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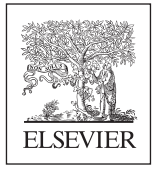

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CHAPTER ONE

Physiological Role of Two-Component Signal Transduction Systems in Food-Associated Lactic Acid Bacteria

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Contents

Abstract

Two-component systems (TCSs) are widespread signal transduction pathways mainly found in bacteria where they play a major role in adaptation to changing environmental conditions. TCSs generally consist of sensor histidine kinases that autophosphorylate in response to a specific stimulus and subsequently transfer the

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phosphate group to their cognate response regulators thus modulating their activity, usually as transcriptional regulators. In this review we present the current knowledge on the physiological role of TCSs in species of the families *Lactobacillaceae* and *Leuconostocaceae* of the group of lactic acid bacteria (LAB). LAB are microorganisms of great relevance for health and food production as the group spans from starter organisms to pathogens. Whereas the role of TCSs in pathogenic LAB (most of them belonging to the family *Streptococcaceae*) has focused the attention, the roles of TCSs in commensal LAB, such as most species of *Lactobacillaceae* and *Leuconostocaceae*, have been somewhat neglected. However, evidence available indicates that TCSs are key players in the regulation of the physiology of these bacteria. The first studies in food-associated LAB showed the involvement of some TCSs in quorum sensing and production of bacteriocins, but subsequent studies have shown that TCSs participate in other physiological processes, such as stress response, regulation of nitrogen metabolism, regulation of malate metabolism, and resistance to antimicrobial peptides, among others.

1. INTRODUCTION

1.1 Two-Component Systems

Regulation of cell physiology in response to changing conditions is a must for survival in the competitive environments that bacteria often face. Signal transduction systems are key players in the regulatory circuits that modulate bacterial physiology. Among them, two-component systems (TCSs) have been subjected to intensive research since their discovery (Nixon, Ronson, & Ausubel, 1986). TCSs are signal transduction pathways typically consisting of a sensor histidine kinase (HK), usually membrane bound, and a cytoplasmic response regulator (RR). Both proteins present a modular structure (Fig. 1). The HK has an N-terminal sensory domain that monitors the environmental signals and two modules involved in the phosphorylation reaction. The first domain holds the phosphorylatable His (histidine-phosphotransfer domain, Dhp). The second domain (CA domain) holds the ATP binding site and catalyzes the phosphorylation of the Dhp domain. The RR presents a conserved receiver domain (REC), where the phosphorylatable Asp residue is located, and a C-terminal effector domain. The domains involved in the phosphorylation reaction are homologous in all TCSs while the sensory and output domains of the HK and RR, respectively, are characteristic for each TCS and determine its specificity.

In general, detection of a specific stimulus triggers the HK autophosphorylation in the conserved His residue at the DHp domain and the subsequent

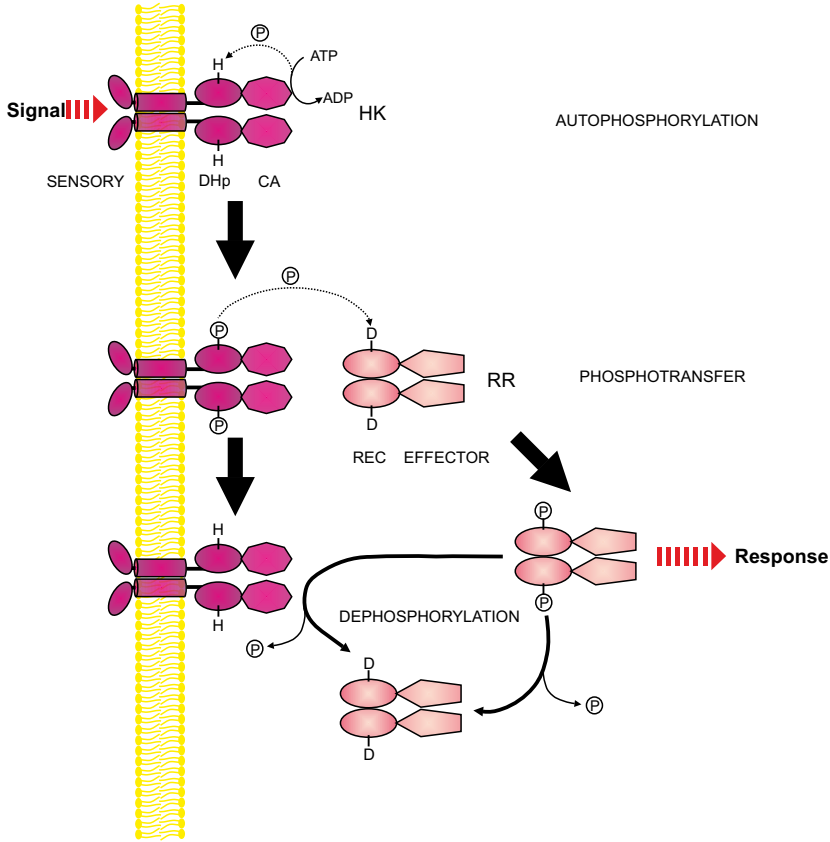

Figure 1 Domain organization of a typical two-component system and signal transduction flow. Histidine kinases (HK) typically consist of a sensory domain, a dimerization and histidine-phosphotransfer (DHp) domain that holds the phosphorylatable His residue, and an ATPase (CA) domain. Response regulators (RRs) consist of a receiver domain (REC) that holds a conserved phosphorylatable aspartate residue and an effector domain that usually is a DNA-binding domain. Signal transduction consists basically of three reactions: autophosphorylation of the HK upon detection of a specific signal whereby the HATPase domain phosphorylates the conserved His residue in the DHp domain at the expense of ATP, phosphotransfer of the phosphate residue to the Asp residue of the REC domain of the cognate RR. Phosphorylation of the RR induces conformational changes that modulate its activity. Signal transduction is usually terminated by spontaneous dephosphorylation of the RR or phosphatase activity of the cognate HK.

transference of the phosphate group to the conserved Asp residue at the REC domain of its cognate RR (Fig. 1). Phosphorylation of the RR modulates its activity, which usually involves transcriptional regulation mediated by its C-terminal effector domain (Stock, Robinson, & Goudreau, 2000). HKs

often also act as phosphatases for their cognate response regulators (Huynh & Stewart, 2011); in other cases, dephosphorylation of RRs is carried out by auxiliary phosphatases (Silversmith, 2010) or spontaneous hydrolysis. The final output response results from the balance of kinase and phosphatase activities. The mechanisms of signal transfer and regulation operated by TCSs will not be covered here as they have been extensively reviewed elsewhere (Casino, Rubio, & Marina, 2010; Galperin, 2010; Gao & Stock, 2009; Groisman, 2016; Huynh & Stewart, 2011; Jung, Fried, Behr, & Heermann, 2012; Krell et al., 2010; Mascher, Helmann, & Unden, 2006; Podgornaia & Laub, 2013; Salazar & Laub, 2015; Stock et al., 2000; Zschiedrich, Keidel, & Szurmant, 2016). These studies have shown that in many cases TCSs are integrated in complex regulatory networks that often involve a number of TCSs as well as other sensors.

The analyses of the HK and RR coding sequences and genetic organization have shown that TCS proteins belong to a limited number of families, which share common ancestry and domain structure (Whitworth & Cock, 2009). This has led to the proposal of a number of classification schemes based on phylogenetic reconstructions of conserved domains (Fabret, Feher, & Hoch, 1999; Grebe & Stock, 1999) or on the domain composition of TCS proteins (Galperin, 2006). Furthermore, TCSs are usually encoded by adjacent genes (although orphan genes, that is, unpaired HK or RR encoding genes, can also be found) and are arranged in the same order and orientation (Koretke, Lupas, Warren, Rosenberg, & Brown, 2000). The evolution of TCS has been the subject of a number of studies that have evidenced that coevolution of HK and RR pairs has been prevalent although examples of recruitment, i.e., duplication of one component and association with a nonorthologous partner, could also be observed (Alm, Huang, & Arkin, 2006; Koretke et al., 2000).

Beyond the basic scheme of signal transfer outlined earlier, more complex phosphotransfer relays also exist, which involve multiple phosphotransfer reactions among domains that can be found on separate polypeptides or as part of multidomain proteins (Appleby, Parkinson, & Bourret, 1996; Zhang, 2005). Besides, other auxiliary proteins can modulate the activities of TCSs (Buelow & Raivio, 2010; Gao & Stock, 2009). Furthermore, TCS can also integrate other signals through additional cytoplasmic sensory domains or through metabolites that can affect the phosphorylation state of the TCS proteins (Gao & Stock, 2009; Krell et al., 2010; Szurmant, White, & Hoch, 2007). Among the cytoplasmic sensory domains identified in HKs, PAS (PER, ARNT, SIM) and GAF (c-GMP—specific and c-GMP—stimulated

phosphodiesterases, *Anabaena* adenylate cyclases and *Escherichia coli* FhlA) are the most common (Galperin, Nikolskaya, & Koonin, 2001; Szurmant et al., 2007). PAS domains can receive signals by several mechanisms including signal binding to the PAS domain cavity, signal perception by cofactor-containing PAS domains, signal binding at the PAS domain–membrane interface, and signal-mediated modulation of inter-PAS domain disulfide bonds (reviewed in Krell et al., 2010). The role of GAF domains in TCS remains undetermined in many cases. It has been shown that GAF domains bind heme in some redox or oxygen-sensing HKs (Kumar, Toledo, Patel, Lancaster, & Steyn, 2007) and cyanobacterial photoreceptor HKs involved in phototaxis covalently bind tetrapyrrole pigments through their GAF domains (Ikeuchi & Ishizuka, 2008). A recent study has shown that the GAF domain of the *Synechocystis* sp. PCC 6803 cytoplasmic HK Hik2 possibly functions as a chloride sensor (Kotajima, Shiraiwa, & Suzuki, 2014). Some metabolites may also affect the phosphorylation state of TCSs. Several RRs have been shown to be phosphorylated by acetyl phosphate (acetyl-P) thus providing a possible link between TCS activity and metabolic state (Wolfe, 2010). Although some studies have noted that HK phosphatase activity may prevent this HK-independent phosphorylation, recent studies support the view of acetyl-P-dependent RR phosphorylation as a mechanism of regulation of TCS activity (Lima et al., 2012; Schrecke, Jordan, & Mascher, 2013). Polyphosphate can also act as a phosphoryl donor for the MprB sensor protein of *Mycobacterium tuberculosis* (Sureka et al., 2007). Finally, other signal transducing pathways, such as those operated by Ser/Thr kinases, can modulate the activity of TCSs via posttranslational modification (Burnside & Rajagopal, 2012).

TCSs participate in most aspects of bacterial physiology, including motility, sporulation, competence, nutrient uptake, stress response, central metabolism, and virulence. TCSs are found in varying numbers in bacteria although, generally, bacteria with larger genomes encode more TCSs (Galperin, 2005; Sheng, Huvet, Pinney, & Stumpf, 2012; Ulrich, Koonin, & Zhulin, 2005; Wuichet, Cantwell, & Zhulin, 2010). In addition, the number of TCSs correlates with ecological niches. Free-living bacteria that inhabit changing or diverse environments usually harbor more TCSs than bacteria that live in constant environments, such as pathogenic bacteria, suggesting a correlation between metabolic versatility and number of TCSs (Capra & Laub, 2012; Galperin, 2005). In contrast to bacteria, TCSs are far less common in eukaryotes and completely absent in mammals. This, together with the role of some TCSs in pathogenesis, has driven their

interest as potential targets for antimicrobial drugs. This situation also reflects on lactic acid bacteria (LAB) where a number of TCSs of pathogenic streptococci have been thoroughly characterized, whereas far less information is available about TCSs of commensal LAB.

1.2 Lactic Acid Bacteria

The term lactic acid bacteria comprises a broad group of microorganisms characterized by their ability to degrade sugars mainly into lactic acid (Orla-Jensen, 1919). Originally classified on the basis of phenotypic traits that led to protracted controversies, the use of phylogenetic techniques based on DNA sequencing has shown that the major group of genera of LAB diverged from a common ancestor (Schleifer & Ludwig, 1995). It is becoming increasingly accepted by the scientific community that LAB species constitute the order *Lactobacillales* (phylum *Firmicutes*) and other species that have been traditionally considered as LAB must not be included in this group (Vandamme, De Bruyne, & Pot, 2014). The order *Lactobacillales* currently consists of six families: *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae*. However, even this family classification is questionable. Phylogenomic analyses have shown that, within the genus *Lactobacillus*, other genera belonging to families *Lactobacillaceae* and *Leuconostocaceae*, such as *Fructobacillus*, *Leuconostoc*, *Oenococcus*, *Pediococcus* and *Weissella*, are grouped within the lactobacilli as subclades (Claesson, van Sinderen, apos, & Toole, 2008; Makarova et al., 2006; Sun et al., 2015; Zhang, Ye, Yu, & Shi, 2011). For the purposes of this review, we will therefore consider the members of families *Lactobacillaceae* and *Leuconostocaceae* as a single phylogenetic unit, and we will refer to them as lactobacilli throughout the text.

LAB have long been used for the transformation of raw foodstuffs into a variety of fermented products as their growth is associated with the acidification and production of antimicrobial substances that prevent the proliferation of pathogenic or spoilage organisms. Furthermore, the enzymatic processes associated to their growth contribute to the characteristic flavor and texture of these products. The continued use of LAB in these processes led to the adaptation of some strains (domestication) to specific food systems through repeated inoculation and selection. This process implied major genomic changes in food LAB strains, mainly loss of gene functions as a consequence of the adaptation to a nutrient-rich environment, but also gene gains associated to relevant technological properties (Douglas & Klaenhammer, 2010; Makarova et al., 2006). Other LAB, naturally

associated to mucosal surfaces of humans and animals, are also used as probiotics since they are attributed health benefits (Tannock, 2004). Lactobacilli, in contrast to streptococci, have been rarely associated to disease (Cannon, Lee, Bolanos, & Danziger, 2005; Kamboj, Vasquez, & Balada-Llasat, 2015), but there is increasing concern about their possible role as reservoirs of potentially transmissible antimicrobial resistance genes (Devirgiliis, Zinno, & Perozzi, 2013; Jaimee & Halami, 2016). This highlights the need to understand not only the antibiotic resistance mechanisms of pathogenic bacteria, but also those present in commensal bacteria that are usually recognized as GRAS/QPS organisms such as most lactobacilli.

Due to its involvement in food production and health, leuconostoc, and specially lactobacilli, have been the subject of intensive research. Despite this, the role of TCSs in their physiology has been somehow neglected and our current knowledge on these systems is rather limited compared to other LAB such as streptococci. The aim of this review is to summarize the available evidence on the physiological role of TCSs in *Lactobacillaceae* and *Leuconostocaceae* as they comprise most food-associated LAB as well as many commensal species associated to plants and animals.

2. NUMBER, DISTRIBUTION, AND CLASSIFICATION OF TCSs IN LACTOBACILLI

To our knowledge, only one study has dealt with the number and classification of TCSs in lactobacilli in an evolutionary setting using 19 genomic sequences of lactobacilli available at the time (Zúñiga, Gómez-Escoín, & González-Candelas, 2011). In this review, we have updated this previous work and used 98 complete genome sequences available at the Microbial Genome Database for Comparative Analysis (MBGD; http://mbgd.genome.ad.jp) (Uchiyama, Mihara, Nishide, & Chiba, 2015). The number of TCS-encoding genes ranges from 4 in *Lactobacillus sanfranciscensis* TMW 1.1304 to 36 in *Lactobacillus rhamnosus* GG (Table S1). The analysis of the 98 genomic sequences reveals a positive correlation between the genome size and the number of TCS-encoding genes (Pearce's correlation coefficient 0.82; $P < .001$; Fig. 2A) as observed for the Bacteria domain as a whole (Capra & Laub, 2012; Galperin, 2005).

It has been pointed out that the number of signal transducing proteins encoded by a strain correlates with the complexity of its lifestyle or the variety of habitats it can colonize (Alm et al., 2006; Capra & Laub, 2012; Galperin, 2005). Therefore, it might be expected that species that colonize

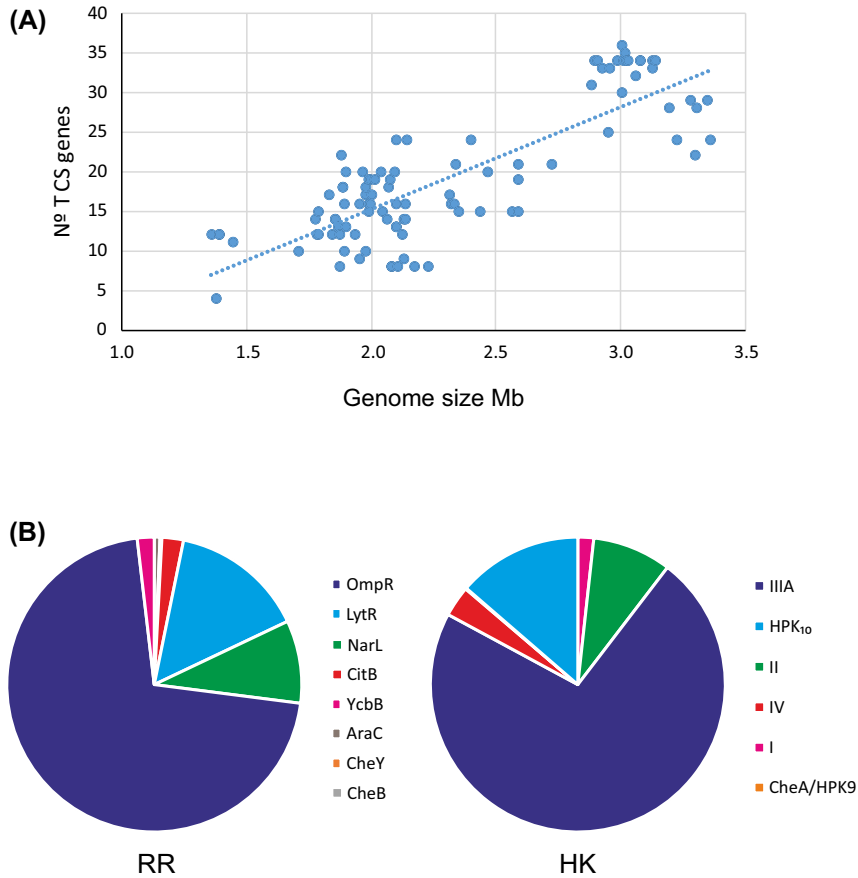

Figure 2 (A) Number of two-component systems (TCSs) genes versus genome size in the 98 lactobacilli strains analyzed. (B) Percentage of response regulator (RR) and histidine kinase (HK) genes belonging to different families encoded by lactobacilli.

specialized habitats would harbor less TCSs than those found in diverse habitats but this trend is not clearly set in lactobacilli. Species with large genomes and more TCSs, such as *Lactobacillus plantarum*, *Lactobacillus casei*, and *L. rhamnosus*, have been found in a variety of habitats, whereas species with small genomes and a low number of TCSs, such as *L. sanfranciscensis* or *Oenococcus oeni*, occupy specialized niches. However, examples of species with large genomes and TCS complements apparently occupying specialized niches as *Lactobacillus koreensis* can also be found. In contrast, other species with widespread distribution, such as *Lactobacillus fermentum* or *Leuconostoc mesenteroides*, possess relatively small genomes and TCS complements. This observation

may be partly explained by the particular evolutionary histories of different species of lactobacilli. For example, a recent study has suggested that the evolutionary history of *L. plantarum* is not related to environmental adaptation, proposing it as an example of nomadic bacterial species (Martino et al., 2016). Similarly, the evolution of *Lactobacillus paracasei* also seems partly unrelated to niche adaptation (Smokvina et al., 2013), whereas the analysis of the closely related species *L. rhamnosus* suggests that niche adaptation has played a relevant role in its evolution (Douillard et al., 2013). Other species of lactobacilli also possess genomic signatures that suggest that niche adaptation has played a major role in shaping their genomes. An example of this is *Lactobacillus reuteri* (Frese et al., 2011). This apparent lack of correlation between amplitude of distribution and number of TCSs in lactobacilli may also reflect the low metabolic diversity within this group and the similarities of their lifestyles. Notwithstanding, there is still limited information about the variety of habitats colonized by many of the recently described species of lactobacilli so that this scenario may change as new information will be available.

The conservation of common domains in HKs and RRs allows their classification on the basis of the similarities found in these domains. Here we have followed the classification scheme used in our previous study (Zúñiga et al., 2011). Briefly, we used Galperin's classification for RR (Galperin, 2006) and Fabret's for HKs (Fabret et al., 1999). Those HKs not fitting in Fabret's scheme were classified according to the classification of Grebe and Stock (HPK_9 and HPK_{10}; Table S1) (Grebe & Stock, 1999). The analysis of orthologous genes reveals a total of 29 different TCSs in lactobacilli (Table S1). There is a strong correlation in the association of families of HKs and RRs, for example OmpR RRs and IIIA HKs that is in accordance with the view of the evolution of TCSs as integral units (Grebe & Stock, 1999; Koretke et al., 2000; Zúñiga et al., 2011). However, two examples of possible nonorthologous displacements have been identified. The Pho RRs encoded by *Lactobacillus farciminis* and *Lactobacillus ginsenosidimutans* (Genbank Acc. N° AKP03461 and AKP68209, respectively) are not identified as orthologous of the other Pho RRs, and the HK of the Wal system encoded by *Lactobacillus ruminis* (Genbank Acc. N° AEN78916) is identified as orthologous of the 460 HKs instead of the Wal HKs (Table S1). Notwithstanding, a proper phylogenetic analysis would be required to confirm this point, and, in any case, the exceptionality of these cases highlights that coevolution of RR and HK partner genes has been prevalent in lactobacilli (Zúñiga et al., 2011). TCSs constituted

by OmpR class RR and IIIA class HK are by far the most abundant in lactobacilli (71.45% of the total TCS-encoding genes; Fig. 2B) and the only ones present in all strains. Most TCS genes are arranged in pairs of cognate RR and HK encoding genes although a few orphan genes can also be identified, but they accounted for a very small fraction (Table S1). Here we have considered as orphan RR or HK genes only those that are not found with a cognate partner in any lactobacilli.

The phylogenetic analysis of OmpR RRs and their cognate IIIA HKs showed that lineage-specific gene expansion by duplication did not occur in lactobacilli after the differentiation of this taxonomical group (Zúñiga et al., 2011). However, this trend may not apply to the Pln group (LytR family RR and HPK_{10} family HK; Table S1) where several paralogs of this system can be found in some strains, mostly those belonging to *L. plantarum* (Table S1). The main conclusion of that study was that lineage-specific gene loss had been the main trend in the evolution of the OmpR/IIIA TCSs in lactobacilli (Zúñiga et al., 2011) in concordance with the general trend of gene loss and genome decay that characterizes the evolution of the *Lactobacillales* (Makarova et al., 2006). Notwithstanding, a detailed analysis of the evolution of the other families of TCSs, particularly those including LytR RRs, would show whether the evolutionary scenario outlined for OmpR/IIIA TCSs can be generalized or if other evolutionary forces have also played a relevant role in the evolution of TCSs in lactobacilli.

3. PHYSIOLOGICAL ROLES OF TCSs IN LACTOBACILLI

TCSs regulate essential physiological processes in many bacteria, and evidence obtained so far indicates that this is also the case in lactobacilli. The first indication on a functional role of TCSs of lactobacilli came from studies of bacteriocin production by lactobacilli. The genetic analyses of bacteriocin gene clusters revealed the presence of TCS-encoding genes in these clusters (Axelsson & Holck, 1995; Diep, Håvarstein, Nissen-Meyer, & Nes, 1994; Hühne, Axelsson, Holck, & Kröckel, 1996). In *Lactobacillus sakei*, it was shown that the HK-encoding gene *sakB* (later named *sapK*) was required for production of sakacin A (Axelsson, Holck, Birkeland, Aukrust, & Blom, 1993) and *sppK* for sakacin P (Hühne et al., 1996). Subsequent studies provided evidence suggesting the involvement of TCSs in the regulation of key physiological processes through the phenotypic analysis of TCS-defective mutants (Alcántara, Revilla-Guarinos, &

Zúñiga, 2011; Morel-Deville, Fauvel, & Morel, 1998). These studies also suggested that there may be important differences in the regulatory networks controlled by homologous TCSs in different species of lactobacilli. For example, Morel-Deville observed that inactivation of the RR *rrp-31* (LSA0277; Cro orthologous group) led to a premature arrest of growth in reference conditions [de Man, Rogosa and Sharpe medium (MRS) at 30°C]; poor growth at high temperature (39°C); sensitivity to heat shock, aeration, and H_2O_2; and higher resistance to vancomycin, whereas it did not show significant differences with the wild-type strain at low pH (Morel-Deville et al., 1998). In contrast, the inactivation of the homologous RR01 (LCABL_02080) in *L. casei* BL23 resulted in normal growth at reference conditions (MRS at 37°C) and sensitivity to acid and vancomycin (Alcántara et al., 2011). The Cro orthologous group corresponds to one of the two TCSs that are present in all lactobacilli included in this study (Table S1) suggesting that it may have a relevant role in lactobacilli physiology.

There is also evidence suggesting that the regulatory networks controlled by some TCSs in lactobacilli may differ markedly from those of other related bacteria. Inactivation of *L. sakei rrp-3* (LSA0077) or the *L. casei* ortholog RR16 (LCABL_30130; Yyc orthologous group, Table S1) did not result in any remarkable phenotypic effect (Alcántara et al., 2011; Morel-Deville et al., 1998). This system is homologous to the YycFK/WalRK system of bacilli and staphylococci where it is essential for growth (Winkler & Hoch, 2008). Within *Lactobacillales*, inactivation of either the RR YycF or the HK YycK could not be accomplished in *Enterococcus faecalis* (Hancock & Perego, 2004), whereas in several streptococci the cognate HK VicK is dispensable (Biswas, Drake, Erkina, & Biswas, 2008; Wagner et al., 2002). Surprisingly, TCS belonging to the Yyc orthologous group constitute the second TCS conserved in all lactobacilli sequenced so far (Table S1). The absence of growth defects under the experimental conditions used for *L. casei* and *L. sakei* does not rule out that YycFG play a relevant role under particular growth conditions. In fact, functional studies have shown that YycFG regulates different sets of genes in different bacteria (Bisicchia et al., 2007; Dubrac, Boneca, Poupel, & Msadek, 2007; Mohedano et al., 2005) but so far, the role of this system in lactobacilli remains undetermined.

The systematic mutation of *L. casei* BL23 genes encoding RRs also revealed that inactivation of RR04 (LCABL_10480, Pho orthologous cluster), RR11 (*prcR*, LCABL_18980; Eta orthologous cluster), and RR12 led to growth defects in the reference conditions assayed in this study (Alcántara et al., 2011). RR04 and its cognate HK are orthologous to the

Bacillus subtilis system PhoR/PhoP involved in the phosphate-deficiency response (Hulett, 1996). Orthologous TCSs in lactobacilli are associated to a gene cluster encoding a putative phosphate transport system suggesting that this system is also involved in the regulation of phosphate metabolism. A defective mutant was also obtained in *L. sakei* (*rrp-2*, LSA500) that displayed premature arrest of growth at 39°C and higher resistance to H_2O_2, but it grew normally in reference conditions (Morel-Deville et al., 1998). Again, remarkable differences were observed with the *L. casei* BL23 mutant, which displayed lower growth rate that the parental strain in reference conditions. Unfortunately, the regulation of phosphate homeostasis in lactobacilli remains largely unknown. *L. casei* BL23 mutants RR11 and RR12 have been characterized with more detail revealing that indeed TCSs regulate key physiological processes in lactobacilli. They will be discussed in subsequent sections of this review.

In summary, evidence available indicates that TCSs may control key physiological processes in lactobacilli but only a few TCSs have been characterized with some detail so far. In the following sections, the current knowledge on these characterized systems will be reviewed.

3.1 TCSs Involved in the Stress Responses of Lactobacilli

As noted previously, the phenotypic analysis of TCS-defective mutants also provided the first clues of the involvement of TCSs in the regulation of stress responses in lactobacilli. Morel-Deville et al. (1998) inactivated five RR in *L. sakei* and found that four of these mutants displayed altered responses toward different stress conditions. Later studies on *Lactobacillus acidophilus* identified a TCS whose inactivation led to diminished acid tolerance and poor acidification rate in milk (Azcárate-Peril et al., 2005) and another one involved in bile tolerance (Pfeiler, Azcárate-Peril, & Klaenhammer, 2007). In *Lactobacillus delbrueckii* subsp. *bulgaricus* CH3, a number of TCS genes were shown to be induced during acid adaptation (Cui et al., 2012). Inactivation of the RR of one of these systems (belonging to the Pln orthologous group; Table S1) resulted in increased acid sensitivity although inactivation of the cognate HK did not (Cui et al., 2012). Finally, a systematic inactivation of all RR encoded by *L. casei* BL23 showed that inactivation of 3 out of 17 RR led to major growth defects under different stress conditions (Alcántara et al., 2011).

These studies in most cases have not clearly established whether the observed growth defects correspond to impaired responses to stress conditions or to altered physiological conditions that result in diminished

ability to respond to stress. As an example of this problem in the interpretation of mutant phenotypic analyses, inactivation of the RR from TCS12 in *L. casei* BL23 led to growth defects under reference conditions and in presence of bile, high temperature and acidic pH (Alcántara et al., 2011). Subsequent analyses showed that a functional TCS12 is required for constitutive high expression of the *dlt* operon among other genes (see further text in the section 'TCS Involved in Resistance to Antimicrobial Peptides') and that the growth defects observed in the RR12 defective mutant were mostly due to decreased expression of the Dlt system (Revilla-Guarinos et al., 2013), responsible for the D-alanylation of the cell wall lipoteichoic acids. Subsequent analyses showed that both the parental strain and the RR12 defective strain induced a growth phase−dependent acid tolerance response thus suggesting that TCS12 was not responding specifically to acid stress (Revilla-Guarinos, Alcántara, Rozès, Voigt, & Zúñiga, 2014). The diminished resistance to acid stress of the RR12-defective strain was attributed to increased permeability of the envelope due to decreased D-alanylation of teichoic acids in this mutant and not to a defective acid stress response (Revilla-Guarinos, Alcántara, et al., 2014). However, in a few examples the control by TCSs on the response to specific stressors has been established. These examples will be discussed in the following sections.

3.2 A TCS Involved in Bile Resistance

A global gene expression study of the response to bile stress of *L. acidophilus* NCFM revealed an operon (LBA1425−LBA1432) significantly induced in the presence of bile (Pfeiler et al., 2007), which contained a TCS consisting of a IIIA HK and an OmpR RR (LBA1430 and LBA1431, respectively; Bil orthologous cluster; Table S1). The other genes of the operon encode for a hypothetical protein with an alpha/beta hydrolase fold (LBA1425), a putative oxidoreductase (LBA1427), a putative OsmC homolog of unknown function (LBA1428), a putative transporter of the major facilitator superfamily (LBA1429), a hypothetical protein with a nucleotidyltransferase domain (LBA1432), and a hypothetical protein with no recognized conserved domain (LBA1426). Inactivation of the RR led to increased induction of the operon in the presence of bile suggesting that the RR acts as a repressor. In contrast, the inactivation of the cognate HK had no effect on the induction of the operon in the presence of bile (Pfeiler et al., 2007). Both mutants displayed reduced survival in oxgall or glycodeoxycholic acid but, whereas the RR-defective mutant was very sensitive to taurodeoxycholic acid, the HK-defective mutant was not

sensitive to this compound. These results suggested that, in addition to its cognate HK, other proteins or compounds can phosphorylate the RR in response to bile (Pfeiler et al., 2007). The analysis of mutants defective in other genes of the operon showed their involvement in bile resistance. Inactivation of genes LBA1429 and LBA1432 resulted in decreased survival in the presence of bile, whereas inactivation of LBA1427 and LBA1428 resulted in increased survival (Pfeiler et al., 2007). Furthermore, gene LBA1425 is homologous to *Lactobacillus salivarius* LSL_1464 whose expression is induced in the presence of bile (Fang et al., 2009) although its function remains undetermined in both organisms.

A survey of genomic sequences using the MBGD tools reveals that this TCS is only present in a limited number of *Lactobacillus* species (Table S1) and the complete operon is only present in *L. acidophilus*, *Lactobacillus amylovorus*, *Lactobacillus crispatus*, *Lactobacillus helveticus*, and *Lactobacillus kefiranofaciens* (Fig. 3). In many cases, the TCS-encoding genes are defective (Table S1 and Fig. 3) suggesting that their regulatory functions are no longer required. In some cases, this loss may be related to the adaptation process to specific niches where bile resistance is no longer required. This might explain the loss of functionality in *L. helveticus* that is restricted to dairy environments but it does not explain the loss in other gut strains such as *L. acidophilus* 30SC or *L. amylovorus*. Unfortunately, information about the role of this TCS in other lactobacilli is lacking.

3.3 TCSs Involved in Resistance to Antimicrobial Peptides

Most living creatures produce antimicrobial peptides (AMPs) and they are seen as an alternative to classic antibiotics due to their broad spectrum of activity, different mechanisms of action, lower toxicity, and scarcity of resistance mechanisms in pathogenic organisms (Brogden, 2005; Hancock & Sahl, 2006; Shah, Hsiao, Ho, & Chen, 2016). Natural AMPs have been the focus of an intense research and have served as models for the development of new synthetic antimicrobials (Fjell, Hiss, Hancock, & Schneider, 2012; Lam et al., 2016). The mechanisms of resistance against AMPs have also been extensively studied (for reviews see Draper, Cotter, Hill, & Ross, 2015; Koprivnjak & Peschel, 2011; Revilla-Guarinos, Gebhard, Mascher, & Zúñiga, 2014). AMPs are usually amphipathic molecules composed of 10–50 amino acids with a net positive charge. AMPs can be divided into nonribosomally synthesized peptides and ribosomally synthesized peptides. The first are produced by bacteria and fungi and are often drastically modified. Ribosomally synthetized peptides are produced by

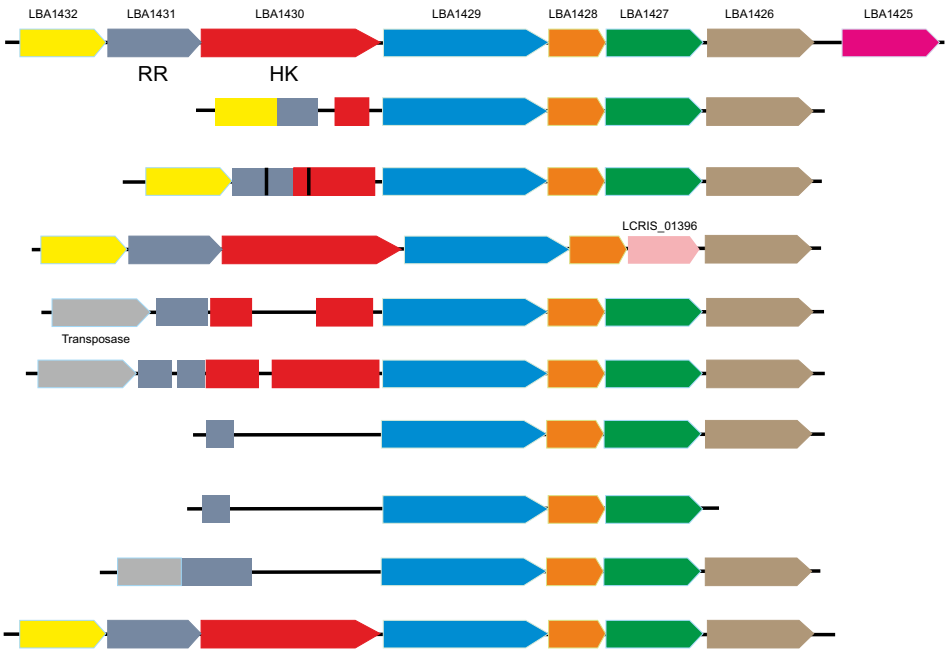

Figure 3 Schematic representation of the genetic organization of clusters homologous to the bile-inducible operon of *Lactobacillus acidophilus* NCFM. Fill-ins indicate homology. Rectangles indicate truncated genes. Vertical black lines indicate translational frameshifts. *HK*, histidine kinase; *RR*, response regulator.

species belonging to all domains of life (Hancock & Chapple, 1999). Examples of the former are bacitracin, gramicidin S, and polymyxin B; examples of the later are mammalian peptides present in the gastrointestinal tract—like defensins (α and β) and cathelicidins, and bacteriocins produced mainly by *Firmicutes*.

AMPs have varied modes of action but the main target of most AMPs is the cell membrane. Notwithstanding, other cellular targets, such as the cell wall or intracellular proteins or nucleic acids, can also be affected by some AMPs (Brogden, 2005; Omardien, Brul, & Zaat, 2016; Shah et al., 2016). The cationic and amphiphilic nature of most AMPs facilitates their interaction with the microbial cell surfaces that usually contain acidic polymers such as teichoic acids. Subsequently, they can interact and insert into the cytoplasmic membrane and, eventually, the membrane can be permeabilized or disrupted. Consequently, protective mechanisms elicited by bacteria often involve changing the properties of the bacterial cell envelope making it less accessible to AMPs and thus reducing their access to the surface targets. In many *Firmicutes*, expression of the Dlt system modulates the negative charge density on teichoic acids by the addition of D-alanyl substituents (Neuhaus & Baddiley, 2003; Neuhaus, Heaton, Debabov, & Zhang, 1996). On the other hand, expression of the MprF (multiple peptide resistance factor) protein modifies anionic phospholipids with L-lysine or L-alanine thereby introducing positive charges into the membrane surface (Ernst & Peschel, 2011; Peschel et al., 2001).

Another relevant mechanism of AMP resistance is mediated by ABC transporters that actively remove the peptides from their sites of action (Bolhuis et al., 1996; Gebhard, 2012; Gebhard & Mascher, 2011; Ohki et al., 2003). Although their mechanism of action is not clearly resolved, it has been hypothesized that some of these transporters can take up AMPs directly from the membrane and release them to the extracellular medium (Bolhuis et al., 1996; Okuda, Aso, Nakayama, & Sonomoto, 2008; Revilla-Guarinos, Gebhard, et al., 2014; Stein, Heinzmann, Solovieva, & Entian, 2003). Some of these transporters, namely BceAB-type, are genetically and functionally associated to TCSs. The BceAB-type transporters are named after BceAB of *B. subtilis*, which confers resistance to bacitracin (Mascher, Margulis, Wang, Ye, & Helmann, 2003; Ohki et al., 2003). A remarkable characteristic of BceAB is that works as a detoxification system and as a sensor, relaying the signal to its cognate HK BceS, which in turn functions as a signal transfer relay to the RR BceR (Rietkötter, Hoyer, & Mascher, 2008). In fact, BceS-like HKs lack of

sensory domains and are not able to directly sense the stimulus (Mascher, 2006, 2014). The mechanism of resistance is as follows: the ABC transporter BceAB detects the stimulus (AMP) and transfers the signal to BceS, which subsequently activates BceR. Activation of BceR then induces the expression of *bceAB*. A recent study has shown that the system responds to changes in activity of the transporters (flux-sensing) rather than to changes in AMP concentration (Fritz et al., 2015). In addition to BceRSAB, homologous systems have been shown to be involved in AMP resistance in *Streptococcus mutans* (Ouyang, Tian, Versey, Wishart, & Li, 2010), *Staphylococcus aureus* (Falord, Karimova, Hiron, & Msadekss, 2012; Hiron, Falord, Valle, Debarbouille, & Msadek, 2011), *Listeria monocytogenes* (Collins, Curtis, Cotter, Hill, & Ross, 2010), and *E. faecalis* (Gebhard et al., 2014), among others. Phylogenetic analyses have shown that BceRS-like TCSs and associated BceAB-like ABC transporters have coevolved underlining the mutual functional dependence between TCSs and cognate ABC transporters (Dintner et al., 2011). Some BceAB-like transporters appear to have developed specific functions. While some display the dual role described earlier, others function only as a sensor or only as a resistance pump (Revilla-Guarinos, Gebhard, et al., 2014).

There is scarce information on the involvement of TCSs in the AMP stress response in lactobacilli. Again, the phenotypic analyses of defective mutants provided the first clues (Alcántara et al., 2011; Morel-Deville et al., 1998). The response of insertional inactivated RR mutants in each of the TCSs present in *L. casei* BL23 against antibiotics (bacitracin, nisin, gramicidin, and vancomycin) was studied (Alcántara et al., 2011). None of the mutants displayed differential sensitivity to gramicidin, but inactivation of RR01, RR04, RR06, RR09, RR10, RR11, RR12, RR15, RR16, RR17 led to differences in sensitivity to the other AMPs relative to the wild type strain (Alcántara et al., 2011). As discussed earlier, it was not determined if the sensitivity phenotypes were due to direct involvement of these TCSs in the antibiotic stress response or to other physiological differences that result in diminished ability to respond to AMP stress. Furthermore, apparently conflicting results suggest that some of these systems may not really be involved in resistance against AMPs. For example, inactivation of RR01 in *L. casei* BL23 led to higher sensitivity to bacitracin, vancomycin, and nisin, whereas the inactivation of the homologous system Rrp-31 (*rrp-31*, LSA0277) in *L. sakei* conferred resistance to vancomycin and teicoplanin (Morel-Deville et al., 1998). Notwithstanding, two of these

RR, RR09 and RR12 were homologous to the *B. subtilis* BceR RR and were further investigated (Revilla-Guarinos et al., 2013).

TCS09 and the paralogous TCS12 are located next to genes encoding ABC09 and ABC12, respectively, which are BceAB-like ABC transporters (Fig. 4). Furthermore, *L. casei* BL23 encodes an additional BceAB-like ABC transporter termed OrABC (Revilla-Guarinos et al., 2013). Inactivation of either RR09 or permease 09 led to higher sensitivity to bacitracin, nisin, plectasin, and subtilin than that observed in the wild-type strain, whereas inactivation of either RR12 or permease 12 resulted in higher sensitivity to bacitracin, nisin, mersacidin, plectasin, subtilin, and vancomycin (Revilla-Guarinos et al., 2013). BceR-like RR recognizes conserved DNA motifs (Dintner et al., 2011); this characteristic was exploited to identify genes under control of module 09 and module 12. The analysis revealed that the promoter regions of genes encoding ABC09, OrABC, MprF, and the *dlt* operon contained putative BceR binding sites (Revilla-Guarinos et al., 2013) (Fig. 4). Subsequent transcriptional analyses showed that TCS09 regulated the expression of ABC09 and TCS12 regulated the expression of OrABC, MprF, and the *dlt* operon. Furthermore, it was shown that inactivation of the RRs or their respective cognate ABC permeases resulted in identical phenotypes and changes in transcript levels, in

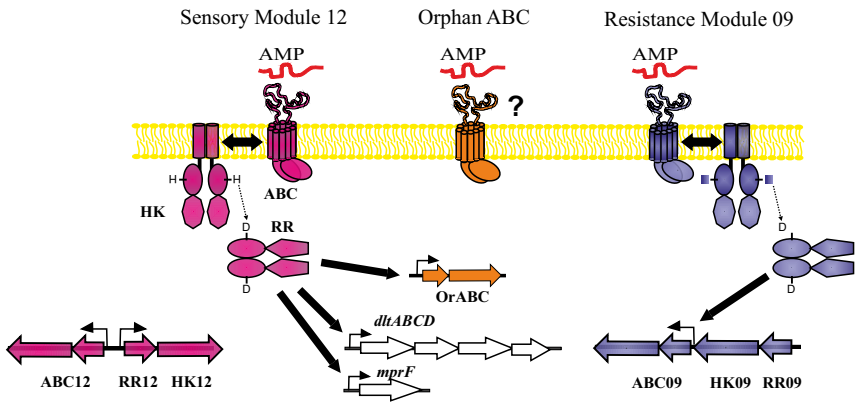

Figure 4 Schematic representation of the signaling network of BceRS/BceAB-like sensory and resistance modules of *Lactobacillus casei* BL23. A generic antimicrobial peptide (AMP) is shown as a substrate interacting with the permeases. Signal transfer between TCS09 and ABC09 and between TCS12 and ABC12 is indicated in the membrane bilayer. Putative phosphotransfer between the histidine kinases (HKs) and response regulators (RRs) is indicated by *black dotted arrows*. Transcriptional activation is indicated by *black arrows*.

agreement with the model outlined earlier where ABC transporters work as signal relays for their cognate HKs. The transcriptional analyses also showed that the module 09 functions as a sensor and resistance system since it was activated in response to nisin, leading to the induction of the expression of ABC09, which conferred resistance to it (Revilla-Guarinos et al., 2013). On the other hand, ABC12 is a sensing transporter that does not confer the actual AMP resistance. The AMP resistance is achieved, mainly, by the regulation of the expression of the Dlt system. In fact, most phenotypic effects of the inactivation of RR12, as the previously mentioned acid sensitivity of ΔRR12 (Revilla-Guarinos, Alcántara, et al., 2014), could be attributed to low expression of the *dlt* operon in this mutant strain (Revilla-Guarinos et al., 2013). In *L. sakei*, inactivation of the RR12 homolog Rrp-1 (*rrp-1*, LSA1454) proved it to be required for the acid tolerance but not for resistance to vancomycin (Morel-Deville et al., 1998). This system presents the same operon organization as Module 12 from *L. casei* BL23. Further studies would be needed to determine if the acid sensitivity is due to the regulation of the surface properties in *L. sakei*, similarly to BL23.

3.4 The Role of TCSs in Quorum Sensing in Lactobacilli

Quorum sensing (QS) signaling comprises mechanisms by which bacteria are able to measure its population density and trigger adequate (transcriptional) responses in a coordinated manner when the population reaches a certain threshold (Fuqua, Winans, & Greenberg, 1994). QS constitutes a kind of intercellular communication mechanism, which is mediated by a variety of secreted signal molecules. These molecules are generally produced at a low level and accumulated in the medium. At a certain concentration, which depends on the reached cell density, they lead to the expression of specific regulons. In Gram-negative bacteria, cellular communication is mediated by the production of N-acyl-homoserine lactones or other molecules derived from S-adenosylmethionine, which act as signals regulating diverse processes in virulence, antibiotic production, conjugation, and bioluminescence (Papenfort & Bassler, 2016). In Gram-positive microorganisms, many processes related to bacterial virulence, competence, and production of AMPs (bacteriocins) are controlled by QS mechanisms that are based on TCS signaling. In this case the signal molecules are generally short peptides (14–27 amino acids, pheromone peptides) that can be post-translationally modified and secreted (Kleerebezem, Quadri, Kuipers, & De Vos, 1997; Nes et al., 1996; Thoendel & Horswill, 2010).

3.5 Regulation of Bacteriocin Production in Lactobacilli

Bacteriocins are ribosomally synthesized AMPs, which generally possess a narrow spectrum of susceptible microorganisms. Many bacteriocins are only active against bacteria phylogenetically related to the producer strain. This suggests that their synthesis mainly aims to eliminate closely related microorganisms that are likely competitors for the same resources in a given ecological niche (Nes et al., 1996). Bacteriocins can be divided into several classes (I, IIa, IIb, IIc, IId, III, and IV) on the basis of their structural characteristics, being bacteriocins of the class II the most common in lactobacilli (Hassan, Kjos, Nes, Diep, & Lotfipour, 2012). It has been reported that synthesis of many class II bacteriocins from this microbial group is transcriptionally regulated by a mechanism of QS, where a bacteriocin-like peptide or the bacteriocin itself acts as the induction factor (Nes et al., 1996). However, for other well-known class II bacteriocins, such as pediocin PA-1, no evidence exists for the activation of bacteriocin gene expression via a QS mechanism (Rodríguez, Martínez, & Kok, 2002).

QS regulation of bacteriocin synthesis is usually mediated by small modified or unmodified peptides that are produced as pro-peptides that exhibit an N-terminal leader secretion signal characterized by the presence of two glycine residues (Fig. 5) (Kleerebezem et al., 1997; Nes et al., 1996). Secretion and hydrolysis of the N-terminal sequence at the level of the Gly−Gly sequence takes place by specialized transporters belonging to the ABC family of transmembrane carriers that carry C39 peptidase domains. Nisin production by *Lactococcus lactis* has become the prototypical example of QS-regulated AMP production system in LAB (Mierau & Kleerebezem, 2005). Nisin is a 34−amino acids peptide belonging to class I bacteriocins, also called lantibiotics, which consist of peptides carrying different posttranslational modifications, including the presence of the amino acid lanthionine. Nisin-producing strains carry a gene cluster that contains the structural gene for nisin (*nisA*), a TCS (*nisKR*), and other genes involved in nisin modification, export, and immunity (Siegers & Entian, 1995). In this system, nisin itself acts as the inducer peptide for NisKR, which activates the induction of the whole *nis* cluster, including *nisA* (Kuipers, Beerthuyzen, de Ruyter, Luesink, & de Vos, 1995). NisR belongs to the OmpR family, and the HK NisK belongs to the IIIA family.

The TCSs regulating the production of class II bacteriocins share distinct features compared to the TCSs controlling nisin production in lactococci. The involved RRs belong to the LytTR family, which carry an N-terminal

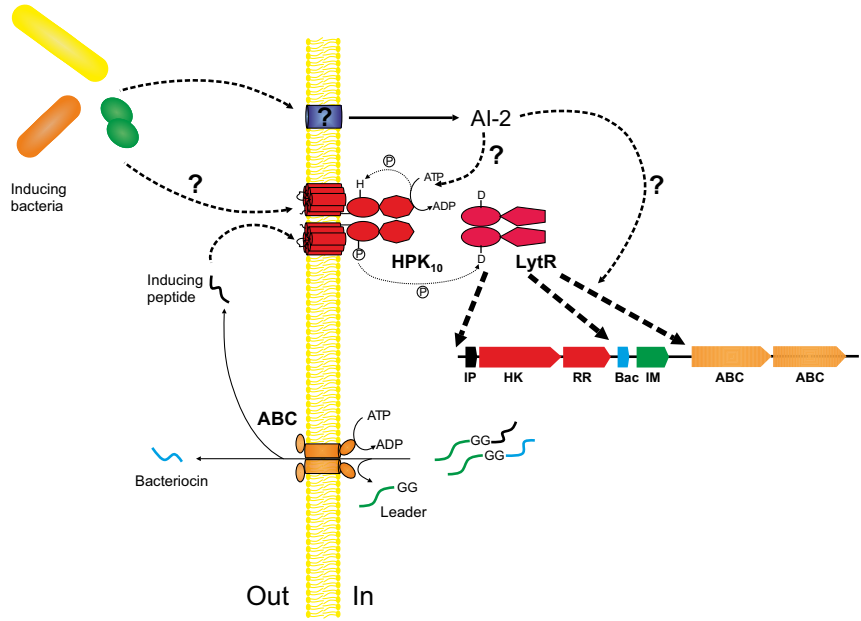

Figure 5 Schematic representation of the regulatory network for class II bacteriocins synthesis. *Thick dashed arrows* indicate gene expression activation; *thin dashed arrows* indicate signal transfer interactions; *dotted lines* indicate phosphotransfer reactions; and *solid lines* indicate hydrolysis reactions and peptide export. *Question marks* indicate the unknown processes by which certain bacteria in coculture with a Bac + strain enhance production of the inducer peptide (IP), where increased synthesis of the AI-2 molecule has been proposed to play a role. A simplified bacteriocin gene cluster is depicted showing the two-component systems mediating quorum sensing, the bacteriocin structural (Bac), and immunity (IM) genes and the ABC transporter responsible for secretion of the IP/bacteriocin and leader sequence (double-glycine type) cleavage. *RR*, response regulator; *HK*, histidine kinase.

DNA-binding domain different to the classical helix-turn-helix structure carried by other RRs (Nikolskaya & Galperin, 2002), and their cognate HKs belong to the HPK_{10} family of sensor kinases. This HK family is almost exclusively found in TCS-mediating QS. HPK_{10} family has many different features compared to other HKs, which include the presence of five to eight predicted transmembrane helices in the N-terminal sensor domain. This differs from the two typical transmembrane helices located at both sides of an extracytoplasmatic loop, which is found in the rest of HKs (Wolanin, Thomason, & Stock, 2002). Furthermore, they have specific sequence signatures in the conserved DHp and CA domains that may help in

identifying other TCSs involved in QS present in bacterial genomes (Sturme, Francke, Siezen, de Vos, & Kleerebezem, 2007).

3.6 Class II Bacteriocin Clusters Regulated by Quorum Sensing in Lactobacilli

Evidence for QS regulation of bacteriocin production in lactobacilli first came from studies on plantaricin A, sakacin A, and sakacin P. The peptide plantaricin A was isolated from culture supernatants of *L. plantarum* C11 producing antibacterial compounds (Nissen-Meyer, Larsen, Sletten, Daeschel, & Nes, 1993). It is a cationic peptide with a bacteriocin-like structure that induces bacteriocin synthesis in *L. plantarum* C11 at a very low concentration but has very low bacteriocin activity (Diep, Håvarstein, & Nes, 1995; Hauge et al., 1998). The *plnA* gene is part of the *pln* regulon of *L. plantarum* C11, which consists of five different operons adjacently located in the chromosome (Diep, Håvarstein, & Nes, 1996; Diep, Straume, Kjos, Torres, & Nes, 2009). Two of the operons (*plnEFI* and *plnJKRH*) code for two different two-peptide bacteriocins, plantaricins EF and JK, that provide the antibacterial activity and their corresponding immunity proteins (Anderssen, Diep, Nes, Eijsink, & Nissen-Meyer, 1998). The *plnGHSTUVW* operon includes genes responsible for bacteriocin extracellular export (*plnGH*) and other unknown functions related to bacteriocin synthesis. The *plnMNOP* operon includes genes encoding a third single peptide bacteriocin (*plnN*) together with putative immunity proteins (encoded by *plnM* and *plnP*). Finally, the *plnABCD* has the structural gene for the inducing peptide (Plantaricin A, *plnA*) and a TCS involved in signaling. Similar *pln* gene clusters are present in other *L. plantarum* strains such as WCFS1 (Kleerebezem et al., 2003), V90 (Diep et al., 2009), NC8 (Maldonado, Ruiz-Barba, & Jiménez-Díaz, 2003), J23 (Rojo-Bezares et al., 2008), J51 (Navarro et al., 2008), and I-UL4 (Tai et al., 2015). The cluster spans a chromosomal region of almost 20 kb in length, and it displays a mosaic structure, where the composition of the different *pln* operons within this region may vary in terms of gene order and content (Diep et al., 2009).

TCS associated to *pln* clusters shares similar characteristics to the rest of systems involved in QS-regulated expression in other Gram-positive bacteria, but some of them have the peculiarity of encoding two different RRs, which display high mutual sequence similarity, in the same operon (PlnC and PlnD). The fact that artificial *plnC* overexpression resulted in an activation of the *pln* genes, whereas *plnD* overexpression resulted in

reduced expression, suggests that one RR acts as a transcriptional repressor, whereas the other acts as an activator (Diep, Johnsborg, Risøen, & Nes, 2001). Some *L. plantarum* strains, e.g., NC8 and J23, only carry a *plnD* homologue, whereas both RR are present in the rest of strains. Binding of PlnC and PlnD to the promoters of the different *pln* operons has been characterized showing that they bind to a conserved motif consisting of two direct repeats of the consensus sequence 5′-TACGTTAAT separated by 12 bp and located upstream of the −10 and −35 *pln* promoter sequences (Risøen, Håvarstein, Diep, & Nes, 1998). This disposition implies that both sites face the same side of the DNA, which may have implications in RR binding through a cooperative process. In fact, the study of PlnC and PlnD binding kinetics revealed that while PlnC strongly and cooperatively binds to the *plnABCD* promoter activating its transcription, it does not bind with high affinity to other *pln* promoters, while the opposite is true for PlnD (Straume, Johansen, Bjørås, Nes, & Diep, 2009). Gel-shift and surface plasmon resonance experiments showed that both PlnC and PlnD bind first to the right repeated sequence present in the promoters, whereas a cooperative binding is found for the left repeat, which is generally less conserved (Risøen et al., 1998; Straume et al., 2009). The differential binding of PlnC and PlnD to the *plnABCD* operon results in an early and strong autoactivation of the regulatory elements of the *pln* regulon and the operon, which codes for bacteriocin secretion elements (*plnGHSTUV*). However, the interplay of PlnC and PlnD to control coordinately the level of expression of *pln* genes at different times and with different strength remains to be completely elucidated.

The effect of phosphorylation on PlnC and PlnD activity has also been explored. Experiments with mutant proteins in the phosphorylatable Asp residues of PlnC and PlnD suggest that binding of both regulators is enhanced by phosphorylation (Diep, Myhre, Johnsborg, Aakra, & Nes, 2003), which presumably is mediated in vivo via the PlnB HK. However, how this phosphorylation takes place and whether PlnC and PlnD are differentially phosphorylated is not known. Topology analysis using alkaline phosphatase, β-lactamase, and β-galactosidase fusions determined that PlnB has seven transmembrane domains and three extracytoplasmatic loops (Johnsborg, Diep, & Nes, 2003). By site-directed mutagenesis, critical residues for PlnA recognition have been elucidated. Most mutations that result in an impaired PlnA recognition and activation of the cytoplasmic kinase domain of PlnB (substitutions of D54, S58, and L61) map to a specific region in the most N-terminal extracellular loop, which seems therefore implicated in PlnA

recognition and activation of the transmembrane signal transduction, as Ala and Val substitutions in these residues resulted in reduced affinity to PlnA or increased basal kinase activity (Johnsborg, Godager, & Nes, 2004). Interestingly, the equivalent region in the homologous HK ComD of *Streptococcus pneumoniae* shows differences among strains (ComD-1 and ComD-2 variants) that differ in their specificity for the peptide pheromones that modulate competence development, supporting the idea that this region is crucial for the determination of receptor specificity (Johnsborg et al., 2004).

Investigations in other lactobacilli have revealed that the production of many other class II bacteriocins is regulated by very similar QS mechanisms to that of plantaricins. These include, among others, gassericin E production by *Lactobacillus gasseri* (Maldonado-Barragán, Caballero-Guerrero, Martín, Ruiz-Barba, & Rodríguez, 2016), lactacin B production by *L. acidophilus* (Dobson, Sanozky-Dawes, & Klaenhammer, 2007), or sakacin production by *L. sakei* (Axelsson & Holck, 1995; Axelsson et al., 1993; Diep, Axelsson, Grefsli, & Nes, 2000; Hühne et al., 1996).

Sakacin A production is mediated by the three components usually involved in pheromone-mediated QS: *orf4*, encoding a 23—amino acids cationic peptide whose deletion abolishes sakacin A production and *sapKR*, encoding the constituents of a TCS (Axelsson & Holck, 1995; Diep et al., 2000). The genetic determinants for the production of sakacin P consist of seven genes (*spp*) encoding all the components necessary for class II bacteriocin production regulated by QS (Hühne et al., 1996). A comparative genomic analysis showed that *spp* genes are found in many sakacin P-negative strains (e.g., strains 23K and Lb790) and that the operons present a mosaic structure in the different strains product of diverse genomic rearrangements (Møretrø et al., 2005). The inability to produce sakacin P is due to frameshift mutations and integrations of insertion elements in the *sppK* gene, encoding de HK. Deletion and frameshift analysis of *spp* genes expressed in a nonproducer *L. sakei* host allowed confirming that *sppK* is necessary for sakacin P production and immunity, whereas deletion of the RR *sppR* did not prevent bacteriocin production (Hühne et al., 1996). This apparently contradictory observation may be explained by complementation via another chromosomal analogue of *sppR* present in the nonproducer host used in this study (Hühne et al., 1996). SppK mutants have been obtained that displayed constitutive activation of the *spp* promoter. Most of the mutations clustered to a region predicted to form a coiled-coil structure, which contains the phosphorylation site and also lies in the interface between HK dimers and a preceding linker region in

SppK (Mathiesen, Axelsen, Axelsson, & Eijsink, 2006). Changes in this region resulted in a constitutive activating phenotype in HKs from different families, suggesting that this region, despite the distinct features of the HPK_{10} family compared to the rest of HK, is crucial for its functionality. Interestingly, some of the identified amino acid changes (namely, N235Y, Q237R, L238P, and D240G) mapped to similar positions identified in ComD mutants of *S. pneumoniae* showing constitutive activation of this HK, further stressing the importance of this region in signal transduction in the HPK_{10} family (Mathiesen et al., 2006).

In *L. gasseri* EV1461 and other gassericin producing strains, such as K7 and LA158, the *gae* regulon consists of three operons, which encompass regulatory genes (autoinducer peptide and a TCS), secretion, and immunity in addition to the gassericin structural genes (Maldonado-Barragán et al., 2016). Similar to *pln* promoters, the three *gae* identified promoters contain two imperfect 10-bp repeats separated by 9 or 10 bp, which are the likely target of the RR GaeR (Maldonado-Barragán et al., 2016).

3.7 Bacteriocin Production Mediated by Quorum Sensing also Responds to the Presence of Other Bacteria in Co-Culture

A number of studies on bacteriocin synthesis have shown that, for some strains, the bacteriogenic activity is only detected in solid medium, when colonies of a bacteriocin producing (Bac +) strain grows on a layer of indicator (sensitive) bacteria or when plates with colonies of a Bac + strain are covered by an agar overlay containing indicator bacteria. On the contrary, liquid cultures often do not render any antimicrobial activity in supernatants (Barefoot et al., 1994; Cintas et al., 1995; Maldonado-Barragán, Ruiz-Barba, & Jiménez-Díaz, 2009; Maldonado, Ruiz-Barba, & Jiménez-Díaz, 2004). Some Bac + lactobacilli can be turned into nonproducing (Bac −) strains when they are inoculated at very low cellular densities (Diep et al., 1995; Eijsink, Brurberg, Middelhoven, & Nes, 1996). These strains can recover the antimicrobial production capacity when they are inoculated from plates at high cellular densities, when supernatants of a Bac + variant (containing the inducer peptides) or the purified inducer peptide are added to the growth medium or when the strains are cocultured with specific bacteria (Maldonado-Barragán et al., 2016; Maldonado, Ruiz-Barba, et al., 2004; Tabasco, García-Cayuela, Peláez, & Requena, 2009).

The effect of cell density on induction of bacteriocin production is not fully elucidated. Bac-derivative strains of Bac + strains, obtained by inoculating at low density in liquid medium, cannot restore the Bac + phenotype

even if they are inoculated at initial high densities, and reversion to the Bac + status can only be achieved if the inducer peptide is added to the liquid growth medium or the cells are grown on agar plates. It has been postulated that growth on solid surfaces (such as agar medium in Petri dishes) resembles the natural growth of bacteria, which in natural environments generally develop in biofilms on surfaces, where they behave differently to planktonic cells (Maldonado-Barragán et al., 2009). These physiological conditions may affect expression of genes related to bacteriocin induction. On the other hand, the limited diffusion of the inducer peptide in a colony grown on solid medium may result in a higher local concentration of the inducer peptide, which cannot be achieved in liquid medium, thus accounting for the different behavior in liquid and solid medium (Saucier, Poon, & Stiles, 1995).

Presence of specific bacteria may also serve as an activating environmental stimulus (Fig. 5; for a review see (Chanos & Mygind, 2016)). In this sense, QS systems regulating class II bacteriocins are somehow able to sense both the population of the producing bacteria (inducer peptides) and that of other possible bacterial competitors. Many Gram-positive bacteria from different genera have been reported to allow or enhance bacteriocin production in liquid coculture, although their inducing ability is not related to their sensitivity toward a given bacteriocin (Maldonado, Ruiz-Barba, et al., 2004; Rojo-Bezares et al., 2008). It has been reported that live bacteria are required for induction (Barefoot et al., 1994; Tabasco et al., 2009), but heat-killed bacteria have also been shown to confer an inducing activity, which is heat resistant and of proteinaceous nature (Maldonado, Ruiz-Barba, et al., 2004). Induction by specific bacteria seems to be widespread among class II bacteriocin producing lactobacilli as it has been reported for Bac + strains of *L. plantarum* (Maldonado-Barragán, Caballero-Guerrero, Lucena-Padrós, & Ruiz-Barba, 2013; Man, Meng, & Zhao, 2012; Rojo-Bezares et al., 2008), *L. acidophilus* (Tabasco et al., 2009), *L. helveticus* (Kos et al., 2011), and *L. gasseri* (Maldonado-Barragán et al., 2016). The presence of inducing bacteria seems to enhance the production of the endogenous inducer peptide (Maldonado, Jiménez-Díaz, & Ruiz-Barba, 2004). Furthermore, it has been observed that coculture induced the expression of the lactacin B structural gene *lbaB* to a higher level than the rest of *lba* genes in *L. acidophilus*, suggesting that an additional regulatory circuit exclusive for expression of the bacteriocin is triggered by the coculture. This is in agreement with the presence of a putative promoter specific for *lbaB* in this strain (Tabasco et al., 2009).

The nature of the inducer factor produced by the bacteria in coculture with the Bac + strain still remains elusive. For example, different *L. plantarum* strains encoding different HK and inducer peptides respond to the same strains in coculture (Maldonado-Barragán et al., 2013). Autoinducer-2 (AI-2), a by-product of the catabolism of S-ribosylhomocysteine synthesized by the action of the product of the *luxS* gene, has been proposed as a signaling molecule mediating induction of bacteriocin production in coculture of Bac + strains with inducer bacteria (Fig. 5). Increased expression of *luxS* has been observed in *L. plantarum* cocultured with *L. sanfranciscensis* or *Lactobacillus rossiae* (Di Cagno, De Angelis, Coda, Minervini, & Gobbetti, 2009), and a correlation between AI-2 activity and bacteriocin production has been observed in *L. plantarum* KLDS1.0391 in coculture with *L. helveticus* KLDS1.9207 (Man, Meng, Zhao, & Xiang, 2014). Notwithstanding, a direct role of AI-2 in bacteriocin production has yet to be established.

3.8 Other Processes Regulated by Quorum Sensing in Lactobacilli

QS regulation in lactobacilli is not restricted to the control of class II bacteriocin expression. In *L. plantarum* two QS mechanisms mediated by HPK_{10}/LytR families TCS have been discovered that are involved in the regulation of the properties of the cell surface that modulate adherence and biofilm formation. The *L. plantarum* WCFS1 *lamBDCA* operon codes for a TCS (LamCA) whose deletion leads to changes in the expression of proteins related to bacterial adherence (Sturme et al., 2005). The inducing peptide, CVGIW, is produced from a peptide precursor of 40 amino acids encoded by *lamD*, and it is processed by the product of *lamB*, which shows 30% identity to the *S. aureus* AgrB protein. AgrB processes the pheromone peptide AgrD that triggers the activation of the *agr* system (Qiu, Pei, Zhang, Lin, & Ji, 2005). As it occurs in the processing of AgrD, LamD processing produces a cyclic thiolactone pentapeptide, LamD558 peptide (Sturme et al., 2005). The *lamBDCA* operon is expressed as a single 2.7 mRNA and it is growth phase−dependent regulated, with maximal expression from mid-exponential phase to early stationary phase. A strain lacking the RR LamA does not form biofilms on glass surfaces, and transcriptional analyses revealed that genes involved in sugar catabolism (e.g., sucrose, cellobiose, trehalose) are downregulated in this strain (Sturme et al., 2005). Other genes downregulated in the mutant comprise genes encoding integral membrane proteins, enzymes for the synthesis of activated sugars,

proteins related to stress response, and the own *lamBDCA* operon. On the contrary, a complete cluster of 14 exopolysaccharide synthesis genes from the *cps2* cluster and *pyr* genes for pyrimidine biosynthesis were upregulated (Sturme et al., 2005).

A paralog of the LamCA TCSs has also been characterized in *L. plantarum* WCFS1. The *lamKR* genes encode a TCS highly homologous to LamCA (Fujii et al., 2008). Transcriptional analyses showed that LamR regulates the same genes that LamA. In fact, the phenotypical effects of LamA inactivation were enhanced in a Δ*lamR*Δ*lamA* double mutant (Fujii et al., 2008). These results indicate that both TCSs work cooperatively in the regulation of the same genes. The analysis of the promoter regions of *lam* genes revealed the presence of imperfect direct repeats separated by 12-bp (5′-TCTT(G/T)AAAT-12nt-TCTTAAA(G/A)-3′) located in the 5′ flanking regions of both promoters, which are similar to targets from related LytTR-type RRs. In *S. aureus*, the *agr* system upregulates many extracellular proteins related to virulence and also modulates cellular metabolism by influencing transcription of *pyr* genes, the UTP-glucose-1-phosphate uridylyltransferase gene, and other genes related to carbohydrate utilization (Dunman et al., 2001). Homologous genes are also regulated by the *L. plantarum lam* systems, which are widespread among *L. plantarum* strains (Fujii et al., 2008). This indicates that these QS systems, which were initially described in pathogenic bacteria, may play a role in host—microbe interactions also in nonpathogenic and commensal lactobacilli. In agreement with this view, a HPK_{10} family kinase (encoded by the *bfrK* gene) was involved in mice forestomach colonization of the rodent isolate *L. reuteri* 100—23 (Frese et al., 2011). Gene *bfrK* is homologous to *lamC* and *lamK* of *L. plantarum*, and it forms an operon together with *bfrR* and *bfrT*, encoding a LytTR-family RR and an ABC transporter with a C39 peptidase domain, respectively. A second homologous TCS is encoded in this organism by the *cemAKR* cluster, which in addition to a TCS carry a gene encoding an autoinducer peptide with a double-glycine leader (*cemA*; IYSLLSL) (Su & Gänzle, 2014). In contrast to *L. plantarum*, mutations in *bfrR* or in *cemAKR* resulted in enhanced capacity for biofilm formation. An autoregulation effect of *bfr* genes was observed and this cluster also functioned in controlling expression of the *cemAKR* genes (Su & Gänzle, 2014). Further transcriptional analyses are needed to disclose the components of the CemR/BfrR regulon. However, these results point toward a cooperative regulation of both RRs, which might involve CemA transport and processing by BfrT. The networks that

regulate in vivo attachment and biofilm formation and the contribution of *bfrKRT* and *cemAKR* to the process are yet to be elucidated.

3.9 TCSs in the Regulation of Nitrogen Metabolism

LAB are fastidious bacteria with a limited capacity to synthesize amino acids, relying on an efficient proteolytic system to fulfill this nutritional requirement (Fernández, Álvarez, & Zúñiga, 2008; Kunji, Mierau, Hagting, Poolman, & Konings, 1996; Savijoki, Ingmer, & Varmanen, 2006). The proteolytic system can be broadly divided into three functional groups: cell wall proteinases that break proteins into peptides, transport systems driving the uptake of the breakdown products, and cytoplasmic peptidases that degrade peptides into free amino acids. The regulation of the expression of the components of the proteolytic system has been studied in detail in lactococci and streptococci where the protein CodY plays a key role. In lactococci, CodY responds to the internal pool of branched-chain amino acids (Guédon, Serror, Ehrlich, Renault, & Delorme, 2001). In this way, the expression of the components of the proteolytic system is repressed when an abundant source of peptides and amino acids, such as casitone, is available in the growth medium (Guédon, Renault, Ehrlich & Delorme, 2001; Marugg et al., 1995). Notwithstanding, the evidence available indicates that CodY is a global regulator with a broader role in the cell physiology as genes not related to peptide and amino acid metabolism have been identified as part of CodY regulons in *La. lactis* (den Hengst et al., 2005; Guédon, Sperandio, Pons, Ehrlich, & Renault, 2005) and several streptococci (Feng et al., 2016; Hendriksen et al., 2008; Huang, Burne, & Chen, 2014; Lemos, Nascimento, Lin, Abranches, & Burne, 2008; Lu, Wang, Wang, & Kong, 2015; Malke, Steiner, McShan, & Ferretti, 2006).

While CodY was well characterized by the mid-2000s, the regulatory mechanisms operating in lactobacilli were largely unknown. Control of the proteinase activity by the concentration of peptides in the growth medium had been reported for *L. helveticus* (Hebert, Raya, & De Giori, 2000), *L. casei* (Paštar, Begović, Lozo, Topisirović, & Golić, 2007), and *L. rhamnosus* (Paštar et al., 2003). The addition of peptides into the growth medium also resulted in down regulation of the oligopeptide transport system Opp, di/tripeptide transport system DtpT, and the peptidase PepT in *L. sanfranciscensis* (Vermeulen, Pavlovic, Ehrmann, Gänzle, & Vogel, 2005). However, CodY is absent in lactobacilli so that regulation in these organisms must differ from that observed in lactococci and streptococci.

The first clue on the involvement of a TCS system in the control of the expression of the proteolytic system in lactobacilli came from the transcriptomic characterization of a *L. acidophilus* NFCM mutant defective in the HK LBA1524 (Eta orthologous group). The analysis revealed that RNA abundance of at least 80 genes was significantly different in the mutant strain (Azcárate-Peril et al., 2005); among them, many genes were involved in peptide transport, proteolysis, and amino acid biosynthesis. Furthermore, the phenotypic analysis of the mutant strain showed that the mutant displayed a deficient growth in milk that was alleviated when milk was supplemented with yeast extract or casamino acids (Azcárate-Peril et al., 2005).

Further evidence came from the characterization of the *L. casei* BL23 ΔRR11 (*prcR*, LCABL_18980), an ortholog of the LBA1524 cognate RR LBA1525 (Zúñiga et al., 2011). This mutant grew slower than the parental strain in MRS (Alcántara et al., 2011), but it acidified milk faster (Alcántara et al., 2016). Subsequent analyses revealed that the mutant strain had higher proteolytic activity than the parental strain *L. casei* BL23 and that this increased activity was due to higher expression of the proteinase encoding gene *prtP* (Alcántara et al., 2016).

The comparison of the transcriptomes of *L. casei* BL23 and ΔRR11 detected 353 genes differentially expressed. The functional classification revealed that a wide variety of cellular functions (Fig. 6A) were affected with the most relevant changes occurring in genes involved in amino acid and peptide metabolism (Alcántara et al., 2016). In particular, in the subset of genes with fold changes greater than 3 (82 genes), 54.88% (45 genes) of them were related to peptide and amino acid metabolism (Fig. 6B). In addition to *prtP* and its cognate maturase, upregulated genes included, among others, 9 putative peptidase encoding genes, 22 oligopeptide ABC transporter subunit encoding genes, and genes encoding enzymes for the biosynthesis of Glu, His, Lys, Thr, and Trp although the biosynthetic pathway for the last one is incomplete in *L. casei*. These genes constitute only a subset of the genes putatively involved in protein degradation and amino acid biosynthesis encoded by *L. casei* although the combined activities of the proteolytic enzymes and amino acid biosynthetic pathways under control of this system may fulfill the amino acid requirements of *L. casei* (Alcántara et al., 2016).

Remarkably, 19 putative regulatory genes were differentially expressed, among them gene *bcaR* (LCABL_27210), which encodes a homologue of the BCARR protein of *L. helveticus* (Wakai & Yamamoto, 2013), a regulator

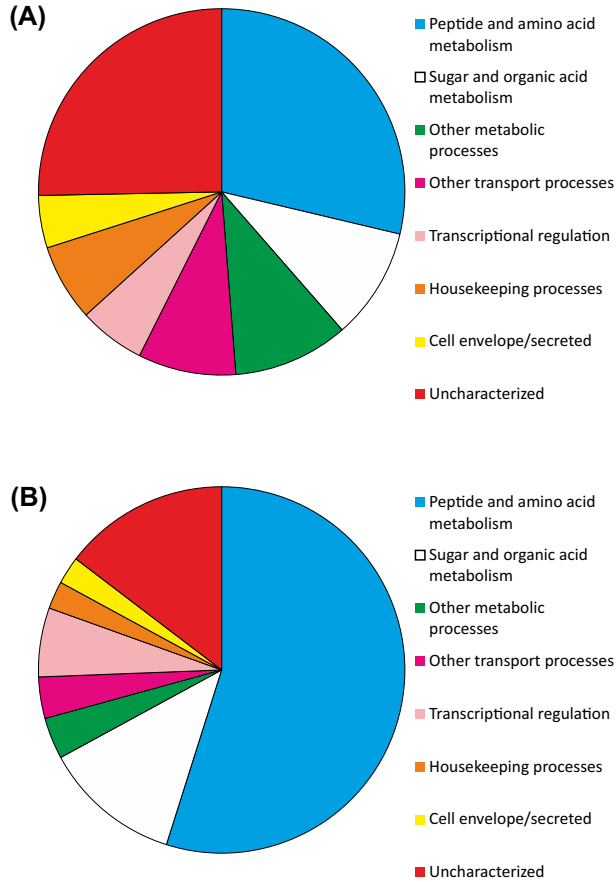

Figure 6 (A) Pie chart of differentially expressed genes (padj < 0.05) in *Lactobacillus casei* RR11 compared to BL23 grouped in functional categories. For ease of visualization, functional categories have been clustered in large groups. (B) Pie chart of differentially expressed genes with fold changes greater than three. *Adapted from Alcántara, C., Bäuerl, C., Revilla-Guarinos, A., Pérez-Martínez, G., Monedero, V., & Zúñiga, M. (2016). Peptide and amino acid metabolism is controlled by an OmpR-family response regulator in* Lactobacillus casei. Molecular Microbiology, 100, *25–41*.

of the expression of peptidase encoding genes in response to the presence of branched-chain amino acids. Also the expression of the gene encoding the sensor kinase of the TC system 11 (LCABL_18970, *prcK*) was 3.1-fold upregulated in the ΔRR11 strain, indicating an autoregulation of expression of this TC system. Differential expression of the homologous TCS was also observed in *L. acidophilus* NCFM (Azcárate-Peril et al., 2005).

The analysis of the PrcR regulon allowed the identification of 12 promoters under direct control of PrcR by electrophoretic mobility shift assays (EMSA; Fig. 7). Among them, the promoter regions of *bcaR* and *prcRK* were recognized by PrcR thus indicating direct control of PrcR on their expression (Alcántara et al., 2016). Additional information was obtained in these assays. First, these promoters corresponded to both upregulated and downregulated genes, suggesting that this RR can function as a repressor or an activator (Alcántara et al., 2016). Second, it was observed that binding of PrcR was enhanced by addition of acetyl-P, which is able to in vitro transfer of the phosphoryl group to the active site in response regulators (Lukat, McCleary, Stock, & Stock, 1992). However, the sequence analysis of the promoter regions recognized by PrcR did not allow identifying any conserved sequence motifs although it could be determined that tracts of four As were involved in the PrcR binding to DNA (Alcántara et al., 2016). This result indicated that PrcR possibly recognizes structural features of the DNA double helix as it has been shown for other OmpR family response regulators such as *E. coli* PhoB (Blanco, Sola, Gomis-Rüth, & Coll, 2002).

The signals to which PrcR may respond were also investigated in this study. Changes in the expression of 12 genes under direct control of PrcR were determined in cells grown in chemically defined medium with or without supplementation with tryptone (Fig. 8). Nine of the thirteen genes assayed were significantly upregulated or downregulated in the parental strain when tryptone was present, whereas they did not show significant differences in the ΔRR11 mutant strain (Fig. 8) indicating that the observed changes in expression depended on the presence of a functional PrcR. Three genes, *bcaR*, *prcK*, and *prtP* displayed small variations in expression (Fig. 8). This difference was attributed to the possible existence of additional regulatory mechanisms. The fact that PrcR bound the *prtP/prsA* and *prcRK* promoter regions even in the absence of acetyl-P (Fig. 7) might indicate phosphorylation-independent binding of PrcR to these promoters in vivo although this point awaits experimental confirmation (Alcántara et al., 2016). Finally, gene *gbuA* was upregulated in both strains, but this gene is not under direct control of PrcR as this protein failed to bind the *gbu* operon promoter region in the EMSA assay (Fig. 7).

All in all, the results of the study of the *L. casei* PrcR protein showed that this protein controls a large regulon, and it responds to the presence of a complex source of amino acids such as tryptone (Alcántara et al., 2016). Furthermore, the wide distribution of orthologs in lactobacilli (Eta orthologs

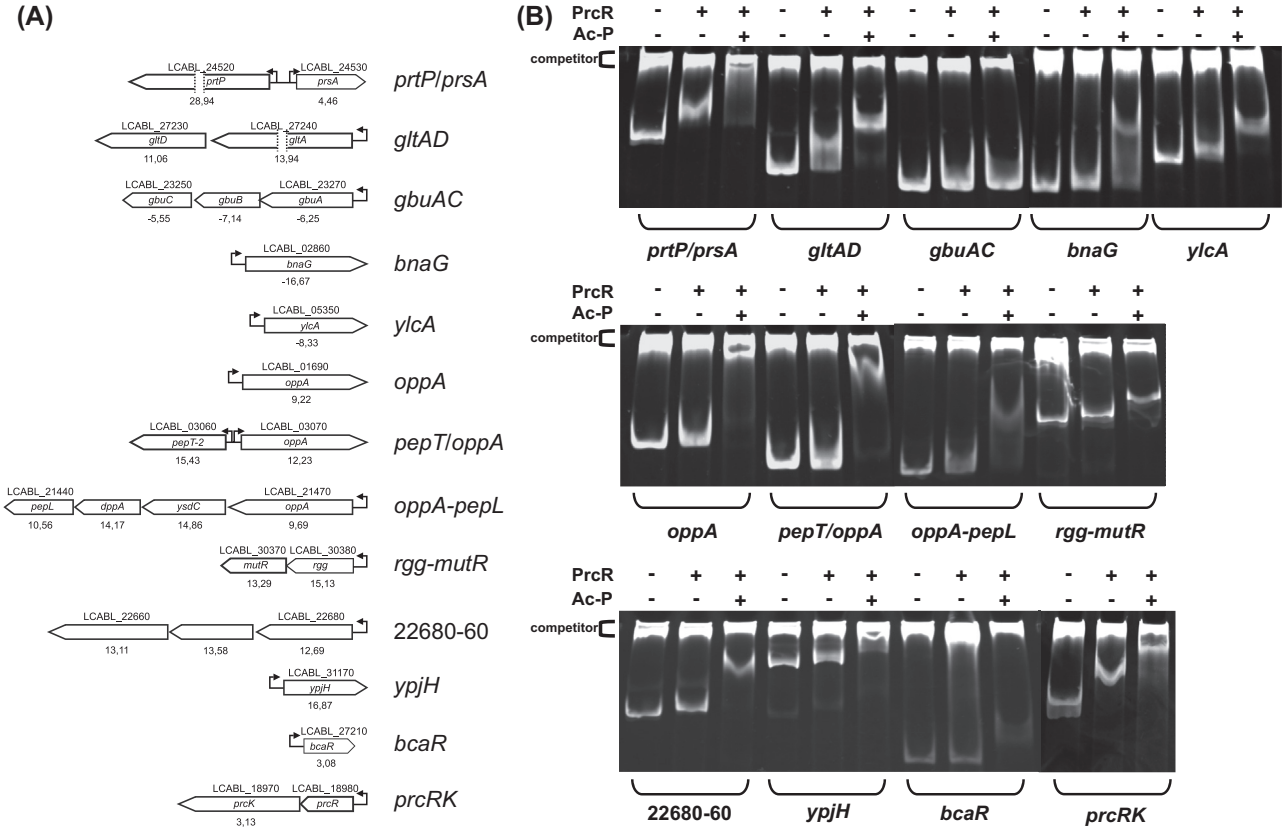

Figure 7 (A) Schematic representation of the gene clusters selected for band mobility shift assays. Fold change values determined for the DRR11 mutant in RNAseq experiments are indicated below each gene. (B) Binding of PrcR to selected promoter regions. Reactions contained 100 ng of target DNA and 1.27 mM of PrcR. The binding reactions were performed in the absence (−) or presence (+) of 10 mM acetyl-P (Ac-P). Competitor indicates the band corresponding to salmon sperm DNA added to the binding reactions. *Reproduced from Alcántara, C., Bäuerl, C., Revilla-Guarinos, A., Pérez-Martínez, G., Monedero, V., & Zúñiga, M. (2016). Peptide and amino acid metabolism is controlled by an OmpR-family response regulator in* Lactobacillus casei. Molecular Microbiology, 100, *25–41*.

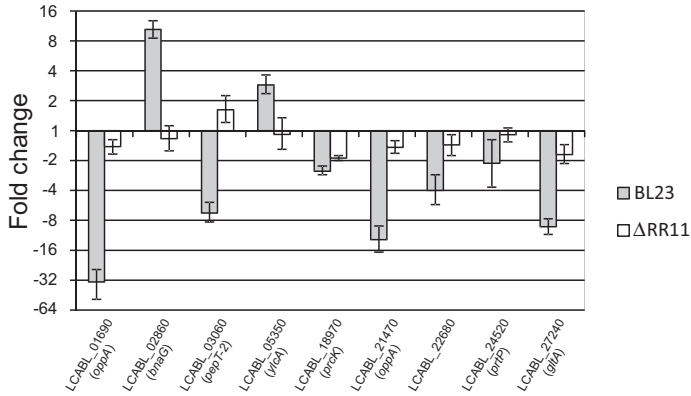

Figure 8 Relative transcript levels in *Lactobacillus casei* BL23 and ΔRR11 strains grown in LcDM plates supplemented with 2% tryptone compared to LcDM plates. Data are shown as means ± standard errors from six independent determinations. *Reproduced from Alcántara, C., Bäuerl, C., Revilla-Guarinos, A., Pérez-Martínez, G., Monedero, V., & Zúñiga, M. (2016). Peptide and amino acid metabolism is controlled by an OmpR-family response regulator in* Lactobacillus casei. Molecular Microbiology, 100, *25–41.*

are only absent in *L. sanfranciscensis*, *Oenococcus kitaharae*, and *O. oeni*), together with the evidence of the characterization of the *L. acidophilus* orthologous system, suggests that this TCS play a major role in the global regulation of peptide and amino acid metabolism in lactobacilli (Alcántara et al., 2016).

Notwithstanding, there remain many questions unanswered on the physiological role of this system in lactobacilli. Genes affected by the inactivation of PrcR encompassed a wide variety of phenotypic functions indicating that the response elicited to the available amino acid sources goes beyond the control of the proteolytic system and the amino acid transport and biosynthesis. This was also observed in *L. acidophilus* (Azcárate-Peril et al., 2005). In this organism, inactivation of the HK LBA1524 affected the survival to low pH, and differential expression of some genes affected by the mutation was observed in cells incubated at different pH values (Azcárate-Peril et al., 2005). The effect of pH on the expression of PrcR-controlled genes has not been determined in *L. casei* BL23, and acid sensitivity was not observed in a PrcR-defective mutant (Alcántara et al., 2011) although the experimental design used in these two studies was different. Most remarkably, the *L. casei* PrcR-defective mutant acidified milk faster than the parental strain (Alcántara et al., 2016), whereas the *L. acidophilus* HK LBA1524-defective strain displayed

a lower acidification rate than the parental strain (Azcárate-Peril et al., 2005). Interestingly, we have very recently obtained an HK PrcK-defective derivative strain of *L. casei* BL23 that also displays lower acidification rate in milk (unpublished results). The fact that inactivation of either the RR or the HK of this TCS results in distinct phenotypes strongly suggests that other regulatory mechanisms beyond phosphorylation of PrcR by its cognate HK are involved in the regulation of PrcR activity.

3.10 TCSs in the Regulation of Malate Metabolism

The metabolism of L-malate by LAB has received considerable attention due to its relevance in the production of fermented beverages, specially wine (Liu, 2002; Lonvaud-Funel, 1999). Most lactobacilli can degrade L-malate to L-lactate by a direct decarboxylation catalyzed by the malolactic enzyme (MLE). The overall pathway works as an indirect proton pump due to the combination of an electrogenic L-malate uptake (Poolman et al., 1991; Salema, Poolman, Lolkema, Loureiro Dias, & Konings, 1994) and the consumption of cytosolic H^+ cations in the decarboxylation reaction (Maloney, 1990). The proton-motive force thus generated can be used for ATP synthesis (Cox & Henick-Kling, 1989) or transport of solutes. A few LAB, however, can utilize the malic enzyme (ME) for L-malate degradation. ME converts L-malate into pyruvate that can be diverted to energy production via glycolysis or enter the gluconeogenic pathway. Therefore, ME enables growth with L-malate as a carbon source (Landete et al., 2010; London, Meyer, & Kulczyk, 1971). This pathway has been studied in *E. faecalis* (Espariz et al., 2011; London & Meyer, 1969; Mortera et al., 2012), *L. casei* (Landete, Ferrer, Monedero, & Zúñiga, 2013; Landete et al., 2010; Schütz & Radler, 1974), *Streptococcus bovis* (Kawai et al., 1996; Kawai, Suzuki, Yamamoto, & Kumagai, 1997), and *Streptococcus pyogenes* (Paluscio & Caparon, 2015). The genes involved in the ME pathway are arranged in two diverging operons, *maePE* and *maeKR*. They encode a putative L-malate transporter (*maeP*), an ME (*maeE*), and a TCS (*maeK* and *maeR*; Mae orthologous group, Table S1). The Mae TCS is present in strains of the *L. casei/rhamnosus* group (Table S1) invariably associated to the *maePE* operon (Fig. 9A). However, a homologous TCS can be found in other species of lactobacilli (Table S1), namely, *L. brevis*, *Lactobacillus* sp. wkB8, and some strains of *Leuconostoc*. In these organisms the TCS-encoding genes are clustered together with a gene encoding a putative D-lactate dehydrogenase and a putative L-malate transporter (Fig. 9A) whose functions remain undetermined.

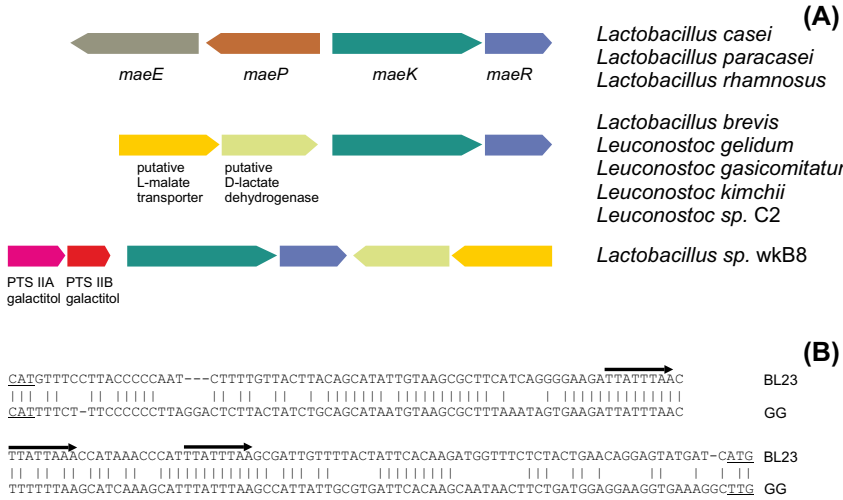

Figure 9 (A) Schematic representation of the genetic organization of Mae orthologous group gene clusters present in lactobacilli. Fill-ins indicate homology. (B) Schematic representation of the *mae* promoter regions of *Lactobacillus casei* BL23 and *Lactobacillus rhamnosus* GG. Translational start sites of *maeP* (5′) and *maeK* (3′) are underlined. MaeR binding sites are indicated by *thick arrows*.

The role of the Mae TCS has been studied in detail in *L. casei* BL23 (Landete et al., 2010, 2013). *L. casei* BL23 can grow with L-malate as a carbon source being the ME encoded by *maeE* essential for this ability (Landete et al., 2010). Transcriptional analyses showed that *maePE* expression was induced in the presence of L-malate and repressed by glucose (Landete et al., 2010, 2013). On the other hand, expression of *maeKR* was also induced by L-malate but was not affected by glucose (Landete et al., 2010). Inactivation of the RR MaeR or the HK MaeK led to the loss of the ability to grow with L-malate as a carbon source (Landete et al., 2010). This effect correlated with the loss of the induction of the expression of *maePE* (Landete et al., 2010). In *E. faecalis* JH2-2, MaeR is also required for *maePE* expression in response to L-malate and expression was repressed by glucose (Mortera et al., 2012). Similarly, expression of *maePE* is induced by L-malate and repressed by glucose in *S. pyogenes* (Paluscio & Caparon, 2015). Interestingly, expression of *maePE* was also induced by low pH even in the absence of L-malate in a MaeK-dependent manner in this organism (Paluscio & Caparon, 2015), indicating that the system responds to both L-malate and low pH. Induction by low pH has

also been observed in *L. casei* BL23 although it was not established if it depended on the cognate TCS (Landete et al., 2013).

The DNA-binding activity of MaeR has also been characterized. EMSA and DNase I footprinting showed that MaeR binds three direct repeats (5′-TTATT(A/T)AA-3′) within the *mae* promoter region (Fig. 9B) although the comparison with the *mae* promoter region of *L. rhamnosus* suggests that more degenerated sites may also be recognized (Fig. 9B). Subsequent EMSA assays showed that the three repeats were required for stable MaeR DNA binding in vitro (Landete et al., 2010). These sets of repeats are conserved in the homologous *mae* promoter regions of *E. faecalis* and streptococci although the spacing of the repeats and the −10 promoter boxes varies in these organisms (Landete et al., 2010), but it remains to be established if these differences affect the regulation of the *mae* gene cluster.

Strains of the *L. casei/rhamnosus* group are remarkable among LAB because they possess the ability to utilize L-malate via the ME or the MLE pathways (Landete et al., 2010, 2013; Schütz & Radler, 1974). The MLE operon consists of three genes encoding MLE (*mleS*), an L-malate transporter (*mleT*), and a LysR-type transcriptional regulator (*mleR*) that controls the transcription of both genes (Landete et al., 2013). Despite both pathways utilize the same substrate, the transcription of the corresponding genes is independently regulated (Landete et al., 2013). The phenotypic analysis of *L. casei* BL23 mutants defective in each L-malate transporters (*maeP* or *mleT*) or both transporters showed that internalization of L-malate was required for MleR induction of *mle* genes but not for the induction of the expression of *mae* genes by the Mae TCS (Landete et al., 2013).

MaeR belongs to Cit family of RRs and MaeK to family IV of HKs. Homologs of this TCS can be found in a wide variety of bacteria usually associated to the regulation of the utilization of dicarboxylates or tricarboxylates (Asai, Baik, Kasahara, Moriya, & Ogasawara, 2000; Bott, Meyer, & Dimroth, 1995; Janausch, Zientz, Tran, Kröger, & Unden, 2002; Landete et al., 2010; Tanaka, Kobayashi, & Ogasawara, 2003; Yamamoto, Murata, & Sekiguchi, 2000; Zientz, Bongaerts, & Unden, 1998). A phylogenetic analysis has shown that *maeR* and *maeK* genes associated to the *maePE* operon of *Lactobacillales* constitute distinctive clusters thus suggesting that they evolved from a common ancestor (Landete et al., 2010). Their closest relatives are TCSs of *Bacillaceae* involved in the regulation of L-malate utilization (Landete et al., 2010; Tanaka et al., 2003). Despite this relatedness, there are functional differences between the MaeKR TCS and the *B. subtilis*

homolog YufLM. Inactivation of the sensor YufL results in constitutive expression of the regulated transporter MaeN (Tanaka et al., 2003), whereas inactivation of MaeK led to complete loss of induction of the expression of *maePE* (Landete et al., 2013). Another remarkable characteristic of some of these TCSs is the use of a cognate transporter as a cosensor. This phenomenon has been thoroughly studied in the C4-dicarboxylate–sensing TCS DcuSR of *E. coli* (Kleefeld, Ackermann, Bauer, Krämer, & Unden, 2009; Unden, Wörner, & Monzel, 2016; Witan, Monzel, Scheu, & Unden, 2012; Wörner et al., 2016). In this organism, deletion of the transporters DctA or DcuB results in the loss of regulation of DcuS activity by C4-dicarboxylates. In contrast, inactivation of the genes encoding L-malate transporters in *L. casei* (*maeP* and *mleT*) did not prevent the induction of the transcription of *maeE* in the presence of L-malate, as noted earlier (Landete et al., 2013). This result suggests that MaeK does not require a cosensor protein for L-malate–dependent induction of the expression of *mae* genes. Therefore, MaeKR TCSs have characteristics that set them apart from other characterized homologous systems of other organisms that await elucidation.

4. CONCLUDING REMARKS

Physiology and genetics of lactobacilli have been an important research area in the past decades due to their relevance in food production and health. However, the study of signal transduction pathways in these organisms has received relatively little attention. The progress made in the study of TCSs in lactobacilli has evidenced that these systems play important roles in the cell physiology of lactobacilli. The role of TCSs in bacteriocin production is relatively well known nowadays, but the studies of other TCSs are only in their preliminary stages and there remain many open questions that need to be addressed to understand how these systems work. Furthermore, information available about most characterized TCSs is limited to a very scarce number of strains.

The wealth of data provided by genome sequencing projects provides a solid basis to identify promising targets for research in this area. For example, the analysis of the genome sequences of lactobacilli has revealed that very few systems have been conserved during the evolution of this group. Conservation of these systems in a bacterial group where genome decay is a major evolutionary trend strongly suggests that they possibly control key

physiological processes. This hypothesis has received strong support for the Eta orthologous group, exemplified by the PrcR/PrcK system of *L. casei* discussed earlier, although studies in other species are needed. For other systems, such as those belonging to the Cro orthologous group, evidence from phenotypic characterization of defective mutants also suggest a relevant role. Other system, the Yyc orthologous group, poses an intriguing riddle, as homologous systems play essential physiological roles in other bacterial groups, whereas inactivation in some lactobacilli has rendered no remarkable phenotypic effect.

Understanding the complexity of regulatory networks involved in the adaptation of lactobacilli to their environments is crucial to improve their performance in industrial processes and to understand their role in the symbiotic relationships that they establish with other bacteria and with their eukaryotic hosts. A detailed knowledge of the TCSs of lactobacilli will be crucial to attain this goal.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/bs.aambs.2016.12.002.

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CHAPTER TWO

Fungal Biorecovery of Gold From E-waste

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Contents

Abstract

Waste electric and electronic devices (e-waste) represent a source of valuable raw materials of great interest, and in the case of metals, e-waste might become a prized alternative source. Regarding gold, natural ores are difficult to mine due to their refractory nature and the richest ores have almost all been exploited. Additionally, some gold mining areas are present in geopolitically unstable regions. Finally, the gold mining industry produces toxic compounds, such as cyanides. As a result, the gold present in e-waste represents a nonnegligible resource (urban mining). Extraction methods of gold from natural ores (pyro- and hydrometallurgy) have been adapted to

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this particular type of matrix. However, to propose novel approaches with a lower environmental footprint, biotechnological methods using microorganisms are being developed (biometallurgy). These processes use the extensive metabolic potential of microbes (algae, bacteria, and fungi) to mobilize and immobilize gold from urban and industrial sources. In this review, we focus on the use of fungi for gold biomining. Fungi interact with gold by mobilizing it through mechanical attack as well as through biochemical leaching by the production of cyanides. Moreover, fungi are also able to release Au through the degradation of cyanide from aurocyanide complexes. Finally, fungi immobilize gold through biosorption, bioaccumulation, and biomineralization, in particular, as gold nanoparticles. Overall, the diversity of mechanisms of gold recycling using fungi combined with their filamentous lifestyle, which allows them to thrive in heterogeneous and solid environments such as e-waste, makes fungi an important bioresource to be harnessed for the biorecovery of gold.

1. INTRODUCTION

1.1 Metallic Waste

Nowadays, metals are omnipresent, from building constructions, machinery, and large equipment, to electric and electronic equipment (EEE). EEE includes any device using electricity for power and such devices are very common and widespread in modern life. However, they often have a short life span and as a result, a large fraction (if not all) ends up sooner or later as waste. This generates a type of waste referred to as electronic waste (e-waste), waste electrical equipment, or electronic scrap (Cui & Zhang, 2008; Robinson, 2009). At present, e-waste represents an ever-growing fraction of the total waste produced worldwide. It is considered that e-waste accounts for 1%–3% of the global municipal waste production (Robinson, 2009). In 2014, 41.8 million tonnes (Mt) of e-waste were produced and it is expected to reach 50 Mt in 2018 due to continuous technological innovation (Baldé, Wang, Kuehr, & Huisman, 2015). In a time when shortage of nonrenewable raw material is expected, accumulating excessive amount of metals into waste is no longer acceptable. In addition to this, the mining of raw material is linked to both environmental and social issues (Jenkins, 2004). Consequently, different approaches to efficiently recycle and/or appreciate the various metals present in e-waste have been developed as a response to the environmental and social footprint of ore mining. These methods consist roughly of physical, chemical, and biological processes. Physical and chemical recycling often poses several environmental concerns and therefore biological methods are preferred.

1.2 Fungi and Metals

Fungi are important biogeochemical agents and are able to both dissolve and immobilize metals. To cope with toxic metal concentrations, they possess several resistance mechanisms (Gadd, 2007). From the outside to the inside of the fungal cell, they are able to (1) immobilize metals outside their biomass with chelating compounds such as organic acids (e.g., citric acid, oxalic acid) or exopolymeric substances (e.g., glycoproteins); (2) bind metals into their cell wall; (3) actively pump metals outside the cell; (4) bind metals on specific intracytoplasmic proteins (e.g., metallothionein, glutathione); or (5) immobilize metals into organelles (mainly into vacuoles). In addition to this, fungi are also able to translocate and accumulate various compounds in specific tissues (e.g., fruiting bodies, rhizomorphs). All these capacities represent interesting tools to be harnessed in biotechnological metal recycling from e-waste.

This review aims at presenting the current context of metal recycling from e-waste and the use of a particular group of microbes, the fungi, for their recycling. As an example of a valuable metal present in e-waste, this review focuses on gold (Au) and how fungi can be used to mobilize as well as immobilize this precious metal.

2. CURRENT CONTEXT OF METAL RECYCLING

2.1 Industrial Metallic Wastes and E-wastes

EEE consists roughly of six categories: (1) temperature exchange equipment, (2) screens and monitors, (3) lamps, (4) large equipment (e.g., washing machines), (5) small equipment (e.g., vacuum cleaners), and (6) IT and telecommunication equipment (Baldé et al., 2015). Each category consists of a unique mixture of chemical components. However, most EEE (and thus e-waste) is composed of metals, plastics, and ceramics in variable proportions (Robinson, 2009). The metal fraction accounts for up to 60% depending on the item (Ari, 2016; Berkhout & Hertin, 2004). The major metallic elements are Fe, Al, Cu, Pb, Zn, Sn, and Ni. Besides these main elements, other metals are present in lower amounts (ppm level) such as heavy metals (e.g., Hg, Pd, Cd); valuable metals (e.g., Au, Ag, Pd); and rare earth elements (Cui & Zhang, 2008; Kaya, 2016). Importantly, it has to be pointed out that the concentration of the different components in EEE/e-waste is extremely variable and usually varies with the type,

age, origin, and manufacturer of the EEE (Cui & Zhang, 2008; Robinson, 2009). Such a feature makes the recycling of e-waste challenging.

2.2 Disposal and Recycling of Metallic Wastes

All components of e-waste, when not disposed properly, represent a potential environmental hazard. As a result, several options are available for the treatment of e-waste: either they are landfilled or incinerated; alternatively, they can be reused or remanufactured; or they can be recycled to reuse their single constituents. Landfilling and incineration are not desired as they pose several environmental concerns through the emission of hazardous compounds such as dioxins and heavy metals (European Parliament, 2012). Reuse and remanufacturing are the preferred options in terms of environmental impact, as these avoid complex treatments that can lead to the release of hazardous compounds in the environment. Recycling of e-waste implies to first disassemble the equipment, then to separate the different types of materials, and finally to recover and refine its single constituents. The recovery and refining of the metals is performed using metallurgical methods (Cui & Zhang, 2008; Glombitza & Reichel, 2014). Such a process typically requires many steps, a great input in energy, and, depending on the method, generates toxic products. As a result, it has both an economic and an environmental cost. The major driver of e-waste recycling is economic. As a matter of fact, the metals present in e-waste represent a nonnegligible resource of valuable materials (e.g., Cu, Ag, Au, Zn, Pd, Ni). In a time of shortage of raw materials and an increased dependency toward geopolitically unstable countries mining these resources, having the ability to obtain these metals from e-waste, a concept coined urban mining, is of great interest (Cui & Zhang, 2008).

Typically, the chemical recovery of metals from solids can be achieved through three main approaches: pyrometallurgy, hydrometallurgy, and biometallurgy. These methods have initially been developed for processing mineral ores and are now being adapted to the treatment of e-waste.

2.2.1 Pyrometallurgical Recovery

Pyrometallurgical processing of e-waste requires reactions performed at very high temperature in a furnace. Briefly, crushed wastes are burned to remove plastics and metals are retrieved through reduction using electrochemical or hydrometallurgical methods. These metals can further be processed to refine them singly. Metals that can be retrieved by pyrometallurgy are Cu, Au, Ag, Pt, Pd, Se, Te, Ni, Zn, Pb, Sb, Bi, Sn, Te, and In. Pyrometallurgy is the most

widely used method for the recovery of metals from e-waste, however it suffers from several limitations. The leftover of pyrometallurgical processing is a slag containing refractory metal oxides (in particular Al and Fe, which are thus lost in this process). In some cases, the slag still contains some precious and base metals that are consequently lost (Cui & Zhang, 2008). In addition to this, the presence of halogenated flame retardants in EEE can lead to the formation of dioxins. Therefore, smelters used in pyrometallurgical processing of e-waste should be adapted and this requires additional investment (Dalrymple et al., 2007).

2.2.2 Hydrometallurgical Recovery

In the first step of hydrometallurgical processing, metals are extracted from their solid matrix by leaching using either acidic or alkaline solutions. Several compounds, including cyanides, halides, thiourea, and thiosulfates are available for this step. In a second step, metals are isolated and concentrated from the leachates by separation and purification procedures. Metals that can be retrieved by hydrometallurgy are Cu, Au, Ag, Ni, Pd, Pt, Sn, Pb, and Ni. In terms of metal recovery, hydrometallurgical processing is more efficient than pyrometallurgy. Larger quantities of metals can be retrieved and the process is more easily controlled. It is thus preferred from an economic point of view. However, regarding environmental issues, hydrometallurgy suffers from the same caveats that exist with pyrometallurgy, i.e., the emission of hazardous compounds in the environment (both in the atmosphere and in the ground), as well as its high demand in energy (Cui & Zhang, 2008).

2.3 Biometallurgy: Microbiological Recycling of Metallic Waste

Currently, large-scale application of microbial metal recycling from e-waste does not exist. However, within the last 10 years, several studies have been published dealing with this particular aspect (Barmettler, Castelberg, Fabbri, & Brandl, 2016; Ilyas & Lee, 2014). Another field where similar microbial processes are already applied is biomining (or biohydrometallurgy), which is used for metal recovery from ores (bioleaching) and heavy metal remediation from waste waters (biosorption) (Glombitza & Reichel, 2014). Traditionally, in the field of biomining, processes using prokaryotes (bacteria and archaea) are more advanced and developed at the industrial scale than those using eukaryotes (algae or fungi). Currently, the most common microbial process in biomining is the extraction by prokaryotic bioleaching of

metals from sulfidic low-grade ores (Schippers et al., 2014). However, recent research demonstrates that both types of organisms can be efficient for metal recovery from different type of matrices (Glombitza & Reichel, 2014; Ilyas & Lee, 2014). Microorganisms can be considered as miniaturized chemical bioreactors. As a result, it is possible to perform virtually any chemical reaction using microorganisms as a catalyst in controlled conditions (temperature, pressure). Currently, there are two types of microbial activities that are exploited for biomining: bioleaching and biosorption. Bioleaching consists in the mobilization of metals promoted by biological activities, whereas biosorption consists in the immobilization of metals on biological templates.

2.3.1 Bioleaching

Bioleaching can be defined as the biological transformation of an insoluble metal compound into a water-soluble form (Schippers et al., 2014). Both prokaryotes and fungi can perform it. The most famous bioleaching process is the solubilization of sulfidic minerals promoted by the activity of Fe(II)- and S-oxidizing autotrophic microbes such as the archaeum *Sulfolobus metallicus* or the bacterium *Acidithiobacillus ferrooxidans* (Rawlings & Johnson, 2007). In this microbial process, solubilization of the metals is promoted by oxidation of the sulfidic moiety either directly or indirectly by the Fe(III) produced by microbial activity. In addition to this, the production of sulfuric acid as a result of sulfide oxidation by acidophilic S-oxidizing microbes can also promote mineral dissolution as demonstrated by Coto, Galizia, Hernandez, Marrero, and Donati (2008) for Ni extraction from laterites.

Another type of bioloeaching consists of the solubilization of metals by heterotrophic microorganisms, given that a suitable carbon source is provided (Schippers et al., 2014). Heterotrophic bioleaching consists of metal solubilization promoted by the excretion of protons (H^+), organic acids, amino acids, peptides, lipids, exopolysaccharidic substances, or cyanides (Brandl, Bosshard, & Wegmann, 2001; Gadd, 2010). Any substance that has a complexing or chelating ability can potentially serve for metal solubilization. All these processes can be observed in fungi during the weathering of mineral substrates in natural environments. Together with bacteria, fungi are essential biogeochemical agents triggering the release of elements from rocks and minerals resulting in the formation of soils (Gadd, 2007; Hoffland et al., 2004; Uroz, Calvaruso, Turpault, & Frey-Klett, 2009).

At present, extraction by prokaryotic bioleaching of Cu and Au from refractory sulfidic ores is applied on an industrial scale and extraction of Co, Mo, Ni, Pb, and Zn from sulfidic ores is technically feasible (Cui & Zhang, 2008). While fungal bioleaching abilities are not harnessed yet on an industrial scale, it can be expected that future developments in biotechnology will develop this aspect linked to heterotrophic bioleaching.

2.3.2 Biosorption

Biosorption is a passive physicochemical process between biological surfaces or components and ions in solution. It can involve both living and nonliving biomass, as well as single organic compounds (Fomina & Gadd, 2014). Many organisms can be used as biosorbing agents from plants and macroalgae to microbes (microalgae, bacteria, archeae, and fungi). Biogenic components such as chitosan from fungal cell walls as well as algal waste (e.g., alginate) can act as biosorbents. As a result, biosorption is a very versatile method and the sole theoretical limitation is to find the type of biomass that has the highest biosorption capacity for a given precious metal (Cui & Zhang, 2008). Besides this, physicochemical parameters such as pH, temperature, and concentration of other interfering ions are the main factors limiting biosorption. Importantly, biosorption using dead biomass is not limited by the toxicity of metals and/or by the production of interfering metabolic products (Gadd, 2001). Biosorption involves mechanisms such as absorption, adsorption, ion exchange, surface complexation, and precipitation (Fomina & Gadd, 2014). Once a metal or a mixture of metals has been biosorbed onto biomass, it has to be recovered. This is usually performed by elution with different solutions containing ethylenediaminetetraacetic acid or salts of sodium, for instance (Gadd, 2001).

Biosorption seems a straightforward process at first sight, however it is a complex approach given the multitude of biosorbents and related mechanisms that exist. As a result, despite the fact that much research has been performed on the phenomenon of biosorption, it is still poorly used at an industrial scale.

Filamentous fungi are very interesting biosorbents as they exhibit a very high surface to volume ratio thanks to their mycelial lifestyle. In addition to this, large amount of fungal biomass can be produced using food and agricultural waste as substrate. As a result, fungi have a large potential in the field of biosorption of heavy and precious metals, as demonstrated from several studies reviewed in Gadd (2009), Dhankhar and Hooda (2011), and Fomina and Gadd (2014). Currently, biotechnological

approaches to recover heavy metals from wastewater and industrial effluents with microbial biomass are being developed using packed- or fluidized-bed reactors (Gadd, 2001). However, the use of biosorption mechanisms to recover precious metals from e-waste is still in its infancy (Creamer, Baxter-Plant, Henderson, Potter, & Macaskie, 2006). In the frame of precious metal recovery, biosorption has been successfully used for the recovery of Au (both as Au(III) and $[Au(CN)_2]^-$), Pd, Pt, and Ag (Cui & Zhang, 2008). Given the high versatility of biosorbing compounds and the fact that food and agricultural waste can be used for this, this biotechnological approach has still the potential to develop in the near future.

2.3.3 Bioaccumulation

Bioaccumulation can be considered as a particular type of biosorption in which metals are incorporated inside living biomass. This incorporation relies mostly on active uptake, but passive uptake can also be involved (Fomina & Gadd, 2014). Metals are incorporated within biomass as a mean of protection against metal toxicity. Metals can be immobilized through complexation with intracellular proteins such as sulfur-containing peptides (metallothionein and phytochelatins) (Harms, Schlosser, & Wick, 2011). Alternatively, in eukaryotic organisms, metals can be stored in intracellular organelles (Gadd, 2010). As a result, bioaccumulation relies strongly on an organism affinity toward a given metal, as well as on the physicochemical conditions influencing metal toxicity (Eh, pH). Metal accumulation into fruiting bodies of mushrooms is a well-known aspect of the fungal storage capabilities for various compounds. For instance, high levels of Pb, Cd, Zn, and Hg are commonly observed in carpophores of macrofungi in polluted areas (Byrne, Ravnik, & Kosta, 1976; Byrne, Tušek-Žnidarič, Puri, & Irgolic, 1991; Gadd, 2007). Accumulation of Au and Sb has also been observed in some fungal species, demonstrating a species-dependent accumulation (Borovička, Řanda, & Jelínek, 2005, 2006). In addition to this, in controlled experiments, bioaccumulation in fruiting bodies of *Agaricus bisporus* has also been shown for Ag (Byrne & Tušek-Žnidarič, 1990). As a result, the ability to accumulate metals in fruiting bodies of macrofungi has been proposed as an easy way to alleviate metal pollution in soils (Das, 2005; Gadd, 2007). However, to the best of our knowledge, this aspect has not yet been investigated to recover metals from e-waste.

3. THE PROBLEM OF GOLD

Gold (Au) is a precious metal and represents a nonrenewable resource. It is extremely rare in the environment and nonessential for life. It occurs in three oxidation states: 0 (Au(0)), +1 (Au(I): aurous gold), +3 (Au(III): auric gold). Au ions are unstable in aqueous solutions under surface conditions (pressure and temperature). Therefore, Au(0) will form metal colloids, whereas Au(I) and Au(III) form complexes with available anions. Au complexes are usually toxic to organisms (Reith, Lengke, Falconer, Craw, & Southam, 2007). Au is used in EEE as an electrical conductor and therefore is omnipresent in e-waste (Robinson, 2009). It is present at the ppm level, which makes it poorly amenable to recycling. However, Au recovery is of prime importance for both economic and environmental reasons. Due to the continuous growth of technological innovation, the demand for Au is expected to grow. Besides this, gold ores usually contain low levels of Au (5 g Au for 1 ton of ore) and the richest ores have already been exploited (Gurung et al., 2012). As a result, having the possibility to recover Au by urban mining is of the upmost interest.

Several studies demonstrate the physical and/or chemical extraction (pyro- and/or hydrometallurgy), separation, and recovery of precious metals from e-waste (e.g., Duan et al., 2009; Gurung et al., 2012; Kaya, 2016; Li, Lu, Guo, Xu, & Zhou, 2007; Li & Xu, 2010). These methods usually require large quantities of hazardous compounds to solubilize the precious metals. In addition to this, the combustion of e-waste produces toxic gases such as HCN, CO, and dioxins, all of which are of concern for the environment and human health (Li et al., 2007). As a result of the hazardous nature of both pyro- and hydrometallurgical processing of precious metals from e-waste, the development of biotechnological alternatives using, bacteria and fungi for instance, is of the highest importance.

3.1 Chemical Recovery of Gold

The chemical recovery of Au is performed using hydrometallurgical processing, which involves solubilization of Au from its solid matrix by chemical leaching and recovery of the soluble Au through several immobilization processes.

Several types of chemical compounds are able to leach Au, such as aqua *regia* (a mixture of three parts of concentrated hydrochloric acid to one part of concentrated nitric acid), cyanides, thiosulfates, and thiourea

Table 1 Summary of the leaching mechanisms used for the hydrometallurgical processing of gold

Leaching solvent	Metals recovered	pH range	Type of process	Environmental hazard
Aqua regia (Cl^-)	Au	<1	Au oxidation by nitric acid and complexation of Au(III) with Cl^-	High. Highly corrosive conditions and production of chlorine gas
Cyanide (CN^-)	Au, Ag, Pd, Pt	10–10.5	Electrochemical	High. Inhibition of cytochrome c oxidase by cyanide
Thiosulfate ($S_2O_3^{2-}$)	Au	9–10	Electrochemical reaction catalyzed by Cu^{2+}	Low, but high consumption of reagents
Thiourea (CH_4N_2S)	Au	1–2	Dissolution of gold at low pH and high redox potential	Medium. Use of sulfuric acid and high consumption of leaching reagents

(Table 1) (Cui & Zhang, 2008; Ha, Lee, Jeong, Hai, & Jha, 2010). Cyanide leaching has been the most used method in the mining industry up to now. In this process, Au is solubilized through an electrochemical process to form $[Au(CN)_2]^-$. While it is a cheap method, accidents at several gold mines causing severe environmental contamination have reduced the interest of this leaching method. Within the last 15 years, thiosulfate leaching has become more and more popular for Au leaching due to its reduced toxicity and its lower cost. Au solubilization in ammoniacal thiosulfate solution is catalyzed by the presence of Cu(II) ions leading to the formation of $[Au(NH_3)_2]^+$ or $[Au(S_2O_3)_2]^{3-}$ (Cui & Zhang, 2008). However, the chemistry of this system is only partly understood and the process is generally slow. In addition to this, thiosulfate leaching requires a high amount of reagents for an efficient Au extraction. As a result, thiosulfate leaching is an alternative to cyanide leaching regarding toxicity, but when leaching efficiency and reagent cost are taken into account, it does not represent an interesting approach. Finally, another leaching agent that can be used for Au solubilization is thiourea. In acidic conditions, Au forms a complex with thiourea, $Au(CS(NH_2)_2)_2^+$. This method is very effective in dissolving Au (up tp 99%) (Hilson & Monhemius, 2006),

but it also requires high amount of reagents reducing the economic interest of this method. However, it has to be pointed out that few large-scale studies have been performed for thiourea leaching, and hence this process has the potential to be improved substantially (Cui & Zhang, 2008).

After leaching, Au has to be recovered. At an industrial scale, several methods exist for this: precipitation (or cementation), solvent extraction, adsorption on activated carbon, or ion exchange resins (Table 2) (Cui & Zhang, 2008). Precipitation is performed mainly using Zn dust leading to the reduction–precipitation of Au from soluble aurocyanides complexes. Other methods exist such as reduction–precipitation of Au from thiourea, thiosulfates, or thiocyanates solutions by sodium borohydride (Awadalla & Ritcey, 1991). Solvent extraction has been extensively studied and many different types of extractants have been proposed (Cui & Zhang, 2008). The aim is to increase the amount of Au recovered with the highest selectivity possible by selecting for an extractant that reacts preferentially with the Au–leachate complex over other metals–leachate complexes. Adsorption on activated carbon and subsequent elution has also been extensively investigated. The aim is to select the best physicochemical conditions (ionic strength, pH, and temperature) in which Au from its leachate complex will be preferentially adsorbed onto activated carbon (and not

Table 2 Summary of the recovery mechanisms used for the hydrometallurgical processing of gold

Method	Commercially applied for the recovery from	Reagent	Type of process
Precipitation/ Cementation	Au–cyanide complexes	Zn dust	Electrochemical, reduction–precipitation of Au
Solvent extraction	Au–cyanide complexes	Commercial extractants: e.g., organophosphorous compounds	Complexation
Activated carbon	Au and Ag–cyanide complexes	Carbon-in-pulp and Carbon-in-leach processes	Adsorption
Ion exchange	Au–thiosulfate complexes	Commercial strong base resins	Ion exchange on a resin followed by sequential elution

other metals) and efficiently leached (Adams & Fleming, 1989). Finally, Au from leachate complexes can be recovered with ion exchange resins. Again, different types of commercial resins have been developed to successfully and selectively isolate pure fractions of Au and other metals (Cui & Zhang, 2008). Overall, these methods still suffer from low selectivity and are therefore rather expensive. Consequently, biorecovery methods have been investigated as a mean to overcome the toxicity related to cyanide leaching and the overall low selectivity of Au recovery (Eisler, 2003).

3.2 Gold Biorecovery

The interaction of Au and prokaryotes has been well described and prokaryotes are believed to be involved in almost every step of the biogeochemical Au cycle (Reith et al., 2007). In addition to prokaryotes, several other organisms have been documented as being able to dissolve or concentrate Au: algae, plants, and fungi. Similarly, organic templates have been described as being able to immobilize Au: peat and crab exoskeletons (Eisler, 2003). As a result, both living and dead biomass of various origins have the potential of being harnessed for the biotechnological recovery of Au.

A well-documented aspect is the implication of prokaryotes in Au biomining through biooxidation. In this process, Fe(II)- or S-oxidizing microbes adapted to acidic and metal-rich conditions degrade recalcitrant gold-bearing arsenopyrite ores leading to the concentration of gold particles (Rawlings, 2002). These microbes do not have a direct effect on Au mobilization but help in its extraction. However, direct examples of the interaction of living organisms with Au are numerous. Microbes present during biomining operations of metal sulfidic ores can be involved in Au mobilization and immobilization. For instance, Fe(III)-reducing bacteria can precipitate Au through its reduction with hydrogen as the electron donor (Kashefi, Tor, Nevin, & Lovley, 2001). Besides this, microscopic algae (both eukaryotes and cyanobacteria) are able to accumulate Au from chloride solutions through biosorption at their outer cell layer (Dyer, Krumbein, & Mossman, 1994; Ting, Teo, & Soh, 1995). Plants have also shown Au accumulation capabilities as described for *Medicago sativa* (Gardea-Torresdey et al., 2000) and *Azolla filiculoides* (Antunes, Watkins, & Duncan, 2001). The ability of bacteria to form Au nanoparticles (AuNPs) has been fairly well described (Focsan, Ardelean, Craciun, & Astilean, 2011; He et al., 2007; Husseiny, El-Aziz, Badr, & Mahmoud, 2007; Pantidos & Horsfall, 2014). Finally, fungi are also important agents of Au mobilization and immobilization as described in the following section.

3.2.1 Mechanisms of Gold Recovery Using Fungi

Several examples of the implication of fungi in Au cycling have been published within the last two decades (Borovička et al., 2005; Das, Liang, Schmidt, Laffir, & Marsili, 2012; Gomes, Camargos, Dias, & Linardi, 1998; Kitching, Ramani, & Marsili, 2015; Niu & Volesky, 1999; Sastry, Ahmad, Khan, & Kumar, 2003; Siddiqi & Husen, 2016; Yadav et al., 2015). In particular, their ability to synthesize AuNPs has been well described (Das et al., 2012; Du, Xian, & Feng, 2011; Kitching et al., 2015; Sastry et al., 2003; Sheikhloo, Salouti, & Katiraee, 2011; Siddiqi & Husen, 2016; Yadav et al., 2015). As a result, recent research demonstrates that fungi are able to both mobilize and immobilize Au using mechanisms such as mechanical attack, bioleaching, biosorption, and biomineralization (Fig. 1).

3.2.1.1 Mechanical Attack on Gold Containing Solids

Fungi are able to mechanically alter mineral substrates (Fomina, Burford, Hillier, Kierans, & Gadd, 2010; Gadd, 2007). They usually do this to either

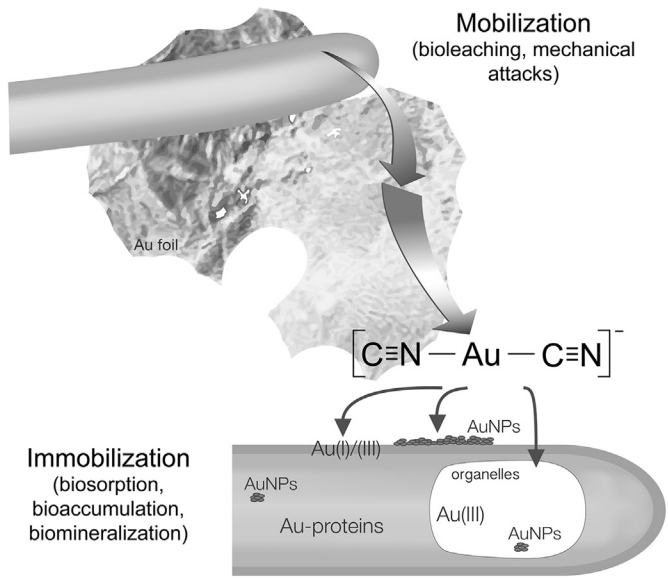

Figure 1 Diagram summarizing the fungal mechanisms of gold cycling. Upper part shows mechanisms of gold mobilization: biochemical bioleaching, e.g., through cyanide complexion and mechanical attack on solid gold. Lower part shows mechanisms of gold immobilization: biosorption of Au to the fungal cell wall, bioaccumulation in the cytoplasm and into organelles such as vacuoles, and biomineralization of gold as gold nanoparticles (either extracellular or intracellular).

explore their environment or to forage for nutrients. For example, when grown on a gold sheet overlying a malt medium (either liquid or solidified with agar), some fungal strains are able to create cracks within this gold sheet. This is probably to access the nutrients present in the growth medium. *Aureobasidium pullulans*, *Epicoccum* sp., *Fusarium oxysporum*, and *Verticillium* sp. were able to create a large quantity of cracks and holes (Fig. 2). *Acremonium* sp., *Aspergillus niger*, *Phoma* sp., *Trichoderma* sp., and *Ulocladium* sp. were also able to create fissures, however with a lower efficiency. In addition to this, scanning electron microscopy observations and associated elemental microanalysis with energy dispersive spectroscopy of fungal biomass grown on gold sheets showed Au accumulation associated to the fungal mycelium (Fig. 3) (Bouquet, 2013).

3.2.1.2 Bioleaching (Biocyanidation, Cyanide Degradation)

Besides their mechanical capabilities, fungi are able to solubilize Au through the production of cyanides. Cyanidation is largely used in the

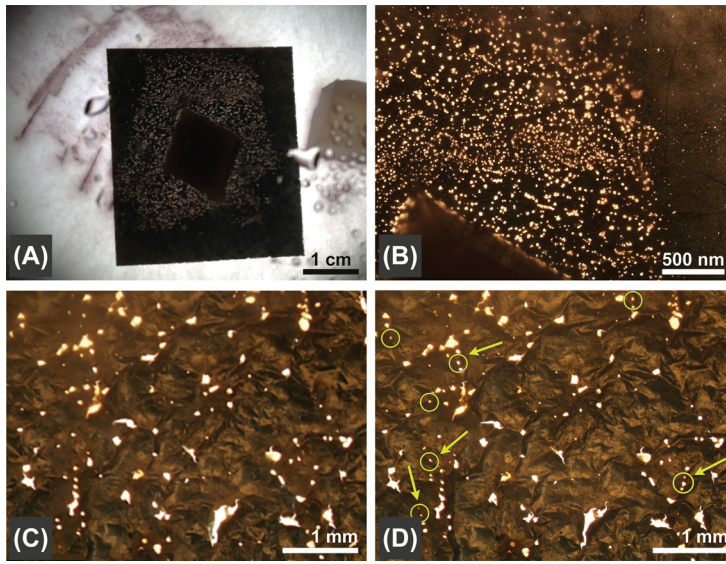

Figure 2 Images showing fissures and cracks created by fungal strains growing over a pure gold sheet overlying a malt agar medium. (A and B) *Fusarium* sp. grown for 83 days, (A) macroscopic photograph and (B) stereoscope observations showing a detail of the right-hand corner of the colonized gold sheet in A. (C and D) Stereoscope observations of the exact same area of a gold sheet colonized by *Aureobasidium pullulans* after (C) 83 days and (D) 167 days, *yellow arrows* show the apparition of new cracks.

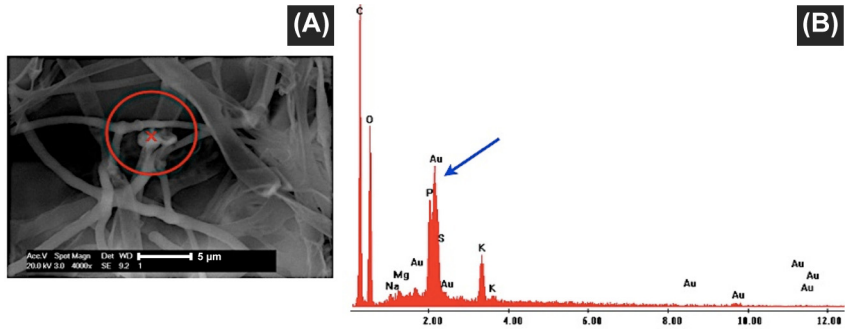

Figure 3 Gold mobilization associated with the presence of the mycelium of *Epicoccum* sp. growing over a pure gold sheet overlying a malt agar medium after 92 days of incubation at room temperature. (A) Scanning electron image of the mycelium, the *red mark* on the image indicates the area analyzed with energy dispersive spectroscopy. (B) Spectrogram showing the presence of gold (*blue arrow*) associated to the fungal hyphae.

hydrometallurgical recovery of gold (Cui & Zhang, 2008). To avoid the toxicity of this compound, a biotechnological approach using cyanogenic organisms has been proposed (Brandl, Lehmann, Faramarzi, & Martinelli, 2008). Natural production of hydrogen cyanide (HCN) by microorganisms seems to be common. In natural systems, HCN is used by microorganisms as an antimicrobial compound against competing microorganisms (Brandl et al., 2008; Knowles, 1976). Microbial HCN has been described for the first time in 1871 by Von Lösecke in the fruiting body of the Basidiomycete fungus *Marasmius oreades* (Knowles, 1976). Since then, several other fungi have been identified as producing HCN, either in their fruiting bodies or associated to their mycelial growth. As reviewed in Knowles (1976), several reports exist on cyanogenic fungi. For instance, several strains of *Clitocybe*, *Pholiota*, *Polyporus*, and *Tricholoma* are mentioned as producing HCN (Bach, 1956) as well as 300 Basidiomycota species from 52 genera (Locquin, 1944). As a result, HCN production seems to be distributed in diverse strains of higher fungi, providing a choice of organisms to be used for the mobilization of Au with microbially produced HCN.

Several cyanogenic bacteria able to mobilize Au and other precious metals from e-waste have also been described, such as *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Escherichia coli* (Brandl et al., 2008). *C. violaceum* has been used in several studies to leach Au from ores (Lawson, Barkhuizen, & Dew, 1999) or from printed circuit boards of mobile phones scraps (Chi, Lee, Pandey, Yoo, & Jeong, 2011).

In liquid cultures amended with solid Au, this bacterium is able to form stable aurocyanide complexes $[Au(CN)_2]^-$ (Aitimbetov, White, & Seth, 2005). In this species, HCN is produced continuously in small amounts with a peak in production at the beginning of the stationary phase. The kinetics of HCN production by four fungal species (*A. niger*, *Pleurotus ostreatus*, *Grifola frondosa*, and *M. oreades*) was different. In *G. frondosa* and *M. oreades*, HCN was produced continuously during 47 days after a lag phase of only 14 h; in *A. niger* and *P. ostreatus*, a continuous HCN production was also observed, however the lag phase was of 18 days (Bouquet, 2014).

Besides their ability to produce HCN, both bacteria and fungi are also able to use HCN as a carbon and nitrogen source despite its toxicity. Several fungal species can degrade cyanide complexes, leading to the release of its metal moiety. For instance, *Fusarium solani* and *Trichoderma polysporum* can use metal cyanides as a nitrogen source to sustain their growth (Barclay, Hart, Knowles, Meeussen, & Tett, 1998). In addition, other fungal species have been described as being able to decompose cyanide complexes: *Rhizopus oryzae* (Padmaja & Balagopal, 1985), *F. oxysporum* (Pereira, Pires, & Roseiro, 1999), *Trichoderma* spp. (Ezzi & Lynch, 2005), *Phanerochaete chrysosporium*, *Pleurotus sajor-caju*, *Trametes versicolor* (Cabuk, Unal, & Kolankaya, 2006), *Ganoderma applanatum*, *Ganoderma lucidum*, *Pleurotus eryngii*, *Polyporus arcularius* (Özel et al., 2010).

Interestingly, when grown in 3.5 mM $KAu(CN)_2$, two strains (*Epicoccum* sp. and *Fusarium* sp.) were able to transform the complexed Au directly into AuNPs as demonstrated by UV-vis spectroscopy and transmission electron microscopy (Fig. 4) (Bouquet, 2014).

As a result, in a biohydrometallurgical process, cyanogenic microorganisms be used to leach Au (Brandl et al., 2008) and cyanotrophic fungi to release Au from aurocyanide complexes (Aitimbetov et al., 2005). Finally, to recover Au(0), Au(I) can further be reduced through a hydrometallurgical physicochemical method. Alternatively, fungal species able to transform Au from aurocyanide complexes directly into AuNPs could represent an interesting biotechnological approach.

3.2.1.3 Gold Biosorption

Biosorption of Au to fungal surfaces has been studied in the frame of metal removal from aqueous solutions and mainly from wastewaters (Ting & Mittal, 2002). Several functional groups present at the outer layer of the fungal cell wall can bind Au(III), such as carboxyls, amines, phosphates, and sulfhydryl groups, for instance (Gadd, 2007). Several studies have

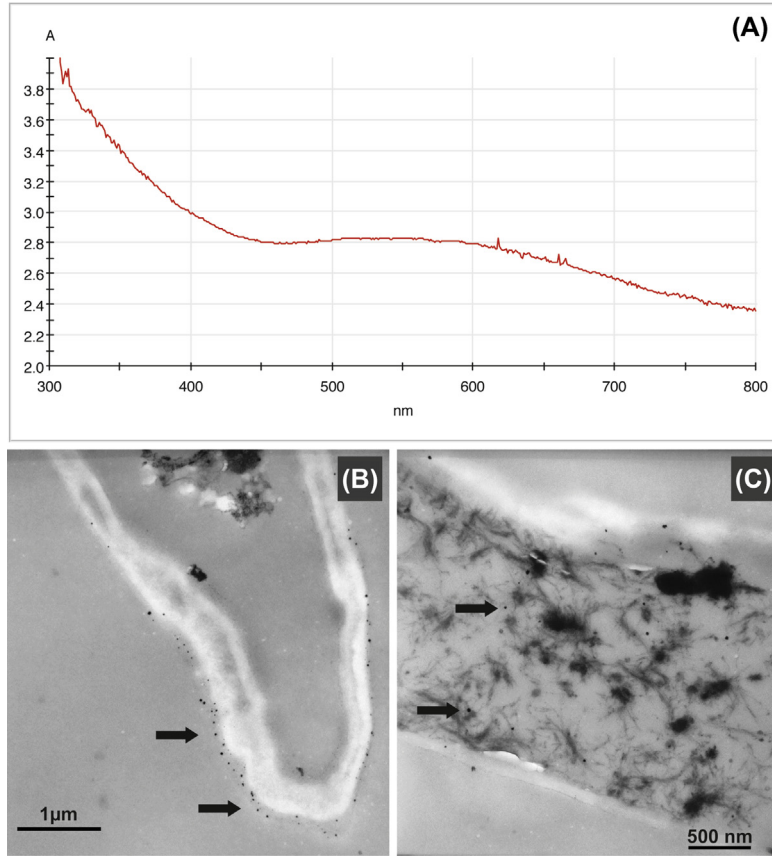

Figure 4 Gold nanoparticle (AuNPs) formation by *Epicoccum* sp. grown in 3.5 mM $Kau(CN)_2$ during 43 days. (A) UV-vis spectrogram showing the characteristic plasmon resonance of AuNPs between 500 and 600 nm. (B and C) Transmission electron microscope micrographs showing AuNPs at (B) the surface of the fungal cell wall and (C) inside the cell (*arrows*).

demonstrated the effectiveness of dead fungal biomass as a biosorbent for Au ions or other precious metals. Alkali-treated biomass of *A. niger* could bind Ag(I) up to 10% of its dry weight (Naseem Akthar, Sivarama Sastry, & Maruthi Mohan, 1995). The biomass of *Cladosporium cladosporioides* mixed with keratinous material and further granulated could bind up to 100 mg of Au(III) from $KAu(CN)_2$ per gram of biosorbent. The pH however was critical to attain this result with an efficient biosorption at a pH range between 1 and 5. In terms of efficiency, 80% biosorption of Au(III) to the fungal keratin beads could be attained in only 20 min

(Pethkar & Paknikar, 1998). Dried and pulverized biomass from the fruiting body of the bracket fungus *Fomitopsis carnea* was used to prepare both polyvinyl alcohol and calcium alginate beads. The initial metal and fungal biomass appear as critical factors for the efficiency of gold biosorption (Khoo & Ting, 2001). pH is also in this case a critical factor to optimize the efficiency of Au(III) biosorption with a maximum observed at pH 3.9 (Ting & Mittal, 2002). Based on the previous examples, biosorption efficiency thus depends upon the concentration of the sorbate and the biosorbent used, as well as on the pH. Other factors that matter are the pretreatment history of the biosorbent, the presence of other metals, as well as temperature. All of which can dramatically alter the biosorption efficiency (Fomina & Gadd, 2014). Besides Au biosorption, these systems should also be easily eluted to recover the biosorbed Au. Naseem Akthar et al. (1995) showed that the bound Ag(I) could be fully recovered by elution with dilute HNO_3. The biomass could be further regenerated using a Ca^{2+}/Mg^{2+} solution. Elution or desorption remains a critical step of metal recovery to avoid the use of toxic compounds as well as to preserve the biosorbent (Fomina & Gadd, 2014).

3.2.1.4 Mechanisms of Gold Nanoparticles Biomineralization by Fungi

Fungi are able to accumulate Au from chlorauric acid solutions ($HAuCl_4$) both intra- and extracellularly. Au(III) reduction in Au(0) nanoparticles (AuNPs) can occur both within the cell wall but also inside cell compartments, such as the plasma membrane, the cytoplasm, and/or the nucleus (Das et al., 2012; Du et al., 2011). For instance, the 10 following species were able to form AuNPs when grown in 2 mM $HAuCl_4$: *A. niger*, *A. pullulans*, *Epicoccum* sp., *F. oxysporum*, *G. frondosa*, *M. oreades*, *Penicillium* sp., *P. ostreatus*, *Rhizopus* sp., *Verticillium* sp. All strains were able to produce AuNPs intracellularly and two strains also produced AuNPs extracellularly (*A. niger* and *Rhizopus* sp.). Intracellular AuNPs had smaller sizes as compared to extracellular AuNPs (Bouquet, 2014) (Table 3; Fig. 5). In *Epicoccum* sp. and *Verticillium* sp. AuNPs synthesis occurred only after 10 min of growth, suggesting a passive mechanism related to the basal fungal metabolism. For all other fungal strains, AuNPs occurred after 3–4 days of growth, indicating that it could be induced by a specific metabolic response. On the other hand, extracellular AuNPs synthesis occurred after a shorter time, i.e., after 18 h. This shorter lag phase combined with larger nanoparticles indicates that the synthesis of these extracellular fungal AuNPs is probably triggered by some extracellular fungal metabolites, but that physicochemical parameters leading to Au precipitation are also essential.

Table 3 Summary of the different fungal species known to synthesize gold nanoparticles (AuNPs) with AuNPs mean sizes, location, and morphologies

Fungal species	AuNPs size (nm) mean	AuNPs location				AuNPs morphology							References
		Intra-cellular	Within cell wall	Cell wall surface	Extra-cellular	Spherical	Trigonal	Rods	Trapezoidal	Rhombohedral	Hexagonal	Aggregated	
Marasmius oreades	10	X	X			X	X						Bouquet (2014)
Fusarium oxysporum	20	X	X			X							
Epicoccum sp.	30	X	X			X							
Grifola frondosa	30	X	X			X	X	X					
Pleurotus ostreatus	30	X	X			X			X				
Rhizopus sp.	30	X	X			X	X	X		X	X		
Rhizopus sp.	130				X							X	
Penicillium sp.	40	X	X			X	X	X			X		
Aureobasidium pullulans	50	X	X			X							
Verticillium sp.	50	X	X			X	X	X					
Aspergillus niger	60	X	X			X	X	X	X	X	X		
A. niger	100				X							X	
A. niger	13				X	X							Bhambure et al. (2009)
Rhizopus oryzae	15	X	X										Das et al. (2012)
Epicoccum nigrum	5—50	X	X			X		X					Sheikhloo et al. (2011)
Verticillium sp.	25	X		X		X					X		Mukherjee et al. (2001)
F. oxysporum	30				X	X	X						Mukherjee et al. (2002)
Trichoderma koningii	35				X	X	X				X		Maliszewska, Aniszkiewicz, and Sadowski (2009)
Penicillium sp.	45	X		X		X							Du et al. (2011)
Penicillium sp.	50				X	X							Du et al. (2011)
Phoma macrostoma	150	X				X							Sheikhloo and Salouti (2012)
Hormoconis resinae	n.d.				X	X					X		Mishra, Bhadauria, Gaur, and Pasricha (2010)

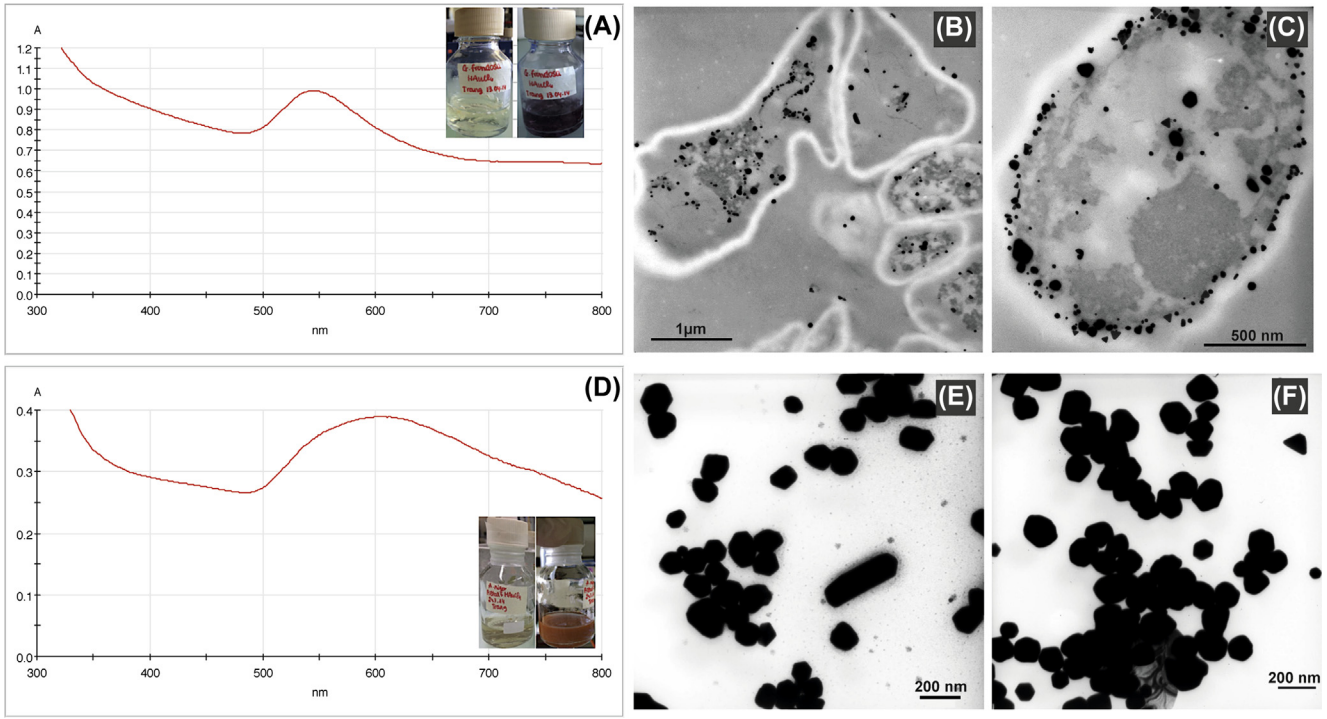

Figure 5 Gold nanoparticle (AuNPs) formation by *Grifola frondosa* (A—C) and *Aspergillus niger* (D—F) grown in 2 mM $HAuCl_4$. (A) UV-vis spectrogram showing the characteristic plasmon resonance of AuNPs between 500 and 600 nm and (B and C) transmission electron microscope micrographs showing intracellular AuNPs formed in *G. frondosa* after 4 days of incubation. (D) UV-vis spectrogram showing the characteristic plasmon resonance of AuNPs between 500 and 600 nm and (E and F) transmission electron microscope micrographs showing extracellular AuNPs formed by *A. niger* after 18 h of incubation. (A and D) Photographs showing the change in color of the culture media as a result of AuNPs synthesis, right-hand side images show culture media with $HAuCl_4$ as a source of Au for AuNPs synthesis and left-hand side images show culture media without $HAuCl_4$.

Two mechanisms of Au(III) reduction through fungal activity have been described for the fungus *R. oryzae*. In the first mechanism, Au(III) ions are adsorbed on the fungal cell wall by electrostatic interactions and are directly reduced by proteins present within the cell wall leading to the synthesis of AuNPs. The reduction of Au(III) to Au(0) seems to occur stepwise. First, Au(III) binding to the cell wall and subsequent reduction to Au(I) seem to occur physicochemically. Further, Au(I) reduction to Au(0) seems to be the result of a biochemically driven pathway. In the second mechanism, Au(III) ions are transported into the cytoplasm and are enzymatically reduced to form AuNPs. In this mechanism, the involvement of Au(I) intermediates is also suggested (Das et al., 2012).

Fungal AuNPs seem to be highly dispersed within fungal biomass, whether they were synthesized inside or outside the cell. Their stabilization seems to occur through organic substances secreted by the fungus (Bhambure, Bule, Shaligram, Kamat, & Singhal, 2009; Du et al., 2011).

The size of fungal AuNPs depends on several factors and varies from 13 to 150 nm. Similarly, AuNPs can exhibit various shapes (Table 3). Availability of Au(III) ions, concentration of $HAuCl_4$, reaction rate, and the fungal species used are the main factors involved for the size and shape of AuNPs (Du et al., 2011). However, high Au(III) ionic strength can have deleterious effect on fungal growth rather than promoting AuNPs synthesis. This is due to toxic effects of Au(III) ions resulting in inhibition of protein synthesis and damage to the cell ultrastructure, directly involved in a subsequent lower AuNPs synthesis as demonstrated by Das et al. (2012). However, when converted to AuNPs, Au does not have any toxic effect anymore as demonstrated by an unaltered growth of fungi-accumulating AuNPs. As a result, AuNPs synthesis is an efficient way of alleviating Au(III) toxicity with great biotechnological potential.

3.2.2 Fungal Biotechnology for Gold Biorecovery in E-waste

A biotechnological approach to effectively use fungi for Au recovery from e-waste should take into account the following points. First, the fungi to be used should be able to cope with a high concentration of other toxic metals, mainly Cu, but also Ni and Pb, for instance. In an experiment targeted at investigating how different fungi could grow in presence of e-waste, several strains were tested for growth in presence of a microprocessor scrap. In liquid media with sodium thiosulfate to enhance Au mobilization and malt as a carbon source, all the strains tested affected the surface aspect of the scrap as compared to the uninoculated control, suggesting that they were able to mobilize part of the scrap components

(Fig. 6). In malt agar media, fungal colonization of microprocessor scraps occurred, even though they did not contain any nutrient, as a result of the fungal exploratory lifestyle (Fig. 7). However, most strains had a very slow growth as a result of high levels of Cu. As a result, this first step requires an intensive screening of fungal strains able to cope with high levels of toxic metals. Second, the fungus or fungi selected should have a high affinity for Au to reach a certain efficiency level of Au biorecovery. For instance, in an experiment looking at fungal growth on microprocessor scrap, two strains, *A. niger* and *G. lucidum*, were able to immobilize Ni as crystals linked to fungal biomass. This demonstrates that each fungal strain has specific capabilities toward metal mobilization and immobilization. Therefore, the second step includes a rigorous screening of fungal affinity toward different metals to select specifically for Au recovery. Alternatively, applying different fungal species with different metal affinity to sequentially recover different metals from e-waste could be another approach. This way, metals could be recovered individually. Finally, the fungus or fungi should be able to first mobilize Au from e-waste through leaching processes and second to ideally immobilize it in a form that is easily recovered through biomineralization processes (AuNPs synthesis) or biosorption.

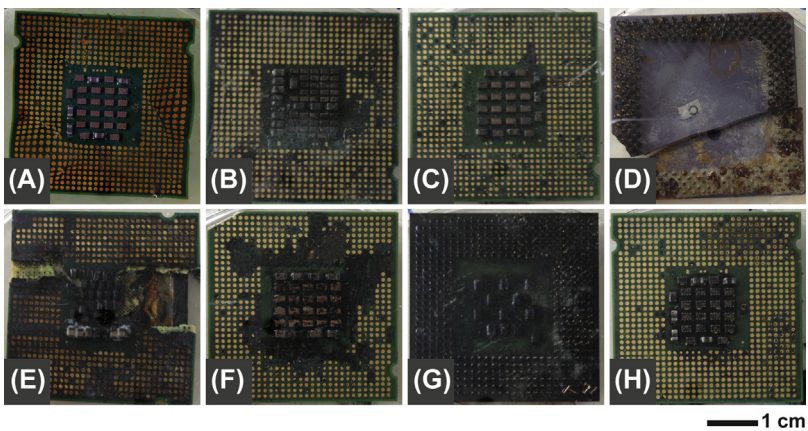

Figure 6 Surface aspect of microprocessors incubated in malt (12 g/L) supplemented with Na-thiosulfate (70 g/L) after 176 days of incubation and showing different degree of fungal colonization as well as color modification of the different materials as a result of fungal metabolism. (A) Uninoculated control, (B) *Acremonium* sp., (C) *Aspergillus niger*, (D) *Beauveria bassiana*, (E) *Fusarium* sp., (F) *Penicillium* sp., (G) *Rhizopus* sp., (H) *Trichoderma* sp.

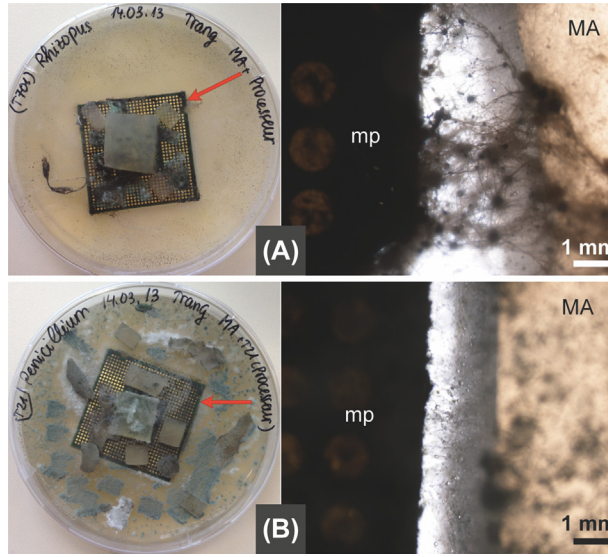

Figure 7 Photographs showing fungal colonization of a microprocessor scrap by hyphae stemming out of a malt agar culture medium after 6 days of incubation. (A) *Rhizopus* sp. and (B) *Penicillium* sp. Left-hand side images show the whole Petri dish setup and right-hand side images show a close-up of the area indicated by a *red arrow*. *MA*, malt agar medium; *mp*, microprocessor.

4. CONCLUSION

The recovery of Au from e-waste is critical for several reasons. First, regarding the environmental impact of both gold ore mining and traditional gold recycling; and second, as a result of the economic and geopolitical importance of this raw material. As compared to physicochemical processing (pyro- and hydrometallurgy), lower recovery rates as well as slower kinetics of the process deter the application of biometallurgical processing. This is a direct result of the physicochemical conditions in which the microorganisms used are developing. For instance, hydrogen cyanide production by cyanogenic microbes is a function of pH, temperature, pO_2, and the presence of glycine as a nutrient (Chi et al., 2011). Therefore, two ways to improve Au biorecovery from e-waste using cyanogenic microorganisms are (1) selecting either a single microorganism, or a complex microbial consortium, that is able to efficiently interact with Au; and (2) selecting the best physicochemical conditions for a given organism (or consortium) to improve both the recovery rate and the kinetics of the process. Besides the use of cyanogenic

microorganisms, cyanide-degrading and AuNPs-synthesizing microorganisms also represent interesting alternatives for the biorecovery of Au from e-waste. As a matter of fact, AuNPs have particular physicochemical properties and therefore represent strategic materials in different fields (medical applications such as drug delivery, electronic conductors, catalysis) (Dykman & Khlebtsov, 2011; Homberger & Simon, 2010). As a result, having the ability to transform Au from e-waste into valuable AuNPs using only microorganisms is of the greatest interest in terms of biotechnology. Using fungi for the biorecovery of gold has several advantages. First of all, filamentous fungi are modular organisms that exhibit a tridimensional lifestyle that is very well adapted to heterogeneous mixtures such as e-waste. Second, fungi represent a very diverse group of microorganisms in which metal tolerance is widespread. In addition to this, it is well known that metal tolerance is an adaptation to the presence of metals in their direct environment (Gadd, 2007). As a result, intensive screening, as well as selection of efficient fungal strains is an easy (though time-consuming) task. Furthermore, since fungi are heterotrophic organisms, a carbon source is required to sustain their growth. Food and agricultural waste could be valorized for this (Fomina & Gadd, 2014). In conclusion, harnessing fungal metabolism, either from single organisms or from complex consortia, for the biorecovery of gold is a promising biotechnological field and surely many new exiting discoveries are ahead within the next decade.

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CHAPTER THREE

Stone-Eating Fungi: Mechanisms in Bioweathering and the Potential Role of Laccases in Black Slate Degradation With the Basidiomycete *Schizophyllum commune*

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Contents

Abstract

Many enzymes, such as laccases, are involved in the saprotrophic lifestyle of fungi and the effects of those may be linked to enhanced bioweathering on stone surfaces. To test this hypothesis, we studied the decomposition of kerogen-enriched lithologies, especially with black slate containing up to 20% of C_{org}. Indeed, a formation of ditches with attached hyphal material could be observed. To address enzymes involved, proteomics was performed and one group of enzymes, the multicopper oxidase family members of laccases, was specifically investigated. A role in bioweathering of rocks containing high contents of organic carbon in the form of kerogen could be shown using the basidiomycete *Schizophyllum commune*, a white rot fungus that has been used as a model organism to study the role of filamentous basidiomycete fungi in bioweathering of black slate.

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1. INTRODUCTION

Bioweathering of rocks is an important field in geomicrobiology (Burford, Fomina, & Gadd, 2003; Gadd, 2010; Konhauser, 2009). The mechanisms involved, such as hyphal penetration resulting in enhanced biomechanical weathering, or the production and excretion of organic and inorganic acids as well as siderophores leading to enhanced biochemical weathering, can effect different rocks and minerals (Favero-Longo, Castelli, Fubini, & Piervittori, 2009; Hoffland et al., 2004; Sterflinger, 2000). However, other mechanisms involved in bioweathering such as the secretion of enzymes and their contribution to weathering are not fully understood and it is not clear, which enzymes may take part in the degradation process. Within the kingdom of fungi, especially basidiomycetes secrete a broad range of enzymes for the degradation of organic matter. They are able to degrade complex structures such as lignocellulose and can cause white or brown rot in wood (Cragg et al., 2015; Fernández-Fueyo et al., 2016; Sánchez, 2009; Sergentani, Gonou-Zagou, Kapsanaki-Gotsi, & Hatzinikolaou, 2016). Since organic fractions in stones such as black slates are forming supramolecular structures resembling lignin, the investigation of the activities of a white rot fungus seems indicated. Thus, it seems appropriate to address enzymes produced when the fungus is growing in the presence of kerogen-containing rock material. A molecular model organism of this class of basidiomycetes is *Schizophyllum commune* (Ohm et al., 2010). A prominent enzyme involved in lignin degradation excreted by *S. commune* is laccase (EC 1.10.3.2). This multicopper oxidase exhibits a broad substrate spectrum and is involved in the oxidative degradation of other substrates beyond lignin. Therefore, laccases may play an active role in the degradation of rocks and minerals.

2. BIOWEATHERING

Living organisms may induce the erosion, decay, and decomposition of rocks and minerals, a process defined as bioweathering (Burford et al., 2003). Rock-inhabiting organisms such as cyanobacteria, algae, fungi, and lichens are well known for their degradation potential (Dakal & Cameotra, 2012). The different mechanisms by which they attack and decompose different rocks can be roughly classified into biomechanical and biochemical weathering.

The first class of bioweathering organisms uses the ability of hyphae to penetrate rocks or mineral surfaces and break down their structures. Since turgor pressure can be created, a mechanical stress on cracks can result in widened spaces. Another component linked to this process is extracellular mucilaginous material (ECMM), basically consisting of polysaccharides, that is spread over the stone surface during growth. When ECMM infiltrates small pores and cavities, its repeated shrinking and swelling upon drying and rewetting induces mechanical stress results in erosion and rock deterioration (Clipson & Gleeson, 2012; Fomina et al., 2007).

Since ECMM can also contain rock degrading substances, biomechanical weathering is usually closely connected to biochemical weathering. The latter is believed to be the major factor in stone biodeterioration. Fungi and bacteria produce and excrete a wide range of substances with which they can attack rocks in a specific or nonspecific process to solubilize minerals, partly for nutrition with, e.g., phosphate. Inorganic acids such as nitric and sulfuric acids as well as organic acids such as citric and oxalic acids are believed to be major components in mineral diagenesis and dissolution (Ehrlich, 1998). Additionally, during microbial respiration, the CO_2 generated can interact with stones and lead to a further acidification of the fungal environment by carbonic acid attack (Gadd, 2007; Sterflinger, 2000).

Metal-complexing metabolites such as siderophores are secreted to bind ferric iron, which can subsequently be reduced and absorbed by fungi. In addition, extracellular enzymes can be involved in rock and mineral degradation containing high amounts of organic matter. The different types of biochemical weathering mainly occur at short distances around the hyphae and allow the organism to take up mobilized nutrients, including carbon compounds and metals, used for fungal nutrition or even energy metabolism. The effect of biochemical weathering leads to changes in the microtopography of minerals and can be seen by etching and pitting.

The emerging tunnel formation is widespread and was demonstrated for several minerals and rocks, e.g., plagioclase feldspars, all minerals in granite, graphite, black slate, and even ivory (Jongmans et al., 1997; Pinzari, Tate, Bicchieri, Rhee, & Gadd, 2013; Romão & Rattazzi, 1996). If the fungus is removed from the mineral, the resulting tunneling structures have shapes similar to fungal hyphae including, for instance, on graphite samples, lateral branches (Fig. 1). The mineral surface can be visualized using vertical scanning interferometry and atomic force microscopy, both revealing

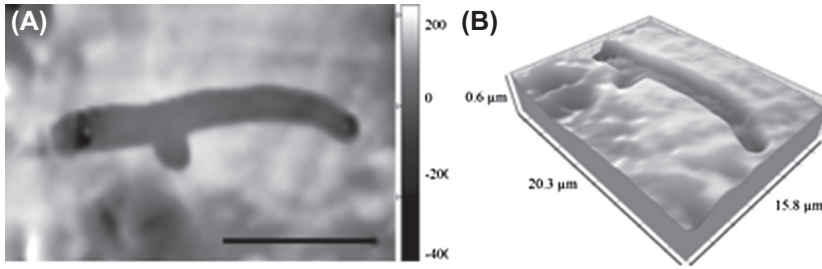

Figure 1 Hyphal tunneling of the fungus *Schizophyllum commune* on graphite surface visualized using vertical scanning interferometry (A) and 3D models (B); scale bar = 10 µm.

extents of etching and pitting as well as resulting changes in the microtopography of the mineral. Interestingly, the wood-rotting basidiomycete *S. commune* showed dissolution effects in the surrounding areas of the hyphae (Fig. 2). This extended biochemical weathering exemplifies the potential significance of secretions from wood-rotting fungi in biogeochemical cycles.

During the growth of *S. commune* on black slate, lateral structures with sizes of 1−2 µm length and 400−500 nm width, which are not representing typical hyphal branches, could be detected (Fig. 3). Partly, they produce elongated excretions which stick to the rock surface. Therefore, it may be assumed that these excretions provide the fungus with a better attachment to the rock surface. With this matrix, a transfer of biochemicals or enzymes needed for mineral dissolution or to promote the fungal uptake of nutrients seems likely.

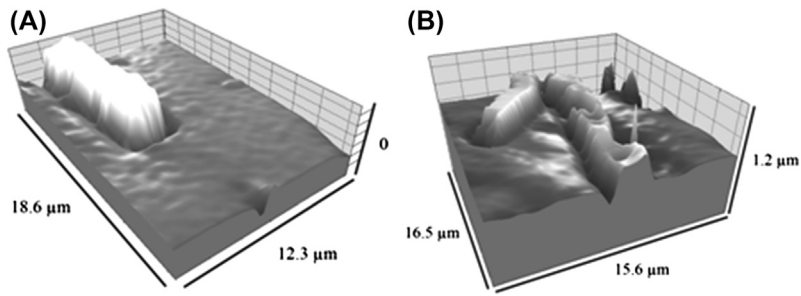

Figure 2 Hyphal tunneling of the fungus *Schizophyllum commune* on graphite (A) and black slate (B) surfaces visualized using vertical scanning interferometry 3D models.

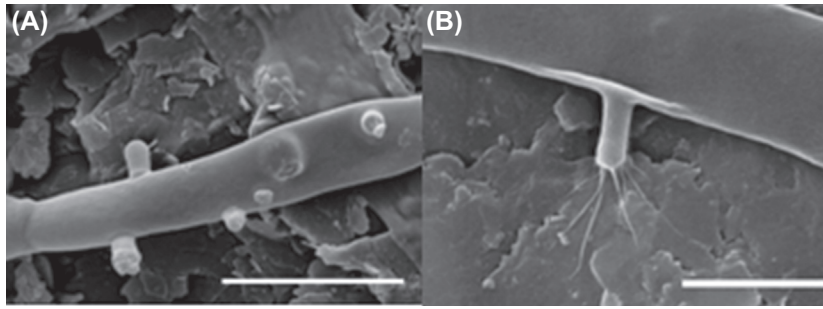

Figure 3 Lateral structures of *Schizophyllum commune* with excretion sticking to black slate surfaces visualized using scanning electron microscopy; scale bars = 5 μm (A) and 2 μm (B).

3. ECOLOGICAL SIGNIFICANCE OF ROCK-INHABITING FUNGI

The importance of organisms and their degradation potential were described for the attack of rocks by lichens, a mutualistic symbiosis of fungi and algae or cyanobacteria (Sollas, 1880). A role of fungi can also be deduced from their occurrence on a wide range of rocks, including limestone, sandstone, marble, granite, and basalt (Diakumaku, Gorbushina, Krumbein, Panina, & Soukharjevski, 1995; Sterflinger, 2000; Verrecchia, 2000). The microbial colonization of rocks depends on various environmental factors such as water availability, pH, climatic exposure, and nutrient sources. Furthermore, petrological parameters such as mineral composition, porosity, and permeability determine the rate of microbial establishment (Warscheid & Braams, 2000). Many organisms adapt to such conditions, and an enhanced bioweathering can be observed (Gorbushina, 2006).

Bioweathering plays considerable roles in the environment. Fungal activity dissolves rocks and minerals and leads to the release of elements and metals, whereby it also influences biogeochemical element cycles of, e.g., C, N, S, and P, and the atmospheric composition through the excretion of metabolic products such as CO_2 and CH_4 (Ehrlich, 1998). A study investigating the bioweathering potential of the basidiomycete fungus *S. commune* demonstrated the release of Fe, Mn, and Ni during black slate degradation (Wengel, Kothe, Schmidt, Heide, & Gleixner, 2006). An additional increase of dissolved organic carbon in the culture media could be observed due to the high amount of organic matter in black slate. But bioweathering can

concomitantly cause the mobilization of nonessential metals which are spread into the environment and may cause contamination of soil and groundwater.

For building stones, fungal contaminations such as mold infestations within residential buildings can cause serious problems for human health, and the fungal attack leads to undesirable mechanical, physical, and chemical modifications on historic monuments and artworks (Dakal & Cameotra, 2012; Gorbushina & Broughton, 2009; Sterflinger, 2010). Fungi have been isolated, often in association with bacteria or as a symbiotic partner in a lichen, from the Roman Catacombs (Italy), the Cathedrals of Salamanca and Toledo (Spain), Mayan buildings (Mexico), the Haji Mehmet Fountain (Turkey), or from the ruins of Angkor temples (Cambodia; Bartoli et al., 2014; Dakal & Cameotra, 2012; Gaylarde, Gaylarde, Guiamet, de Saravia, & Videla, 2001).

In addition to biodeterioration, an accompanying fungal induced discoloration, due to biogenic oxidation of mineralic iron or manganese, or staining, as a result of biogenic pigments (e.g., melanin), can occur on the surface of building stone material and reduce the aesthetic property of historic buildings (Grbić, Vukojević, Simić, Krizmanić, & Stupar, 2010; Warscheid & Braams, 2000). To preserve historic sites and to reduce their decay and the release of toxic elements into the environment, a closer look on the mechanisms involved in the dissolution of rock material is necessary.

4. GENES INVOLVED IN ROCK DEGRADATION

Two fungal strains isolated from brown coal, one producing Mn-peroxidase and the other one laccases and peroxidases, were shown to secrete oxidative enzymes shortly before the coal started to dissolve (Willmann & Fakoussa, 1997). The addition of coal powder to the growth medium enhanced fungal growth, implicating that nutrients and organic carbon were mobilized from the coal and utilized for fungal metabolism. This demonstrates the role of laccases and peroxidases as microbial enzymes in coal degradation. However, the addition of one purified exoenzyme failed to lead, in the absence of the fungus, to a solubilization of coal. Based on this, the authors concluded that either more than one enzyme causes coal degradation, or the investigated enzymes act as mediators and coactivate other enzymes.

Other enzymes studied with bacteria have also been shown to play a role in bioweathering. A silicate weathering *Bacillus mucilaginosus* was used to study molecular mechanisms in potassium mineral weathering (Xiao, Lian, & Shao, 2012). There, secreted proteins (glycine hydroxymethyltransferase, phosphoserine aminotransferase, ketol-acid reductoisomerase, and an ABC transporter) were induced in presence of K-containing rock powder. These changes in metabolism in *B. mucilaginosus* show a direct correlation between secreted proteins and mineral weathering. A similar response to the addition of stone could be shown with the fungus *S. commune,* where an aminotransferase and an ABC transporter were solely produced in the presence of black slates (data not shown).

Another study investigated changes in gene expression of carbonic anhydrase and cysteine synthase, when the ascomycetous fungus *Aspergillus niger* was grown with K-bearing silicate minerals (Sun et al., 2013). An upregulation of both genes was demonstrated in presence of silicate minerals. For *Aspergillus fumigatus*, upregulation of genes involved in carbon consumption (24%), stress response (4%), acid production (8%), metal ion binding (6%), nitrogen metabolism (8%), cytochromes or oxidoreductases (7%), and genes participating in nucleic acid metabolism (3%) was shown (Xiao, Lian, Sun, & Shao, 2012). The upregulation of carbonic anhydrase supports the view that *A. fumigatus* accelerates the weathering of minerals through carbonic acid production. The induction of proteins involved in stress response and metal binding regulates the absorption of metal ions and promotes their storage.

Although it is known that excreted enzymes take part in bioweathering of rocks and minerals, less information is available on the types of participating enzymes. To discover genes and proteins and to determine the molecular mechanisms involved in the biodegradation processes, *omics* techniques hold great potential (Haferburg & Kothe, 2010). Genes involved in bioweathering of basalt were investigated using the heavy metal—resistant bacterium *Cupriavidus metallidurans* (Olsson-Francis, Van Houdt, Mergeay, Leys, & Cockell, 2010). Porins and transporters, both presumably involved in Fe^{2+} uptake, were found to be induced by basalt. Furthermore, genes participating in biofilm formation and heavy metal resistance were upregulated.

To identify new genes for bioweathering with *S. commune,* microarrays were used based on the genome of this fungus which has revealed multiple enzymes for the degradation of complex biomolecules including lignin (Ohm et al., 2010). This makes it an ideal organism to study its molecular

mechanisms during the degradation of black slate. In total, 13,181 genes were tested for differential regulation during growth on black slate. About 730 genes (5.5%) were solely expressed in the presence of black slate, 362 genes (2.7%) were upregulated, and 400 genes (3.0%) downregulated when *S. commune* was grown with black slate (cutoff with a fold-change ≥ 4; Table 1).

Most upregulated genes belong to oxidoreductase and hydrolase families, enzymes known to be required for lignin and cellulose degradation, respectively (Baldrian & Valášková, 2008; Martínez et al., 2005). Within oxidoreductases and hydrolases, genes such as a multicopper oxidase (MCO), an intradiol ring—cleavage dioxygenase distantly related to a laccase from ascomycetes, a phenol 2-monooxygenase, and different glycoside hydrolases were identified. These results suggest that enzymes involved in lignocellulose degradation are related to enzymes needed for black slate degradation. Interestingly, a lectin, which is responsible for sugar moiety recognition and is involved in attachment and penetration of organic matter (Imberty, Mitchell, & Wimmerová, 2005; Khan & Khan, 2011), was found to be upregulated indicating its potential role in organic matter decomposition and fungal adhesion to the stone surface.

Other than these, genes potentially involved in organic acid production were detected, some of which involved in the citric acid cycle (D-aspartate oxidase, holo-[acyl-carrier protein], aconitase, and isocitrate dehydrogenase). Citrate, aconitate, isocitrate, succinate, fumarate, and malate produced in the citric acid cycle may be participating in acidic bioweathering of black slates, while the holo-[acyl-carrier protein] is involved in coenzyme A biosynthesis, a cofactor for, e.g., citric acid cycle enzymes. D-aspartate oxidase catalyzes the formation of oxalate as an anapleurotic reaction by generating oxaloacetate, ammonia, and hydrogen peroxide. These products are known to attack, oxidize, and even dissolve minerals and metal ions from rocks and ore surfaces (Ehrlich, 2006; Gadd, 2010; Nooshabadi & Rao, 2014). In addition, CO_2 is produced which, in turn, might promote a further degradation of black slate by the formation of carbonic acid (Gadd, 2016).

Other upregulated genes involved in acid production independent of the citric acid cycle were identified with arginase and urease. Arginase hydrolyzes agmatine to putrescine and urea. The latter is further hydrolyzed by urease to ammonia and carbamate, which in turn can be degraded to ammonia and carbonic acid as well. The exceeding production of inorganic and organic acids leads to an acidification within the fungal cell. To stabilize

Table 1 Selected genes upregulated during black slate degradation (more than fourfold upregulation)

Enzyme	Function
Oxidoreductases	
D-aspartate oxidase	Production of oxalacetic acid, NH_3, H_2O_2
Fatty acid desaturase	Environmental stress resistance
Glutaredoxin	Defense against oxidative stress
Intradiol ring—cleavage dioxygenase	Degradation of aromatic compounds
Isocitrate dehydrogenase	Citric acid cycle and carbohydrate metabolism
Isoflavone reductase	Fatty acid and biotin biosynthesis
Isopenicillin N synthase	Oxidation of an organic substrate; Fe binding and/or substrate binding; oxalate oxidase activity
L-iditol 2-dehydrogenase	Zn ion binding; fructose and mannose metabolism
Monodehydroascorbate	2 iron, 2 sulfur cluster binding
Multicopper oxidase	Cu ion binding; substrate oxidation
Phenol 2-monooxygenase	Hydroxylation of phenol, chloro- and methyl-phenol and naphthol; gamma-hexachlorocyclohexane, toluene and xylene degradation
Phosphoadenosine phosphosulfate reductase	Reduction of activated sulfate into sulfite
Holo-[acyl-carrier protein] synthase	Mg ion binding; pantothenate and CoA biosynthesis
Transferases	
Arginase	Formation of putrescine and urea
Hydrolases	
D-tyrosyl-tRNA (Tyr) deacylase	Broad substrate specificity; detoxification
Formamidopyrimidine-DNA glycolase	Metal ion binding; damaged DNA binding
Glycoside hydrolase, family 5	Degradation of cellulose and xylans
Glycoside hydrolase, family 20	N-glycan degradation
Metallophosphoesterase	Metal binding
Peptidase M1	Metal binding
Peptidase M36, fungalysin	Metal binding
Peptidase S10	Metal binding
Urease	Ni cation binding; formation of NH_3 and carbamate
Molybdopterin cofactor biosynthesis protein MoaC	Metal binding; C, N, and S metabolism

(*Continued*)

Table 1 Selected genes upregulated during black slate degradation (more than fourfold upregulation)—cont'd

Enzyme	Function
Lyases	
Aconitase	Organic acid production
Hydroxymethylglutaryl-CoA lyase	Catalyzes the transformation of HMG-CoA into acetyl-CoA and acetoacetate
Ricin B lectin	Carbohydrate binding; attachment and binding to targets
Triosephosphate isomerase	Carbon fixation; energy production
Isomerases	
Phosphoribosylamine-glycine ligase	Mg ion binding; Mn ion binding; purine biosynthesis
Ligases	
Cl- channel CLC-3 and related proteins	Chloride transport
Others	
HCO_3 transporter	pH regulation
Iron transporter	Iron transport

the acid—base balance, an HCo_3^- transporter is upregulated in presence of black slate. This Na^+-independent Cl^-/HCo_3^- antiporter plays a critical role in intra- and extracellular pH regulation (Alper, 2009; Liu, Yang, & Chen, 2015).

S. commune attacks the rock to gain carbon and nutrients for its growth. In presence of black slates, released elements lead to an increased metabolic activity. This can be seen through the enhanced gene expression of L-iditol 2-dehydrogenase, molybdopterin cofactor biosynthesis protein MoaC and triosephosphate isomerase for an enhanced fructose and mannose, carbon, nitrogen, sulfur, and glucose metabolism (Compagno et al., 2001; Mendel & Bittner, 2006). During the decomposition, many essential and nonessential metals are released from black slate, and *S. commune* requires multiple defense mechanisms to cope with toxic metal stress. Thus, as a response, upregulations of a fatty acid desaturase, involved in environmental stress resistance, and glutaredoxin, participating in oxidative stress defense, were

identified from the microarray data. L-iditol 2-dehydrogenase, monodehydroascorbate, arginase and formamidopyrimidine-DNA glycolase are known for their metal ion binding capacities and molecular repair mechanisms of damaged DNA as well.

5. MULTICOPPER OXIDASES IN THE ENVIRONMENT

White rot fungi are known to secrete high amounts of enzymes including MCOs to degrade lignin. These enzymes are also involved in attacking rocks containing high yields of organic carbon or kerogen (Hoegger, Kilaru, James, Thacker, & Kües, 2006). MCOs, and especially laccases, act unspecifically on a wide variety of substrates and therefore participate in many ecological processes including adsorption of metals, dissolution of existing and formation of new minerals, detoxification of organic and inorganic pollutants, pigment formation, or the transformation of soil organic matter (Baldrian, 2003; Henson, Butler, & Day, 1999; Kunamneni, 2007; Madhavi & Lele, 2009). Through catalyzed oxidation reactions, phenolic molecules can be polymerized, depolymerized, or transformed, and since phenols are toxic for many microbes, their oxidation can affect the activity and *composition of soil microbial communities (Sinsabaugh, Zak, Gallo, Lauber, & Amonette, 2004)*. In addition, laccases are involved in fungal morphogenesis, e.g., vegetative growth and fruiting body formation as well as in stress response and plant pathogenesis (Madhavan, Krause, Jung, & Kothe, 2014; Thurston, 1994). Due to their wide substrate specificity, laccases can also be used in pulp, paper, and textile industries as well as in bioremediation to remove organic pollutants such as PAHs and herbicides (Gianfreda, Xu, & Bollag, 1999; Gochev & Krastanov, 2007; Maruyama, Komatsu, Michizoe, Ichinose, & Goto, 2006; Wu, Teng, Li, Liao, & Luo, 2008).

Laccases catalyze a one-electron oxidation of substrate molecules, accompanied by a simultaneous reduction of molecular oxygen to water (Fig. 4). Since laccases have a very broad substrate spectrum, they can also utilize inorganic compounds, aromatic diamines, polyphenols, and polyamines as a substrate (Baldrian, 2006; Kües & Rühl, 2011). To date, over 100 laccases have been purified from fungal cultures, and the substrate range varies from one enzyme to another (Baldrian, 2006). This, and overlaps in substrate specificity to other enzymes, makes it difficult to differentiate laccases by its reducing substrate. The active center of laccases contains four Cu atoms which are arranged in a three-domain structure.

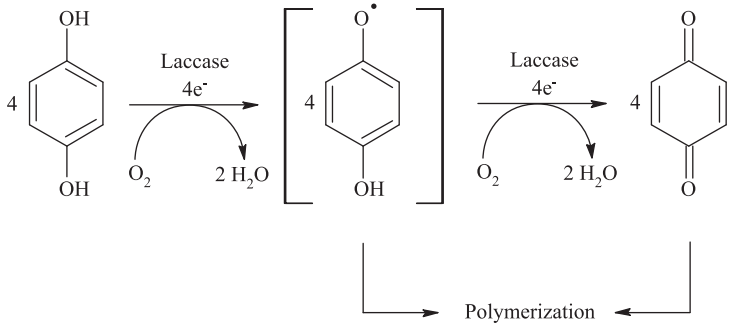

Figure 4 Laccase reaction scheme.

The three different Cu binding sites can be distinguished according to their electron paramagnetic resonance (EPR) (Giardina et al., 2010). Type 1 consists of one Cu atom (Cu1) which exists in its oxidized form and is EPR detectable. It is characterized by a strong absorption (about 600 nm) and gives the laccase its characteristic blue color. Type 2 also contains one Cu atom (Cu2), is colorless and shows only a weak absorption with a different EPR spectrum compared to Cu1. Type 3 is composed of two Cu atoms (Cu3) which are antiferromagnetically coupled ions and EPR-silent. Type 2 and Type 3 are close to each other and form a trinuclear cluster involved in the catalytic mechanism of a laccase reaction (Gochev & Krastanov, 2007). During a laccase-catalyzed reaction, Cu1 serves as the primary electron acceptor site and oxidizes the substrate. The extracted electron is first transferred to Type 1 and then through a Cys—His pathway to the trinucleate cluster which reduces O_2 to H_2O (Solomon, Augustine, & Yoon, 2008).

6. LACCASES IN SOIL AND INTERACTION WITH MINERALS

Soil basidiomycetes, and specifically their laccases, have been shown to play a role in the decomposition of leaf litter and the oxidation and degradation of soil organic matter (Chen, Su, He, Liang, & Wu, 2013; Courty et al., 2009; Luis, Walther, Kellner, Martin, & Buscot, 2004). In soil, minerals and organic matter exhibit strong binding capacities for immobilizing extracellular enzymes (Zimmerman & Ahn, 2010). In particular, soil iron and aluminum adsorb high amounts of laccase produced by the white rot fungus *Trametes versicolor* (Wu et al., 2014). The adsorbed

laccase shows a higher catalytic activity, an extended life span, an enhanced stability to acidic pH, and is more resistant to protease attack compared to an unbound laccase. Bacterial MCOs also participate in metal oxidation. For example, MCOs in *Leptothrix discophora* and *Escherichia coli* were shown to catalyze the oxidation of Mn^{2+} resulting in precipitation of Mn^{3+} and Mn^{4+} oxides (Brouwers et al., 2000; Zhang et al., 2015), the conversion of Cu^{1+} to less toxic Cu^{2+} and a *Pseudomonas aeruginosa* MCO was found to be involved in the oxidation of Fe^{2+} to Fe^{3+} (Huston, Jennings, & McEwan, 2002). Similar features can also be associated with fungi and are linked to the oxidation of Mn^{2+}-bearing rhodochrosite, where attack leads to an accelerated dissolution and surface modification (Tang, Zeiner, Santelli, & Hansel, 2013). Phenol oxidizing enzymes, especially laccases, are produced by a wide range of lichens as well, and a role in phenolic compound transformation and humus formation/pedogenesis has been shown (Laufer, Beckett, Minibayeva, Lüthje, & Böttger, 2009; Zavarzina & Zavarzin, 2006).

7. STRESS RESPONSE IN BIOWEATHERING

Fungi are well known to inhabit extreme environments such as contaminated sites, deserts, and rocks. Minerals in these rocks can also contain toxic metals which are released during fungal attack. Several studies showed a correlation between an increased extracellular laccase activity and heavy metal stress (Baldrian & Gabriel, 2002; Lorenzo, Moldes, & Sanromán, 2006). Since the active center of a laccase contains Cu atoms, it is likely that the addition of Cu ions promotes laccase production. This could be shown for *Trametes pubescens*, which produces extracellular laccase stimulated by Cu^{2+} or Mn^{2+}, while Ag^{+}, Cd^{2+}, Hg^{2+}, and Zn^{2+} had the opposite effect (Galhaup & Haltrich, 2001). *T. versicolor* showed differential response to Cu^{2+}, with high Cu^{2+} or Cd^{2+} concentrations inhibiting the formation of the enzyme (Lorenzo, Moldes, Couto, & Sanromán, 2005). In *Ganoderma lucidum*, laccase activity was enhanced at low concentrations of Ca^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} and inhibited in the presence of Fe^{2+} (Murugesan, Kim, Jeon, & Chang, 2009). From these investigations, a species-dependent effect of metals on extracellular laccase production can be deduced.

To cope with environmental stress, such as a limited amount of nutrients available, or metal stress, fungi are able to produce pigments in a defense

mechanism. Although various biosynthetic pathways exist for the production of pigments, laccases were found to be involved (Bell & Wheeler, 1986; Clutterbuck, 1990; Gochev & Krastanov, 2007). Zhao and Kwan (1999) suggested that laccases may catalyze the formation of extracellular pigments by oxidative polymerization. Many fungi are known to produce a variety of pigments in response to environmental stress including the exposure to UV radiation, extreme temperatures, toxic metals, bacterial attack, and nutrient deficiency (Bell & Wheeler, 1986; Pagano & Dhar, 2015; Velíšek & Cejpek, 2011). Melanins (brown, black) and carotenoids (yellow, orange, red) are widespread among fungi for protection against radiation and to scavenge the toxic singlet oxygen (Griffin, 1996). Melanin can be found in spores, the hyphal cell wall, or as an extracellular polymer to improve survival under extreme environmental conditions, such as high radiation, oxidizing agents, and dehydration (Fogarty & Tobin, 1996; Pagano & Dhar, 2015).

Many fungi are known to have a high biosorption capacity to heavy metals. In the past, numerous studies showed a connection between melanin production and biosorption of metals. Gadd and de Rome (1988) demonstrated a higher uptake of Cu^{2+} in melanized than in albino biomass as well as a reduction in metal toxicity in melanized strains. In presence of melanin, higher adsorption capacities could also be found for U^{6+} and Cd^{2+} (Mowll & Gadd, 1984; Sakaguchi & Nakajima, 1987). It is assumed that pigments bind to metal ions and immobilize them. Extracellular pigments thus can prevent metals from entering the fungal cell, whereas intracellular pigments are thought to inhibit further transport of metals within the mycelium (Grishkan, 2011).

8. CONCLUSIONS

The microarray analysis clearly shows how *S. commune* responds to black slate and accelerates weathering through changed gene expression. A variety of proteins, including multicopper oxidases, are produced to attack the rock and cause weathering. Molecular mechanisms involved in the production of organic acids or other metabolites exhibiting rock degrading potential were elucidated. To cope with the consequences of black slate degradation such as the release of toxic metals, genes involved in stress resistance were shown to be upregulated. The microarray analysis has shown to be a powerful tool to understand, exactly how fungi are responding to the

rock and which molecular principles may be involved in rock weathering processes. Thus, new insights into bioweathering systems and mechanisms in biodeterioration could be revealed.

Laccases, involved in many environmental interactions such as the degradation of brown coal or black slate containing high amounts of organic matter, were among the enzymes with enhanced expression when the fungus was grown with black shale. Since the degradation of rocks often coincides with the release of toxic metals, protective measures were seen as well. Laccase-producing fungi seem to better withstand the resulting harsh conditions due to the involvement of laccases in resistance against (heavy) metal stress occurring directly or indirectly via the production of pigments. Consequently, laccases turned out to be enzymes of particular importance in bioweathering.

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CHAPTER FOUR

The Arsenic Detoxification System in *Corynebacteria*: Basis and Application for Bioremediation and Redox Control

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Contents

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Abstract

Arsenic (As) is widespread in the environment and highly toxic. It has been released by volcanic and anthropogenic activities and causes serious health problems worldwide. To survive arsenic-rich environments, soil and saprophytic microorganisms have developed molecular detoxification mechanisms to survive arsenic-rich environments, mainly by the enzymatic conversion of inorganic arsenate (As^{V}) to arsenite (As^{III}) by arsenate reductases, which is then extruded by arsenite permeases. One of these Gram-positive bacteria, *Corynebacterium glutamicum*, the workhorse of biotechnological research, is also resistant to arsenic. To sanitize contaminated soils and waters, *C. glutamicum* strains were modified to work as arsenic "biocontainers." Two chromosomally encoded *ars* operons (*ars1* and *ars2*) are responsible for As resistance. The genes within these operons encode for metalloregulatory proteins (ArsR1/R2), arsenite permeases (Acr3-1/-2), and arsenate reductases (ArsC1/C2/C1′). ArsC1/C2 arsenate reductases are coupled to the low molecular weight thiol mycothiol (MSH) and to the recently discovered mycoredoxin-1 (Mrx-1) present in most *Actinobacteria*. This MSH/Mrx-1 redox system protects cells against different forms of stress, including reactive oxygen species (ROS), metals, and antibiotics. ROS can modify functional sulfur cysteines by oxidizing the thiol (-SH) to a sulfenic acid (-SOH). These oxidation-sensitive protein cysteine thiols are redox regulated by the MSH/Mrx-1 couple in *Corynebacterium* and *Mycobacterium*. In summary, the molecular mechanisms involved in arsenic resistance system in *C. glutamicum* have paved the way for understanding the cellular response against oxidative stress in *Actinobacteria*.

1. GENERAL INTEREST IN *CORYNEBACTERIUM GLUTAMICUM*

The genus *Corynebacterium* belongs to the *Actinobacteria* phylum. Two main groups can be distinguished: (1) highly pathogenic or emerging pathogenic members and (2) nonpathogenic microorganisms of basic research and industrial interest. The pathogenic species include *Corynebacterium diphtheriae*, the causative agent of diphtheria, as well as *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*, the causative agents of caseous lymphadenitis and diphtheria-like diseases, respectively, and many emergent opportunistic pathogens detected in immunosuppressed patients, among them, *Corynebacterium amycolatum*, *Corynebacterium urealyticum*, *Corynebacterium xerosis*, *Corynebacterium striatum*, and *Corynebacterium freneyi* (Bernard, 2012; Funke, Von Graevenitz, Clarridge, & Bernard, 1997; Letek, Ordóñez, Fernández-Natal, Gil, & Mateos, 2006; Mateos et al., 2011). The genomes of several of these pathogenic *Corynebacterium* species have been sequenced. Phylogenetic and pan-genomic analyses have illustrated the molecular mechanisms underlying the survival of these bacteria (Letek, Fiuza, Villadangos, Mateos, & Gil, 2013).

Nonpathogenic *Corynebacterium* species are mostly represented by *Corynebacterium glutamicum*. This bacterium was identified in the 1950s during screening for natural producers of amino acids (Kinoshita, Udaka, & Shimono, 1957). Since then, it has been extensively used not only for the industrial production of amino acids and nucleotides (Hirasawa & Shimizu, 2016; Mateos et al., 1994) but also for the biosynthesis of many other primary and secondary metabolites (Becker & Wittmann, 2012). Because of its economic importance and its generally recognized as safe (GRAS) (Meiswinkel, Rittmann, Lindner, & Wendisch, 2013) status, intensive efforts have been initially performed to developing efficient genetic manipulation tools for *C. glutamicum*, to the sequencing of its genome (Kalinowski et al., 2003), and to genomic analyses of different *Corynebacterium* species. The results of these studies were of great advantage for proteomic/transcriptomic studies and metabolomic/fluxomic developments, which resulted in establishing the systems biology of *C. glutamicum* (Franzel et al., 2010) and allowed for the genetic manipulation of its metabolism. For instance, with the development of "genome breeding" techniques, the production of amino acids was greatly improved (Ohnishi, Hayashi, Mitsuhashi, & Ikeda, 2003; Ohnishi et al., 2002).

Additionally, nonpathogenic *Corynebacterium* species are of environmental interest because of the ability of some of them to degrade/assimilate a broad variety of aromatic compounds (Shen, Zhou, & Liu, 2012). The potential of these species in the bioremediation/bioaccumulation of highly toxic heavy metals from water or soil, including inorganic arsenic species, has been demonstrated in several studies (Feo et al., 2007; Ordoñez et al., 2012; Villadangos et al., 2014) and is discussed in detail in Section 4.

2. ARSENIC AND ITS ROLE IN NATURE

Many heavy metals that are commonly present in the Earth's crust are nonetheless toxic for biological systems, even in very low amounts, as is the case for cadmium (Cd), mercury (Hg), silver (Ag), lead (Pb), uranium (U), and arsenic (As), the focus of this review. Bacteria and many other living organisms have developed molecular strategies to counteract heavy metal toxicity by hampering their uptake and/or by increasing metal secretion.

Arsenic is one of the most prevalent toxic metal/loids in the world, present in soils and environments containing arsenic-based minerals (arsenopyrite, realgar, orpiment, etc.). For more than 20 years, it has ranked first on the priority list of hazardous substances from the Agency for Toxic Substances and Disease Registry (http://www.atsdr.cdc.gov/spl/), based on its environmental ubiquity, toxicity, and potential hazards for humans.

Arsenic is also an important carcinogenic agent as reported by the International Agency for Research on Cancer and many scientific studies (Beyersmann & Hartwig, 2008).

The presence of As in different habitats originates from both natural and anthropogenic activities. Arsenic's occurrence in the natural environment, mainly as arsenic sulfides or oxides, reflects its abundance not only in the Earth's crust, but also in the troposphere, the world's oceans, and continental waters, albeit in smaller amounts in these latter sources (Zhu, Yoshinaga, Zhao, & Rosen, 2014). Natural arsenic, which represents one-third of the As present in the atmosphere, is released from volcanic eruptions, groundwater, lixiviation, and other processes. Anthropogenic origins of As are mainly industrial, as the metal is used in a broad range of manufacturing processes (timber processing, paint manufacture, and the synthesis of alloys, agricultural pesticides, antimicrobial agents, etc.), or released as a waste product (mining, coal-fired power plants, etc.) (Mukhopadhyay, Rosen, Phung, & Silver, 2002). In general, the amount of As in air and seawater is usually low (less than 1 part per billion, excluding highly industrial or volcanic areas), higher in lakes and rivers, and extremely high (up to 100–200 ppb) in underground water from areas rich in As-containing rocks or minerals. In aquatic environments, both inorganic and, to a lesser extent, organic forms of As are detected. Arsenic from solid structures (rocks/minerals or soils) can be transported over short or long distances by wind and/or water. The deposition of atmospheric arsenic (volcanic or anthropogenic origin) in different environments is facilitated by rainfall.

For humans, drinking water and food are the main sources of As exposure/intake. Air can contain As associated to particles (less than 1% from the global average; except for smokers where the value moves to more than 10%), drinking water can represent 5%–30%, whereas food can represent 50%–90% (as informed by the environmental agency: Science Report SC020104/SR4). The allowed concentration of As in food and water differs from country to country. The World Health Organization (WHO), which settles national regulations and standards for water/food safety in support of public health, has established the legal limit of arsenic in water and food to a level of 10 ppb (http://www.who.int/topics/arsenic/en/). However, the current standard for determination of As concentration is frequently addressed independently of the As origin (organic or inorganic) and its species (trivalent or pentavalent), and therefore the previously indicated limits are mainly referring to the highly toxic inorganic As^{III}.

The oxidation state of the inorganic forms of arsenic can be 0 (elemental arsenic, As^0), +5 (arsenate, As^V), or +3 (arsenite, As^{III}). As^V, the only As species that is a true heavy metal, is predominant in aerobic environments, while As^{III} is the most abundant in reduced environments. In addition to being much more toxic than As^V, As^{III} has greater mobility and bioavailability (Yamamura & Amachi, 2014). Its origin can be traced to that of the planet Earth itself, but as the atmosphere became oxygenated it was progressively replaced by As^V. Microorganisms in soil and water play a key role in the As biogeochemical cycle, as they participate in the mobilization of this metal from biotic to abiotic compartments and vice versa (Lièvremont, Bertin, & Lett, 2009). They are also involved in the generation of methylated forms of arsenic (mono-, di-, trimethyl-As^{III}/As^V) and the synthesis of organic As derivatives. These compounds enter waterways and are often ultimately detected in marine organisms, as shown by the greater As content in marine organisms (Bosch, O'Neill, Sigge, Kerwath, & Hoffman, 2016; Zhu et al., 2014). In solution, triprotic arsenic acid (AsO_4H_3) is detected in one of three equilibrium states depending on its pK_a value: $AsO_4H_2^-$ (2.2), AsO_4H^{2-} (6.9), and AsO_4^{3-} (11.5).

Both As^V (arsenate) and As^{III} (arsenite) are able to enter prokaryotic and eukaryotic cells. As^V is structurally a tetrahedral oxyanion that can act as a phosphate analog. It is taken up by cells via the phosphate transport systems, although the smaller sized phosphate is favored over the larger arsenate. Due to its structural similarity, As^V interferes in processes where phosphate is involved, such as protein phosphorylation, and therefore causes toxicity (Beyersmann & Hartwig, 2008). At neutral pH, As^{III} is imported via aquaglyceroporins (Rosen & Tamás, 2010) because the structure of trivalent As^{III} resembles that of glycerol. Once inside the cell, As^{III} is able to bind to the Cys-thiol groups (R-SH) of proteins, thereby disturbing redox homeostasis (Beyersmann & Hartwig, 2008). The toxic properties of inorganic As (mostly As^{III}) are well known and have been exploited in the treatment of promyelocytic leukemia and protozoan diseases (Rosen & Tamás, 2010).

3. MOLECULAR MECHANISMS OF ARSENIC DETOXIFICATION: *ars* OPERONS

In the majority of species, arsenic uptake results in cellular toxicity. Arsenic metabolism seems to be a niche-specific process limited to prokaryotes that utilize As^{III} and As^V as an electron donor (chemolithotrophic

organisms) and electron acceptor (dissimilatory processes), respectively, during energetic metabolism (Lièvremont et al., 2009).

To avoid arsenic toxicity, both prokaryotes and eukaryotes have developed resistance mechanisms mostly based on intracellular biochemical reactions that result in arsenic detoxification. These reactions are mediated by enzymes encoded within clusters of genes known as *ars* operons (see Fig. 1).

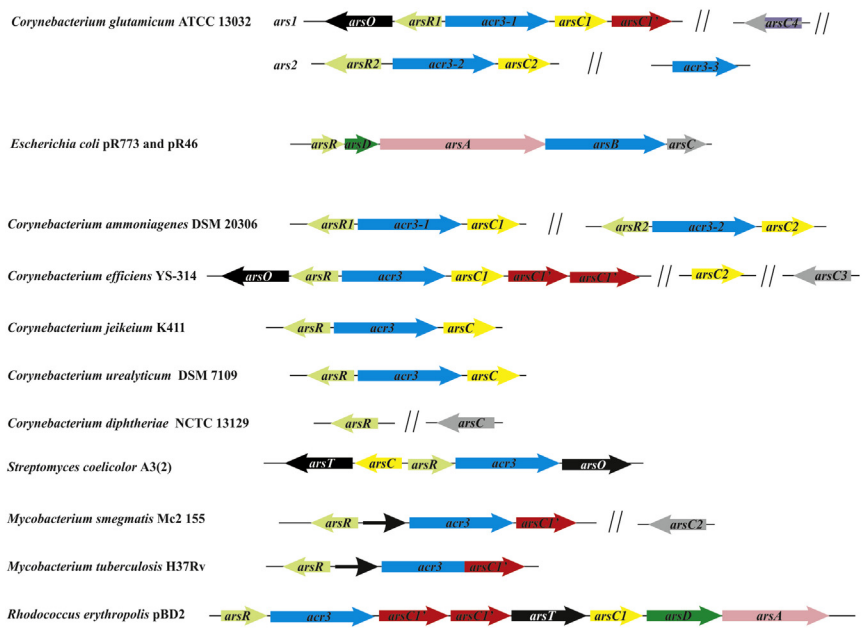

Figure 1 ***Genes and operons involved in arsenic resistance in*** **Corynebacterium glutamicum** ***and members from the*** **Actinobacteria** ***group.*** The three components *ars1* and *ars2* operons from *C. glutamicum* ATCC 13032 and accessory genes are shown at the top (Ordóñez et al., 2005); the *Escherichia coli ars* operon from plasmids R773 and R46 as an example of the five-component system is at the second line (Silver & Phung, 1996). The *ars* systems from *Corynebacterium ammoniagenes* DSM 20306, *Corynebacterium efficiens* YS-324, *Corynebacterium jeikeium* K411,*Corynebacterium urealyticum* DSM 7109, *Corynebacterium diphtheriae* NCTC 13129, *Streptomyces coelicolor* A3(2), *Mycobacterium smegmatis* Mc^2 155, *Mycobacterium tuberculosis* H37Rv, and *Rhodococcus erythropolis* pBD2 are obtained by homology search using the NCBI (National Center for Biotechnology Information) genome database. *Arrows* indicate genes for (1) light green: arsenic transcriptional repressor (*arsR*); (2) blue: arsenite permeases (*acr3* and *arsB*); (3) yellow: arsenate reductases hypothetically MSH/Mrx-1 dependent *(arsC*); (4) red: Trx-dependent arsenate reductases (*arsC*); (5) gray: GSH/Grx-dependent arsenate reductases; (6) dark green: metallochaperone (*arsD*); (7) pink: ATPase pump (*arsA*); (8) black: accessory arsenic genes (*arsT*, *arsO*).

Microbial *ars* operons have been identified either on the chromosome or on transmissible plasmids (Silver & Phung, 1996). They consist of three (*arsRBC*) or five (*arsRDABC*) genes organized into single transcriptional units. Comparisons of these units across many bacterial species have identified a minimum set of common genes. A three-gene *ars* operon is present not only on the chromosome of many members of the enterobacteria (including *Escherichia coli*) and *Pseudomonas aeruginosa*, but also on that of *C. glutamicum* and in plasmids pI258 and pSX267 from *Staphylococcus* (Ji & Silver, 1992). The *arsR*, *arsB*, and *arsC* genes encode a metalloregulatory protein, an arsenite permease, and an arsenate reductase, respectively, as discussed in detail in Section 3.1. The enlarged five-gene operon (*arsRDABC*) is much less frequent than the three-gene unit but it confers higher levels of arsenic resistance. In the *arsRDABC* operon, in addition to the three previously mentioned enzymes, *arsD* encodes a metallochaperone and *arsA* encodes an ATP-based pump. These two genes are usually adjacent to each other within the operon (Fig. 1) (Lin, Walmsley, & Rosen, 2006).

Indeed, *C. glutamicum* contains two *ars* operons (Fig. 1), as does multidrug-resistant *Pseudomonas putida* KT2440 (Páez-Espino, Durante-Rodríguez, & de Lorenzo, 2015). For the Gram-negative bacterium *Ochrobactrum tritici* SCII24T the presence of two *ars* operons was initially described (Branco, Chung, & Morais, 2008), although a new *ars3* operon has been recently reported (Sousa, Branco, Piedade, & Morais, 2015). By contrast, in pathogenic microorganisms (such as pathogenic or emerging pathogens of *Corynebacteria*), *ars* operons can be present only as remnants or as scattered functional or nonfunctional *ars* genes, as exposure to As of these animal host—associated microorganisms is rare.

In *C. glutamicum*, additionally to the presence of two *ars* operons, there are two nonoperon chromosomal genes, *acr3-3* and *arsC4*, that code for hypothetical arsenite permease and arsenate reductase proteins, respectively (Ordóñez et al., 2005), and which functionally have been analyzed (see further). Other possible *ars* genes (operon enclosed or not) encode accessory arsenic resistance functions, including ArsH (As-organic oxidase, mostly converting trivalent methyl-As to pentavalent methyl-As), ArsN (putative acetyltransferase), ArsP (As-organic permease), ArsTX (thioredoxin complex), and ArsO (flavin-dependent redox enzyme) (Andres & Bertin, 2016). Moreover, within the *ars* complex there are single genes coding for a single protein with two different activities (but formerly encoded by separated genes). This is the case in *Mycobacterium tuberculosis*, which contains

the fused gene cluster *acr3-arsC* encoding arsenite permease and arsenate reductase activities, respectively (Fig. 1) (Wu, Song, & Beitz, 2010).

As soil saprophytic bacteria, nonpathogenic *Corynebacterium* is highly exposed in As-contaminated environments and not surprisingly, the genomes of these species typically include *ars* genes/operons. In fact, the presence of *ars* genes/operons in the genome of environmental prokaryote members is very common, even with higher frequencies than the genes for certain amino acid biosynthetic pathways. This suggests that the arsenic-resistance system is very ancient, near the time of the origin of life (Mukhopadhyay et al., 2002).

3.1 The *ars* Operons of *Corynebacterium glutamicum*

In *C. glutamicum*, the *ars1* operon consists of $^{5'}$-*arsO-arsR1-acr3/1-arsC1-arsC1′*-$^{3'}$, which in the previously described model is essentially *arsRBC*. Besides the enzymes encoded by the *arsR*, *acr3-1* (an *arsB* from a different family), and *arsC* genes, *arsO* encodes a hypothetical redox enzyme and *arsC1′*, an arsenate reductase (Fig. 1). The *ars2* operon, $^{5'}$-*arsR2-acr3/2-arsC2*-$^{3'}$, has the *arsRBC* three-gene structure, as *acr3-2* gene, such as *arsB*, encodes an arsenite permease. The *ars1* and *ars2* operons possess several noteworthy features, including the following: (1) the opposite direction of transcription in the *ars1* operon of *arsR1-arsO* and in the *ars2* operon of *arsR2* relative to the other genes in the respective operons and (2) the presence in *ars1* of two genes encoding arsenate reductases (*arsC1* and *arsC1′*) but belonging to different families. The *ars1* operon is responsible for most of the cellular As resistance, as shown by gene/operon deletion and complementation analyses (Ordóñez et al., 2005). Cloning the whole *ars2* operon (using bifunctional pECM2-derivatives vectors) into a *C. glutamicum* strain lacking both *ars* operons resulted in lower As^{III} resistance scores than obtained for the *ars1* operon. This may be caused by the lower expression of the *ars2* operon than that of the *ars1* operon. Additional analysis of the individual genes from the *ars2* operon revealed that *acr3-2* is the main bottleneck for *C. glutamicum* arsenic resistance (Ordóñez et al., 2005; Villadangos et al., 2012).

3.1.1 ArsR *Metallorepressors*

More than 3000 members of the ArsR/SmtB family have been identified in prokaryotic genomes. These small metalloregulatory proteins act as sensors of heavy metals (As^{III}, Sb^{III}, Cd^{II}, Pb^{II}, Zn^{II}, Co^{II}, and Ni^{II}),

transition metals, and metalloids, and thus control the expression of genes involved in cellular resistance. As homodimeric repressors, ArsR/SmtB proteins bind to DNA in the absence of metal/loid inducers but dissociate from the DNA in the presence of an inducer (Busenlehner, Pennella, & Giedroc, 2003) presumably via a conformational change that leads to the derepression of the downstream genes. ArsR/SmtB repressors have in common two dimerization loops and a helix-turn-helix motif in the DNA binding region. There is a considerable plasticity in the inducer binding sites. The regulatory region coordinates a metal binding site via three or four cysteines. In contrast to the conserved winged helix DNA binding proteins, ArsRs show broad plasticity. For example, (1) in the *E. coli* ArsR repressor from plasmid R773, As^{III} (and Sb^{III}) binds to the three Cys residues from the sequence Cys^{32}-Val-Cys^{34}-Asp-Leu-Cys^{37}, present on each monomer of the homodimeric repressor, in a very specific three-coordinate binding site for the trivalent metalloid As^{III} (Fig. 2A) (Shi, Wu, & Rosen, 1994); (2) ArsR from *Acidithiobacillus ferrooxidans* has three cysteine residues (Cys^{95}, Cys^{96}, and Cys^{102}) at the repressor dimer interface that form a three-coordinate sulfur binding site for As^{III} on the same monomer molecule (Fig. 2A) (Qin et al., 2007); (3) in *C. glutamicum*, ArsR1 and ArsR2 have 66% sequence identity and both have three cysteine residues, N-terminally, Cys^{15}-Cys^{16}, and near the putative DNA binding domain, Cys^{55} (Fig. 2A). Both ArsR1 and ArsR2 are homodimers in vivo, with two As^{III} binding sites associated with Cys^{15} and Cys^{16} from one monomer subunit and with Cys^{55} from another monomer subunit in an intermolecular interaction (Ordóñez et al., 2008). In *C. glutamicum*, Sb^{III} does not interact with ArsR and does not induce the operon. The difference in the regulatory binding sites of *C. glutamicum* ArsR compared to other well-characterized ArsR/SmtB family members demonstrates the convergent and parallel evolution of metal/loid binding sites. It has thus been hypothesized that the inducer binding sites of ArsR/SmtB homologues evolved in diverse locations on the surface of the protein in response to environmental pressures that resulted in the spatially distinct positioning of residues to form three- or four-coordinate metal/loid binding sites (Ordóñez et al., 2008). There is a crystal structure of *C. glutamicum* ArsR1, in which the three cysteines were mutated to serine (Cys15/16/55-Ser) (Santha, Pandaranayaka, Rosen, & Thiyagarajan, 2011). However, additional information is needed for a complete understanding of the mechanism.

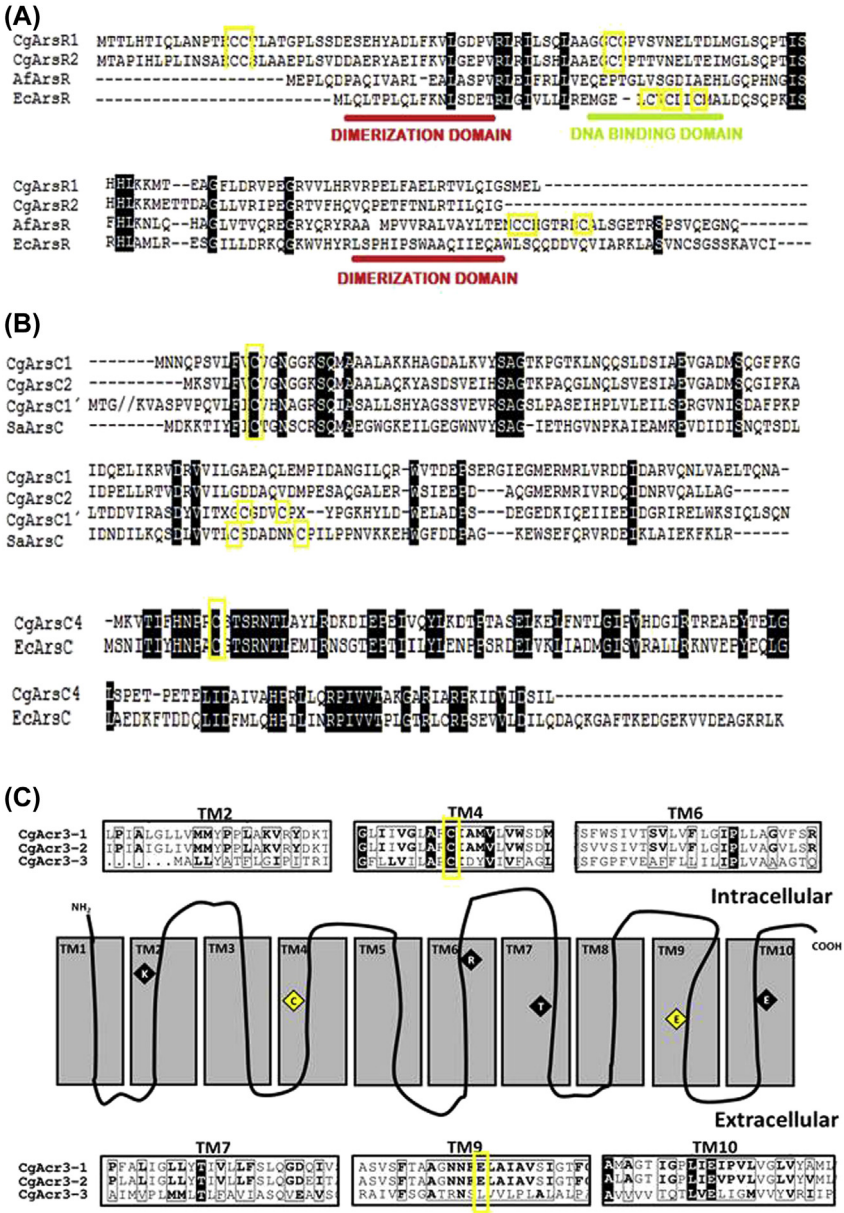

Figure 2 (A) Alignment of *Corynebacterium glutamicum* ATCC 13032 homologue proteins for metalloregulators ArsR1 (CgArsR1) and ArsR2 (CgArsR2), using the *Acidithiobacillus ferrooxidans* and *Escherichia coli* ArsRs (AfArsR and EcArsR, respectively) for analysis; DNA binding and dimerization domains are indicated, in addition to the Cys residues for As^{III} binding. (B) Alignment of *C. glutamicum* homologue proteins for the arsenate

3.1.2 ArsC Cytoplasmic Arsenate Reductases

Once inorganic As^V enters the cell, it undergoes rapid enzymatic reduction to trivalent As^{III}, which then becomes substrate for multiple modes of detoxification. Protein tyrosine phosphatase homologues evolved to reduce As^V and are known as arsenate reductases (ArsCs) (Messens & Silver, 2006). ArsCs are small cytoplasmic redox enzymes belonging to several families of evolutionarily related sequences. The differences in the structural fold and enzymatic mechanism of the different ArsCs suggest an independent evolution of these enzymes. However, the activity of all ArsCs is associated with cellular redox systems based on thiol−disulfide exchange reactions, including the sequential involvement of three different thiolate nucleophiles functioning as a redox cascade (Messens et al., 2002; Zegers, Martins, Willem, Wyns, & Messens, 2001).

Based on the mechanism used to reduce As^V to As^{III}, two distinct ArsC classes were defined: (1) The thioredoxin (Trx)-coupled class, represented by ArsCs from *Staphylococcus aureus* pI258 and *Bacillus subtilis* (Messens et al., 2002; Zegers et al., 2001). These arsenate reductases have a structural fold of low molecular weight (LMW)−tyrosine phosphatase, containing three nucleophilic thiolates on the same molecule, and they depend on Trx to start a second catalytic cycle. (2) The glutathione (GSH)/glutaredoxin-coupled ArsCs are found in a broad diversity of organisms, including *E. coli* (ArsC from plasmid R773, which structurally resembles glutaredoxin) and the eukaryotes *Leishmania major* and *Saccharomyces cerevisiae* (named Acr2p) with a rhodanase/Cdc25 phosphatase fold (Mukhopadhyay & Rosen, 2002; Oden, Gladysheva, & Rosen, 1994). These arsenate reductases have a thiolate nucleophile at the N-terminal end of an α-helix. (3) In *C. glutamicum*, our team discovered a third group of arsenate reductases, ArsC1 and ArsC2 (Fig. 2B), encoded respectively by *arsC1* and *arsC2* (66% sequence identity) (Ordóñez et al., 2009). *C. glutamicum* ArsC1/C2

reductases ArsC1, ArsC2, and ArsC1′. ArsC from *Staphylococcus aureus* (SaArsC) was included to be compared with ArsC1′; both reductases contain three Cys and are activated by the Trx/TrxR system. Alignment of the (orphan) *C. glutamicum* ArsC4 (CgArsC4) was feasible when aligned to the *E. coli* ArsC (EcArsC) by means of the GSH/Grx system. (C) Schematic representation of the arsenite permeases transmembrane domains (TM1-10) corresponding to the *C. glutamicum* Acr3-1 and Acr3-2 and Acr3-3; only amino acid sequences (in *boxes*) for domains containing potential key amino acids (inside black or yellow *romboids* figure) are indicated. The Acr3-3 permease lacks the TM1 domain, half of the TM2, and the glutamic acid (E) residue from TM9.

are mycothiol (MSH)- and mycoredoxin-1 (Mrx-1)-dependent arsenate reductases, as we were able to show by "in vitro" reconstitution of the electron transfer pathway (Ordóñez et al., 2009). This reductase system requires, in addition to ArsC1 (or ArsC2), the LMW−thiol mycothiol (MSH; 1-d-myo-inosityl 2-(N-acetyl-l-cysteinyl)-amido-2-deoxy-α-d-glucopyranoside), mycoredoxin-1 (Mrx-1, a recently described actinobacterial redoxin), and mycothione/mycothiol disulfide reductase (Mtr), with the latter enzyme-reducing mycothione (MS-SM, the oxidized disulfide form of MSH) for recycling, as described in detail in Section 5. This group of arsenate reductases (ArsC1/C2) has an LMW-PTPase structural fold [equivalent to the Trx-dependent class (1)], but with only one thiolate located at the active loop (Fig. 2B) (Ordóñez et al., 2009; Villadangos et al., 2011).

In *C. glutamicum*, the presence of MSH is linked to the level of arsenate resistance, as the bacterium contains the genes for the MSH biosynthesis pathway (MshA/B/C/D enzymes) but not for the synthesis of GSH (Feng et al., 2006). The reduction of As^{V} to As^{III} is performed after the formation of an As^{V}-MSH adduct which is resolved by coupling to the Mrx-1/Mtr redox system. The ArsC1/C2 enzymes facilitate the adduct formation by lowering the energy barrier between arsenate and MSH, which was demonstrated by an increased electron transfer and by the production of arsenite in the presence of MSH and Mrx-1 (Ordóñez et al., 2009). In previous studies, Newton and Fahey (2002) showed that in *Mycobacterium* the activities of MshA and MshC, but not of MshB and MshD, are essential for MSH production. In *C. glutamicum*, the As^{V} resistance level of the MshA or MshC mutants was analogous to that of the double-disrupted *C. glutamicum* mutant ArsC1-C2 whereas As^{III} resistance was the same as that of the wild-type (WT) strain (Villadangos et al., 2011).

In *C. glutamicum* cells, millimolar concentrations of MSH seem to constitute the first line of defense against arsenate (As^{V}) uptake because in this bacterium only arsenite (As^{III}) induces the *ars* operon, by releasing the ArsR repressor from the *ars* operator region (Ordóñez et al., 2005). In the absence of ArsC, MSH is only minimally able to reduce As^{V} to As^{III}, probably via the formation of an MS-As^{V} adduct that is subsequently reduced by the mycoredoxin (Mrx-1) to generate As^{III} and mycothione (MSSM). The latter is reduced by the flavoenzyme Mtr, using nicotinamide adenine dinucleotide phosphate (NADPH) as the ultimate electron donor. The reduction of MS-As^{V} proceeds more efficiently in the presence

of Mrx-1. The postulated mechanism is the nucleophilic attack of the MS-As^V adduct by the N-terminal Cys of Mrx-1, resulting in the release of As^{III} and the formation of an Mrx-1-S-SM mixed disulfide complex that is re-generated via the MSH/Mtr/NADPH pathway (Ordóñez et al., 2009).

The mechanism underlying the formation of As-MSH intermediates in *C. glutamicum* resembles the one proposed for *E. coli* R773 ArsC, which is coupled to GSH/Grx. However, in the *E. coli* enzyme, a monohydroxy positively charged arsenite intermediate is formed and As^{III} is released only after its hydroxylation. The mechanism underlying *C. glutamicum* ArsC differs in that the active-site thiolate in ArsC lowers the energy barrier to facilitate adduct formation between As^V and MSH, with the release of arsenite after the nucleophilic attack of Mrx-1 (Ordóñez et al., 2009).

A comparison of the kinetic constants of ArsC1 and ArsC2 from *C. glutamicum* with those of other small cytoplasmic arsenate reductases showed that both ArsCs are very slow (Ordóñez et al., 2009), although many GSH/Grx-coupled arsenate reductases are similarly characterized by a low specificity constant. The slow reaction rate may reflect the sequential involvement of the three different thiolate nucleophiles on three different molecules functioning as a redox cascade (see alignment of Fig. 2B). In contrast, the catalytic efficiency is higher when all three thiolate nucleophiles are present in the same enzyme (as is the case for pI258 ArsC from *S. aureus*), with a specificity constant that is several orders of magnitude higher (Ordóñez et al., 2009; Zegers et al., 2001).

The *ars1* operon of *C. glutamicum* also encodes a third arsenate reductase, ArsC1′ (Figs. 1 and 2B), responsible for a minor level of arsenate resistance. The enzymatic mechanism coupled to ArsC1′ is based on the Trx/TrxR system (Villadangos et al., 2011). The orphan ArsC4 was apparently a GSH/Grx-dependent arsenate reductase, as deduced by amino acid sequence identity (Villadangos et al., 2011). However, it is likely that ArsC4 is not functional: (1) when *arsC4* was heterologously expressed in *E. coli* (where the system GSH/Grx is present), the recombinant strain did not increase the arsenate resistance compared to the control strain; (2) in addition, the ArsC4 protein expression in *C. glutamicum* was not detectable on western blot with His-tagged antibodies against the His-ArsC4-fused recombinant protein (Villadangos et al., 2011).

As^{III} is much more toxic than As^V. However, the reduction of As^V in the detoxification mechanism is evolutionarily explained by the absence of phosphate-releasing pumps and the presence of As^{III} efflux pumps that work in concert with ArsCs (Villadangos et al., 2012).

3.1.3 Acr3 Cytoplasmic Arsenite Permeases

Membrane As^{III} transporters allow for the bacterial resistance to environmentally high As^{III} concentrations. These proteins are responsible for its fast extrusion. In fact, As^{III} efflux pumps are often the main arsenite resistance mechanism expressed by cells; as such it displays considerable diversity and several arsenite transporter families have been recently described (Yang, Wu, Lilley, & Zhang, 2015). However, three families of As^{III} transporters detoxifying As^{III} have been traditionally proposed: (1) the ArsB family; (2) the multidrug resistance—associated protein (MRP) family; and (3) the Acr3 (arsenic resistance-3) family. One additional family of arsenite permeases (major intrinsic proteins) includes aquaglyceroporins, the main entry pathway for As^{III}, that can also be involved in As^{III} transport out of the cell, as described for SmAqpS from *Sinorhizobium meliloti* (Yang, Cheng, Finan, & Rosen, 2005; Yang et al., 2015). The ArsB family of arsenite permeases was the first to be identified (Chen, Dey, & Rosen, 1996) and its members are ubiquitously present in bacteria and archaea. ArsB transporters have 12 transmembrane spanning regions and are similar to members of the major facilitator superfamily (Yang et al., 2015). The best-studied ArsB transporter is that of *E. coli*, which catalyzes the efflux of As^{III} and Sb^{III}, with higher affinity for Sb^{III}. *E. coli* ArsB acts as an $As(OH)_3/H^+$ and $Sb(OH)_3/H^+$ antiporter or, in the presence of the ArsA ATPase, an ATP-dependent pump. The ATP-coupled ArsAB efflux pump confers higher levels of As^{III} and Sb^{III} resistance than ArsB alone (Dey & Rosen, 1995; Yang, Fu, Lin, & Rosen, 2012).

MRP family proteins are ABC-ATPase transporters. They are widely distributed in prokaryotes and eukaryotes and constitute one of the most abundant families of membrane proteins. In eukaryotes, MRPs sequester As^{III} or Sb^{III} in vacuoles or other cellular compartments, and cells extrude these metals by complexing them to thiols such as GSH (Leslie, Deeley, & Cole, 2001).

Acr3 arsenite permeases belong to the BART (bile/arsenite/riboflavin transporter) superfamily, whose members are found in bacteria, archaea, and fungi. Thus, these enzymes are much more widely distributed than ArsB (Yang et al., 2012). Acr3 transporters are smaller than ArsB, and in the literature they have sometimes been erroneously annotated as ArsB, despite the absence of sequence similarity. This was the case for *C. glutamicum* (Ordóñez et al., 2005) until further analysis revealed the

relationship of its Acr3 with the Acr3s from *B. subtilis* and *Saccharomyces cerevisiae* (Aaltonen & Silow, 2008). In contrast to ArsB, which recognizes both As^{III} and Sb^{III}, Acr3 members are As^{III}-specific transporters and mostly mediate As^{III} resistance, as shown in the Acr3s of *B. subtilis*, *Alkaliphilum metalliredigens*, and *Saccharomyces douglassi* (Aaltonen & Silow, 2008; Fu et al., 2009). However, the Acr3 from *S. cerevisiae* may also have transport affinity for Sb^{III}, albeit with a threefold lower affinity rate (Maciaszczyk-Dziubinska, Wawrzycka, & Wysocki, 2012).

Analysis of the *C. glutamicum* genome identified three potential arsenate reductases, two of them located in the *ars* operons (*acr3-1*, *acr3-2*) and the third (*acr3-3*) as an orphan gene scattered on the chromosome (Fig. 1). As^{III} resistance and accumulation studies in gene-disrupted mutant strains of *C. glutamicum* showed that only Acr3-1 and Acr3-2 are functional arsenite permeases (Fig. 2C) (Ordóñez et al., 2005; Villadangos et al., 2012). In homologous and heterologous complementation analyses of the *acr3s* genes (expressed under their own promoters) in *C. glutamicum* and *E. coli* mutant strains lacking the *ars* operon/s, each of the permease pumps conferred different levels of As^{III} resistance, with Acr3-1 responsible for ~80% of arsenite permease activity and Acr3-2 for the remaining (Villadangos et al., 2012). The orphan Acr3-3 permease protein was not functional, nor was it adequately expressed or detected in western blot assays of *C. glutamicum* proteins (Villadangos et al., 2012). This lack of functionality was attributed to the lack of transmembrane domain TM-1 and (partially) TM2 (Fig. 2C), compared with the other two *C. glutamicum* Acr3 proteins, and to the lack of its regulation by the metalloregulators ArsR1/R2.

The specificity of Acr3-1 and Acr3-2 was demonstrated in in vivo analyses of As^{III} resistance and accumulation in *C. glutamicum* cells and in in vitro studies of As^{III} and/or Sb^{III} uptake using everted membrane vesicles from *E. coli* and *C. glutamicum*. Neither permease catalyzed the efflux of Sb^{III}. In addition, As^{III} uptake via Acr3-1 in everted membrane vesicles prepared from *C. glutamicum* was unaffected by the presence of different oxyanions (AsO_4^{3-} and PO_4^{3-}, among others) confirming *C. glutamicum* Acr3-1 as a specific As^{III} permease (Villadangos et al., 2012).

It was shown that the *C. glutamicum* Acr3-1 pump, the most active arsenite permease in *C. glutamicum*, is coupled to the proton-motive force. Assays of everted membranes vesicles from *E. coli* and *C. glutamicum* expressing *acr3-1* demonstrated that in the presence of NADH (the energy source) As^{III} accumulated inside the vesicles. The addition of carbonyl

cyanide m-chlorophenylhydrazone (CCCP), which disrupts the entire proton motive force (Δp), caused a rapid reversal of As^{III} accumulation. Together, these results indicate that metalloid transport by *C. glutamicum* Acr3-1 is coupled to the NADH electrochemical proton gradient (Villadangos et al., 2012). To determine whether the Acr3-1 pump acts as a uniporter for the arsenite anion (AsO_2^-) or transports the neutral species $As(OH)_3$ in exchange for protons, artificial potassium and sodium gradients were created using everted membranes, but neither of them drove the uptake of As^{III}. The addition to Acr3-1-expressing everted membranes of the permeant anion thiocyanate (SCN^-) and the permeant weak base ammonium (NH_4^+), which dissipate, respectively, the positive interior Δψ and the acidic interior ΔpH, uncoupled As^{III} transport, indicating the involvement of these components of the electrochemical proton gradient in As^{III} transport. Evidence suggesting that Acr3-1 is an electrophoretic metalloid-proton exchanger with $As(OH)_3/H^+$ antiporter activity has been described (Villadangos et al., 2012). Also, the putative topology of the *C. glutamicum* Acr3-1 permease pump was deduced based on the Acr3 transmembrane protein of *Alkaliphilus metalliredigens*; 10 conserved transmembrane domains were proposed. From these, six hydrophilic, conserved, and transmembranal residues of *C. glutamicum* Acr3-1 were examined by site-directed mutagenesis: Lys^{72}, Cys^{129}, Arg^{210}, Thr^{241}, Glu^{305}, and Glu^{332} (Fig. 2C). Only substitutions of Cys^{129} and Glu^{305} by alanine altered As^{III} resistance in homologous (*C. glutamicum*) and heterologous (*E. coli*) expression analyses, suggesting a key role for these two amino acids in As^{III} translocation and cellular release (Villadangos et al., 2012). For microbial ArsB arsenite permeases, no conserved cysteines associated with transmembrane domains were identified, consistent with the lack of Cys-thiolate involvement in As^{III} translocation (Kuroda, Dey, Sanders, & Rosen, 1997).

3.2 Origin of the *ars* Operons

The *ars* operon was initially discovered in plasmids R773 and R46 from *E. coli* and then on plasmid pKW301 from *Acidiphilium multivorum* (Suzuki, Wakao, Kimura, Sakka, & Ohmiya, 1998). Based on the heterogeneity of the *ars* operons, as described previously, we performed a comparative analysis between a member from the coryneform group using the *ars* genes present in the *C. glutamicum* ATCC 13032 genome [*arsR* (metallorepressor), *arsB/acr3* (arsenite permease from different families), and *arsC* (arsenate reductase)], those in different *Corynebacterium* species (most of them

opportunistic pathogens) and those members of the Mycolata group (*Mycobacterium* and *Streptomyces*). The results of these analyses of the extent of shared synteny and conserved gene orders in *C. glutamicum* and other microorganisms are provided in Fig. 3.

Pairwise comparisons of the DNA sequences from different *Corynebacterium* members were carried out using the Artemis comparison tool (Carver et al., 2005). The two *ars* operons from *C. glutamicum* are likely to have been horizontally acquired. The *ars1* operon in *C. glutamicum* has landed in a well-conserved region in corynebacteria mostly involved in the biosynthesis of cysteine (Fig. 3A). The *ars1* operon is also present in the closely related specie *C. efficiens* (locus tags CE0873-8; Fig. 3A), but in a different genome region,

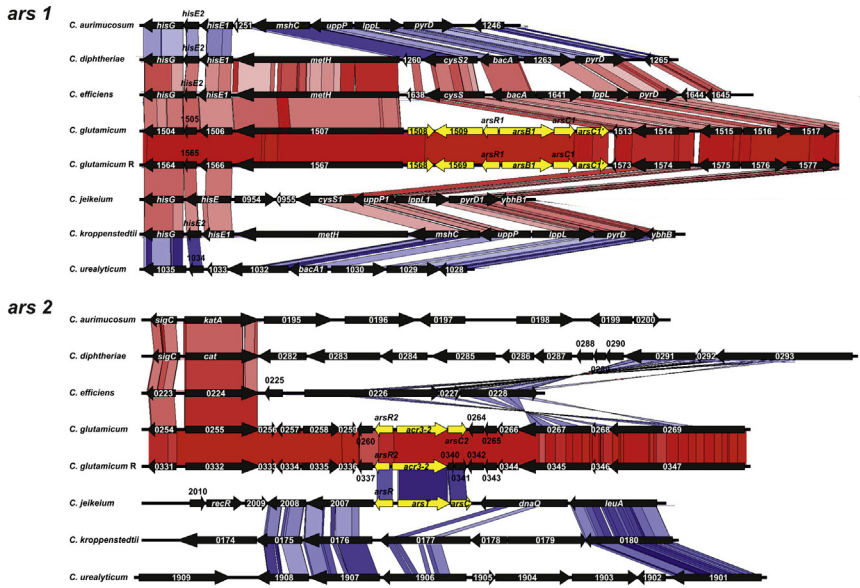

Figure 3 ***Origin of the* ars *operons from* Corynebacterium glutamicum *ATCC 13032.*** (A) Genetic organization and ACT (Artemis comparison tool) (Carver et al., 2005) pairwise comparisons of the *ars1* operon homologues in different corynebacterial genomes. Genes are represented by *arrows* indicating the direction of transcription. Regions with significant similarity (tBLASTx) are connected by *colored lines* (red, sequences in direct orientation; blue, sequences in reverse orientation). The intensity of the color indicates the strength of the sequence homology (pink/light blue, lowest; red/deep blue, highest). (B) Genetic organization and ACT pairwise comparisons of the *ars2* cluster in different actinobacterial genomes. Description for genes (*arrows*) and colors are as mentioned in panel A. *Corynebacterium glutamicum* R is a *C. glutamicum* strain isolated from soil in Japan and used for industrial processes (Yukawa et al., 2007).

suggesting that the operon was acquired independently from *C. glutamicum* and very recently during evolution.

Moreover, an analysis of the *C. glutamicum* genome using the "Alien Hunter software" (Vernikos & Parkhill, 2006) indicated that the *ars1* operon was indeed DNA acquired by horizontal gene transfer (HGT). This HGT region also includes two genes of unknown function located upstream of *arsR1*. One is referred to as *arsO* (coexpressed with *arsR1*, the gene for the *ars* metallorepressor; Fig. 3A) because of the homology of the encoded protein with an oxidoreductase enzyme, although its relation to arsenic resistance in *Corynebacterium* is unclear. The additional upstream gene (number 1508 in *C. glutamicum*; Fig. 3A) seems to be expressed (if at all) independently of *arsR1–arsO* and has no apparent homology with previously described genes/proteins. The *ars2* operon of *C. glutamicum* is not conserved with respect to its genomic location in other *Corynebacterium* species (Fig. 3B), although it shares homology with the single *ars* operon of *Corynebacterium jeikeium*, located in an adjacent region of the genome. In *Corynebacterium aurimucosum*, *Corynebacterium kroppenstedtii*, and *C. urealyticum*, *ars* operons are scattered in different areas of the respective genomes (cauri_1868–70, ckrop_1615–7, and cu0032–34, respectively). The frequent location of the *ars2* operon adjacent to transposase or recombinase genes suggests its horizontal acquisition.

In conclusion, although the origin of the *ars* operon is unknown, it seems to have been independently acquired at different times during evolution via horizontal gene transfer from a common pool of *ars* operons in the *Corynebacterium* environment (based on the C + G percentage) and not from other *Actinobacteria*, including *Mycobacterium* and *Streptomyces*. The fact that *Corynebacterium* species possess two operons likely reflects the high selective pressure exerted by the presence of arsenic in the natural niche of these bacteria, especially the soil saprophyte *C. glutamicum* (Mateos et al., 2011). Equipped with these two operons and thus well-armed against the noxious effects of different arsenic species, *C. glutamicum* is an excellent choice for the development of novel bioremediation tools, as discussed in the following section.

4. BIOREMEDIATION OF ARSENIC: *CORYNEBACTERIUM GLUTAMICUM* AS AN EXAMPLE

There are many microbial-based approaches to the remediation of metal/loid/s from polluted soils, freshwater, and marine water bodies. The advantages of using microorganisms include their higher surface

area/weight (compared to larger cells), their relatively low environmental impact, their metabolic capacity, and the frequent simplicity and low cost of microbially dependent processes (Tsai, Singh, & Chen, 2009). Heavy metal contamination is generally anthropogenic, and its microbial remediation proceeds via several key cellular stages.

1. In this stage, both the cell wall and the cytoplasmic membrane act as chelants of the target metal, either by cell-surface adsorption (without uptake) or cellular uptake (absorption). Specific or nonspecific metal adsorption can be genetically improved by the cellular overexpression of particular biopolymers, which can later be collected. In this case, the use of bacteria for remediation purposes is favored by their higher surface area/weight ratio (compared to larger cells) and their relatively low environment impact (Tsai et al., 2009). Heavy metal uptake by cells constitutes a trigger for further cellular toxicity reactions. For the uptake of toxic metals whose structure and/or functions mimic those of essential metals, the overexpression of specific protein permeases can improve bioaccumulation.
2. The second stage involves the intracellular fate of the metal after its uptake. This includes linkage of the toxic metal to cytoplasmic chemical compounds, its chemical modification to a different metallic species, and/or its physical sequestration in cellular compartments or inert structures (such as inclusion bodies). Natural cellular chelants of heavy metals that confer cellular protection against metallic stress and are thus of interest for remediation purposes include LMW proteins with a high content of Cys residues, such as metallothioneins and phytochelatins; the small peptide GSH, and CoA-SH. However, toxic metal uptake might also alter cellular processes, by interacting with functional sites (frequently carboxyl, sulfhydryl/thiol, amino, phosphate, and sulfate groups, among others) (Wang & Chen, 2009), leading to further metabolic deregulation, oxidative stress, and cell death.
3. Metal release from cells for detoxification purposes can operate at two levels, either extrusion by arsenite permeate pumps or by metal adsorption to the external cellular layers when the metal is released; a schematic view of the postulated processes is depicted in Fig. 4.

Inorganic As remediation has been carried out using eukaryotic systems, mostly plants (phytoremediation) and either filamentous (mycoremediation) or unicellular (mostly *Saccharomyces*) fungi. For example, *Arabidopsis thaliana* and the As-hyperaccumulating fern *Pteris vittata* have been used in the bioremediation of contaminated soils (Huang, Miyauchi, Inoue, & Endo, 2016). The remediation of arsenic has also been achieved using microorganisms,

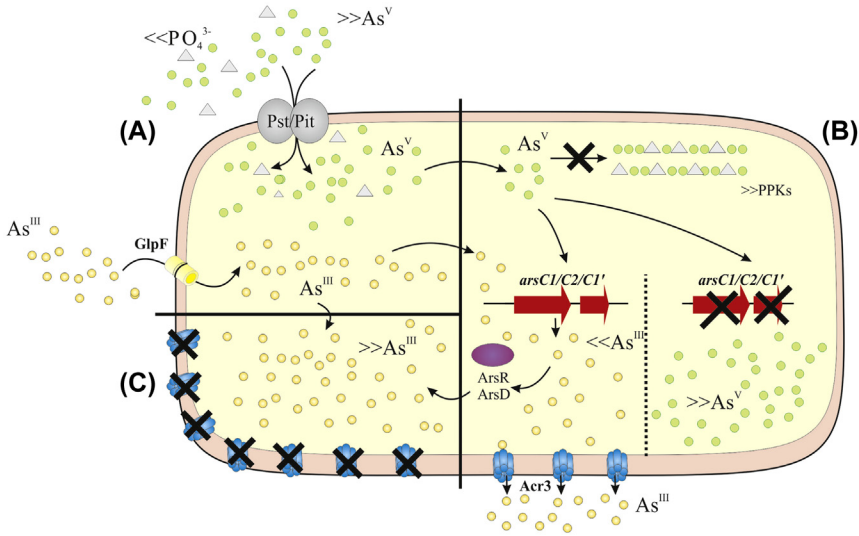

Figure 4 Schematic representation of the stages for *Corynebacterium glutamicum* arsenic bioremediation; (A) Uptake of As^{V} depends on phosphate specific/inorganic transporters (Pst/Pit); for (As^{III}) uptake an unknown yet to be discovered mechanism seems to be involved, although expression of certain aquaglyceroporins (GlpF) improves the uptake of As^{III}. (B) As^{V} is detoxified to As^{III} by arsenate reductases (ArsC1/C2/C1′); however, in absence of reductases, bioaccumulation of As^{V} can be obtained. Overexpression of enzymes involved in poly-phosphate biosynthesis (Ppk's) did not improve As^{V} accumulation. When ArsCs are functional, reduction of As^{V} to As^{III} is very efficient. (C) The cytoplasmic As^{III} must be released because of its toxicity, but mutant strains lacking the arsenite permease activities Acr3-1 and Acr3-2 were efficient As^{III} "biocontainers."

mostly genetically modified bacteria. For example, recombinant *E. coli* strains overexpressing the homologous gene for the permease aquaglyceroporin-mediating As^{III} uptake (*EcglpF*) and heterologous genes for metallothioneins accumulated 10-fold more As^{III} and As^{V} than control strains (Singh, Mulchandani, & Chen, 2008). Also in *E. coli*, heterologous expression of genes encoding phytochelatins and the enzymes needed for glutathione synthesis from *Schizosaccharomyces pombe* (*SpPCS* and *Spgsh*, respectively) increased bacterial As accumulation by up to 36-fold, with an 80-fold improvement following the removal of the endogenous arsenite permease genes (Singh, Kang, Lee, Mulchandani, & Chen, 2010).

Recently the use of the Gram-negative bacteria *Ochrobactrum tritici* for arsenic biocontention has been described (Sousa et al., 2015). *O. tritici*

present three chromosomal genes encoding for arsenite permeases: ArsB, Acr3-1, and Acr3-2, although only the two former are involved in bacterial arsenite resistance. Authors describe the construction of the double mutant strain *O. tritici* ArsB/Acr3-1 that is able to accumulate eightfolds more arsenite (17 ppm) than the control WT strain (2 ppm), when assayed at moderate arsenite concentrations (Sousa et al., 2015).

4.1 Arsenic Bioremediation Using *Corynebacterium glutamicum* Strains

Based on the attributes of *C. glutamicum* discussed throughout this review, including its two *ars* operons, genetic plasticity, and its GRAS status, this bacterium is of strong interest in the bioremediation of As. Moreover, we were able to optimize *C. glutamicum* at all of the previously described stages yielding an effective microbial "biocontainer." As^{III} uptake was improved by the heterologous cloning of aquaglyceroporins (*glpF*) genes from closely related microbial species. For the second stage, bacterial metabolism involved in arsenic detoxification was modified by inactivating arsenate reductase activities and by constructing mutants with altered MSH or Mrx-1 synthesis/activity. Finally, for the third stage, we generated mutants lacking the functional arsenite permease activities, which prevented the cellular release of As^{III} (Mateos, Ordóñez, Letek, & Gil, 2006).

4.1.1 Optimization of Arsenic Uptake

Two main strategies for the optimization of As uptake were followed, depending on the As species to be removed (Fig. 4A). Thus, for As^{V}, the culture medium was low in a phosphate environment because phosphate competition for the same import system significantly hampers As^{V} uptake. In fact, when the phosphate concentration in the environment or culture medium is reduced to one-tenth of the original concentration, As^{V} uptake improved 10-fold. By contrast, As^{III} uptake was barely affected by the presence of phosphate (Feo et al., 2007), as in aqueous solution (neutral pH) it enters bacterial cells via aquaglyceroporins (GlpF). However, in WT *C. glutamicum*, homologues of *glpF* are absent, which accounts for the relatively high resistance (up to 12 mM As^{III}) of the bacterium to otherwise highly toxic As^{III}. Nonetheless, even in the absence of GlpFs, a certain amount of As^{III} is incorporated into WT *C. glutamicum* by an as yet unknown mechanism. Our attempts to increase the cellular As^{III} uptake involved the heterologous expression in *C. glutamicum* of *glpF* genes from the actinobacteria *Corynebacterium diphtheriae* NCTC 13129 (*CdglpF*;

DIP2236) and *Streptomyces coelicolor* A3(2) (*ScglpF*; SCO1659). The success of this strategy was evidenced by the decreased resistance to As^{III} of the *CdglpF*- and *ScglpF*-overexpressing cells (to 6 and 8 mM, respectively), which clearly correlates with their increased As^{III} uptake rates (2.6-fold and 2.1-fold, respectively) (Villadangos et al., 2014). Moreover, this result demonstrated the involvement of GlpF in the As^{III} uptake, as was also observed in other bacteria and in eukaryotes (Liu et al., 2002; Meng, Liu, & Rosen, 2004).

4.1.2 Intracellular Arsenic Accumulation

To improve As accumulation by *C. glutamicum*, its genes encoding the conversion of As^{V} to As^{III} (either those of the arsenate reductases ArsC1/C2 or *msh*, required for MSH synthesis) were inactivated. Alternatively, we overexpressed the genes encoding for the synthesis of chelants [in this case, poly-phosphate (poly-P)] (Fig. 4B). Accordingly, three *C. glutamicum* mutant strains carrying single or double mutations of *C. glutamicum* (ArsC1, ArsC2, and ArsC1-C2) were tested for As^{V} accumulation in the presence of 0.1 mM As^{V}. After a 2-h incubation, As^{V} levels were 28-fold higher in *C. glutamicum* ArsC1-C2 than in the control WT strain, whereas in the remaining mutants the accumulation of As^{V} was unaltered (Ordóñez et al., 2009). The mutant *C. glutamicum* 2Δ*ars*, lacking both the complete *ars1* and *ars2* operons and therefore all important As-detoxification genes, accumulated the same amount of As^{V} as the ArsC1-C2 mutant (Ordóñez et al., 2005; Villadangos et al., 2014). The latter mutant was also used for arsenic speciation, as it was able to discriminate between the two inorganic As species (Villadangos et al., 2010). Even in the presence of a 10-fold higher concentration of As^{III} in the assayed solution, there was no effect on As^{V} accumulation by the ArsC1-C2 mutant because the amount of retained arsenic was the same as in the absence of As^{III}. The incorporated As^{III} was probably rapidly removed from the cell by the arsenite permeases Acr3-1/Acr3-2, whereas As^{V} accumulated because of the absence of functional arsenate reductases in *C. glutamicum* ArsC1-C2 (Villadangos et al., 2014).

Given the critical role played by MSH in protecting cells against oxidative stress and providing electrons for As^{V} reduction by arsenate reductases, mutants for *mshA* and *mshC* were also analyzed for As^{V} accumulation. Both were very sensitive to As^{V}, accumulating amounts equivalent to those (or even slightly higher, up to 30-fold) of the *C. glutamicum* ArsC1-C2 and 2Δars mutants. This experiment corroborated

the role of MSH as a cofactor of the *C. glutamicum* arsenate reductases ArsC1 and ArsC2 (Ordóñez et al., 2009).

Long polymers of poly-phosphates (poly-P) are typical of coryneform bacteria, including *C. glutamicum* (Lindner, Vidaurre, Willbold, Schoberth, & Wendisch, 2007). By sequestering metal cations, poly-P alleviates metal-derived stress (Alcántara, Blasco, Zúñiga, & Monedero, 2014; Pan-Hou, Kiyono, Omura, Omura, & Endo, 2002). The genes encoding the two (main) enzymes required for poly-P biosynthesis in *C. glutamicum*, PPK2A (NCgl0880) and PPK2B (NCgl2620), were overexpressed in the ArsC1-C2 and 2Δars mutants and the modifications of As^V resistance and/or retention was then analyzed. Neither recombinant strain showed improved As^V retention compared to the background strains, suggesting that poly-P is not involved in As^V resistance or accumulation in *C. glutamicum* (Villadangos et al., 2014).

4.1.3 Arsenite Release

The bioremediation potential of mutants unable to synthesize Acr3 arsenite permeases (and therefore to expel As^{III}) strains overexpressing genes encoding aquaglyceroporins (*glpF*) or As^{III}-chelating agents (such as the *C. glutamicum* endogenous metalloregulators ArsR1/2), and strains heterologously expressing the ArsD chaperone from *E. coli* were also tested.

The natural resistance of *C. glutamicum* strains to As^{III} can be ascribed to the two functional arsenite permeases (Acr3-1 and Acr3-2), which actively extrude As^{III} from the cells (Villadangos et al., 2012) and to the lack of endogenous *glpF* genes mediating As^{III} uptake (Fig. 4C). Thus, mutant 2Acr3 (lacking both Acr3-1 and Acr3-2 activities) and mutant 2Δars (lacking the *ars1* and *ars2* operons) were evaluated for As^{III} resistance/accumulation in "resting cells" incubated for 2 h in the presence of 1 mM As^{III}. The mutant strains are slightly more sensitive than the control WT strain to As^{III}, but they differ in the amount of retained As^{III}: 7.2-fold for mutant 2Acr3 versus fourfold for the mutant 2Δars strain versus the control WT strain. When the 2Acr3 strain, which has functional ArsCs, was assayed only in the presence of As^V, As retention (expected to be As^{III}) reached 15-fold versus the WT strain, reflecting its ability to incorporate both As^{III} and As^V (Feo et al., 2007; Villadangos et al., 2014); in this case, the As^{III} accumulation is lower than in the ArsC1-C2 mutant strain, when As^V was added, indicating that As^{III} is a more toxic specie and is therefore less accumulated by 2Acr3 cells.

Expression of the aquaglyceroporin genes *CdglpF* or *ScglpF* in mutant strains 2Acr3 and 2Δars led to additional As^{III} accumulation: (2-fold and 1.5-fold, respectively) compared to that by the background strains. As^{III} accumulation is highest (11-fold vs. the control WT strain) in the 2Acr3 strain harboring *CdglpF*-containing plasmids (Villadangos et al., 2014).

Additional strategies to increase As^{III} accumulation in *C. glutamicum* include the cellular expression of biosorbents (Wang & Chen, 2009). As^{III} has a high affinity for the thiol groups of cysteines. In *E. coli*, among the well-known proteins capable of binding As^{III} are the metallochaperone ArsD. The genes *EcarsD109*, a truncated functional *arsD* gene fragment from the *E. coli* R773 *ars* operon (GenBank: U13073.1) (Yang, Abdul Salam, & Rosen, 2011), and *arsR1/R2* from *C. glutamicum* (NCgl1452/NCgl0257) were overexpressed against the background of the mutant strain *C. glutamicum* 2Δars, but no improved As^{III} accumulation was observed compared to the unmodified 2Δars strain. This can be explained because the overexpression of *arsR1/arsR2* genes in *C. glutamicum* 2Δars is apparently toxic, as showed from the lower growth kinetics in the presence of As^{III} (Villadangos et al., 2014).

5. ALLEVIATION OF OXIDATIVE STRESS IN *CORYNEBACTERIUM GLUTAMICUM* BY THE REDOX SYSTEM MSH/Mrx

One of the most important and ubiquitous system for maintaining both the intracellular reduced state and redox homeostasis, essential for protection against oxidative stress, is that of the universal thioredoxin (Trx) and thioredoxin reductase (TrxR) (Lu & Holmgren, 2014). In eukaryotes and Gram-negative bacteria, disulfide stress is also averted by the LMW-thiol tripeptide GSH, coupled to glutaredoxins (Grxs) and GSH disulfide-reductase (GR) (Fahey, 2013). Gram-positive bacteria such as *S. aureus* and *Bacillus cereus* produce coenzyme A (CoA-SH) as an abundant thiol (Van Loi, Rossius, & Antelmann, 2015). CoA-SH is coupled to CoA disulfide reductase (CoADR) in an additional redox system (DelCardayré, Stock, Newton, Fahey, & Davies, 1998). In *Bacillus/Clostridium* and *Staphylococcus* species, bacillithiol (BSH) (Gaballa et al., 2010), and in members of *Corynebacterium* (and in *Actinobacteria* in general), the pseudodisaccharide mycothiol (MSH) perform similar functions (Sharma, Van Laer, Messens, & Hamilton, 2016; Van Laer, Hamilton, & Messens, 2013). In its reduced state, MSH contains a cysteine moiety as a reactive thiol

whereas MS-SM, the oxidized form of MSH, is reduced by the disulfide reductase Mtr in an NADPH-dependent reaction, as noted in a previous section (Newton, Buchmeier, & Fahey, 2008). In *E. coli*, detoxification/protection against alkylating and oxidative agents (including heavy metals) and certain antibiotics is predominantly mediated by the GSH/Grx redox system (Masip, Veeravalli, & Georgiou, 2006).

The cysteine residues of cellular proteins are frequent targets of reactive chemical species, which promote reversible oxidations to sulfenic acid in addition to reacting with other thiols to form intramolecular and intermolecular protein disulfides. One of the most common intermolecular disulfides involves a mixed disulfide between the LMW-thiol and a protein Cys-SH, which generally protects the protein cysteines from irreversible oxidations (sulfinic and sulfonic acids). These reactions can also exert transcriptional control of gene expression in the presence of certain stresses (Antelmann & Helmann, 2011) and may participate in the posttranslational regulation of cellular activities. By making use of S-thiolation/dethiolation reactions, reductive enzymes such as the LMW-thiol redoxins provide redox-coupling mechanisms for a reducing cytoplasmic environment.

Our team was the first to describe an MSH-dependent redoxin (mycoredoxin-1; Mrx-1) coupled to ArsC1/C2, and its role in arsenate detoxification of *C. glutamicum* (Ordóñez et al., 2009). Here, MSH behaves as a cofactor for these arsenate reductases, which operate in tandem with Mrx-1/Mtr, reducing As^{V} to As^{III}, as one of the steps of the arsenic detoxification mechanism. Additional roles for MSH, the main nonenzymatic antioxidant in *Actinobacteria*, have recently been reported (Chi et al., 2014; Feng et al., 2006; Liu et al., 2013), including detoxification via the scavenging of free radicals or reactive oxygen species, serving as a cysteine reservoir than preserves the cellular reducing environment, as described for GSH (Jozefczak, Remans, Vangronsveld, & Cuypers, 2012; Newton et al., 2008). In addition, by forming mixed disulfides with proteins/enzymes (Chi et al., 2014), MSH prevents further target damage by oxidizing agents and "labels" certain toxins for removal as MSH-S-toxin conjugates, similar to its role in *Mycobacterium* and *Streptomyces* (Newton, Av-Gay, & Fahey, 2000; Park & Roe, 2008).

An understanding of the functions of MSH and the recently described Mrx-1 proteins from *C. glutamicum* in S-thiolation/dethiolation processes has led to the discovery of proteins or intermediate metabolites that are activated/deactivated by the MSH/Mrx-1 sensor system. In one report,

the *C. glutamicum* S-mycothiolome was analyzed in the presence or absence of the oxidizing agent sodium hypochlorite (Chi et al., 2014). Using mass spectrometry, the authors identified S-mycothiolated proteins containing MSH-mixed-protein disulfides that formed under normal or oxidative stress conditions. More than 20 *C. glutamicum* proteins were S-mycothiolated at cysteine residues of NaOCl-exposed cells; changes in the ratio of nonmycothiolated to S-mycothiolated proteins occurred in response to stress, indicating a protective role for MSH in S-mycothiolated proteins (Chi et al., 2014). Some of the mycothiolated proteins were involved in peroxidase activities, including thiol peroxidase (Tpx) and mycothiol peroxidase (Mpx), others in intermediate carbohydrate metabolism (e.g., hexulose kinase, fructose-bisphosphate aldolase, maltodextrin phosphorylase) or amino acid or nucleotide biosynthesis pathways (homoserine dehydrogenase, methionine synthase, and sulfoxide reductase, phosphoglycerate dehydrogenase, etc.), while others were associated with ribosomal proteins or elongation factors (Chi et al., 2014; Van Loi et al., 2015).

Some of the previously mentioned S-mycothiolated proteins obtained from the mycothiolome have been further studied in depth to determine whether they are substrates of Mrx-1. A schematic representation of the process is depicted in Fig. 5. *C. glutamicum* Tpx (an atypical 2-cys peroxiredoxin) was shown to be demycothiolated by the corresponding Mrx-1 (Chi et al., 2014). In the di-thiol system of *C. glutamicum*, Tpx presents active peroxidatic Cys^{60}, whereas Cys^{94} is the resolving Cys using a typical di-thiol system (Lu & Holmgren, 2014). When Tpx is S-mycothiolated (by a mixed disulfide) it loses Trx-dependent peroxidase activity, which is restored when Tpx is demycothiolated by the redox couple MSH/Mrx-1 (Chi et al., 2014). Mpx from *C. glutamicum* is a cysteine mycothiol peroxidase (a member of the CysGPx family) that controls cellular oxidative stress during S-thiolation. With its active peroxidatic Cys^{36} and resolving Cys^{79}, Mpx serves as a dithiol system allowing the recovery of the reduced state. The transient sulfenic acid from Cys^{36} can also reacts with MSH, forming a mixed disulfide (S-mycothiolation) that can be reduced both by Mrx-1(MSH/Mrx-1/Mtr) and Trx, although Mrx-1 reduction is 100-fold faster (Pedre et al., 2015). Expression of the *C. glutamicum mpx* gene was directly activated by the stress-responsive extracytoplasmic factor SigH (Si, Xu, et al., 2015). A role for overexpressed *C. glutamicum* Mpx in reducing intracellular ROS levels (induced by the acid-stress SigH-controlling factor) has also been demonstrated (Wang et al., 2016).

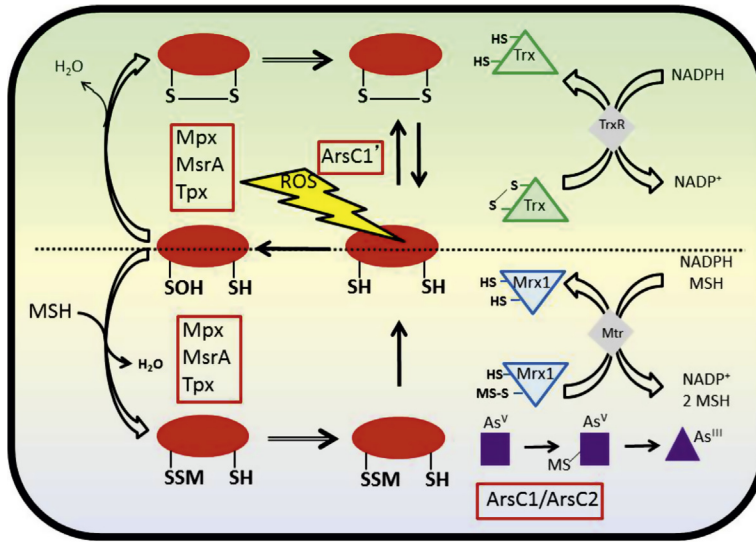

Figure 5 Schematic representation of the *Corynebacterium glutamicum* redox mechanisms involved in stress alleviation of proteins/enzymes containing thiol functional groups. After certain stresses (oxidative, metallic, etc.), the cytoplasmic environment can change from reducing to oxidizing, and the sensitive thiol groups can be oxidized to sulfenic acid (S-OH). Several cellular mechanisms protects proteins from overoxidation by the formation of disulfide bonds (if an additional protein thiol group is around), or by S-thiolation (mixed disulfide) with the millimolar concentrations of MSH present in these cells. Some protein thiols are recovered with the Trx/TrxR redox system, which reduces the disulfide bond, others through the MSH/Mrx-1 redox system. Some enzymes, such as MsrA (Tossounian et al., 2015) and Mpx (Pedre et al., 2015), couple to both the Trx/TrxR and MSH/Mrx-1 systems. ArsC1/ArsC2 uses the MSH/Mrx-1 redox system, whereas ArsC1′ uses Trx/TrxR.

The enzyme MsrA (methionine sulfoxide reductase A) also plays a pivotal role in cellular redox signaling and was detected in its mycothiolated form in the S-mycothiolome of *C. glutamicum* (Chi et al., 2014). *C. glutamicum* MsrA belongs to the 3-Cys family of MsrAs involved in oxidative stress resistance and is induced under certain stresses via the sigma factor SigH (Si, Zhang, et al., 2015). This MsrA contains the peroxidatic Cys^{56} and the resolving Cys residues Cys^{204} and Cys^{213}, which form an intramolecular disulfide bond. MsrA has been independently studied in *C. glutamicum* and *C. diphtheriae* (Si, Zhang, et al., 2015; Tossounian et al., 2015). The two MsrAs are closely related by both protein sequence identity and cysteines located in equivalent positions; in *C. diphtheriae* the MSH redox relay pathway starts from catalytic Cys^{52} toward Cys^{206} and Cys^{215}

(Tossounian et al., 2015) and it is expected an equivalent mechanisms of action for the *C. glutamicum* MsrA. The cellular oxidation of methionine to methionine sulfoxide (MetO) is reversed by methionine sulfoxide reductases from the unrelated classes MsrA and MsrB. Following the oxidation of MsrA, the enzyme is restored to its reduced state by either the Trx/TrxR redox system, resolving the intracellular disulfide bond from MsrA under nonstressed conditions, or the MSH/Mrx-1/Mtr redox pathway (Si, Zhang, et al., 2015; Tossounian et al., 2015).

In their study of the MSH/Mrx-1 redox system in *Actinobacteria* members, Hugo et al. (2014) described the alkyl-hydroperoxide reductase AhpE (a 1-Cys peroxiredoxin lacking a resolving Cys) from *M. tuberculosis*, which detoxifies peroxinitrite and other substrates. During the catalytic cycle of AhpE, peroxidatic Cys^{45} was oxidized to a sulfenic acid, but shown to be reduced by Mrx-1 or S-mycothiolated and further regenerated by coupling to the MSH/Mrx-1/Mtr system. A very recent study reported a new mechanism for the regeneration of the oxidized Cys^{45} (peroxidatic) from AhpE, based on the action of bare MSH and completely independent of Mrx-1 (Kumar, Balakrishna, Nartey, Manimekalai, & Grober, 2016). Kinetic studies and computational approaches to the crystallographic structure of *M. tuberculosis* AhpE showed the special orientation of the protein's active site residues such that peroxide reactions are favored (Pedre et al., 2016).

6. FUTURE PERSPECTIVES

Our review of the arsenic resistance mechanisms of *C. glutamicum* shows details on all the involved proteins. We also describe the use of different mutant/recombinant strains as As biocontainers, based on our own work, and the mechanisms underlying the MSH/Mrx-1 system that mediate As^V detoxification, including the *C. glutamicum* proteins protecting cells against oxidative stress via MSH-mixed disulfides. *C. glutamicum* has become a workhorse in biotechnological processes, and knowledge of its systems biology has been exploited to solve challenging biological problems using metabolic engineering (Wendisch, 2014). As a GRAS bacterium, *C. glutamicum* can be used in the production of many metabolites, facilitated by its relatively small genome (around 3.2 Mbp) (Kalinowski et al., 2003). Finally, *C. glutamicum* is phylogenetically closely related to important pathogens such as *C. diphtheriae* and *M. tuberculosis* (Krawczyk, Kohl, Goesmann, Kalinowski, & Baumbach, 2009) suggesting its importance for virulence/toxicity studies.

The resistance of *C. glutamicum* to many adverse chemical and physical environmental conditions is in part due to an additional layer external to the peptidoglycan cell wall and including corynomycolic acids (Tropis et al., 2005). This physical property and the robust antioxidant system, based on the redox couples Trx/TrxR and MSH/Mrx-1, suggest the potential of *C. glutamicum* for bioremediation purposes when the latter system was disrupted/disengaged, as discussed herein for the metalloid arsenic. Although we also assayed *C. glutamicum* WT strains for cobalt (Co) accumulation/remediation analyses, the accumulated Co was rapidly released from the cells, contrary to what was observed for *B. subtilis* WT strains (Mateos et al., 2016). However, *C. glutamicum* strains might be genetically manipulated to make them suitable as general toxic metal accumulators.

The presence of MSH, as the major LMW-thiol in members of Actinobacteria, offers an excellent mechanism protecting cells against stress. The intracellular concentrations of MSH differ among the various Actinobacteria but the overexpression in *Corynebacterium* of the enzyme involved in MSH recycling (Mtr) confers greater cellular resistance to stress conditions (Si et al., 2016). Thus far, only Mrx-1 in *Corynebacterium* and *Mycobacterium* has been described but its function as a mycoredoxin resembles that of glutaredoxins in the reduction of MSH-mixed protein disulfides. Important to note, the kinetics of Mrx1-based As^V reduction associated with ArsC1/C2 are relatively slow (Ordóñez et al., 2009), suggesting the presence of uncharacterized mycoredoxin-like proteins which might be involved in the As^V reduction pathway. Future work will involve discovery and characterization of unknown mycoredoxins supposedly implicated in arsenic detoxification and/or cellular stress survival.

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