammatory response that manifests in reduced severity of colitis (Katakura et al. 2005). Taken together, these data suggest that the physiological interaction of TLR9 with bacterial CpG DNA is essential for the maintenance of intestinal homeostasis by inducing counter-regulatory, anti-inflammatory mechanisms.

While pDCs selectively sample the gut lumen and activate adaptive immune responses, epithelial cells are continuously exposed to the luminal microbiota and their products. TLR9 has also been shown to modulate both pro- and anti-inflammatory signaling in intestinal epithelial cells. Like gastric epithelial cells, intestinal epithelial cells express TLR9 in both the apical and basolateral compartments (Schmausser et al. 2004; Lee et al. 2006). When polarized intestinal epithelial cells were challenged with CpG DNA at either the apical or basolateral surface, only basolateral challenge resulted in NF-κB activation and IL-8 secretion. Apical stimulation failed to induce NF-κB activation due to an inability to degrade β-TrCP ubiquitinated proteins (such as p105/NF-κB1). Consequently, when the genes induced by apical versus basolateral stimulation were examined, less than half of them were shared. Although apical stimulation did not result in a canonical NF-κB mediated anti-microbial response, it did induce targets of the Wnt pathway with anti-microbial activity, particularly cryptidins. Additionally, apical TLR9 stimulation promotes immune tolerance and can inhibit pro-inflammatory IL-8 secretion resultant from basolateral challenge with TLR9 agonists, as well as TLR2, TLR3, or TLR5 agonists. Basolateral TLR9 activation also resulted in a tolerogenic response when challenged repeatedly. Compared to the polarized intestinal epithelial cells, non-polarized cells induce a pro-inflammatory phenotype similar to that of basolaterally challenged cells (Lee et al. 2006). This suggests that cell polarization can modify the signaling pathway of TLR9 in the intestinal epithelium. These studies highlight the importance of TLR9 in maintaining gut homeostasis and

provide insight into possible mechanisms of how TLR9 deficiency or site-specific activation may contribute to IBD or colitis.

How TLR9 regulates its response to DNA remains controversial; some studies posit that the micro-environment may regulate the type of response (Hiramatsu et al. 2013), while others suggest that the response is regulated by signal localization (either apically or basolaterally) (Lee et al. 2006). Another hypothesis suggests that the response is sequence-/structure-dependent since some commensal DNAs have been shown to be anti-inflammatory compared to pro-inflammatory pathogenic DNAs (Jijon et al. 2004; Rachmilewitz et al. 2004; Rakoff-Nahoum et al. 2004; Ghadimi et al. 2010). Despite its mode of action, TLR9 signaling has been shown to be of great importance in maintaining gut homeostasis and has vast potential as a therapeutic target in diseases where the inflammatory response is dysregulated.

4 DNA Translocation Strategies

Bacterial T4SSs are responsible for the mobilization of macromolecules including monomeric proteins, multimeric toxins, and DNA-protein complexes across the envelopes of Gram-negative or Gram-positive bacteria (Cascales and Christie 2003; Alvarez-Martinez and Christie 2009; Fronzes et al. 2009a; Backert et al. 2015). T4SSs can be categorized into three groups: conjugation systems, effector translocation systems, and DNA release/uptake systems (Alvarez-Martinez and Christie 2009). Although there is extensive versatility in bacterial T4SSs, the most common and well-studied subset of bacterial T4SSs is the conjugative system. The conjugative T4SSs are of significant medical relevance due to their capacity for widespread transmission of antibiotic resistance genes or virulence genes through plasmid or chromosomal DNA transfer (Cascales et al. 2013). Conjugative bacterial T4SS are not only capable of translocating DNA out of donor cells, but also they can take up DNA from the extracellular milieu. Additionally, organisms can use the DNA release/uptake T4SSs to release DNA substrates into the extracellular milieu in a contact independent manner (Cascales and Christie 2003). DNA transfer events between bacterial species or into the extracellular milieu are very common; however, transkingdom DNA transfer events are rare. To date, there are only a few bacterial species capable of translocating DNA into a eukaryotic host including: *Agrobacterium tumefaciens*, *E. coli* (Waters 2001) (Fernandez-Gonzalez et al. 2011), *Bartonella henselae* (Schroder et al. 2011), and most recently *H. pylori* (Varga et al. 2016).

4.1 DNA Translocation Through the T4SS

A. tumefaciens uses a VirB/D4 conjugation system to deliver oncogenic DNA (T-DNA) in conjunction with effector chaperone proteins into susceptible plant

cells, resulting in crown gall disease. This bacterial T4SS has served as the canonical conjugative secretion system and has been studied most extensively. The *A. tumefaciens* T4SS is composed of 12 proteins, VirB1-11, and VirD4 (Schroder and Lanka 2005). Most bacterial T4SS, including the *H. pylori cag* T4SS and the *comB* T4SS, share some homology in sequence or function to the VirB/D4 proteins of this archetypal secretion system (Frick-Cheng et al. 2016).

Conjugation can be categorized into three distinct biochemical reactions. First, DNA transfer and replication (Dtr) proteins bind to its cognate origin of transfer (*oriT*) sequences and initiate the processing of DNA for transfer (de la Cruz et al. 2010; Zechner et al. 2012) (Wong et al. 2012). Second, the Dtr-*oriT* complex (relaxosome) binds to the type IV coupling protein (T4CP) (Gomis-Ruth et al. 2002). Lastly, the T4CP delivers the DNA substrate to the transenvelope channel composed of mating pair formation (mpf) proteins that shuttle DNA across the cell membrane (Schroder and Lanka 2005; Wallden et al. 2010). The process of DNA transfer is energized by the ATPases VirD4, VirB11, and VirB4 components (Christie and Cascales 2005; Schroder and Lanka 2005; Fronzes et al. 2009a). In Gram-negative bacteria such as *A. tumefaciens* or *E. coli*, the central hub protein, VirB10, spans both the inner and outer membranes and is decorated by structural proteins VirB7 and VirB9 in a 1:1:1 ratio to form a 14-fold symmetrical outer membrane pore which termed the core complex (Fronzes et al. 2009b; Low et al. 2014). This core complex serves as the structural scaffold for the biogenesis of the outer membrane pore and the pilus (composed mostly of VirB2 and few VirB5 proteins) (Schroder and Lanka 2005; Fronzes et al. 2009a; Low et al. 2014). The channel gate opens as a result of structural changes of VirB10 in response to ATP utilization by VirD4 and VirB11, as well as DNA substrate docking (Cascales et al. 2013). Once the channel is open, the DNA substrate is able to move to the bacterial cell surface (Cascales et al. 2013). The pilus and core complex have been well characterized to date; however, the structure of the interacting proteins outside of the core complex, termed the inner membrane complex (IMC), are less well defined. In the *E. coli* R388 encoded T4SS, the core complex is connected to the IMC via a central stalk region (Low et al. 2014). At the distal end of the stalk, there are two barrel-shaped densities that display C_2 symmetries composed mostly of VirB4 (Low et al. 2014). The structure of the *E. coli* R388 system is suggestive of a two-step DNA transfer process in which VirD4 pumps the relaxosome-DNA substrate into the periplasm or by an IMC-mediated passage through the inner membrane. Once in the periplasm, the DNA substrate can then be secreted via the core complex (Low et al. 2014).

4.2 The H. pylori cag *Pathogenicity Island (PAI)*

The *cag*PAI is a 40kb gene locus that encodes proteins required to assemble a type IV secretion system (T4SS). The *H. pylori cag* T4SS retains significant homology to the archetypal T4SS of *A. tumefaciens* and has been shown to translocate the

effector molecules CagA and peptidoglycan into host cells and has recently been shown to translocate DNA as well (Odenbreit et al. 2000; Viala et al. 2004; Varga et al. 2016).

The primary effector of the *cag* T4SS is CagA, a bacterial oncoprotein that is injected into host cells upon bacterial attachment. Following translocation into host epithelial cells, CagA can either be phosphorylated by Src and Abl kinases at specific EPIYA amino acid motifs at the 3′ terminus or can remain un-phosphorylated (Mueller et al. 2012). Phosphorylated CagA activates multiple cellular phosphatases which can subsequently lead to morphological changes and induce responses similar to those of growth factor stimulation (Selbach et al. 2009). Similarly, unmodified CagA has been shown to induce mitogenic and pro-inflammatory responses as well as weaken cell-cell junctions to induce a loss of cellular polarity (Amieva et al. 2003; Bagnoli et al. 2005; Suzuki et al. 2005; Murata-Kamiya et al. 2007; Saadat et al. 2007; Franco et al. 2008).

4.3 Structure of the H. pylori Cag *T4SS*

In contrast to the conjugative T4SS of *A. tumefaciens* or *E. coli* R388 which can translocate DNA substrates into eukaryotic hosts (Chilton et al. 1977; Waters 2001), the *H. pylori* T4SS has been categorized as an effector/translocator T4SS because until recently, only CagA and peptidoglycan have been shown to be translocated (Odenbreit et al. 2000; Viala et al. 2004). Compared to the conjugative T4SS, relatively little is known about the structure of effector translocation T4SSs (Frick-Cheng et al. 2016). The *H. pylori cag* pathogenicity island is composed of 18 genes required for T4SS-dependent phenotypes (Fischer et al. 2001). While some of these genes share sequence relatedness to other bacterial species, the sequence conservation is quite low, and 9 of the 18 genes are fundamentally unique to *H. pylori* and do not display any homology to other bacterial proteins (Frick-Cheng et al. 2016). Recent studies have shown that the *H. pylori cag* T4SS core complex is composed of proteins unrelated to components of T4SS in other bacteria, and similarly, the architecture of this T4SS is vastly different from those of other T4SS (Frick-Cheng et al. 2016). The *cag* core complex is 41 nm in diameter and is composed of 5 *cag*-encoded proteins (Cag3, CagT, CagM, CagX, and CagY) (Frick-Cheng et al. 2016). In contrast, the *A. tumefaciens* core complex has a diameter of only 20 nm and is composed of 3 proteins, VirB7, VirB9, and VirB10 (Chandran et al. 2009; Fronzes et al. 2009b). Of note, CagX and CagY share some homology to VirB9 and VirB10, respectively, and both core complexes share a 14-fold symmetry (Frick-Cheng et al. 2016). The *H. pylori cag* island also encodes for a T4SS pilus; however, its composition and structure are not well defined. Upon host cell contact, multiple *cag*-encoded structural proteins have been reported to localize to the *cag* pilus, as well as the effector protein CagA (Kwok et al. 2007; Backert et al. 2008; Jimenez-Soto et al. 2009; Barrozo et al. 2013). Importantly, *H. pylori cag* mutants that fail to form pili are also incapable of translocating CagA,

suggesting an important role for pili in T4SS function (Kwok et al. 2007; Jimenez-Soto et al. 2009; Shaffer et al. 2011; Johnson et al. 2014). Furthermore, the *H. pylori cag*-encoded pilus protein CagL has been proposed to interact with beta-1 integrin receptors on the host cell surface to facilitate effector molecule translocation (Conradi et al. 2012; Barden et al. 2013). In contrast, *A. tumefaciens* does not require biogenesis of the *vir* T4SS or host receptor contact to deliver its oncogenic T-DNA effector into plant cells (Cascales and Christie 2004; Jakubowski et al. 2009; Garza and Christie 2013). Taken together, these data suggest that the *H. pylori cag* T4SS is highly unique, and this notion is further evidenced by the fact that *H. pylori cag* T4SS is the only known T4SS to date that is capable of translocating a protein substrate (CagA), non-proteinaceous substrate (peptidoglycan), and a DNA substrate (Christie and Cascales 2005; Varga et al. 2016).

4.4 H. pylori *T4SS ComB*

The *H. pylori comB* T4SS is primarily involved in natural competence and is functionally independent of the *cag* T4SS (Israel et al. 2000). Generally, competence systems share some homology with proteins involved in the T4SS pilus formation or type II secretion systems (Chen and Dubnau 2004). Indeed, the *H. pylori comB* system is composed of many conserved T4SS components, and its proteins have thus been named after their orthologues in the *A. tumefaciens* VirB/D4 system (Karnholz et al. 2006). The *comB* system shares a similar gene cluster organization of the VirB/D4 system and only lacks the VirB1, VirB5, and VirB11 components (Fernandez-Gonzalez and Backert 2014). ComB2 and ComB3 are orthologous to VirB2 and VirB3 in secondary structure despite having only 27.2% or 19.5% sequence homology, respectively (Karnholz et al. 2006). Although *comB* systems are nearly ubiquitous in *H. pylori* strains, they are unique from other DNA uptake T4SSs in that: they incorporate DNA in a pilus-independent manner, their DNA uptake efficiency varies between strains, and their DNA uptake is saturable, sensitive to length, symmetry, and the strandedness of the DNA substrate (Hofreuter et al. 1998; Levine et al. 2007). The VirB2 protein, and by association the ComB2 protein, is a subunit of the surface-exposed pilus structure of the VirB/D4 system. One study has suggested that the ComB2 protein may be a component of an incomplete "stump structure" in the cell envelope required for DNA uptake (Schroder and Lanka 2005). Unlike the VirB2 component, the functions of VirB3 homologues are unknown except that they are essential for T4SS function (Karnholz et al. 2006). The *H. pylori* ComB DNA uptake system is energized by ComB4, an orthologous protein to VirB4 (Hofreuter et al. 2001). The remaining components of the *H. pylori* ComB system are the ComB6–10 proteins that together form the core complex of the import channel. ComB6 is a polytopic membrane protein essential for T4SS function (Karnholz et al. 2006), while ComB7 is a non-essential membrane-attached protein that stabilizes the central channel structure (Hofreuter et al. 2003). ComB8 is a membrane-associated protein that

contains a periplasmic N-terminus, ComB9 is a periplasmic protein anchored to the membrane, and lastly, ComB10 is also mostly periplasmic with its N-terminus anchored to the cytoplasmic membrane (Hofreuter et al. 2003). Based on its homology to the archetypal T4SS of *A. tumefaciens*, a two-step DNA uptake model has been proposed (Stingl et al. 2010). The ComB T4SS facilitates double-stranded DNA uptake into the periplasm, and a second, ComEC system is then utilized to incorporate the periplasmic DNA across the cytoplasmic membrane and into the host cell (Stingl et al. 2010; Kruger et al. 2016).

4.5 tfs3 *and* tfs4 *Secretion Systems*

In addition to the *comB* and *cag* systems, a novel 16.3 kb gene cluster was recently discovered in one of three plasticity regions of the *H. pylori* genome (Kersulyte et al. 2003). First described in *H. pylori* strain PeCan18B, this gene cluster houses 16 ORFs, 7 of which share homology to the *virB/D4* operon (Kersulyte et al. 2003). Based on these data, this gene cluster is speculated to represent a third T4SS involved in conjugative DNA transfer and has thus been named *tfs3*. Furthermore, this gene cluster has a lower G + C content compared to the rest of the *H. pylori* genome, and it has been suggested that *tfs3* was acquired by a horizontal gene transfer event from a different bacterial species (Kersulyte et al. 2003). When 94 clinical *H. pylori* isolates were screened for the presence of *tfs3*, 19% of the strains contained either the full-length genes or truncated genes (Kersulyte et al. 2003). Upon further investigation into the *tfs3*, significant sequence variation and number of genes were present among different isolates, and subsequently re-classified the *Tfs3* into two sub-groups based upon their homology, termed *tfs3a* and *tfs3b* (Yamaoka 2010). However, *tfs3a* and *tfs3b* were clearly distinct, and *tfs3b* was renamed to *tfs4*, in reference to a fourth T4SS (Fischer et al. 2010; Fernandez-Gonzalez and Backert 2014).

The *tfs4* gene cluster was first observed and subsequently characterized in *H. pylori* strain P12 (Fischer et al. 2010). Similar to *tfs3*, *tfs4* was found in another plasticity zone of the *H. pylori* genome and contained enough orthologues of the *A. tumefaciens virB/D4* genes to constitute a complete T4SS (Fischer et al. 2010). The *tfs4* cluster contained the disease marker *dupA*, a *virB4* homolog that has been affiliated with increased risk for duodenal ulcer (Lu et al. 2005) and a TraG/*VirD4* homolog which encodes a type IV coupling protein independent of the established VirD4 protein in the *cag* T4SS (Selbach et al. 2002). In vitro experiments have shown that *tfs4* encodes for proteins that can excise the gene cluster and facilitate its horizontal transfer to recipient cells via the Tfs4 T4SS (Fischer et al. 2010). Additional studies have shown that *H. pylori* can translocate genomic DNA to another bacterial species, *Campylobacter jejuni*, in a unidirectional manner on agar plates (Oyarzabal et al. 2007). This transfer was independent of the *comB*, *cag*, and *tfs3* genes in the donor (Oyarzabal et al. 2007), and thus leaves the open possibility that *tfs4* genes could facilitate genomic (gDNA) transfer events (Fernandez-Gonzalez and Backert 2014).

5 *H. pylori*/TLR9 Interactions

Studies investigating the role of TLR9 in gastric cancer suggest that it is up-regulated in cancer tissue and may play a role in cancer progression (Schmausser et al. 2004, 2005; Wang et al. 2014). Similarly, TLR9 SNPs have been implicated in increasing gastric cancer risk (Hold et al. 2009; Wang et al. 2013; Trejo-de la et al. 2015). Given the highly established relationship between *H. pylori* and gastric cancer, we and others have sought to determine the effects of *H. pylori* on TLR9-mediated responses and its possible implications in gastric carcinogenesis.

5.1 TLR9-Mediated Responses to H. pylori

In the human gastric niche, TLR9 expression is primarily localized to the apical surface epithelium; however, *H. pylori*-induced chronic active gastritis alters the expression pattern to a basolateral only pattern (Schmausser et al. 2004). As mentioned previously, in polarized intestinal epithelial cells, TLR9 activation in the basolateral compartment has been linked to pro-inflammatory signaling; however, the effects of TLR9 localization in gastric epithelial cells have not been elucidated (Schmausser et al. 2005; Lee et al. 2006). Previous studies have shown that *H. pylori* can induce gastric epithelial cell expression of Cox2 in a TLR2-/TLR9-dependent manner. In this study, the authors show that TLR2/TLR9 signaling in gastric epithelial cells induce MAPKs and subsequently allow transcription factors binding to both the CRE and AP-1 sites within the Cox2 promoter. As a result of Cox2 expression, prostaglandin E_2 is released and promotes gastric cancer cell invasion and angiogenesis (Chang et al. 2005). The oncogenic potential of *H. pylori*-TLR9 interactions was also exemplified by a study in which purified *H. pylori* DNA induced invasion of gastric epithelial cells in vitro, an effect that could be partially reduced with the endosomal inhibitor chloroquine (Kauppila et al. 2013). In murine models of *H. pylori* infection, Rad et al. have demonstrated that TLR9 detects *H. pylori* in vivo and induces pro-inflammatory responses (Rad et al. 2009). These studies are complicated by more recent investigations in which TLR9 was shown to promote anti-inflammatory signaling during the acute phase of *H. pylori* infection, which was mediated by type I IFNs (Otani et al. 2012). Moreover, purified *H. pylori* DNAs have been shown to alleviate DSS-induced colitis in mouse models (Luther et al. 2011; Owyang et al. 2012). Collectively, these data highlight the dichotomous role of TLR9 during *H. pylori* infection. *H. pylori* may utilize TLR9 signaling to dampen the inflammatory response during the acute phase to establish infection; however, in an inflammatory micro-environment in which cells have lost their polarity, TLR9 may execute pro-inflammatory cascades and further exacerbate the progression toward gastric cancer.

Although both murine and human TLR9 recognize CpG DNA motifs, caution must be taken in translating findings from mouse studies into human disease. TLR9

receptors from mice and humans recognize different DNA ligands, and mouse TLR9 is expressed in a broader number of cell types compared to humans. Human TLR9 is expressed in epithelial cells, B cells, neutrophils, and pDCs whereas murine TLR9 is expressed in those same cell types and additionally in myeloid DCs, as well as macrophages (Bauer et al. 2001; Rehli 2002). These data suggest that the DNA sequences or structures that activate murine TLR9 may not elicit the same responses in humans while the more abundant localization of TLR9 in mouse species suggests that it may be exposed to more and varied ligands. Together, the differences in DNA sequence recognition and cellular distribution may account for the contrasting observations between human and murine responses to TLR9 activation and must be considered in translating into human studies or contemplating possible therapeutic targets.

5.2 H. pylori *Activates TLR9 via the T4SS*

As studies of *H. pylori*-TLR9 interactions have generated mixed results, we sought to determine how *H. pylori* could engage this endosome-bound innate immune receptor (Varga et al. 2016). Since *H. pylori* primarily interact with the epithelial cell surface, we focused our studies on possible modalities of DNA transfer into host epithelial cells. Through the use of a TLR9 reporter assay, we found that *H. pylori* strains could activate TLR9 in vitro, and that the activation was independent of the ComB, Tfs3, and Tfs4 secretion systems. Once these known DNA transfer T4SSs had been eliminated as possible candidates for TLR9 activation, we shifted our focus to the *cag* PAI. We tested *H. pylori cag* isogenic mutants and found that an intact, functional *cag* T4SS was required for TLR9 activation. Since some bacterial T4SSs, like that of *Neisseria gonorrhoeae* use the T4SS to translocate DNA into the extracellular milieu (Hamilton et al. 2005), we also tested *H. pylori* conditioned media for its capacity to activate TLR9. Bacterial cell free, *H. pylori* conditioned media had no effect on TLR9 activation at concentrations ranging up to 30%. Lastly, although rare, *H. pylori* have the capacity to invade host epithelial cells. Thus, we performed gentamicin protection assays to determine whether the *cag* isogenic mutants could be endocytosed efficiently compared to wild-type *H. pylori* as a means to explain the differences observed in TLR9 activation. We found that there were no significant differences in the degree of endocytosis of wild-type versus *cag* mutant bacteria in our reporter cell line. Together, these data suggest that TLR9 activation is mediated directly through the *cag* T4SS. To confirm and validate these results, we cultured *H. pylori* in media enriched with the DNA nucleoside thymidine analog, bromodeoxyuridine (BrdU). In this manner, the microbial DNA can be easily discerned from host cell DNA. We challenged the labeled bacteria with AGS gastric epithelial cells with labeled *H. pylori,* and through structured illumination confocal microscopy, we were able to directly observe *H. pylori* DNA within host cells (Fig. 2), a result that was further validated by flow cytometry.

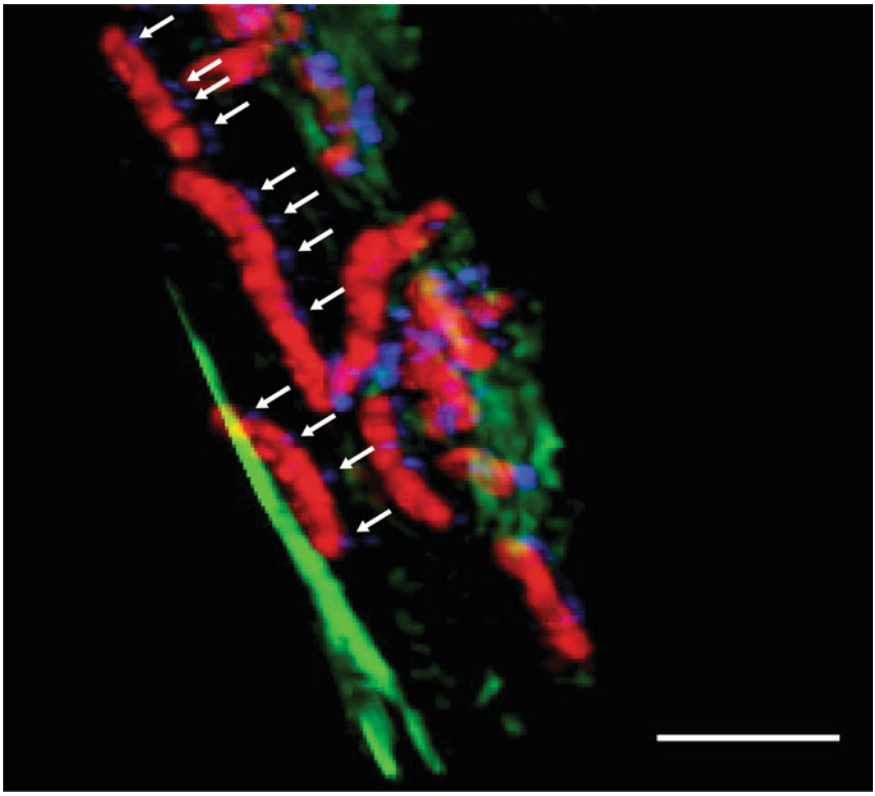

Fig. 2 *H. pylori* DNA translocation: Confocal micrograph demonstrating *H. pylori* DNA translocation in the presence of host epithelial cells. *H. pylori* is shown in *red*, DNA in *blue*, and actin filaments in *green*. *Arrows* indicate areas of DNA exiting *H. pylori.* Scale bar, 2μm. Image acquired by Drs. Carrie Shaffer and Maria Hadjifrangiskou

The ability of the *cag* T4SS to translocate DNA is a unique finding, but not without precedent as both *A. tumefaciens* and *B. henselae* can also translocate plasmid DNA into eukaryotic hosts. However, the *H. pylori* system is different from the two aforementioned species because we observed TLR9 activation with *H. pylori* strains that were devoid of plasmid DNA, and the *H. pylori cag* T4SS is the first known T4SS that can translocate different classes of substrates including protein (CagA), non-protein (peptidoglycan), and DNA. We therefore wanted to next examine whether *H. pylori cag* T4SS-mediated DNA translocation mirrored that of *A. tumefaciens* (Cascales and Christie 2004; Jakubowski et al. 2009; Garza and Christie 2013). Similar to *Agrobacterium*, we found that DNA translocation required de novo protein synthesis, and that DNA was both protected and exposed to the extracellular milieu during transport.

To expand our findings into the natural niche of *H. pylori*, TLR9 expression and localization were examined in human gastric biopsies obtained from patients

residing within either a high-risk or low-risk region of gastric cancer. Both populations share a similar incidence of *H. pylori* infection (90%); however, patients residing within the low-risk region displayed significantly less TLR9 expression compared to those within the high cancer-risk region. Moreover, when *H. pylori* strains isolated from patients in the low-risk region were tested for their ability to activate TLR9 in our in vitro reporter assay, they induced significantly less activation compared to strains isolated from the high-risk region.

6 Conclusions and Outlook

H. pylori has co-evolved with its human host over the course of millennia, and its prolonged colonization can be attributed to its ability to modulate the host immune system. Upon first encounter, *H. pylori* contacts the gastric epithelium and receptors of the innate immune system. Thus, it is critical that *H. pylori* harbors the ability to evade these innate immune responses to establish infection and persist for the lifetime of the host. *H. pylori* has adopted mechanisms to either hide or modulate its potentially immunogenic components such as altering its LPS to avoid TLR4 detection or harboring amino acid substitutions in its flagella to subvert the TLR5 immune recognition. In addition to those established mechanisms, we have recently shown that *H. pylori* can also translocate its gDNA through the *cag* T4SS to activate TLR9. Due to the dual roles that TLR9 plays in facilitating both pro- and anti-inflammatory responses, *H. pylori*'s ability to engage this receptor suggests yet another way in which this pathogen can manipulate the host response to maintain its persistence within the gastric niche.

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Exploiting the Gastric Epithelial Barrier: *Helicobacter pylori*'s Attack on Tight and Adherens Junctions

Steffen Backert, Thomas P. Schmidt, Aileen Harrer and Silja Wessler

Abstract Highly organized intercellular tight and adherens junctions are crucial structural components for establishing and maintenance of epithelial barrier functions, which control the microbiota and protect against intruding pathogens in humans. Alterations in these complexes represent key events in the development and progression of multiple infectious diseases as well as various cancers. The gastric pathogen *Helicobacter pylori* exerts an amazing set of strategies to manipulate these epithelial cell-to-cell junctions, which are implicated in changing cell polarity, migration and invasive growth as well as pro-inflammatory and proliferative responses. This chapter focuses on the *H. pylori* pathogenicity factors VacA, CagA, HtrA and urease, and how they can induce host cell signaling involved in altering cell-to-cell permeability. We propose a stepwise model for how *H. pylori* targets components of tight and adherens junctions in order to disrupt the gastric epithelial cell layer, giving fresh insights into the pathogenesis of this important bacterium.

Contents

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1 Introduction

1.1 The Polarized Epithelium as the First Barrier for H. pylori *Colonization*

Helicobacter pylori colonizes the gastric mucosal epithelium in the hostile environment of the human stomach. This epithelium represents a highly organized and essential cell monolayer controlling important digestive, absorptive and secretory functions. However, this epithelium also forms a first barrier against the microbiota and pathogenic microbes such as *H. pylori*. The discovery of *H. pylori* in gastric biopsies by Robin Warren and Barry Marshall, more than 33 years ago, radically changed the view on understanding and treatment of gastric disorders as an infectious disease (Marshall and Warren 1984). Today, we know that about half of the human world population carries *H. pylori*, causing chronic gastritis in all carrying persons, and more severe gastric disease in about 10–15% of infected individuals (Amieva and El-Omar 2008; Atherton and Blaser 2009; Polk and Peek 2010; Salama et al. 2013; Yamaoka and Graham 2014). Colonization by *H. pylori* commonly appears early in childhood, and if not treated by antimicrobial therapy, the bacteria can persist lifelong. Although *H. pylori* colonization is frequently associated with a strong inflammatory reaction, which is controlled by the host innate and adaptive immune systems, the bacteria are not eliminated. Various mechanisms of immune evasion have been reported, and *H. pylori* became a prime example of a persistent bacterial pathogen causing chronic infections (Ramarao et al. 2000; Gobert et al. 2001; Wunder et al. 2006; Patel et al. 2013; Foegeding et al. 2016). Evolutionary analyses revealed that *H. pylori* has been associated with modern humans over at least 100,000 years, possibly after the bacterium was first acquired by a single host jump from a yet unknown carrier (Moodley and Linz 2009). Because of this long time of coevolution, it has been proposed that *H. pylori* colonization may have been beneficial for the human host and hence provided a selective advantage (Backert and Blaser 2016). In the modern world, however, these infections cause a heavy burden of morbidity and mortality as a consequence of peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Correa and Houghton 2007; Atherton and Blaser 2009;

Salama et al. 2013). In the last few years, the cellular and molecular mechanisms utilized by *H. pylori* to subvert host defences have been studied thoroughly (Amieva and El-Omar 2008; Salama et al. 2013; Backert et al. 2015; Caron et al. 2015). These investigations showed that the clinical outcome of infection with *H. pylori* is controlled by a highly sophisticated host–pathogen crosstalk. Multiple factors determine disease outcome including the bacterial genotype, genetic susceptibility of the host as well as environmental parameters.

H. pylori strains are remarkably diverse both in their genetic content and pathogenicity. Dozens of factors were identified to affect the pathogenesis of *H. pylori.* These determinants can be classified as virulence factors and pathogenicity-associated factors. There are two classical virulence factors encoded by *H. pylori*, the vacuolating cytotoxin (VacA) and the effector protein CagA. VacA is categorized as a pore-forming toxin, and many of its activities are associated with the formation of membrane channels in target cells. The best studied VacA activity is vacuole formation. However, the toxin has many other effects on host cells including the induction of apoptosis in epithelial cells or blocking the proliferation of immune cells (Foegeding et al. 2016). VacA genes are harbored by virtually all *H. pylori* isolates worldwide, but exhibit considerable sequence variation. *VacA* is present in several alleles that have been identified in the signal region (s1 and s2), mid-region (m1 and m2) and others, occurring in multiple combinations. The type s1/m1 VacA molecules produce an extensive cell vacuolation phenotype, while S2-type VacAs are inactive in such assays (Foegeding et al. 2016). The second factor is CagA, which is encoded by the *cag* (cytotoxin-associated genes) pathogenicity island (*cag*PAI) present in highly virulent *H. pylori* strains and being absent in less virulent isolates. The *cag*PAI encodes a type IV secretion system (T4SS), which is induced upon host contact and forms a syringe-like pilus structure for the export of virulence factors such as the CagA effector protein into host target cells (Tegtmeyer et al. 2011a; Backert et al. 2015). After delivery, CagA becomes tyrosine phosphorylated at EPIYA motifs by Src and Abl kinases (Lind et al. 2014; 2016) and mimics a host cell factor for triggering intracellular signaling cascades affecting membrane dynamics, disruption of cell–cell junctions as well as pro-inflammatory, cell cycle-related and anti-apoptotic transcriptional responses (Tegtmeyer et al. 2011b; Mueller et al. 2012; Zhang et al. 2015). Other known pathogenicity-associated phenotypes include flagella-driven motility in the mucus layer, acid neutralization by the urease (UreA, UreB and accessory proteins), adhesion to gastric epithelial cells mediated by adhesins (BabA, SabA, OipA, AlpA/B, HopQ, HopZ and others) as well as proteolytic targeting of host receptors such as E-cadherin by the secreted serine protease HtrA (Aspholm et al. 2006; Dubois and Boren 2007; Roure et al. 2012; Posselt et al. 2013; Yamaoka and Graham 2014). In addition, specific polymorphisms in human genes involved in inflammatory and immune-regulatory processes such as interleukin-1β (IL-1β), Toll-like receptors (TLRs) or NOD (nucleotide oligomerization domain) receptors as well as lifestyle properties (diet, smoking, alcohol consumption, etc.) have also been linked to an increased risk of developing gastric disease including cancer (Amieva and El-Omar 2008; Polk and Peek 2010).

Disruption of the epithelial barrier and in particular opening the intercellular adhesions is an important hallmark of *H. pylori*-dependent inflammation and neoplastic transformation. In principle, these individual processes can interfere with the functional cell-to-cell contacts independently of each other, but the combination of diverse temporary and locally regulated mechanisms reveals a dynamic and complex network. Here, we summarize our current knowledge on the multiple functions of *H. pylori* factors on exploiting the epithelial barrier and discuss the multitude of involved host signaling cascades with focus on their importance in pathogenesis.

1.2 Mechanisms of H. pylori*-Triggered Reprogramming of Epithelial Cell Differentiation*

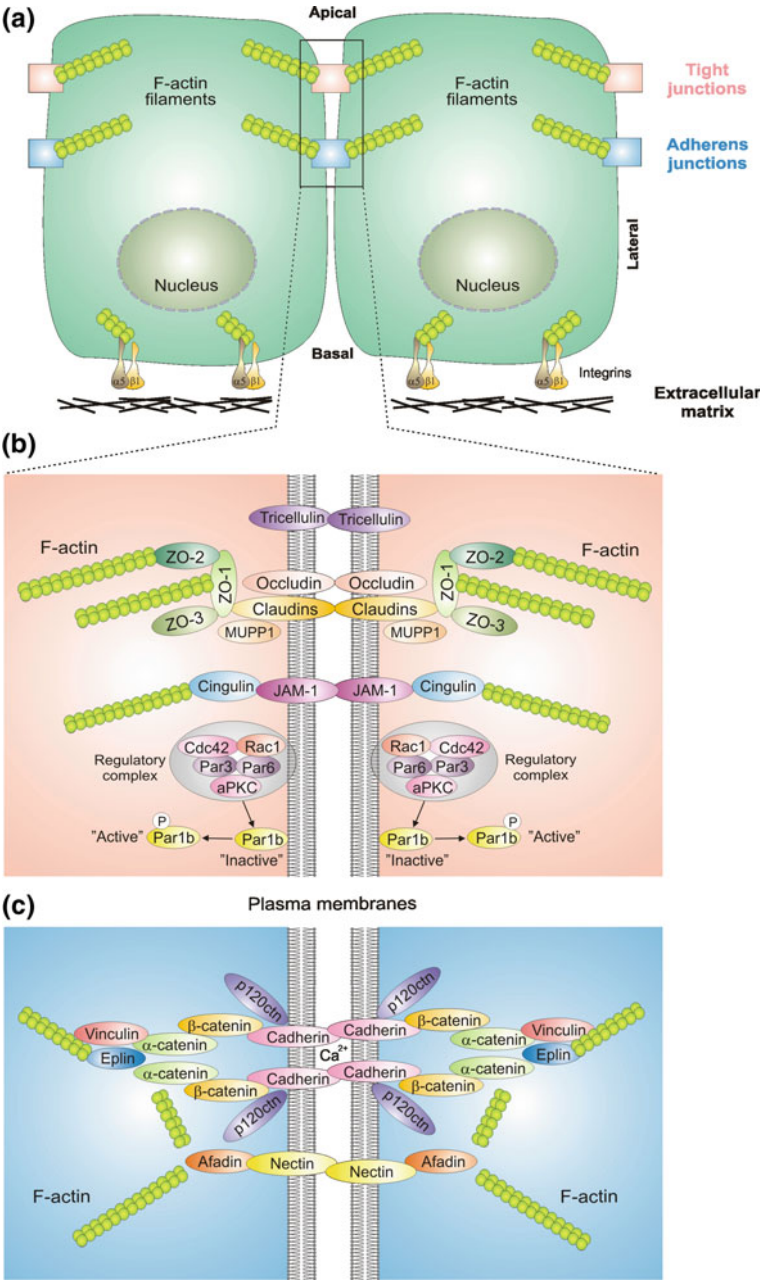
One virulence factor that clearly determines a high risk of gastric cancer development is the *cag*PAI encoding CagA. CagA itself has originally been recognized as an immuno-dominant antigen. Patients who are sero-positive for *H. pylori* and CagA exhibit a 5.8-fold higher risk of developing intestinal and diffuse gastric adenocarcinoma as compared to uninfected persons, whereas individuals infected with CagA-negative strains are only at a 2.2-fold higher risk of developing distal gastric adenocarcinoma compared to uninfected control persons (Parsonnet et al. 1997). Thus, the *cag*T4SS was established as a strong predictor of severe disease outcome. CagA-positive wild-type *H. pylori* can induce pre-malignant and malignant pathologies in the Mongolian gerbil infection model system. Four weeks after infection, almost each animal exhibited gastric dysplasia, and by eight weeks about two-thirds revealed gastric adenocarcinoma (Franco et al. 2005). Another straightforward experiment confirming the role of CagA in gastric cancer progression in vivo was provided by establishing CagA-expressing transgenic C57BL/6 J mice (Ohnishi et al. 2008). After 72 weeks, these transgenic mice developed gastric epithelial hyperplasia, while some animals revealed polyps and adenocarcinomas in the stomach. Moreover, systemic expression of CagA in mice leads to the development of leukocytosis with IL-3/GM-CSF hypersensitivity, while various animals displayed B-cell lymphomas and myeloid leukemias (Ohnishi et al. 2008). Based on the above findings, CagA was described as the first bacterial oncoprotein. These studies were confirmed by two other transgenic model organisms. A transgenic drosophila model demonstrated that CagA functions as a mimetic for the eukaryotic adaptor protein Gab1 (Botham et al. 2008). Expression of CagA in drosophila and zebrafish exhibited significantly enhanced levels of downstream c-Jun N-terminal kinase (JNK) phosphorylation and Wnt target gene induction, leading to proliferation of intestinal epithelial cells and growth of small cell carcinoma and adenocarcinoma (Wandler and Guillemin 2012; Neal et al. 2013). Altogether, the above studies established conclusively that *H. pylori* can induce the generation of gastric adenocarcinoma in gerbils and other model organisms. In addition, transgenic expression

of CagA alone appears to be sufficient for the development of severe malignant lesions in various transgenic animals.

A hallmark of gastric cancer development is the strong inflammatory response phenotype (Backert and Naumann 2010). Chronic inflammation and late stages of cancer are often accompanied by disruption of the proper architecture in the gastric epithelium, and *H. pylori* have been found intercellular and intracellular in gastric cancer biopsy samples (Necchi et al. 2007). In fact, infection with CagA-positive *H. pylori* as an important disease-associated feature has been clearly implicated in the disruption of the epithelial layer contributing to inflammatory gastric diseases (Polk and Peek 2010; Salama et al. 2013; Yamaoka and Graham 2014; Caron et al. 2015). Based on histology parameters, gastric cancer can be categorized into diffuse or intestinal types and both are associated with chronic infection by *H. pylori*. While we know very little about the pathogenesis of diffuse-type carcinoma, the intestinal type usually comprises a set of well-known steps. In the latter model, chronic active inflammation induced by *H. pylori* corresponds to the initial phase of disease progression followed by the loss of gastric glands, development of atrophy and hyperproliferation (Amieva and El-Omar 2008; Polk and Peek 2010). This might be dominated by specific alterations in cell cycle, apoptosis rates and cell proliferation. Finally, there is a progressive loss of differentiation leading to the invasive growth of individual neoplastic cells.

2 Structure and Composition of Polarized Cell Monolayers in the Healthy Epithelium

In order to understand how *H. pylori* can disrupt epithelial barrier functions, we must first take a look at the organization of healthy epithelia. An intact epithelial barrier requires tightly controlled cell architecture to provide effective protective functions. A complex network of diverse regulatory structures is necessitated in the establishment and maintenance of the protective epithelium. Besides a strictly regulated intracellular cytoskeleton and cell-to-ECM (extracellular matrix) interactions, epithelial cell-to-cell adhesions are crucially important and involve tight junctions (TJs) and adherens junctions (AJs) (Yu and Elble 2016; Sumigray and Lechler 2015; Rodriguez-Boulan and Macara 2014). The structure of the healthy epithelium is normally maintained by the integrity of the apical–basal polarity, a highly organized actin cytoskeleton and junctional complexes exhibiting tumor-suppressive and/or anti-metastasis properties (Fig. 1a). Importantly, junctional complexes are based on the extensively studied lateral cell-to-cell contacts including TJs (Fig. 1b) and E-cadherin-based AJs (Fig. 1c) as well as desmosomes, gap junctions and others, which are discussed elsewhere (Wei and Huang 2013). As shown in Fig. 1, TJs mark the apical–basolateral border of polarized cells and establish a highly selective barrier to prevent leakage and paracellular diffusion of small molecules. Importantly, functional TJs build up cell polarity through


(a)
Apical
F-actin filaments
F-actin filaments
Tight junctions
Adherens junctions
Lateral
Nucleus
Nucleus
Basal
Integrins
Extracellular matrix
(b)
Tricellulin
Tricellulin
F-actin
ZO-2
ZO-1
Occludin
Occludin
Claudins
Claudins
ZO-3
MUPP1
ZO-1
ZO-2
F-actin
ZO-3
MUPP1
Cingulin
JAM-1
JAM-1
Cingulin
Regulatory complex
Cdc42
Rac1
Par3
Par6
aPKC
Rac1
Cdc42
Par6
Par3
aPKC
Regulatory complex
"Active"
P
Par1b
Par1b
"Inactive"
Par1b
P
Par1b
"Active"
"Inactive"
(c)
Plasma membranes
p120ctn
β-catenin
Cadherin
Vinculin
α-catenin
Eplin
Ca2+
p120ctn
β-catenin
Cadherin
Vinculin
α-catenin
Eplin
α-catenin
Cadherin
β-catenin
Cadherin
α-catenin
β-catenin
p120ctn
p120ctn
Afadin
Nectin
Nectin
Afadin
F-actin
F-actin

◂**Fig. 1** Model for the organization of polarized epithelial cells and composition of intercellular junctions. **a** Simplified schematic presentation of the polarized cell layer in a healthy epithelium. Two important types of intercellular junctions, the tight junctions (TJ, *orange*) and adherens junctions (AJ, *blue*), are indicated and exhibit specific localization at the apical lateral borders. The basal focal adhesions are composed of integrins such as α5β1 integrin and connect the extracellular matrix with the intercellular actin cytoskeleton. Gap junctions and (hemi)desmosomes are other examples, but they are not discussed in this chapter. **b** TJs are key protein complexes in establishing and maintaining epithelial cell polarity. They are crucial for the tight sealing of the cellular sheets, which control the paracellular ion flux and maintain tissue homeostasis. TJs are localized at the apical side of the lateral membrane keeping barriers between the apical and basal compartments of the plasma membranes as indicated. TJs comprise at least four types of transmembrane proteins: junctional adhesion molecules (JAMs), claudins, occludin, tricellulin and various indicated cytoplasmic proteins. While the transmembrane proteins mediate cell-to-cell adhesion, the cytosolic TJ platform bears several other protein types (e.g., PDZ proteins, such as the zonula occludens (ZO) protein-1/-2/-3, MUPP1 or cingulin) which connect the TJ transmembrane proteins to the associated cytoskeleton. These molecules can also engage other signaling factors, including small GTPases, kinases, phosphatases and transcription factors. The integrity of TJs is maintained by a regulatory complex including atypical PKC (aPKC), Cdc42, Rac1, Par3 and Par6. aPKC can phosphorylate and activate Par1b kinase at threonine residue 595. Activated Par1b specifically localizes to the basal and lateral membranes to regulate cell polarity. **c** AJs are positioned immediately below TJs and form a complex of membrane proteins and associated factors which ensure the mechanical adhesion between two neighboring cells. AJs assemble via calcium-dependent homophilic interactions between the extracellular domains of E-cadherin connecting the cells as indicated. E-cadherin not only acts as an adhesive molecule, but also plays important roles as a suppressor of growth development and carcinogenesis. The calcium-dependent integrity of AJs is stabilized by binding of E-cadherin to intracellular catenins. The proximal carboxy-terminal domain of E-cadherin interacts with the cytoplasmic protein β-catenin. $p120^{ctn}$ can interact with the juxtamembrane part of E-cadherin, further stabilizing the entire adherens junction platform. The E-cadherin–β-catenin complex is connected to the actin cytoskeleton via binding to α-catenin, EPLIN (epithelial protein lost in neoplasm) and vinculin as shown. Nectin and afadin contribute to the organization of E-cadherin-mediated AJ functions in epithelial cells

impeding lateral diffusion of membrane proteins between the apical and basolateral domains of the epithelium leading to special characteristics and functions of the cell surface. Further functions include the control of epithelial proliferation and differentiation (Balda and Matter 2016; Martin 2014; Aijaz et al. 2006). The structure of TJs involves different transmembrane proteins (e.g., occludin, claudins, junctional adhesion molecules [JAMs]) connecting adjacent cells (Fig. 1b). Occludin consists of four transmembrane domains, two extracellular loops and two intracellular domains. Their functions appear to be crucially important in epithelial differentiation, but not in the establishment of the barrier (Schulzke et al. 2005). Although no significant sequence similarity to occludin exists, claudin proteins also contain four transmembrane domains, two extracellular loops and two intracellular domains (Krause et al. 2015). The human claudin family contains 27 members (Krause et al. 2015) and is important in the establishment and maintenance of the barrier function (Inai et al. 1999). Members of the JAM family include JAM-A, JAM-B, JAM-C and JAM4/JAML (Garrido-Urbani et al. 2014) and have a single transmembrane domain, an extracellular domain with two Ig-like motifs, and a

cytoplasmic domain (Kostrewa et al. 2001). JAM proteins are required for intercellular adhesion as well as for maintaining cell polarization (Garrido-Urbani et al. 2014). In the cytoplasm, TJs form an intracellular plaque that is composed of a complex network of scaffolding and adaptor proteins connecting the actin cytoskeleton and intracellular signaling molecules (Fig. 1b). Zonula occludens-1 (ZO-1), ZO-2 and ZO-3 are scaffolding proteins that interact directly with claudins and occludin (Runkle and Mu 2013). Other cytoplasmic proteins are cingulin, Rab13, afadin, membrane-associated guanylate kinase with inverted orientation-1 (MAG proteins) and multi-PDZ domain protein 1 (MUPP-1), etc. A variety of signaling molecules, such as small GTPases of the Rho family (Rho, Rac and Cdc42), ZO-1-associated kinase, PKCζ, etc., complete the functional structure of TJs (Denker and Nigam 1998).

In a given polarized epithelium, AJs are located directly beneath TJs. The main function of AJs is the formation of intercellular adhesions. The integrity of AJs is established by the homophilic interactions of members of the cadherin family of proteins (Niessen and Gottardi 2008; Gumbiner 2005). E-cadherin (Cdh1) is an N-glycosylated transmembrane protein composed of an extracellular (EC) domain, transmembrane domain and an intracellular (IC) domain. The extracellular domain contains five repetitive amino acid sequences (EC1–EC5) with calcium-binding motifs located between the individual EC domains (Ringwald et al. 1987; Harrison et al. 2010). Binding of calcium ions is a requirement for the formation of interactions between the EC domains. The flexible three-dimensional E-cadherin structure is pushed into a rigid, while rod-like assembly upon calcium binding allowing interactions of EC1 and EC3 in *cis* and in *trans* (Niessen and Gottardi 2008; Ozawa et al. 1990; Takeda et al. 1999; Pokutta et al. 1994). As shown in Fig. 1c, the IC domain of E-cadherin interacts with members of the catenin family, in particular β-catenin and p120-catenin ($p120^{ctn}$). Binding of β-catenin to the IC bridges E-cadherin to the actin cytoskeleton through the interaction with α-catenin. In turn, α-catenin can bind actin either directly or via other proteins like EPLIN (epithelial protein lost in neoplasm) or vinculin (Abe and Takeichi 2008; Hazan et al. 1997; Meng and Takeichi 2009). Together with β-catenin, interaction of $p120^{ctn}$ with the juxtamembrane part of E-cadherin stabilizes the integrity of AJs and contributes to the regulation of the turnover of E-cadherin (Yap et al. 1998; Gooding et al. 2004).

3 Depolarization of Epithelial Cells by *H. pylori* Involves Alterations in Tight and Adherens Junctions

Infections with *H. pylori* are accompanied by alterations of the cell architecture leading to depolarization of the epithelium. In particular, the intercellular junctions are direct targets. The functionality of AJs can be disrupted by multiple mechanisms. Importantly, gastric cancer of the diffuse type is strongly correlated with

severe interference of AJ function through loss of function mutations or (epi)genetically downregulation of the *cdh1* gene. Additionally, ectodomain shedding of E-cadherin by upregulated matrix metalloproteases (MMPs) is intensively discussed in the literature to dysregulate E-cadherin and to increase the malignancy of gastric cancer as the consequence of the loss of adhesive properties of AJs to increase metastasis of tumor cells (Margineanu et al. 2008; Chan 2006). Upon disruption of AJs, the catenins can aggravate this phenomenon. Besides their function in the stability and integrity of AJs, β-catenin and $p120^{ctn}$ are implicated in the tumor-suppressive function of E-cadherin. Destabilization of the AJ complex can lead to the release of β-catenin and $p120^{ctn}$. Non-junctional cytoplasmic β-catenin is constantly degraded by the proteasome after phosphorylation by a multiprotein complex comprising glycogen synthase kinase 3β (GSK-3β), casein kinase 1 (CK1), adenomatous polyposis coli (APC) and axin to prevent nuclear functions of β-catenin (He et al. 2004). An activated Wnt signaling pathway can result in an inhibition of β-catenin phosphorylation followed by the stabilization of β-catenin (MacDonald et al. 2009). Accumulated β-catenin can translocate into the nucleus, where it interacts with T-cell-specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) family to activate the expression of cancer-associated Wnt target genes, such as *c-myc* or *cyclin d1* (MacDonald et al. 2009; McCrea and Gottardi 2016). Similarly, nuclear $p120^{ctn}$ protein physically interacts with Kaiso to relieve Kaiso-mediated inhibition transcription of canonical Wnt target genes, including *cyclin d1* or *mmp-7* (Daniel and Reynolds 1999; Park et al. 2005; Spring et al. 2005). In conclusion, intact AJ complexes are crucially important in the establishment of intercellular adhesions and in tumor suppression. However, *H. pylori* developed fascinating mechanisms to disrupt intercellular adhesions (Wessler and Backert 2008), which are summarized in Table 1 and discussed in the following text.

3.1 Direct Targeting of Tight Junction Factors by H. pylori *Effector Proteins*

3.1.1 Selective Opening of Tight Junctions by *H. pylori* VacA

Early studies have provided hints that *H. pylori* can target TJs in the gastric epithelium. The first implicated bacterial factor was VacA. Papini et al. (1998) reported that treatment of various polarized cell lines with acid-activated VacA increased the cell permeability for low molecular weight (<350–440 Dalton) molecules and ions such as iron (Fe^{3+}) and nickel (Ni^{2+}). In accordance with these data, the transepithelial electrical resistance (TER) was decreased by VacA treatment. However, high-resolution immunofluorescence analyses of VacA-treated cells failed to reveal alterations of junctional proteins, including ZO-1, occludin and E-cadherin. The authors proposed that VacA induces a selective permeabilization of

Table 1 Reported activities of *H. pylori* factors targeting tight and adherens junction proteins

Bacterial factor	Proposed function	Interaction partner	Experimental evidence	Host cells used	*H. pylori* strain infection	Applied methods	References
VacA	Increase of cell permeability	n.d.	Treatment with enriched VacA	MDCK, T84, epH4, HeLa	None	CLSM, TER, WB, NRU, ^{125}I-EGF degradation	Papini et al. (1998)
VacA	Increase of cell permeability	n.d.	Infection	MDCK	CCUG 17874, SPM326, 95-54	TER, WB, QAB, NRU, PF	Pelicic et al. (1999)
VacA	Cell cytoskeletal disruption	n.d.	Treatment with VacA-containing supernatants	AGS, HeLa, RK13	J116, 60190, 60190v1, M99, Tx30a, M99v1	BFM, WB, CLSM, AA, TEM, PCR	Bebb et al. (2003a, b)
VacA	Apical membrane disruption	Calpain, ezrin	Treatment with enriched VacA	Rabbit gastric primary cells	None	CLSM, WB, EM, APUA, Ca^{2+} influx, IVP, PCR, PA	Wang et al. (2008)
CagA	Disruption of tight junctions	ZO-1, JAM	Infection	MDCK, AGS	G27	CLSM, MF, WB, TEM, LBSAB, CSA, DA	Amieva et al. (2003)
CagA	Activation of β-catenin	n.d.	Gerbil infection, transfection, proteomics	AGS, MG-262	7.13, B127, J166, J68	CLSM, IHC, WB, PCR, LA, ELISA	Franco et al. (2005)
CagA	Loss of cell polarity	ZO-1	Transfection of CagA-GFP	MDCK, AGS, HEK-293	None	CLSM, MIA, PCR, TLIA, IP	Bagnoli et al. (2005)
CagA	Activation of β-catenin	E-cadherin	Transfection of CagA	MKN28, MKN45	None	CLSM, IP, WB, LA, cDNA-MA, FC, NB	Murata-Kamiya et al. (2007)
CagA	Loss of cell polarity	MARK2/ Par1b	Transfection of CagA	COS-7, AGS, MDCK	None	CLSM, TER, MS, in vitro KA, WB, IP	Saadat et al. (2007)
CagA	Loss of cell polarity	MARK2/ Par1b	Infection, transfection of CagA-GFP	MDCK, AGS	G27	CLSM, IP, WB, CF, RT-PCR, iTRAQ, COA, SCX, LC-MS/MS, CSA, DRMP	Zeaiter et al. (2008)

(continued)

Table 1 (continued)

Bacterial factor	Proposed function	Interaction partner	Experimental evidence	Host cells used	*H. pylori* strain infection	Applied methods	References
CagA	Regulation of MMP-7 expression	E-cadherin, p120	Infection, siRNA knockdown	MKN28, Phoenix 293	SS1, 7.13	CLSM, RT-PCR, RT, ChIP, SCF, WB, LA, QAB, DA	Ogden et al. (2008)
CagA	Invasion of epithelial cells	E-cadherin, c-Met, p120	Infection, transduction of human E-cadherin	AGS, NCI-N87, IPA220	26695, 60190	CLSM, IP, WB, MIA, ICC	Oliveira et al. (2009)
CagA	Increase of claudin-2 expression	Cdx2	Infection	AGS	P1	SEM, TEM, WB, EMSA, qPCR, sqPCR, CLSM, MIA	Song et al. (2013)
CagA	Snail mediates EMT via GSK-3 depletion	GSK-3	Infection, transfection of CagA	MKN28, AGS, 293	60190	CLSM, IP, WB, IHC, in vitro KA, qPCR, RGA, MIA, PCR, IMA, IFM	Lee et al. (2014)
CagA	Targeting of claudin-7	β-Catenin, Snail	Infection, microinjection	Gastroids, MKN28	7.13, 60190	CLSM, FC, IHC, WB, CF, LA, siRNA, EdUA, DA, RT-PCR	Wroblewski et al. (2015)
CagA	MMP activation	n.d.	Infection, siRNA knockdown	AGS, MKN45, MKN28, KATO-III	P12, 26695, 60190, 84183	CLSM, microarray, WB, qPCR, siRNA, MIA, CZ, PCR	Sougleri et al. (2016) Costa et al. (2016)
Urease	Targeting of occludin via NH_3	n.d.	Infection, treatment with ammonium	Caco-2, MDCK, Jurkat	P12	CLSM, WB, TER, PF, RT-PCR, DA	Lytton et al. (2005)
Urease	Targeting TJs via MLCK	n.d.	Infection, MLCK inhibitor treatment	MKN28	7.13, 60190	CLSM, IHC, WB, TER, CF, AA, DA	Wroblewski et al. (2009)

(continued)

Table 1 (continued)

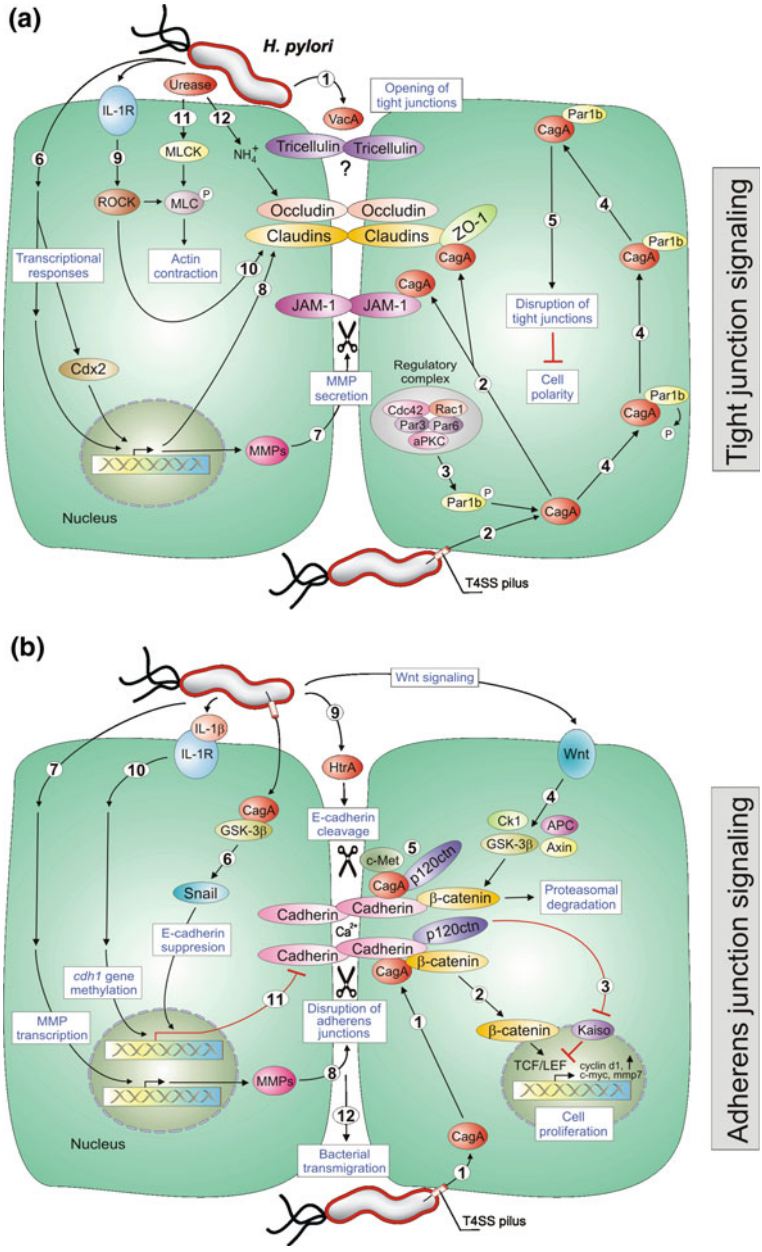
Bacterial factor	Proposed function	Interaction partner	Experimental evidence	Host cells used	*H. pylori* strain infection	Applied methods	References
HtrA	Opening of AJs	E-cadherin	Infection and in vitro cleavage	MKN28, MDCK, INT-407	P12, 26695	CLSM, WB, CZ, PCR, GPA, MS	Hoy et al. (2010, 2012)
HtrA	Opening of AJs	E-cadherin	Infection and in vitro cleavage	MKN28	SS1, B38, G27, J99, B8, 35A+ 15 clinical strains	WB, CZ, PCR	Tegtmeyer et al. (2016)
HtrA	Opening of AJs	E-cadherin	Infection and in vitro cleavage	MKN28, AGS, NCI-N87	P12, 26695	SPR, TER, WB, MS, HPLC, PS, NTS	Schmidt et al. (2016a, b)

[a]**Abbreviations:** ^{125}I-EGF degradation (^{125}I-iodination epidermal growth factor degradation); AA (apoptosis assay); APUA (aminopyrine uptake assay); BFM (bright field microscopy); cDNA-MA (cDNA microarray); CF (cell fractionation); ChIP (chromatin immunoprecipitation); CLSM (confocal laser scanning microscopy); COA (collagen overlay assay); CSA (calcium switch assay); CZ (casein zymography); DA (densitometric analysis); DRMP (detergent-resistant membrane preparation); EdUA [EdU(5-ethynyl-2′-deoxyuridine)-assay]; ELISA (enzyme-linked immunosorbent assay); EM (electron microscopy); EMSA (electrophoretic mobility shift assay); FC (flow cytometry); GPA (gentamicin protection assay); HPLC (high-performance liquid chromatography); ICC (immunocytochemistry); IFM (immunofluorescence microscopy); IHC (immunohistochemistry); IMA (invasion and migration assay); in vitro KA (kinase assay); IP (immunoprecipitation); iTRAQ (isobaric tags for relative and absolute quantitation); IVP (in vitro proteolysis); LA (luciferase assay); LBSAB (leakage of bovine serum albumin biotin); LC-MS/MS (liquid chromatography-mass spectrometry/mass spectrometry); MF (membrane fractionation by iodixanol gradients, OptiPrep™); MIA (Matrigel™ invasion assay); MS (mass spectrometry); n.d. (not determined); NB (northern blotting); NRU (neutral red uptake); NTS (N-terminal sequencing); PA (permeabilization assay); PCR (polymerase chain reaction); PF (paracellular flux); PS (peptide synthesis); QAB (quantification of adherent bacteria assay); qPCR (quantitative real-time PCR); RGA (reporter gene assay); RT (retroviral transduction); RT-PCR (real-time reverse transcriptase PCR); SCF (subcellular fractionation); SCX (strong cation exchange chromatography); SEM (scanning electron microscopy); siRNA (small interfering RNA); SPR (surface plasmon resonance); sqPCR (semiquantitative PCR); TEM (transmission electron microscopy); TER (transepithelial electrical resistance); TLIA (time-lapse imaging analysis); WB (Western blotting)

the paracellular epithelial route to certain molecules and ions, which may serve to acquire nutrients to support *H. pylori* survival and growth in vivo (Papini et al. 1998). Further work showed that infection of Madin-Darby canine kidney (MDCK) monolayers with *H. pylori* also resulted in a decrease in TER, while isogenic Δ*vacA* mutants did not (Pelicic et al. 1999). A similar effect was observed with various VacA-producing isolates, including those expressing m2-type toxins that exhibit no vacuolating activity, suggesting that vacuole formation per se is not required for the response. Later, it was postulated that VacA permeabilizes the apical membrane of gastric parietal cells and induces hypochlorhydria (Wang et al. 2008). Using freshly isolated rabbit gastric glands and cultured parietal cells, it was shown that VacA induces an influx of extracellular calcium (Ca^{2+}), followed by activation of the protease calpain and subsequent cleavage of ezrin, a regulator of filamentous actin (F-actin) in cell junctions (Selbach et al. 2004), which results in the liberation of ezrin from the apical membrane of parietal cells (Wang et al. 2008). Electron microscopic examination revealed that VacA treatment disrupts the radial arrangement of F-actin filaments in apical microvilli due to the loss of ezrin integrity in parietal cells (Wang et al. 2008). Further studies showed that VacA-containing culture supernatants disrupt the actin cytoskeleton of epithelial cell lines, leading to cell rounding and apoptosis through anoikis (Bebb et al. 2003a). However, the role of VacA is not yet fully clear as other data have shown that isogenic Δ*vacA* mutants behaved like wild-type *H. pylori* and changed TER during infection (Wroblewski et al. 2009).

3.1.2 Disruption of Tight Junctions by Ectopic CagA

Another bacterial effector protein involved in the alteration of TJs by *H. pylori* is CagA. A pioneering study has shown that CagA mediated the redistribution of TJ proteins in MDCK cells. Amieva et al. (2003) showed that translocated CagA associates with ZO-1 and JAM, causing an ectopic assembly of TJ components at sites of bacterial adherence, thus altering composition and function of the apical–junctional complex. Further studies have indicated that ectopic expression of CagA can disrupt the cell-to-cell junctions and this depends on the phosphorylation state of CagA and specific CagA domains (Bagnoli et al. 2005). Transfection experiments showed that CagA expression is not only sufficient to disrupt the apical junctions, but also perturbs epithelial cell differentiation. CagA-expressing cells lose their polarity and cell-to-cell adhesion, acquiring an invasive cell phenotype (Bagnoli et al. 2005). Expression of the carboxy-terminal EPIYA-containing CagA domain stimulates pseudopodial activity, but is not sufficient to trigger cell migration. Importantly, the amino terminus of CagA targets the protein to the apical cell junctions. However, neither domain alone is sufficient to change cell polarity or cell adhesion, but when coexpressed in *trans*, the amino terminus determines the localization of both polypeptide chains (Bagnoli et al. 2005). It appears that the first 200 amino acids of CagA are implicated in an inhibition of certain CagA functions, in particular diminishing cell elongation and apical surface constriction (Pelz et al. 2011).


(a)
H. pylori
Urease
IL-1R
MLCK
ROCK
MLC
Transcriptional responses
Actin contraction
Cdx2
Nucleus
MMPs
MMP secretion
VacA
Opening of tight junctions
Tricellulin
Occludin
Claudins
JAM-1
ZO-1
CagA
Par1b
Regulatory complex
Cdc42
Rac1
Par3
Par6
aPKC
Disruption of tight junctions
Cell polarity
T4SS pilus
Tight junction signaling
(b)
Wnt signaling
IL-1β
IL-1R
HtrA
Wnt
CagA
GSK-3β
E-cadherin cleavage
Ck1
APC
Axin
c-Met
p120ctn
Snail
Cadherin
β-catenin
Proteasomal degradation
E-cadherin suppresion
Ca2+
cdh1 gene methylation
MMP transcription
Disruption of adherens junctions
Kaiso
TCF/LEF
cyclin d1, t c-myc, mmp7
Cell proliferation
Bacterial transmigration
Adherens junction signaling

◂**Fig. 2** Model of *H. pylori*-induced epithelial barrier disruption by specific targeting of tight and adherens junction proteins. Schematic presentation of two junctional complexes and particular signaling pathways, which are induced during infection with *H. pylori* and/or treatment with purified proteins. For simplification, only targeted factors are displayed. **a** TJs are hijacked by purified VacA, which can selectively open the TJs by a yet unknown mechanism (*1*). *H. pylori* translocates CagA proteins into the host cell cytoplasm via the T4SS pilus. CagA has been shown to colocalize with ZO-1 and JAM proteins (*2*). The overall integrity of TJs is maintained by a regulatory complex (gray circle) including atypical PKC (aPKC), Cdc42, Rac1, Par3 and Par6. aPKC can phosphorylate Par1b kinase at threonine residue 595. Activated Par1b specifically localizes to the basal and lateral membranes to regulate cell polarity (*3*). Transfected or translocated CagA binds Par1b and thereby inhibits aPKC-mediated phosphorylation of Par1b (*4*). The CagA-Par1b complex mislocalizes to TJs and apical membranes (*4*). This signaling results in the disruption of TJs and loss of cell polarity (*5*). *H. pylori* also stimulates host nuclear responses (*6*). In this way, various matrix metalloproteases (MMPs) are transcriptionally upregulated, which can be secreted and cleave TJ proteins (*7*). Another target is transcription factor Cdx2, which upregulates the expression of claudin-2 (*8*). *H. pylori* can also induce IL-1 receptor (IL-1R) phosphorylation, playing a role in ROCK kinase activation (*9*) and subsequently claudin-4 disruption (*10*). The *H. pylori* urease enzyme also affects TJ proteins in two ways, first by activating myosin light chain (MLC) phosphorylation by MLC kinase (*11*) or by elevating free $NH4^+$ levels resulting in occludin fragmentation by a yet unknown mechanism (*12*). **b** AJs are targeted by *H. pylori* in multiple ways. The bacteria translocate CagA proteins into the host cell which may interact with E-cadherin directly (*1*). This interaction results in the release of β-catenin from the E-cadherin complex and subsequently translocation of β-catenin into the nucleus. In this way, β-catenin acts as cofactors for TCF/LEF transcription factors to stimulate the expression of various proliferative target genes such as the proto-oncogenes *cyclin d1* and *c-myc* (*2*). This response can be enhanced by p120-catenin ($p120^{ctn}$) translocating to the nucleus where it interacts with Kaiso to relieve Kaiso-mediated inhibition of TCF/LEF transcription (*3*). Deregulation of the Wnt pathway including glycogen synthase kinase 3 beta (GSK-3β), adenomatous polyposis coli (APC), casein kinase 1 (CK1) and Axin feeds into the same pathway, affecting β-catenin phosphorylation, nuclear localization or degradation (*4*). Another report showed that CagA forms a complex with E-cadherin, c-Met and $p120^{ctn}$ affecting cell migration and invasion (*5*). Intracellular CagA can also bind GSK-3, resulting in reduced GSK-3 activity. In this way, CagA stabilizes Snail, a transcriptional repressor of E-cadherin expression (*6*). *H. pylori* also activates MMP transcription (*7*), leading to elevated MMP secretion and AJ protein cleavage (*8*). In addition, *H. pylori* secretes the serine protease HtrA, which can cleave E-cadherin directly (*9*). It was also shown that *H. pylori* activates the IL-1 receptor (IL-1R) by upregulating IL-1β, which results in E-cadherin gene (*cdh1*) methylation (*10*), suppression of E-cadherin translation and downregulation at the protein level (*11*). The result of these processes is a local epithelial disruption allowing some *H. pylori* entering the intercellular space and reaching basal membranes (*12*). In this manner, the bacteria could probably access the basal integrin receptor and translocate CagA. The position of CagA translocation and the sequence of various indicated events are hypothetical in this model and were not yet proven experimentally

The detailed interplay between the inhibitory N-terminal part of CagA and the tyrosine-phosphorylated C-terminal part needs to be investigated in future. Further studies showed that CagA specifically interacts with PAR1/MARK kinase, which has an essential role in controlling epithelial cell polarity (Saadat et al. 2007; Nesic et al. 2010; Zeaiter et al. 2008). Association of CagA inhibits PAR1 kinase activity and prevents atypical protein kinase C (aPKC)-mediated PAR1 phosphorylation, which dissociates PAR1 from the membrane, collectively causing junctional and polarity defects (Fig. 2a). Taken together, these data suggest that CagA induces a

morphogenetic program in polarized epithelial cells resembling an epithelial mesenchymal transition (EMT) phenotype, which may be an early event in *H. pylori*-induced carcinogenesis. Further support for these ideas came by studies on gastroids that developed into a self-organizing differentiation axis. Infection of these gastroids showed that *H. pylori* induced the mislocalization of claudin-7 and increased cell proliferation in a CagA- and β-catenin-dependent fashion (Wroblewski et al. 2015). In another publication, it was reported that CagA targets Cdx2 (caudal-related homeobox 2) during an infection of AGS cells with *H. pylori* wild type and Δ*cagA* as control (Song et al. 2013). Cdx2 is an intestine-specific transcription factor highly expressed in multistage tissues of dysplasia and cancer. One specific target of Cdx2, claudin-2, is involved in the regulation of TJ permeability. It was shown that Cdx2 upregulated the expression of TJ factor claudin-2 both at transcriptional and at translational levels (Song et al. 2013). However, AGS cells do not form polarized cell monolayers due to the lack of proper junctions; thus, further studies with polarized cell lines are required. In another study, it was demonstrated that *H. pylori* can disrupt claudin-4 by a Rho kinase (ROCK)-dependent pathway in human HGE-20 gastric epithelial cell monolayers, but this occurs independently of CagA and VacA and without altering claudin-4 transcription (Lapointe et al. 2010). Additional experiments revealed that *H. pylori* induced IL-1 receptor type I (IL-1RI) phosphorylation playing a role in ROCK activation and claudin-4 disruption. Taken together, these findings identify a novel pathophysiological mechanism by which *H. pylori* disrupts gastric epithelial barrier structure via IL-1RI-dependent activation of ROCK, which in turn mediates claudin-4 disruption in TJs (Lapointe et al. 2010).

3.1.3 Urease-Dependent Targeting of Tight Junctions via MLCK and MLC

Urease is another effector protein of *H. pylori*, which has been shown to target TJs, presumably by two independent mechanisms. The urease produces ammonium (NH_3/NH_4^+), which is elevated in the gastric aspirates of *H. pylori*-infected patients and has been implicated in the disruption of TJ functional integrity and the induction of gastric mucosal damage during infection. Lytton et al. (2005) have reported that acute exposure to ammonium salts or NH_3/NH_4^+ derived from urea metabolism by wild-type *H. pylori* resulted in a 20–30% reduction in TER. In contrast, cultures that were exposed to supernatants derived from Δurease mutant *H. pylori*, showed no significant decrease in TER. Occludin-specific immunoblots revealed the expression of a low molecular weight form of occludin at 42 kDa after NH_3/NH_4^+ treatment, but its origin is yet unknown (Lytton et al. 2005). A few years later Wroblewski et al. (2009) demonstrated that phosphorylation of myosin regulatory light chain (MLC) by MLC kinase (MLCK) regulates TJ function during *H. pylori* infection. MLCK was activated by *H. pylori* and the progressive loss of barrier function that was attenuated by inactivation of *ureB*, but not *cagA*, *cagE* or *vacA* genes (Wroblewski et al. 2009). Decrease in TER was also dependent on functional urease activity, and this was significantly decreased by inhibition of

MLCK or Rho kinase or by loss of UreB. In addition, *H. pylori* infection of either cultured monolayers or hypergastrinemic INS-GAS mice induced occludin endocytosis, reflecting the disruption of TJs. Taken together, these results indicate that modulation of TJ functions by *H. pylori* involves various bacterial factors which target individual TJ components by different pathways.

3.2 H. pylori *Actively Disrupts Adherens Junctions to Induce an EMT-like Phenotype*

The loss of E-cadherin functionality is associated with the EMT process through which epithelial cells can convert to motile and invasive growing cells during the progression of gastric carcinogenesis (Yilmaz and Christofori 2010; Huang et al. 2015). Early studies indicated that *H. pylori* infections are significantly associated with the loss of E-cadherin expression and/or functions. Several modes of action have been described that trigger both intracellular signal transduction through the translocated CagA effector and extracellular modifications of E-cadherin through proteases epithelial and bacterial origin. Implicated bacterial factors and altered signaling pathways are highlighted in Fig. 2b and summarized in the following section.

3.2.1 Interference of *H. pylori* CagA with the Integrity of Adherens Junctions

The early observation that *H. pylori* induces a CagA-dependent EMT-like scattering phenotype led to the initial suggestion that CagA is directly responsible for the disruption of lateral AJs through the regulation of intracellular signal transduction pathways. Correspondingly, a physical interaction between ectopically expressed CagA and the IC domain of E-cadherin was shown. CagA binding to the IC domain of E-cadherin was proposed to compete with β-catenin interaction in a CagA phosphorylation-independent manner leading to an increase of cytoplasmic β-catenin (Murata-Kamiya et al. 2007). Mislocalization of β-catenin and internalization of E-cadherin in the cytoplasm upon infection with *H. pylori* wild type, but not with the Δ*cagA* mutant strain, indicated a pleiotropic effect by CagA through binding to Crk adaptor proteins (Suzuki et al. 2005). In addition to the CagA/E-cadherin interaction, it was further postulated that CagA can bind to $p120^{ctn}$ and c-Met, which leads to a suppression of the *H. pylori*-driven cell-invasive phenotype (Oliveira et al. 2009). Ectopically expressed CagA requires the EPIYA motif-containing sequence for binding to E-cadherin, which has been also identified as a multimerization domain within the CagA molecule (Kurashima et al. 2008; Ren et al. 2006). However, it is not entirely clear if there is a direct interaction betweeen CagA and the IC domain of E–cadherin, but may involve additional signaling

molecules such as the PAR1 kinase (Kurashima et al. 2008; Saadat et al. 2007). These data are partly in accordance with a previous work showing the release of β-catenin from the membrane after infection with a CagA-positive *H. pylori* strain in the *Mongolian gerbil* infection model (Franco et al. 2005). Once released from the AJ complex, β-catenin is constantly degraded by the proteasome after phosphorylation by GSK-3β/CK1/APC/axin complex (He et al. 2004). In *H. pylori*-colonized MDCK cells, suppression of β-catenin phosphorylation and degradation was observed as a CagA-independent process that was regulated by GSK-3β and Akt1 kinases (Sokolova et al. 2008), indicating that β-catenin is not only released from the AJ complex, but exhibits additional functions in the nucleus as a cofactor of TCF/LEF transcription factors, which are discussed in Sect. 3.3.

The intracellular CagA-mediated deregulation of AJ functions is controversial in the literature. Analysis of the EMT-like phenotype of *H. pylori*-infected cells revealed that cell motility and elongation are induced via different signal transduction pathways. Obviously, cell motility and loss of cell-to-cell adhesion are independent of CagA delivery, while cell elongation requires CagA translocation and tyrosine phosphorylation (Moese et al. 2004, Tegtmeyer et al. 2009). Biopsy samples of *H. pylori*-positive patients also demonstrated a *cag*PAI-independent reduction of junctional β-catenin expression (Bebb et al. 2006). Accordingly, the disintegration of E-cadherin-mediated AJs was shown as a CagA-independent process as demonstrated by isogenic *H. pylori* mutants (Sokolova et al. 2008; Weydig et al. 2007). These data imply that CagA-independent mechanisms must exist which can deregulate AJ functions in gastric epithelial cells in response to *H. pylori* infections. Further studies are necessary to clarify these important questions.

3.2.2 Disruption of E-Cadherin-Mediated Adherens Junctions by Secreted HtrA

Apart from possible destabilization of the E-cadherin complex by intracellular CagA (Murata-Kamiya et al. 2007; Oliveira et al. 2009), downregulation of E-cadherin expression or promoter hypermethylation (Chan et al. 2003), also proteolytic cleavage of E-cadherin upon infection with *H. pylori* has been consistently reported (Weydig et al. 2007; Schirrmeister et al. 2009). Ectodomain shedding represents an important mechanism in E-cadherin regulation of the healthy epithelium, but is also a very frequent event in cancer progression and is often associated with a poor prognosis due to the high capability to metastasize. Several host cell proteases have been described to cleave E-cadherin on the cell surface, including matrix metalloprotease (MMP)-3, 7, 9 and ADAM (a disintegrin and metalloprotease)-10 and 15 as well as plasmin and kallikrein 7 (Maretzky et al. 2005; Ryniers et al. 2002; Johnson et al. 2007; Davies et al. 2001; Noe et al. 2001; Covington et al. 2005; Symowicz et al. 2007). In fact, *H. pylori* has been shown to upregulate several E-cadherin proteases, including MMP-1 (Pillinger et al. 2007), MMP-3 (Sougleri et al. 2016), MMP-7 (Ogden et al. 2008; Yin et al. 2010; Bebb et al. 2003b), MMP-9 (Kundu et al. 2006), MMP-10 (Costa et al. 2016) or

ADAM-10 (Hoy et al. 2010; Schirrmeister et al. 2009). Correspondingly, an increase in serum level of the extracellular domain of E-cadherin has been detected in *H. pylori*-infected patients by O'Connor et al. (2011), which might serve as a biomarker or prognostic marker of gastric cancer.

It is unequivocally clear that host proteases are upregulated and activated in response to *H. pylori* infection, which are implicated in E-cadherin shedding. However, siRNA-mediated downregulation and pharmacological inhibition of various MMPs and ADAM proteases revealed that additional proteases must be involved in this process (Hoy et al. 2010; Schirrmeister et al. 2009). Interestingly, it was found that a soluble factor of *H. pylori* is sufficient to efficiently disrupt E-cadherin-based AJs (Weydig et al. 2007). The serine protease high-temperature-requirement A (HtrA) of *H. pylori* was finally identified as a secreted serine protease that directly and selectively targets E-cadherin in vitro and on gastric epithelial cells (Hoy et al. 2010). Generally, HtrA is expressed as a periplasmic protein, but is also secreted into the environment and was found in outer membrane vesicles (Bumann et al. 2002; Lower et al. 2008; Hoy et al. 2010; Olofsson et al. 2010; Boehm et al. 2013; Turner et al. 2015). Interacting with E-cadherin at the molecular level, HtrA targets amino acid stretches containing the [VITA]↓[VITA]-x-x-D-[DN] motif within the E-cadherin molecule as preferred cleavage positions (Schmidt et al. 2016b). HtrA cleavage sites are positioned between the five individual extracellular repeats (EC1-EC5), which are important calcium-binding motifs. Functional E-cadherin-mediated AJs require calcium binding to form functional homophilic interactions between the EC domains in *cis* and *trans*. Therefore, in a physiological context, these sites are only partially accessible in calcium-bound E-cadherin. It is hypothesized that calcium binding to the HtrA-targeted E-cadherin cleavage sites limits the HtrA-mediated E-cadherin and explains why a stable 90-kDa fragment is observed during infection with *H. pylori* instead of a fragment ladder (Schmidt et al. 2016a). These data point to a tightly controlled E-cadherin shedding mechanisms probably involving both host and bacterial proteases.

If HtrA-mediated E-cadherin shedding does not only open intercellular adhesion, but also destabilize the intracellular E-cadherin complex to release β-catenin, $p120^{ctn}$ has not been investigated yet. However, the consequences of the disruption of AJs by *H. pylori* are potentially substantial for the gastric epithelium and might be one aspect to permit *H. pylori* transmigration across this barrier (Hoy et al. 2010, 2012; Schmidt et al. 2016b). In *H. pylori*, HtrA showed a remarkable stability under extreme conditions (high temperature, high salt concentrations, etc.), which is certainly beneficial in the gastric environment (Hoy et al. 2013). The HtrA protease is highly conserved in *H. pylori* strains across the world and is absolutely essential for *H. pylori* survival (Tegtmeyer et al. 2016) underlining the importance of HtrA in *H. pylori* physiology and pathogenesis. In contrast to many other bacteria, it was not yet possible to create an *htrA*-deletion mutant in *H. pylori* (Tegtmeyer et al. 2016; Salama et al. 2004). Hence, the development and optimization of HtrA inhibitors are currently of high interest (Geppert et al. 2011; Lower et al. 2011; Perna et al. 2014, 2015). The application of an HtrA-specific small molecule

inhibitor indicated that HtrA significantly contributes to *H. pylori*-mediated E-cadherin ectodomain shedding and consequently, bacterial transmigration across an intact epithelial barrier (Hoy et al. 2010; Boehm et al. 2012). Based on the above HtrA cleavage sites in E-cadherin, a substrate-derived peptide inhibitor was also developed that selectively bound and inhibited HtrA, thereby blocking transmigration of *H. pylori* (Schmidt et al. 2016b). These studies imply that HtrA-mediated E-cadherin cleavage is a crucial step in the infection of *H. pylori* by opening intercellular adhesions locally allowing *H. pylori* access to the basolateral membranes of a polarized epithelium, where it can interfere with different host factors, which are necessary to promote the infection.

3.3 Direct Targeting of Tight and Adherens Junction Proteins by Changing Nuclear Responses

In *H. pylori*-infected patients with early-onset gastric cancer, somatic *cdh1* gene mutations and cytoplasmic β-catenin localization were observed indicating that E-cadherin-mediated AJs in gastric cancer patients were disrupted (Saito et al. 1999). Mislocalization of E-cadherin and catenins was also found in cultured and primary human epithelial cells through the detection of E-cadherin, β-catenin, α-catenin and $p120^{ctn}$ in intracellular vesicles upon infection with *H. pylori* leading to a destabilization of cell adherence (Conlin et al. 2004; Weydig et al. 2007; Krueger et al. 2007). This might be further supported by alterations of the E-cadherin expression. In patients, downregulation of E-cadherin expression was significantly associated with *H. pylori* infection in antral biopsy sections (Terres et al. 1998). Similar observations were made for α-catenin. Associated with an infection with *H. pylori*, mRNA levels of α-catenin were reduced in gastric cancer tissues (Yu et al. 2000). Together with additional cell adhesion molecules, E-cadherin expression was downregulated as monitored by RT-PCR and Western blot analyses (Lim et al. 2003). The downregulation of E-cadherin was further connected with promoter methylation in gastric mucosae from intestinal metaplasia, primary and metastatic cancer indicating that *H. pylori*-mediated promoter methylation might occur early in carcinogenesis (Chan et al. 2003; Perri et al. 2007). Eradication of *H. pylori* by antibiotics reverted hypermethylation in patients with chronic gastritis (Chan et al. 2006) and gastric cancer (Leung et al. 2006). The transcriptional downregulation of E-cadherin expression was also observed in cells, which were infected for 24 h with *H. pylori* in vitro (Lee et al. 2014b). Long-term infections stabilized the zinc-finger transcription factor and EMT marker protein Snail, which has been implicated in E-cadherin suppression via its binding to E-cadherin proximal promoter (Lee et al. 2014a). In MKN28 cells, *H. pylori* induced the suppression of claudin-7 that was regulated by elevated β-catenin and Snail levels. Comparably, Snail expression was elevated and claudin-7 levels were downregulated in *H. pylori*-infected gastric patients (Wroblewski et al. 2015).

Mechanistically, the transcriptional repressor Snail is stabilized in response to CagA/GSK-3 interaction through which GSK-3 activity is inactivated (Ngo et al. 2016; Sougleri et al. 2016).

Besides the transcriptional control of E-cadherin expression, impairing the E-cadherin-mediated AJ complex function activates cancer-associated signal transduction pathways. Cytoplasmic accumulated β-catenin can translocate into the nucleus where it can function as a cofactor for TCF/LEF transcription factors. Nuclear localization of β-catenin in *H. pylori*-infected AGS cells and after over-expression of CagA indicated that CagA might have a regulating effect in β-catenin-dependent signal transduction (Franco et al. 2005). Whether this reflects a physiological effect is questionable as AGS cells do not express E-cadherin, but show abnormal β-catenin localization. However, enhanced transactivation of *cdx1* in MKN28 cells (Murata-Kamiya et al. 2007) or *cyclin d1* in MDCK cells (Sokolova et al. 2008) was described acting as proliferation markers indicating that *H. pylori* infection can induce β-catenin-mediated TCF/LEF transactivation in E-cadherin-positive cells. However, the majority of reports indicate that CagA is not implicated in this process. Accordingly, β-catenin-mediated up-regulation of TCF/LEF-dependent transcription in MDCK cells was described as T4SS dependent, but CagA independent (Sokolova et al. 2008). A similar observation was made in a study investigating $p120^{ctn}$ functions in *H. pylori*-infected cells. Nuclear translocation of $p120^{ctn}$ relieved Kaiso-mediated transcriptional repression of *mmp-7*. This mechanism required a functional *cag*PAI, but not CagA expression (Ogden et al. 2008). If the increase of MMP-7 expression enhances E-cadherin shedding needs to be investigated in future, but represents a possible scenario.

In summary, these data imply that not only the function of E-cadherin within the AJ complex can be affected, but also its expression at the transcriptional level. Loss of function and downregulated expression of E-cadherin are strongly associated with invasive growth of gastric tumor cells, and *H. pylori* can deregulate various factors controlling E-cadherin and catenin functions. These findings indicate a coordinated deregulation of E-cadherin expression and function in *H. pylori*-infected gastric epithelial cells.

4 Multistep Model for *H. pylori*-Induced Epithelial Barrier Disruption

Through the expression of manifold pathogenic and virulence factors, *H. pylori* can colonize the gastric epithelial cells and interfere with host cell functions at several levels. Early events, such as bacterial motility, adherence and colonization, are indispensable as very first steps in the infection process and are discussed in other excellent reviews (Caron et al. 2015; Aspholm et al. 2006; Dubois and Boren 2007). Importantly, the progression of gastric cancer in *H. pylori*-infected patients is accompanied by the loss of cell polarity and the disruption of the epithelial

architecture, processes which are closely linked with cancer-associated signal transduction pathways. In this context, disruption of TJs and AJs is a hallmark of *H. pylori* infections and involves a combination of different bacterial factors that interact with host cell elements in a locally coordinated and phased manner assembling a complex multistep infection process.

Depolarization of *H. pylori*-infected epithelial cells requires efficient T4SS-mediated translocation of the oncoprotein CagA. The question whether CagA translocation occurs apically or basolaterally is still not answered. In vivo CagA translocation is difficult to prove. Hence, most studies describe an epidemiological association between CagA presence and *H. pylori*-dependent disorders. The knowledge of CagA-triggered depolarization and disruption of the epithelial barrier function mainly came from a series of studies on cultured non-polarized or only partially polarized tumor cells limiting the functional investigations of CagA. Until very recently, it was assumed that *H. pylori* can translocate CagA at the apical surface of non-polarized epithelial tumor cells without the requirement of a host receptor. In agreement with this idea, apical CagA delivery via phosphatidylserine and cholesterol was suggested as a possible mechanism (Murata-Kamiya et al. 2010) that together with infection and transfection studies in vitro implicated that CagA can directly target and disrupt intercellular junctional functions (Fig. 2). However, tyrosine-phosphorylated CagA reflecting its intracellular occurrence was primarily connected with the interaction of the T4SS adhesin CagL with the basolateral receptor $\alpha_5\beta_1$-integrin leading to the hypothesis that *H. pylori* needs to contact the basolateral membrane (Kwok et al. 2007; Saha et al. 2010; Tegtmeyer et al. 2010, 2014; Conradi et al. 2012a, b; Barden et al. 2013, 2014). Later on, additional T4SS components (CagY, CagI and CagA) were identified as β_1-integrin-interacting proteins (Jimenez-Soto et al. 2009) further supporting this hypothesis. However, whether apical and/or basolateral CagA translocation occurs in combination remains yet unknown. In this review, we proposed a complex multistep model covering several sophisticated processes to open intercellular TJs and AJs allowing the access of *H. pylori* to $\alpha_5\beta_1$-integrins summarizing CagA-dependent and CagA-independent mechanisms contributing to the loss of intercellular adhesions and epithelial depolarization.

5 Concluding Remarks

Gastric carcinogenesis is associated with *H. pylori*-induced signaling leading to depolarization of the epithelium. According to the multistep model, we hypothesize that the loss of intercellular adhesion is an important event and facilitates invasive growth of tumor cells. Mechanistically, disruption of TJs and AJs might allow efficient injection of the bacterial effector and oncoprotein CagA to derail cancer-associated signal transduction. Of course, *H. pylori*'s attack on intercellular adhesion is only one piece in the complex pathogenesis scenario, and the knowledge about the mechanisms is steadily increasing. It would also import to investigate in future whether and how other TJ and AJ components such as ZO-2, ZO-3,

cingulin, MUPP1, α-catenin, EPLIN, nectin or afadin may be affected during infection (Fig. 1). A combination of different bacterial factors is involved in gastric barrier disruption, and the direct interference of soluble *H. pylori* factors with components of the TJs and AJs came to attention in the last years as they represent highly attractive drug targets for novel intervention strategies to combat *H. pylori* infections. In addition to the apical–junctional complex, desmosomes and gap junctions are also important constituents, which impact to cell-to-cell interactions (Wei and Huang 2013). Future studies should also consider the investigation of these junctional protein platforms and their potential role in *H. pylori* infections.

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Helicobacter pylori-Induced Changes in Gastric Acid Secretion and Upper Gastrointestinal Disease

Adam J. Smolka and Mitchell L. Schubert

Abstract Appropriate management of *Helicobacter pylori* infection of the human stomach is evolving and remains a significant clinical challenge. Acute infection results in hypochlorhydria, whereas chronic infection results in either hypo- or hyperchlorhydria, depending upon the anatomic site of infection. Acute hypochlorhydria facilitates survival of the bacterium and its infection of the stomach. Interestingly, most patients chronically infected with *H. pylori* manifest a pangastritis with reduced acid secretion due to bacterial virulence factors, inflammatory cytokines, and various degrees of gastric atrophy. While these patients are predisposed to develop gastric adenocarcinoma ($\sim$1%), there is increasing evidence from population studies that they are also protected from gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), and esophageal adenocarcinoma (EAC). Eradication of *H. pylori*, in these patients, may provoke GERD in predisposed individuals and may be a contributory factor for the rising incidence of refractory GERD, BE, and EAC observed in Westernized societies. Only $\sim$10% of chronically infected patients, mainly the young, manifest an antral predominant gastritis with increased acid secretion due to a decrease in somatostatin and increase in gastrin secretion; these patients are predisposed to develop peptic ulcer disease. *H. pylori*-induced changes in acid secretion, in particular hypochlorhydria, may allow ingested microorganisms to survive transit through the stomach and colonize the distal intestine and colon. Such perturbation of gut microbiota, i.e. dysbiosis, may influence human health and disease.

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Contents

1 Gastric Acid Secretion

1.1 Introduction

Helicobacter pylori is a Gram-negative bacterium that infects about half the world's population, colonizing the gastric epithelium and inducing chronic inflammation. Most of those infected persons are asymptomatic, but ~10% develop peptic ulcer disease, and ~1% progress to gastric adenocarcinoma (Polk and Peek 2010). The severity and pathological sequelae of diffuse acute and later chronic inflammation are determined by bacterial virulence, host genetics and environmental factors. One function of the stomach, to regulate and sustain acid secretion at levels sufficient to sterilize ingested nutrients, is impaired in the setting of mucosal inflammation. In chronic infection, the degree of impairment and whether acid secretion is increased or decreased depends on the predominant anatomic focus of the infection (Dixon et al. 1996). Infection of the gastric antrum (i.e., pyloric mucosa) is associated with acid hypersecretion that is driven by an increase in gastrin secretion as well as effects of cytokines derived from infiltrating proinflammatory neutrophils and macrophages. Infection of the gastric body and fundus (i.e., oxyntic mucosa) is associated with acid hyposecretion that is driven by *H. pylori*-induced suppression of H,K-ATPase (the parietal cell proton pump) expression as well as effects of cytokines. *H. pylori*-induced changes in gastric acid secretion can have deleterious as well as beneficial clinical consequences in humans. Deleterious outcomes include duodenal ulcer disease, autoimmune and atrophic gastritis, intestinal metaplasia, and gastric adenocarcinoma. Beneficial outcomes include protection from gastroesophageal reflux disease (GERD), Barrett's esophagus (BE),

esophageal adenocarcinoma (EAC), and rebound hypersecretion after acute cessation of proton pump inhibitors (PPIs) (Reimer et al. 2009; Backert and Blaser 2016).

1.2 Physiology of Gastric Acid Secretion

1.2.1 Neural, Hormonal, and Paracrine Regulation

The major physiological stimulants of acid secretion are histamine, secreted from oxyntic enterochromaffin-like (ECL) cells (paracrine pathway); gastrin, secreted by antral G cells (hormonal pathway) (Beales et al. 1997; DelValle et al. 1987); and acetylcholine, secreted from oxyntic and antral intramural postganglionic neurons (neural pathway). The parietal cell expresses specific receptors for each of these secretagogues (H_2, gastrin or CCK_2, and M_3, respectively). However, it is currently thought that gastrin, the main hormonal stimulant during meal ingestion, activates acid secretion mainly by releasing histamine from ECL cells. Histamine H_2 receptors are coupled predominantly to adenylate cyclase which catalyzes generation of adenosine 3′,5′-cyclic monophosphate (cAMP). However, in rat hepatoma-derived cells transfected with canine histamine H_2 receptor, histamine also elicits concurrent transient elevation of intracellular calcium ($[Ca^{2+}]_i$) with generation of inositol trisphosphate (IP_3) (DelValle et al. 1992). Gastrin, acting via CCK_2 receptors (formerly termed gastrin receptors) coupled to an increase in $[Ca^{2+}]_i$, stimulates the parietal cell directly and, more importantly, indirectly by releasing histamine from ECL cells. Acetylcholine stimulates parietal cells directly through M_3 subtype muscarinic receptors coupled to an increase in $[Ca^{2+}]_i$ and indirectly by inhibiting somatostatin secretion via M_2 and M_4 receptors. The principal inhibitor of acid secretion is somatostatin, released from oxyntic and antral D cells. Oxyntic (gastric body and fundus) somatostatin cells are anatomically and functionally coupled to ECL and parietal cells, whereas antral (pyloric) somatostatin cells are anatomically and functionally coupled to G cells. During the basal state, somatostatin exerts a tonic restraint on acid secretion from the parietal cell, histamine secretion from the ECL cell, and gastrin secretion from the G cell. Removing this restraint (i.e., disinhibition) by activation of cholinergic neurons is an important physiological mechanism for stimulating acid secretion (Fig. 1).

1.2.2 H,K-ATPase: The Parietal Cell Proton Pump

The final step in gastric acid secretion is mediated by the enzyme H,K-ATPase which is expressed in cytoplasmic tubulovesicles and canalicular membranes of parietal cells (Forte et al. 1989; Sachs 1987). Functional H,K-ATPase is a heterotetrameric ($\alpha_2\beta_2$) assembly of catalytic α subunits (HKα) and glycosylated β subunits (HKβ). HKα and HKβ mRNAs are translated on endoplasmic

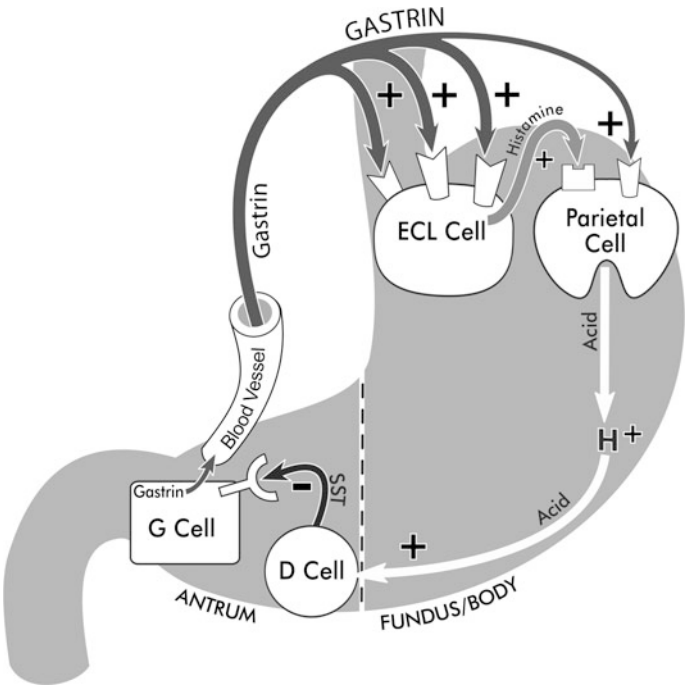

Fig. 1 Regulation of gastrin and acid secretion in health and disease. (i) Between meals, acid secretion is inhibited by somatostatin (SST)-secreting D cells on adjacent gastrin-secreting G cells. This inhibition is complemented by unbuffered luminal acid which also stimulates SST. (ii) During a meal, food buffers the secreted acid and the increased pH inhibits SST, stimulating gastrin secretion. Distension and nutrient protein also stimulates gastrin. Gastrin stimulates parietal cell acid secretion and mediates histamine release from ECL cells. Gastrin also stimulates proliferation of ECL cells, which also occurs during long-term PPI treatment, particularly at high dose. (iii) Most patients chronically infected with *H. pylori* manifest a pangastritis and are hypochlorhydric, due in part to gastric atrophy. In such patients, *H. pylori* may protect against GERD, Barrett's esophagus, and esophageal adenocarcinoma. Eradication of *H. pylori*, especially in patients on long-term PPIs, may lead to acid hypersecretion caused by hypergastrinemia-induced increase in ECL mass. (iv) About 10% of patients with chronic *H. pylori* infection exhibit hyperchlorhydria, have antral predominant inflammation, and are vulnerable to duodenal ulcer disease. The increased acid secretion is due to reduced antral SST secretion and increased basal and stimulated gastrin secretion

reticulum-bound ribosomes into integral membrane polypeptides that are targeted to cytoplasmic tubulovesicles. HKα subunits carry out the transport function of the enzyme, whereas HKβ subunits mediate assembly of functional H,K-ATPase, delivery of the complex to the tubulovesicular and canalicular compartments, and protect the enzyme complex from degradation (Asano et al. 2000; Bakkelund et al. 2010; Spicer et al. 2000). In the absence of luminal K^+, the enzyme complex is inactive. Enzyme activity is stimulated by secretagogue-mediated increases in intracellular cAMP (e.g., histamine) and activation of calcium-dependent signaling

pathways (e.g., acetylcholine) that activate downstream protein kinases, ultimately leading to fusion of tubulovesicles with collapsed invaginations of the parietal cell apical membrane which expand into the extensive microvillous secretory canalicular network characteristic of actively secreting parietal cells. Potassium channels in the canalicular membrane (KCNQ1, its β-subunit KCNE2, and Kir4.1) supply and regulate flow of cytoplasmic K^+ to luminal K^+-binding sites on the HKα catalytic subunit, thereby initiating electroneutral 1:1 exchange of luminal K^+ for cytoplasmic protons (Roepke et al. 2006; Song et al. 2009b, 2011). Under maximal secretory drive, H,K-ATPase activity establishes a million-fold proton gradient across the canalicular membrane (Black et al. 1980; Smolka et al. 1983; Soroka et al. 1993).

PPIs such as omeprazole, lansoprazole, rabeprazole, and esomeprazole are substituted benzimidazoles that potently inhibit acid secretion by covalently binding to luminally exposed cysteine residues on the HKα subunit. PPIs have revolutionized the treatment of GERD, peptic ulcer disease, and Zollinger-Ellison syndrome (gastrinoma). More recently, the potassium-competitive acid blocker Vonoprazan, a lipophilic, weak base pyrrole derivative that accumulates in parietal cell canaliculi and prevents proton pump activation by competing with K^+ on the luminal surface of HKα, offers the potential for more potent and sustained acid suppression with more favorable pharmacokinetics and toxicological profile than conventional delayed-release PPIs (Hunt and Scarpignato 2015; Otake et al. 2016).

2 *H. pylori* Infection of the Stomach

2.1 Colonization of Favored Gastric Niches

The human stomach is an inhospitable environment for microorganisms, including bacteria. High acidity (160 mM hydrochloric acid or pH 0.8), in combination with pepsin and lipase, kills ingested microorganisms. In order to establish gastric colonization, *H. pylori* must minimize its exposure to lethal concentrations of acid (i.e., low pH). Bacterial urease catalyzes the hydrolysis of ambient gastric urea into carbon dioxide and ammonia (Hidaka et al. 2001). Basic ammonia (NH_3) neutralizes protons diffusing into *H. pylori*'s periplasmic space via conversion to ammonium (NH_4^+), thereby maintaining the bacterial microenvironment at a pH compatible with optimal growth and survival. The efficiency of this process is such that *H. pylori* is able to sustain proton motive force across its inner membrane, essential for ATP synthesis, at external pH ranging from 3.5 to 8 (Meyer-Rosberg et al. 1996). The local increase in pH also effects a gel-sol transition in the 200-μm-thick gastric mucus layer that overlies the mucosal epithelium (Bansil et al. 2013; Celli et al. 2009), allowing *H. pylori* to penetrate the mucus gel by virtue of its spiral morphology and flagellar activity. Surface epithelial cells actively secrete bicarbonate, establishing a pH gradient across the mucus gel, although there is little

evidence that mucus itself poses a significant diffusion barrier to hydrogen ions (Schubert and Kaunitz 2015; Tanaka et al. 2002). Within this extracellular niche, immediately adjacent to surface epithelial cells and their tight junctions with neighboring cells, *H. pylori* is well situated to (i) evade humoral and cellular immune defenses and (ii) modulate gastric acid secretion. *H. pylori* inhibits acid secretion directly by interfering with parietal cell proton pump expression, and indirectly by activating neural pathways coupled to stimulation of somatostatin and inhibition of histamine and acid secretion. Other constituents and products of the bacterium [e.g., acid inhibitory factor, vacuolating cytotoxin, and lipopolysaccharide (LPS)] as well as proinflammatory cytokines (interleukin-1β, interleukin-2, tumor necrosis factor-α, and interferon γ) are also capable of directly inhibiting parietal cell secretion (Beales and Calam 1998, 2001; Gooz et al. 2000; Hoffman et al. 1995; Kobayashi et al. 1996; Padol and Hunt 2004; Saha et al. 2010a; Schepp et al. 1998). In rat oxyntic mucosa mounted in Ussing chamber, acute perfusion of the mucosal surface with *H. pylori* derived from humans with duodenal ulcer activates intramural calcitonin gene-related peptide (CGRP) sensory neurons coupled to stimulation of somatostatin and thus inhibition of histamine and acid secretion (Zaki et al. 2013). Activation of neural pathways may explain how initial patchy colonization of the superficial mucosa by the bacterium can acutely and profoundly inhibit acid secretion.

Gastric *H. pylori* colonization is usually accompanied by inflammation, the severity of which is correlated with *H. pylori* density (Alam et al. 1992; Khulusi et al. 1995). Although *H. pylori* can be detected throughout the stomach, the antrum is initially more susceptible to infection and inflammation than the body (Bayerdorffer et al. 1989, 1992; Louw et al. 1993; Satoh et al. 1991). The determinants of *H. pylori* colonization of particular anatomic regions of the stomach are not fully understood, but may include chemosensing of localized gastric epithelial cell secretion of urea, mediated by the bacterial chemoreceptor TipB (Huang et al. 2015). Gastric acid secretory status also plays a role in colonization. The efficiency of *H. pylori*'s defenses against low pH notwithstanding, the organism favors colonization of the antrum, whose mucosal branched glands lack parietal cells and consequently do not secrete acid. Antral predominant infection is associated with a decrease in antral somatostatin content and a corresponding increase in gastrin and acid secretion; such patients are predisposed to duodenal ulcer disease. The decrease in somatostatin secretion may be mediated by proinflammatory cytokines derived from the inflammatory infiltrate (e.g., interferon γ and tumor necrosis factor-α) (Calam 1998). Hypergastrinemia may not only be mediated by decreased somatostatin secretion, but there is evidence that certain cytokines (e.g., interleukin-8 and platelet activating factor) are capable of directly stimulating gastrin as is CagL, an *H. pylori* protein considered to be a component of its type IV secretion system (T4SS) (Beales 2001; Cover 2012; Wiedemann et al. 2012).

With time, exacerbated by the use of antisecretory medications, *H. pylori* infection with inflammation migrates proximally from antrum to the oxyntic mucosa (body and fundus) of the stomach. Most patients chronically infected with *H. pylori* manifest a pangastritis and produce less than normal amounts of acid.

Reduced acid secretion initially is due to suppression of parietal cell H,K-ATPase expression by products of *H. pylori* as well as the inflammatory infiltrate. Subsequently, an advancing front of atrophic gastritis ensues with loss of parietal cells and multifocal areas of intestinal metaplasia. It is upon this background of atrophic pangastritis with intestinal metaplasia that gastric cancer develops (Plottel and Blaser 2011; Spicer et al. 2000; Zavros et al. 2005).

2.2 Immune Response

H. pylori resides in proximity or attached to surface epithelial cells but can penetrate deeper into gastric pits and glands, ultimately breaching tight junctions to gain access to basolateral membrane receptors. The gastric immune response to *H. pylori* is necessarily complex (Hunt et al. 2015) as it serves as much to provide an hospitable environment for continued colonization and infection as it does to protect the host from the more damaging potential sequelae of *H. pylori*'s delivery of virulence factors into gastric epithelial cells. The immune response includes both innate (epithelial, neutrophil, macrophage, dendritic cell) and adaptive (B and T cell) components. The innate responses are particularly relevant to acid secretory regulation as *H. pylori*-induced secretion of IL-8 from epithelial cells mediates chemotactic recruitment of neutrophils and macrophages into glandular mucosa where their release of IL-1β (Cullen et al. 2015) potently inhibits acid secretion (Beales and Calam 1998; Wallace et al. 1991). Ingestion of *H. pylori* or its cellular debris by antigen-presenting cells in the lamina propria promotes activation of the adaptive response which leads to diverse cytokine production by helper T cells (Th), including interferon-γ (Th1 cells) and interleukin-17 (Th17 cells), while regulatory T cells (Tregs) mediate tolerance. The Th cytokines stimulate epithelial cell production of chemokines which drive neutrophil and macrophage secretion of reactive oxygen and nitrogen species (ROS and RNS). Hydroxyl radicals enhance IL-8-induced gastrin-stimulated acid secretion (Yakabi et al. 2003), and exposure of gastric epithelial cells to hydrochloric acid itself induces mitochondrial superoxide production, which then triggers cellular lipid peroxidation and apoptosis (Matsui et al. 2011). Production of these mediators of gastritis, which would otherwise promote an unfavorable environment for continued *H. pylori* colonization, is counteracted by anti-inflammatory responses conferred by *H. pylori*-induced activation of a subset of Tregs that express FOXP3, a transcription factor that regulates Treg development (Fontenot et al. 2005) and increase mucosal levels of IL-10 and TGF-β1 (Arnold et al. 2011; Kabisch et al. 2014; Kandulski et al. 2008). These and other cytokines are now understood to play an important role in persistence of *H. pylori* infection (Harris et al. 2008; Rad et al. 2006; Koch et al. 2015). Interestingly, ROS production has been reported to promote glucose-stimulated somatostatin secretion in rat gastric primary D cells, thereby inhibiting acid secretion and favoring *H. pylori* persistence (Li et al. 2010).

3 *H. pylori*-Induced Changes in Gastric Acid Secretion

3.1 Acute Hypochlorhydria

Acute infection with *H. pylori* results in hypochlorhydria, whereas chronic infection results in either hypo- or hyperchlorhydria, depending upon the predominant anatomic site of infection. Many clinical studies, including reports of accidental *H. pylori* inoculation through contaminated gastric endoscopes and nasogastric tubes, have associated acute *H. pylori* infection with transient hypochlorhydria. Gastric pH one to four weeks after initial infection was reported to range from 6.4 to 7.6 (Gledhill et al. 1985; Morris and Nicholson 1987; Ramsey et al. 1979; Sobala et al. 1991), with acid secretion returning to baseline levels within a few weeks or months (El-Omar et al. 1997; Gledhill et al. 1985; Graham et al. 1988; Harford et al. 2000; Marshall 1995; Morris and Nicholson 1987; Ramsey et al. 1979; Sobala et al. 1991). The hypochlorhydria associated with acute infection is thought to facilitate survival of the bacterium and its colonization of the stomach (Merchant 2005). Acute *H. pylori*-induced acid inhibition is not caused by parietal cell loss or atrophy as normal numbers of parietal cells are reported in the stomachs of acutely infected Mongolian gerbils as well as gastric biopsies of inadvertently infected patients (Graham et al. 1988; Ramsey et al. 1979; Takashima et al. 2001).

Soon after the discovery of *H. pylori* as an etiologic agent of gastritis and peptic ulceration, in vitro studies showed that interaction of parietal cells with bacteria and/or with secreted bacterial products was sufficient to inhibit acid secretion (Cave and Vargas 1989; Defize et al. 1989; Hoffman et al. 1995). Human gastric mucosal ultrastructural studies revealed the presence of *H. pylori* in the immediate vicinity of parietal cells and even sequestered within parietal cell secretory canaliculi (Bjorkholm et al. 2000; Chen et al. 1986; Tagkalidis et al. 2002; Tricottet et al. 1986). In animal studies, dogs and ferrets became achlorhydric after infection with *Helicobacter* species (Fox et al. 1993; Lee et al. 1992), and acid secretion by isolated rabbit and guinea gastric cells, as measured by [^{14}C] aminopyrine accumulation, was reduced after *H. pylori* infection (Cave and Vargas 1989; Kobayashi et al. 1996). In human parietal cells, *H. pylori* inhibits histamine-, carbachol-, and dibutyryl cyclic AMP-stimulated acid secretion (Jablonowski et al. 1994a, b). The acute inhibitory effects of *H. pylori* on acid secretion are transitory and normal acid secretion can be restored after *H. pylori* is eradicated (Furuta et al. 1999).

H. pylori interferes with transcription of the parietal cell HKα gene. In gastric AGS cells transfected with human or rat HKα 5′-flanking DNA sequence fused to a luciferase reporter plasmid (Gooz et al. 2000), histamine elicits a dose-dependent increase in cAMP, $[Ca^{2+}]_I$, and HKα promoter activity. Addition of *H. pylori* of the transfected cells dose-dependently inhibited basal and histamine-stimulated HKα promoter activity as well as HKα activity induced by phorbol myristate acetate or the diacylglycerol analog 1-oleoyl-2-acetyl-*sn*-glycerol that was sensitive to staurosporine and calphostin C. The data indicate that *H. pylori* inhibits HKα gene expression via intracellular pathways involving protein kinases A and C

(Gooz et al. 2000). Interestingly, the study also showed that a human HKα-*Luc* reporter construct transfected into human gastric epithelial cells was far more sensitive to *H. pylori*-induced repression than the rat HKα reporter, consistent with the fact that, with the exception of non-human primates (Dubois et al. 1994), the human stomach is the only substantial reservoir of *H. pylori*. A later study assessed the biochemical and physiological consequences of *H. pylori*-mediated HKα gene repression (see below) (Saha et al. 2010b). Taken together, the data confirmed that *H. pylori*-induced repression of HKα promoter-reporter constructs in AGS cells is recapitulated in acutely isolated, *H. pylori*-infected human gastric biopsies, where the effects of infection are manifest as significant attenuation of HKα mRNA, virtual disappearance of HKα protein subunit, and concomitant inhibition of gastric acid secretion. The data are consistent to a certain degree with a microarray analysis of parietal cell DNA from germ-free and *H. pylori*-infected mice that showed 5.3-fold down-regulation of H,K-ATPase α-subunit expression by *H. pylori* (Mills et al. 2001). However, the relatively comprehensive *H. pylori*-induced elimination of HKα protein and acid secretory capacity observed in human biopsies (Saha et al. 2010b) suggests that in addition to HKα transcriptional regulation, *H. pylori* also targets posttranscriptional phases of H,K-ATPase synthesis and activation.

The evidence and mechanistic basis for *H. pylori*-induced interference with HKα gene expression was recently reviewed (Smolka and Backert 2012). Pathogenic *H. pylori* strains that express a T4SS with pilus can inject virulence factors into gastric epithelial cells. Structural T4SS proteins are encoded by a cytotoxin-associated gene (*cag*) pathogenicity island (PAI). The *H. pylori* T4SS protein, CagL, interacts with host cell $\alpha_5\beta_1$ integrin (Kwok et al. 2007; Conradi et al. 2012; Barden et al. 2013) facilitating injection of the oncogenic bacterial protein CagA which can activate multiple signaling pathways including the proinflammatory transcription factors NF-κB (Backert and Selbach 2008) (Fig. 2). *H. pylori* infection of gastric epithelial cells in vitro has been shown to inhibit HKα gene expression by ERK 1/2-mediated NF-κB p50 homodimer binding to HKα promoter (Saha et al. 2008). Acute *H. pylori* infection causes CagL to dissociate ADAM17 from $\alpha_5\beta_1$ integrin, activating ADAM17-dependent, NF-κB-mediated repression of HKα promoter (Saha et al. 2010a). *H. pylori cag*PAI isogenic mutants (Δ*cagE*, Δ*cagM*, and Δ*cagL*) failed to repress HKα, confirming the need for T4SS integrity (Saha et al. 2010b). *H. pylori* is also implicated in posttranslational regulation of HKα expression. *H. pylori* infection has been reported to up-regulate gastric epithelial cell microRNA (miR-1289), which in turn binds to a highly-conserved HKα 3′-UTR binding site, repressing HKα mRNA translation (Zhang et al. 2014). In the same study, CagA and bacterial soluble lytic transglycosylase (SLT) (Hammond et al. 2015; Viala et al. 2004) were also implicated in HKα-specific miR-1289 up-regulation. More recently, virulence factors other than those mediating CagA translocation or IL-8 induction have been reported to participate in HKα repression by activating NF-κB (Hammond et al. 2015). AGS cells transfected with HKα promoter-*Luc* reporter constructs containing an intact or mutated NF-κB binding site were infected with wild-type *H. pylori* strain 7.13, isogenic mutants lacking *cag*PAI genes responsible for CagA translocation and/or IL-8 induction (*cagA*, *cag*, *cag*, *cagZ* and *cagβ*), or deficient in

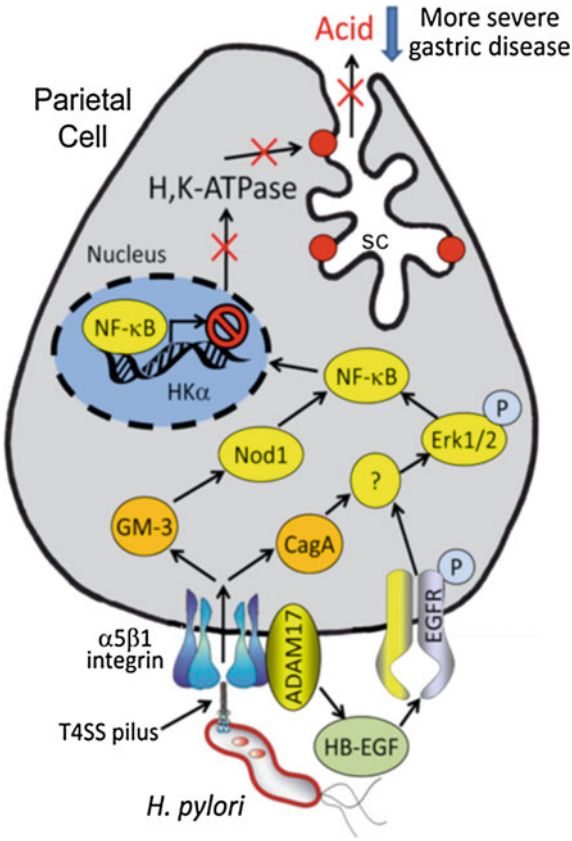

Fig. 2 Mechanistic basis of *H. pylori*-induced acid inhibition. Schematic diagram of a gastric parietal cell showing the *H. pylori* T4SS pilus interacting with α5β1 integrin on the basal lateral membrane to facilitate delivery of CagA and SLT-derived glycosylated tripeptide GM-3 into the parietal cell. Subsequent activation of diverse host signaling pathways mobilizes nuclear factor-κB (NF-κB) p50 homodimers to the nucleus resulting in transcriptional repression of the H,K-ATPase α subunit gene. *H. pylori* interaction with host cell integrin also activates ADAM17, leading to generation and binding of heparin-binding epidermal growth factor (HB-EGF) to EGF receptor (EGFR) and synergistic ERK-mediated mobilization of NF-κB (*sc* secretory canaliculus)

genes encoding two peptidoglycan hydrolases (*slt* and *cagγ*). Measurement of *H. pylori*-induced AGS cell HKα promoter activity, translocated CagA, IL-8 secretion and acid secretion in human oxyntic biopsies showed that HKα repression is independent of IL-8 expression and that CagA translocation together with *H. pylori* transglycosylases encoded by *slt* and *cagγ* participates in NF-κB-dependent HKα repression and acid inhibition (Hammond et al. 2015).

Although virulence factors secreted by *H. pylori* as well as inflammatory cytokines are capable of directly inhibiting parietal cell secretion, such inhibition may be restricted to inter-prandial periods when parietal cells are in a resting state, and bacterial penetration deep into oxyntic glands is not impeded by the relatively

high intraglandular pressures generated during active secretion (Holm et al. 1992). In rat oxyntic mucosa mounted in Ussing chamber, it was shown that acute perfusion with *H. pylori* activates CGRP sensory neurons coupled to stimulation of somatostatin and inhibition of histamine secretion (Zaki et al. 2013). Activation of neural pathways may explain how initial patchy colonization of superficial gastric mucosa can acutely and profoundly inhibit acid secretion.

3.2 Chronic Hypochlorhydria

Most patients chronically infected with *H. pylori* manifest a pangastritis with hypochlorhydria. Reduced acid secretion is mainly due to functional inhibition of parietal cell secretion by products of the bacterium as discussed above, and/or the inflammatory infiltrate, specifically the cytokine IL-1β. Polymorphisms in the promoter region of the host *IL-1B* gene significantly increase neutrophil production of IL-1β in the lamina propria of *H. pylori*-infected gastric mucosa, leading to more profound inhibition of acid secretion. Such prolonged inhibition, coupled with the permanent loss of acid secretory capacity because of chronic inflammatory damage to parietal cells, leads to corpus atrophy with loss of parietal cells and reduced acid secretion (Furuta et al. 2002). This progression to chronic hypochlorhydria may be entirely or partly reversible with eradication of the organism, particularly if atrophy is incomplete. The proinflammatory genotypes of the *IL-1B* gene have been shown to reduce the risk of GERD and complications of erosive esophagitis, an association whose mechanistic basis may be induction of gastric atrophy with reduced acid secretion (Ando et al. 2006).

3.3 Hyperchlorhydria

Hormonal (somatostatin and gastrin) and neural (CGRP) mechanisms are understood to underlie gastric hyperchlorhydria induced by chronic *H. pylori* infection of gastric antral mucosa. Gastrin secretion by antral G cells is regulated by a negative feedback pathway predicated on intraluminal pH, such that gastrin secretion is inhibited at low pH (high acidity) and stimulated at relatively high pH (low acidity); the pathway involves somatostatin. High concentrations of acid activate sensory CGRP neurons that, via an axon reflex, stimulate somatostatin secretion and thus inhibit gastrin secretion, whereas low concentrations of acid, for example, by antisecretory medications such as PPIs or gastric atrophy, fail to stimulate or inhibit somatostatin secretion, and patients develop hypergastrinemia (Brand and Stone 1988; Manela et al. 1995; Schubert et al. 1988). It is conceivable that *H. pylori*'s capacity to alkalinize its immediate environment by virtue of urease hydrolysis of gastric urea creates a localized high pH in the vicinity of antral somatostatin-containing D cells, resulting in decreased somatostatin and increased gastrin

secretion; the latter driving parietal cell trophism and increased acid secretion (Levi et al. 1989). In addition, *H. pylori*-mediated release of proinflammatory cytokines is capable of inhibiting somatostatin secretion and stimulating gastrin secretion (Zavros et al. 2002; Moss et al. 1992; Odum et al. 1994). Finally, neural involvement in *H. pylori*-induced hyperchlorhydria was inferred from a study in which the inhibitory effect on gastric acid secretion induced by antral distension was absent in chronically *H. pylori*-infected patients whether or not they had duodenal ulcer disease (Olbe et al. 1996).

4 *H. pylori* in Gastric and Esophageal Diseases

4.1 Gastric Atrophy, Intestinal Metaplasia, and Adenocarcinoma

Gastric cancer claimed 723,000 lives globally in 2012, making it the third-highest mortality cancer after lung cancer (1.6 million lives) and liver cancer (745,000 lives) (Ferlay et al. 2015). Currently, four subtypes of gastric adenocarcinoma are recognized based on comprehensive molecular evaluation of tumors: Epstein-Barr virus-positive tumors; unstable microsatellite tumors; genomically stable with diffuse-type histology; and chromosomally unstable with intestinal-type histology (Cancer Genome Atlas Research Network 2014). *H. pylori* infection is necessary, but not sufficient, for the development of non-cardia gastric cancer; infection confers a small, but measurable, ∼threefold risk (Helicobacter and Cancer Collaborative Group 2001; Rugge et al. 2016). For the most part, the rates of infection and gastric cancer are concordant. However, in Africa as well as in coastal Colombia, the rate of infection with *H. pylori* is high, but the frequency of gastric cancer is low (Correa et al. 1976; Holcombe 1992). The etiology of gastric cancer is multifactorial and involves environmental and genetic risk factors. Risk factors include *H. pylori* strain, genetically determined host inflammatory responses, specific interactions between *H. pylori* virulence factors and the host, gender, ethnicity, and cigarette smoking (Amieva and Peek 2016; Ernst et al. 2006; Rugge 2015). It has recently been proposed that *H.pylori*-induced changes in gastric commensal flora might play a role in carcinogenesis.

In susceptible individuals, bacterial T4SS-mediated delivery of the oncogenic protein CagA, expressed by *cag*PAI-positive *H. pylori* strains, into host antral epithelial cells and glandular stem cells, triggers multiple signaling cascades that increase the risk for distal gastric cancer compared with strains that lack this locus (Amieva and Peek 2016; Mueller et al. 2012; Sigal et al. 2015). CagA also reduces epithelial cell apoptosis, reduces signaling through cell junctions, and enhances oncogenic wound healing responses (Amieva and Peek 2016). There is recent evidence that carcinogenesis may be promoted by (i) integration of bacterial DNA into the human somatic genome (Riley et al. 2013) and (ii) dysbiosis, manifest as growth of non-*Helicobacter* bacteria within the hypochlorhydric stomach (Correa

2004; He et al. 2016). In support of the latter, transgenic, insulin-gastrin mice develop atrophic gastritis, achlorhydria, overgrowth of non-Helicobacter microbiota, and intraepithelial neoplasia at a high rate after *H. pylori* infection compared with germ-free controls (Lofgren et al. 2011).

It has been proposed that *H. pylori* infection initiates a series of events starting with chronic inflammation that, over years in susceptible individuals, leads to atrophic gastritis (including autoimmune gastritis with pernicious anemia), intestinal metaplasia (IM), dysplasia, and eventually gastric adenocarcinoma (Uemura et al. 2001). Problems with this multistep model include the relatively poor inter- and intra-observer agreement on the diagnosis of atrophic gastritis as well as the fact that both atrophy and IM are typically multifocal and commonly missed; yields increase with more extensive biopsy sampling (Capelle et al. 2010; El-Zimaity and Graham 1999; Lim et al. 2013). If a sufficient number of biopsies are obtained, virtually all patients chronically infected with *H. pylori* may have histological evidence of some degree of atrophy and IM (de Vries et al. 2010; Lim et al. 2013; El-Zimaity and Graham 1999; Satoh et al. 1998; Sugano et al. 2015). In reality, atrophy and IM usually coexist and IM is often included in the definition of gastric mucosa atrophy (Rugge et al. 2016). Very few practitioners follow the Sydney System recommendation of obtaining five gastric biopsy specimens: antrum, (greater and lesser curvature), incisura, and corpus greater and lesser curvature. Even if followed, these specimens may still be insufficient for an adequate diagnosis of premalignant lesions (El-Zimaity and Graham 1999; Guarner et al. 2003). The presence of extensive IM (and for that matter, extensive atrophy), however, may portend a significantly higher risk of gastric cancer (Cassaro et al. 2000). IM may be defined as replacement of native gastric glands by intestinal-type glands containing enterocytes, goblet cells, and Paneth cells. Pseudo-pyloric metaplasia, currently termed spasmolytic polypeptide-expressing metaplasia, is the replacement of oxyntic glands by mucin-secreting antral-like glands; it is found in virtually all forms of atrophic gastritis (Rugge et al. 2016).

The lifetime risk of acquiring non-cardia gastric adenocarcinoma is ~0.24% for the general population, and modeling suggests that it is only reduced by 0.2% with *H. pylori* screening/treatment (Yeh et al. 2016). Although all individuals chronically infected with *H. pylori* develop gastric inflammation, only a small fraction (<1%) develop gastric adenocarcinoma (Kodaman et al. 2014). In the Netherlands, patients with premalignant histology still have a relatively low annual incidence of gastric cancer: ~0.1% for atrophic gastritis and 0.25% for intestinal metaplasia (de Vries et al. 2008). In the USA, the annual incidence rate of gastric cancer is only 0.07% in patients with IM (Li et al. 2016). Although eradication of *H. pylori* can resolve gastric inflammation, there is insufficient evidence in Western societies with low rates of gastric cancer as well as some Asian societies with high rates of gastric cancer, that eradication of *H. pylori*, once atrophy or IM occurs, can prevent subsequent cancer (Ford et al. 2015; Kawanaka et al. 2016; Lee et al. 2016; Wong et al. 2004). Consequently, present data do not support routine endoscopic surveillance (or for that matter, *H. pylori* eradication) to prevent gastric cancer, at least in the USA. Such practices may be considered in those patients with additional

risk factors such as Hispanics, Asians, extensive IM, or family history of gastric cancer (Choi et al. 2015; Kim et al. 2016). Although *H. pylori* is conceptually easy to treat, antibiotic resistance and patient nonadherence to complex antibiotic regimens are common causes of treatment failure. Standard initial treatment of *H. pylori* infection with triple therapy consisting of a PPI, amoxicillin, and clarithromycin for 10–14 days is presently suboptimal (<80%), mainly due to an increase in prevalence of clarithromycin resistance (Shiota et al. 2015). Due to "indiscriminate" use of antibiotics, the prevalence, in the USA, of resistance to clarithromycin is 16%, to metronidazole is 20%, and to levofloxacin is 31%. Currently recommended empiric therapies consist of complex 14-day regimens of concomitant and bismuth quadruple therapies (Fallone et al. 2016; Graham and Laine 2016). Other adverse effects of antibiotics are dysbiosis (including *clostridium difficile* infection) and emergence of resistance, not only in *H. pylori*, but also in commensal and other pathogenic bacteria.

4.2 Esophageal Gastroesophageal Reflux Disease, Barrett's Esophagus and Adenocarcinoma

GERD is a common malady affecting 25–40% of the population. GERD refers to troublesome symptoms and/or tissue damage that occurs from the backflow of gastric contents, chiefly acid, into the esophagus. GERD can cause inflammation, metaplasia, dysplasia, and cancer of the esophagus. Patients with GERD do not necessarily secrete increased amounts of acid but rather are thought to have increased dwell times in the esophagus due to hiatal hernia, obesity, transient lower esophageal sphincter (LES) relaxation, reduced LES pressure, and impaired esophageal clearance or acid hypersensitivity (Mitchell et al. 2016). The incidence of EAC has increased sharply, more rapidly than any other malignancy in the USA. Most, if not all cases of EAC arise from preexisting BE. BE is thought to develop, in predisposed individuals, when GERD damages the esophageal mucosa and the injury heals through a metaplastic process in which the normal squamous epithelium is replaced by specialized intestinal epithelium. Not only is the incidence of EAC rising coincident with that of BE and GERD, but the incidence of refractory or PPI-unresponsive GERD is rising and now represents ~25% of GERD patients (Vela 2014). Importantly, eradication of *H. pylori* may be a contributing factor in the increasing incidence of routine GERD, refractory GERD, BE, and esophageal adenocarcinoma.

A substantial body of data supports the notion that *H. pylori* may protect against GERD/BE/EAC by reducing gastric acid secretion and that eradication of the bacteria may increase acid secretion and promote these conditions in predisposed individuals and make them more difficult to manage. Although acid reflux occurs most commonly after a meal, at a time when nutrient buffering of gastric acidity to a pH > 4.0 is greatest, regional differences in postprandial gastric acidity have been reported (Beaumont et al. 2010; Pandolfino et al. 2007), especially immediately

distal to the cardia where pH = 1.6 has been recorded (Fletcher et al. 2001). Thus, factors that compromise LES function are likely to expose the esophageal mucosa to localized highly acidic gastric refluxate. If gastric acid secretion is inhibited by chronic *H. pylori* infection (or by PPI treatment), the esophageal mucosa is exposed to lower concentrations and volumes of acid, a situation that promotes healing of esophagitis and explains the inverse relationship between *H. pylori* and GERD/BE/EAC reported in numerous population studies (Fischbach et al. 2012; Raghunath et al. 2003; Rokkas et al. 2007). As the prevalence of *H. pylori* infection is decreasing in Caucasian adults in developed countries, there is a parallel increasing incidence of GERD/BE/EAC (Labenz and Malfertheiner 1997). In addition, in those with GERD, *H. pylori*-infected patients have significantly less severe esophagitis compared to uninfected patients (Wu et al. 2000). As discussed earlier, most patients chronically infected with *H. pylori* exhibit reduced acid secretion due to products of the bacteria, inflammatory cytokines, and various degrees of gastric atrophy. After eradication, there is amelioration of inflammation and a partial restoration of gastric acid secretion (>fivefold increase in basal and stimulated secretion) (Annibale et al. 2000). When patients with duodenal ulcer but without reflux esophagitis at the time of *H. pylori* treatment were followed up prospectively for up to three years, the incidence of reflux esophagitis was 26% (n = 244) in those cured of infection versus 13% (n = 216) in those with persistent infection ($P < 0.001$) (Labenz et al. 1997a). Patients who developed GERD after eradication have a more severe body gastritis before cure.

Not only may eradication of *H. pylori* promote GERD in predisposed individuals, but it may make it more recalcitrant to treatment with PPIs. The healing rate of reflux esophagitis at 8 weeks is directly related to the duration that gastric pH is >4 over a 24-h period (Bell et al. 1992). In order to achieve >80% healing of erosive esophagitis, pH must be maintained >4 for at least 15 h of the day; this can only be achieved with PPIs, not histamine H_2-receptor antagonists (H_2RAs). Twenty-four-hour intragastric pH studies were performed in 18 *H. pylori* positive subjects before and after a one week course of a PPI, omeprazole (20 mg daily), and repeated after the infection had been cured (Verdu et al. 1995). During omeprazole treatment, 24-h pH values were ~2 logs higher before cure than after cure of infection (5.4 vs. 2.6); this effect persisted when retested 1 year after cure on infection (Labenz et al. 1997b). Thus, the pH achieved with omeprazole represented the sum of the acid inhibitory effect of the PPI as well as that of *H. pylori*; eradication of the bacteria caused the PPI to be less effective. Similar findings, but less pronounced, were reported during 24 h pH recording during treatment with an H_2RA, ranitidine (300 mg hs), before and 4–6 weeks after cure of *H. pylori* infection (Labenz et al. 1997c). The clinical correlate of the greater increase in intragastric pH during treatment with an antisecretory agent in *H. pylori*-infected versus non-infected individuals is improved overall healing of reflux esophagitis in those infected. In 971 patients with endoscopically verified reflux esophagitis treated with a PPI, pantoprazole (40 mg daily) for 4 weeks, overall healing rates were 87% in those infected with *H. pylori* versus 76% in those non-infected (P = 0.0005) (Holtmann et al. 1999). Thus, eradication of *H. pylori* may be one

factor responsible for the rising incidence of PPI-unresponsive GERD. Preliminary data suggest that *H. pylori* may also protect against developing eosinophilic esophagitis (von Arnim et al. 2016).

In *H. pylori* negative patients, discontinuance of long-term (>8–12 weeks) PPIs may result in rebound acid hypersecretion that may exacerbate GERD, particularly in patients with large hiatal hernias (Gillen et al. 1999; Inoue et al. 2004). The phenomenon is due to hypergastrinemia-induced increases in parietal and ECL cell masses and persists for ~8 weeks (Fossmark et al. 2005). The reason rebound hypersecretion does not occur in *H. pylori*-infected individuals who discontinue PPIs may be due to the fact that the bacteria as well as products of the inflammatory infiltrate inhibit acid secretion and thus mask the phenomenon.

5 Concluding Remarks

As discussed, *H. pylori* clearly modulates gastric acid secretion in multiple ways. Whether the patient develops hypo- or hyperchlorhydria depends on the timeline of infection, the predominant anatomic site of infection, the spectrum of virulence factors expressed by the bacterium, and host genetic and immunologic responses. Since microbiota destined for distal intestinal colonization must first transit the hostile barrier of the stomach, *H. pylori*-induced changes in gastric acid secretion may play a role in gut microbial homeostasis. Perturbation of the gut microbiota (dysbiosis) is increasingly understood to impact human health. Given the overwhelming superiority in bacterial cell and gene numbers compared to human cells (tenfold and 400-fold respectively), the genetic potential of gut microbiota to influence human health and disease is massive (Sommer and Backhed 2013). Dysbiosis may be an etiologic factor in diabetes, obesity, metabolic syndrome, heart disease, allergic disorders, and infections (particularly with *C. difficile*) (Wu and Lewis 2013). Significant associations have been reported between *H. pylori* infection and distal intestinal inflammation, in particular inflammatory bowel disease (IBD) (Lidar et al. 2009; Luther et al. 2010; Song et al. 2009a). Additionally, bacterial species in Crohn's disease, ulcerative colitis, and healthy fecal samples were separately clustered by principal components analysis (Qin et al. 2010), and global microbiome data distinguished IBD patients from healthy controls (Frank et al. 2007). Interestingly, Firmicutes are the most abundant gastric bacterial phylum in the absence of *H. pylori* infection in humans, but Proteobacteria predominate in *H. pylori*-infected humans (Sheh and Fox 2013). Thus, studies of the impact of *H. pylori* gastric infection on the abundance and diversity of the colonic microbiota in humans, and the consequences of such impact in terms of susceptibility and/or resistance to disease, should be accorded high priority.

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Impact of the Microbiota and Gastric Disease Development by *Helicobacter pylori*

Teresa Alarcón, Laura Llorca and Guillermo Perez-Perez

Abstract Microorganisms in humans form complex communities with important functions and differences in each part of the body. The stomach was considered to be a sterile organ until the discovery of *Helicobacter pylori*, but nowadays, it is possible to demonstrate that other microorganisms beyond *H. pylori* can colonize the gastric mucosa and that the diverse microbiota ecosystem of the stomach is different from the mouth and the esophagus, and also from the small intestine and large intestine. *H. pylori* seems to be the most important member of the gastric microbiota with the highest relative abundance when present, but when it is absent, the stomach has a diverse microbiota. *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* are the most abundant phyla in both *H. pylori*-positive and *H. pylori*-negative patients. The gastric commensal flora may play some role in the *H. pylori*-associated carcinogenicity, and differences in the gastric microbiota composition of patients with gastric cancer, intestinal metaplasia, and chronic gastritis are described. The gastric microbiota changed gradually from non-atrophic gastritis to intestinal metaplasia, and to gastric cancer (type intestinal).

Contents

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1 Introduction

Microorganisms present in a given environment have been defined as the microbiota of this ecological niche and in the past were classically studied by culture methods. Nowadays, most studies are performed by using sophisticated molecular methods, so the term microbiome is also used, and it refers to the genes that are contained within the microorganism cells, as each sequence detected is assigned to a specific microbial taxa (Ursell et al. 2012). The human microbiota describes the microbial taxa associated with humans and consists of as much as 10–100 trillion microbial cells harbored by each person in the different parts of the body (Ursell et al. 2012). These microorganisms in the human body form complex communities with important differences in each part of the body, such as skin, mouth, respiratory tract, vagina, and gastrointestinal tract. Bacteria comprise the vast majority of the biomass and diversity in the human gut, though small numbers of archaea, viruses, and eukaryotes are also present (Ursell et al. 2012). The microbiota of each organ differs in the number of bacteria and the composition. The highest number of bacteria can be found in the gut microbiome, although each part is a clear ecosystem with different number of bacteria (Ursell et al. 2012; Turnbaugh et al. 2007; Peterson et al. 2009; Qin et al. 2010).

Nowadays, there is enough scientific evidence to consider humans to be superorganisms composed of human and microorganism cells: the number of the microorganism cells is higher than the human cells (in proportion 10:1), and the number of microorganism genes is also higher than human genes (in proportion 150:1) (Abdo et al. 2006).

Microbiota plays an important role in human health, and an increasing number of studies have shown that changes in the composition of human microbiota may correlate with different diseases. According to Blaser et al. (2016), humans and

microbes had coevolved along the history and microbes play essential roles in the whole human life. Human microbiome has important effects in health, as likely affects several organs through the immune, circulatory, and nervous systems (Fujimura et al. 2010), and the microbiome disruptions (dysbiosis) may play a role in modulating associations between diet and disease in malnutrition and obesity (Blaser et al. 2016).

The human digestive tract has important differences in the number of bacteria: The oral cavity has a high number; then, the esophagus and the stomach have the lowest number of bacteria, the number increased in the small intestine and reach the highest number in the colon with nearly a 10^{10}-fold increase in number compared with the stomach (Hattori and Taylor 2009; Arweiler and Netuschil 2016) (Fig. 1). The gut microbiota contains 100 trillion microorganisms, including at least 1000 different species of bacteria comprising more than 3 million genes. By comparison, bacteria from oral cavity belong mainly to *Firmicutes*, *Proteobacteria,* and *Actinobacteria,* while in the colon, *Firmicutes* and *Bacteroidetes* are the most abundant.

Gastrointestinal microbiome contributes to different biological functions, such as protection against colonization by pathogens (Sekirov et al. 2010), digestion of complex carbohydrates (Musso et al. 2011), regulation of mucosal immune system

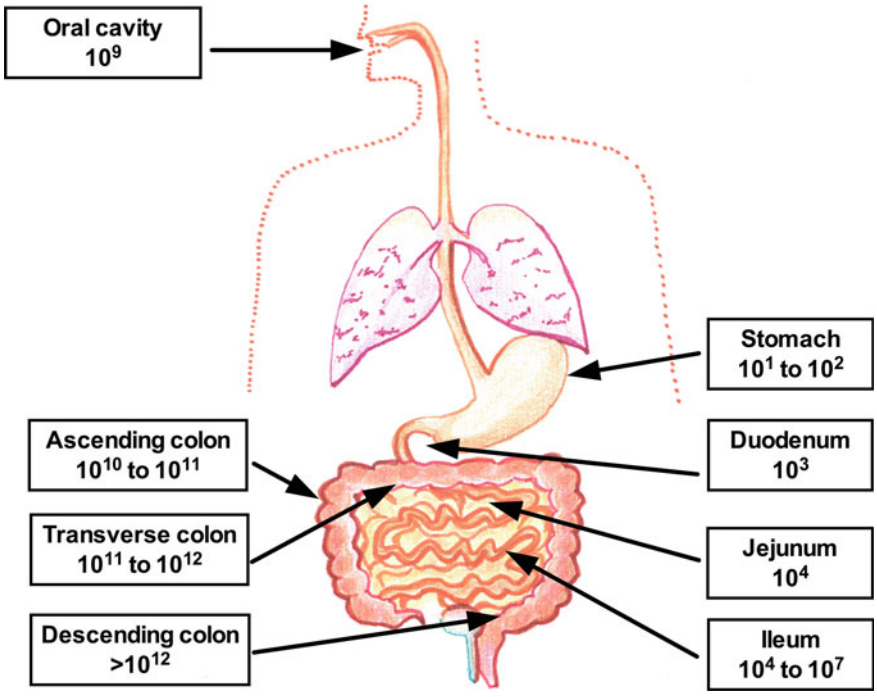

Fig. 1 Bacteria density in different regions of the human gut. A number of bacteria per gram of cellular component are given (*courtesy* F.J. Alarcón)

(Chung et al. 2012), and maturation of the immune response (Ivanov et al. 2009) or regulation of the central nervous system (Cryan and Dinan 2012). Moreover, there is more and more evidence to consider the role of microbiome in diseases such as obesity (Zhao 2013), diabetes (Hartstra et al. 2015), or inflammatory bowel disease (Matsuoka and Kanai 2015).

2 The Stomach

For a long time, the stomach was considered to be a sterile organ until the discovery of *Helicobacter pylori*, but nowadays, it is known that a diverse microbiota of the stomach might exist (Yang et al. 2016). Consequently, an interest in this microbial ecosystem has arisen, thanks to the development of culture-independent methods (Delgado et al. 2013). This is particularly interesting since other microbial communities could be able to survive in an acidic environment as well as *H. pylori* does (Bik et al. 2006; Maldonado-Contreras et al. 2011). Non-*H. pylori* bacteria must survive in the acidic conditions of the stomach, but also the reflux of bile in the stomach, the thickness and density of the mucus layer, and the effectiveness of gastric peristalsis (Nardone and Compare 2015). *Streptococcus*, *Neisseria,* or *Lactobacillus* are some of the acid-resistant bacterial strains detected in the stomach (Nardone and Compare 2015).

Bacteria could reach the stomach from the oral cavity by constantly swelling but also from the duodenum by reflux (Nardone and Compare 2015). However, the human stomach has a microbial ecosystem different from the mouth and the esophagus, and also from the small intestine and large intestine. *Streptococcus* species (*S. mitis*, *S. parasanguinis,* and *S. anginosus*) are frequently isolated in the stomach of non-*Helicobacter* patients (Khosravi et al. 2016), and they are commonly found in the human oral cavity and also in the lower gut (Van Den Bogert et al. 2013; Hirsch et al. 2012).

3 How We Can Assess the Composition of Microbiota

As describe above, human's bodies are heavily colonized by bacteria and this microbiota composition is associated with different states of health or disease; therefore, it is very important to identify the bacterial composition of our microbiota. In order to know the microbial communities living all over the body, currently, there exits different methodologies to carry out this analysis, ranging from culture to metagenomics approaches.

3.1 Culture

The first studies of human microbiota employed culture-dependent methods involving isolation and culture of microorganisms prior to identification (Moore and Holdeman 1974; Fuchs et al. 1976). However, as only a small proportion of them seem to be cultivable, most recent studies have used high-throughput DNA sequencing to detect all the species, although they were present at a low concentration. However, this molecular approach has also some limitations, and recently, it is becoming popular in the use of culture and the subsequent use of molecular methods based on DNA or protein analysis. So, culturomics has been defined as an approach allowing culture and extensive assessment of the microbial composition by high-throughput sequencing and MALDI-TOF MS (matrix-assisted laser desorption ionization–time-of-flight mass spectrometry) identification (Greub 2012). Another possible workflow of culture-dependent methods is based on targeted phenotypic culturing linked to large-scale whole-genome sequencing (Browne et al. 2016). The main advantages of using culture to analyze human microbiota are the possibility to detect minority populations with the use of selective media and detect only viable bacteria. However, this method misses the presence of non-cultivable bacteria. It is estimated that up to 99% of observable microorganisms in nature cannot be grown by standard techniques in laboratories (Streit and Schmitz 2004). However, Browne et al. describe in their recent publication that a considerable proportion of the bacteria within the fecal microbiota can be cultured with a single growth medium (Browne et al. 2016).

3.2 Molecular Methods

The other strategies to assess a given bacterial composition are DNA-based methods. The emergence of molecular techniques has led to a profound change, allowing the identification of non-culturable microorganisms and establishing phylogenetic relationships between them. In this group, there are several possibilities, such as microarrays, sequencing using cloning vectors, next-generation sequencing technologies, or whole-genome shotgun.

Many molecular methods used nowadays are based on the analysis of the 16S rRNA gene. This gene is highly conserved and universally present in all bacteria, although it has some hypervariable regions that allow to distinguish bacteria even at specie level. The 16S gene is amplified, and the sequences are clustered according to similarity. A threshold of 97% identity is commonly used to define operational taxonomic units (OTUs) that is equivalent to different species (Nguyen et al. 2016).

– High-density 16S rRNA gene microarrays are DNA microarrays consisting of rRNA-targeted oligonucleotide probes that were designed according to the "multiple probe concept." PhyloChip allows to detect microbial sequences from any sample in a parallel and very fast, high-throughput way (Nikolaki and

Tsiamis 2013). A single array can contain thousands of DNA sequences with a high degree of specificity. Thus, this technology is able to create a profile of microbial species present in a sample. Nowadays, there are two generations of PhyloChip. The G2 PhyloChip microarray contains 297,851 probes targeting 16S rRNA genes representing 8741 taxa. This assay was based on over 30,000 16S rRNA gene sequences retrieved from the "Greengenes" database in March 2002 (Maldonado-Contreras et al. 2011). An updated version of this microarray is the G3 PhyloChip which is able to categorize prokaryotes (bacteria and archaeal OTUs) into over 50,000 taxa using 1,100,000 25-mer probes that target variations in the 16S rRNA gene (Hazen et al. 2010).

– Cloning vector sequencing: This is a highly efficient strategy based on direct insertion of amplified products into a given cloning vector for subsequent sequencing. Consequently, a cloning vector is a DNA molecule that carries external DNA into a host cell, replicates inside a bacterial cell, and produces lots of copies of itself and the foreign DNA. Therefore, a strategy is needed that allows the suitable insertion or removal of DNA fragment in or out of the vector, usually by treating the vector and the foreign DNA with restriction enzymes to generate complementary ends, then ligating the fragments together.

There are several types of cloning vectors. However, small high-copy plasmids are the most commonly used. Plasmids most commonly utilized can replicate in *Escherichia coli.* Other types of cloning vectors are bacteriophages, cosmids, and bacterial artificial chromosomes (BACs) (Preston 2003).

Bik et al. used this strategy to assess bacterial composition, and they found that the gastric bacterial community was dominated by five major phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria*. In this study, they found 128 phylotypes, and 64 (50%) were derived from uncultivated bacteria (Bik et al. 2006).

– Next-generation sequencing (NGS): A major revolution has come with the development of next-generation sequencing methods, mainly due to the huge amount of reads generated per experiment. This allows knowledge improvements about bacterial composition in diverse environments of human microbiome (Caporaso et al. 2011; Yoon et al. 2015). Currently, there are several NGS platforms available. These platforms might be of second or third generation if a step of clonal amplification prior to sequencing or not is required. In the second-generation NGS, clonal amplification may be performed by emulsion PCR (454 Roche, SOLID) or by bridge (Illumina).
  Pyrosequencing (454) was the first NGS method commercially available. It is based on detecting the release of pyrophosphate when nucleotides are incorporated by luminescence (Shendure and Ji 2008). Illumina sequencing is based on reversible chain-terminating nucleotides that enable the identification of single bases as they are introduced into DNA strands. The reversible chain-terminating nucleotides are released when the nucleotide-specific fluorescence label is removed by washing (Hutchison 2007). The sequencing

chemistries of these two systems are "sequencing-by-synthesis" approach. Besides this sequencing methodology, there is another mechanism, "sequencing-by-ligation." Conducted by supported oligonucleotide ligation and detection (SOLID) sequencing, where instead of using DNA polymerase to incorporate nucleotides to the elongating strand, uses the enzyme ligase (Huang et al. 2012).

With respect to third-generation NGS, new platforms are currently being developed. One of them is HeliScope (Helicos BioSciences), which is based on the sequencing of billions of unique DNA molecules attached to a solid surface. The procedure is similar to Illumina, but only one fluorophore is used and nucleotides are individually provided in a predetermined order. Another option is PacBio (Pacific Biosciences) characterized by the length of the readings, which can reach more than 1000 nucleotides.

- Whole-genome sequencing: This analysis is accomplished by unrestricted sequencing of the genome of all microorganisms present in a given sample. Sequencing the entire microbial genome is central for making accurate reference genomes, for the identification of microorganisms, and to carry out comparative studies of bacterial communities. One of the main advantages of this approach is that is able to characterize functional activity of the microorganisms living in our body.
- Visualizing and tracking bacteria within a host. Recently, two experimental new approaches allow visualizing and tracking bacteria in animals for exploring interactions among bacteria at a deeper level. How they assemble, exchange metabolites, and interact with their host are analyzed (Propheter and Hooper 2015).

  Using group-specific fluorescence in situ hybridization (FISH) probes and a computer algorithm that overlaps fluorescent images into a continuous image (BacSpace), it is possible to quantify spatial relationships among the bacteria and between bacterial cells and the host surface. With this approach, the initial insights into the social behavior of intestinal bacteria in their native gut habitat were acquired (Earle et al. 2015). Geva-Zatorsky et al. (2015) introduced a fluorescent bacterium (*Bacteroides fragilis*) into the intestine of mice. Using flow cytometry and fluorescence microscopy, they were able to track how microbiota can interact with each other and with the cells and tissues of the immune system.

3.3 Methods of Analysis

In addition to the sequencing methods, it is also important to note the different ways of displaying the results once analyzed the data obtained after sequencing. The first step is to recognize the concept of bacterial diversity. The measure of diversity is important to understand the structure of microbial communities. In this sense,

Whittaker introduced the terms of alpha and beta diversity that are currently being used to compare the diversity of different microbial communities (Whittaker 1972). Alpha diversity can be defined as the species richness in a community considered as homogeneous (Lozupone and Knight 2008). This diversity can be measured by the number of observed species or according to the phylogenetic distance between these species. Regarding beta diversity, this method measures the differences in the bacterial composition of two or more samples. This analysis can be carried out quantitatively or qualitatively. The first considers the abundance of each taxon, while the second one only takes into account the presence or absence of data (Lozupone et al. 2007).

Another interesting point is the taxonomic description of the bacterial profile that integrates a community, being able to distinguish between levels of phylum to genus level. In this line, it is possible to describe whether there are significant differences between groups using a multivariate statistical technique called LDA effect size (LEfSe). It is an algorithm for biomarker discovery and identification of genomic features (genes, pathways, or taxa) studying the differences between two or more biological conditions (Segata et al. 2011).

4 Microbiota Composition of the Stomach

Microbial cultures of gastric juice or mucosa biopsies were used in several studies to identify the gastric microbiota and have characterized several members of the *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Fusobacteria* phyla. In these studies, yeasts are found in relatively low abundance (Savage 1977; Adamsson et al. 1999).

Some of the phylotypes identified in the stomach are described in specimens from the human mouth (Kazor et al. 2003), such as *Veillonella*, *Lactobacillus,* and *Clostridium*, and when found in gastric juice, these could be just transient bacteria ingested with food, drinks, or saliva (Zilberstein et al. 2007). Transient bacteria could exist as small groups of bacteria, for a brief period, without colonizing the gastric mucosa. Thus, the study of bacteria found in gastric juice alone may underestimate the real presence of bacteria (Nardone and Compare 2015).

Gastric fluid samples are commonly dominated by *Firmicutes*, *Bacteroidetes,* and *Actinobacteria,* while in gastric mucosal samples, the most abundant ones are *Firmicutes* and *Proteobacteria* (Bik et al. 2006). The dominant genera found in the gastric mucosa of healthy individuals were studied by several authors (Table 1).

4.1 Differences in Antrum and Corpus

When the microbiome was compared in antrum and corpus mucosa, the species richness was higher in corpus samples than in antral samples (according to Chao1

Table 1 Analysis of dominant genera in the gastric mucosa

Reference	Subjects	Dominant genera
Bik et al. (2006)	23	*Streptococcus* *Prevotella* *Rothia* *Fusobacterium* *Veillonella*
Dicksved et al. (2009)	6	*Streptococcus* *Lactobacillus* *Veillonella* *Prevotella*
Li et al. (2009)	10	*Streptococcus* *Prevotella* *Neisseriae* *Haemophilus* *Porphyromonas*
Engstrand and Lindberg (2013)	13	*Prevotella* *Streptococcus* *Veillonella* *Rothia* *Pasteurellaceae*
Delgado et al. (2013)	12	*Streptococcus* *Propionibacterium Lactobacillus*
Llorca et al. (2016a)	33	*Pseudomonas* *Lactobacillus* *Bacteroides* *Acinetobacter* *Comamonas*

index); however, no differences were found according to the mean of OTUs neither by Shannon nor Simpson diversity (Jo et al. 2016). Moreover, differences were detected in corpus samples according to *H. pylori*-positive and *H. pylori*-negative groups, with a more diverse microbiome in *H. pylori*-negative subjects than in *H. pylori*-positive subjects. The beta diversity analysis showed that *H. pylori* infection status was more important than antrum or corpus for cluster of population communities (Jo et al. 2016). A study of gastric microbiota in healthy subjects with different ethnic groups and from different geographical origin found similar composition (Engstrand and Lindberg 2013).

4.2 Modification of Gastric Microbiome

The composition of gastric microbiota could be modified by some factors such as *H. pylori* colonization, dietary habits (Chan et al. 2013; Fan et al. 2014; Goldsmith and Sartor 2014; Salonen 2014; David et al. 2014), medication use, age, and inflammation of the gastric mucosa.

The use of antacid compounds could affect the composition of the gastric microbiota. Furthermore, in the last decade, proton pump inhibitor (PPI) therapy has been used by most physicians on patients with non-ulcer dyspepsia. Patients on PPI therapy had significant reduction on gastric pH, and the gastric microbiota had more abundant bacteria similar to oropharyngeal or fecal bacteria than those patients on H_2 antagonists and untreated control (Sanduleanu et al. 2001). The bacterial overgrowth could occur at basic gastric pH (>3.8) (Vesper et al. 2009).

Antibiotic consumption has also effects on gastrointestinal microflora. Mason et al. (2012) showed that treatment with cefoperazone produced alteration in gastric microbiota, with overgrowth of *Enterococci* and a reduction of *Lactobacilli* (Nardone and Compare 2015).

Differences among microbiome composition from different studies could be explained by different dietary habits or perhaps different ages (Yang et al. 2013; Wu et al. 2014). Although several studies were performed in healthy subjects or in patients with digestive diseases, those results are still controversial. Up to now, the studies for microbiota analysis performed are not homogeneous according to the methods used, and there are differences in environmental factors such as diet, age, lifestyle, geography, and ethnicity. Although *H. pylori* was thought to be the only relevant bacterial colonizer of the human gastric mucosa, several studies have shown that bacteria other than *H. pylori* can also be detected, but the role of these bacteria is unclear (Yang et al. 2016).

5 Differences in Gastric Microbiota in *H. pylori*-Positive and *H. pylori*-Negative Subjects

The existence of a gastric core microbiome in patients with or without *H. pylori* was suggested (Schulz et al. 2015, Engstrand and Lindberg 2013), although differences in gastric microbiota between *H. pylori*-positive and *H. pylori*-negative patients were reported in other studies (Andersson et al. 2008, Maldonado-Contreras et al. 2011). For a long time, it was thought that the stomach is a sterile organ mainly due to low pH associated with acid production. However, with the discovery of *H. pylori,* the scientific community refuted this dogma, and currently, thanks to the new sequencing techniques, it is possible to demonstrate that other microorganisms beyond *H. pylori* can colonize the gastric mucosa (Nardone and Compare 2015). The influence of *H. pylori* on the composition of human gastric microbiota it is a topic that we are obtaining more information, but there is still much to discover.

H. pylori seems to be the most important member of the gastric microbiota with the highest relative abundance when present (Bik et al. 2006). However, some studies using 16S rRNA sequencing showed in very low numbers *H. pylori* sequences even in *H. pylori*-negative subjects (Bik et al. 2006; Kim et al. 2015; Kienesberger et al. 2016). They proposed a cutoff value for *H. pylori* colonization in gastric mucosa samples by the pyrosequencing method.

Proteobacteria, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Fusobacteria* were described as the most abundant in both *H. pylori*-positive and *H. pylori*-negative patients (Jo et al. 2016), although previous studies reported *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* as the most abundant in *H. pylori*-negative stomachs (Bik et al. 2006; Andersson et al. 2008; Li et al. 2009).

Andersson et al. (2008) showed that *H. pylori* sequences dominated the samples in positive patients, but when it is absent, the stomach has a diverse microbiota. Maldonado-Contreras et al. (2011) also showed that the stomach is dominated by *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, but *H. pylori* positive had an increased abundance of *Proteobacteria*, *Spirochetes,* and *Acidobacteria*, while there was a decreased abundance of *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*.

When the gastric microbiome was studied in pediatric patients, the most abundant phylum, in *H. pylori*-positive patients, was *Proteobacteria* (69.3%), *Firmicutes* (14.3%), *Bacteroidetes* (8.2%), and *Actinobacteria* (6%) (Fig. 2a). The high frequency of *Proteobacteria* was due to the increased frequency of *Helicobacter* genus in these samples. Bacterial communities in the group of *H. pylori*-negative patients were mainly dominated by the same phylum but with different percentages of relative abundance: *Proteobacteria* (52.6%), *Firmicutes* (26.4%), *Bacteroidetes* (12%), and *Actinobacteria* (6.4%) (Llorca et al. 2016a).

Hu et al. (2012) used MALDI-TOF MS to identify the non-*H. pylori* bacteria in gastric biopsies from *H. pylori*-positive patients and described *Streptococcus*, *Neisseria*, *Rothia*, and *Staphylococcus* as the major species detected in human gastric biopsies of patients with symptoms.

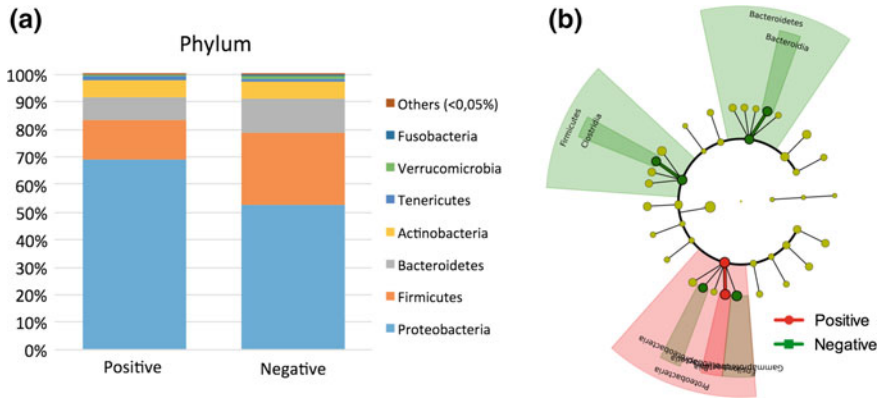

Fig. 2 Bacterial composition and differences in the gastric microbiota of *H. pylori*-positive and *H. pylori*-negative patients. **a** Relative abundance at phyla level in the subsets of *H. pylori*-positive and *H. pylori*-negative patients. In the group of *H. pylori*-positive patients, there was a predominance of *Proteobacteria* phylum (68.7%). **b** LEfSe analysis at class level of the differences in gastric microbiota in relation to *H. pylori* status

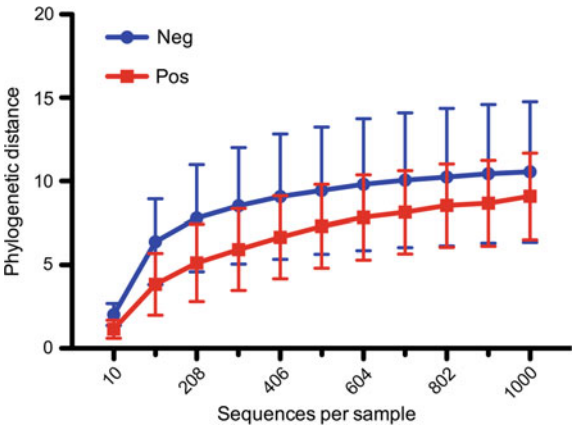

Fig. 3 Measurement of alpha diversity through rarefaction curves calculated for phylogenetic distance. *H. pylori*-positive patients showed lowest alpha diversity compared with *H. pylori*-negative patients. The difference was not statistically significant

Regarding the influence of *H. pylori* presence on the species diversity in gastric microbiome, several authors published that alpha diversity is lower in patients colonized by *H. pylori* than in the group of patients negative for *H. pylori* (Bik et al. 2006; Andersson et al. 2008). We have also consistently observed that alpha diversity is lower in *H. pylori*-positive subjects than in *H. pylori*-negative subjects (Fig. 3). Studying the beta diversity based on *H. pylori* status, we observed that the presence of this microorganism in the gastric mucosa is a clear predominance of this bacteria, which alters the microbial composition of patients colonized by *H. pylori* (Llorca et al. 2016b).

6 Effect of the Histopathological Changes in the Gastric Mucosa Associated with *H. pylori* Over the Microbiota

There are serious limitations to perform longitudinal studies to assess changes in the gastric microbiota associated with the progressive histological changes that occur in patients colonized by *H. pylori*. Most of the published studies are based on cross-sectional comparison of subjects with and without histological changes in the gastric mucosa independently of the *H. pylori* status or comparing infected versus uninfected subjects. We have performed the multivariate statistical analysis with the technique called LEfSe comparing children with and without *H. pylori* colonization that allows us to determine major differences in gastric microbiota composition between groups (Fig. 2b). In *H. pylori*-positive patients, only *Epsilonproteobacteria* was statistically associated, while *Clostridia*, *Bacteroidia*, *Gammaproteobacteria,* and *Betaproteobacteria* were associated significantly with *H. pylori* negative.

6.1 Gastritis

A decrease of *Proteobacteria* and an increase of *Firmicutes* were observed in *H. pylori*-positive patients with antral gastritis versus *H. pylori*-negative subjects (Li et al. 2009). Similarly, an increase of *Streptococcus* and a decrease of *Prevotella* were found in patients with atrophic gastritis, versus healthy subjects (Engstrand and Lindberg 2013).

6.2 Peptic Ulcer

The prevalence of non-*H. pylori* bacteria was higher in patients with non-ulcer dyspepsia than in those with gastric ulcer. The gastric microbiota was studied in Malaysian patients by MALDI-TOF MS and demonstrated that there was no difference between *H. pylori*-positive and *H. pylori*-negative patients, but the isolation of streptococci was correlated with peptic ulcer and similar results were found in the same study but using 16S rRNA sequencing (Khosravi et al. 2014). In another study based on culture, the non-*H. pylori* bacteria showed higher colonization in the group with non-ulcer dyspepsia than in the gastric ulcer group (Hu et al. 2012).

6.3 Gastric Cancer

H. pylori is considered a carcinogen of type 1 by the IARC (2012), although the mechanisms involved in the gastric carcinogenesis are still not well defined. As discussed in Chaps. "Exploiting the Gastric Epithelial Barrier: *Helicobacter pylori's* Attack on Tigh and Adherens Junctions", "Pathogenesis of Gastric Cancer: Genetics and Molecular Classification", and "*Helicobacter pylori*-Mediated Geneti Instability and Gastric Carcinogenesis", *H. pylori* infection initiates the inflammation of gastric mucosa, leading to atrophy and maybe followed by cancer development. The *H. pylori* strain variation, host responses, and environmental factors are considered to represent key factors for the development of gastric cancer (Amieva and Peek 2016; Polk and Peek 2010). Recently, thanks to the studies on gastric microbiota, it is possible to outline the hypothesis that microbes from the gastric or intestinal niche may contribute to the transformation of gastric cells (Abreu and Peek 2014).

Bacterial overgrowth in the stomach has been found in various precancerous conditions, such as hypochlorhydria and mucosal atrophy. Chronic *H. pylori*-induced gastritis may produce a glandular atrophy and a reduction in the acid production and consequently in gastric pH value. Colonization of the gastric mucosa by other bacteria, viruses, or fungi is facilitated by acid decrease (Schulz et al. 2015). These bacteria can promote the production of nitrite, followed by an accumulation of carcinogenic N-nitroso compounds, and can contribute to the development of gastric cancer. In fact, the microbial diversity, and structure and

composition of gastric microbiota can affect the gastric cancer development (Wang et al. 2016). Moreover, Wang et al. found a similar number of bacterial species in the microbiota between gastric cancer and chronic gastritis, while by Principal Coordinates Analysis (PCoA, a method to explore and visualize similarities or dissimilarities of the data), a scattered pattern in gastric cancer was found in contrast to chronic gastritis, suggesting the presence of a diversified microbial community in gastric cancer (Wang et al. 2016). The bacterial overgrowth could be due to the hypochlorhydria produced by *H. pylori* infection but also as a consequence of cancerous mucosa that favors the bacteria proliferation (Wang et al. 2016).

Wang et al. (2016) found that the composition of microbiota in gastric cancer was similar to chronic gastritis at the phylum level; however, some bacterial genera were found in gastric cancer, including *Lactobacillus*, *Escherichia/Shigella*, *Nitrospirae*, and *Burkholderia.* The relationship between the increase in the abundance of *Lactobacillus* and gastric cancer development requires further studies. Interestingly, several species of *Lactobacillus* have been used as probiotics, as they may prevent infection by pathogens (Gotteland et al. 2006), palliate the inflammation, and produce modulation of the microbiota (Čitar et al. 2015), but on the other hand, some *Lactobacillus* are able to induce inflammation (Lukic et al. 2013). An increased abundance of *Escherichia/Shigella* has also been reported in colorectal cancer (Leung et al. 2015), and a toxin produced by *E. coli* was described as the promotor for the development of colon cancer in mice (Arthur et al. 2012).

Eun et al. (2014) showed differences in the gastric microbiota of patients with gastric cancer, intestinal metaplasia, and chronic gastritis and suggested that the gastric commensal flora may play some role in the carcinogenicity of *H. pylori*. However, another possible explanation is that the gradual changes in gastric acidity as a result of the histological changes induced by *H. pylori* make easier the colonization of the gastric epithelium by other microorganisms. Aviles-Jimenez et al. (2014) reported that the gastric microbiota changed gradually from non-atrophic gastritis to intestinal metaplasia, and to gastric cancer (type intestinal). Diversity and richness of gastric microbiome from patients with chronic gastritis, intestinal metaplasia, and gastric cancer showed an increase in the bacilli class in gastric cancer compared with other groups (Nardone and Compare 2015).

The relative abundance of the *Streptococcaceae* family in gastric cancer patients with *H. pylori* compared with controls was described by Eun et al. (2014), while no differences in microbial composition was found by Jo et al. (2016). In this last study, a high proportion of *Actinobacteria* in the cancer groups was found independently of *H. pylori* status.

Besides a large number of *Streptococcus*, a large number of *Bifidobacteria*, *Lactobacilli,* and *Veillonella* were found in patients with gastric cancer in a culture-based study (Sjöstedt et al. 1985; Sjöstedt et al. 1988). However, studies not based on culture did not show differences between cancer patients and controls (Dicksved et al. 2009; Lawson and Coyle 2010; Engstrand and Lindberg 2013).

Studies in animal models as well as in humans showed that the outcome of *H. pylori* infection could be modified when bacteria from the lower bowel colonize the stomach. An increase in the risk of gastric cancer was also found when the

stomach is colonized by these bacteria having an adjuvant effect in *H. pylori* infection, and they contribute to the development of gastrointestinal neoplasia (Lofgren et al. 2011; Lertpiriyapong et al. 2014), although it is not known whether it is due to an increase or a decrease of the bacterial diversity (Aviles-Jimenez et al. 2014; Martin and Solnick 2014).

Are these observations a clear indication that the gut microbiome has a real carcinogenic effect? Or are the modifications in the gut microbiome the result in changes in the gastric environment? Studies using germfree mice have been used, and they have provided some answers to those questions as discussed below.

In a germfree mouse model of gastric cancer, gastric neoplasia developed quicker when the mice were infected with *H. pylori* plus a normal complex gastric microbiota compared with *H. pylori* alone (Lofgren et al. 2011). Moreover, treatment with antibiotic was able to modify the evolution of gastric neoplasia in mice without *Helicobacter* and without specific pathogen (Lee et al. 2009).

The gastrointestinal microbiota strongly accelerated the induction of gastric preneoplastic lesions produced by *H. pylori* in a gastric carcinogenesis mouse model (Lofgren et al. 2011). The addition of a select intestinal microflora also accelerated this process (Lertpiriyapong et al. 2014; Yang et al. 2016).

Colonization with other *Helicobacter* species in extragastric niches may also affect gastric carcinogenesis in a mouse models. Mice infected with *Helicobacter bilis* or *Helicobacter muridarum* before giving *H. pylori* significantly reduced the severity of gastric inflammation induced by *H. pylori*, while colonization with *Helicobacter hepaticus* increased gastric injury by *H. pylori* (Ge et al. 2011). So, it seems important to know the relationships between *H. pylori* and gastrointestinal microbiome to understand better the gastric carcinogenesis (Amieva and Peek 2016).

6.4 Mechanisms of Carcinogenesis

Several bacteria could participate in the carcinogenesis through the production of nitrate/nitrite and their metabolites. These compounds are associated with several functions: Acidified nitrite was described as capable of killing other bacteria (Rao et al. 2006). However, nitrate can act as a source of energy and modified the intestinal microbiota (Winter et al. 2013), nitric oxide is formed as a final product of nitrite reduction, and it can be involved in the protection of mucosal integrity (Lundberg and Weitzberg 2013); the N-nitroso compounds formed in the metabolisms of nitrate/nitrite are important carcinogens (Forsythe and Cole 1987; Stockbrugger et al. 1982).

Bacteria other than *H. pylori* may influence the development of gastric disease, modulate inflammation, or produce N-nitroso compounds (Mowat et al. 2000; Sanduleanu et al. 2001). Bacteria such as *Clostridium*, *Veillonella*, *Haemophilus*, *Staphylococcus*, or *Neisseria* may be implicated in the formation of these compounds (Calmels et al. 1990; Mowat et al. 2000; Williams and McColl 2006; Hu

et al. 2012), suggesting that they can increase the risk of cancers (Mowat et al. 2000). In the gastric microbiome, it is possible to find nitrosating bacteria and also nitrate-reducing bacteria (Jo et al. 2016). *E. coli*, *Lactobacillus*, and *Nitrospirae* are described as more abundant in gastric cancer and play a role in the metabolisms of nitrate/nitrite (Winter et al. 2013; Wang et al. 2016).

A study was carried out in two areas in Colombia–Túquerres in the Colombian Andes and Tumaco, a town on the coast (Yang et al. 2016). Túquerres has a higher risk of gastric cancer than Tumaco (25-fold), although the prevalence of *H. pylori* was similar. *H. pylori* status, as well as *cag* pathogenicity island and phylogeographic groups, was studied. Results showed that gastric microbiota composition was highly variable between individuals, but with a high correlation with the town of origin. Two OTUs, *Leptotrichia wadei* and a *Veillonella* sp., were more abundant in Túquerres, while as many as 16 OTUs, including *Staphylococcus*, were more abundant in Tumaco. Moreover, no correlation of microbiota composition with *H. pylori* phylogeographic population or *cag* PAI was found (Yang et al. 2016). However, we need more studies that may help to explain mechanistic the role of gastric microbiota and its correlation with *H. pylori* colonization.

7 Effect of *H. pylori* Infection in the Intestinal Microbiome

Hypochlorhydria and hypergastrinemia produced by *H. pylori* may produce changes in the large intestine microbiota (Schulz et al. 2015; Heimesaat et al. 2014). An altered composition of the microbiota was suggested in a Mongolian gerbil model after infection with *H. pylori,* analyzing the relative abundance of different bacteria along the entire gastrointestinal tract by quantitative polymerase chain reaction (qPCR) (Osaki et al. 2012). However, another study in Mongolian gerbils showed differences in the distal, but not in the proximal inflamed gastrointestinal tract microbiota (Schulz et al. 2015). Moreover, antibiotic treatment for *H. pylori* eradication produced a significant change in the human throat and gut microbiomes with a reduction in bacterial diversity after eradication (Jakobsson et al. 2010).

8 Effect of Probiotics

Probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Schulz et al. 2015; Homan and Orel 2015), and they are used in the management of *H. pylori* infection generally used in parallel with the antibiotics treatment. Probiotics are used with two different aims: to increase the eradication rate and to reduce gastrointestinal adverse events produced by antibiotic treatment. They can be used as single therapy (although still experimental) or associated with standard eradication therapy (Homan and Orel

2015). Patients on treatment for *H. pylori* eradication had antibiotic-associated diarrhea less frequently when they received probiotic therapy (Schulz et al. 2015).

The influences of antibiotics and their combination with probiotics on the composition of the gut microbiota were analyzed by Oh et al. (2016). In the gut microbiota of all investigated subjects, the three most abundant phyla were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. *Firmicutes* decreased, whereas *Proteobacteria* increased after the treatment in both groups (group treated with general therapy versus group with general therapy and probiotic), but the proportion in the antibiotics group was higher than that in the probiotics group. Moreover, antibiotic-resistant bacteria were higher in the antibiotics than in the probiotics group (Oh et al. 2016).

9 Concluding Remarks

Human microbiota plays an important role in human health, and changes in microbiota composition may have negative consequences in humans. Nowadays, the identification of non-culturable microorganisms and establishing phylogenetic relationships between them are possible thanks to the development of molecular tools that has revolutionized human microbiome study. Several bioinformatic tools are available to study microbiome composition, being the measure of alpha and beta diversity, as well as bacterial compositions, important to understand the structure of microbial communities.

Gastric physiology determines a particular niche, where *H. pylori* is a main actor and its presence may or may not affect the composition of the gastric microbiota in humans. Moreover, gastric microbiota is different in patients with gastric cancer, intestinal metaplasia, and chronic gastritis, but its role in gastric cancer has not been determined. Despite that in the gastric microbiome, it is possible to find bacteria which produce nitrates that can increase the risk of cancers, and its presence is not determinant.

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Pathogenesis of Gastric Cancer: Genetics and Molecular Classification

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Abstract Gastric cancer is the fifth most incident and the third most common cause of cancer-related death in the world. Infection with *Helicobacter pylori* is the major risk factor for this disease. Gastric cancer is the final outcome of a cascade of events that takes decades to occur and results from the accumulation of multiple genetic and epigenetic alterations. These changes are crucial for tumor cells to expedite and sustain the array of pathways involved in the cancer development, such as cell cycle, DNA repair, metabolism, cell-to-cell and cell-to-matrix interactions, apoptosis, angiogenesis, and immune surveillance. Comprehensive molecular analyses of gastric cancer have disclosed the complex heterogeneity of this disease. In particular, these analyses have confirmed that Epstein–Barr virus (EBV)-positive gastric cancer is a distinct entity. The identification of gastric cancer subtypes characterized by recognizable molecular profiles may pave the way for a more personalized clinical management and to the identification of novel therapeutic targets and biomarkers for screening, prognosis, prediction of response to treatment, and monitoring of gastric cancer progression.

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Contents

1 Gastric Cancer Epidemiology

Gastric cancer is the fifth most incident malignancy in the world, with 952,000 new cases (6.8% of the total number of cancers) estimated to have occurred in 2012 (Ferlay et al. 2013). Of these, 823,000 cases were at non-cardia anatomic subsites. The incidence greatly varies between countries, and the majority of cases are registered in developing countries, half of them occurring in Eastern Asia. Gastric cancer incidence is twice as high in men than in women, suggesting that environmental, hormonal, and/or genetic exposures may affect risk.

Globally, gastric cancer is the third most common cause of death by cancer, representing 723,000 deaths (8.8% of all cancer deaths) in 2012 (Ferlay et al. 2013). The mortality rate is quite similar to the incidence rate with the highest estimated mortality rates observed in Eastern Asia. The high mortality rates of gastric cancer are due to the paucity of clinical symptoms, consequent late diagnosis, absence of screening, and ineffective treatments. Thus, the majority of patients present advanced-stage tumors at the point of diagnosis. The five-year survival of gastric cancer patients of all stages is about 25%, and the median overall survival is less than one year (Gastric Group et al. 2013).

Gastric cancer incidence and mortality have been declining over the past decades, and this has been related to a decrease in the prevalence of *Helicobacter pylori*, probably as a result of improved living conditions, hygiene, and sanitation, as well as to the widespread use of antimicrobials (Ferro et al. 2014). Nevertheless, and despite the declining rates, the global burden of gastric cancer is expected to increase in the coming years due to the demographic effects of growth and aging of the population worldwide (Ferlay et al. 2013).

2 Histological Phenotypes of Gastric Cancer

From the morphologic standpoint, gastric cancer is very heterogeneous, which is reflected by the diversity of histopathological classifications (Carneiro et al. 1995; Lauren 1965; Ming 1977; Lauwers et al. 2010). The most widely used classification was proposed by Lauren, in which gastric cancer is divided in two main types—intestinal and diffuse—which also differ from each other clinically and epidemiologically (Lauren 1965). Intestinal-type tumors are usually exophytic, often ulcerating, and are associated with intestinal metaplasia of the stomach. Diffuse-type tumors are poorly differentiated infiltrating lesions, which lead to the thickening of the stomach (*linitis plastica*). Patients with diffuse-type tumors appear to have a worse prognosis than those with intestinal-type tumors. Intestinal-type cancers are more common in proximal (fundus) location, while diffuse-type tumors predominate in younger patients (Lo et al. 1996). Interestingly, diffuse-type tumors demonstrate a nearly equal sex ratio, compared with the male preponderance in the intestinal-type.

Correa proposed a model of gastric carcinogenesis according to which the intestinal-type carcinoma represents the end product of a cascade of sequential changes in gastric mucosa including superficial gastritis, chronic atrophic gastritis, small intestinal metaplasia, colonic metaplasia, and adenomatous (flat or polypoid) dysplasia (Correa 1988, 1992). The sequence of histogenetic changes leading to diffuse-type cancer is less well defined. A further complication for these models of precancerous stages is that a substantial fraction of gastric cancers are characterized by the coexistence of both intestinal-type and diffuse-type morphologic features (Carneiro et al. 1995).

3 *Helicobacter pylori* Infection and Gastric Cancer

Helicobacter pylori infection is the major risk factor for gastric cancer and has been considered as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC 1994). A re-evaluation incorporating a substantial amount of new available data provided support for an association between *H. pylori* infection and non-cardia gastric cancer (IARC 2011). Recent estimates show that 89% of all non-cardia tumors (about 780,000 cases) are attributable to *H. pylori* infection, making this bacterium responsible for at least 6.2% of all cancer cases worldwide (Plummer et al. 2015). It must be noted, however, that the detection methods may not be sensitive enough and that the true fraction of attributable cases may actually be higher than 89%.

Adding to the geographic overlap between the prevalence of *H. pylori* infection and the incidence of gastric cancer, epidemiological data from cohort studies, case–control studies, and meta-analyses also evidenced the carcinogenicity of chronic *H. pylori* infection in humans (IARC 2011). Risk estimates from a combined analysis of 12 independent prospective cohort studies showed an association

between *H. pylori* seropositivity and non-cardia tumors with an overall odds ratio of 2.97 (95% CI 2.34–3.77), but not with cardia tumors (Helicobacter and Cancer Collaborative Group 2001). The magnitude of the risk is higher, reaching 21-fold, when more sensitive assays are used for determining the *H. pylori* status (Gonzalez et al. 2012; Ekstrom et al. 2001). The risk associated with infection with CagA-positive *H. pylori* strains is also higher than that of infection with CagA-negative strains (Huang et al. 2003; Kamangar et al. 2006; Palli et al. 2007). Moreover, *H. pylori* eradication reduces the incidence of gastric cancer. In a meta-analysis of six randomized eradication trials, including 6497 participants, *H. pylori* eradication was superior to placebo or no treatment in preventing gastric cancer, with a risk ratio of 0.66 (95% CI 0.46–0.95) (Ford et al. 2015).

Noteworthy, not all of the *H. pylori*-infected individuals will develop gastric cancer, which points to a multifactorial etiology of this disease. Indeed, host genetic susceptibility factors that influence the inflammatory response to the infection and lifestyle factors such as smoking and diet with a high-salt content are associated with increased gastric cancer risk (Figueiredo et al. 2002; Gonzalez et al. 2003; Persson et al. 2011; Peleteiro et al. 2011). The mechanisms of malignant transformation mediated by *H. pylori* infection are discussed in Chaps. "Human and *Helicobacter pylori* Interactions Determine the Outcome of Gastric Diseases", "DNA Transfer and Toll-Like Receptor Modulation by *Helicobacter pylori*"–"Impact of the Microbiota and Gastric Disease Development by *Helicobacter pylori*" and "*Helicobacter pylori*-Mediated Genetic Instability and Gastric Carcinogenesis".

4 Epstein–Barr Virus Infection and Gastric Cancer

Epstein–Barr virus (EBV) is almost ubiquitous in the human population, primarily maintained as a latent infection in a subset of B lymphocytes comprising roughly 0.001% of peripheral blood mononuclear cells. In vitro, EBV infection of B cells is highly efficient with most B cells becoming immortalized after infection. In B cells, the viral envelope glycoprotein gp350 binding to the B cell surface coreceptor CD21 tethers the virus to the host cell surface, and subsequent binding to human leukocyte antigen (HLA) class II facilitates fusion of viral and cell membranes, releasing the viral capsid into the cytoplasm (Fingeroth et al. 1984; Li et al. 1997; Nemerow et al. 1987; Tanner et al. 1987). HLA binding and fusion are mediated by a different set of envelope glycoproteins: gH, gL, gp42, and gB (the fusion core complex) (Hutt-Fletcher 2007). gp42 is the main HLA class II-binding protein bridging between a gH/gL heterodimer and HLA. gH/gL binds gB, the main effector of viral fusion (Backovic et al. 2007a, b).

On the other hand, epithelial cells are nearly refractory to EBV, and susceptibility to infection is facilitated only under special experimental conditions, e.g., after ectopic expression of CD21 or after B cell-mediated transference of viral particles (Borza et al. 2004; Shannon-Lowe et al. 2006; Shannon-Lowe and Rowe 2011). In epithelial cells, EBV entry is thought to be mediated by integrins. Several

interactions have been described, including gH/gL with the integrin receptors αvβ5, αvβ6, and αvβ8, and the viral envelope glycoprotein BMRF2 with the cellular transmembrane receptor integrins αv, α3, α5, and β1 (Chesnokova et al. 2009; Hutt-Fletcher and Chesnokova 2010; Chesnokova and Hutt-Fletcher 2011; Tugizov et al. 2003; Xiao et al. 2008). Viral fusion also requires binding to the cellular transmembrane glycoprotein neuropilin 1 (Wang et al. 2015).

EBV released by B cells and that by epithelial cells bear distinctive envelopes displaying different tropisms. gH/gL/gp42 trimers are enriched in epithelial cell-derived viruses, while gp42 is lacking on virus released by B cells (Chen et al. 2012; Wang et al. 1998). Since gp42 mediates binding to HLA class II, B cell-originated virus has an enhanced tropism for epithelial cells and epithelial cell-originated virus for B cells. This switching tropism has important implications at an individual level. Integrins are exclusively expressed at the basolateral face of polarized epithelia, the side-contacting circulating B cells (Lee and Streuli 2014; Manninen 2015). On the contrary, virus present in saliva contains gp42 exhibiting an enhanced tropism for B cells (Jiang et al. 2006). Thus, persistent infection of epithelia most probably follows virus released by reactivated B cells in chronically infected hosts. Also, epithelial infection is more permissive for IgA- and CD21-bound virus, and after formation of B cell–epithelial cell conjugates (Shannon-Lowe et al. 2006; Shannon-Lowe and Rowe 2011; Sixbey and Yao 1992), all mechanisms favored at the basolateral face. All these data support a model of viral transmission in which EBV-infected B cells circulating close to the basolateral side would be reactivated by stimuli that are still not clear, producing viral particles with an increased capacity to infect epithelial cells.

In B cells, viral expression starts with the EBV nuclear antigens (EBNAs), EBNA-LP (leader protein), EBNA-2, EBNA-1, the EBNA-3 family (EBNA-3a, EBNA-3b, and EBNA-3c) and the latent membrane proteins (LMP), LMP-1, LMP-2A, and LMP-2B (the latency III program). In vivo, EBV switches between different gene expression programs (latencies), presumably to counteract immune cell responses. In immune-competent hosts, the germinal center model argues that infection guides the B cell into a series of stages that mimic antigen activation and formation of long-lived memory B cells (Thorley-Lawson et al. 2013). While passing through the germinal center, viral gene expression is reduced to EBNA-1, LMP-1 and LMP-2A (latency II), EBNA-1 only (latency I), and no protein expression (latency 0). Latency 0 is found in memory cells and is considered the ultimate state of EBV persistence (Babcock et al. 2000). During these latency programs, EBV also expresses several untranslated RNAs: the EBER-1 and EBER-2 (EBV-encoded small RNAs) and about 25 pre-micro-RNAs (pre-miRNAs). Expression of EBNA-1, LMP-2A, and noncoding RNAs with or without LMP-1 has been demonstrated in epithelial cells in vitro and in vivo.

EBV is considered to be a human carcinogen linked to the development of several types of lymphoma and carcinoma, accounting for about 200,000 new cancer cases annually worldwide. In particular, this virus was first detected (via polymerase chain reaction) in a gastric tumor in 1990 (Burke et al. 1990). Approximately 9% of gastric cancer cases have latent EBV infection in every tumor

cell (Murphy et al. 2009). This fraction is relatively constant in both high- and low-gastric cancer incidence populations worldwide. Consequently, EBV-related gastric cancer is the most common malignancy associated with EBV infection, exceeding even EBV-related nasopharyngeal carcinoma in number of cases annually (Cohen et al. 2011).

Since EBV infection is so common in humans, presence of the virus in tumor tissue is needed to implicate the infection in gastric carcinogenesis. However, the tissue inflammation often present in gastric cancer may lead to infiltration of incidental EBV-infected leukocytes as a non-specific source. Conventionally, EBV is localized to tumor cells by in situ hybridization for the abundant EBER-1 and EBER-2 transcripts of unknown function. Next-generation sequencing provides a versatile alternative for identification of EBV-positive tumors (Camargo et al. 2016a). In viral-positive tumors, the nucleic acids are present as circularized episomes and chromosomal integration does not appear to occur. The episomes are monoclonal with uniform terminal repeats in a given tumor, indicating infection was present at the time of transformation in the clonal progenitor cell.

EBV-infected lymphocytes in inflamed gastric mucosa may represent the route of introduction into the gastric epithelium (Ryan et al. 2012). Nevertheless, studies are inconsistent about the presence of EBV in epithelial cells of premalignant lesions, including intestinal metaplasia and dysplasia (Gulley et al. 1996). In prospective follow-up of a high-risk population, serologic markers of viral reactivation were associated with gastric preneoplastic lesions and risk of progression (Schetter et al. 2008).

EBV genome sequences vary among isolates, with two recognized major types based primarily on variation of EBNA-2 and EBNA-3 genes (Palser et al. 2015). Furthermore, some strains have greater propensity for infecting epithelial cells, which may be indicative of carcinogenic competence (Tsai et al. 2013). However, there is no known association to date of particular viral genotypes with EBV-positive gastric cancer.

EBV reactivation from latency is a postulated mechanism for the development of EBV-associated malignancies. Supporting this hypothesis, serologic studies have found significantly higher immunoglobulin G antibody titers against the viral capsid and early antigens (although not against EBNA) in patients with EBV-positive tumors as compared to EBV-negative cases (Levine et al. 1995; Shinkura et al. 2000).

There is limited evidence on the possible interaction or antagonism between EBV and *H. pylori* in gastric carcinogenesis. In a study comparing *H. pylori* serologic profiles of patients with EBV-positive and EBV-negative non-cardia tumors, antibody levels to 15 *H. pylori* proteins did not vary by tumor EBV status (Camargo et al. 2016b). This similarity of host response indicates that *H. pylori* plays an essential role in the etiology of EBV-positive tumors as well.

Taken together, much has been learned about EBV-positive gastric cancer since it was first documented, but important knowledge gaps remain. It is still unclear when and how the virus appears in the gastric epithelium prior to tumor emergence. Elucidating the viral contribution to gastric cancer pathophysiology could lead to

novel strategies for prevention and treatment, with possible extension to other EBV-related malignancies.

5 Molecular Phenotypes of Sporadic Gastric Cancer

It has long been recognized that gastric cancer, like other cancers, harbors genetic and epigenetic alterations that contribute to the cancer development, progression, and response to therapy (Peltomaki 2012). Previous to the genome-wide characterization of gastric cancer, several molecular phenotypes, chromosome instability (CIN), microsatellite instability (MSI), CpG island methylator phenotype (CIMP), and stable or diploid tumors were already described and widely accepted (Ottini et al. 2006). The advent of high-throughput genome-, transcriptome-, and proteome-wide approaches confirmed the remarkable genetic and epigenetic intertumoral heterogeneity of gastric cancer and highlighted the molecular complexity of this disease.

Profiling of gastric tumors also led to the emergence of several molecular classification schemes. Gene expression profiling has been used for the discovery of subtypes that may predict patient survival and response to therapeutic modalities. Unsupervised hierarchical clustering analysis of data obtained with cell lines identified two major gastric cancer subtypes with distinct genomic signatures: one associated with intestinal tumors and enriched in biologic functions related to carbohydrate and protein metabolism and cell adhesion, designated G-INT, and one associated with diffuse tumors and enriched in cell proliferation and fatty acid metabolism functions, designated G-DIF (Tan et al. 2011). Although related to Lauren's histotypes, the overall concordance was only 64%, and thus, G-INT and G-DIF molecular subtypes were considered distinct. In fact, and unlike Lauren's classification, the G-INT and G-DIF gene expression patterns were prognostic of survival in various patient cohorts, and patients with G-DIF gastric cancer had worse survival outcomes. Furthermore, distinct in vitro sensitivities of G-INT cell lines to chemotherapeutic agents 5-fluorouracil and oxaliplatin and of G-DIF cell lines to cisplatin were observed, and patients with G-INT tumors benefited from adjuvant 5-fluorouracil-based therapy.

Following this early discovery study, gene expression profiling of gastric tumors led to the identification of proliferative, metabolic, and mesenchymal gastric cancer subtypes (Lei et al. 2013). Proliferative subtype tumors are mostly of the intestinal-type and have high levels of genomic instability with recurrent *ERBB2*, *KRAS*, *CCNE1*, and *MYC* gene amplification, DNA hypomethylation, and high levels of tumor suppressor gene *TP53* mutations. Mesenchymal subtype tumors are mostly of the diffuse-type, contain cells with cancer stem cell features, and are particularly sensitive to inhibitors of the PI3K-AKT-mTOR kinase pathway. Metabolic subtype tumors include both diffuse and intestinal histological types and show high expression of genes associated with metabolic pathways as well as features of spasmolytic polypeptide-expressing metaplasia (SPEM). Metabolic subtype tumor cells were sensitive to 5-fluorouracil in vitro, which concurred with

the findings that metabolic subtype gastric cancer patients from two independent cohorts had benefit from 5-fluorouracil treatment in terms of cancer-specific and disease-free survival. However, there were no major differences in survival among these three gastric cancer subtypes.

A more comprehensive analysis of gastric cancer by The Cancer Genome Atlas (TCGA) network which studied a series of 295 untreated, surgically resected tumors with array-based somatic copy number analysis, whole-exome sequencing, array-based DNA methylation profiling, mRNA sequencing, micro-RNA sequencing, and reverse-phase protein array led to the proposal of four molecular subtypes (Fig. 1a): tumors with CIN, tumors with MSI, genomically stable (GS) tumors, and EBV-positive tumors (Cancer Genome Atlas Research 2014). Using this classification, no significant differences in patient survival and tumor recurrence rates were observed between subtypes, but the follow-up time of the cohort was limited at the time of analysis.

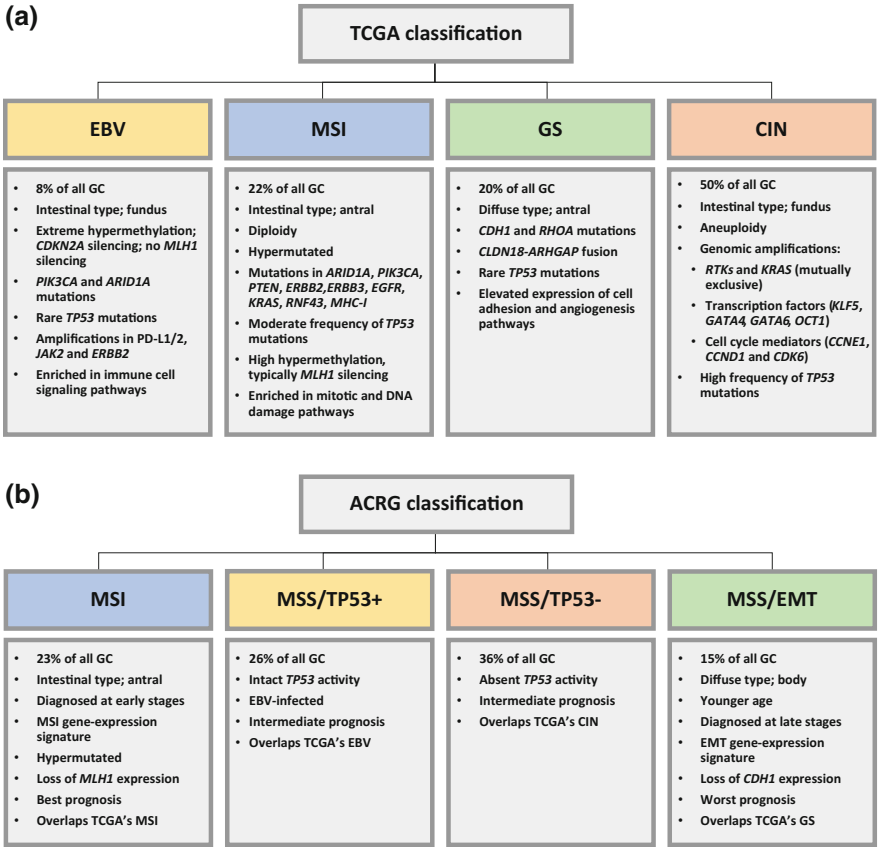

Fig. 1 Current gastric cancer classifications based on the comprehensive molecular analyses. The main features of each subtype are highlighted in the boxes below each subtype. **a** TCGA classification (Cancer Genome Atlas Research 2014). **b** ACRG classification (Cristescu et al. 2015)

The Asian Cancer Research Group (ACRG) used gene expression data to define four gastric cancer molecular subtypes that were linked to distinct patterns of molecular alterations, disease progression, and prognosis (Fig. 1b): the epithelial-to-mesenchymal transition (EMT)/microsatellite-stable (MSS) subtype, the MSI subtype, the MSS/*TP53*+, and the MSS/*TP53*−, the last two based on the status of *TP53* activation (Cristescu et al. 2015). EMT/MSS tumors are mainly of the diffuse type and occur at an earlier age, and patients have the worst prognosis and the highest recurrence rate of the four subtypes. MSI tumors are hypermutated, occur frequently in the antrum, and are mostly of the intestinal histotype, and patients have the best overall prognosis and the lowest recurrence rate. Patients with MSS/*TP53* + and MSS/*TP53*− tumors have intermediate prognosis and recurrence rates, the former showing better prognosis. EBV infection occurred more frequently in the MSS/TP53+ subtype. For the sake of an easier correspondence with the classical gastric cancer phenotypes, we will now refer to a more thorough discussion of each of the phenotypes: gastric cancer with CIN, gastric cancer with MSI, genomically stable gastric cancer, and EBV-positive gastric cancer.

5.1 Tumors with Chromosomal Instability

CIN is one of the major genomic instability pathways involved in gastric carcinogenesis, characterized by losses or gains of whole chromosomes resulting in altered chromosome copy number (aneuploidy). CIN may also involve changes in portions of chromosomes, which include allelic losses (LOH), gene deletions, and/or amplifications (Lengauer et al. 1998; Ottini et al. 2006).

In the TCGA series, CIN subtype tumors represent about 50% of the gastric cancers and are localized mainly in the gastroesophageal junction/cardia (Cancer Genome Atlas Research 2014). These tumors have marked aneuploidy and harbor a very high frequency of *TP53* mutations (71%). CIN subtype tumors frequently harbor gene amplifications.

One of the major functional groups showing gene amplifications in gastric cancer is receptor tyrosine kinases (RTKs). While *ERBB2 (*HER2*)*, *EGFR*, and *MET* amplifications have long been described in gastric cancer, *FGFR2* has only been detected quite recently (Lemoine et al. 1991; Tsugawa et al. 1993; Hara et al. 1998; Sakakura et al. 1999; Graziano et al. 2011; Zang et al. 2011; Deng et al. 2012; Dulak et al. 2012; Wang et al. 2014; Wong et al. 2014; Cancer Genome Atlas Research 2014).

FGFR2 has been recently described as amplified in about 4–10% of tumors, with no association with histological type (Deng et al. 2012; Jung et al. 2012; Su et al. 2014). Dysregulation of the FGFR signaling pathway is associated with cancer development and progression, and in preclinical models of gastric cancer, amplification of *FGFR2* is associated with increased tumor cell proliferation and survival (Xie et al. 2013). A large international multicentric study showed that *FGFR2* amplification is related to poor prognosis in patients with resectable tumors,

irrespective of ethnic origin and underlying significant differences in clinico-pathological parameters, survival, and treatment (Su et al. 2014). Importantly, gastric cancer cell lines and patient-derived xenograft models carrying *FGFR2* amplification exhibit sensitivity to *FGFR* inhibitors, thus supporting this type of therapeutic intervention (Deng et al. 2012; Xie et al. 2013).

Interestingly, amplifications in RTK genes appear to be mutually exclusive (Deng et al. 2012; Dulak et al. 2012; Das et al. 2014; Wong et al. 2014). RTK amplification (either of *FGFR2*, *ERBB2*, *EGFR*, or *MET*) was a predictor of poor prognosis, but considering each of the RTKs as independent factors, patients with *MET*-amplified tumors and *ERBB2*-amplified tumors were the ones with the worst prognosis (Deng et al. 2012). After adjustment for tumor stage and grade, *ERBB2* amplification had the strongest prognostic impact. *ERBB2* amplification and high HER2 immunohistochemical overexpression have already been used as stratification biomarkers for anti-HER2 therapies in gastric cancer (Bang et al. 2010; Satoh et al. 2014).

KRAS amplification has only been recently detected in about 10% of gastric tumors and appears to be mutually exclusive to RTK gene amplification (Deng et al. 2012; Dulak et al. 2012). Patients with tumors exhibiting *KRAS* amplification have significantly worse prognosis compared with patients with tumors lacking either *KRAS* or RTK amplification (Deng et al. 2012).

Overall, amplification in this group of genes is present in around 30% to 40% of the gastric cancer cases (Deng et al. 2012; Dulak et al. 2012). Considering that several inhibitors that target components of the RTK/RAS signaling pathways are currently in clinical trials, this subgroup of patients may benefit from stratification based on the presence of these genetic alterations.

Genes that encode transcription factors also exhibit amplification in gastric tumors. *MYC* amplification and overexpression in gastric cancer are predictors of poor clinical prognosis, especially in patients that have tumors of the intestinal-type (Kozma et al. 2001; Burbano et al. 2006). In addition to *MYC* and *GATA4* that were previously shown to be amplified in gastric cancer, more recent studies detected amplifications in *GATA6* and *KLF5* transcription factors (Weiss et al. 2004; Deng et al. 2012; Dulak et al. 2012; Cancer Genome Atlas Research 2014; Cristescu et al. 2015). *KLF5*, *GATA4*, and *GATA6* are each amplified in about 10% of tumors, and although their amplification occurs in an independent pattern, they are coexpressed in gastric cancer, especially of the intestinal-type (Cancer Genome Atlas Research 2014; Chia et al. 2015). Recently, Chia et al. showed that *KLF5*, *GATA4*, and *GATA6* functionally cooperate to regulate common downstream pathways and to promote gastric tumorigenesis (Chia et al. 2015). Interestingly, they identified hepatocyte nuclear factor-4α (HNF-4α) as a direct target of KLF5/GATA4/GATA6. HNF-4α is present at high levels in intestinal-type tumors with *KLF5/GATA4/GATA6* amplifications, is required for gastric cancer proliferation, and can be targeted by the antidiabetic drug metformin. These findings forecast a therapeutic opportunity for *KLF5/GATA4/GATA6*-amplified tumors, which together represent about 30% of gastric cancer cases.

The octamer transcription factor 1-encoding gene (*OCT1*) is also recurrently amplified and upregulated in gastric cancer and associated with poor prognosis of patients (Qian et al. 2015). Functionally, *OCT1* amplification contributes to ERK/MAPK activation leading to accelerated tumor growth. Interestingly, *OCT1* amplification displays mutual exclusivity with amplifications in *KRAS* and *FGFR2* (Qian et al. 2015).

Amplifications in genes encoding cell cycle regulators are also observed in gastric cancer. Amplifications of *CCNE1* and *CCND1*, which encode cyclin E1 and cyclin D1, are observed, respectively, in 15 and in 10% of tumors (Akama et al. 1995; Bizari et al. 2006). *CCNE1* amplification is associated with the presence of lymph node metastasis (Akama et al. 1995). *CDK6*, encoding cyclin-dependent kinase 6, is also found amplified in about 15% of tumors (Isinger-Ekstrand et al. 2010). A recent high-resolution profiling of copy number alterations in a panel of 233 gastric cancers confirmed amplifications in *CCNE1*, *CCND1*, and *CDK6* and, most interestingly, detected a pattern of co-occurrence of amplifications in the same tumor between *CCNE1* and *ERBB2* (Deng et al. 2012). This pattern of amplification co-occurrence has been previously associated with resistance to anti-HER2 therapies in several cancers (Kim et al. 2014b) and may partially explain the modest efficacy of HER2-directed therapies in gastric cancer (Bang et al. 2010; Satoh et al. 2014).

VEGFA is also reported to be amplified in gastric cancer (Dulak et al. 2012; Andreozzi et al. 2014). *VEGFA* encodes vascular endothelial growth factor A, a growth factor that contributes to tumor-induced angiogenesis that is a critical step in tumor growth and metastasis (Ellis and Hicklin 2008). This may represent an interesting biomarker candidate for predicting clinical outcome in patients with advanced gastric cancer treated with bevacizumab (Van Cutsem et al. 2012).

In the context of CIN, translocations, amplifications, and rearrangements may result in gene fusions. For example, an oncogenic fusion of *CD44* and the glutamate transporter *SLC1A2* gene was identified in a small subset (1–2%) of gastric tumors (Tao et al. 2011). Interestingly, silencing of *CD44-SLC1A2* sensitized cells to cisplatin, a chemotherapeutic agent commonly used in gastric cancer, suggesting that this gene fusion represents a potential drug target.

A novel fusion transcript between dihydrouridine synthase 4-like and B-cell receptor-associated protein 29 (*DUS4L-BCAP29*) that shows functional tumorigenic potential was also described in both gastric cancer cell lines and tumors (Kim et al. 2014a). Additional rare gene fusions involving *AGTRAP-BRAF, ERBB2-CDK12, ERBB2-NEUROD2, SLC34A2-ROS1, SNX2-PRDM6*, and *MLL3-PRKAG2* have been detected in gastric cancer (Palanisamy et al. 2010; Zang et al. 2011; Lee et al. 2013; Yao et al. 2015). Whether they are functionally relevant or can identify novel subclasses of patients that may clinically benefit from targeted therapies remains to be determined.

5.2 Tumors with Microsatellite Instability

The MSI phenotype results from the accumulation of numerous mutations across the genome in short repetitive sequences called microsatellites, due to a defective DNA mismatch repair (MMR) system (Velho et al. 2014). The MMR system corrects base–base mismatches and insertion/deletion mispairs generated during DNA replication and recombination, to prevent mutations from becoming permanent in dividing cells and thus preserve genomic stability (Li 2008). Epigenetic silencing of *MLH1* gene by promoter hypermethylation is the main mechanism leading to MMR deficiency in sporadic gastric cancer, accounting for 75–100% of the cases (Leung et al. 1999; Pinto et al. 2000; Leite et al. 2011).

Tumors can be classified as MSI with high instability (MSI-H), MSI with low instability (MSI-L), or microsatellite stable (MSS) (Umar et al. 2004). It is still not clear in gastric cancer whether MSI-L and MSS represent separate groups, as both appear to have similar phenotypes (Pinto et al. 2000). For the sake of simplicity, hereafter MSI stands for MSI-H.

Tumors with MSI phenotype represent up to 37% of all stomach cancer cases and constitute a well-defined subset of tumors (Zhu et al. 2015; Cancer Genome Atlas Research 2014). MSI gastric cancer is characterized by antral tumor location, intestinal histological type, expanding growth pattern, absent or minimal desmoplastic response, abundant lymphoid infiltration, diploid DNA content, female gender, old age at presentation, and better prognosis (dos Santos et al. 1996; Yamamoto et al. 1999; Falchetti et al. 2008). No significant associations were observed between the MSI phenotype and anatomic location, Lauren's classification, and lymphocytic infiltration in the TCGA series (Cancer Genome Atlas Research 2014). Divergent studies regarding the prognostic value of MSI in gastric cancer exist, but two recent meta-analyses confirmed that patients with MSI tumors have better prognosis than those with MSS tumors (Choi et al. 2014; Zhu et al. 2015).

Next-generation sequencing (NGS)-based studies provided a comprehensive view of the mutations present in MSI tumors. MSI gastric cancers are characteristically hypermutated tumors with an estimated mutation rate of 31.6 mutations/megabase (Mb) in comparison with 3.3 mutation/Mb in MSS tumors (Wang et al. 2011). Moreover, MSI tumors also portray a distinctively high C-to-T transition signature compared to other gastric cancer molecular subtypes that have enrichment of the C-to-A transversion signature (Yamamoto et al. 1999; Wang et al. 2011; Nagarajan et al. 2012; Liu et al. 2014; Wang et al. 2014; Cancer Genome Atlas Research 2014).

Exome sequencing studies that characterized the mutational repertoire of the MSI gastric cancer subset identified *ARID1A*, *PIK3CA*, *PTEN*, *ERBB2*, *ERBB3*, *KRAS*, *RNF43*, and *TP53*, among other genes as significantly mutated (Wang et al. 2011; Zang et al. 2012; Wang et al. 2014; Cancer Genome Atlas Research 2014; Liu et al. 2014; Cristescu et al. 2015).

Somatic inactivating mutations in *ARID1A*, encoding the AT-rich interactive domain-containing protein 1A, are consistently identified more frequently mutated in MSI than in MSS tumors (Wang et al. 2011; Zang et al. 2012; Cancer Genome Atlas Research 2014). The *ARID1A* mutation spectrum also differs between gastric cancer subtypes, and while the great majority of mutations in MSI are frameshift due to insertions or deletions (indels) involving short mononucleotide repeats, in MSS tumors mutations are both indels and single-nucleotide variations. The high rate of *ARID1A* mutations is consistent with a driver gene being targeted by the MSI mechanism, since it was 12- to 61-fold higher than the global background mutation rate of somatic indels at mononucleotide tracts of similar length in MSI tumors (Wang et al. 2011). Reduced or absent ARID1A protein expression is observed in the majority of *ARID1A*-mutated samples, and loss of *ARID1A* expression is more frequent in MSI than in MSS tumors (Wang et al. 2011; Zang et al. 2012; Abe et al. 2012). While no significant differences in disease-free or overall survival are observed in the MSI subtype with mutated or wild-type *ARID1A*, in MSS tumors loss of *ARID1A* expression significantly correlates with worse disease-free and overall survival (Abe et al. 2012). In the MSI subset, mutations in *ARID1A* are significantly associated with activating *PIK3CA* mutations, but not with *TP53* mutations, suggesting that *ARID1A* and *TP53* mutations drive alternative subsets of gastric cancer (Wang et al. 2011).

In MSI tumors, *PIK3CA* activating mutations are frequent and are mainly localized in the kinase and helical domains (Cancer Genome Atlas Research 2014) and this may represent a target for PI3K inhibitors.

RNF43, encoding an E3 ubiquitin ligase, was identified as frequently mutated in the MSI gastric cancer setting and was also found frequently mutated in MSI colorectal and endometrial cancers (Giannakis et al. 2014). *RNF43* is expressed in Lgr5-positive stem cells and negatively regulates Wnt/β-catenin signaling. In the pancreatic tumor model, *RNF43* inactivating mutations lead to aberrant Wnt activation. Moreover, in preclinical models, blocking of Wnt inhibited the growth of *RNF43*-mutant pancreatic tumors in vivo, suggesting the use of *RNF43* mutations as a biomarker for patient selection for treatment with Wnt inhibitors (Jiang et al. 2013).

Remarkably, in the TCGA series, analysis of genes mutated within MSI tumors revealed frequent alterations in major histocompatibility complex class I genes, including *HLA-B* and *B2M* (Cancer Genome Atlas Research 2014). Mutations affecting *B2M* and *HLA* inactivation have been previously associated with the MSI phenotype in gastric cancer (Hirata et al. 2007). In colorectal cancer, β2-microglobulin deficiency reduces antigen presentation and generates tumor phenotypes that are able to escape immune surveillance (Bernal et al. 2012).

In gastric cancer, and in addition to *MLH1*, other genes can be silenced by promoter hypermethylation, including genes involved in DNA repair, such as *MGMT*, cell–cell and cell–matrix interactions, such as *CDH1* and *TIMP3*, cell cycle regulation, such as *CDKN2A/P16INK4A*, and transcriptional regulation, such as *RUNX3* (Qu et al. 2013).

Frequently, MSI tumors show concurrent hypermethylation in multiple *loci* and this has been termed as CpG island methylator phenotype (CIMP). Controversy exists as to the full overlap between the MSI and the CIMP in gastric cancer (Lee et al. 2004; Zouridis et al. 2012; Moarii et al. 2015). A genome-wide characterization of the DNA methylation events associated with gastric cancer disclosed a subgroup of tumors characterized by a CIMP, which was associated with widespread hypermethylation, young patient age, and adverse outcome in a disease-stage-independent manner (Zouridis et al. 2012). Interestingly, it was also observed that cell lines with the CIMP were sensitive to treatment with 5-Aza-2′-deoxycytidine, a DNA methylation inhibitor. This suggests that pharmaceutical interventions with demethylating drugs toward CIMP gastric cancer may be a possibility.

5.3 Genomically Stable Tumors

According to the TCGA study, GS tumors have an enrichment of the diffuse histological type and are diagnosed at an earlier age. Compared to CIN subtype, GS tumors have a low degree of aneuploidy and lower frequency of *TP53* mutations. These tumors frequently harbor alterations in the gene encoding the cell–cell adhesion molecule E-cadherin *(CDH1)* and in the gene encoding the small GTPase that regulates cell motility Ras homolog gene family, member A (*RHOA*) (Corso et al. 2013; Cancer Genome Atlas Research 2014). Mutations in *RHOA* are almost exclusively found in GS tumors. Accordingly, others have identified *RHOA* mutations in 14–25% of diffuse-type but not in intestinal-type tumors (Wang et al. 2014; Kakiuchi et al. 2014). *RHOA* mutations occur at hotspots that cluster at functional domains that are important to the interaction of RhoA with its effectors. Functional analyses revealed that hotspot mutants had defects in RhoA-mediated signaling which can contribute to the lack of cell adhesion and to the invasive growth pattern of diffuse-type tumors (Wang et al. 2014; Cancer Genome Atlas Research 2014).

Interestingly, recurrent structural genomic alterations that are implicated in dysregulated Rho signaling were also found enriched in the GS subtype. *CLDN18-ARHGAP26* and *CLDN18-ARHGAP6* gene fusions that juxtapose the tight junction protein claudin 18 with GTPase-activating proteins (GAPs) that are RhoA regulators have been identified (Cancer Genome Atlas Research 2014). Functional analyses showed that the *CLDN18-ARHGAP26* fusion impairs epithelial integrity by reducing cell–cell and cell–extracellular matrix adhesion and by increasing cell invasion (Yao et al. 2015). *CLDN18-ARHGAP* fusions were mutually exclusive with *RHOA* mutations, and within the TCGA GS subtype, RHOA and *CLDN18-ARHGAP* alterations represent about 30% of cases.

In accordance with the aforementioned genetic alterations, integrated pathway analysis revealed that cell adhesion was the most enriched biologic pathway in GS subtype tumors (Zang et al. 2012; Cancer Genome Atlas Research 2014).

5.4 EBV-Positive Tumors

EBV-positive tumors differ from other gastric cancer types, exhibiting distinct histopathological and epidemiological features. EBV-positive tumors tend to occur in non-antral anatomic subsites and more frequently exhibit diffuse-type histology; tumor EBV positivity is twofold higher in males and fourfold higher in postsurgical remnant tumors (Murphy et al. 2009; Lee et al. 2009). Since gastric cancer overall is twice as frequent in men than in women, the twofold sex difference in EBV positivity implies that the incidence of EBV-positive tumors is approximately 4 times higher in males than in females. This relative protection of females is, as yet, unexplained, but similar sex disparities are apparent in other EBV-associated cancers, including Hodgkin's lymphoma, Burkitt's lymphoma, and nasopharyngeal carcinoma. Regarding behavioral risk factors, tumor EBV positivity has been associated with cigarette smoking but not with alcohol drinking (Camargo et al. 2014b).

Apart from an etiologic contribution in EBV-positive gastric cancer, viral presence may influence clinical progression. In a large, multicenter series, EBV positivity was associated with 30% lower mortality adjusted for stage and other confounders (Camargo et al. 2014a). Thus, EBV-positive tumors appear to have a more favorable clinical course than gastric cancer overall.

Based on the comprehensive molecular analysis performed by the TCGA, EBV-positive tumors were characterized by extreme CIMP. Notably, EBV-positive tumors had a higher prevalence of DNA hypermethylation than any other subtypes, showing *CDKN2A/p16INK4A* promoter hypermethylation, while lacking *MLH1* hypermethylation that is otherwise characteristic of MSI-associated CIMP. Additionally, EBV-positive tumors are characterized by frequent *PIK3CA* and *ARID1A* mutations, absence of *TP53* mutations, and recurrent amplifications of the chromosome 9 locus containing *JAK2*, and the immunomodulatory *PD-L1* and *PD-L2* genes (Cancer Genome Atlas Research 2014; Wang et al. 2011).

PIK3CA mutations were present in 80% EBV-positive tumors in comparison with 42% of the MSI tumors, and in the former, mutations were dispersed throughout the gene, contrasting with the preference for the kinase and helical domains in EBV-negative tumors (Cancer Genome Atlas Research 2014).

The viral genes with the highest levels of expression in EBV-positive tumors exhibit a uniform transcription pattern, primarily of sequences encoded in the *BamH1A* gene region of the genome (Cancer Genome Atlas Research 2014). However, other genes are more variable, with about half of EBV-positive gastric cancers exhibiting moderate expression of BNLF2a, a virally encoded inhibitor of antigen peptide transport (Strong et al. 2015). Viral transcripts and their protein products represent candidate targets for functional studies to explore the mechanisms of viral carcinogenesis.

6 Hereditary Gastric Cancer

While the great majority of gastric cancers are sporadic, familial aggregation occurs in about 10% of the cases, and of these, only 1–3% clearly constitute hereditary forms. Hereditary gastric cancers include syndromes such as hereditary diffuse gastric cancer (HDGC), gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS), and familial intestinal gastric cancer (FIGC). Gastric cancer has also been identified as part of the tumor spectrum of other hereditary cancer syndromes such as hereditary non-polyposis colorectal cancer (HNPCC), Li–Fraumeni syndrome (LFS), familial adenomatous polyposis (FAP), and Peutz–Jeghers syndrome (PJS). The clinical features, genetics, and pathogenesis of the hereditary gastric cancer syndromes have been reviewed recently (Oliveira et al. 2015).

HDGC is one of the best genetically characterized forms of hereditary gastric cancer. Heterozygous germline *CDH1* (E-cadherin) mutations, including frameshifts, splice site, nonsense and missense mutations, as well as large rearrangements, were until recently the only known causative alterations of HDGC, representing up to 40% of patients belonging to families that fulfill the clinical criteria for HDGC (van der Post et al. 2015).

Efforts conducted to identify alternative causative genes for the remaining HDGC that do not harbor *CDH1* alterations enabled the identification of a germline truncating mutation in the α-1-catenin (*CTNNA1*) gene (Majewski et al. 2013). This mutation, owing to a two-base deletion in *CTNNA1* exon 2, was detected in ten individuals with invasive tumors or intramucosal signet ring cells that were part of a large non-*CDH1* HDGC pedigree. Loss of α-1-catenin expression was observed in tumor cells, along with reduced levels of E-cadherin, consistent with the prediction that the deletion removes key functional domains of the α-1-catenin, compromising the binding with β-catenin and the formation of the E-cadherin complex. In another study, two novel germline truncating *CTNNA1* mutations, one frameshift and one nonsense, were identified in two individuals of unrelated families out of 144 non-*CDH1* HDGC probands (Hansford et al. 2015). Immunohistochemistry of the tumors from the two *CTNNA1* mutation-positive patients showed loss of α-1-catenin expression, suggesting the occurrence of an as-yet-unidentified second-hit event at the *CTNNA1 locus*. Contrasting with previous findings, E-cadherin expression was preserved in tumors of *CTNNA1* mutation-positive patients. These data support that germline *CTNNA1* mutations cause HDGC and strengthen the inclusion of *CTNNA1* testing in non-*CDH1* HDGC families. Screening of mutations in catenin family member genes in 22 non-*CDH1* HDGC families did not reveal mutations in *CTNNA1*, *CTNNB1*, or *CTNND1* and found two non-synonymous variants in the γ-catenin-encoding gene *JUP* (Schuetz et al. 2012).

Other pathogenic germline mutations in known gastric cancer-predisposing genes, such as *BRCA2* and *STK11*, and in other cancer-susceptibility genes, such as *PRSS1*, *PALB2*, *ATM*, *MSR1*, and *SDHB*, have been identified (Hansford et al. 2015). In other HDGC families, screenings of *APC*, *BRCA1*, *BRCA2*, *STK11*, and

TP53 did not identify novel variants that matched the expected inheritance pattern (Majewski et al. 2013). Thus, their role in HDGC needs further investigation.

Germline mutations in *MAP3K6* were also identified upon examination of a large family with a history of familial gastric cancer (FGC) without *CDH1* mutations, with many features of HDGC, but with diversity in clinical presentation and advanced age of onset (Gaston et al. 2014). Four individuals with gastric cancer and five of the 27 currently unaffected relatives harbored a germline truncating (p.P946L) *MAP3K6* mutation. A de novo second-hit somatic mutation in *MAP3K6* was further detected in tumor DNA from one of the p.P946L carriers. In an additional screening of 115 individuals from unrelated non-*CDH1* FGC families, four additional *MAP3K6* variants, one truncating and three missense, as well as the p.P946L variant, were identified. Within this cohort, DNA hypermethylation of the *MAP3K6* gene was observed as a second hit in tumor DNA of one affected individual. Taking together, these observations with the evidence that *MAP3K6* (also known as *ASK2 or MEKK6*) functions as tumor suppressor gene in vivo and is mutated in primary tumors and gastric cancer cell lines reinforce the notion that *MAP3K6* is a strong susceptibility gene candidate predisposing to FGC (Iriyama et al. 2009; Zang et al. 2011).

Germline mutations in *INSR* (insulin receptor), *FBXO24* (F-box protein 24), and *DOT1L* (DOT1-like histone H3K79 methyltransferase) have been identified in three affected members of a family with six cases of diffuse-type gastric cancer in two generations (Donner et al. 2015). Analysis of tumor samples of the three patients did not show LOH in any *loci* of the variants. These variants and the adjacent regions were screened in an additional 26 gastric cancer patients with a confirmed or suspected family history of DGC, but no other non-synonymous or additional mutations were identified, warranting further validation of these mutations in other HDGC families.

Regarding GAPPS, germline point mutations in the promoter 1B of the *APC* gene were recently identified in six families (Li et al. 2016). The three point mutations identified were located within a *Ying Yang* transcription factor (*YY1*) binding motif, which inhibits the binding of *YY1* to the *APC* promoter, leading to reduced APC expression. LOH or somatic APC-truncating mutation second hits were found in the majority of the polyps of the fundic glands that are typical of GAPPS, although in only a small number of cells. This fact led authors to suggest that *APC* haploinsufficiency is responsible for the fundic gland polyposis in GAPPS, and the second *APC* hit might be the driver of dysplasia. No germline mutations have been identified so far in FIGC.

7 Concluding Remarks

The most recent results on the characterization and biology of gastric cancer lent further support to the prevailing concept that this disease is highly heterogeneous. While these findings may help explain the ineffectiveness of a one-size-fits-all

approach to treating this disease, they certainly open the door for a more precise clinical management. Future clinical approaches will certainly take into consideration the specific features of the different subgroups. The prognostic significance of the various gastric cancer types may emerge as more data accumulate. Researchers and clinicians should take advantage of the information provided by these studies to both design and test new targeted therapeutic approaches.

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Helicobacter pylori-Mediated Genetic Instability and Gastric Carcinogenesis

Takahiro Shimizu, Tsutomu Chiba and Hiroyuki Marusawa

Abstract *Helicobacter pylori* infection is the most important cause of human gastric cancer worldwide. Gastric cancer develops over a long time after *H. pylori* infection via stepwise accumulation of genetic alterations and positive selection of cells with growth advantages. *H. pylori* itself and the resultant chronic inflammation lead to the emergence of genetic alterations in gastric epithelial cells via increased susceptibility of these cells to DNA damage. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) in inflammatory and gastric epithelial cells, as well as the expression of cytidine deaminase in gastric epithelial cells, may link *H. pylori*-related inflammation and DNA damage. Recent comprehensive analyses of gastric cancer genomes provide clues for the possible molecular mechanisms of gastric carcinogenesis. In this chapter, we describe how genetic alterations emerge during gastric carcinogenesis related to *H. pylori* infection.

Contents

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1 Genetic Abnormalities in Gastric Cancer Tissues Related to *H. pylori* Infection

Gastric cancer is generally classified by histologic classification systems, including those of Lauren (1965) and the World Health Organization (WHO) (2010; www.who.int/en/). The Lauren classification includes two subtypes of gastric cancer, the intestinal type and the diffuse type, and the WHO classification has four subtypes, including papillary, tubular, mucinous and poorly cohesive types. Intestinal-type gastric cancers represent cohesive tumor cells with a glandular architecture. These cancer types are typically generated from *Helicobacter pylori*-infected gastric mucosa with chronic gastritis, atrophy and metaplastic changes (Correa 1988). While intestinal metaplasia has been focused as a precursor to gastric cancer, spasmolytic polypeptide-expressing metaplasia (SPEM) has also been highlighted as another metaplastic lesion (Goldenring et al. 2010). SPEM generates via the trans-differentiation of chief cells following parietal cell loss due to *H. pylori* infection (Nam et al. 2010). In addition, SPEM gives rise to intestinal metaplasia in animal models (Yoshizawa et al. 2007; Choi et al. 2016) and also progresses to further aberrant and invasive phenotypes in *H. pylori*-infected Mongolian gerbil models (Shimizu et al. 2016). Therefore, SPEM is thought to be the initial pre-neoplastic metaplasia predisposing to gastric cancer. Diffuse-type gastric cancer, by contrast, is composed of scattered, poorly cohesive cells with poor cellular differentiation. This type of cancer develops in *H. pylori*-infected mucosa with or without atrophic and metaplastic changes, as well as in the mucosa unaffected by *H. pylori* infection.

On the other hand, classification of gastric cancers based on comprehensive genome analyses has been recently proposed (Cancer Genome Atlas Research Network 2014; Cristescu et al. 2015). Cancer is a disease of genetic abnormalities (Stratton et al. 2009). Whole-genome sequencing and whole-exome sequencing which targets coding exons of genes using next generation technologies have been conducted on various cancer types and have identified numerous genetic alterations in cancerous tissues (Lawrence et al. 2013). The Cancer Genome Atlas (TCGA)

project revealed that the gastric cancer genome has, on average, 11.4 mutations per megabase (Cancer Genome Atlas Research Network 2014). Although most of these genetic alterations may be passenger mutations that do not contribute to carcinogenesis, 2–6 mutations on average per each cancer tissue could be oncogenic driver mutations (Kandoth et al. 2013). Based on the abundant information of genetic changes in tumors, TCGA research network demonstrated that gastric cancer is subdivided into four subtypes: tumors positive for Epstein-Barr virus (EBV), tumors with microsatellite instability (MSI), tumors with chromosomal instability (CIN) and genomically stable (GS) tumors. EBV-positive cancer shows extreme DNA hypermethylation, *PIK3CA* mutations and amplification of *JAK2*, *PD-L1*, and *PD-L2* genes. The characteristics of MSI tumors are epigenetic silencing of *MLH1*, one of the DNA mismatch repair genes, in the context of a CpG island methylator phenotype (CIMP). *MLH1* silencing can lead to subsequent genetic changes in hundreds to thousands of genes. The frequency of MSI is reportedly higher in intestinal-type gastric cancer, older females and distal gastric cancer (Kim et al. 2011). Early gastric cancer genomes with MSI show a level of mutations comparable to that of advanced MSI gastric cancer in terms of the number, sequence composition, and functional consequences of mutations (Kim et al. 2014). These findings suggest that genetic or epigenetic alterations characterized as MSI are already present in early gastric cancer genomes. CIN tumors account for 50% of gastric cancers, and most of them are histologically of the intestinal-type. This type of cancer typically has tumor protein p53 (*TP53*) mutations and chromosomal aberrations, including marked aneuploidy, and focal amplification, such as receptor tyrosine kinases. *TP53* mutations are frequently seen in non-cancerous gastritis mucosa with *H. pylori* infection (Shimizu et al. 2014), and various chromosomal aberrations are present in gastric adenoma (Uchida et al. 2010). These findings suggest that *TP53* mutations and various chromosomal alterations are early events during *H. pylori*-related gastric carcinogenesis with atrophy-metaplasia-dysplasia sequence. GS tumors that lack these specific features are predominantly of the diffuse histologic subtype, and half of them harbor mutations or fusion in E-cadherin (*CDH1*) or Rho GTPase family genes (Wang et al. 2014; Kakiuchi et al. 2014).

Combined histologic and genetic analyses are essential for understanding the process of gastric cancer development. Although each cancer has a very different profile, these analyses elucidate several possible processes from early genetic events to progression in *H. pylori*-related gastric carcinogenesis. In addition to these approaches for uncovering the process of gastric carcinogenesis, molecular mechanisms by which genetic alterations generate and accumulate during *H. pylori* infection are also important. Two main types of factors can influence the generation of genomic abnormalities: one that induces DNA damage and another that repairs damaged DNA (Fig. 1), which will be discussed in this chapter.

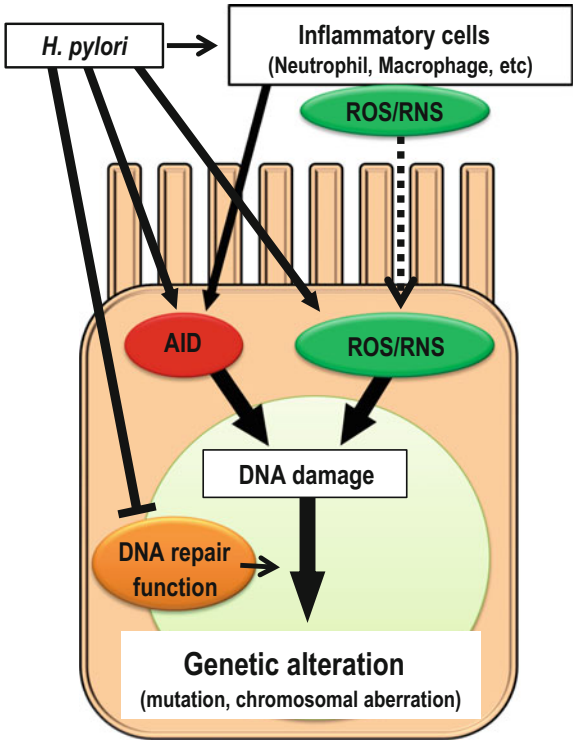

Fig. 1 The mechanisms how genetic alterations generate during *H. pylori*-related gastric carcinogenesis. *H. pylori* itself and the resultant chronic inflammation induce DNA damages in gastric epithelial cells via the expression of reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well as activation-induced cytidine deaminase (AID). In addition to these genotoxic or genome editing agents, the alteration of DNA repair function induced by *H. pylori* infection also influences the generation of genetic alterations

2 Genotoxic Mediators: ROS/RNS

2.1 ROS/RNS in H. pylori*-Infected Gastric Mucosa*

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated in human tissues are considered potential genotoxic factors (Hussain et al. 2003). The high expression levels of ROS and RNS in the gastric mucosa of *H. pylori*-infected patients correlate well with histologic mucosal damage (Suzuki et al. 1996; Davies et al. 1994). Sources of ROS and RNS are inflammatory cells such as neutrophils, as well as gastric epithelial cells (Hardbower et al. 2014). In general, the oxidative burst from phagocytes in neutrophils is the main innate mechanisms of immunity against pathogenic bacteria (Naito and Yoshikawa 2002). In neutrophils, ROS production is catalyzed by nicotinamide adenine dinucleotide phosphate oxidase on

the membrane (Handa et al. 2011). Upon recognition of pathogenic bacteria, neutrophils immediately engulf the bacteria, form phagosomes and kill the bacteria by ROS production. In addition, nitric oxide (NO) is produced by macrophages as a normal host immune response against *H. pylori*. In *H. pylori*-infected gastric mucosa, however, ROS and RNS cannot kill these bacteria because of bacterial defense mechanisms (Gobert and Wilson 2016). On the one hand, ROS and RNS derived from neutrophils and macrophages increase in *H. pylori*-infected gastric mucosa, and on the other hand, the bacterial cytotoxin-associated gene A(CagA) protein stimulates multiple responses in gastric epithelial cells including oxidative stress (Backert et al. 2015). In gastric epithelial cells, *H. pylori* CagA induces the expression of spermine oxidase (SMOX), an enzyme for the back-conversion of spermine to spermidine (Xu et al. 2004; Chaturvedi et al. 2011). This reaction leads to the production of H_2O_2 as a by-product. Increased H_2O_2, however, causes ROS accumulation via mitochondrial membrane depolarization and the activation of caspase-mediated apoptosis (Chaturvedi et al. 2004). In fact, gastric epithelial cells in individuals infected with CagA-positive *H. pylori* express high SMOX levels (Chaturvedi et al. 2011).

2.2 *DNA Damage Induced by ROS/RNS in* H. pylori*-Infected Gastric Mucosa*

ROS and RNS induce various types of DNA damage, including point mutations, DNA adducts and single- or double-strand DNA breaks (DSBs). Among these, 8-hydroxydeoxyguanosine (8-OHdG), which is the main oxidatively modified product of DNA, is significantly expressed in gastric cancer tissues as well as in adjacent tissues in humans (Lee et al. 1998). Also, NO produced in *H. pylori*-infected gastric mucosa is highly reactive and rapidly reacts with superoxide (O_2^-) to produce highly toxic peroxynitrite ($ONOO^-$), inducing nitrative and oxidative DNA damage, such as the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine and 8-nitroguanine, which are used as biomarkers of oxidative or nitrative DNA damage (Handa et al. 2011; Borrego et al. 2013). These damaged guanines preferentially lead to G > T transversion mutations during the genome replication process, although repair systems are closely involved in this formation as described later (Bruner et al. 2000). ROS and RNS are considered to induce single- or double-strand DNA breaks. Indeed, *H. pylori* infection leads to significantly increased levels of phosphorylated histone H2A variant X (H2AX), a marker of DSBs in gastric epithelial cells (Toller et al. 2011). In addition to ROS or RNS, the involvement of some repair systems is needed for the formation of DSBs as well as resultant chromosomal aberrations.

Oxidative stress also induces apoptosis or autophagy in gastric epithelial cells (Cover and Blanke 2005; Tsugawa et al. 2012). Therefore, oxidative stress has a mutagenic role as well as a preventive role in carcinogenesis. Recent studies demonstrated that cancer stem cells possess enhanced mechanisms for protection against oxidative stress (Tsugawa et al. 2012). Expression of a variant 9 form of the

receptor CD44 (CD44v9), a possible cancer stem cell surface marker (Lau et al. 2014) contributes to ROS defense via up-regulation of the synthesis of reduced glutathione (GSH), the primary intracellular antioxidant. CD44v9 interacts with and stabilizes xCT, a subunit of the cysteine-glutamate transporter xc(-), and thereby promotes cysteine uptake for GSH synthesis (Ishimoto et al. 2011). Cancer stem cells able to defend against ROS due to CD44v9 expression are thus thought to drive tumor growth, chemoresistance and metastasis. In *H. pylori*-infected gastric mucosa, SPEM, which is considered a precancerous lesion, expresses high levels of CD44v9 (Wada et al. 2013). These findings suggest that the balance of the accumulation of genetic alterations by ROS and cell survival via protection against ROS is important for the generation of cancer cells in *H. pylori*-infected gastric mucosa.

3 Genome Editing Enzyme: Cytidine Deaminase

3.1 APOBEC Family

Human beings have several kinds of genome editing enzymes. Among them, the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) family, represents cytidine deaminases that convert cytosine (C) to uracil (U) (Cascalho 2004). Most APOBEC family members act against foreign genomes, such as those of some intruding viruses. For example, APOBEC3G inactivates the viral function of human immunodeficiency virus (HIV) or hepatitis B virus (HBV) via editing their genomes (Harris et al. 2003; Noguchi et al. 2005). In contrast, activation-induced cytidine deaminase (AID), APOBEC3A, and APOBEC3B induce genetic alterations in human DNA sequences. AID is normally expressed in activated B cells and is a key molecule for generating immune diversity via inducing both somatic hypermutation, which occurs in variable regions of the immunoglobulin genes, and class-switch recombination, which occurs in switch regions of the immunoglobulin genes. AID appears to act on single-stranded DNA that is generated during the transcriptional stage (Matsumoto et al. 2015a, b) in the form of transcription bubbles, resulting in the conversion of C to U. The generated U:G mismatches can usually be repaired to C:G by the high-fidelity repair system (Liu and Schatz 2009). If the U:G mismatch is not repaired before the onset of DNA replication and is replicated, it gives rise to C:G to T:A transitions. Alternatively, the removal of the uracil by uracil-DNA glycosylase (UNG) or the recognition by mismatch repair proteins such as MSH2 and MSH6 and error-prone translesion polymerases can induce various types of mutations. Various mutations in variable regions of the immunoglobulin genes result in increased antigen-binding affinity. In addition, nicks in the near sites of both strand sequences of switch regions are generated by the repair process of AID-induced U:G mismatches, resulting in DNA DSB (Stavnezer 2011). Recombination of DSB by non-homologous end joining (NHEJ) contributes to the class-switch recombination. APOBEC3A and 3B also have the capacity to edit the human genome. Although their functions in normal

conditions are unknown, recent reports demonstrated that high expression of APOBEC3A and 3B is linked with the mutation signatures of several cancer types, including breast cancer and lung cancer (Burns et al. 2013a, b). In fact, in vitro experiments showed that these enzymes induce mutations in human genomes (Shinohara et al. 2012; Burns et al. 2013a, b). As with AID, deamination of these enzymes and subsequent repair processes induce various mutations. The target sequences of these enzymes differ: APOBEC3A and 3B favor C residues flanked by 5′-T, and AID exhibits a strong preference for deaminating C residues flanked by a 5′-purine (*G* or *A*) (Schmitz and Petersen-Mahrt 2012; Beale et al. 2004). Although recent detailed analyses of these mutation signatures identified more complicated target motifs (Chan et al. 2015), mutator enzymes as well as various repair processes are deeply related to mutation patterns.

3.2 The Role of AID in Gastric Carcinogenesis

AID protein is aberrantly expressed in a substantial proportion of *H. pylori*-associated human gastric epithelium and gastric cancer tissues, although no AID expression is observed in normal gastric mucosa (Matsumoto et al. 2007). In particular, mononuclear cell infiltration and intestinal metaplasia correlate with AID expression (Nagata et al. 2014). After eradication of *H. pylori*, AID expression is significantly decreased but still higher than that in *H. pylori*-negative gastric mucosa. Intriguingly, infection with *cag*PAI-positive *H. pylori* ectopically induces high expression of AID in human gastric epithelial cell lines, but *cag*PAI-negative *H. pylori* has no effect on AID expression (Matsumoto et al. 2007). Also, inflammatory cytokines such as tumor necrosis factor (TNF)-α increase the expression of endogenous AID protein in gastric epithelial cells via the nuclear factor (NF)-κB pathway. Furthermore, aberrant AID expression in gastric epithelial cells induced by these stimuli causes a number of somatic mutations in tumor-related genes, including the tumor-suppressor gene *TP53*, and knockdown of endogenous AID significantly reduces the number of *TP53* mutations observed in *H. pylori*-infected cells. AID transgenic mice that have constitutive and ubiquitous AID expression develop malignant lymphoma as well as various epithelial tumors, including gastric cancer (Okazaki et al. 2003; Morisawa et al. 2008). These findings suggest that aberrant AID expression in gastric epithelial cells induces mutations via a genome editing function. In addition, AID expression in gastric epithelial cells causes chromosomal aberrations, mainly submicroscopic deletions, at various chromosomal loci (Matsumoto et al. 2010). Among these deleted loci, the recurrently deleted chromosomal regions harbor the tumor-suppressor cyclin-dependent kinase inhibitor genes *CDKN2A/CDKN2B*. In *H. pylori*-infected wild-type mice, the copy numbers of the *Cdkn2b-Cdkn2a* locus in the gastric mucosa are reduced, whereas no such changes are observed in the gastric mucosa of *H. pylori*-infected AID-deficient mice. These findings suggest that AID induces point mutations as well as chromosomal aberrations in gastric epithelial cells.

4 Guardian of DNA Damage: DNA Repair System

4.1 Overview of the DNA Repair System

In general, the high-fidelity DNA repair system in humans has an important role in preventing the generation of genetic abnormalities. The spontaneous mutation rate during DNA replication is very low, typically $<10^{-9}$ per base pair per cell division (Lange et al. 2011). Defects in DNA repair function, however, can induce many mutations that result in cancer initiation (Lange et al. 2011; Eso et al. 2015). In fact, individuals with mutations of the mismatch repair gene *MUTYH* develop familial adenomatous polyposis and multiple cancers in the gastrointestinal tract (Nielsen et al. 2007). Some types of cancer have mutations or methylated silencing of DNA repair genes such as *MLH1* and polymerase ε (*POLE*) (Imai and Yamamoto 2008; Rayner et al. 2016).

The DNA repair system generally involves the removal of damaged or incorrect bases and DNA re-synthesis by DNA polymerases (Lange et al. 2011). Briefly, base excision repair (BER) mediates the removal of a single base residue by a specific DNA glycosylase, the incision of the resultant abasic site by an apurinic/apyrimidinic (AP) endonuclease, and DNA re-synthesis by DNA polymerase β. Nucleotide excision repair (NER) can remove various helix-distorting adducts caused by ultraviolet (UV), cisplatin, and others, followed by the re-synthesis of the resulting 27–29 nucleotide gap by polymerase δ, ε or κ. Mismatch repair (MMR) is an excision repair process that removes mismatched bases. Some mismatch repair proteins such as MSH2 and MSH6 can recognize mismatch regions, and a segment of DNA is excised between the mismatch and a nearby nick, followed by filling of the resultant gap by DNA polymerase δ.

DNA DSBs are cytotoxic lesions that promote carcinogenesis or are lethal if they are left unrepaired or inappropriately repaired. The presence of DSBs is first recognized by the MRE11-RAD50-NBS1 (MRN) complex (Stracker and Petrini 2011), and this complex and activated ataxia telangiectasia mutated kinase (ATM) induce the activation of downstream DNA repair genes and a cell cycle checkpoint such as checkpoint kinase 2 (CHK2) (Shiloh and Ziv 2013; Bartek and Lukas 2003). DSBs are repaired by two major pathways: homologous recombination (HR) and NHEJ (van Gent et al. 2001). HR occurs between two homologous sequences, usually two sister chromosomes, after DNA replication, and the BRCA2-RAD51 complex has a central role in HR (Esashi et al. 2005). On the one hand, HR is relatively error-free, while on the other hand, NHEJ is the simple ligation between two DNA ends independently of the chromosome locus, and is therefore relatively error-prone and sometimes results in chromosomal aberrations. In fact, NHEJ contributes to class-switch recombination in the immunoglobulin gene loci of activated B cells. Therefore, in addition to genotoxic factors, the alteration of some repair functions can contribute to the induction of genetic aberrations or chromosomal aberrations during tumorigenesis.

4.2 *Dysfunction of the DNA Repair System in* H. pylori*-Infected Gastric Epithelial Cells*

H. pylori infection has several effects on alterations of the DNA repair function. *H. pylori* infection in cultured gastric epithelial cells down-regulates the proteins involved in MMR and BER (Machado et al. 2009; Kim et al. 2002). In addition, down-regulation of MMR proteins occurs both in an *H. pylori*-infected mouse model and in human cases (Machado et al. 2009; Park et al. 2005). A combination of reduced expression of these repair genes with increased expression of genotoxic factors could enhance the accumulation of somatic mutations in gastric epithelial cells. A recent paper demonstrated that DSBs are introduced by NER, including endonucleases XPG and XPF, rather than BER (Hartung et al. 2015). Also, DSBs trigger a damage signaling and repair response involving ATM and its downstream target genes such as *53BP1* and *MDC1* (Hanada et al. 2014; Toller et al. 2011), but *H. pylori* infection induces the down-regulation of some components of several DNA repair pathways such as ATR, ATRIP, MRE11, and NBS1, which are involved in DSB repair (Koeppel et al. 2015). Interestingly, *H. pylori*-induced DSBs are repaired via NHEJ rather than HR, possibly due to the up-regulation of NHEJ-related genes and the down-regulation of HR-related genes (Hartung et al. 2015). These findings suggest that various alterations of DNA repair functions are closely linked with the formation of DSBs as well as chromosomal aberrations during the development of *H. pylori*-associated gastric cancers.

5 Molecular Mechanisms of DNA Aberrations

5.1 *Footprint of Carcinogenesis Process Determined by Comprehensive Genome Analyses*

Comprehensive cancer genome analyses reveal not only genetic abnormalities of the cancer genome, but also footprints of its carcinogenesis process. Each cancer type has its own specific dominant mutation signatures. Recent studies revealed that the mutation signature that accumulates in tumor tissues provides a clue to identifying the cause of genetic alterations during tumor development (Alexandrov et al. 2013; Helleday et al. 2014; Matsumoto et al. 2015a, b). Loss of function in DNA repair genes represents specific patterns of genomic alterations. As mentioned above, tumors with MSI in many cancer types have numerous substitutions, and small insertions and deletions due to defects of mismatch repair functions (Imai and Yamamoto 2008; Shah et al. 2010). Tumors with mutations in *POLE* or *POLQ*, which produce DNA polymerases with proofreading functions, have very large numbers of mutations (Heitzer and Tomlinson 2014). In tumors with inactivating mutations of HR-related genes *BRCA1* or *BRCA2*, such as some pancreatic cancers, substantial numbers of larger deletions (up to 50 bp) with overlapping

microhomologies at breakpoint junctions are found (Alexandrov et al. 2013). By contrast, some extrinsic mutagens have specific mutation signatures. UV light, a well-known extrinsic mutagen, induces mainly C:G > T:A transitions in the dipyrimidines. This mutation pattern is predominantly observed in melanoma and basal cell carcinoma, providing evidence that UV light plays a role as a causative factor in the development of these tumors (Krauthammer et al. 2012; Jayaraman et al. 2014). Benzo[a]pyrene, one of the convincingly established carcinogens contained in tobacco, typically causes C:G > A:T transversions. This mutation pattern is dominantly observed in lung cancers, especially those associated with smoking (Pfeifer et al. 2002; Alexandrov et al. 2013). As described above, intrinsic mutagens such as oxidative factors and APOBEC family members also have specific mutation signatures. Oxidative stress generally causes C:G > A:T transversions (Bruner et al. 2000). APOBEC3A, APOBEC3B, and AID are probably related to the development of various cancer types based on their expression levels and mutation signatures (Burns et al. 2013a, b; Schmitz and Petersen-Mahrt 2012). Thus, comprehensive cancer genome analyses can reveal the actual mechanisms of carcinogenesis in human tissues, which cannot be identified in cultured cells or animal models. Several mutation patterns, however, currently have unknown origins and may eventually elucidate novel mechanisms of carcinogenesis (Alexandrov et al. 2013).

5.2 *Exploring the Molecular Mechanisms of Gastric Carcinogenesis According to Mutation Signature (Fig. 2)*

In gastric cancers, the most common mutation pattern is the C:G > T:A transition, more than half of which occurs in XpCpG trinucleotides (Wang et al. 2011; Zang et al. 2012; Nagarajan et al. 2012; Cancer Genome Atlas Research Network 2014; Shimizu et al. 2014). This pattern is the prominent mutation signature in many cancer types and is probably related to the spontaneous deamination of 5-methylcytosine (Alexandrov et al. 2013; Pfeifer 2006). In particular, this mutation signature is prominently observed in gastrointestinal cancers and therefore seems to be linked with inflammation-associated carcinogenesis (Burns et al. 2013a, b). In addition, gastric cancers have numerous C:G > T:A transitions at non-CpG sites that occur preferentially at GpCpX or ApCpX sequences (Nagarajan et al. 2012; Shimizu et al. 2014). This mutation pattern corresponds well with the mutation signature induced by AID activity (Olivier et al. 2014), suggesting the involvement of AID-mediated cytidine deamination in the induction of somatic mutations during gastric carcinogenesis. As mentioned above, DNA repair systems are deeply involved in the formation of mutations after DNA damage by AID activity, suggesting that MSI status could contribute to preserve the mutation signature induced by AID activity. Consistently, C:G > T:A transitions are more prominently

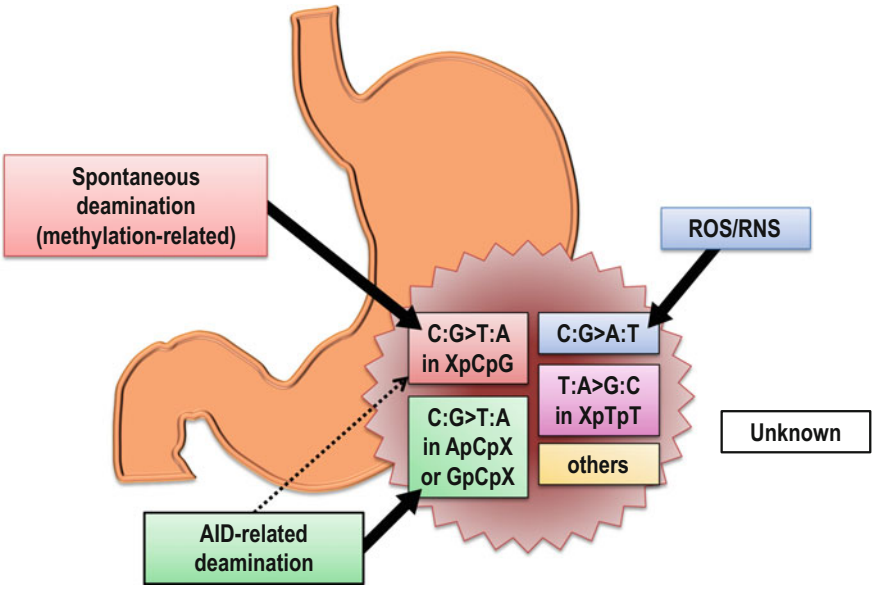

Fig. 2 Gastric carcinogenesis process according to mutation signatures of gastric cancer genome. Mutation signatures of gastric cancer genome represent the footprint of carcinogenesis. C:G > T:A transitions in XpCpG are most common pattern, indicating methylation-related spontaneous deamination. C:G > T:A transitions in ApCpX or GpCpX and C:G > A:T transversions suggest the involvement of AID-related deamination and ROS/RNS, respectively. Some patterns, including T:A > G:C transversions in XpTpT, suggest that unknown mechanisms still remain

observed in MSI gastric cancers and occur preferentially at XpCpG as well as at GpCpX or ApCpX sequences that are target sequences preferred by AID (Cancer Genome Atlas Research Network 2014, Shimizu et al. 2014). Recent reports demonstrated that the AID/APOBEC family also deaminates 5-methylcytosine or 5-hydoxymethylcytosine during the process of DNA demethylation (Bhutani et al. 2010; Popp et al. 2010). The deamination of 5-methylcytosine can induce C:G > T:A transitions in XpCpG sequences if the subsequent repair system does not work. Therefore, overexpression of AID also seems to be related to the emergence of C:G > T:A transitions in XpCpG sequences. More recently, deep sequencing of selected cancer-related genes, such as *TP53* in non-tumorous gastritis mucosa, revealed that C:G > T:A transitions at GpCpX sequences were strongly preferred, like those in gastric cancer tissues (Shimizu et al. 2014). Interestingly, human *TP53* knock-in (Hupki) mice with AID overexpression also had *TP53* mutations that led to C:G > T:A transitions at GpCpX sequences (Shimizu et al. 2014). Taken together, these findings indicate that AID is deeply involved in the emergence of mutations during gastric cancer development.

C:G > A:T transversion is also a mutation pattern frequently seen in gastric cancer genomes. This is the typical pattern induced by oxidative stress (Bruner et al. 2000). Considering the experimental data just discussed, oxidative stress is also an

important factor for inducing DNA damage during gastric carcinogenesis. In addition, T:A > G:C transversions at XpTpT sequences are unique patterns of gastric cancer genomes (Cancer Genome Atlas Research Network 2014). Esophageal adenocarcinoma has the same mutation patterns (Agrawal et al. 2012; Dulak et al. 2013; Nones et al. 2014), but another type of esophageal cancer, squamous cell carcinoma, for which the risk factors are tobacco and alcohol, does not have this mutation pattern (Song et al. 2014; Lin et al. 2014; Gao et al. 2014). Because esophageal adenocarcinoma is caused by duodeno-gastro-esophageal reflux, T:A > G:C transversions at XpTpT sequences may be linked with a currently unknown mediator of mutation induction.

Chromosomal aberrations are very important genetic alterations in gastric cancer, particularly CIN-type gastric cancer. As mentioned above, ROS and AID are possible inducers of DSBs during gastric carcinogenesis. In addition, the DNA repair system is deeply involved in the emergence of DSBs as well as in the repair of DSBs. Interestingly, CIN-type gastric cancers often have *TP53* mutations in addition to various chromosomal aberrations (Cancer Genome Atlas Research Network 2014). Many reports indicate that the functions of TP53 include the regulation of HR, repair genes, cell cycles, and others (Nicolai et al. 2015; Wang et al. 2015). Therefore, loss-of-function mutations of *TP53* could accelerate the emergence of DSBs as well as chromosomal aberrations.

6 Concluding Remarks

To fully understand the process of gastric carcinogenesis, precise molecular mechanisms of malignant transformation from gastric epithelial cells to cancer cells should be elucidated. Thus, we must know “what cells” are the origin of cancer, “what mutations” must occur, and “how” these cells can obtain these mutations. Recent comprehensive genome analyses revealed “what mutations” the gastric cancer genome possesses; however, it remains unknown “what cells” acquire “what mutations” for malignant transformation. Some reports demonstrated that normal stem cells can become cancer-initiating cells, while other reports indicated that differentiated cells may be better candidates for the origin of cancer cells (Beck and Blanpain 2013; Rycaj and Tang 2015; Brungs et al. 2016). The dynamic changes in the gastric glands during long-term *H. pylori* infection complicate the understanding of this process. Another difficulty is the lack of animal models that mimic human gastric carcinogenesis, although *H. pylori*- or *H. felis*-infected animal models are well established for the study of gastritis (Hayakawa et al. 2013). Mutation signatures in gastric cancer tissues can also provide information to help uncover actual mechanisms, showing “how” gastric epithelial cells acquire mutations. Some mutators, such as cytidine deaminase and oxidative stress, have been well investigated as key molecules involved in gastric carcinogenesis, but it is clear that unknown mechanisms still remain. For example, why are C:G > T:A transitions at CpG sites frequently observed? What induces T:A > G:C transversions at

XpTpT sites? How does the DNA repair system influence on the acquisition of mutations? Epigenetic alterations in gastric epithelial cells could also be involved in the induction of mutations. Further investigations will provide new insights toward understanding the whole process of gastric carcinogenesis.

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Helicobacter pylori and Extragastric Diseases

Andreas Kyburz and Anne Müller

Abstract The Gram-negative bacterium *Helicobacter pylori* is predominantly known for its tight association with peptic ulcer disease and gastric cancer development. However, recent epidemiological and experimental evidence suggests that chronic infection with *H. pylori* may at the same time be beneficial to the host by conferring protection against gastroesophageal diseases, asthma, other allergic disease manifestations and inflammatory bowel diseases (IBD). In this chapter, we summarize the epidemiological data that are available to date to support or refute a possible inverse correlation of *H. pylori* infection with various extragastric diseases. We further examine and discuss the experimental evidence, generated mostly in mouse models of allergic diseases and IBD, showing that these disorders fail to develop in the presence of *H. pylori*. The proposed mechanisms of the protective effects of *H. pylori*, which appear to involve the induction of regulatory T-cells (Tregs) with highly suppressive activity, are presented and explained.

Contents

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1 *H. pylori* in Gastric and Extragastric Health and Disease

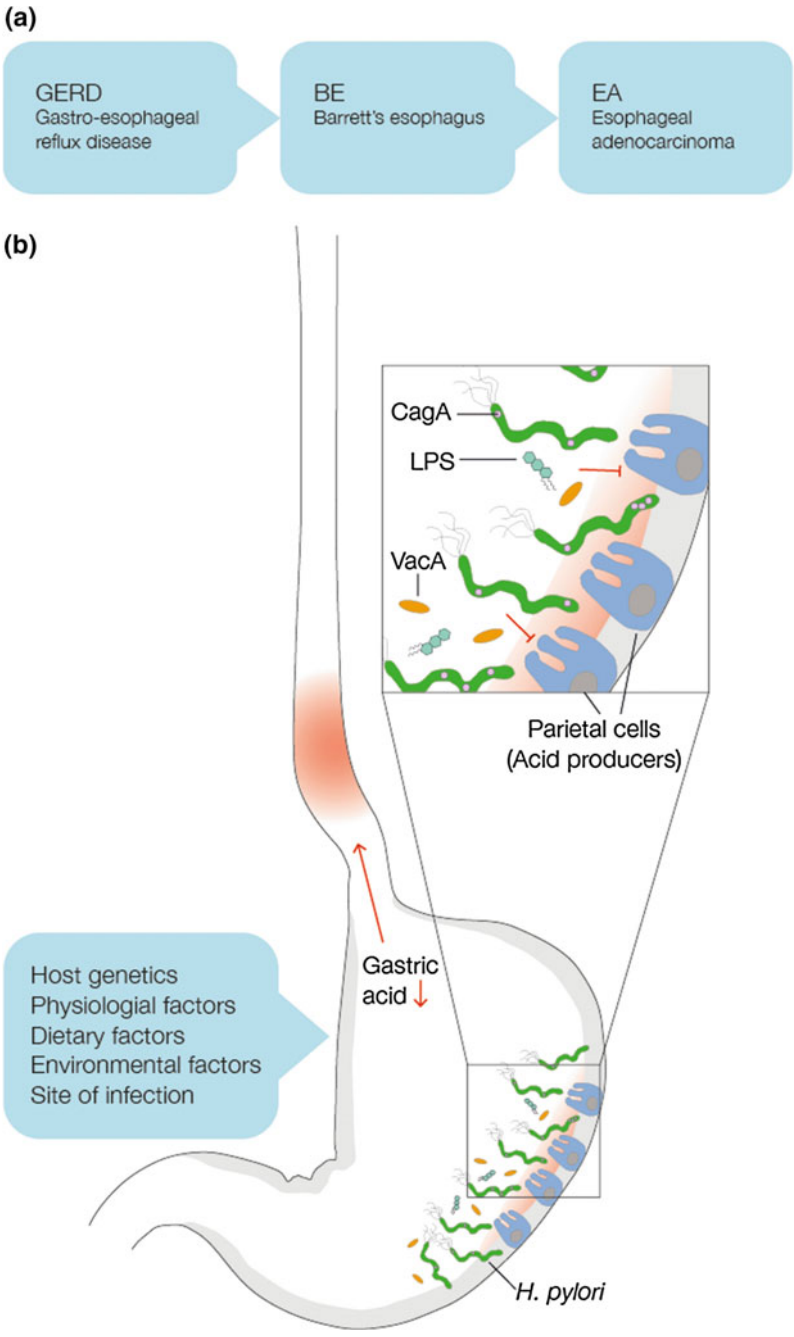
Helicobacter pylori is a highly successful pathobiont of humans that infects roughly 50% of the world population. Since its discovery in the early 1980s (Marshall and Warren 1984), *H. pylori* has been linked to a variety of gastric and extragastric disease manifestations (Salama et al. 2013; Pritchard and Crabtree 2006; Cover and Blaser 2009). *H. pylori* resides exclusively in the human stomach, where it colonizes the mucus layer overlying the gastric mucosa; minor populations adhere to gastric epithelial cells and colonize the glands of both the antrum and the corpus of the stomach (Salama et al. 2013). *H. pylori* causes histologically evident gastritis (Marshall and Warren 1984), which remains asymptomatic in the majority of infected individuals (Cover and Blaser 2009). Chronic *H. pylori* infection can result in gastric and duodenal ulcers and is the single most important risk factor for the development of gastric adenocarcinoma and gastric B-cell lymphoma, the so-called mucosa-associated lymphoid tissue (MALT) lymphoma (Herrera and Parsonnet 2009; Parsonnet et al. 1991, 1997, 1994; Parsonnet and Isaacson 2004; Cover and Blaser 2009). Bacterial virulence factors, host genetic predisposition and environmental factors such as lifestyle and diet have all been linked to an individual carrier's risk of developing disease (Pritchard and Crabtree 2006; Cover and Blaser 2009). *H. pylori* strains expressing virulence factors such as the cytotoxin-associated gene A (CagA) and the *cag* pathogenicity island (PAI) as well as toxic versions of the vacuolating cytotoxin (VacA) are more tightly associated with peptic ulcer disease and gastric cancer than Cag/VacA-negative strains (Cover and Blaser 2009; Pritchard and Crabtree 2006; Backert and Blaser 2016). More recently, the asymptomatic carrier state versus clinically evident disease has been attributed to *H. pylori*-specific T-cell responses: carriers with peptic ulcer disease are more likely to launch T-helper 1 (Th1)- and Th17-biased responses to *H. pylori*, whereas asymptomatic carriers exhibit regulatory T-cell (Treg)-predominant responses (Robinson et al. 2008). Similarly, asymptomatic children are more likely to generate Treg-dominated anti-*Helicobacter* responses than (symptomatic) adults (Harris et al. 2008; Serrano et al. 2013). The differential responses of young versus adult, experimentally infected mice, mirror the observations made in humans and have been linked to the development of Treg-mediated peripheral immune tolerance (Arnold et al. 2011c). As a consequence, neonatally infected mice are largely protected against the characteristic Th1- and Th17-driven gastric immunopathology that virulent strains elicit in mice and that is reminiscent of the gastric preneoplastic pathology of a minority of infected humans (Arnold et al. 2011c). A large body of evidence now suggests that *H. pylori* has both pathogenic and strong immunomodulatory properties, with the latter potentially conferring beneficial effects to the human host. Although the *H. pylori* field has been driven mostly by the quest to understand the pathogenic traits of *H. pylori* and especially its pro-carcinogenic activities, investigating the immunomodulatory and other protective properties of *H. pylori* may be equally worthwhile. *H. pylori* is an ancient member of the human gastric microbiota and has co-evolved with humans for at

least 60,000 years (Linz et al. 2007). Most humans are colonized with the same strain for life in an asymptomatic fashion. The intimate co-existence between *H. pylori* and its human host provides a context in which host and bacteria may benefit from one another.

The prevalence of *H. pylori* infection has decreased dramatically in the twentieth century from >50 to ~10% (Blaser and Falkow 2009) and parallels that of other infectious diseases (Bach 2002). As a consequence, gastric cancer rates and the associated mortality have declined steadily in countries from which *H. pylori* has disappeared (Forman 2005). In the same time frame, the incidence of immunological disorders such as autoimmune diseases (e.g., multiple sclerosis and type I diabetes), inflammatory bowel diseases (IBDs), asthma and other allergies has strongly increased (Bach 2002; Eder et al. 2006). Esophageal diseases such as gastro-esophageal reflux diseases (GERD), Barrett's esophagus and esophageal carcinoma are also increasingly common in Western societies from which *H. pylori* is disappearing (Pohl and Welch 2005). Whether the inverse trend of *H. pylori* prevalence and the prevalence of extragastric diseases is merely coincidental, or causally linked, has lately been the focus of increasingly sophisticated epidemiological and, to some extent, experimental research. This chapter aims to discuss the epidemiological and experimental evidence for (and against) a direct causal link between *H. pylori* and a variety of these extragastric diseases; we will begin by discussing the link to esophageal diseases, followed by dedicated sections on *H. pylori* and IBDs and allergies, respectively. Populations from various geographical areas of the world and from various age groups will be discussed separately wherever possible or necessary.

2 Esophageal Diseases and *H. pylori*

In the 1990s, several interventional trials implied that curing *H. pylori* infection in patients with duodenal ulcers might provoke reflux esophagitis (Labenz et al. 1997). Although many studies have since addressed the topic, the beneficial role of *H. pylori* in GERD and the serious sequelae Barrett's esophagus (BE) and esophageal adenocarcinoma (EA) remains controversially discussed and requires further clinical and experimental confirmation. Overall, most but by far not all clinical, epidemiological and experimental literature supports a positive and preventive role for *H. pylori* in esophageal diseases. The term GERD on the one hand refers to several related disorders that include BE and EA, but is on the other hand also thought to be the clinical starting point of a pathogenic sequence that can result in BE and EA (Fig. 1a) (Shaheen and Ransohoff 2002). GERD is characterized by esophageal acid exposure that is caused by the reflux of gastric contents and the failure of the esophagus to clear by means of peristaltic contractions. The severity of the disease depends strongly on the pH of the refluxed gastric juice (Collen et al. 1994; Gardner et al. 2004). BE is characterized by the replacement of the stratified squamous epithelium with a metaplastic columnar epithelium and is most likely caused by chronic GERD. The


(a)
GERD
Gastro-esophageal reflux disease
BE
Barrett's esophagus
EA
Esophageal adenocarcinoma
(b)
CagA
LPS
VacA
Parietal cells
(Acid producers)
Gastric acid
Host genetics
Physiologial factors
Dietary factors
Environmental factors
Site of infection
H. pylori

◂**Fig. 1** Esophageal diseases and *Helicobacter pylori*. **a** Pathogenic sequence of GERD to BE to EA. GERD patients are more prone to develop BE due to increased esophageal acid exposure. BE patients in turn are characterized by an inflamed esophageal, fast-dividing columnar tissue, which subsequently may shift to EA. **b** In an inflamed *H. pylori*-infected stomach, acid production by parietal cells is likely inhibited by bacterial LPS and the virulence/persistence factor VacA. Gastric atrophy resulting from chronic inflammation further decreases acid levels and eventually results in achlorhydria. Consequently, the lower pH is believed to relieve gastric esophageal reflux symptoms and disease progression. This phenomenon is predominantly associated with CagA-positive *H. pylori* strains

characteristic secretion of bicarbonate and mucus by Barrett's epithelium is believed to represent a protective adaptation against continued gastric acid exposure. Chronic acid exposure and the resulting inflammation appear to drive the sustained proliferation of BE cells, thus promoting the development of EA (Lao-Sirieix et al. 2008). The prevalence of BE is approximately 1–2% in patients subjected to endoscopy for any indication. This percentage increases up to 5–15% in patients with GERD symptoms. Strikingly, even though EA is a rare malignancy, the risk of EA among patients with BE is assumed to be 30- to 125-fold higher than that of the general population (Runge et al. 2015). Moreover, the prevalence of EA has increased dramatically in recent decades (Brown et al. 2008).

Several epidemiological studies and meta-analyses have addressed a possible inverse correlation of *H. pylori* infection with the incidence of a broad range of non-malignant GERDs. In a first meta-analysis of European studies published in 2003, Raghunath et al. showed that *H. pylori* infection is significantly inversely linked to GERD as defined as abnormal esophageal pH or erosive esophagitis (Raghunath et al. 2003). However, significance was lost after removal of a single dominant outlier study, thereby challenging the reliability of the analysis (Kandulski and Malfertheiner 2014). Similarly, Nordenstedt et al. (2007) found that there was no negative association of GERD symptoms with *H. pylori* infection except in patients with reduced pepsinogen levels and gastric atrophy (Nordenstedt et al. 2007). In 2013, Rubenstein et al. confirmed the lack of association of GERD symptoms with *H. pylori* infection, irrespective of the CagA status of the colonizing strains. In contrast, this analysis found an inverse correlation of *H. pylori* with erosive esophagitis, especially in patients harboring CagA-positive strains (Rubenstein et al. 2014). This evidence was further supported by Korean and Japanese studies in which *H. pylori* could be negatively linked with the risk and severity of erosive esophagitis (Chung et al. 2011; Minatsuki et al. 2013). Numerous studies and meta-analyses have indicated a negative correlation of *H. pylori* infection with BE and EA. In a systemic review in 2007, Rokkas et al. (2007) found a lower prevalence of *H. pylori* in patients with either BE or EA, which was more significant for infections with CagA-positive strains (Rokkas et al. 2007). In 2012, Fischbach et al. arrived at a similar conclusion when investigating four methodologically comparable studies, and also documented a decreased risk of EA predominantly in patients infected with CagA-positive *H. pylori* strains (Fischbach et al. 2012). A similar result was obtained in a recent US-based

case–control study (Rubenstein et al. 2014). In addition, Fischbach and co-workers recently confirmed a negative association of *H. pylori* with the risk of BE, which was particularly evident in participants with likely low gastric acidity due to corpus atrophy or anti-secretory drug use (Fischbach et al. 2014). The association of *H. pylori* infection with a decreased risk of BE and EA thus is rather consistent, but seems to generally be more pronounced in Eastern compared to Western countries (Thrift et al. 2012; Xie et al. 2013a, b). In a recent meta-analysis, Nie et al. (2014) speculated that CagA-positive strains might even have opposing associations with another esophageal malignancy, esophageal squamous cell carcinoma (ESCC) in Asian and non-Asian populations; however, these authors also found that CagA-positive *H. pylori* strains were associated with a decreased risk of EA in all populations, irrespective of geographical location (Nie et al. 2014). Numerous studies have addressed whether *H. pylori* eradication promotes the development of GERD or associated diseases. Several studies have reported an aggravation of symptoms of GERD or BE after eradication of the bacterium (Ahmed and Sechi 2005; Carroll et al. 2004; Tanaka et al. 2004; Fallone et al. 2000; Haruma 2004). Some literature even suggests that successful eradication serves as the starting point of GERD in certain cases (Nakajima and Hattori 2003; Sakata and Fujimoto 2005). This was particularly evident in Asian populations (Xie et al. 2013b; Cremonini et al. 2003). A recent study conducted in Japan reported that healthy asymptomatic *H. pylori*-infected individuals have a lower prevalence of reflux esophagitis than those subjected to eradication therapy (Minatsuki et al. 2013). Additionally, a study from Taiwan reported increased morbidity associated with reflux esophagitis upon eradication (Lee et al. 2013). On the other hand, two studies have failed to detect an effect of eradication therapy on the subsequent development of GERD, BE or similar (Saad et al. 2012; Laine and Sugg 2002). Vaira et al. (2003) even found an amelioration of GERD symptoms after *H. pylori* eradication. Two other meta-analyses similarly concluded that eradication does not influence the incidence of reflux symptoms or esophagitis (Qian et al. 2011; Yaghoobi et al. 2010). Possible explanations for the discrepancies have raised issues of ethnic differences and the reasons for why eradication therapy was prescribed in the first place (Iijima et al. 2015).

Little definitive mechanistic data are available to explain putative protective or detrimental effects of *H. pylori* on GERD, BE or EA. On the one hand, *H. pylori* has been implicated in raising gastric acid secretion by promoting the destruction of somatostatin-secreting D-cells in antrum-predominant gastritis, resulting in increased parietal cell mass, hyperchlorhydria and an aggravation of GERD symptoms (Kamada et al. 1998). On the other hand, pangastritis, which is mostly associated with CagA and VacAs1 bearing strains, results in the destruction of acid-secreting parietal cells, causing hypo- or achlorhydria due to gastric atrophy and an amelioration of GERD-associated symptoms (Ghoshal and Chourasia 2010). VacA is believed to directly disrupt the apical membrane–cytoskeletal interaction in

parietal cells and thereby lower acid secretion (Wang et al. 2008). Bacterial lipopolysaccharides may also dampen esophageal acid exposure through the prostaglandin system and by inhibition of the enzymatic function of the H/K ATPase (Tsuji et al. 1992; Helmer et al. 2004). In a recent publication, Gall et al. reported in a BE cohort that infection with the bacterium was associated with a decreased incidence of genomic instability which predicts progression to EA. The authors also detected *H. pylori* at esophageal sites, which raise the possibility that the bacterium directly impacts the survival and proliferation of esophageal epithelial cells (Gall et al. 2015). When investigating the role of *H. pylori* in BE and EA formation in a rat model, Liu et al. (2011) found that the bacteria reduce BE severity when the infection site is restricted to the stomach, but promote an increase in inflammation and incidence of BE and EA when colonizing the esophagus (Liu et al. 2011).

Various host physiological and genetic determinants but also environmental and dietary factors have been implicated in playing a critical role in shaping the outcome of GERD diseases in the context of *H. pylori* infections (summarized in Fig. 1b) (Chourasia and Ghoshal 2008; Ghoshal and Chourasia 2010). Moreover, the difference in responsiveness between Eastern and Western countries has been explained by the fact that *H. pylori* infection does not lead to a significant change in gastric acid secretion in Europeans and Americans, whereas a strong decrease is observed in Asians (Iijima et al. 2015). However, the cellular and molecular mechanisms behind this finding remain to be elucidated.

In summary, epidemiological studies have repeatedly described a negative association between *H. pylori* infection and erosive esophagitis, BE and EA, but not between *H. pylori* and GERD symptoms. Infection with CagA-positive strains in particular appears to protect the distal esophagus by causing fundic gland atrophy and impairing gastric acid secretion. Although several early reports have suggested the development of erosive esophagitis after *H. pylori* eradication, more recent studies have failed to corroborate an important clinical impact on GERD of *H. pylori* eradication. As in many other disorders, the patient's ethnic, genetic, physiological and pathological background as well as dietary habits appears to play a crucial role in shaping disease risk.

3 Inflammatory Bowel Diseases and *H. pylori*

Inflammatory bowel diseases (IBDs) are chronic relapsing disorders of increasing incidence that affect the gastrointestinal tract. The two main forms of IBD, Crohn's disease and ulcerative colitis, are both characterized by intestinal inflammation and epithelial injury, but differ in terms of their clinical and histopathological features, suggesting that they represent independent clinical entities. In Crohn's disease,

inflammation is discontinuous and can affect any part of the gastrointestinal tract and all layers of the bowel wall. The transmural nature of inflammation accounts for the serious complications associated with Crohn's disease such as fibrostenosis, abscesses and fistula formation. In contrast, ulcerative colitis is confined to the superficial layer of the mucosa and expands continuously from the rectum, with progressive inflammation and ulceration of the distal colon in more advanced forms. Histological features of IBD further include disruption of the intestinal epithelium with goblet cell depletion, decreased mucus production and hyperplasia (Xavier and Podolsky 2007). The precise etiology of IBD is unclear, but appears to involve a complex combination of host genetic, microbial and environmental factors. Chronic inflammation arises from an abnormal immune response against the microorganisms of the intestinal flora in genetically susceptible individuals and results in the breakdown of intestinal homeostasis (Maloy and Powrie 2011). Both dysregulated innate and adaptive immune pathways contribute to the chronic inflammatory response in patients with IBD (Geremia et al. 2014). Interestingly, both ulcerative colitis and Crohn's disease patients have altered microbial communities, with reduced diversity in major phyla such as Firmicutes (including *Clostridium*) and Bacteroidetes (including *Bacteroides fragilis*), and increased numbers of adherent-invasive strains of the Enterobacteriaceae. These observations suggest a direct link between the presence of specific bacterial products and the maintenance of major anti-inflammatory pathways in the gut. Changes in the human microbiota composition arising from modern hygienic practices and diet have been proposed to account for the increasing incidence of IBD in Western societies (Baumgart et al. 2011). However, whether the dysbiosis observed in IBD patients represents a primary predisposing factor or results from the combination of other deficiencies is still unclear.

An inverse correlation between *H. pylori* infection and IBD in its various manifestations has long been suspected by gastroenterologists. The initial sporadic observations by clinicians were examined more systematically in a series of epidemiological studies initiated in the mid-1990s; of the roughly 10 studies published between 1994 and 2004, the vast majority found a lower seroprevalence of *H. pylori* infection in patients with IBDs relative to an age-matched control population. Crohn's disease and ulcerative colitis patients were both less likely to be seropositive for *H. pylori* than healthy controls; Crohn's disease patients had an even lower prevalence of *H. pylori* infection than ulcerative colitis patients (el-Omar et al. 1994; Halme et al. 1996; Parente et al. 1997). Later studies in which active *H. pylori* infection was detected by urea breath test rather than serum IgG or IgA also unequivocally confirmed a lower prevalence of *H. pylori* in IBD patients (Pearce et al. 2000; Pronai et al. 2004; Piodi et al. 2003). All early studies were conducted on European populations, in which IBDs are much more common than in other geographical areas of the world; however, several more recent studies have

since confirmed the same trends in Asian and American populations (Wu et al. 2015; Sonnenberg and Genta 2016; Jin et al. 2013). A recent study of pediatric IBD patients confirmed the trend seen in adults, i.e., children with newly diagnosed Crohn's disease or ulcerative colitis were significantly less likely to harbor *H. pylori* than non-IBD controls from the same general population (Roka et al. 2014). Several of the more recent studies have used multivariate logistic regression analyses to adjust for gender, ethnicity, age, income, ZIP code and other measures of socioeconomic status, but found the trends to hold true irrespective of these parameters (Sonnenberg and Genta 2016, 2012; Castano-Rodriguez et al. 2015). Moreover, studies that include very large numbers of subjects such as one analysis of the surgical pathology files of 65,515 patients (1061 IBD patients and 64,451 controls) confirm the low prevalence of *H. pylori* infection among patients with IBD (Sonnenberg and Genta 2012).

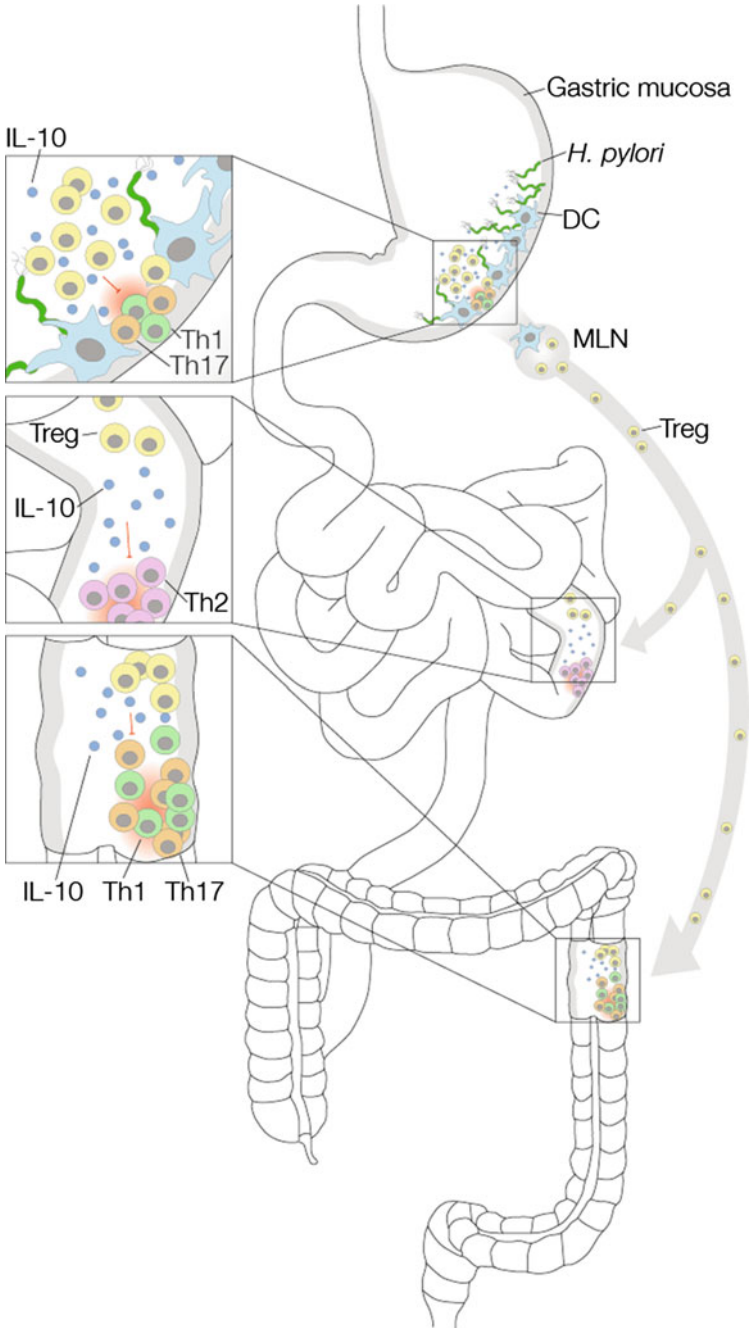
Several meta-analyses have been conducted on the topic in recent years. A meta-analysis of ten studies involving 1299 Asian IBD patients and 1817 controls showed infection rates of 24.9% in IBD patients relative to 48.3% in the controls, with a resulting pooled risk ratio of 0.48 for *H. pylori* infection in IBD patients (Wu et al. 2015). Other meta-analyses have reached similar conclusions: For example, a meta-analysis of 23 studies conducted in 2010 (5903 subjects in total) found that overall, 27.1% of IBD patients had evidence of infection with *H. pylori* compared to 40.9% of patients in the control group (relative risk of 0.64). A more recent meta-analysis of 33 eligible studies that included 4400 IBD patients and 4763 controls (the vast majority being non-Asian) found that 26.5% of IBD patients were *H. pylori*-positive, compared to 44.7% of individuals in the control group (risk ratio of 0.62) (Rokkas et al. 2015). In the most comprehensive meta-analysis to date, with data from 40 studies, Crohn's disease and ulcerative colitis patients were evaluated together as well as separately (Castano-Rodriguez et al. 2015). The entire study population included 6130 patients with IBD and 74,659 non-IBD controls. The overall calculated risk ratio for *H. pylori* infection was 0.43; stratification by patient age revealed an even lower risk ratio for pediatric populations (0.24 relative to 0.45 in adults). Crohn's disease patients had a lower risk ratio than ulcerative colitis patients (0.38 relative to 0.53), and Eastern populations had a lower risk ratio than Western populations (0.35 relative to 0.46) (Castano-Rodriguez et al. 2015). The same meta-analysis found a positive association between infection with enterohepatic *Helicobacter* species and *Campylobacter* species and IBD, suggesting that closely related bacteria can have vastly different effects on this disease group (Castano-Rodriguez et al. 2015). All meta-analyses and almost all original articles covering the topic thus consistently find a strong negative association between *H. pylori* colonization and IBD.

3.1 H. pylori *and Host Factors Determine Protection Against IBDs*

Several pieces of experimental evidence are now available to support a direct contribution of *H. pylori* to protection against the chronic intestinal inflammation that is the main histopathological hallmark of IBD. Several complementary models have been used to investigate protection against IBD by live *H. pylori* and purified components of the bacteria. For example, Higgins et al. (2010) examined the effects of *H. pylori* infection on *Salmonella typhimurium*-induced chronic colitis, a model for Crohn's disease. *H. pylori* co-infection suppressed the Th17 response to *S. typhimurium* in the mouse cecum and reduced the histopathological symptoms associated with this model (Higgins et al. 2010). The beneficial effects could be linked to IL-10 production in the mesenteric lymph nodes (MLNs) of the co-infected animals (Higgins et al. 2010) and to *H. pylori* DNA, which contains a high ratio of immunoregulatory to immunostimulatory sequences, especially relative to other Gram-negative bacteria such as *Escherichia coli* (Luther et al. 2011). Oral administration of *H. pylori* DNA was sufficient to protect against the histopathological symptoms of dextran sodium sulfate (DSS)-induced colitis in acute and chronic models of the disease (Luther et al. 2011). Further work identified a specific immunoregulatory sequence, 5'-TTTAGGG, as being unique to *H. pylori* genomes and particularly active in suppressing DCs (Owyang et al. 2012). Work by the same group and others has revealed a role for TLR2 signaling in the suppression of DC activation, the induction of Treg-biased T-helper cell responses and protection against IBD (Sun et al. 2013; Koch et al. 2015). *H. pylori* expresses TLR2 ligands that dominate the bacteria's interaction with DCs, and other innate and adaptive immune cell compartments, including B-cells (Rad et al. 2009; Sun et al. 2013; Sayi et al. 2011; Koch et al. 2015). TLR2 signaling thus presumably drives a tolerogenic response in DCs that directs Treg-biased responses to *H. pylori* antigens and suppresses T-effector responses to the bacteria (Koch et al. 2015). The host benefits from this regulatory response due to protection from gastric immunopathology even in the face of high-level colonization (Koch et al. 2015). Interestingly, responses to unrelated (bystander) T-cell antigens are suppressed as well, including allergen-specific and maybe autoantigen-specific immune responses (Koch et al. 2015; Oertli et al. 2012; Arnold et al. 2011a). This finding has been used to explain why *H. pylori*-infected individuals are less likely to develop allergic disease manifestations (Blaser et al. 2008; Chen and Blaser 2007, 2008; Reibman et al. 2008), celiac disease (Lebwohl et al. 2013) and possibly autoimmune diseases (Cook et al. 2015) (as well as IBD), as discussed below.

TLR2 signaling is required for the *H. pylori*-induced production and secretion of IL-10 (Sun et al. 2013; Sayi et al. 2011), a well-studied cytokine with a plethora of anti-inflammatory and regulatory activities. It is also required for the priming of

inflammasome activation, a critical event during the *H. pylori*/host interaction (Kim et al. 2013; Koch et al. 2015). *H. pylori* exclusively activates the NLRP3 inflammasome; in contrast, other cytoplasmic innate immune sensors such as AIM2, NLRP6 and NLRC4 do not contribute measurably to inflammasome and caspase-1 activation (Koch et al. 2015; Semper et al. 2014; Kim et al. 2013). NLRP3 inflammasome activation is preceded by a "priming" event, which allows cells to upregulate NLRP3 transcription in a TLR2-dependent manner (Kim et al. 2013; Koch et al. 2015). DCs lacking TLR2 are incapable of NLRP3 transcriptional activation and caspase-1 auto-proteolysis and activation, and therefore fail to process and secrete the caspase-1-dependent cytokines IL-1β and IL-18 (Kim et al. 2013; Koch et al. 2015). Both have critical roles in the *H. pylori*/host interaction, with IL-1β driving Th1- and Th17-polarized T-cell responses and *H. pylori* control, and IL-18 providing regulatory activity (Hitzler et al. 2012). The lack of mature IL-18 in particular recapitulates the phenotypes of TLR2 deficiency and NLRP3 deficiency: mice lacking either the cytokine or its receptor control *H. pylori* more efficiently due to unrestricted Th1 and Th17 responses, but suffer from severe infection-associated immunopathology (Hitzler et al. 2012; Oertli et al. 2012). The critical contribution of the TLR2/NLRP3/caspase-1/IL-18 signaling axis to immune tolerance induced by *H. pylori* hinted at a role of this pathway also in protection against IBD. Indeed, confirming earlier data, *H. pylori* protected effectively against DSS-induced colitis not only via its DNA as shown previously (Luther et al. 2011), but also in the context of experimental infection (Engler et al. 2015). Infection of mice during the neonatal period, when their predisposition to develop tolerance to foreign antigens is at its peak, alleviated DSS colitis symptoms later in life (Engler et al. 2015). The effect of live infection could be mimicked by regular doses of *H. pylori* extract, administered orally or intraperitoneally starting from the neonatal period onwards (Engler et al. 2015). The effects of live infection and extract treatment required NLRP3 and IL-18, and were attributed to the production of copious amounts of mucus in NLRP3/IL-18 proficient animals (Engler et al. 2015). Mucus production was detectable by endoscopic procedures as well as at the transcriptional level (the main intestinal mucin is Muc2, which was strongly upregulated upon infection or extract treatment) (Engler et al. 2015) and likely explains the resistance to barrier destruction by DSS that is the underlying cause of colitis in this model. Overall, there is now more and more convincing experimental evidence supporting a protective role of *H. pylori* on IBD development. Combined with the epidemiological data in humans documenting an inverse correlation of IBD risk with *H. pylori* prevalence, it appears likely that direct effects (via the regulatory activity of *H. pylori* DNA, NLRP3 ligands and potentially other immunomodulators) of *H. pylori* on immune cells, mainly DCs and Tregs, account for its beneficial effects (summarized in Fig. 2).


Gastric mucosa
H. pylori
DC
MLN
Treg
IL-10
Th1
Th17
Treg
IL-10
Th2
IL-10
Th1
Th17

◂**Fig. 2** *H. pylori* in its relationship to intestinal diseases. *H. pylori* exclusively inhabits the gastric mucosa of humans. 10–20% of infected individuals will develop gastric infection-associated diseases, such as chronic gastritis and gastric ulcers, that are driven by pathogenic T-cells polarized to express Th1 and Th17 cytokines. The majority (greater than 80% of the infected population) will remain asymptomatic throughout life despite harboring high levels of *H. pylori*. Asymptomatic carriers mount a Treg-predominant response to the infection (upper inset); Tregs (in *yellow*) suppress Th1, Th17 and Th2 responses, both locally in the gastric mucosa and at other sites of the GI tract (shown here are the small and large intestine). *H. pylori*-induced Tregs are believed to contribute to the alleviation of colitis symptoms in models of inflammatory bowel disease (lower two insets). Treg- and DC-derived IL-10 contributes to *H. pylori*-specific immunomodulation

4 *H. pylori* and Allergic Disease Manifestations

The severity and incidence of allergic asthma and other atopic diseases have increased dramatically in developed countries over the last decades. Allergic diseases thus follow two major trends that have dominated public health in developed countries since the second half of the twentieth century: The incidence of infectious diseases has declined sharply in that time frame, whereas immunological disorders such as multiple sclerosis (MS), type I diabetes, the aforementioned IBDs and allergies have dramatically increased in incidence over the same time period (Bach 2002). Numerous epidemiological studies have addressed, and demonstrated, an inverse association of *H. pylori* infection with asthma and other allergies with respiratory tract manifestations (Blaser et al. 2008; Chen and Blaser 2007, 2008; Reibman et al. 2008; Amberbir et al. 2011). This inverse association was particularly strong in children and adolescents and in individuals with early onset allergies and asthma (Blaser et al. 2008; Chen and Blaser 2007, 2008; Reibman et al. 2008; Amberbir et al. 2011). The chronic inflammatory skin disease atopic dermatitis/eczema has also been inversely linked to *H. pylori* infection in studies including over 3000 German school children and almost 2000 Japanese university students (Herbarth et al. 2007; Shiotani et al. 2008). Two meta-analyses have since been conducted that have investigated a possible inverse association of *H. pylori* with allergic asthma. Wang et al. (2013) retrieved 19 studies conducted up until 2012 (nine cross-sectional studies, seven case–control studies and three prospective cohort studies) and from these calculated a pooled OR for the association between asthma and *H. pylori* infection of 0.81. A second meta-analysis—also published in 2013—which included 14 studies involving 28,283 patients also found a significantly lower rate of *H. pylori* infection in the asthmatics than in the controls (OR = 0.84, P = 0.013) (Zhou et al. 2013). Following up on the various observational studies in human populations, mechanistic studies in experimental models have examined a possible protective effect of experimental *H. pylori* infection in animal models of allergic asthma. In a murine model of allergic asthma induced by ovalbumin or house dust mite antigen sensitization and challenge, *H. pylori* infection confers almost complete protection against the airway hyper-responsiveness, broncho-alveolar eosinophilia, lung inflammation and goblet cell metaplasia that are hallmarks of asthma in humans and mice (Arnold et al. 2011a). The protective effects are

particularly pronounced in animals that have been experimentally infected during the neonatal period (Arnold et al. 2011a), i.e., at an age when humans typically contract the infection from their mothers (Weyermann et al. 2009). Asthma protection conferred by *H. pylori* is abolished by antibiotic eradication therapy prior to allergen challenge and depends critically on regulatory T-cells (Tregs, Fig. 3) (Arnold

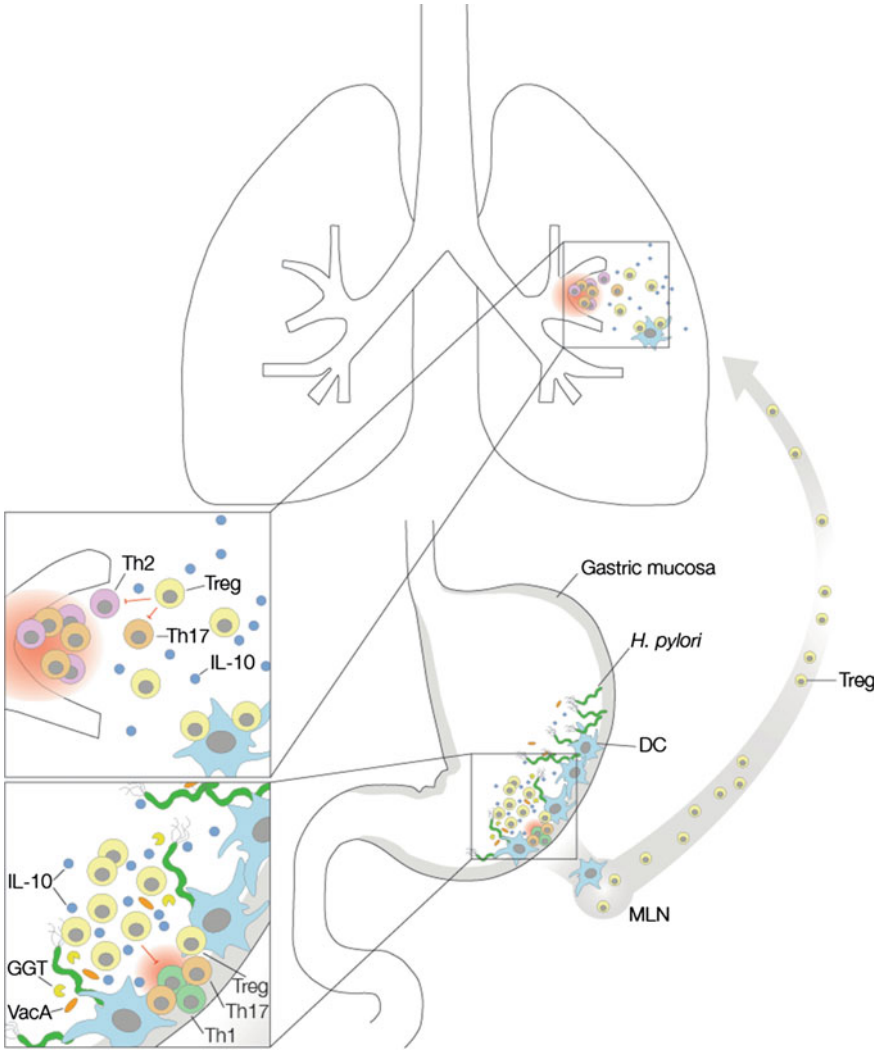


Fig. 3 Gastric *H. pylori* colonization protects against allergic asthma. Despite exclusively colonizing the gastric mucosa, *H. pylori* has robust systemic effects on T-cell responses in other organs. The *H. pylori* persistence factors γ-glutamyl-transpeptidase (GGT) and vacuolating cytotoxin (VacA) promote chronic infection by tolerizing DCs and thereby promoting local Treg differentiation (lower inset). *H. pylori*-induced Tregs and DC/Treg-derived IL-10 are required for the suppression of allergen-specific Th2 and Th17 responses in the lung (upper inset). Children and young adults are more likely than older hosts of *H. pylori* to benefit from the infection in terms of their individual allergy risk

et al. 2011a). The systemic depletion of Tregs abrogates asthma protection, and conversely, pure populations of Tregs are sufficient to transfer protection from neonatally infected donors to naive recipients. These results are in line with earlier observations that neonatal infection with *H. pylori* induces Treg-mediated immune tolerance to the bacteria (Arnold et al. 2011c) and that mice can be actively "tolerized" against *H. pylori* by vaccination (Arnold et al. 2011b). Interestingly, the suppressive activity of Tregs in the asthma model depends on interleukin-18 proficiency of the donor (Oertli et al. 2012), which is reminiscent of the prerequisites of protection against chronic intestinal inflammation (see above). In the absence of IL-18 signaling, neonatal tolerance to the infection cannot be established; Tregs derived from IL-$18^{-/-}$ or IL-$18R^{-/-}$ donors are not protective against asthma (Oertli et al. 2012). Further work has shown that IL-18 is produced by DCs upon exposure to *H. pylori* infection (Oertli et al. 2012). IL-18 production by DCs appears to be required for *H. pylori*-specific tolerance. IL-18 proficiency is required both in DCs derived from bone marrow and DCs isolated immunomagnetically from mesenteric lymph nodes for the conversion of naive $CD4^+$ T-cells into $CD25^+FoxP3^+$ Tregs (Oertli et al. 2012). In line with these observations, $FoxP3^+$ Treg numbers in the MLNs of both infected IL-$18^{-/-}$ and IL-$18R^{-/-}$ mice are significantly lower than those of infected wild-type mice (Oertli et al. 2012).

Pro-IL-18 is processed by caspase-1 to yield the mature cytokine. Several recent publications have identified the NLRP3 inflammasome as the predominant type of inflammasome to become activated upon *H. pylori* exposure of murine DCs (Semper et al. 2014; Kim et al. 2013; Koch et al. 2015). TLR2 proficiency was found to be a clear prerequisite of NLRP3 inflammasome activation, as $TLR2^{-/-}$ DCs failed to activate caspase-1 and secrete caspase-1-dependent cytokines (Koch et al. 2015). The available evidence thus points to a critical role of the TLR2/NLRP3/caspase-1/IL-18 axis in *H. pylori*-specific immune modulation, with TLR2 signaling leading to the transcriptional activation of NLRP3, which then assembles with pro-caspase-1 and the adaptor protein ASC to form the functional NLRP3 inflammasome, auto-proteolytically activate caspase-1 and process the caspase-1-dependent cytokines IL-18 and IL-1β (Koch and Muller 2015). Accordingly, TLR2-deficient mice are not protected against allergic asthma induced by house dust mite allergen (Koch et al. 2015).

Several *H. pylori* determinants have been implicated in immune tolerance, the differentiation and function of suppressive Tregs, and the protection against allergic disease manifestations. In particular, the persistence factors and *H. pylori* immunomodulators vacuolating cytotoxin (VacA) and γ-glutamyl-transpeptidase (GGT) are known to be required for persistent high-level colonization on the one hand, and protection against allergic asthma on the other hand (Fig. 3) (Oertli et al. 2013). VacA- or GGT-deficient mutants fail to colonize at wild-type levels, which correlates with higher numbers of Th1 and Th17 cells, higher expression of IFN-γ and IL-17 by restimulated mesenteric lymph node (MLN) single cell preparations, and lower numbers of $FoxP3^+$ $CD25^+$ regulatory T-cells in MLNs (Oertli et al. 2013). VacA in particular appears to bias T helper cell responses toward Tregs, which could be attributed to VacA's effects on DCs. DCs that were

immunomagnetically purified based on their CD11c expression from the MLNs of wild-type-infected mice induced FoxP3 and CD25 expression in co-cultured naive $CD4^+$ T-cells ex vivo (Oertli et al. 2013). This was not observed with DCs from uninfected animals or from mice infected with a *vacA* mutant (Oertli et al. 2013). Interestingly, both GGT and VacA can be administered to mice in purified form and confer a level of protection against allergen-induced asthma that is comparable to the live infection (Engler et al. 2014). Administration of several doses of VacA protects efficiently against allergen-induced asthma, especially if the protein is provided in the neonatal tolerance window (Engler et al. 2014). This time frame constitutes a period in both mice and humans in which immune tolerance to antigens is readily established; therefore, it is perhaps not surprising that VacA acts most potently during this time. Active tolerization against allergens using VacA requires its interaction with DCs (Fig. 3), as mouse strains lacking IL-10 expression in the DC compartment cannot be tolerized with VacA (Engler et al. 2014). Administration of several doses of VacA protects efficiently against allergen-induced asthma, especially if the protein is provided in the neonatal tolerance window (Engler et al. 2014).

In conclusion, substantial epidemiological evidence is available to support the idea that *H. pylori* is not just a pathogen, but in its function as a normal, ancient member of the gastric microbiota may also contribute to esophageal health, protection against allergies and IBDs, and possibly against auto-immune diseases although the evidence toward this end remains less well documented. There is hope that the immunomodulatory properties of *H. pylori* can be separated from its pathogenic properties to enable its future exploitation for therapeutic purposes in one or more of these disease areas.

5 Concluding Remarks

Controlling the epidemic increase in allergic, chronic inflammatory and auto-immune diseases is by many accounts one of the great public health challenges of this century. An overly "sterile" life style, exaggerated use of antibiotics in childhood and many other sanitary and behavioral practices common in developed countries are known to contribute to the rise in incidence in these "immunological" disorders (Bach 2002). The gradual disappearance of our normal, "ancestral" microbiota has been blamed by some investigators for this trend (Blaser and Falkow 2009). Epidemiological and experimental data point to a special role of *H. pylori*, a dominant component of the normal flora until half a century ago, in this context due to its strong immunomodulatory capacity. More work is clearly required to gain a detailed understanding of the mechanistic basis of *H. pylori*-specific immune tolerance and identify the *H. pylori* factors involved in immunomodulation, until it will be possible to harness the immunomodulatory properties of *H. pylori* for the purpose of *H. pylori*-specific tolerization against asthma and allergies, IBDs and possibly auto-immune diseases.

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