ADVANCES IN

APPLIED MICROBIOLOGY

VOLUME 68

Academic Press is an imprint of Elsevier
525 B Street, Suite 1900, San Diego, CA 92101-4495, USA
30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
32, Jamestown Road, London NW1 7BY, UK

First edition 2009

ISBN: 978-0-12-374803-4
ISSN: 0065-2164

Printed and bound in the USA
09 10 10 9 8 7 6 5 4 3 2 1

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CHAPTER 1

Bacterial L-Forms

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Contents

Abstract

L-forms are "cell wall-deficient" bacteria which are able to grow as spheroplasts or protoplasts. They can be differentiated into four types depending on their ability to revert to the parental, cell-walled form and to the extent of their cell-wall modification. L-forms are significant in modern science because of their contributions to an improved understanding of principal questions and their interactions with eukaryotes. This review particularly focuses on research using stable protoplast-type L-forms which have contributed to a better understanding of the structural and functional organisation of the cytoplasmic membrane and of cell division. These L-forms, which have only a single surrounding bilayer

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Advances in Applied Microbiology, Volume 68
ISSN 0065-2164, DOI: 10.1016/S0065-2164(09)01201-5

membrane, also represent a unique expression system for production of recombinant proteins. A large proportion of L-form publications concern their putative role in human disease and its therapy, a topic which is discussed briefly. L-forms have also been used to form intracellular associations with plant cells and have been shown to elicit induced disease resistance offering a novel method for plant protection. The recent decline in active research on L-forms is a concern as knowledge and experience, as well as unique L-form strains which have been maintained for decades, are being lost.

ABBREVIATIONS

CL	cardiolipin
CM	cytoplasmic membrane
CWD	cell wall-deficient
CWDB	cell wall-deficient bacteria
FA	fatty acids
LPS	lipopolysaccharide
MPLS	molecular phospholipid species
Omp	outer membrane protein
SAK	staphylokinase

I. INTRODUCTION

L-form bacteria are unfamiliar to many microbiologists and the last 20 years has seen a reduction in the number of L-form research groups and consequently in L-form-related publications. There are several reasons for this decline. Although L-forms play a role in human and animal disease, their significance as causative agents remains unclear. Furthermore, L-forms and results obtained with them sometimes challenge current hypotheses, leading to controversy over some fundamental concepts, for example, cell envelope organisation and cell division. Finally, L-forms are, in general, difficult to both isolate and induce with their cultivation being much more laborious than that of typical cell-walled eubacteria and hence progress is often slow. This, in turn, makes repetition of experiments and verification of results by different laboratories difficult. Consequently, although L-form research is viewed by most as interesting, many view it with scepticism. This can cause difficulties for funding and dissemination of results.

This review focuses on the literature of the last 20 years, much of which discusses results with stable protoplast-type L-forms. This highlights the relevance of L-forms, which do not have a cell wall, for studying their interactions with eukaryotic hosts. It also shows how the contemporary methods of molecular biology, biochemistry and biotechnology have enhanced our knowledge of L-form bacteria *per se* and how L-form research may influence our understanding of other biological problems. A major difficulty in reviewing any publications concerning L-forms is the diversity of descriptions, or indeed lack of characterization, of the organisms with respect to their stability and state of their cell wall. In this review, a nomenclature is suggested to avoid future ambiguity. Needless to say, material is cited as originally presented although on occasion some papers have been interpreted in the light of our many years experience of these intriguing organisms.

II. DEFINITION AND CHARACTERISTICS OF L-FORMS

A. Definition of L-forms

Originally observed and named by Emmy Klieneberger (1935) (''L'' was used in honour of the Lister Institute, London, where she worked), the most popular definition of L-forms has been that of Madoff (1986) describing them as a special type of growth derived or induced from a bacterium following suppression of the rigid cell wall. This, however, does not precisely define the organisms whose nomenclature is confused by the different names and phenomena that are considered as L-forms. Thus, the literature uses numerous terms such as L-phase, L-variants, L-organisms, cell wall-deficient (CWD)-forms, as well as others (Gumpert and Taubeneck, 1983; Madoff and Lawson, 1992; Mattman, 2001; Onwuamaegbu *et al.*, 2005). CWD-forms is an all-inclusive term encompassing spheroplasts and protoplasts which are not able to divide and those which can. However, only spheroplasts and protoplasts capable of growth and cell division should be called L-forms. The term L-form encompasses different phenomena. On one hand, there are phenotypic variants of bacteria caused by reversible alterations in cell wall organisation resulting in unstable L-forms. On the other hand, there are irreversible alterations in cell wall organisation, caused by mutations in the genome and resulting in stable L-form strains.

L-forms can be differentiated into four types: unstable and stable spheroplast L-forms and unstable and stable protoplast L-forms (Fig. 1.1). Cells of **spheroplast-type L-forms** still possess some cell wall structure while cells of **protoplast-type L-forms** (Fig. 1.2) are free of any cell wall structure, that is, cell wall-less (Gumpert and Taubeneck, 1983;

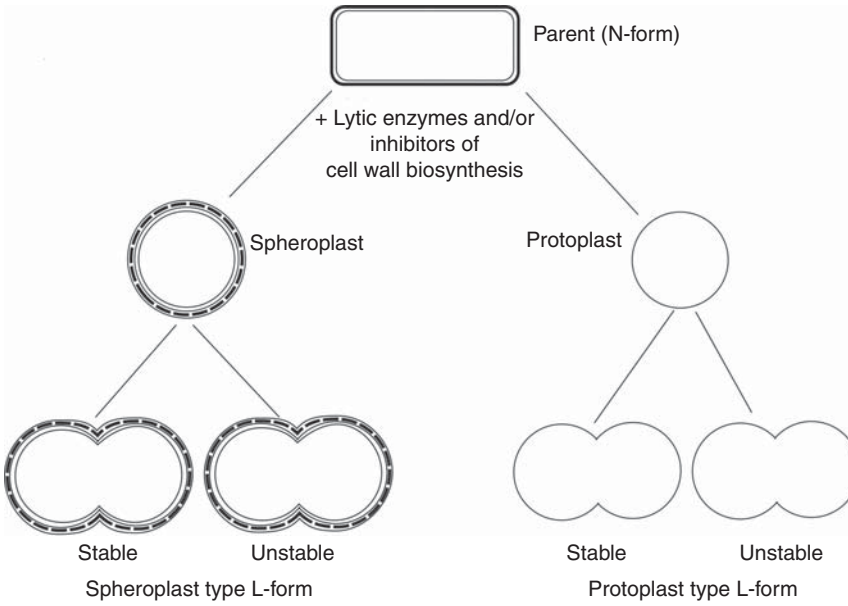

FIGURE 1.1 Schematic diagram showing the derivation of cell wall-deficient bacteria capable of growing as four types of L-forms. Spheroplast-type L-forms have remnants of the cell wall while protoplast-type L-forms do not. Stable L-forms will, unlike unstable forms, not revert to the parental form (N-form) when the inhibitors of cell wall biosynthesis are removed from the growth medium.

Hofschneider and Martin, 1968). Protoplasts are defined *per se* as cells without a cell wall structure as recognized by ultrathin sections and electron microscopy. **Unstable** L-forms can revert to normal walled parent bacteria (N-form), when inducing agents such as penicillin are omitted. They are considered to be genetically identical to the N-form although they may have lost some function, for example, ability to be attacked by bacteriophages (Waterhouse *et al.*, 1994a). **Stable** spheroplast and protoplast-type L-forms are not able to revert to the parental N-form. They have to be considered as genetically altered from the parent strains, as stable mutants, showing extreme pleotropic changes in their characteristics. These changes concern an altered colony and cell morphology (Fig. 1.2), the inablility to form intact cell walls, capsules, flagella and pili, changes in lipid and protein components of the cytoplasmic membrane (CM), reduction or absence of extracellular proteolytic activities, resistance against bacteriophages and β-lactam antibiotics and no or low toxic and pathogenic effects on laboratory animals (Domingue, 1982; Gumpert and Taubeneck, 1983; Gumpert *et al.*, 2002; Madoff, 1986; Mattman, 2001). All four L-form types can be isolated from the same

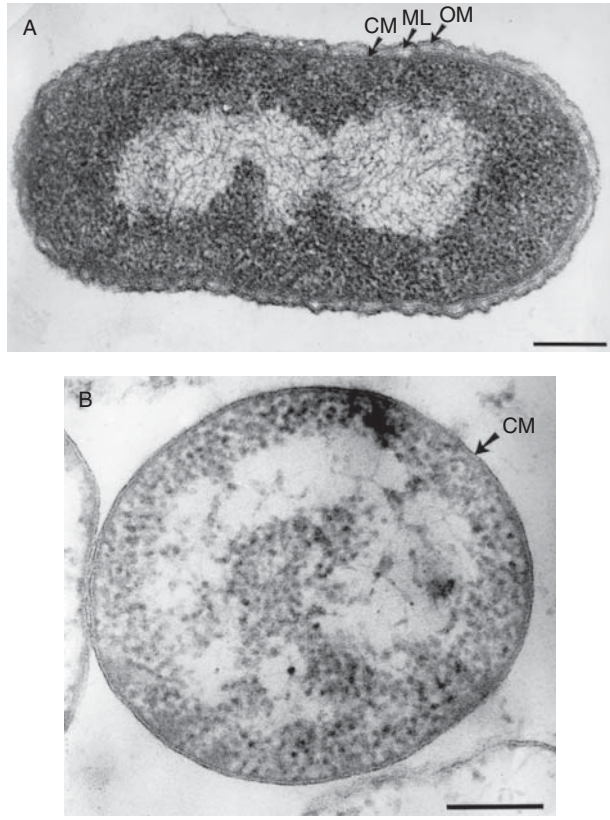

FIGURE 1.2 Ultrathin sections of a cell of the N-form *Proteus mirabilis* VI (A) and its derived protoplast-type L-form *P. mirabilis* LVI (B). CM, cytoplasmic membrane; ML, peptidoglycan layer; OM, outer membrane. Bar: 0.2 μm.

bacterial species. This has been shown for *Proteus mirabilis* and *Escherichia coli* (Gumpert and Taubeneck, 1979, 1983; Hofschneider and Martin, 1968). Usually, stable L-forms have been isolated by applying selective and adaptative procedures (see Section II.B).

The term ''L-phase'' has also been used by some authors as a natural and transient CWD stage that can occur as a survival strategy to environmental stress (Horwitz and Casida, 1978). The terms ''L-phase'' and indeed ''L-phase variants'' are in fact rarely used in modern literature and Madoff (1986), in her excellent book on L-forms, described them as misleading because of the implication that they reflect a natural state (i.e. a phase) universal to all bacteria. Thus, although the concept of loosing the cell wall in order for a cell to survive is fascinating, it is considered that much more scientific evidence is required in order to re-instate the use of these terms.

Many published reports do not characterize L-forms according to the aforementioned four types. Such descriptions often lack information about the presence or absence of the cell wall and consequently results have to be interpreted with care. Although it has been suggested that the terms CWD and L-form can be used interchangeably (Onwuamaegbu *et al.*, 2005), it is not recommended, as the ability of L-forms to divide is an important criterion for differentiating them from some CWD-forms.

B. Induction and cultivation

Prior to the 1940s and the advent of antibiotics, L-forms had been observed *in vivo* with some being isolated in pure culture. Since then, researchers have tended to induce the L-form state experimentally using the wide range of cell wall inhibitors now available. This has been achieved for many eubacteria and some authors consider that conversion to the L-form state may be an universal property (Gumpert and Taubeneck, 1983; Madoff and Lawson, 1992). Indeed, L-forms have been obtained from both Gram-positive and Gram-negative species as well as filamentous bacteria such as *Streptomyces hygroscopicus* (Hoischen *et al.*, 1997a) and *Streptomyces viridifaciens* (Innes and Allan, 2001). Most stable L-forms have been isolated by selective cultivation, following the four-step process of (1) *Induction*: growth of cells with inhibiting concentrations of substances that interfere with cell wall synthesis, (2) *Selection*: transfer of single colonies onto fresh media with and without cell-wall inhibitors, (3) *Stabilization*: selection of L-form colonies from medium without cell-wall inhibitors and (4) *Adaptation*: whereby the selected cells can be grown in different media under different growth parameters to improve the stability and quality of the cells and cultures.

Although it takes much time and detailed work, it is relatively straightforward to achieve a stable L-form line and there is a wide supporting literature for specific bacteria (Lederberg and St.Clair, 1958; Madoff, 1986; Schuhmann and Taubeneck, 1969). Having said this, experience has shown that the induction method for one particular species is not always successful for a different strain of that species. Indeed, in some cases, L-form induction and cultivation appears to be impossible. Induction is basically achieved by growing the cell walled (N-form) on media with agents (either singly or in combination) that affect the cell wall such as antibiotics, lytic enzymes and some amino acids, which interfere with the peptidoglycan cross-linkage (Schmidtke and Carson, 1999; Strang *et al.*, 1991). For survival of the cells and growth in a wall-less state, osmotic protection has usually to be provided by supplementation with sucrose and/or salt, although depending on the L-form strain, osmostabilization is not always required. Animal serum, typically horse serum,

is often used as an additional supplement and although it may not be required for the induction process *per se*, it improves the quality of growth and is often necessary for the growth of protoplast L-form strains on agar media. Direct induction is hence achieved by plating onto/into media containing inducing agents and osmotic protection. Indirect induction is often very successful whereby samples of protoplasts or spheroplasts are plated, usually at high cell numbers. Solidified media (often of low gel strength) are most commonly used for induction since the separation of N-forms from the slower growing L-forms tends to be more difficult in liquid culture. The dilemma of L-form induction and cultivation continues in a way that some bacteria are only inducible in liquid media and others on solidified media. Some authors have obtained excellent induction by using a solid–liquid interface (Paton and Innes, 1991). L-form induction, even when following published methods, often consists of many trial and error scenarios until the correct combination of inducing agents, gel strength, plating cell density, etc. is obtained and one occasionally wonders whether this reflects expertise or serendipity. Once obtained, L-forms have to be nurtured often with frequent subculture and the ''push-block'' technique (Allan, 1991) has been commonly used. The development of a stable cell line follows the route whereby the concentration of inducing agents is gradually reduced. The number of transfers for obtaining a stable L-form strain depends on the species and the selection conditions (composition of the medium, transfer rate per week). It needed 10–30 transfers for *Bacillus subtilis* (Allan, 1991) and *P. mirabilis* and more than 100 for *E. coli* (Schuhmann and Taubeneck, 1969). On occasion, no stable L-form could be isolated from some bacteria, for example, *Pseudomonas aeruginosa* and *Pseudomonas syringae* pv. *phaseolicola* in spite of several hundreds of transfers and various selection procedures (E. Schuhmann, C. M. J. Innes, unpublished results).

Many experiments to isolate stable protoplast L-forms by treatment with mutagenic substances and X-rays, followed by subculture under specific growth conditions, have been unsuccessful (Gumpert and Taubeneck, 1983). One exception seems to be the creation of a stable protoplast L-form of *E. coli* NC-7 obtained by treatment with *N*-methyl-*N*′-nitrosoguanidine and lysozyme (Onoda *et al.*, 1987). On the other hand, genetically stable spheroplast L-form strains of *E. coli* and *P. mirabilis* were successfully isolated after treatment with mutagens. These mutants were characterized by their L-form-like colony morphology and ability to revert to the N-form, for example, by addition of diaminopimelic acid (DAP) indicating a block in DAP synthesis (Lederberg and St.Clair, 1958; Martin *et al.*, 1974). The appearance of L-form colonies in *B. subtilis* after transformation with DNA of its stable L-form may be a further way to create L-form strains (Wyrick *et al.*, 1973).

Frequent and careful microscopic observation of L-forms is required during their isolation and this is achieved with or without staining. One of the most commonly used stains is that of Dienes (1967) although many other staining procedures are available (Mattman, 2001). Allan *et al.* (1992) used a modified Gram stain which is particularly useful during the induction of Gram-positive cocci which lose the typical Gram stain on loss of the cell wall allowing easy differentiation between the cell-walled and L-form state. Experience in our laboratories show that direct observation using unstained materials in conjunction with good quality phase and interference contrast microscopy is both quick and reliable. Observation of cultures allows the differentiation of L-forms by their pleomorphic shape and often with the appearance of vacuoles and granular material. The DNA binding fluorescent dye, DAPI (diamidino-2-phenylindole), has proven very useful for observing L-forms in plant cells (Aloysius and Paton, 1984). The occurrence of vacuoles presents an intriguing property of L-forms which questions one of the dogmas of microbiology in that the process of fluid-phase endocytosis was considered to be a unique feature of eukaryotes. Oparka *et al.* (1993), however, showed that this process, that is invagination of the plasma membrane to form a closed vesicle containing some of the extracellular fluid surrounding the cell, did indeed occur in a stable, protoplast-type, *B. subtilis* L-form. These authors speculated that the evolutionary development of fluid-phase endocytosis in eukaryotes may lie in the loss of the cell wall accompanied by the loss of turgor pressure.

During *B. subtilis* and *S. viridifaciens* L-form growth, the cell diameter increases as the number and size of vacuoles increases indicating that some L-forms may go through a ''developmental cycle'' (Allan *et al.*, 1993; Innes and Allan, 2001). As these cell lines aged, the cells appeared to lyse although successful subculture could still be achieved from very old shake flasks that appeared to only contain cell debris. Interestingly, the *B. subtilis* L-form also formed increasing numbers of so-called granules during its growth. Such granules can accumulate within cells and become released after lysis and they are considered by some authors as elementary reproductive units. It has been hypothesized that these granules, also referred to as ''dense elementary bodies,'' that form inside L-form cells may be regarded as ''stem cells'' which are able to produce new L-form or N-form cells (Domingue and Woody, 1997). It is well documented that L-forms may pass through membranes with small pore sizes (0.22 μm) (Madoff, 1986; Mattman, 2001) and some authors believe that granules are responsible for this (Darwish *et al.*, 1987). Similarly, Paton (1988) speculated that these reproductive granules allow dissemination of L-forms through plants. The authors of this review have never found any indication for the existence of such elementary reproductive units during studies with a wide range of L-forms and consider that such structures are formed by storage and degradation processes. The ability of L-forms to

pass through filters that normally act as a barrier to N-forms is probably a reflection of the flexibility of the cell membrane and/or the production of minute functional cells rather than the alternative suggestion of a different bacterial reproductive system incorporating granules or ''dense elementary bodies.'' Although some L-forms have a distinctive cellular shape some others are much less defined (see e.g. Mattman, 2001). Indeed, it would seem that the better an L-form strain is adapted to growth in solid or liquid media, the more uniform the cells become with respect to their spherical shape, size and absence of intracellular granules and vacuoles. Thus, the identification of L-forms by shape or colony morphology cannot be universally applied.

Isolation and cultivation of L-forms follow a strategy of adaptation. Most important processes are (1) adaptation of the organism to grow as CWD cells. This means especially strengthening the CM and modification of processes of cell division, transport and osmoregulation; (2) selection of mutants which have an irreversible block in cell wall biosynthesis, resulting in stable L-forms; and (3) further adaptation procedures to improve growth rates in various liquid media and under fermentative regimes, for example, to increase the ability to resist shear forces and to grow with or without various supplements.

III. SIGNIFICANCE OF L-FORMS

The significance of L-forms may be seen in four areas:

- as research tools which allow a better understanding of the structural and functional organisation of bacterial cells,
- as a unique expression system for use in biotechnology and medicine,
- as commensal, pathogenic and symbiotic organisms associated with animals including humans which may also be exploited towards disease therapy and
- as artificially associated organisms with plants that confer novel attributes such as disease resistance.

To highlight only four areas of significance, though pertinent to this review, is perhaps supercilious and like any list, additions or deletions can be made. The loss of the cell wall has been advocated, as mentioned earlier, as a survival strategy. In their paper on biological significance of L-forms, Pachas and Madoff (1978) advocated this concept as a means for preservation of bacterial life in both the natural environment and *in vivo*. Unfortunately, some 30 years later, the addition of this idea to the above list cannot be justified since further research is still required.

Pachas and Madoff (1978) also suggested that L-forms may play a role in evolution with respect to the development of mycoplasma

from bacteria. Since then, genome sequencing data from L-forms and changes in their cell wall, CM and physiological properties show clearly that genetic alterations occur during long-term cultivation in the L-form state (see below). In nature and especially in associations with eukaryotic hosts, the modification and acquisition of new properties through environmental influences, including horizontal gene transfer, are countless. Thus, L-forms may also play a role in evolution of mycoplasmas, non-culturable bacteria, nanobacteria (Wainwright, 1999) and indeed, eubacteria. Moreover, work on L-form bacteria and their interactions with plants (see Section III.D.4) indicates that L-forms can enter into the cytoplasm of plant cells and so, it is not surprising that Paton (1987) speculated as to whether this could suggest a mechanism for the evolution of the mitochondrion or chloroplast.

A. Contributions to bacterial cell division

Cell division in *E. coli* takes place precisely at the mid-cell of the dividing cells. Septum formation between the replicated and segregated daughter nucleoids involves the invagination of all three envelope structures, the CM, the murein sacculus and the outer membrane (OM). Meanwhile at least 10 essential division proteins are known to participate in the constriction of the septum by formation of a contractile Z ring that interacts with the CM and components of the periplasm. The coordinated interaction of all divisome components, that is the cytosolic proteins, the CM spanning and membrane anchored periplasmic proteins, the invaginating and stabilizing murein sacculus and regulatory factors (e.g. MinCDE), is essential for proper cell division. Despite the rapid growth of data there are still several unanswered questions concerning bacterial cell division (Goehring *et al.*, 2006; Gumpert, 1993; Lutkenhaus, 2007; Margolin, 2000, 2005; Norris *et al.*, 1999a).

Stable protoplast L-forms can be used to elucidate some of these questions. These cells (Fig. 1.2) are able to divide by a process akin to binary fission even although they do not have any cell wall and are surrounded only by the CM (Gumpert and Taubeneck, 1983). Nonetheless, the stabilizing peptidoglycan layer has been postulated to be necessary for proper cell division (Joseleau-Petit *et al.*, 2007; Li *et al.*, 2007) and thus, stable protoplast L-forms challenge fundamental concepts regarding cell division. In addition, the concepts that chromosomes segregate by zonal growth of the cell envelope between putative attachment sites of the chromosomal DNA or that of an active in-growth of the cell envelope are clearly not the processes used by L-forms. With respect to these issues and considering cell division as a comprehensive entity, two alternative concepts have been devised: the ''nucleoid-associated compartmentation'' concept (Gumpert, 1983, 1993) and the ''enzoskeleton-hyperstructure'' concept (Norris *et al.*, 1996, 1999b, 2007).

The ''nucleoid-associated compartmentation'' concept combines known facts of bidirectional DNA replication, the supercoil packaging of DNA in the nucleoid body and the formation of a network consisting of open DNA loops, of coding and non-coding RNA and of proteins around the nucleoid. Light microscopic and electron microscopic analyses showed characteristic changes in the nucleoid body: I, Y, V and U forms (Gumpert, 1993). These forms reflect the ordered packaging of chromosomal DNA during replication in individual nucleoids in the course of cell division. The formation of an individual network around each daughter nucleoid would lead to an inner compartmentation that differentiates the cell content into two (or more) entities and is according to this concept, one of the driving forces in segregation of nucleoids and finally in their separation and cell division. For this early stage of cell division no Z ring is necessary. It forms after the nucleoids are separated and drives the septum formation. This basic process does not involve a peptidoglycan septum and the cell wall (Gumpert, 1983, 1993).

The ''enzoskeleton-hyperstructure'' concept (Norris *et al.*, 1996, 1999b, 2007) follows the idea that the cell cycle and the cell division processes are controlled and determined by hyperstructures. These are large-scale structures of DNA, cytoplasm and membranes, that is, the replication complex or proteolipid domains in the CM. The presence of kinases, phosphorylated proteins and FtsZ-MreB filaments represents, according to this concept, an enzoskeleton-like structure in L-form cells. The concept postulates that many hyperstructures are involved in cell division (Norris *et al.*, 1996, 1999b). The presence of several phosphorylated proteins (DnaK, S1, SucD, TypA, Dps, YfiD) in the *E. coli* L-form NC-7 was discussed with respect to their possible role in cellular integrity and cell division (Freestone *et al.*, 1998). Such phosphoproteins might regulate and enforce a putative enzoskeleton in L-form cells. However, there are no conclusive data how these hyperstructures interact and result in a precise cell division (Norris *et al.*, 2007).

Data arising from Siddiqui *et al.* (2006) at the Fritz-Lipmann Institute of Age Research, Jena, Germany (former Institute of Molecular Biotechnology) support the idea that bacterial cell division is possible with a rudimentary Z ring and without a peptidoglycan layer. Comparative DNA sequencing of PCR products of genes of the *dcw* cluster in *E. coli* LWF+ and its cell-walled parent N-form showed that all the important cell division genes are present in the L-form. Identical findings were obtained for the protoplast L-form of *B. subtilis* L170 (R.A. Siddiqui, personal communication). However, in *E. coli* LWF+ *mraY* and *ftsQ* are mutated to an extent that their products cannot be functional anymore. The nonsense mutation in *ftsQ* (W132Stop) results in a truncated protein lacking the 145 C-terminal amino acids. The truncated region of FtsQ is essential for localisation at the Z ring and interaction with various downstream

recruited Z ring components (Goehring *et al.*, 2007). In contrast to *E. coli* LWF+ , the cell-walled *E. coli* K12 is not viable with a truncated FtsQ lacking the 140 C-terminal amino acids (D'Ulisse *et al.*, 2007). Probably, the cells cannot divide because several components of the divisome, which interact with periplasmic proteins can no longer localised at the Z ring. Missense mutations causing amino acid substitutions were observed in *ftsA*, *ftsW* and *murG*. It is not clear whether these alterations are irrelevant for their function or they modulate the protein products allowing a better function in the cell division machinery of L-form cells.

The most important finding is the frameshift mutation in *mraY*, which results in a truncated, probably inactive MraY protein (Siddiqui *et al.*, 2006). The product of the *mraY* gene is an integral membrane protein. It catalyzes the binding of building blocks for cell wall synthesis, for example, UDP-MurNAc-pentapeptide, teichoic acids, O-antigen determinants and capsular polysaccharides to bactoprenol, a C_{55} isoprenoid alcohol lipid, which translocates these building blocks through the CM. If this process cannot take place, the synthesis of the murein sacculus and other extracellular polysaccharides (LPS, capsules) can also not take place. The consequences are among others, inhibition of cell wall biosynthesis and cell wall-less cells. It seems that alterations in the nucleotide sequence of the *mraY* gene are indeed one of the crucial steps in the creation of the stable protoplast L-form *E. coli* LWF+.

E. coli L-forms have been investigated for the presence of FtsZ, the main component of the Z ring and for the formation of Z rings. Onoda *et al.* (2000) estimated a five-fold lower FtsZ level per unit of protein in L-form lysates of the slow growing *E. coli* NC-7 than in the parent strain and thus postulated that this FtsZ content was too low for activities in cell division. They showed furthermore that growth of their L-form cells specifically requires calcium and concluded that cells possess an enzoskeleton which is partially regulated by calcium. These cells have never been investigated by cell biological methods for the presence of Z rings. In contrast to NC-7, however, the *E. coli* L-form LWF+ exhibits a high level of FtsZ obviously sufficient for Z ring formation. Using fluorescence microscopical approaches *in situ* with FtsZ specific antibodies and *in vivo* with green fluorescent protein (GFP)-tagged FtsA, ring structures of FtsZ and FtsA could unequivocally be observed in cells of *E. coli* LWF+ (C. Hoischen, unpublished results). These rings are contractile and seem to drive septum formation during cell division. The relatively short doubling time of about 50–70 min supports the idea of a Z ring-driven septum formation.

In a recent paper, Joseleau-Petit *et al.* (2007) have described ''L-form like cells'' that still contain a low amount of peptidoglycan and require peptidoglycan synthesis for growth as demonstrated by chemical analysis. These cells, which still possessed an OM, were induced by overnight

cultivation in the presence of cefsoludin, a potent inhibitor of cell wall synthesis. In the absence of this selective antibiotic the cells revert to normal N-forms. In concurrence with these results the authors and commentators (Casadesus, 2007; Holzman, 2007; Young, 2007) speculated that possibly all L-forms have residual peptidoglycan synthesis which is essential for their growth and cell division. This necessitates some emphatic supplementary remarks. Similar experiments using a strain of *E. coli* K12 1655 have previously been undertaken by Schuhmann and Taubeneck (1969). In the presence of β-lactam antibiotics mucoid colonies were produced on agar media. Cells in these colonies were pleomorphic, surrounded by remaining cell wall material and a slime layer and showing reversion to the parent rod-shaped bacterium in the absence of the selective antibiotic (Gumpert and Taubeneck, 1974; Gumpert *et al.*, 1971b). Obviously, the mucoid colonies and ''L-form-like'' cells described by Joseleau-Petit *et al.* (2007) represent similar unstable spheroplast L-forms. The presence of peptidoglycan in such cells is not surprising.

In contrast, stable protoplast L-form strains do exist, for example, those of *E. coli* LWF+, *P. mirabilis* LVI and *B. subtilis* L170, which have been grown for decades in the wall-less L-form state without any observed reversion. Their cells show no cell wall or septum-like peptidoglycan structure as shown by ultrathin sections (Gumpert *et al.*, 1971a; Hofschneider and Martin, 1968) and freeze-fracture electron microscopy (Hoischen *et al.*, 2002). In addition, these protoplast L-forms are in general insensitive to phage adsorption (Gumpert and Taubeneck, 1983; Gumpert *et al.*, 1971a). DAP and muropeptides were also absent (Geuther and Tkocz, 1972; Gumpert and Taubeneck, 1983; V. Höltje, unpublished results). Furthermore, the mutation in the cell division gene *mraY* which catalysis an early step in peptidoglycan synthesis indicates that *E. coli* LWF+ should have no residual peptidoglycan outside the CM. So, the conclusion that peptidoglycan synthesis is essential for bacterial cell division does not apply to stable protoplast L-forms of *E. coli* LWF+ and *P. mirabilis* LVI and it can be surmised that this is probably true for all stable protoplast L-forms.

Recently, *B. subtilis* L-forms have been generated from a strain, M96, where expression of the *murE* operon was controlled by an inducible promoter (Leaver *et al.*, 2009). Sustained proliferation of L-form-like cells was maintained and although the strain did not require penicillin for growth, a low reversion frequency was observed. It was considered that a single point mutation in the *yqiD* gene, which is homolog to the *ispA* gene of *E. coli* (involved in formation of essential lipids in peptidoglycan and teichoic acid synthesis), was responsible for L-form generation. This strain, in contrast to the results of the Jena group, was able to divide independently of Fts-Z albeit with relatively long doubling times, although in contrast to Joseleau-Petit *et al.* (2007) did not require residual peptidoglycan.

It is obvious that protoplast L-form cells divide without peptidoglycan structures. It is further obvious that the chromosomal DNA is the functional and structural centre of the cell that also follows physical and spatial rules. So with respect to cell division, the chromosome should be regarded not only as a sequence of nucleotides and division genes, but also as a substantial mass, the nucleoid body. Here, the DNA is highly ordered and can dynamically interact with the cytoplasmic components and the CM and thus plays a substantial role in cell division.

B. Contributions to membrane organisation

As previously stated, cells of protoplast L-forms are surrounded only by a typical bilayer membrane, representing the CM (Fig. 1.2). Thus, it has to carry out all functions in the processes of osmoregulation, transport, energy transfer, signal transduction and cell division without the help of the cell wall and the periplasm. The CM of adapted L-form strains, especially of those able to grow in fermenters, has higher mechanical strengths, elasticity and functionality than the CM of their parent N-form cells (Domingue, 1982; Klessen *et al.*, 1989). Comparative biochemical and ultrastructural studies of the CMs of *E. coli* WF+, *P. mirabilis* VI and *S. hygroscopicus* 33–354 and of their L-forms have been undertaken by the group in Jena to determine whether these properties are associated with changes in lipid and protein components (Gumpert *et al.*, 2000; Gura, 1998; Hoischen *et al.*, 1997a,b).

1. Lipid components

Lipids play a key role in the structural and functional organisation of biomembranes. The lipid fractions, phospholipid classes, fatty acids (FA) and molecular phospholipid species (MPLS) were analysed by thin layer chromatography, high-performance liquid chromatography (HPLC), gas chromatography and electrospray ionization coupled with collision-induced mass spectrometry (ESI-MS–CID-MS).

One common result was that L-form membranes of all three species contained the same phospholipid classes, FA and the same MPLS than their N-form membranes. No sterols or additional glycolipids were detected. Although Nishiyama and Yamagushi (1990) reported sterols in CM of *Staphylococcus aureus* L-forms there is some debate about a possible incorporation from the growth medium. In the CMs of most protoplast L-forms there are no qualitative changes in the lipid components. Obviously, the lipid metabolism and structural genes for phospholipid synthesis are not altered. This interpretation is supported by the proof of intact lipid genes after sequencing the L-form genome of *E. coli* LWF+ (R. A. Siddiqui, personal communication).

Another result common for all three bacteria was the elevated content of total extractable lipid (two- to four-fold) and cardiolipin (CL) (five- to seven-fold) in L-form membranes. The higher lipid content would suggest that there is more space in the CM where lipid–lipid interactions predominate and this may contribute to higher mechanical strengths, better lateral diffusion and formation of lipid domains. The higher proportion of CL confirms earlier reports, for example, for staphylococcal L-forms (Hayamy *et al.*, 1979). More CL should stabilize the membrane by its binding of divalent cations and by supporting membrane curvature (de Kruijff *et al.*, 1997).

Further quantitative differences have been observed which, however, were not common for all three investigated species. They were specific for *S. hygroscopicus* on the one hand and for *E. coli* and *P. mirabilis* on the other. The L-form membranes of *S. hygroscopicus* showed more phospholipids (+20 mol%), more PIM (phosphatidylinositol) (+8%), less PE (phosphatidylethanolamine) (−10%), more anteiso-branched FA (aiFA; +10–12%) and more MPLS containing aiFA. The L-form membranes of *E. coli* and *P. mirabilis* showed less phospholipid (−8%), more saturated FA (+8–10%), less cyclopropanated FA (−8%) and more MPLS composed of two saturated acyl chains (two- to seven-fold).

The comparative analyses of MPLS in this study showed for the first time an unexpected extraordinary diversity among the lipid molecules in bacterial membranes. In *E. coli* and *P. mirabilis*, the number of different MPLS was 11–16 for PE, 10–14 for PG (phosphatidylglycerol) and 29–30 for CL. Because the majority of MPLS contain two different FA which can bind to the *sn*-1 and *sn*-2 position as well and because the glycolipid and the neutral lipid fractions show similar complex FA pattern, more than 200 different lipid molecules can be expected. The content of MPLS which are composed of two saturated FA acyl chains was two- to three-fold higher in the PL classes of *E. coli* LWF+ and up to seven-fold higher in those of *P. mirabilis* LVI. A similar increase was observed for MPLS composed of one saturated and one unsaturated acyl chain (two- to four-fold) in both L-form membranes. *S. hygroscopicus* contained eight phospholipid classes with a similar pattern of FA in each class. Up to 400 different lipid molecular species can be expected in these membranes.

Each lipid molecule has its own individual molecular configuration and phase behaviour. The transition temperatures from gel to liquid phase and from lamellar to non-lamellar phase determine strengths and fluidity of the membrane (de Kruijff *et al.*, 1997; Dowhan, 1997). In addition, many MPLS interact specifically with membrane proteins. The availability of so many diverse lipid molecules and the regulation of their quantities is obviously a prerequisite for a well functioning membrane and might be essential for the CM of wall-less L-form cells.

Whatever this complexity means and what functions each of these molecules serves is widely unknown (Cronan, 2003; Dowhan, 1997). It is clear, however, that L-form cells are using their sets of molecular lipid species to establish such quantitative proportions which allow fluidity, molecular packaging, permeability and mechanical strength in the bilayer system which is sufficient for cell integrity and growing in a cell wall-less state. It is apparent that the stress of a life without the cell wall and the periplasmic compartment does not induce the formation of new phospholipids and PLMSs, but does affect the proportion being produced. Such characteristic quantitative changes in lipids, especially in FA and MPLS should be regarded as an explanation for the special properties of L-form membranes and as a general principle of adaptation and evolution.

2. Proteins

Like N-forms, L-form membranes contain very many proteins as documented by gel electrophoresis (one-dimensional, *P. mirabilis* LVI, Karch and Nixdorff, 1980) and very impressively by two-dimensional PAGE which differentiated about 500 proteins in CMs of *E. coli* LWF+ (Gumpert *et al.*, 2000). Of special interest was the detection of OM proteins in the CM of *E. coli* LWF+. OmpA, OmpC, OmpF, OmpT and LambB have been identified by their position in 2D-PAGE gels and partially by N-terminal sequencing. They can occur in considerable amounts and may play a role in the structural and functional organisation of the L-form membrane.

L-form membranes can incorporate its own and foreign membrane proteins after overproduction of recombinant genes (see Section III.C). The *OmpA* gene from *E. coli* could be expressed in L-forms of *E. coli* LWF+ and *P. mirabilis* LVI. Surprisingly, it is stably overexpressed in considerable amounts by the L-form of the Gram-positive bacterium *B. subtilis* L170 and a certain portion remains bound to (maybe even incorporated into) the CM (Fritsche, 2001; Gumpert *et al.*, 2002). It should be pointed out, that in general *B. subtilis* degrades recombinant OmpA immediately after synthesis.

Further examples are membrane-spanning sequences of integral membrane proteins LacY, SecY and CcmA, which can be integrated into the L-form membranes. Interestingly, the overproduction of homologous and heterologous membrane proteins OmpA, LacYH1-sak, CcmAH1-sak and the incorporation into the L-form membrane was stably maintained for 10–50 generations. It had no negative effect on growth and on the stability and functionality of the membrane (Fritsche, 2001; Gumpert *et al.*, 2002; Hoischen *et al.*, 2002).

3. Ultrastructure

The structural organisation of membranes can be studied by using electron microscopic techniques, especially ultrathin-sectioning, freeze-fracturing and immunolabelling. In ultrathin sections no differences were seen in the CMs of protoplast L-form and N-form cells (Gumpert and Taubeneck, 1983; Gumpert *et al.*, 1971a; Hofschneider and Martin, 1968; Sieben *et al.*, 1998). They are typical trilamellar structures with an inner and an outer dark leaflet, more or less similar in thickness, separated by a contrast-less layer, representing the acyl chain region of lipid molecules (Fig. 1.2).

Freeze-fracturing splits the two leaflets of the membrane, allowing views onto the inner and outer leaflet. In general, freeze-fracture micrographs are again similar in L-form and N-form membranes. The inner leaflet is characterized by a more or less dense cover of granules (about 10 nm in diameter) which represent integral membrane proteins. The outer leaflet contains only a few of such particles which can be concentrated in heaps. However, L-form membranes often show structural peculiarities. The density of intramembrane particles can vary considerably. Particle-free areas representing lipid domains, which seem to be free of integral membrane proteins, are formed more frequently in L-form membranes.

An unusual tetragonal wafer pattern of periodically curved bilayer areas was observed in membranes of *S. hygroscopicus* (Gumpert and Taubeneck, 1983; Meyer *et al.*, 1990). A high diversity of such structures were obtained in L-form membranes and liposomes prepared from extracted lipids. Obviously, these structures are ''infinite periodic minimal surface structures'' (IPMS), resulting from special arrangements of bilayer-forming and non-bilayer-forming lipid molecules. This first report of such structures in bacterial membranes shows how freeze-fracture investigations of L-form membranes contribute to new aspects concerning the formation of lipid domains and processes of membrane curvature and phase transition (Meyer and Richter, 2001).

Further interesting results were obtained by studying L-form membranes by freeze-fracture replica-labelling electron microscopy. This method allows the localisation of biomolecules in the inner or outer leaflet of a membrane using immunogold labelled antibodies (Fujimoto, 1997). Most important results have been the detection of LPS molecules in the outer leaflet of the CM of *P. mirabilis* LVI (M. Westermann, unpublished results), the localisation of OmpA in the outer leaflet of CM from *P. mirabilis* LVI, *E. coli* LWF+ and *B. subtilis* L170 (Fritsche, 2001) and the anchoring of the surface-displayed staphylokinase (SAK)-protein in the CMs of *P. mirabilis* LVI and *E. coli* LWF+ (Hoischen *et al.*, 2002).

These comparative studies showed that in L-forms the CM is a highly complex system, composed of several hundreds of different lipid and

protein molecules. On one hand, L-form membranes seem to be a conservative, stable system. The ultrastructure and the overall lipid and protein composition of L-form CMs are similar to the CMs of walled parent cells. However, simultaneously, it is a variable dynamic system, able to change its lipid and protein pattern. As shown for *P. mirabilis* LVI, the CM of Gram-negative L-form strains can contain permanently LPS, Omp's and it can incorporate foreign proteins after overproduction. There is some indication that they also incorporate lipids from media components into their membranes (FA, cholesterol; Gmeiner and Martin, 1976). One can speculate that these remarkable changes contribute to the higher mechanical strengths, elasticity and functionality. Further studies with CM's of protoplast L-forms might give more insights in the relationships between membrane composition, membrane structure and membrane function.

C. Biotechnology

The first approaches for a biotechnological use of L-forms arose in the 1970s and the work that followed was mainly done with the stable protoplast L-forms of the Jena group. The idea was that L-forms might show advantages over conventionally used bacteria due to the reduced contents of LPS, antigens and toxic components (Taubeneck *et al.*, 1986).

In the early 1980s, protocols for successful transformations of protoplast L-forms by plasmid DNA had been developed based on PEG (polyethylene glycol)-mediated procedures (Gumpert *et al.*, 1987, 2002; Klessen *et al.*, 1989; Mahony *et al.*, 1988), electrotransformation (Katenkamp *et al.*, 1992) and transconjugation (Tölg *et al.*, 1993). From this time, the L-form strains could be used for the production of heterologous proteins. Besides *P. mirabilis* LVI, which appeared to be, by far, the most suitable strain, *E. coli* LWF+ and *B. subtilis* L170 were used for overexpression studies.

For construction of expression plasmids constitutive promoters (*sak*, *speA*), many inducible promoters (*lac*, *tac*, *tetA*, T7) and the usual origins of replication could be used. Resistance genes for antibiotics interfering with the cell wall synthesis (e.g. penicillins) were not suitable, while those for erythromycin, kanamycin, neomycin, nourseothricin and oxytetracyclin were suitable (Gumpert *et al.*, 2000, 2002).

L-forms were grown in liquid complex growth media such as BHIB (brain heart infusion broth), TSB (tryptic soy broth) and LFSB (L-form standard broth made from beef extract; Klessen *et al.*, 1989), all supplemented with 0.5–0.8% yeast extract. The media had to be modified with further supplements depending on the strains used and the peculiarities of the protein product (Gumpert *et al.*, 1987, 2000). A culture of *P. mirabilis* LVI typically grows with a log phase of 6–12 h, a stationary phase without lysis of 30–60 h and generation times between 60 and 120 min up to a cell density of 10^8–10^9 cells ml^{-1} (2–4 g dry weight L^{-1}). The amount of

inoculum is a critical point. It needs 10^6 cells ml^{-1} for *P. mirabilis* LVI and 10^7 cells ml^{-1} for *E. coli* LWF+ for successful growth (Gumpert and Hoischen, 1998; Gumpert *et al.*, 2002).

By long-term adaptation, strains of *P. mirabilis* LVI, *E. coli* LWF+ and *B. subtilis* L170 were generated, which were able to grow under conditions of fermentation in 2–100 L scale. Procedures for obtaining well growing L-form strains of *P. mirabilis* in laboratory fermenters have been described in patents (Gumpert *et al.*, 1987, 2000). Stirred fermenters (BIOSTAT B Braun/Melsungen, Germany) allowed the investigation of growth parameters and the optimization of growth and product yields (Gumpert *et al.*, 2000, 2002). The parameters for prochymosin production in 2–40 L scale were 37 °C, pH 6.5, stirring frequency 300–800 rpm, aeration rate 0.6–1.0 volume air per volume LFSB medium supplemented with yeast extract 0.5%, sucrose 0.2% and NaCl 0.05% (Klessen *et al.*, 1989).

More than 30 different protein products, among them enzymes, enzyme activators and antibodies have been synthesized using the L-form expression system. Summarized, it was shown (for detailed information, see reviews of Gumpert and Hoischen, 1998; Gumpert *et al.*, 2002) that the L-forms have a potential to be an alternative suitable expression system, which might in certain cases have advantages over standard bacterial expression systems. Using appropriate signal sequences from Gram-negative as well as from Gram-positive bacteria, for example, those from *OmpA*, *speA*, *sak*, *hemA*, *pac* and *phoA* (Bushueva *et al.*, 1998; Gumpert *et al.*, 1996, 2002; Klessen *et al.*, 1989; Kujau *et al.*, 1998; Laplace *et al.*, 1989a,b; Rippmann *et al.*, 1998), soluble cytosolic proteins were secreted into the extracellular medium. For this, the proteins were transported via a Sec-dependent secretion process including the activity of a leader peptidase. The secretion is not due to an uncontrolled lysis of the L-form cells (Gumpert and Hoischen, 1998; Gumpert *et al.*, 2002). In walled cells of *E. coli*, proteins with a signal sequence are secreted into the periplasm. This difference turned out to be a true advantage of the L-form expression system. Compared to the restricted volume of the periplasmic space in walled N-form cells, the area outwith the CM of L-forms is more or less unlimited. This means, the concentrations of secreted proteins in the periplasm and of unsecreted proteins in the cytoplasm are much higher even when the total amount of expressed proteins is the same as in L-forms. This strongly increases the risk for formation of inclusion bodies as could be shown for miniantibodies (Kujau *et al.*, 1998) and several scFv antibodies (Rippmann *et al.*, 1998).

The total yields of expressed proteins were comparable to *E. coli* producer strains. In some cases, the amounts of functional active proteins were relatively high in L-forms. Maximum yields were, for example, 70 mg L^{-1} prochymosin and 200 mg L^{-1} SAK and GFP (Bushueva *et al.*, 1998; Fritsche, 2001; Klessen *et al.*, 1989; Kujau *et al.*, 1998; Rippmann

et al., 1998). The lack of extracellular proteases decreases the risk of product degradation. As shown for PAC (penicillin G acylase), miniantibodies and scFvs-antibodies the proteins were correctly folded and modified after expression (Gumpert *et al.*, 1996; Kujau *et al.*, 1998; Rippmann *et al.*, 1998).

Also, OM proteins of Gram-negative bacteria have been synthesized with the L-form expression system. After overexpression, recombinant OmpA from *E. coli* was cleaved by a leader peptidase and secreted. About 50% of the protein was released into the medium and 50% remained bound to the CM. These findings were observed in all three L-form producer strains, *E. coli* LWF+ , *P. mirabilis* LVI and *B. subtilis* L170 (Fritsche, 2001; Gumpert *et al.*, 2002). Immunogold labelling and freeze-fracture electron microscopy showed that OmpA molecules are bound to the outer leaflet of the L-form membrane (Fritsche, 2001). L-form cells overexpressing OmpA were viable and showed no increased lysis. As a further example, ShlB, a hemolysin (60 kDa) located in the OM of *Serratia marcescens*, was overexpressed and localised in the CM of *P. mirabilis* LVI (Sieben *et al.*, 1998).

Finally, *P. mirabilis* LVI and *E. coli* LWF+ have been established as a system for bacterial surface display (Fritsche, 2001; Gumpert *et al.*, 2002; Hoischen *et al.*, 2002). For surface display, cells synthesize foreign proteins and locate them via various mechanisms at their outside surface (Ståhl and Uhlen, 1997). In contrast to regular surface display systems the proteins are anchored in the CM of L-form cells by transmembrane domains of integral membrane proteins. In walled Gram-negative N-forms, proteins are usually anchored in the OM or bound to surface components such as pili or flagella, whereas in Gram-positive bacteria the proteins are bound to protein components of the cell wall. SAK was used as a model protein and surface displayed by *P. mirabilis* LVI and *E. coli* LWF+. When the *sak* gene was fused to DNA sequences encoding hydrophobic transmembrane domains (e.g. helix 1 and helices 1–3 from the lactose permease LacY, the preprotein translocase SecY, or the curved cell morphology protein CcmA), the fusion proteins were synthesized and 80–100% were found to be membrane-bound at the outer surface of the cells. Detailed studies of cells and isolated membranes (ultrasonication, solubilization with detergents, digestion with trypsin, western blot, SDS-PAGE, functional milk test, immunogold labelling, freeze-fracture electron microscopy) showed that SAK molecules were tightly bound to the outside surface of the L-form membrane, that they were functionally active and were accessible for interaction with other agents (Fritsche, 2001; Hoischen *et al.*, 2002).

In summary, studies show that the L-form expression system can be advantageous in comparison to typical bacterial host systems. It provides an opportunity to overcome problems due to intracellular localisation of gene products, formation of inclusion bodies, burdening of isolation

procedures by endotoxins and immunoreactive substances and degradation of gene products due to extracellular proteolytic activities. Of special interest is the potential of overexpressing membrane proteins and fusion proteins for surface display (Hoischen *et al.*, 2002). This may allow the generation of protein-membrane preparations for application in vaccination, diagnosis, therapy and biotechnological processes. Following adaptative strategies, it should be possible to produce recombinant proteins by L-forms of *P. mirabilis* VI and *E. coli* WF+ in large-scale fermenters (Gumpert *et al.*, 2000).

D. L-form interaction and association with eukaryotes

1. Interaction with animals

The role of the cell wall in determining bacterial virulence and pathogenicity is well established and it is not surprising that there has been speculation concerning the role of L-forms in animal and human disease and its therapy. Similarly, the bacterial cell wall is crucial to many plant interactions, especially in those concerning pathogenicity and so its alteration or loss is also likely to have consequences.

CWDB have been detected in insects, birds, invertebrates and mammalia. Some of these may be non-culturable ''mycoplasma-like organisms'' (Lee and Davis, 1992) while others may be L-forms. This detection has resulted in much discussion concerning the roles of L-forms in pathogenicity and disease therapy. Although there are several reports of L-forms existing in eukaryotes their presence may, in many situations, be a consequence of exposure to antibiotics and other inducing agents which in turn results in resistance to therapy. A good example is the dairy cow which can suffer from inflammation of the udder (mastitis) with dramatic effects on milk yield and milk quality. In severe cases, death can occur. In order to prevent mastitis, the udder is treated prophylactically with long-term antibiotics during the drying-off period. The major cause of mastitis is *S. aureus* although many other organisms can be implicated. Based on the studies on *S. aureus* L-forms, Owens (1987) noted that L-forms may play a role in a variety of human diseases. He, like others, postulated that L-forms could represent a transient form allowing the organism to escape antibiotic therapy and re-emerge, under more favourable conditions, as the N-form (pathogen). He showed that L-forms occurred in cattle infected experimentally with *S. aureus*, which had been treated with penicillin. In later work, this group investigated the effects of different antibiotics on *S. aureus* L-form induction, showing the occurrence of a wide diversity of colony characteristics and the ability of the L-forms to revert hence suggesting that L-forms could act as transient forms *in vivo* (Owens, 1988; Owens and Nickerson, 1989). Similar results were found by Sears *et al.* (1987). Such studies intrigued veterinary

scientists although Buswell and O'Rourke (1995) concluded that L-forms are not of practical significance in mastitis since they can only be isolated on hypertonic medium, a condition that does not exist in the udder. Such comments are difficult to discount when dealing with unstable L-forms in natural situations and the challenge of identifying L-forms *in vivo* cannot be underestimated.

Kiessling *et al.* (1990) undertook an in-depth study concerning the problems of resistance of *Salmonella typhimurium* var. *copenhagen* to therapeutic and vaccination programmes in domesticated pigeons. They recovered extremely high levels of different types of L-forms from domesticated, free-living pigeons and their eggs. It was concluded that L-form occurrence resulted from over-use of antibiotics by pigeon breeders. The occurrence of L-forms in wild birds led the authors to agree with others (Domingue, 1982) that the interaction of antibiotics, complement, phagocytes, lysozyme and other enzymes may result in L-form induction.

In studies on fish, the L-form of *Aeromonas salmonicida*, *Yersinia ruckeri* (Gibb *et al.*, 1996) and *Lactococcus garvieae* (Schmidtke and Carson, 1999) have similarly been implicated in the recurrence of disease. Interestingly, Schmidtke and Carson (1999) presented evidence that salmonids have sites with suitable osmolality for L-form survival and that stable wall-less L-forms of *L. garvieae* persist without reversion and expression of disease. It has also been suggested that vaccines based on L-form cells may enhance protection compared to those prepared from N-forms (McIntosh and Austin, 1993).

An interesting example of L-forms occurring in another eukaryotic group is that of insects. L-forms of group D *Streptococcus faecalis* have been found to occur in *Drosophila paulistorum* and *Ephestia kuenela* (Ehrman *et al.*, 1990; Somerson *et al.*, 1984). These L-forms are endosymbionts and are involved in male fertility.

2. Clinical significance and role in diseases

Interactions of L-forms with mammalia and especially with humans have many facets. On one side, they may lead to immunological and pathogenic reactions in the host. On the other hand, such interactions may be considered as a continual biological process leading to genetically altered bacteria, as also discussed for the evolution of mycoplasmas (Domingue and Woody, 1997). Large bodies of data support the concept that L-forms can be induced in eukaryotic organisms, can persist there for long times, can produce pathogenic effects and can be the cause for recurrent infections after apparently successful chemotherapy.

The clinical significance of L-forms, however, is not clear. Data and problems are discussed in several monographs (Domingue, 1982; Madoff, 1986; Mattman, 2001) and reviews (Beaman and Beaman, 1994; Beran *et al.*, 2006 [this also summarizes research from the former Soviet Union];

Clasener, 1972; Domingue and Woody, 1997; Gumpert and Taubeneck, 1983; Onwuamaegbu *et al.*, 2005). Our understanding remains as controversial as 50 years ago with all authors agreeing that more serious studies are necessary to clarify the clinical significance of L-forms. Detailed comments on the numerous papers published over the last 20 years would exceed the space of the article. Furthermore, many texts are in Russian or Chinese which do not allow an easy critical valuation. For that reason we would like to make only some remarks concerning the present situation. There are three groups of thought: (1) L-forms possess very important clinical significance, (2) L-forms may have clinical significance, (3) L-forms do not have clinical significance.

1. An important role in human diseases is postulated over recent years especially by A. Proal and B. Marshall, offered on the internet (http://bacteriality.com). The authors have propounded that L-forms grow within cells of the human immune system and other tissues; that they can persist there for long periods and produce metabolites which cause symptoms of diseases. They also state that L-forms are causative agents of most chronic diseases and these can be treated successfully by following the so-called ''Marshall Protocol.'' This therapy combines long-term antibiotic treatment (e.g. minocycline), while stimulating the immune system by Benicar, an angiotensin II receptor blocker. The statement that nearly all chronic diseases, including sarcoidosis and Alzheimer's disease, are caused by bacterial L-forms is highly speculative. In particular, the statements that L-forms are the cause of many diseases of aging, ranging from artheriosclerosis to dementia and that they can be vertically transmitted within families are problematic and unlikely. Nevertheless, this L-form campaign may have positive aspects, namely, if the ''Marshall Protocol'' becomes an effective therapy for specific chronic diseases, research on L-forms may be stimulated.
2. The majority of papers postulate that L-forms may have clinical relevance while their role as either the causative agent or as an attendant agent is mostly unclear. These concern Crohn's disease (Greenstein, 2003), tuberculosis (Doroshkova *et al.*, 1989, 1995; Gerasimov *et al.*, 2003; Michailova *et al.*, 2005), sarcoidosis (Alavi and Moscovic, 1996; Almenoff *et al.*, 1996; El-Zaatari *et al.*, 1996), urinary and gastric diseases (Domingue and Woody, 1997; Mattman, 2001; Wang and Chen, 2004; Yu *et al.*, 2003), Lyme disease and Alzheimer's disease. Concerning the last two diseases, some authors believe that a relationship may exist between the occurrence of L-forms of *Borrelia burgdorferi* in the cerebrospinal fluid and multiple sclerosis and Alzheimer's disease (Broxmeyer, 2004; Fritzsche, 2004; MacDonald, 2006, 2007; Hermanowska-Szpakowicz *et al.*, 2003). Plaque diversity in Alzheimer's parallel variable cystic diameter of *B. burgdorferi* and MacDonald (2006) hypothesized that

rounded cystic cells, presumably L-forms of *B. burgdorferi*, are the root of the plaques in the Alzheimer's brain.

One special aspect for L-forms having a clinical significance is in their colonisation of biomaterials and medical devices (catheters, transplants, injectors). Bacteria adhering to such materials and forming biofilms are known to be less susceptible to antibiotic therapy than free-living planktonic bacteria and they can be changed to CWD forms or persist as L-forms. They thus become involved in bacteraemia and chronic diseases (Haley *et al.*, 1990; Hibma *et al.*, 1996, 1997; Woo *et al.*, 2001). *Enterococcus faecum* L-forms could attach and form biofilms on silastic rubber surfaces (Jass *et al.*, 1994) while *Listeria monocytogenes* L-forms were shown to attach and remain viable in biofilms on food, stainless steel and intravenous tubing (Hibma *et al.*, 1996).

3. Opinions which deny any clinical significance of L-forms are based on reports about results which could not be confirmed by repeated experiments or on new studies. They concern, for example, sarcoidosis (Brown *et al.*, 2003; Milman *et al.*, 2004), Crohn's disease (Beran *et al.*, 2006), infective endocarditis and urinary diseases (Onwuamaegbu *et al.*, 2005). As a result of critical evaluation of randomized controlled trials, Onwuamaegbu *et al.* (2005) concluded that the clinical significance of CWDB in diseases ''is not compelling.''

3. Immunomodulating activity and potential for therapy

Besides their negative role, L-forms might also play a positive role in human health by their immunomodulating activities and therapeutic potential. The antigenic characteristics of L-forms and the cellular and humoral immune response are discussed in several papers (Banai *et al.*, 2002; Domingue, 1982; Grichko and Glick, 1999; Madoff, 1986; Mattman, 2001). In the case of spheroplast-type L-forms, whose cells still possess remnants of the cell wall, they show immunogenic activities similar to those of N-forms.

However, in the case of stable protoplast L-forms unusual immunomodulating activities have been detected, mainly by a Bulgarian group at the Institute of Microbiology of the Bulgarian Academy of Sciences in Sofia (E. Ivanova, A. Toshkow, R. Toshkova). They used cells or purified CMs from stable protoplast L-forms of *E. coli* LWF+ and LB, *P. mirabilis* LVI and *L. monocytogenes* for intraperitoneal (i.p.) or intraveneous (i.v.) administration to mice, rats and hamsters. Both cells and membranes were found to cause a two- to four-fold enlargement of the spleen over a period of 20–40 days. This reaction demonstrated a general and long lasting stimulation of the immune response. A two- to five-fold increase in the number of peritoneal macrophages and of antigen binding T- and B-lymphocytes was obtained in mice and hamsters after application of

L-form membranes (Ivanova *et al.*, 1991, 1997b, 2000, 2002). The macrophages isolated from membrane-treated animals showed higher immunological activities such as phagocytic index, mitogen response, migration activity and chemoluminescent activity (Ivanova *et al.*, 1990, 2000, 2002). Electron microscopic observations illustrated long lasting activated cell surfaces (formation of pseudopodes and lamellipodes) of macrophages from membrane-treated mice (Ivanova *et al.*, 1997b, 2000).

Further studies showed that membranes of *E. coli* LWF+ induced protective effects to sublethal infections of mice with *S. aureus* (four-fold higher survival compared with control; Ivanova *et al.*, 1991) and that they restored the immune functions of macrophages and lymphocytes (e.g. mitogen and migration activity) after infection with *Yersinia pseudotuberculosis* (Ivanova *et al.*, 1997a) and Influenza A H3N2 virus (Ivanova *et al.*, 1992). L-form membranes from *L. monocytogenes*, *Staphylococcus pyogenes* and *Streptococcus agalacticae* showed protective effects on survival of hamsters transplanted with the myeloid Graffi tumour (Ivanova and Toshkova, 1999; Toshkova *et al.*, 1997).

It is not clear which components in the L-form membranes are responsible for the extraordinary immunomodulating activities. With respect to the unexpected intensities of the observed effects, the authors initially speculated that it was mainly LPS (Ivanova *et al.*, 1990). In the case of *P. mirabilis* LVI, indeed LPS cannot be excluded since the CM of this L-form contains certain amounts. But, the immunostimulation of the *P. mirabilis* LVI membranes is much higher than comparable doses of purified LPS from walled *E. coli* (Karch and Nixdorf, 1980). In the case of membranes from *E. coli* LWF+, however, no LPS could be detected (O. Holst, personal communication) indicating that proteins and other components may be involved.

The lack of the cell wall makes membrane proteins of L-forms, which are normally masked in N-form cells, accessible to immune cells (Kuwano *et al.*, 1993; Madoff, 1986). Akashi *et al.* (1996) described two proteins (30 and 36 kDa) in the CM of an unstable L-form of *S. aureus*, which induced the production of TNF-α and activated human immunodeficiency virus. Kita *et al.* (1995) supposed that the expression of an hsp65-like heat shock protein is involved in intracellular survival of *S. typhimurium* L-forms in macrophages and in long-lived immunity to murine typhoid. In all probability, it is the special organisation of the L-form membrane as a highly complex mixture of lipids, proteins and sugars in a stable bilayer system and of the aforementioned special proteins which are able to cause the extraordinary immunomodulating effects.

L-forms might have a potential for therapeutic use. Cells and membranes of protoplast L-forms are not or much less toxic than those of the N-forms. This has also been shown for *E. coli* LWF+ and *P. mirabilis* LVI when cells or membranes have been applied i.v. or i.p. into mice and rats

(Ivanova *et al.*, 1993; J. Gumpert, unpublished results). This fact offers the opportunity to use such L-form preparations as new therapeutic agents. Such agents could be nonviable L-forms or their membranes. Indeed, genetically engineered L-forms could be developed to contain membrane-bound or surface-displayed antigens for vaccination purposes or for drug delivery, for example, antibodies (Hoischen *et al.*, 2002). Such L-form preparations should be distributed within the human body after i.v. or i.p. application and they may find target tissues when equipped with target-specific antibodies.

Another opportunity would be to construct L-form strains which can produce and secrete recombinant proteins even within the human body. The safety implications of any recombinant therapy cannot be underestimated and a novel product incorporating L-forms would require equal and probably even more consideration.

4. Association with plants

A retrospective examination of the literature would suggest that L-forms, although not discovered at that time, were first postulated to occur in plant cells in the 1920s, with later reports implicating their role in disease (see references in Jones and Paton, 1973). Almost 40 years ago, a plant bacteriologist, Professor Alan M. Paton, University of Aberdeen, investigated the problem of latency in black leg disease of potato, which led to the formation of a research group dedicated to L-forms. His initial studies developed methods for L-form induction, cultivation and their association with plants (Jones and Paton, 1973). Observations were supported with pioneering techniques of the time, that is, immunofluorescence and the use of optical brighteners (Paton and Jones, 1971) to observe L-forms *in vitro* and *in planta*. Although it is now known that L-forms are not involved in the intracellular location of the pathogens of black leg disease, this work led to the hypothesis that N-form pathogens could be induced into the L-form state and thereby enter into the cytoplasm of viable plant cells and remain there, without any signs of pathogenicity, to promote a period of latent infection. At some point, due to some unknown environmental trigger, the L-form could revert, resulting in the formation of the N-form pathogens, which had the potential to initiate outbreaks of disease.

Research work was extended to determine whether L-form associations could be made with a range of different plants. Observations were mostly made on fresh tissue after injecting unstable and stable L-form suspensions into plant parts, by inoculating germinating seeds with drops of L-form suspensions and by co-cultivation of L-forms with plant cell suspensions. It was concluded that plant-L-form associations were universal being independent of both the type of bacteria and plant (Aloysius and Paton, 1984; Paton, 1987, 1988; Paton and Innes, 1991). Initially, the term somatic association was used to describe the contact

between the L-form and eukaryotic cell (Paton, 1988), but in later work this was referred to as a symbiotic relationship since the L-forms were confirmed to be situated within the cytoplasm in an intimate association. Indeed, the L-forms benefit from new ecological niches within the plant which provide protection for their fragile state and the plant can be conferred with the benefit of protection from disease.

The concept that bacterial L-forms could form benign associations, by entering into plant cells, was novel and attracted the interests of Professor Don Braben, the creator and Director of the Venture Research Unit (VRU) throughout its 10-year life. This unique unit was sponsored by BP International and operating internationally, concentrated on providing radical academic researchers in any field with total freedom. Initial work showed the universal nature of the association with L-forms being able to associate with both mono- and dicotyledonous plants (Paton, 1987, 1989). Immunofluorescence and light microscopy also showed that the L-forms were located within the cytoplasm of the plant cell in approximately 1% of the host cells (Paton and Innes, 1991) suggesting that the plant may restrict the size and dissemination of the L-form population. An important development came from the knowledge that L-forms can pass through filter membranes (0.45 and 0.22 μm pore diameter). Thus, filtrates of L-form-associated plant material could be used as effective inoculants for establishing new associations in the same and different species of plants. A major advantage of this method was that the plant filtrates could be frozen for long periods, re-thawed and used as required. This characteristic led to the development of a new method for associating plant material. Thus, stable and unstable L-forms as well as N-forms in the state of induction were placed on sterile membrane filters (0.45 and 0.22 μm) supported on water-based agar. After 1–2 days incubation, the filter would be carefully removed and seeds allowed to germinate on the same location. After a few days incubation, plant parts such as root hairs could be examined, to confirm the L-form association.

Initially, newly induced L-form cultures were used to form L-form plant associations (Paton and Innes, 1991). These were derived by either plating N-forms onto medium containing different levels of cell wall-inhibiting antibiotics to gain populations of unstable spheroplast-type L-forms that were continually subcultured or by using N-forms that were treated with penicillin for only 3 h to obtain at least 40% CWD cells. These suspensions were either used directly or re-suspended in mannitol before being injected into plants, or imbibed by germinating seeds. An interesting aspect was that irrespective of whether unstable or stable L-forms were used, the L-forms, once associated were maintained *in planta* without reversion (Paton, 1988; Paton and Innes, 1991). Although this observation has, over the years, been confirmed in the Aberdeen laboratories, it must be said that if high populations of unstable and

particularly newly induced L-forms are used, there can be massive reversion within hours of the co-association. However, if this does not occur, then the L-form association does appear to be stable and in contrast to human and animal cells, there does not appear to be any reversion. Having said this, the ability of L-forms *in planta* to never revert cannot be authenticated or relied upon especially when using plant pathogens. The inability of L-forms *in planta* to revert may reflect the relatively stable environmental (laboratory) conditions in which the research was undertaken or indeed our inability to detect very small bacterial numbers. Reversion would after all, be required in order to fulfill Patons' concept that L-forms have a role in the latency of plant diseases.

Unstable spheroplast L-forms derived from the plant pathogen *Ps. syringae* pv. *phaseolicola*, the causative organism of halo blight disease in beans, became a favoured cell line for the research group. Waterhouse and Glover (1994) monitored bioluminescence during the initial stages of L-form induction of a *lux*-marked N-form of *Ps. syr. phaseolicola* with results suggesting that major mutations and genetic loss did not occur during the induction process. This confirms that unstable L-forms are not the result of mutation but are a result of reversible alterations in cell wall organisation. Since many detection systems would not discriminate between parent N-form, reverted N-form, or unstable L-form, much discussion concerning the association centred on whether the bacteria existed *in planta* as L-forms. Much time and effort was spent confirming the association of the unstable *Ps. syr. phaseolicola* L-form and this was achieved using high quality immunofluorescence in *Phaseolus vulgaris* (Paton and Innes, 1991), DNA hybridisation in its non-host plant *Brassica campestris* spp. *pekinensis* (Waterhouse *et al.*, 1994b) and comparison of re-isolation of the N-form and L-form (Daulagala and Allan, 2003).

A crucial observation was that L-forms of *Ps. syr. phaseolicola*, derived from this renowned plant pathogen, did not cause any disease when associated with host plants. Amijee *et al.* (1992) confirmed this observation by undertaking detailed glasshouse experiments. They showed that, unlike N-forms, unstable *Ps. syr. phaseolicola* L-forms did not elicit a hypersensitive response in tobacco leaves and when associated with bean plants during seed germination, seedling emergence and plant growth were not affected. Most interesting, however, was that plants associated with this L-form and subsequently challenged by the cell-walled pathogen, were protected with significantly lower disease symptoms than non-associated plants (Amijee *et al.*, 1992). Indeed, results indicated that this was due to a host response since stem extracts from associated plants were inhibitory to both the N- and L-form. This provides a further indication that the L-form was in some way protected from this response when associated with the plant, presumably by living within a plant cell compartment. These spectacular results were at that

time completely novel as the idea that plants were capable of showing ''immunity'' against bacterial pathogens was not generally believed. Further corroboration of these results was achieved by Waterhouse *et al.* (1996) who not only detected the activity of *lux*-marked L-forms derived from *Ps. syr. phaseolicola* in Chinese cabbage plants but also showed that once associated, these plants conferred resistance to the heterologous plant pathogen *Xanthomonas campestris*. At the time of his death in 1994, Paton had filed two patents which contained many research results particularly showing examples of L-form/plant associations and their ability to protect plants against disease (Paton, 1989, 2002).

Further work showed that L-forms derived from non-pathogenic organisms, that is, stable protoplast *B. subtilis* L-forms, could also be associated with plants (Walker *et al.*, 2002). These were associated with Chinese cabbage during seed germination and could be detected at different stages of plant growth, that is at 12, 21 and 35 days after L-form treatment. Importantly, when the cotyledons were infected with conidia of the fungal pathogen *Botrytis cinerea* there was a significant reduction in conidial germination on L-form-associated plants compared to non-associated plants (Walker *et al.*, 2002). These results supported the speculation that L-form bacteria could evoke a broad spectrum resistance against plant pathogens and funding was obtained from a commercial strawberry grower, Mr. Ken Muir, England. An L-form selective, but not specific, ELISA was developed to detect the association (Ferguson *et al.*, 2000). It showed that L-forms persisted in different parts of mature strawberry plants and that if injected into the stolon could move for long distances (38–42 cm) from the injection site. *B. subtilis* stable L-forms, marked with the *gus* gene, were detected in Chinese cabbage seedlings (Tsomlexoglou *et al.*, 2003) thus emphatically establishing the existence of L-form plant associations. This work coincided with a surge in interest in plant endophytes and plant-induced systemic disease resistance (Pieterse *et al.*, 2002).

As a consequence of the above results, L-form bacteria were advocated as novel biocontrol agents offering advantages over surface inoculated biocontrol agents, of long-term persistence in the plant. Such biocontrol agents are likely to target the pathogen better and are less likely to lead to the development of resistance. The mode of action of the L-form biocontrol agent remained unclear with both antibiosis and/or induced resistance being suggested. In 2003, it was shown that Chinese cabbage plants associated with *Ps. syr. phaseolicola* L-forms had significantly higher level of chitinases than those that did not (Daulagala and Allan, 2003). The induction of this family of pathogenesis-related proteins would explain how L-form-associated plants had an increased resistance to infection by *B. cinerea*. It has been suggested that this represents a new process of inducing host defences which may be associated with the ability of the L-forms to colonise the plant (Hammerschmidt, 2003). These experiments again indicated that the

L-forms persisted and moved in the plant with the bacteria being detected in the root, cotyledons and leaves 31 days after their original inoculation via the seed. Although Hammerschmidt has suggested this as a novel system only for L-forms derived from plant pathogenic bacteria, for example *Ps. syringae*, the fact that symbiotic L-forms derived from non-pathogens, such as *B. subtilis*, also proffer plant protection (Walker *et al.*, 2002) would suggest that this is a much more general inducible defence system. The ability of the L-form to produce antibiotics would be a further possible means of enhancing biocontrol efficacy.

Elvira-Recuenco and van Vurde (2003) induced and cultivated *Ps. syringae* pv. *pisi* L-forms in order to assess its potential for biocontrol of pea bacterial blight but unfortunately lacked funding to progress into the plant system. In Aberdeen, commercial work on strawberry disease protection was aimed at protecting plants and their fruit against general spoilage organisms and specifically against the pathogen/spoilage organism, *B. cinerea* (Paton, 2002). In itself, strawberry is a rather challenging plant as its fruit is highly perishable and several plants are picked to fill a given fruit container. Walker *et al.* (2002) showed that not all strawberry plants injected with L-forms maintained their association and hence the plant protection phenomenon could not be anticipated to be at a sufficient level for commercial use. When micropropagated strawberry plants were associated with *B. subtilis* L-forms, they offered significant protection against challenge by *B. cinerea* (E. J. Allan, unpublished results). However, although the parent plants could be selected for the presence of L-form bacteria using techniques such as the aforementioned ELISA, not all daughter plants maintained the association. Funding a novel research area which has commercial potential but lacks some fundamental understanding, in this case as to why some plants maintained the association and other did not, remains a challenge and it is hoped that future research could determine the basic nature of how the L-form associates and distributes itself within plants. There is no doubt that L-form/plant research poses some intriguing scientific questions and hopefully will evoke future scientific interest.

Results on L-form/plant associations allow the conclusion that among the many types of ''mycoplasma-like'' organisms observed in plants (Lee and Davis, 1992), L-forms derived from eubacteria might also exist. A second conclusion is that plants may be engineered by L-forms. When L-form cells can persist in plants then the somatic associative technology, perhaps in supplement with gene technology, can be used to improve the resistance against pathogens and to introduce new metabolic activities (Paton, 1987). Such work would require a considerable advance in our knowledge of the behaviour of L-forms *in planta*. Paton (1987, 1988) also indicated that associations could be made between L-form and fungi but there is little detailed work published.

IV. CONCLUSIONS

L-form bacteria are interesting and important variants of ordinary eubacteria with modified or no cell walls. As **unstable L-forms** they represent transient states able to grow as CWD cells (spheroplasts or protoplasts). They can be induced and persist in eukaryotic hosts. In some cases, it is well documented that L-forms of Gram-negative and Gram-positive pathogenic bacteria can act as etiological agents in animal and human diseases and play a role in recurrent infections. Their putative role in some important chronic diseases, however, is unclear and needs more careful and long-lasting investigation.

As **stable L-forms** they represent mutants with highly pleotropic changes in comparison to their parent strains. Of special interest are stable protoplast-type L-form strains. They are important as subjects to contribute to a better understanding of processes such as cell division, structural and functional organisation of the cell wall and CM, genetic plasticity and strategies of adaptation. They, like unstable L-forms, can also form associations with plants and animals and confer protection against pathogens. Well established strains can be used in biotechnology, especially as unique expression systems for production of recombinant proteins. Engineered L-form strains might also have potential in therapy of diseases. It is evident that L-forms have much worth and have contributed to a greater understanding. However, there is an indication that practical experience of L-form bacteria is diminishing due to the loss of active researchers. It is hoped that this review may encourage a renewed investigation into L-forms as there is no doubt that the tools of modern science would be beneficial in unraveling the mysteries of these important forms of eubacteria.

ACKNOWLEDGMENTS

We thank our many co-workers and colleagues who have been involved in our endeavours with L-form bacteria particularly those, past and present, in our laboratories at Aberdeen and Jena. This work was supported by grants of the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 197) and of the German Ministry of Science and Education.

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CHAPTER 2

Biochemistry, Physiology and Biotechnology of Sulfate-Reducing Bacteria

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Advances in Applied Microbiology, Volume 68
ISSN 0065-2164, DOI: 10.1016/S0065-2164(09)01202-7

Abstract

Chemolithotrophic bacteria that use sulfate as terminal electron acceptor (sulfate-reducing bacteria) constitute a unique physiological group of microorganisms that couple anaerobic electron transport to ATP synthesis. These bacteria (220 species of 60 genera) can use a large variety of compounds as electron donors and to mediate electron flow they have a vast array of proteins with redox active metal groups. This chapter deals with the distribution in the environment and the major physiological and metabolic characteristics of sulfate-reducing bacteria (SRB). This chapter presents our current knowledge of soluble electron transfer proteins and transmembrane redox complexes that are playing an essential role in the dissimilatory sulfate reduction pathway of SRB of the genus *Desulfovibrio*.

Environmentally important activities displayed by SRB are a consequence of the unique electron transport components or the production of high levels of H_2S. The capability of SRB to utilize hydrocarbons in pure cultures and consortia has resulted in using these bacteria for bioremediation of BTEX (benzene, toluene, ethylbenzene and xylene) compounds in contaminated soils. Specific strains of SRB are capable of reducing 3-chlorobenzoate, chloroethenes, or nitroaromatic compounds and this has resulted in proposals to use SRB for bioremediation of environments containing trinitrotoluene and polychloroethenes. Since SRB have displayed dissimilatory reduction of U(VI) and Cr(VI), several biotechnology procedures have been proposed for using SRB in bioremediation of toxic metals. Additional non-specific metal reductase activity has resulted in using SRB for recovery of precious metals (e.g. platinum, palladium and gold) from waste streams. Since bacterially produced sulfide contributes to the souring of oil fields, corrosion of concrete, and discoloration of stonework is a serious problem, there is considerable interest in controlling the sulfidogenic activity of the SRB. The production of biosulfide by SRB has led to immobilization of toxic metals and reduction of textile dyes, although the process remains unresolved, SRB play a role in anaerobic methane oxidation which not only contributes to carbon cycle activities but also depletes an important industrial energy reserve.

ABBREVIATIONS

APS	adenylylsulfate
AOM	anaerobic oxidation of methane
BTEX	benzene, toluene, ethylbenzene and xylene
DMRB	dissimilatory metal-reducing bacteria
DSR	dissimilatory sulfite reductase
EPR	electron paramagnetic resonance
EPM	extracellular polymeric matrix
MIC	microbially influenced corrosion
NR-SOB	nitrate-reducing, sulfide-oxidizing bacteria
SRB	sulfate-reducing bacteria
TCE	trichloroethene
TNT	trinitrotoluene
PCE	tetrachloroethene
A.	*Archaeoglobus*
Dsm.	*Desulfomicrobium*
Dst.	*Desulfotomaculum*
D.	*Desulfovibrio*
Dsf.	*Desulfuromonas*

I. INTRODUCTION

The study of sulfate-reducing bacteria (SRB) evolved from seeking a solution to a practical problem. While looking for a process to remove calcium sulfate from water in Dutch canals and reduce the mineral level of water for use in steam boilers, M.W. Beijerinck discovered the biological activity of ''sulfide ferment'' which we now refer to as ''dissimilatory sulfate reduction'' (Kluyver, 1995; Postgate, 1993). Beijerinck (1895) published several articles describing the characteristics and activity of his newly isolated *Spirillum desulfuricans.* By the middle of the twentieth century, the economic effects of SRB were recognized in the following areas: pollution and toxicity attributed to bioproduction of H_2S, biocorrosion of metals, food spoilage due to endospore-producing thermophiles and financial expenses in the oil industry attributed to sulfate reducers. The role of SRB in biotechnology was discussed in previous reviews (Barton and Tomei, 1995; Hockin and Gadd, 2007; Postgate, 1979). It is interesting to note that even now, SRB are of economic importance in biocorrosion and biofilm development in oil fields. In the last few decades, development of new technology for cultivation of anaerobes and for molecular characterization of strains has enabled many new genera and species to be characterized.

In dissimilatory sulfate reduction (also called ''sulfate respiration''), microorganisms utilize inorganic sulfate as an external electron acceptor in the oxidation of energy substrates resulting in the production of hydrogen sulfide (Fauque *et al.*, 1991; LeGall and Fauque, 1988; Muyzer and Stams, 2008; Rabus *et al.*, 2006). Sulfate can be utilized as a terminal electron acceptor both by members of the bacteria and the archaea. Most prokaryotes with sulfate-reducing capability are bacteria and this supports our decision to use the term SRB instead of sulfate-reducing prokaryotes in this chapter.

Developments in microbial ecology have prompted microbiologists to consider using SRB in bioremediation of toxic compounds in the environment and pursue biocontrol of SRB in corrosion. While the focus of this review is on the role of SRB on economic acitivites of industrial microbiology and on environmental studies, we consider it is important to initially provide an overview of the biochemical and physiological activities of SRB. Biotechnology studies involving SRB are almost exclusively with mixed cultures and in some instances require a separation of abiotic and enzymatic processes. As the reader will notice, some of the biotechnical activities of SRB can be related to the unique molecules of the anaerobic electron transport systems.

II. DIVERSITY OF SRB

A. Distribution in the environment

SRB are of major numerical and functional importance in many ecosystems including marine sediments, polluted environments such as anaerobic purification plants, cyanobacterial microbial mats, oil fields environments, rice fieds, deep-sea hydrothermal vents and even in human diseases (Fauque, 1995; Loubinoux *et al.*, 2002; Muyzer and Stams, 2008; Ollivier *et al.*, 2007; Rabus *et al.*, 2006). Organisms included as SRB are phylogenetically and metabolically versatile and may represent the first respiring microorganisms with subsequent role to be played in the biogeochemistry of the various environments they inhabit. SRB have successfully adapted to almost all the ecosystems of the planet, including the deep extreme niches such as the deep-sea hydrothermal vents and the oil-field environments. In these ecosystems, SRB have to cope with drastic physico-chemical conditions (e.g., high temperature and high pressure). SRB contribute to the complete oxidation of organic matter and participate through sulfide production and/or metal reduction to the overall biogeochemistry of these extreme environments.

More than 220 species of 60 genera of SRB have been described until now. They belong to five divisions (phyla) within the bacteria

(the spore-forming *Desufotomaculum*, *Desulfosporomusa* and *Desulfosporosinus* species within the Firmicutes division, the *Deltaproteobacteria*, the *Thermodesulfovibrio* species within the Nitrospira division and two phyla represented by *Thermodesulfobium narugense* and *Thermodesulfobacterium/Thermodesulfatator* species) and two divisions within the archaea (the euryarchaeotal genus *Archaeoglobus* and the two crenarchaeotal genera *Thermocladium* and *Caldivirga*, affiliated with the *Thermoproteales*) (Castro *et al.*, 2000; Itoh *et al.*, 1998, 1999; Mori *et al.*, 2003; Muyzer and Stams, 2008; Ollivier *et al.*, 2007; Rabus *et al.*, 2006).

The complete genome sequences of nine SRB have been deposited in public databases to date: *Archaeoglobus* (*A.*) *fulgidus* (Euryarchaeota), *Caldivirga maquilingensis* (Crenarchaeota), the Gram-positive *Desulfotomaculum* (*Dst.*) *reducens* (Firmicutes) and six Gram-negative *Deltaproteobacteria*: *Desulfobacterium autotrophicum*, *Desulfovibrio* (*D.*) *vulgaris* Hildenborough, *Desulfovibrio vulgaris* subsp. *vulgaris* DP4, *Desulfovibrio desulfuricans* G20, *Desulfotalea psychrophila* and *Syntrophobacter fumaroxidans* (Rabus and Strittmatter, 2007). The genomes of the two archaea, *A. fulgidus* (~2.2 Mb) and *C. maquilingensis* (~2.1 Mb), are much smaller than those of the SRB (~3.5–5.6 Mb). A low similarity exists between the genomes of *A. fulgidus* and *D. psychrophila*, mainly between the genes that encode proteins which are involved in dissimilatory sulfate reduction (Rabus and Strittmatter, 2007).

B. Major characteristics

Initially, SRB were believed to utilize a limited range of substrates as energy sources (e.g., lactate, molecular hydrogen, pyruvate, ethanol, etc.) but recent microbiological and biochemical studies have greatly extended the number of electron acceptors and donors known to be used by SRB (Rabus *et al.*, 2006). SRB may have an heterotrophic, autotrophic, lithoautotrophic, or respiration-type of life under anaerobiosis and their possible microaerophilic nature has also been discussed recently (Cypionka, 2000; Fauque and Ollivier, 2004). A few species of SRB first considered as strict anaerobes were able to perform a microaerobic respiration coupled to energy conservation. More than one hundred compounds including sugars (e.g., fructose, glucose, etc.), amino acids (glycine, serine, alanine, etc.), monocarboxylic acids (e.g., acetate, propionate, butyrate, etc.), dicarboxylic acids (fumarate, succinate, malate, etc.), alcohols (e.g., methanol, ethanol, etc.) and aromatic compounds (benzoate, phenol, etc.) are potential electron donors for SRB (Fauque *et al.*, 1991; Rabus *et al.*, 2006).

SRB are the microorganisms that reduce the greatest number of different terminal electron acceptors including inorganic sulfur compounds and

various other organic and inorganic compounds (Fauque, 1995; Fauque and Ollivier, 2004; Fauque *et al.*, 1991; LeGall and Fauque, 1988; Muyzer and Stams, 2008; Rabus *et al.*, 2006). This suggests that their ecological and metabolic function in nature is of great importance. SRB are widely distributed in terrestrial, substerrestrial and marine ecosystems. Their contribution to the total carbon mineralization process in marine sediments, where sulfate is not limiting, was estimated to be up to 50% (Fauque, 1995; Rabus *et al.*, 2006). They can also grow in different physico-chemical conditions, thus inhabiting the most extreme environments of our planet such as the saline, hot, cold and/or alkaline ecosystems. Dissimilatory sulfate reduction has evolved by 3.5 billion years, as indicated by stable sulfur isotopes. SRB should be considered as ancestral microorganisms, which have contributed to the primordial biogeochemical cycle for sulfur soon as life emerged on the planet (Shen and Buick, 2004).

III. CENTRAL METABOLIC PATHWAYS OF SRB

The most extensive biochemical and physiological researches have been done with SRB members of the genus *Desulfovibrio*, which are the most easily and rapidly cultured sulfate reducers. Dissimilatory sulfate reduction in *Desulfovibrio* species is linked to electron transport-coupled phosphorylation because substrate level phophorylation is inadequate to support their growth (Peck, 1959). The SRB belonging to the genus *Desulfovibrio* possess a number of unique biochemical and physiological characteristics such as the requirement for ATP to reduce sulfate (Peck, 1959), the cytoplasmic localization of two key enzymes [adenylylsulfate (APS) reductase and bisulfite reductase] involved in the pathway of respiratory sulfate reduction (Kremer *et al.*, 1988), the periplasmic localization of some hydrogenases (Fauque *et al.*, 1988) and the abundance of multihemic *c*-type cytochromes (Fauque *et al.*, 1991; LeGall and Fauque, 1988; Pereira and Xavier, 2005; Pereira *et al.*, 1998).

A. Sulfur metabolism

The investigation of the mechanism of dissimilatory sulfate reduction has been undertaken mostly with *Desulfovibrio* species (Fauque and Ollivier, 2004; Fauque *et al.*, 1991; LeGall and Fauque, 1988; Rabus *et al.*, 2006). Four cytoplasmic enzymes are sufficient for reduction of sulfate to sulfide in an eight electron reduction process.

1. Sulfate activation and reduction of sulfate to sulfite

Owing to its chemical inertia, sulfate needs first to be activated to APS (adenylyl sulfate) by consumption of ATP (Peck, 1959). The ATP sulfurylase (EC 2.7.7.4; ATP sulfate adenylyltransferase) forms PPi (inorganic pyrophosphate) and APS from sulfate and ATP:

$$SO_4^{2-} + ATP + 2H^+ \rightarrow APS + PPi,\ \Delta G^{\circ'} = +46\,kJ/mol$$

The ATP sulfurylase has been purified and characterized from *Desulfovibrio gigas and D. desulfuricans* ATCC 27774; it is a novel metalloprotein containing cobalt and zinc (Gavel *et al.*, 1998).The formation of PPi being thermodynamically unfavourable, the reaction needs to be pulled to completion by a second enzyme, an inorganic pyrophosphatase (EC 3.6.1.1; pyrophosphate phosphohydrolase) which hydrolyzes PPi according to the following reaction:

$$PPi + H_2O \rightarrow 2Pi,\ \Delta G^{\circ'} = -22\,kJ/mol$$

The reduction of APS to bisulfite and AMP, catalyzed by APS reductase is the first redox reaction and is more exergonic than the pyrophosphate cleavage:

$$APS + H_2 \rightarrow HSO_3^- + AMP + H^+,\ \Delta G^{\circ'} = -69\,kJ/mol$$

APS reductase (EC 1.8.99.2) has been purified and characterized from several *Desulfovibrio* species (Lampreia *et al.*, 1994; Lopez-Cortés *et al.*, 2005) and from *A. fulgidus* (Lampreia *et al.*, 1991). APS reductase is a cytoplasmic iron–sulfur flavoprotein containing one FAD and eight iron atoms arranged as two different [4Fe–4S] centers (Lampreia *et al.*, 1994). The specific electron donor required for the reduction of APS to bisulfite is yet unknown.

2. Sulfite reduction to sulfide

The six-electron reduction of sulfite to sulfide, catalyzed by sulfite reductase (EC 1.8.99.1) must compensate the energy investment of sulfate activation and yield additional ATP for growth. The standard free energy change of sulfite reduction to sulfide, with hydrogen as electron donor, is −174 kJ/mol. and this could allow the regeneration of at least two ATP. The pathway of bisulfite reduction to hydrogen sulfide is somewhat controversial and two mechanisms have been proposed. In the first mechanism, also called the trithionate pathway, bisulfite is reduced to sulfide in three steps via the free intermediates, trithionate and thiosulfate (Cypionka, 1995; Rabus *et al.*, 2006). The second mechanism is the direct six-electron reduction of bisulfite to sulfide in one step, catalyzed by the dissimilatory sulfite reductase (DSR),

without the formation of free intermediates. Arguments against and for a trithionate pathway have been discussed earlier (LeGall and Fauque, 1988) but only the isolation of mutants that will be altered with respect to one or both reductase activities would provide definitive informations on the true bisulfite reduction mechanism.

Two types of sulfite reductases can be defined in SRB on the basis of physiological function. The first type comprises the high-spin bisulfite reductases (EC 1.8.99.1), which possess a large molecular mass (around 200 kDa) and a complex structure with at least two different polypeptides in an $\alpha_2\beta_2$ tetramer containing [4Fe–4S] centers and siroheme. DSR has been detected in all sulfate-reducing species investigated, so far. Four different enzymes belonging to the high-spin bisulfite reductase class have been purified and characterized from different genera of sulfate-reducing eubacteria (Fauque *et al.*, 1991; LeGall and Fauque, 1988). The green protein, desulfoviridin, is the DSR characteristic of the genus *Desulfovibrio* but it has also been found in some species of the genera *Desulfococcus*, *Desulfonema*, *Desulfomonile* and *Desulforegula* (Moura *et al.*, 1988a,b; Rees and Patel, 2001). The red brown protein, desulforubidin, belongs to the genera *Desulfomicrobium*, *Desulfosarcina*, *Desulfobulbus*, *Desulfobacter* (DerVartanian, 1994) and to the newly described genus *Desulfocurvus* (G. Fauque, M.-L. Fardeau and M. Magot, unpublished results). The dark brown-colored protein, desulfofuscidin, is the DSR of thermophilic sulfate-reducing eubacteria such as two *Thermodesulfobacterium* species and *Thermodesulfovibrio hydrogeniphilus* (Fauque *et al.*, 1990; Haouari *et al.*, 2008; Hatchikian, 1994). P-582-type bisulfite reductase is only present in several species of the spore-forming genus *Desulfotomaculum* (Fauque *et al.*, 1991; Rabus *et al.*, 2006). These four enzymes differ mainly by their behaviour of siroheme moieties, reaction with CO, major optical absorption and electron paramagnetic resonance (EPR) spectra (Fauque *et al.*, 1991; LeGall and Fauque, 1988).

An archeal DSR has been purified and characterized from the extremely thermophilic SRB *A. fulgidus*; it is an $\alpha_2\beta_2$ tetramer of molecular mass 178 kDa and it contains two sirohemes and six [4Fe–4S] clusters per molecule (Dahl *et al.*, 1993, 1994). The second type is constituted by the low-spin sulfite reductases, also called assimilatory-type sulfite reductases. They have a low molecular mass (27 kDa), one polypeptide chain and contain a single [4Fe–4S] cluster coupled to a siroheme in a low-spin state (Moura and Lino, 1994). The physiological role of the low-spin sulfite reductases in *Desulfovibrio* species is still not understood. APS reductases and DSR have analogous functions as cytochrome oxidases in aerobic respiration even if they are soluble proteins in contrast to the tightly membrane-bound cytochrome oxidases.

3. Elemental sulfur reduction

Different genera, of domain bacteria and archaea, are able to gain energy for growth by a dissimilatory reduction of elemental sulfur to sulfide in a respiratory type of metabolism (Fauque *et al.*, 1991, 1994; Le Faou *et al.*, 1990; Rabus *et al.*, 2006; Widdel and Pfennig, 1992). The facultative sulfur-reducing eubacteria, such as the SRB, use elemental sulfur as a respiratory substrate in the absence of other possible terminal electron acceptors such as sulfate, sulfite, thiosulfate, nitrite, or nitrate. Even if most of SRB cannot grow by elemental sulfur reduction, some thiophilic sulfate reducers, belonging to the genera *Desulfomicrobium* and *Desulfovibrio*, utilize sulfur as an alternative electron acceptor (Biebl and Pfennig, 1977). The tetraheme cytochrome c_3 is the constitutive elemental sulfur reductase in several species of *Desulfomicrobium* and *Desulfovibrio* from which the sulfur reductase activity can be copurified with the tetrahemoprotein (Fauque, 1994; Fauque *et al.*, 1979a). A mechanism of attack of colloidal sulfur by the *Desulfomicrobium* (*Dsm.*) *baculatum* Norway 4 (formerly *D. desulfuricans* Norway 4) tetraheme cytochrome c_3 has been proposed and it might involve insoluble S_8 molecules as intermediates (Cammack *et al.*, 1984). Membranes isolated from *D. gigas* and *Dsm. baculatum* Norway 4 contained *c*-type cytochromes and hydrogenase and catalyzed the dissimilatory sulfur reduction. Membranes of *D. gigas* were able to couple esterification of orthophosphate to electron flow from hydrogen to elemental sulfur (Fauque *et al.*, 1980).

B. Nitrogen metabolism

1. Fixation of molecular nitrogen

Fixation of molecular nitrogen has been demonstrated in SRB species of the genera *Desulfobulbus*, *Desulfobacter*, *Desulfotomaculum* and *Desulfovibrio* (Lespinat *et al.*, 1987; Postgate *et al.*, 1988; Rabus *et al.*, 2006). The *nifH* gene, coding for the Fe protein of the nitrogenise system has been sequenced in *D. gigas* (Postgate *et al.*, 1988).

2. Dissimilatory reduction of nitrate and nitrite

The disimilatory reduction of nitrate and (or) nitrite to ammonia (also called ammonification) can function as sole energy conserving process in some SRB. Nitrate is reduced to ammonia (with nitrite as intermediate) by a few strains belonging mainly to *D. desulfuricans* but also by *Desulfovibrio oxamicus*, *Desulfovibrio termitidis*, *Desulfovibrio furfuralis*, *Desulfovibrio profundus* and *Desulfovibrio simplex* (Lopez-Cortès *et al.*, 2006; Moura *et al.*, 2007; Seitz and Cypionka, 1986). A dissimilatory nitrate reduction has also been reported with *Desulfotomaculum thermobenzoicum*, *Desulfobulbus propionicus*, *Desulfobacterium catecholicum*, *Desulforhopalus singaporenssi*,

Thermodesulfovibrio islandicus and *T. narugense* (Mori *et al.*, 2003; Moura *et al.*, 2007). Depending on the organism, nitrate or sulfate may be the preferred electron acceptor. Nitrate reductase is inducible by nitrite or nitrate, whereas nitrite reductase is synthesized constitutively in *D. desulfuricans* Essex 6 (Seitz and Cypionka, 1986). A vectorial proton translocation during nitrate or nitrite reduction has been demonstrated with whole cells of *Desulfovibrio* species (Cypionka, 1995). ATP synthesis coupled to the reduction of nitrite to ammonia was obtained with membranes of *D. gigas* (Barton *et al.*, 1983).

A novel type of metabolism connecting the sulfur and nitrogen cycles has been reported in *D. desulfuricans* CSN which is able to oxidize thiosulfate and sulfite with nitrate and nitrite as electron acceptors (Krekeler and Cypionka, 1995). Nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) can inhibit the growth of SRB in the presence of nitrate (Haveman *et al.*, 2005). This inhibition could be due to the production of nitrite by the NR-SOB or to an increase in redox potential. Nitrite (but not nitrate) is known to inhibit the last step in the dissimilatory sulfate reduction pathway (reduction of sulfite to sulfide by DSR) (Haveman *et al.*, 2004). The production of hydrogen sulfide by SRB in oil reservoirs (souring) and the microbially influenced corrosion (MIC) can be controlled through nitrate or nitrite addition (see Section V). *D. desulfuricans* subsp. *desulfuricans* DSM 6949 (ATCC 27774) is the ammonifying strain of SRB that has been the most characterized from a biochemical and physiological point of view. The biochemical, genetical and spectroscopical characterization of *D. desulfuricans* ATCC 27774 nitrate and nitrite reductases has been extensively described by Moura *et al.* (2007).The nitrate reductases catalyze the two-electron reduction of nitrate to nitrite:

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O,\ E^\circ = +420\,\text{mV}$$

The *D. desulfuricans* ATCC 27774 nitrate reductase is a periplasmic enzyme with a molecular mass of 74 kDa, containing one molybdenum atom and one [4Fe–4S] cluster by molecule, which exhibits EPR signals assigned to Mo(V) (Moura *et al.*, 2007). It is the first periplasmic nitrate reductase to have its crystal structure determined; it is a monomeric protein organized in four domains, all involved in cofactor binding (Dias *et al.*, 1999).

The multiheme nitrite reductases [(EC 1.7.2.2 nitrite reductase) (cytochrome; ammonia-forming)] act on the dissimilative ammonification process, where they catalyze the reduction of nitrite to ammonia in a unique six-electron step:

$$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O,\ E^\circ = +330\,\text{mV}$$

The *D. desulfuricans* ATCC 27774 nitrite reductase is a membrane-associated high molecular mass (890 kDa) oligomer cytochrome *c*

containing two types of subunits of 60 and 20 kDa (Liu *et al.*, 1994; Moura *et al.*, 1997, 2007). Its X-ray structure was solved at 2.3 Å (Cunha *et al.*, 2003).

A membrane-associated cytochrome *c* nitrite reductase has also been characterized from *D. vulgaris* Hildenborough, a non-ammonifying sulfate reducer. This membrane-bound complex of 760 kDa contains two cytochrome *c* subunits of 18 and 56 kDa and has both sulfite and nitrite reductase activities (Pereira *et al.*, 2000).

C. Hydrogen metabolism

Molecular hydrogen is (besides acetate) a key intermediate in the metabolic interactions of a wide-range of microorganisms. Hydrogen plays a central role in the energy metabolism of SRB of the genus *Desulfovibrio*, which can either utilize or produce hydrogen depending on the growth conditions (Fauque *et al.*, 1988; Rabus *et al.*, 2006). Hydrogenases have a central function in the process of interspecies hydrogen transfer that occurs in the fermentation of organic matter in the anaerobic microbial ecosystems (Fauque, 1989; Peck and Odom, 1984). Hydrogenases (hydrogen: oxidoreductase EC.1.12) constitute a class of enzymes that are highly diversified in their active center composition and structure. These enzymes catalyze the reversible oxidation of the dihydrogen molecule to electrons and protons (Fauque, 1989; Fauque *et al.*, 1988; Moura *et al.*, 1988a,b). SRB of the genus *Desulfovibrio* contain three classes of hydrogenases ([Fe], [NiFe], [NiFeSe]), which differ in their H_2-uptake and H_2-evolving activities, subunit composition and metal structure, amino acid sequence, localization, gene structure, catalytic properties (sensitivity to CO, NO, nitrite) and immunological reactivities (Fauque *et al.*, 1988, 1991). The three classes of hydrogenases are not uniformly distributed among the different *Desulfovibrio* species and the [NiFe] hydrogenase is the most represented (Voordouw *et al.*, 1990). The *D. vulgaris* Hildenborough genome encodes six different hydrogenases. Four of them are periplasmic (one [Fe] hydrogenase, one [NiFeSe] hydrogenase and two [NiFe] isoenzymes) and two multisubunit membrane-associated [NiFe] hydrogenases (Heidelberg *et al.*, 2004).

Two mechanisms are possible for the formation of a proton gradient in *Desulfovibrio* species: a vectorial electron transport linked to the oxidation of hydrogen by hydrogenases and a proton translocation coupled to the reduction of specific substrates (Cypionka, 1995; LeGall and Fauque, 1988). In the first mechanism, also called the obligate H_2-cycling, hydrogen is formed from lactate and pyruvate in the cytoplasm by a cytoplasmic hydrogenase; then it diffuses into the periplasmic space where it is oxidized by a periplasmic hydrogenase (Peck and Odom, 1984; Peck *et al.*, 1987). This process generates a membrane potential and a transmembrane pH gradient without pumping protons across the cell membrane.

This mechanism is somewhat controversial even if a direct demonstration of hydrogen cycling has been made employing membrane-inlet mass spectrometry during the metabolism of pyruvate plus sulfate by washed intact cells of *D. vulgaris* Hildenborough (Peck *et al.*, 1987). The finding of periplasmic hyrogenases in SRB of the genus *Desulfovibrio* is in favour of energy conservation by vectorial electron transport, the simplest transmembrane process generating a proton gradient for chemiosmotic ATP synthesis. In this mechanism, SRB are able to pump protons across the cell membrane, performing the classical type of vectorial proton translocation. *D. desulfuricans* strain Essex 6, grown on H_2 and sulfate, generates a proton-motive force by classical proton translocation using the reductant pulse method (Cypionka, 1995).

D. Oxygen metabolism

Prior to 1990, SRB were considered as strict anaerobes. Then it has been shown that they are able to tolerate the transient presence of oxygen (Cypionka, 2000; Dilling and Cypionka, 1990; Dolla *et al.*, 2006, 2007; LeGall and Xavier, 1996; Santana, 2008). The capability of true aerobic respiration coupled to energy conservation was detected in *D. propionicus*, *Desulfococcus multivorans*, *D. autotrophicum* and in several strains of *Desulfovibrio* species (Dilling and Cypionka, 1990). Usually SRB do not grow with molecular oxygen as electron acceptor but growth of *D. desulfuricans* ATCC 27774 at low oxygen levels has been reported (Lobo *et al.*, 2007). Aerobic respiration by SRB is microaerophilic and not sensitive to cyanide and azide (Dilling and Cypionka, 1990). SRB obviously contain terminal oxidases different from those of aerobic bacteria. Different oxygen-reducing systems are present in *Desulfovibrio* species. A NADH oxidase activity entirely responsible for the oxygen reduction to water was found in some *Desulfovibrio* species (Cypionka, 2000). In *D. termitidis*, *D. vulgaris* and *D. desulfuricans*, oxygen reduction was coupled to ATP conservation and proton translocation. In these last three species, tetraheme cytochrome c_3 and periplasmic hydrogenase play a major role in oxygen reduction (Cypionka, 2000). SRB uses superoxide reductases as one component of an alternative oxidative stress protection system that catalyzes reduction rather than disproportionation of superoxide to hydrogen peroxide. Two classes of superoxide reductases are present in SRB containing one (neelaredoxin) or two (desulfoferrodoxin or rubredoxin oxidoreductase) iron centers (Kurtz and Coulter, 2002). A membrane-bound terminal oxygen reductase of the cytochrome *bd* family has been characterized from *D. gigas* and shown to completely reduce oxygen to water (Lemos *et al.*, 2001).The structural characteristics of the two classes of *Desulfovibrio* superoxide reductases and the mechanistic aspects of biological superoxide anion reduction have been very recently reviewed (Pereira *et al.*, 2007a,b).

E. Fermentation of organic substrates

Some *Desulfovibrio* species are able to ferment malate and fumarate with the formation of succinate, acetate and carbon dioxide (Rabus *et al.*, 2006). In the absence of sulfate, many species belonging to the genera *Desulfovibrio*, *Desulfobacterium*, *Desulfococcus*, *Desulfotomaculum* and *Desulfobulbus* ferment pyruvate with acetate, carbon dioxide and hydrogen appearing as major end products of metabolism (Rabus *et al.*, 2006). *Desulfovibrio aminophilus* ferment pyruvate, peptone, casamino acids, glycine, serine, threonine and cysteine (Baena *et al.*, 1998). Some genera of SRB can also carry out a propionic fermentation which has been studied in detail in *D. propionicus* (Rabus *et al.*, 2006). Four unidentified saccharolytic Gram-negative non-sporulating mesophilic sulfate-reducing strains fermented fructose, sucrose or glucose to acetate, carbon dioxide and hydrogen (Joubert and Britz, 1987). *Desulfovibrio fructosovorans* can ferment fructose to succinate and acetate with the production of small amounts of ethanol (Ollivier *et al.*, 1988). Fermentation of lactate to acetate, carbon dioxide and hydrogen has been reported with *D. vulgaris* Marburg (Pankhania *et al.*, 1988) even if this reaction normally does not allow growth, since lactate oxidation to pyruvate ($E'^{\circ} = -190$ mV) requires energy-dependent reverse electron transport, probably catalyzed by a membrane-bound enzyme. SRB of the genus *Desulfovibrio* that cannot grow by fermentation of lactate, ethanol, or choline may grow with these substrates in the absence of sulfate in syntrophic cocultures with hydrogen-scavenging methanogenic bacteria (Rabus *et al.*, 2006).

F. Fermentation of inorganic sulfur compounds

1. Disproportionation of elemental sulfur

A disproportionation of elemental sulfur to sulfate and sulfide is thermodynamically unfavourable (Rabus *et al.*, 2006). However, *D. propionicus* DSM 2032 has been reported to dismutate elemental sulsur, even though growth with sulfur as the electron donor and Fe(III) as electron acceptor (or sulfide sink) was very slow (Lovley and Phillips, 1994a).

2. Dismutation of sulfite and thiosulfate

A novel type of energy metabolism involving fermentation of inorganic sulfur compounds has been reported in *Desulfovibrio sulfodismutans* (Bak and Pfennig, 1987) which is able to conserve energy for anaerobic growth by disproportionation (or dismutation) of sulfite or thiosulfate to sulfate and sulfide according to the following reactions:

$$4SO_3^{2-} + H^+ \rightarrow 3SO_4^{2-} + HS^-,\ \Delta G^{\circ'} = -235\text{kJ/mol}$$
$$S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+,\ \Delta G^{\circ'} = -21.9\text{kJ/mol}$$

The free energy change of thiosulfate disproportionation is very low (−21.9 kJ/mol) and cannot always permit growth. The capacity of thiosulfate and sulfide dismutation is constitutively expressed. The enzymes required for the disproportionations appear to be the same as for sulfate reduction (Kramer and Cypionka, 1989). Evidence has been shown that during inorganic sulfur compounds fermentations, sulfate is formed not by sulfite oxidoreductase but via APS reductase and ATP sulfurylase. Reversed electron transport is necessary to enable the reduction of thiosulfate or sulfite with the electrons derived from APS reductase (Kramer and Cypionka, 1989).

G. Carbon metabolism

Species of *Desulfovibrio*, *Desulfomicrobium*, *Desulfobulbus*, *Desulfdococcus* and *Thermodesulfobacterium* oxidize their substrates, such as lactate, incompletely to acetate. Further species and genera of SRB were later described and they were able to oxidize acetate, higher fatty acids, or aromatic compounds (Rabus *et al.*, 2006). Two different mechanisms exist for the complete oxidation of acetate to carbon dioxide by SRB. In *Desulfobacter* species, a tricarboxylic acid cycle is operative even if it is slightly different from the cycle present in aerobic bacteria. Completely oxidizing SRB other than *Desulfobacter* species, such as *Desulfobacterium* species and *Desulfotomaculum acetoxidans*, do not possess a complete citric acid cycle but are able to oxidize acetate by the carbon monoxide dehydrogenase pathway (Rabus *et al.*, 2006). *D. autotrophicum* and *Desulfoarculus baarsii* (formerly *D. baarsii*) are also able to grow with carbon monoxide as a major or sole carbon source (Rabus *et al.*, 2006).

IV. CHARACTERISTICS OF ELECTRON TRANSFER PROTEINS

From a biochemical point of view, SRB are a gold mine, containing a diversified and complex electron carrier system. A characteristic feature of the sulfate reduction electron transfer pathway is the involvement of multiheme *c*-type cytochromes and iron–sulfur proteins of low redox potentials (LeGall and Fauque, 1988; Matias *et al.*, 2005; Pereira and Xavier, 2005; Pereira *et al.*, 1998).

A. Soluble electron transfer proteins

1. Cytochromes

Desulfovibrio species contain different *c*-type cytochrome compositions. At least 17 periplasmic or membrane-bound *c*-type cytochromes are present in *D. vulgaris* Hildenborough, some of which belonging to the cytochrome c_3 family (Matias *et al.*, 2005; Pereira *et al.*, 2007a,b).

a. Monoheme c-type cytochrome (methionine–heme–histidine) A monoheme *c*-type cytochrome, known as cytochrome c_{553}, is present in several *Desulfovibrio* species and in *Desulfomicrobium norvegicum* (formerly *D. desulfuricans* Norway 4) (Fauque *et al.*, 1979b, 1991). This small periplasmic monomeric hemoprotein (9 kDa) contains a single heme coordinated by a histidine and a methionine residue. It has a redox potential in the range of 0–50 mV and its physiological role remains unknown. The three-dimensional structures of cytochrome c_{553} have been reported for two strains of *D. vulgaris* (Matias *et al.*, 2005).

b. Multiheme c-type cytochromes Tetraheme cytochrome c_3 is the predominant cytochrome in SRB (LeGall and Fauque, 1988). Tetraheme cytochrome c_3 belongs to the class III cytochromes characterized by the presence of hemes in a low-spin state with bis-histidinyl coordination and quite negative redox potentials. Tetraheme cytochrome c_3 is the only hemoprotein found in all *Desulfovibrio* species and it is characteristic of this genus although it has also been reported in *Dsm. norvegicum*, *Desulfobulbus elongatus* and in two *Thermodesulfobacterium* species (Fauque *et al.*, 1991; Matias *et al.*, 2005; Pereira *et al.*, 1998). Tetraheme cytochrome c_3 is a small (106–118 amino acid residues) soluble monomeric protein located in the periplasmic space. Tetraheme cytochrome c_3 plays a fundamental role in the bioenergetics of dissimilatory sulfate reduction, mediating the flow of electrons from periplasmic hydrogenases to respiratory transmembrane electron transport complexes linked to the transfer of protons (Matias *et al.*, 2005; Pereira *et al.*, 2007a,b). There are two classes of tetraheme cytochrome c_3. The Type I-c_3 has a molecular mass of 13 kDa and contains four low redox potential hemes (-120 to -400 mV). It may simultaneously capture protons and electrons, which could be crucial for its biological function. The X-ray crystallographic three-dimensional structure of Type I-c_3 has been determined in five *Desulfovibrio* species and in *Dsm. norvegicum* (Matias *et al.*, 2005). A dimeric cytochrome c_3 [formerly named cytochrome c_3 (Mr = 26,000) or cc_3], containing two tetraheme subunits similar to TpI-c_3, has been purified, characterized and crystallized from *D. gigas* and *Dsm. norvegicum* (Matias *et al.*, 2005).

The Type II-c_3 represents another group of tetraheme cytochrome c_3 with genetic, structural and reactivity characteristics different from Type I-c_3 (see Section IV.B). In addition to the three cytochromes discussed above (Type I-c_3, Type II-c_3 and cc_3), the genome of *D. vulgaris* Hildenborough encodes for five other tetrahemic cytochromes. A Split-Soret cytochrome has been isolated from *D. desulfuricans* ATCC 27774; it is a dimer of a diheme subunit (Pereira *et al.*, 1998). A monomeric nine-heme cytochrome *c* has also been purified from *D. desulfuricans* ATCC 27774 and its three-dimensional structure determinated (Matias *et al.*, 1999) (see Section IV.B).

2. Flavoproteins

Two types of flavoproteins have been purified and characterized in SRB, mainly of the genus *Desulfovibrio*: a flavodoxin and a flavoredoxin. Flavodoxins constitute a group of small monomeric electron carrier flavoproteins (Mr = 15–22 kDa) containing a single molecule of non-covalently bound riboflavin 5′-phosphate (FMN). Flavodoxin is not found in all *Desulfovibrio* species (LeGall and Fauque, 1988; Vervoort *et al.*, 1994). The spectrum of biological activities of flavodoxin is very similar to that of ferredoxin; flavodoxin is able to replace ferredoxin in both hydrogen-utilizing and hydrogen-producing reactions (Fauque *et al.*, 1991). There is an interesting redox analogy between flavodoxin and the oligomeric ferredoxin system of *D. gigas* in that flavodoxin has also two stable redox states at −440 and −150 mV (LeGall and Fauque, 1988). Flavoredoxin has been isolated from *D. gigas*; it is a 40-kDa homodimer, containing one FMN molecule per monomer. A deletion of flavoredoxin gene in *D. gigas* reveals its participation as an electron carrier in thiosulfate reduction and not in sulfite reduction, as previously suggested (Broco *et al.*, 2005).

3. Iron–sulfur proteins

The structure, the spectroscopic and magnetic properties of simple and complex iron–sulfur proteins from SRB, mainly from the genus *Desulfovibrio*, have been extensively reviewed by Moura *et al.* (1999); it is the reason why we have decided to present here only a short summary of these characteristics.

Rubredoxins are the smallest monomeric iron–sulfur proteins (Mr = 6 kDa) containing a single iron atom per polypeptidic chain. They are present as cytoplasmic proteins in all *Desulfovibrio* species so far investigated (Fauque *et al.*, 1991; Moura *et al.*, 1999). The iron atom is coordinated by four cysteine residues and is stabilized in two redox states. Rubredoxins from *Desulfovibrio* species have a relatively high redox potential (between 0 and −50 mV).

Desulforedoxin is a new type of non-heme iron protein, related to rubredoxin, isolated from *D. gigas*; it is a dimer (Mr = 8 kDa) consisting of two identical subunits with one iron and four cysteine residues per monomer (Moura *et al.*, 1999).

Desulfoferrodoxin is a fusion protein isolated from *D. desulfuricans* ATCC 27774 containing a small N-terminal desulforedoxin-type domain and a larger C-terminal domain similar to neelaredoxin. This monomeric protein (Mr = 16 kDa) contains two iron atoms per molecule and can be purified in two distinct redox states: the fully oxidized (gray) form and the half-reduced (pink) form (Moura *et al.*, 1990, 1994; Tavares *et al.*, 1994). The function of desulfoferrodoxin is still unknown.

Rubrerythrin is a fusion protein containing an N-terminal diiron-binding domain and a C-terminal domain homologous to rubredoxin. Rubrerythrin has been characterized from *D. vulgaris* Hildenborough and *D. desulfuricans* ATCC 27774; it is constituted by two identical subunits of 22 kDa and has a midpoint redox potential of +230 mV (Moura *et al.*, 1999). The major function of rubrerythrin could be in the protection against deleterious effects of molecular oxygen.

Ferredoxins are small molecular weight (6 kDa) iron–sulfur proteins with low redox potentials. Ferredoxins are very common in SRB and they contain four types of cluster arrangement: [3Fe–4S], [4Fe–4S], [3Fe–4S] + [4Fe–4S] and 2 × [4Fe–4S] centers (Fauque *et al.*, 1991; Moura *et al.*, 1999). A common structural feature shared by these clusters is that each iron atom is tetrahedrally coordinated and contains bridging inorganic sulfur atoms. Two forms of ferredoxins exist in *D. gigas*: the ferredoxin I is a trimer containing a [4Fe–4S] center with a redox potential of −440 mV; the ferredoxin II is a tetramer with a [3Fe–4S] center and a redox potential of −130 mV. These two proteins differ in their biological reactivity: the ferredoxin I is fully active in the phosphoroclastic reaction (hydrogen production from the oxidation of pyruvate) and the ferredoxin II functions as an electron donor in the reduction of bisulfite to sulfide (hydrogen oxidation).The three- and four-non-heme iron centers can be easily chemically (i.e., *in vitro*) interconverted (LeGall and Fauque, 1988).

Fuscoredoxin, a new iron–sulfur protein brown-colored containing two [4Fe–4S] centers, without known function, has been isolated from *D. vulgaris* Hildenborough and *D. desulfuricans* ATCC 27774 (Hagen *et al.*, 1989; Moura *et al.*, 1999).

4. Other redox proteins and electron carriers

A flavohemeprotein, named ROO (for rubredoxin: oxygen oxidoreductase) was purified in *D. gigas*. It is an 86-kDa homodimer flavohemeprotein containing two FAD molecules and two unique hemes per monomer (one mesoheme IX and one Fe-uroporphyrin I) (Gomes *et al.*, 1997).

Adenylate kinases have been purified from *D. gigas* and *D. desulfuricans* ATCC 27774 and they were biochemically and spectroscopically characterized in the native and fully zinc- or cobalt-substituted forms. These proteins are the first reported adenylate kinases that bind either cobalt or zinc and their electronic absorption spectra are consistent with tetrahedral coordinated cobalt (Gavel *et al.*, 2008).

SRB of the genus *Desulfovibrio* contain several proteins with modified porphyrins, some of them unusual. Some examples include bacterioferritin of *D. desulfuricans* ATCC 27774 containing an iron-coproporphyrin III cofactor (Romao *et al.*, 2000) and the *D. gigas* rubredoxin oxygen oxidoreductase that contains iron uroporphyrin I (Timkovich *et al.*, 1994).

Proteins containing cobalt-porphyrin are also present in *D. gigas* and *Dsm. norvegicum* (Hatchikian, 1981; Moura *et al.*, 1980).

Several molybdenum-containing proteins are present in SRB of the genus *Desulfovibrio* (Fauque *et al.*, 1991; Moura *et al.*, 1999; Thapper *et al.*, 2006). *D. desulfuricans* ATCC 27774 grown with nitrate as electron acceptor generates a complex enzymatic system containing three different molybdenum enzymes: (a) a formate dehydrogenase oxidizing formate to CO_2, (b) an aldehyde oxidoreductase converting aldehydes to carboxylic acids and (c) a nitrate reductase reducing nitrate to ammonium (Moura *et al.*, 2007). A tungsten-containing formate dehydrogenase has been isolated from *D. gigas*; it is a heterodimer (92 and 29 kDa subunits) containing tungsten and two [4Fe–4S] clusters (Almendra *et al.*, 1999).

Menaquinones are present in all SRB so far examinated suggesting that they seem to be the obligate components of their electron transport chains. The most frequently found menaquinones in SRB are MK-6 and MK-7 (Rabus *et al.*, 2006).

B. Membrane-associated electron transport complexes

The description of different respiratory membrane complexes present in SRB was reported very recently by Pereira (2008) and we will only summarize these results here.

Only two transmembrane redox complexes are conserved in all SRB, Dsr and Qmo complexes, suggesting that they play an essential function in dissimilatory sulfate reduction, most likely as electron donors to the bisulfite and APS reductases, respectively (Pereira, 2008). The Dsr complex isolated from *D. desulfuricans* ATCC 27774 contains several iron–sulfur centers and hemes of the *b* and *c*-type (Pires *et al.*, 2006). The Dsr complex is probably involved in electron transfer to the dissimilatory bisulfite reductase because its genes are part of a locus including the genes for sulfite reductase in several microorganisms (Pereira, 2008). The Qmo complex (for quinone-interacting membrane-bound oxidoreductase complex) is composed of three subunits and contains two hemes b low-spin, two FAD groups and several iron–sulfur centers (Pires *et al.*, 2003). The Qmo complex could be involved in electron transfer to APS reductase. Three other respiratory membrane complexes (Hmc, 9Hc and Tmc complexes) are only found in *Desulfovibrio* species (Pereira, 2008).

The largest multiheme cytochrome *c* found so far in *Desulfovibrio* species is a monomer containing 16 hemes, also known as the high molecular mass cytochrome *c* (HmcA), isolated and crystallized from *D. vulgaris* Hildenborough (Czjzek *et al.*, 2002; Matias *et al.*, 2002). This Hmc complex (for heterodisulfide reductase-like menaquinol-oxidizing

enzyme complex) could be involved in the transfer of electrons from the periplasmic hydrogen oxidation to the cytoplasmic reduction of sulfate (Matias *et al.*, 2005; Santos-Silva *et al.*, 2007).

The transmembrane redox complex (9Hc) from *D. desulfuricans* ATCC 27774 that lacks heme b, but contains the nine-heme cytochrome *c* and a periplasmic iron–sulfur subunit (Matias *et al.*, 2005; Saraiva *et al.*, 2001). The Tmc complex contains four units, including the Type II-c_3, an integral membrane cytochrome *b* and two cytoplasmic proteins (Matias *et al.*, 2005). The Type II-c_3 is a membrane-bound cytochrome c_3 and it has been found in three *Desulfovibrio* species (Matias *et al.*, 2005; Valente *et al.*, 2001). The Tmc complex could act as a transmembrane conduit for electrons resulting from periplasmic hydrogen oxidation (Pereira, 2008). Future studies are required to answer some important questions remaining in the metabolism of SRB, such as the exact physiological function of all the respiratory membrane complexes described, the mechanisms of proton translocation and the nature of the electron donors to APS and bisulfite reductases.

V. ENVIRONMENTAL IMPACT OF SRB

A. Biocorrosion of ferrous metals

Corrosion of ferrous pipes and supports is a significant expense for many industries and countries. While chemical activities can contribute to corrosion, about 15% of these cases are attributed to bacterial action and are designated as MIC. In the oil and gas industry, the cost attributed to MIC may account for about 0.5% of the GNP which in the USA would be hundreds of millions of dollars annually (Beech and Sunner, 2007). Bacteria are the principle organisms contributing to MIC and several recent reviews have focused on the role of SRB on corrosion of ferrous metals (Beech and Sunner, 2007; Cord-Ruwisch, 2000; Hamilton, 2003; Muyzer and Stams, 2008). SRB are not limited to corrosion of ferrous iron but their activity on CrMoAl steel (Wang and Liang, 2007) and NiCr stainless steel (Lopes *et al.*, 2006) is also reported.

Over the years there has been considerable interest in MIC and especially the role of SRB in corrosion of ferrous metals. The model used to characterize metal corrosion by SRB was initially suggested in 1934 by Von Wolzogen Kuhr and Van der Vlugt and a simplified version is given in Fig. 2.1. The pitting of metal due to electrons consumed as they are released from iron atoms and the ferrous atoms are deposited along the surface of the metal near the site where metal solubilization occurs. To emphasize the electroconductivity of the metal oxidation process, one region is referred to as the anode and another is the cathode. For

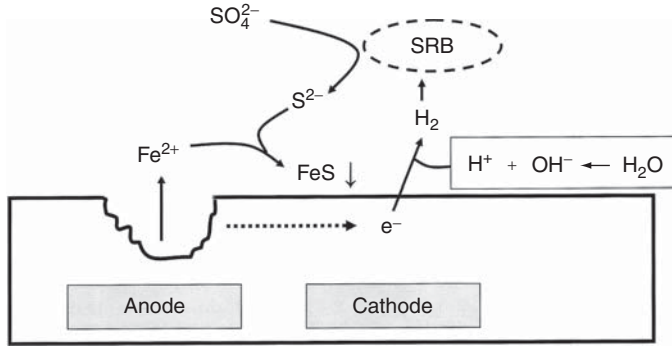

FIGURE 2.1 Model for corrosion of ferrous metal by SRB. Consumption of H_2 by SRB accounts for cathodic depolarization. Fe^{2+} is released from the anode, a pit in the metal results and insoluble FeS is produced. H^+ from ionization of water combines with electrons to produce H_2.

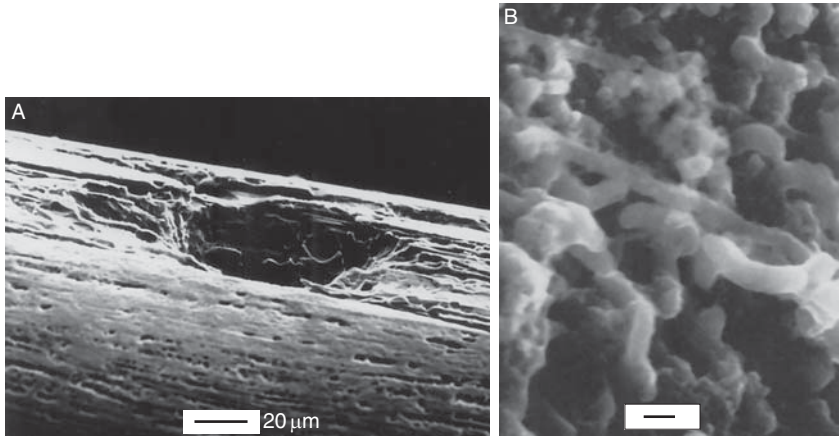

FIGURE 2.2 Ferrous corrosion by SRB as observed with an environmental scanning electron microscope. (A) Ferrous wire with a pit attributed to action by *D. desulfuricans*. Bar = 20 μm. (B) Biofilm of *D. desulfuricans* on the ferrous wire. Bar = 1 μm. Electron micrographs provided by L.L. Barton.

reference, Fig. 2.2 provides electron micrographs of SRB corrosion of ferrous wire. As summarized by Beech and Sunner (2007), there are several mechanisms involved in SRB-influenced corrosion:

1. *Biofilm formation and fixing the anodic site.* Mixtures of environmental bacteria including SRB will collect on the metallic surface and we assume that SRB employ quorum sensing to optimize the oxidation process. Bacteria become localized on the metal and it is at this site

where the pit is developed in the ferrous surface. In non-microbial influenced corrosion, anodic sites tend to move around leading to a more generalized corrosion.

2. *Cathodic depolarization.* This mechanism is based on the idea that the rate limiting step in corrosion of iron is the dissociation of hydrogen from the cathodic site. It is through this activity that SRB consume H_2 by hydrogenase and thus depolarize the cathode with an acceleration of corrosion. Electrons from this process would be used by SRB to promote growth and other physiological activities (Cord-Ruwisch and Widdel, 1986; Hardy, 1983; Pankhania *et al.*, 1986).
3. *Formation of iron sulfide deposits on the iron surface.* The release of hydrogen gas on the surface is normally the rate limiting step in corrosion. The release of hydrogen is accelerated by the deposition of iron sulfides formed by the action of SRB on the metal surface and the surrounding medium. The conductive iron sulfide matrix provides an increased surface area for the release of hydrogen. It is the consumption of hydrogen by SRB and not the surface area alone that accelerated corrosion.
4. *Formation of occluded area on metal surface.* As bacteria grow, they form colonies on the surface of iron in non-uniform layers. The site selected for colony formation may be related to metallurgical features, preexisting corrosion rates, inclusion in the iron material, or surface charge. Once the colony has formed, extracellular polymeric matrix (EPM) is produced which attracts and binds other biological and non-biological materials.

Clearly, the process of biocorrosion by SRB reflects a summation of complex environmental activities and new understanding may result from studies in microbial ecology. As suggested by Hamilton (2003), metallic ions other than iron in the corrosion environment can participate in oxidation–reduction activities and oxygen may to some degree stimulate biocorrosion even if the exposure to oxygen is only transient.

The role of oxygen in the metabolism of SRB is being examined especially with respect to biofilms and the cultivation of SRB in aerobic environment (Beech and Sunner, 2007). New information about the mechanism of oxidation of metallic iron may be developed from studies on the newly isolated bacteria that grow faster that existing SRB strains by acquiring electrons form corrosion of iron by a process other than using hydrogenase (Dinh *et al.*, 2004). In light of electrochemical development in microbial ecology with electron conducting nanowires on the surface of bacterial cells (Rabaey *et al.*, 2007) and extracellular electron movement by secreted quinones acting as shuttle compounds (Marsili *et al.*, 2008; Newman and Kolter, 2000), there is a great

potential that new information relevant to SRB biocorrosion may emerge. Although the presence of exopolymeric substances (EPS) in corrosion environments and microbial communities with SRBs (Braissant *et al.*, 2007) has been noted for many years, additional studies on production and regulation of EPS may provide additional information on this material.

B. Corrosion of concrete and stonework

Concrete pipes and conduits are subject to biocorrosion and this activity is initiated by SRB. Bacteria grow in the sediment and water that collects in the bottom of concrete pipes and hydrogen sulfide is produced. The aerobic sulfide-oxidizing bacteria convert the gaseous hydrogen sulfide to sulfuric acid. As this end product of metabolism accumulates, sulfuric acid slowly dissolves the concrete structure. A model depicting this activity is given in Fig. 2.3.

Porous stone structures positioned in loose soil and especially nutrient-rich mud in tropical regions are subject to biocorrosion. When the production of H_2S by SRB reaches the aerobic zone, the sulfur-oxidizing bacteria produce sulfuric acid which slowly dissolves stonework. As reported by Postgate (1979), stone statues in the temples of Cambodia have been subjected to corrosion involving activities of SRB.

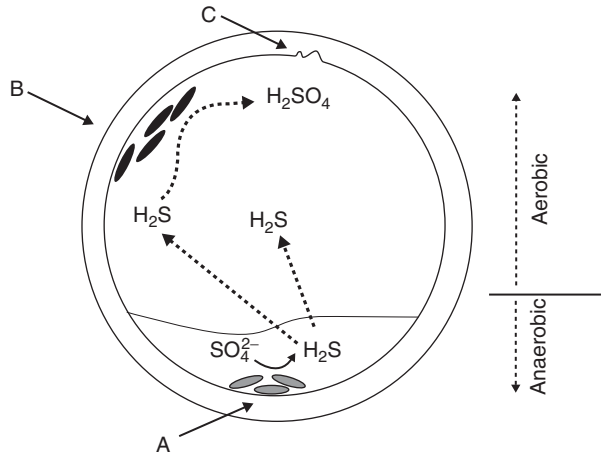

FIGURE 2.3 Model indicating biocorrosion of concrete pipes attributed to bacteria metabolizing sulfur compounds. (A) SRB grows in the anaerobic sediment and releases H_2S as a result of their metabolism. (B) *Acidithiobacillus* sp. present in the aerobic region of the pipe oxidize H_2S to sulfuric acid which (C) dissolves various minerals in the wall of the concrete pipe. The result of this action weakens the structure of the pipe.

C. Impact on the petroleum industry

1. Biocide use

Souring of oil fields, which refers to the release of hydrogen sulfide into the oil field, is a result of either abiotic or SRB activity. It is a common practice in the pumping of oil from fields to inject water into the subsurface and if seawater is used, the well receives about 300 mM sulfate. Indigenous SRB oxidize various components in the crude oil with the production of hydrogen sulfide and the souring of oil and gas fields leads to corrosive activities that may cause reservoir plugging (Hamilton and Lee, 1995). As reviewed by Voordouw (2008), the DSR in *D. vulgaris* Hildenborough is markedly inhibited by nitrite. When SRB are grown in the presence of a nitrate-respiring bacterium, nitrite generated will inhibit sulfate respiration and upregulate the nitrite reductase but downregulate the sulfate reduction enzymes in *D. vulgaris* Hildenborough (Haveman *et al.*, 2005). Thus, the addition of nitrate to oil reservoirs will reduce the souring attributed to SRB because environmental nitrate-reducing bacteria will produce nitrite.

Biofilms in oil field pipes is detrimental to the petroleum process and various treatments have been employed to control the growth of SRB (Cullimore, 1999). Over the years many different inhibitors have been introduced into the wells to prevent biofouling and examples of these include formaldehyde, glutaraldehyde, chlorine, or quaternary ammonium compounds. In a recent study, Greene *et al.* (2006) report that the inhibition of SRB-mediated production of sulfide is attributed to a synergistic inhibition resulting from combined use of nitrite and various biocides (e.g., glutaraldehyde, formaldehyde, benzyalkonium chloride, cocodiamine, tetrakishydroxymethylphosphonium sulfate and bronopol).

In controlling the activity of SRB in oil field pipelines, the biocide must be effective in both the bulk fluid and the biofilm. A mathematical model has been employed by Vilcaez *et al.* (2007) to assess the effectiveness of various biocides. They concluded that treatment of water in the pipelines was dependent on the concentration of the biocide, the disinfection coefficient and the decay rate coefficient but not affected by the SRB concentration in the biofilm or rate of inactivation of the biocide on the surface of the biofilm.

Perhaps one of the more interesting biocides to be used for inhibition of SRB in biofilms is produced by genetically engineered bacteria. Suspension cultures of *D. gigas* and *D. vulgaris* were readily inhibited by known antibiotics (e.g., gramicidin S, gramicidin D and polymyxin B) (Jayaraman *et al.*, 1999). Additionally, they report that the cationic peptides indolicidin and bactenecin from bovine neutrophils were also effecting in killing these SRB. Genes for indolicidin and bactenecin were cloned into *Bacillus subtilis* and the expressed proteins inhibited corrosion of

stainless steel by SRB cultures. Additionally, bacteriocidal activity against SRB was demonstrated when *B. subtilis* carrying the genes for indolicidin and bactenecin were incorporated in an *in vivo* biofilm. While the introduction of genetically engineered bacteria into the environment is problematic, augmentation of oil fields with native bacteria producing antibiotics effective against SRB would be useful. This area of research could provide a new approach to control ferrous corrosion by SRB.

2. Methane oxidation

As a result of decomposition of organic material on ocean floor, methane is produced by archeal methanogens and it is evident that a few meters below the seafloor, there is anaerobic oxidation of methane (AOM). As reviewed by Parks and Sass (2007), there is considerable evidence for the involvement of SRB in AOM because the reduced concentration of sulfate and increased concentration of iron sulfide closely parallels the reduction of methane in anaerobic sediments. High rates of AOM have been observed associated with gas seeps of the Northwestern Black Sea shelf, mud volcanoes in the North Atlantic and methane hydrates at Hydrate Ridge in the North Pacific (Widdel *et al.*, 2007). In a study of the methane hydrate formation of the Cascadia Margin accretionary system in the Pacific Ocean (Cragg *et al.*, 1996), the rate of AOM was greater than in other regions and in the area of methane hydrates the populations of prokaryotic organisms was also increased. SRB isolated from methane environments are from the deltaproteobacterial genera including *Desulfovibrio*, *Desulfomicrobium* and from the Firmicutes division with the sporulated genera *Desulfosporosinus* and *Desulfotomaculum* (Parks and Sass, 2007).

Thus far, no single SRB species has been isolated that oxidizes methane. It has been suggested by Hoehler *et al.* (1994) that a consortium consisting of a methanogen and SRB account for AOM. The methanogen would oxidize methane by a reversal of reactions accounting for production of methane and the SRB would serve as the electron acceptor of the system. Widdel *et al.* (2007) have reevaluated the possibilities of a syntrophic consortium and suggest three possibilities:

1. Reducing equivalents are transferred from the archaeal member oxidizing methane and electrons are transferred to the SRB. There would not be H_2 production and the mechanism for movement of electrons is unknown and may even be attributed to nanowires (Gorby *et al.*, 2006).
2. The methane-oxidizing partner would produce an organic compound that would be used by the SRB. The production of CO_2 derived from methane would be attributed to the second partner in this syntrophism.
3. The archaeal cells would account for both methane oxidation and sulfate reduction. Growth of SRB would be attributed to unknown nutrients released from the archaea.

The process of SRB and archaeal cell growth is extremely slow with generation time estimated at 7 months (Girguis *et al.*, 2005). The quantity of methane beneath the seafloor may exceed that of fossil fuel (Kvenvolden, 1992). With the demands for energy increasing and the possibility of using marine derived methane as fuel, it is important to have a better understanding of the AOM process.

VI. BIOTECHNOLOGY OF SRB

A. Bioremediation of organic compounds

The concern for a clean environment has prompted scientists to consider the use of bacteria in a variety of remediation applications and SRB are appropriate candidates. Specific isolates of SRB have the capability of using complex organic molecules as either electron donors or electron acceptors.

1. Oxidation of monoaromatic hydrocarbons

While SRB were considered earlier to use principally lactate and pyruvate as electron donors, it is now recognized that this physiological group of bacteria can grow with a variety of organic compounds. It is now estimated that SRB can oxidize over 100 different organic compounds but each species of bacteria is limited to using a few different organic compounds as electron donors (Fauque *et al.*, 1991). Contamination due to petroleum spills are difficult to remediate with aerobic bacteria because they are frequently subsurface in anaerobic zones. The activities of SRB in remediation of the hydrocarbon environment have been summarized in several reviews (Ensley and Suflita, 1995; Widdel *et al.*, 2007). Benzene, toluene, ethylbenzene and xylene (BTEX) (structures in Fig. 2.4) are the principal compounds in aromatic fuel hydrocarbons and various species of sulfate reducers have been reported to oxidize these compounds (Table 2.1).

Bacteria capable of metabolizing hydrocarbons in contaminated soils and aquifers are broadly distributed in the terrestrial environment. The physiological type of bacteria involved in oxidation of petroleum hydrocarbon in anaerobic environments reflects the characteristics of the specific site (Cunningham *et al.*, 2001; Heider *et al.*, 1998). If the conditions are appropriate for denitrification, BTEX compounds are degraded by organisms of the *Azoarcus/Thauera* group. If conditions are appropriate for iron reduction, various species of *Geobacter* would be active. Alternately, if the environment contains adequate levels of sulfate, BTEX molecules would be oxidized by SRB. Energetics of reactions used by SRB oxidizing BTEX compounds are given in Table 2.2.

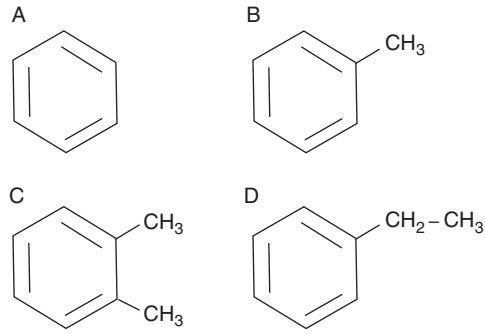

FIGURE 2.4 Structures of important compounds included as BTEX: (A) benzene, (B) toluene, (C) *o*-xylene and (D) ethylbenzene. Other compounds in BTEX include *m*-xylene, *p*-xylene and 1,2,4-trimethylbenzene.

The effectiveness of resident SRB in contaminated soil or aquifer for degradation of BTEX compounds support the concept of natural attenuation as a treatment of aromatic hydrocarbons (Edwards *et al.*, 1992; Kleikemper *et al.*, 2002; Seagren and Becker, 2002). Using bacterial consortia from ground water at petroleum spill sites from Alaska and California, the degradation of BTEX in 14 days under sulfate-reducing condition were degraded 22%, 38% 42% and 39%, respectively (Chen and Taylor, 1997). With the addition of ^{14}C labeled compounds to the consortia, it was determined that in the biodegradation of toluene and benzene water soluble products were produced and very little was mineralized to CO_2. Recently, it has been suggested that models of sulfur and oxygen isotope fractionation, while SRB are degrading BTEX compounds, are useful to evaluate the potential of natural attenuation at field sites (Knöller *et al.*, 2006). If the petroleum hydrocarbon contaminated ground water contains oxygenated additives (e.g., ethanol or methyl *tert*-butyl ether) these compound may delay the rate of BTEX bioremediation because the oxygenated additives may be degraded before the petroleum compounds. While methyl *tert*-butyl ether (structure in Fig. 2.5) is decomposed by bacteria, SRB have not been reported to participate in this activity. Another parameter that may be of importance in the rate of bioremediation of BTEX compounds is the prevention of toxic accumulation of hydrogen sulfide in the ground water by the addition of ferrous salts which precipitates sulfide as FeS (Jin *et al.*, 2007). If soil is contaminated with petroleum products, slurry treatments may be conducted with the inocula for the bioreactors taken near the site of the petroleum spills.

The mechanism for oxidation of benzene by SRB is now well understood. It has been suggested by Musat and Widdel (2008) that methylation or hydroxylation reactions are not involved but perhaps degradation of benzene proceeds through conversion to benzoate. Although it has not

TABLE 2.1 Sulfate reducers that metabolize environmentally important organic compounds

Substrate	Organism	Reference
A. Electron donors		
Benzene	Consortium	Phelps *et al.* (1998)
Crude oil	*D. desulfuricans* M6	Kim *et al.* (1990)
Crude oil	Enrichment from oil tank sediment	Eckart *et al.* (1986)
Ethylbenzene	SRB strain EbS7	Kniemeyer *et al.* (2003)
Toluene	*Desulfobacula toluolica*	Knöller *et al.* (2006)
	Desulfobacula phenolica	Rabus *et al.* (1993)
	Desulfosarcina cetonica	Harms *et al.* (1999)
	Desulfobacterium phenolicum	Rabus *et al.* (1993)
m-Xylene	SRB strain mXyS1	Harms *et al.* (1999)
o-Xylene	SRB strain oXyS1	Harms *et al.* (1999)
p-Xylene	Enrichment culture	Morasch and Meckenstock (2005)
B. Electron acceptors		
3-Chlorobenzoate	*Desulfomonile tiedjei*	El Fantroussi *et al.* (1999)
Chloroethene respiration:		
Tetrachloroethene and/or polychloroethenes	*Desulfitobacterium frappieri* TCE1	Gerritse *et al.* (1999)
	Desulfitobacterium frappieri TCE1	Drzyzga *et al.* (2001)
	Desulfitobacterium frappieri PCP1	Dennie *et al.* (1998)
	Desulfitobacterium hafniense Y51	Nonaka *et al.* (2006)
	Desulfitobacterium sp. KBC1	Tsukagoshi *et al.* (2006)
	Desulfitobacterium sp. PCE-1	Gerritse *et al.* (1996)
	Desulfitobacterium sp. Viet1	Loeffler *et al.* (1999)
	Desulfitobacterium sp. Y51	Suyama *et al.* (2001)

(*continued*)

TABLE 2.1 (*continued*)

Substrate	Organism	Reference
Nitroaeromatic respiration:		
2,4,6-Trinitrotoluene	*Desulfobacterium indolicum*	Boopathy *et al.* (1993)
	SRB isolate	Preuss *et al.* (1993)
	Coculture of:	Boopathy and Manning (1996)
	D. gigas	
	D. vulgaris	
	D. desulfuricans strain A	
	D. desulfuricans strain B	
2,4-Dinitrophenol	*D. desulfuricans* strain B	Boopathy *et al.* (1993)
2,4-Dinitrotoluene	*D. desulfuricans* strain B	Boopathy *et al.* (1993)
2,6-Dinitrotoluene	*D. desulfuricans* strain B	Boopathy *et al.* (1993)

been established which strain of SRB metabolizes benzene, Da Silva and Alvarez (2007) suggest that *Desulfobacterium* sp. clone OR-M2 is one of the organisms prevalent in a benzene-degrading consortium. As proposed by Safinowski and Meckenstock (2006), the initial steps in degradation of naphthalene by SRB is by methylation to 2-methylnaphthalene followed by the oxidation to 2-naphthoic acid. While metabolism of aromatic hydrocarbons by anaerobes contributes to the economic loss of oil reservoirs (Aitken *et al.*, 2004), some of the details of these carbon pathways by SRB remain to be established.

2. Dehalorespiration

Contamination of the environment by solvents is an important aspect resulting from improper handling and disposing of chloroethenes such as tetrachloroethene (PCE) or trichloroethene (TCE). Specific strains of SRB are capable of using chloroethenes as electron acceptors in sulfate limited environments (see Table 2.1). The contamination of sediments and ground waters is an environmental problem and efforts are being directed to evaluate persistence and effectiveness of dechlorinating SRB in settings where indigenous bacteria are present. Using a coculture system with *Desulfitobacterium frappieri* TCE1 and *Desulfovibrio* sp. strain SULF1,

TABLE 2.2 Energetics of the oxidation reactions of hydrocarbons by SRB

Hydrocarbon	Reactions	$\triangle G^{\circ\prime}$ (kJ/mol)[a]
Methane	$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$	−16.6
Benzene	$4C_6H_6 + 15SO_4^{2-} + 12H_2O \rightarrow 24HCO_3^- + 15HS^- + 9H^+$	−186.2
Toluene	$2C_7H_8 + 9SO_4^{2-} + 6H_2O \rightarrow 14HCO_3^- + 9HS^- + 5H^+$	−204.8
Ethylbenzene	$4C_8H_{10} + 21SO_4^{2-} + 12H_2O \rightarrow 32HCO_3^- + 21HS^- + 11H^+$	−240.3
m-Xylene	$4C_8H_{10} + 21SO_4^{2-} + 12H_2O \rightarrow 32HCO_3^- + 21HS^- + 11H^+$	−228.0

[a] Based on 1 mol of hydrocarbon used. From Widdel *et al.* (2007).

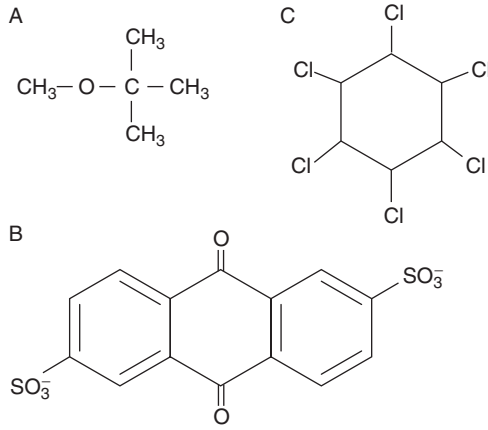

FIGURE 2.5 Organic compounds associated with pollution or extracellular electron shuttle activity: (A) methyl *tert*-butyl ether, (B) anthraquinone-2,6-disulfonate [AQDS] and (C) lindane. Lindane is 1,2,3,4,5,6-hexachlorocyclohexane and the gamma isomer is the active structure in the insecticide. The *cis–trans* position of Cl in the gamma isomer is 1,2,4,5/3,6.

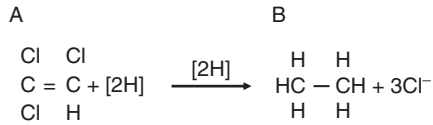

FIGURE 2.6 A dehalogenation reaction involving biological reduction. Reducing equivalents as electrons and accompanying protons from the cell are indicated as [2H]: (A) trichloroethene and (B) ethane.

Drzyzga *et al.* (2001) observed that H_2 produced by *Desulfovibrio* sp. from fermentation of lactate under sulfate-limiting conditions was used by the dehalogenating bacteria. The reaction showing reductive release of chloride from TCE is given in Fig. 2.6. In fact a syntrophic association between these bacterial types resulted in greater dehalogenation when both were present than when *D. frappieri* was the only bacterium present. As discussed by Drzyzga *et al.* (2002), sulfidic conditions in contaminated sediments are considered beneficial for bioremediation of chloroethenes. In pure culture, the metabolism of PCE by *D. frappieri* results in the accumulation of *cis*-dichloroethene but the dehalogenation of PCE by indigenous bacteria in contaminated sediment under sulfate-reducing conditions resulted in the production of 55% ethene and 45% ethane. For development of bioremediation of chloroethenes, it would be useful to promote both sulfate-reducing activity and other anaerobic bacteria.

From the report by Boyle *et al.* (1999), there is an indication that SRB may be useful in the transformation of lindane (structure in Fig. 2.5.). Gamma-hexachlorocyclohexane is the isomeric form of the active insecticide and it has a very long residence time in soil. Mixed cultures of anaerobes from marine sediments converted dechlorinated lindane to produce benzene and monochlorobenzene. Similarly, cell suspensions of *Desulfovibrio africanus, Desulfovibrio gigas* and *Desulfococcus multivorans* were shown to dechlorinate lindane. Additional studies are needed to provide information about the mechanism of this transformation of lindane.

3. Nitroaromatic respiration

Trinitrotoluene (TNT) is commonly found in soil and ground water near sites where explosives and propellants are manufactured. Efforts directed to bioremediation of nitroaromatics by SRB have been reviewed by Boopathy (2007). Several strains of *Desulfovibrio* will use TNT and several other nitroaromatics as the electron acceptor under sulfate-limiting conditions (see Table 2.1). The initial steps in the reduction of TNT by bacteria are given in Fig. 2.7. With TNT as the sole carbon source, 27% of ^{14}C-TNT

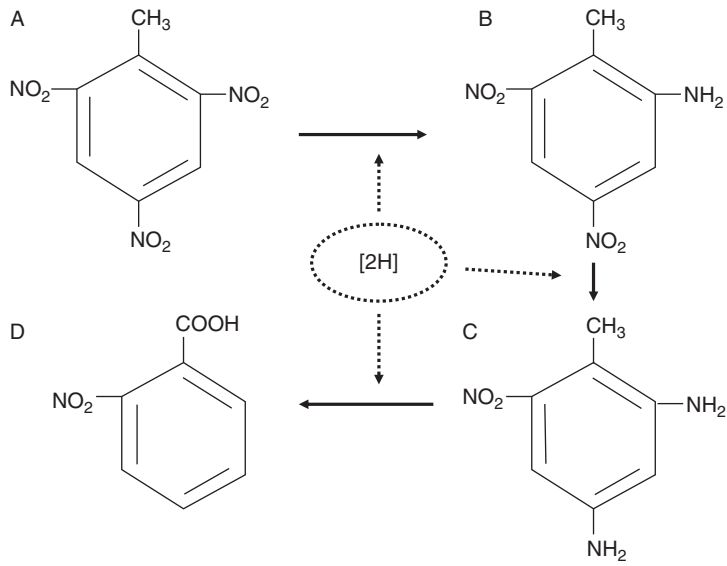

FIGURE 2.7 Initial steps in the bacterial reduction of TNT: (A) TNT, (B) 2-amino-4,6-dinitrotoluene (DNP), (C) 2,4-diamino-6-nitrotoluene and (D) nitrobenzoic acid. Several additional enzymatic steps are required to convert nitrobenxoic acid to acetic acid. The initial step in reduction of one of the nitro groups on TNT could result in either the production of 2-amino-4,6-DNM or 4-amino-2,6-DNP.

was converted to biomass and 49% of TNT was acetic acid but no CO_2 was released. Intermediates in the SRB culture with TNT as the carbon source included nitrobenzene, cyclohexanone, butyric acid, 2-methyl pentanoic acid, 4-amino-2,6-dinitrotoluene, 2-amino-4,6-dinitrotoluene and 2,4-diamino-6-nitrotoluene. If TNT is used as the electron acceptor with pyruvate as the carbon and electron donor, TNT is converted quantitatively to CO_2. TNT can be used as the nitrogen source by SRB following reduction of nitro groups reduced to amines with liberation of ammonia by reductive deamination. It remains unresolved if this reduction of nitro groups to amino groups is attributed to nitrite reductase which is commonly found in SRB (Moura *et al.*, 2007).

B. Immobilization of toxic metals

1. Precipitation as metal sulfides

It has been estimated that billions of tons of organic and inorganic hazardous wastes are produced annually around the world. Over a thousand pounds of metals (Pb, Cd, As and Zn) can be released into the environment from a single metallurgical plant; petroleum refining industry contributes tons of lead and chromium into the environment annually and acidic water from a single coal mine releases hundreds of tons of toxic metals into the environment (Ehrlich and Holmes, 1986). Hydrogen sulfide produced by the bacteria will react with the cationic metals to give highly insoluble metal sulfides (Hockin and Gadd, 2007). SRB are important as biocatalysts to remediate metal-containing streams because the bioremediation with hydrogen sulfide is relatively inexpensive. The microbial process for remediation of soils contaminated with toxic metals has been discussed in previous reports (Luptakova and Kusnierova, 2005; White *et al.*, 1998). Bioaugmentation is not required for metal remediation of mine tailings by SRB because these bacteria are indigenous in soils around the world even in northern regions of Siberia (Karnachuk *et al.*, 2005). Several examples of *in situ* bioremediation of mines with removal of toxic metals are given in Table 2.3. Various types of bioreactors have been used and generally the principal addition is an energy source for the SRB. One of the systems for immobilization of toxic metals is the use of permeable reactive barriers. Permeable barrier technology has evolved from a concept in the 1970s into a viable *in situ* passive mitigation process designed to treat contaminated groundwaters (Benner *et al.*, 2002). Heavy metal sulfides are immobilized by SRB and the permeable barrier can retain pollutants without significantly altering the hydraulic gradient.

An important commercial process developed by PAQUES BV is used in Balk, The Netherlands, to treat groundwater from the Budelco zinc refinery (Hockin and Gadd, 2007). The initial phase of the treatment process uses H_2 as the electron source for the SRB to precipitate Zn^{2+} as

TABLE 2.3 Case studies of bioremedation of acid mine drainage using SRB

Technology	Location	Site	Metals removed	Website	Reference
1. Compost bioreactor	Yellow Creek, Pennsylvania	Abandoned underground coal mine	Fe, Al	http://www.kcstreamteam.org; http://www.ogm.utah.gov/amr/nammip; http://2005.treatminewater.com/Presentations/PDFs/2cGusek.pdf	Doshi (2006)
2. Compost bioreactor	Carnon Valley, Cornwall, UK	Wheal Jane tin mine	Zn, Fe		Halberg and Johnson (2003), Whitehead *et al.* (2005)
3. Compost bioreactor	Reynolds County, Missouri	Doe Run West Fork Mine (active underground Pb–Zn mine)	Pb, Zn		Doshi (2006)
4. *In situ* bioreactor	Elliston, Montana	Lilly/Orphan Boy Mine	Al, As, Cd, Cu, Zn	http://www.epa.gov/ORD/NRMRL/std/mtb/mwt/annual/annual2004/adwt/sulfatereducingbacteriademo.htm	Doshi (2006)
5. Compost bioreactor	Ellison, Montana	Surething amandoned mine	Al, As, Cd, Cu, Fe, Pb, Mn, Zn	http://www.epa.gov/ORD/NRMRL/std/mtb/mwt/annualannual2004/adwt/passivebiotreat.htm	Bless *et al.* (2006)
6. Compost-free bioreactor	Markleeville, California		Al, Cu, Fe, Ni, Cd, Cr, Pb, Se, Zn		Bates *et al.* (2006)

(*continued*)

TABLE 2.3 (*continued*)

Technology	Location	Site	Metals removed	Website	Reference
		Leviathan amandoned mine			
7. Permeable reactive barrier	Sudbery, Ontario, Canada	Nickel Rim abandoned Ni–Cu mine	Al, Cu, Ni, Zn	http://www.rtdf.org/PUBLIC/permbarr/prbsumms/profile.cfm?mid=41	Benner *et al.* (2002)
8. Permeable reactive barrier	Shoshone County, Idaho	Success Mine and Mill	Cd, Pb, Zn		Conca and Wright (2006)
9. In-lake treatment	Black Hills, South Dakota	Ancher Hill Pit Lake	Al, As, Cd, Cu, Fe, Se, Zn	http://www.epa.gov/region8/superfund/giltedge/gltfactsht.html	Doshi (2006)
10. THIOPAQ®	Balk, The Netherlands	Budelco zinc refinery	Zn	http://www.paques.nl/paques/	Hockin and Gadd (2007)

ZnS. In the second phase, excess sulfide is oxidized to elemental sulfur by an aerobic process. This process can handle large volumes in that about 400 m^3/h of contaminated groundwater can be treated on a routine basis.

2. Reduction of toxic metals

A few bacteria have the capability of reducing toxic heavy metals by a process that couples electron transport to reduction of oxidized metals and the organisms that carry out this process are referred to as dissimilatory metal-reducing bacteria (DMRB). Included as DMRB are members of the SRB and in this physiological group *Desulfovibrio* are the most prominent species. The initial observation by Woolfolk and Whiteley (1962) indicated that *D. desulfuricans* was capable of reducing several metals. However, it was not until the reports by Lovley and Phillips (1992) and Lovley *et al.* (1993b) concerning the reduction of uranium by *Desulfovibrio* species that great interest was shown by the scientific community in metal reduction by SRB for bioremediation of toxic environments or application in biotechnology (see the following section). Currently, it has been reported that cells or electron transport proteins from SRB reduce several different metals (see Table 2.4). With respect to bacterial nutrition, some of the metals listed have no role in cellular metabolism while others (Fe, Mn, Se, Mo and Cu) are required only at trace levels but are inhibitory at elevated concentrations.

Since SRB are capable of reducing a large number of metals, the mechanism of dissimilatory metal reduction has been examined in several cell-free systems. One of the first demonstrations of metal reduction by a protein was the report of Lovley *et al.* (1993a) concerning the reduction of uranyl ion by tetraheme cytochrome c_3 from *D. vulgaris* Hildenborough. We now understand that cytochromes other than tetraheme cytochrome c_3 are capable of metal reduction that several metals are reduced by cytochromes and that Fe–S clusters in non-heme iron proteins can also reduce metals. Several of these metal reductions by proteins from SRB are given in Table 2.5. It is rather interesting that in SRB no one protein is capable of reduction of a specific metal but several proteins are capable of reducing numerous metals. The requirement appears to be that the heme or Fe–S cluster has the appropriate energetics and availability for metal reduction. With the periplasmic located tetraheme cytochrome c_3 having no apparent role in metabolism, it may be that its primary activity is metal detoxification.

a. The microbiology of DMR by SRB The location of metal reduction by SRB is not as well substantiated as with other DMRB. It has been well established that *Geobacter* and *Shewanella* have cytochromes in the outer membrane responsible for metal reduction and the toxic metal never enters the periplasm. While there has been the suggestion that heme is

TABLE 2.4 Dissimilatory metal reduction by cells of SRB

Redox couple	Organism	Reference
AsO_3^-/As^{3+}	*Desulfotomaculum auripigmentum*	Newman *et al* (1997)
	Desulfovibrio strain Ben-RA	Macy *et al.* (2000)
$AuCl_4^-/Au^0_{(S)}$	*Desulfovibrio desulfuricans*	Deplanche and Macaskie (2008)
CrO_4^{2-}/Cr^{3+}	*D. vulgaris* Hildenborough	Wang and Shen (1997)
	D. desulfuricans	Tucker *et al.* (1998)
	Dst. reducens	Tebo and Obraztsova (1998)
Fe^{3+}/Fe^{2+}	*Desulfobacter postgatei*, *Desulfobacterium autotrophicum*, *Desulfobulbus propionius*, *Desulfovibrio baculatus*, *Desulfovibrio baarsii*, *Desulfovibrio vulgaris*, *Desulfovibrio sulfodismutans*, *Desulfotomaculum acetoxidans*	Lovley *et al.* (1993b)
MnO_2/Mn^{2+}	*Desulfotomaculum acetoxidans*	Nealson and Saffarini (1994)
	Desulfobacterium autotrophicum	Lovley (1995)
	Desulfomicrobium baaculatum	Lovley (1995)
	Desulfotomaculum reducens	Tebo and Obraztsova (1998)
MoO_4^{2-}/Mo^{4+}	*D. desulfuricans*	Tucker *et al.* (1997, 1998)
$Pd^{2+}/Pd^0_{(S)}$	*D. desulfuricans*	Lloyd *et al.* (1998)
$Pt^{2+}/Pt^0_{(S)}$	*D. desulfuricans*	Vargas *et al.* (2005)
$Rh^{+7}/Rh^0_{(S)}$	*D. desulfuricans*	Xu *et al.* (2000)
$SeO_4^{2-}/Se^0_{(S)}$	*D. desulfuricans*	Tomei *et al.* (1995)
$SeO_3^{2-}/Se^0_{(S)}$	*D. desulfuricans*	Tucker *et al.* (1998)
	D. desulfuricans	Tomei *et al.* (1995)
$TcO_4^-/TcO_{2(S)}$	*D. desulfuricans*	Lloyd *et al.* (1999)
	D. fructosovorans	

(*continued*)

TABLE 2.4 (*continued*)

Redox couple	Organism	Reference
		De Luca *et al.* (2001)
$TeO_3^{2-}/Te_{(S)}$	*D. desulfuricans*	Woolfolk and Whiteley (1962)
$UO_2^{2+}/UO_{2\ (S)}$	*D. desulfuricans*	Tucker *et al.* (1998)
	D. desulfuricans strain 20	Payne *et al.* (2002)
	Desulfovibrio strain UFZ B490	Pietzsch *et al.* (1999)
	D. vulgaris	Lovley *et al.* (1993b), Tucker *et al.* (1998)
	Dst. reducens	Tebo and Obraztsova (1998)
	D. baarsii, *D. sulfodismutans* and *D. baculatus*	Lovley *et al.* (1993b)
	D. gigas	Barton *et al.* (1996)
$VO_3^-/VO_{2(S)}$	*D. desulfuricans*	Woolfolk and Whiteley (1962)

present in the outer membrane of *D. vulgaris* (Van Ommen Kloeke *et al.*, 1995), it is possible that cytochrome or a heme protein from the periplasm may be co-isolated with the outer membrane. In outer membrane fraction isolated from *D. desulfuricans* ATCC 27774, proteomic studies revealed that a subunit of nitrite reductase is isolated along with the outer membrane (Barton *et al.*, 2007) and it remains to be established if nitrite reductase plays a role in metal reduction. Reduced metals may occur on the cell surface or collect in the EPM. The composition of the EPM from *Desulfovibrio* is highly diverse and it is considered to contain polysaccharides, uronic acid polymers, proteins and nucleic acids (Beech and Sunner, 2007). Braissant *et al.* (2007) measured the buffering activities of EPM from several SRB and found the charged groups to be attributed to amino groups ($pK_a = 8.4–9.2$), carboxyl groups ($pK_a = 3.0$) and sulfur-containing groups including thiols ($pK_a = 7.0–7.1$). The presence of numerous charge groups in the EPM would account for the binding of various metals.

b. Biological reduction of chromium from industrial waste streams Industrialized countries use chromium in many processes including paint pigments, leather tanning, electroplating of metal surfaces, or cleaning

TABLE 2.5 Proteins of SRB associated with metal reduction

Protein	Metals reduced	Organism	Reference
Cytochromes:			
Cyto c_3	Cr(VI)	*D. vulgaris* Hildenborough	Lovley and Phillips (1994b)
	Fe(III)	*D. vulgaris* Hildenborough	Lojou *et al.* (1998a,b)
	Se(VI)	*D. vulgaris* Hildenborough	Abdelouas *et al.* (2000)
	U(VI)	*D. vulgaris* Hildenborough	Lovley *et al.* (1993a)
	Fe(III)	*D. gigas*	Lojou *et al.* (1998a)
	Fe(III)	*D. desulfuricans* Norway	Lojou *et al.* (1998a)
	Fe(III)	*D. vulgaris* Hildenborough	Lovley *et al.* (1993a)
	Tc(VII)	*D. fructosovorans*	De Luca *et al.* (2001)
Cyto c_7	V(VI)	*Desulfuromonas*[a] (*Dsf.*) *acetoxidans*	Lojou *et al.* (1998b)
	Mn(IV)	*Dsf. acetoxidans*	Lojou *et al.* (1998b)
	Fe(III)	*Dsf. acetoxidans*	Lojou *et al.* (1998a, b)
	Cr(VI)	*Dsf. acetoxidans*	Lojou *et al.* (1998b)
Cyto c_{553}	Fe(III)	*D. vulgaris* Hildenborough	Lojou *et al.* (1998a)
Nonheme iron (Fe–S) proteins:			
Hydrogenase	Pd(II)	*D. desulfuricans* G20[b]	Lloyd *et al.* (1999)
Hydrogenase	Tc(VII)	*D. desulfuricans* G20	Lloyd *et al.* (1998)
[Fe] hydrogenase	Cr(VI)	*D. vulgaris* Hildenborough	Michel *et al.* (2001)
[Fe] hydrogenase	Tc(VII)	*D. fructovorans*	De Luca *et al.* (2001)
[NiFe] hydrogenase	Tc(VII)	*D. fructosovorans*	De Luca *et al.* (2001)
[NiFe–Se]	Cr(VI)	*Dsm. norvegicum*	Michel *et al.* (2001)
Ferredoxin	Cr(VI)	*D. gigas*	Chardin *et al.* (2003)

[a] *Dsf. acetoxidans* is a sulfur-reducing bacterium and not a SRB.
[b] May be more similar to *D. aleskensis* than *D. Desulfuricans*.

solutions for glass and electrical components. As a result of these various uses, chromium contamination in soil or ground water is an environmental problem and a serious health risk. Bioremediation of chromium contaminated sites with SRB is effective and has been used for several sites (De Filippi, 1994). While waste streams from Cr-related industries may use alkaline treatment to generate $Cr(OH)_3$, the formation of Cr_2S_3 is of interest due to the greater insolubility of the metal sulfide.

c. Microbiology of chromate reduction Based on electrochemical studies and kinetic analysis, Chardin *et al.* (2003) suggests that the principal protein in the periplasm for Cr(VI) reduction is the periplasmic hydrogenase. If the toxic metal enters the periplasm before reduction occurs, a mechanism for export of the reduced metal from the cell would be required. Chromate stress response has been studied in *D. vulgaris* and Wall *et al.* (2007) report that 337 genes had changes in expression and that the Fur regulon may be involved in metal stress response.

d. Biological reduction of uranium in the environment With the initial demonstration of uranium reduction by bacteria by Lovley and colleagues (Gorby and Lovley, 1992; Lovley *et al.*, 1991), there was considerable interest in extending the laboratory-based results to environmental sites and especially to uranium mill tailings. As reviewed by Landa (2005), uranium mill tailings were a result from the processing of the low grade uranium ore. In many operations, the uranium ores were treated with sulfuric acid to extract uranium and ammonia was added to the soluble uranium to generate insoluble ammonium diuranate also known as yellow cake. Over time indigenous bacteria oxidized ammonium to nitrate. Commonly, uranium ore contained oxy-anions of selenium, vanadium, molybdenum and arsenic, which were extracted by sulfuric acid in addition to uranium. Thus, ground water from uranium mill tailings potentially contained toxic levels of metals and elevated nitrate as well as sulfate. SRB were one of the candidate organisms for bioremediation because of their tolerance to various heavy metals and capability to reduce uranium. The general goal of bioremediation of uranium-containing ground water was to use SRB to convert soluble uranyl $[U(VI)O_2^{2-}]$ to insoluble uraninite $[U(IV)O_2]$.

As discussed by Landa (2005), the activity of SRB in ground water from mill tailings or from leaching piles may result in mobilization of elements in the ^{236}U decay chain. Radium (^{226}Ra) may be insoluble as $Ba(Ra)SO_4$ but the utilization of sulfate by SRB would result in solubilization and increased mobility of Ra. In contrast to the action of SRB, sulfur-oxidizing (*Thiobacillus* sp. or *Acidithiobacillus* sp.) promote the formation of the interaction of alkaline earth elements with radium and sulfate to promote immobilization of Ra. Plutonium (^{210}Po) may be solubilized by

the action of SRB but under appropriate conditions may be precipitated as a sulfide or converted to dimethyl plutonium.

SRB have been detected in ground water of several sites where uranium processing had been conducted. At the Uranium Mill Tailings site near Shiprock, New Mexico, USA, molecular probes were employed to detect the genes for [NiFe] hydrogenase and for dissimilarity sulfite reductase gene (Chang *et al.*, 2001). The most abundant SRB were the spore-forming *Desulfotomaculum* and *Desulfotomaculum*-like sequence clusters. Additionally, SRB were present in ground water from uranium mill tailing sites at Bowman, North Dakota; Tuba City Arizona; Falls City, Texas; and Cannonsberg, Pennsylvania (Barton *et al.*, 1996). Thombre *et al.* (1996) used a column system with a matrix of various cellulosic materials (sawdust, wheat straw, or alfalfa hay), a feed solution with a chemical composition simulating the plume water at the uranium mill tailing site near Shiprock, New Mexico and the bacterial source was ground water from the Shiprock site. When following effluent from the column, the concentration of sulfate and uranium levels did not decline until after nitrate and nitrite levels were no longer detected. A microbial community had established in the column and over a period of 70 days, only 11% of the cellulosic material had been metabolized by the bacteria. In related studies, cells of *D. desulfuricans* that were immobilized in acrylamide displayed greater removal of chromate, uranyl and selenate than for molybdate (Tucker *et al.*, 1998). The products of Cr(VI), U(VI) and Mo(VI) were respective sulfides and there was considerable stability of metals immobilized by *in situ* microbial reduction (Simonton *et al.*, 2000; Thomson *et al.*, 2001).

No doubt the reduction of metals by bacteria deprives the cell from using electrons for energy production. When *D. desulfuricans* was grown in the presence or absence of sub-lethal concentrations of uranyl ion, the growth yield (g cell dry wt/mole pyruvate used) was less in the presence of uranium as compared to growth response in the absence of uranium (Tucker *et al.*, 1996). This response of *D. desulfuricans* to uranyl ion suggests that there is a regulatory activity that preferentially detoxifies the environment from metals. The utilization of respiratory electrons to reduce uranium may account for the absence of viable SRB in ground water containing elevated levels of soluble uranyl ion.

e. Other redox active metals In SRB, the reduction of mercury ion is considered to be by the *mer* operon found in many other bacteria. From analysis of the genomes of several of the *Desulfovibrio* sp., merA and merP are present. There is also the possibility that Hg(II) may be immobilized by the formation of an insoluble metal sulfide if SRB generate a strong sulfidic environment. While Th(IV) and Am(III) are not considered amenable to bacterial reduction (Lloyd, 2005), the reduction of Pu(V) to Pu(IV)

and Pu(VI) to Pu(III) may be within the capabilities of SRB because certain of these reductions have been reported for Fe(III)-reducing bacteria. Reduction of Nb(V) to Nb(IV) has been reported for *Shewanella putrefaciens* but not observed with SRB (Lloyd, 2005).

C. Reduction of azo dyes

As outlined in the United Nations Environment Progamme there is considerable interest in persistent organic pollutants. Organic compounds that have a long residence time in the environment include organic pesticides, herbicides, azo dyes, polychlorinated dioxins, polyaromatic hydrocarbons and polychlorinated dibenzofurans. The quantity of pigmented dyes used is considerable with over 85,000 tones used in Western Europe and 668,000 tonnes used by the world in 1991 (Øllgaard *et al.*, 1998). Few of these xenobiotic compounds are degraded rapidly in the environment and several of these organic pollutants are associated with mutagenic and carcinogenic activity. Frequently, these compounds are swept into the rivers and groundwater where the pollutants collect in anaerobic regions.

Azo dyes are used in the textile industry and are characterized as having the N=N (azo) bond. Various bacteria will utilize the dye as carbon and energy sources with complete mineralization of the dye molecules (Stolz, 2001). Several azo dyes have been reported to be degraded in biodigestors containing municipal sewage sludge where H_2S is formed as an end product of respiration of SRB (Yoo *et al.*, 2000). Solutions of Orange II, Reactive Black 5, Reactive Red 120, Reactive Brilliant violet 5R (azo dyes) and Reactive blue 2 (an anthraquinone dye) have been reported to be reduced in biodigestors under sulfidogenic environments (Togo *et al.*, 2008). The decolorization of Congo Red, a sulfonated azo dye, is reduced by H_2S and not by extracts of *Desulfovibrio* sp. (Diniz *et al.*, 2002). The action of biogenic H_2S on azo dyes is to attack the N=N bond with the formation of colorless aromatic amines. An example of azo dye reduction is given in Fig. 2.8 with sulfanilic acid and 1-amino-2-naphthol being generated from Acid Orange 7. This activity is distinct from the reduction of artificial electron carriers (e.g., methyl viologen or benzyl viologen) which are readily reduced by cells and/or cell extracts of sulfate reducers.

Considerable evidence is available for the chemical reduction of azo dyes with biologically produced H_2S and recent information suggests that hydrogenase may have a role in decolorization and degradation of textile dyes (Mutambanengwe *et al.*, 2007). In an anaerobic sludge blanket reactor, the addition of anthaquinone 2,6-disulfonate (AQDS, structure in Fig. 2.5) and riboflavin was reported to enhance the decolorization rate of azo dyes (dos Santos *et al.*, 2007). It has been suggested that AQDS

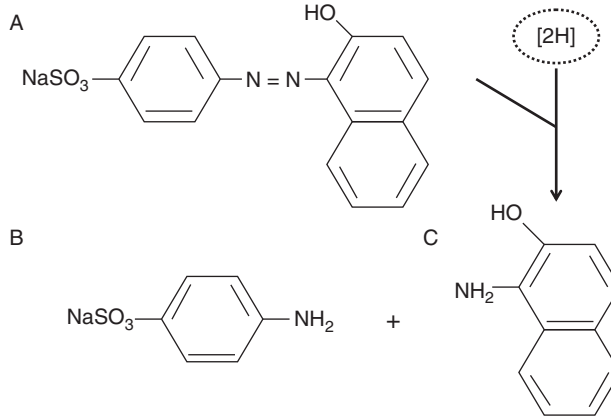

FIGURE 2.8 Bacterial decolorization of a monoazo dye: (A) Acid Orange 7, (B) sulfanilic acid and (C) 1-amino-2-naphthol. Reducing power from the bacterial electron transport system is represented as [2H].

functions to transfer electrons to the dyes' molecules from hydrogenase with H_2 as the electron donor (Van der Zee *et al.*, 2001). The specific bacteria associated with reduction of AQDS in the bioreactors remains to be established; however, there is considerable potential for the use of sludge granules containing SRB for treatment of waste waters from industrial dye facilities.

While robust information has not been reported for respiratory-coupled reduction of azo dyes in SRB, information concerning azo respiration in *Shewanella decolorationis* has been provided by Hong *et al.* (2007). *S. decolorationis* was reported to grow using the monoazo dye amaranth as the sole electron acceptor and formate, lactate, pyruvate, or H_2 as electron donors. The end products of amaranth reduction were 1-naphthylamine-4-sulfonic acid and 1-napthylamine-2-disulfonic acid which accumulated in the medium. Through the use of inhibitors, it was demonstrated that the electron transport system involved in amaranth reduction included hydrogenase, cytochromes and other electron transport components. Similar experiments concerning electron transport for azo dye reduction by SRB would be useful.

D. Recovery of precious metals

As discussed previously in this review, there have been several reports on metal reduction and there is some interest in using SRB to recover precious metals at industrial sites. Two of the platinum group metals, platinum and palladium, are used in automobile catalytic converters and a

biorecovery process for recovery of Pt and Pd from spent catalysts would be attractive. Lloyd *et al.* (1998) initially reported that *D. desulfuricans* can reduce $Pd(NH_3)_4CL$ or Na_2PdCl_4 to produce Pd^0 deposits on the surface of the cell that were approximately 50 nm in diameter. Additionally, this activity was remarkably stable in that it was not inactivated by transient exposure to air and it would proceed at pH 2–3. Involvement of a periplasmic hydrogenase was demonstrated by inhibition of Pd(II) reduction by Cu^{2+}, a known inhibitor of periplasmic hydrogenase and from the use of mutants of *D. fructosvorans* lacking hydrogenases (Creamer *et al.*, 2006). According to De Vargas *et al.* (2004), the reduction of Pd(II) at pH 2–3 by hydrogenase follows the attachment of Pd^{2+} to amine groups on the cell surface. The formation of the Pd^0 nanoclusters on the cell surface stabilizes the periplasmic hydrogenase for several hours at pH 2 (Mikheenko, 2004). Crystal growth with concomitant reduction of Pd(II) proceeds in the presence of H_2 or formate (Yong *et al.*, 2002). Cells of *D. desulfuricans* with Pd^0 on the surface are referred to as palladised cells and are useful in recovery of Pd and Pt from acidic waste streams (Mabbett *et al.*, 2005). At pH 3, *D. desulfuricans* will bind Pd and Pt onto the cells to produce 19% and 9%, respectively, of the biomass dry weight. Using a simulated system, Yong *et al.* (2003) found that palladised cells of *D. desulfuricans* is an effective system for recovery of precious metals from spent automotive catalytic converters.

Gold is another precious metal that can be collected from industrial waste streams with the aid of SRB. According to Deplanche and Macaskie (2008), *D. desulfuricans* will reduce Au(III) to elemental Au^0 using H_2 as the electron donor with nanocrystals of Au^0 collecting on the cell surface and in the periplasm. The mechanism of bioreduction of Au(III) at pH 2–3 appears to be unlike that for the reduction Pd(II) in which cationic gold prefers thiol groups as coordinating ligands (Mirkin *et al.*, 1996) while Pd(II) prefers amine groups for the initial cellular binding. Quantitative recovery of gold from pure solutions using $HAuCl_4$ or leached jewellery wastes could be achieved using *D. desullfuricans* (Deplanche and Macaskie, 2008).

The remarkable stability of the metal reductase activity of *D. desulfuricans* at pH 2–3 enables recovery of precious metals from dilute *aqua regia* (Deplanche and Macaskie, 2008). A multistep process has been proposed by Creamer *et al.* (2006) for the recovery of Au, platinum group metals and Cu(II) from electronic scrap leachate. This biorecovery process for precious metals is desirable because it is not practicable to use chemical methods to collect Au, Pt and Pd at the low concentrations found in dilute industrial waste streams.

Another activity of SRB is the potential for gold recovery in heap leaching processes involving *Acidithiobacillus*. As reviewed by Reith *et al.* (2007), sulfur-oxidizing bacteria may produce thiosulfate from sulfide

minerals in nature and gold may be solubilized with the formation of Au(I/III). Gold thiosulfate compounds would be important complexes that would contribute to mobility of gold. An enrichment of SRB from the Driefontein Consolidated Gold Mine, Witwatersrand Basin, Republic of South Africa was found to precipitate gold from gold thiosulfate, $Au(S_2O_3)_2^{3-}$, producing nanoparticles of Au^0 outside of the bacterial cells (Lengke and Southam, 2006). While this may suggest the use of bacteria to recover gold from waste streams from heap leaching, studies are needed to see if this approach would be feasible.

VII. PERSPECTIVE

The application of SRB to biotechnology with an emphasis on economic activities is a complex issue. One requirement is the presence of appropriate biochemical capabilities which is frequently achieved by isolation of new genera and species. Since there is a wealth of information concerning the activities of key enzymes and electron transport molecules, many of the recent bioremediation aspects with SRB can be readily appreciated. Additionally, the multifunctional activity of electron transport proteins and *c*-type cytochromes with exposed redox centers provides for redox activites. It will be exciting to follow some of the emerging areas where SRB can have a positive impact on diverse bioremediation processes.

The activities of SRB with respect to corrosion, oil fields souring and bioremediation are influenced by activities of other bacteria in the immediate environment. Apart from reports concerning the commensalistic activities between SRB and methanogens, relatively little is known about controlling the interaction of SRB with anaerobes in biofilms and anaerobic digestors. Obviously, the synergism between SRB and other anaerobes is desirable for robust bioremediation and future studies are needed to optimize these environmental systems.

ACKNOWLEDGMENTS

We dedicate this chapter to the memory of Professors Harry D. Peck, Jr., Jean LeGall and Antonio V. Xavier for their great contribution to our knowledge of the physiology, biochemistry and biotechnology of SRB. We were very pleased and honoured to have collaborated with these three pioneers in the work on dissimilatory sulfate reduction.

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CHAPTER 3

Biotechnological Applications of Recombinant Microbial Prolidases

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Contents

Abstract

Prolidase is a metallopeptidase that is ubiquitous in nature and has been isolated from mammals, bacteria and archaea. Prolidase specifically hydrolyzes dipeptides with a prolyl residue in the carboxy

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Advances in Applied Microbiology, Volume 68
ISSN 0065-2164, DOI: 10.1016/S0065-2164(09)01203-9

terminus (NH_2–X–/–Pro–COOH). Currently, the only solved structure of prolidase is from the hyperthermophilic archaeon *Pyrococcus furiosus*. This enzyme is of particular interest because it can be used in many biotechnological applications. Prolidase is able to degrade toxic organophosphorus (OP) compounds, namely, by cleaving the P–F and P–O bonds in the nerve agents, sarin and soman. Applications using prolidase to detoxify OP nerve agents include its incorporation into fire-fighting foams and as biosensors for OP compound detection. Prolidases are also employed in the cheese-ripening process to improve cheese taste and texture. In humans, prolidase deficiency (PD) is a rare autosomal recessive disorder that affects the connective tissue. Symptoms of PD include skin lesions, mental retardation and recurrent respiratory infections. Enzyme replacement therapies are currently being studied in an effort to optimize enzyme delivery and stability for this application. Previously, prolidase has been linked to collagen metabolism and more recently is being associated with melanoma. Increased prolidase activity in melanoma cell lines has lead investigators to create cancer prodrugs targeting this enzyme. Thus, there are many biotechnological applications using recombinant and native forms of prolidase and this review will describe the biochemical and structural properties of prolidases as well as discuss their most current applications.

I. INTRODUCTION

Proteases are defined as enzymes that are able to catalyze the hydrolysis of proteins into smaller peptide fractions and amino acids. There are very few proteases that can cleave a peptide bond adjacent to a proline residue because of the conformational constraint that the structure of proline puts on the backbone of a peptide bond. Because of its cyclic nature, proline is one of the most unique of the 20 amino acids (Cunningham and O'Connor, 1997). The cyclic structure limits the angle of rotation about the α-carbon and nitrogen so that it introduces a fixed bend into the peptide chain (Cunningham and O'Connor, 1997) (Fig. 3.1). Physiologically, proline is important due to its location within the peptide chain, which is thought to protect biologically active peptides against excessive degradation (Cunningham and O'Connor, 1997; Vanhoof *et al.*, 1995). Creating a polypeptide is a very ordered process involving endopeptidases to cleave precursors at specific sites and exopeptidases for trimming the polypeptide chains to their functional lengths (Cunningham and O'Connor, 1997). Proline residues are often found near the amino termini of many biologically active peptides, which suggests again that they could play a role in regulating proteolytic

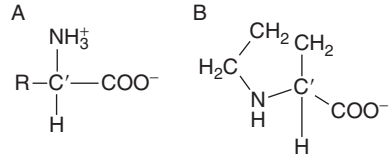

FIGURE 3.1 The structure of a basic R-group amino acid with chiral α-carbon (C′) connected to an amino and carboxyl group (A) and the cyclic structure of the amino acid proline (B).

degradation (Bradbury *et al.*, 1982; Mentlein, 1988; Persson *et al.*, 1985; Yaron, 1987). Because of the uniqueness of proline, there are few enzymes that can cleave proline-containing peptides (Walter *et al.*, 1980). Enzymes that are able to cleave a proline bond are very rare and specific for the substrate they target. These enzymes include proline-specific endopeptidases, which hydrolyze peptides on the carboxyl side of the proline residue within a polypeptide (–X-Pro–/–X–); prolyl aminopeptidase, which cleaves the N-terminal amino acid and a penultimate proline in both short and long peptides (NH_2–X–/–Pro-X–); proline iminopeptidase, which cleaves the N-terminal proline residue from any length polypeptide (Pro–/–X–); proline-specific C-terminal exopeptidase, which releases an amino acid from the C terminus of a peptide with a penultimate proline (–X-Pro–/X–COOH); and prolidase (NH_2–X–/–Pro–COOH) (Ghosh *et al.*, 1998) (Fig. 3.2). Prolidase is a proline-specific peptidase that can hydrolyze dipeptides with proline at the C-terminus and a nonpolar amino acid at the N-terminus (X-Pro). Some prolidases have also shown the ability to hydrolyze substrates with proline in the N-terminus as well as the C-terminus.

Prolidases are ubiquitous in nature and can be found in archaea (Ghosh *et al.*, 1998), bacteria (Fernandez-Espla *et al.*, 1997; Fujii *et al.*, 1996; Kabashima *et al.*, 1999; Park *et al.*, 2004; Suga *et al.*, 1995) and eukarya (Browne and O'Cuinn, 1983; Endo *et al.*, 1989; Jalving *et al.*, 2002; Myara *et al.*, 1994; Sjostrom *et al.*, 1973). In archaea and bacteria, the role of prolidase is not well understood; however, it has been suggested that it aids in protein degradation and could be responsible for the recycling of proline (Ghosh *et al.*, 1998). Due to its reaction mechanism, it could also play a role in regulating biological processes. In humans, it is known that prolidase is involved in the final stage of the degradation of endogenous and dietary protein and is important in collagen catabolism (Endo *et al.*, 1989; Forlino *et al.*, 2002). Prolidase deficiency (PD) in humans is a recessive disorder and is characterized by skin ulcerations, mental retardation and recurrent infections of the respiratory tract (Endo *et al.*, 1989; Forlino *et al.*, 2002).

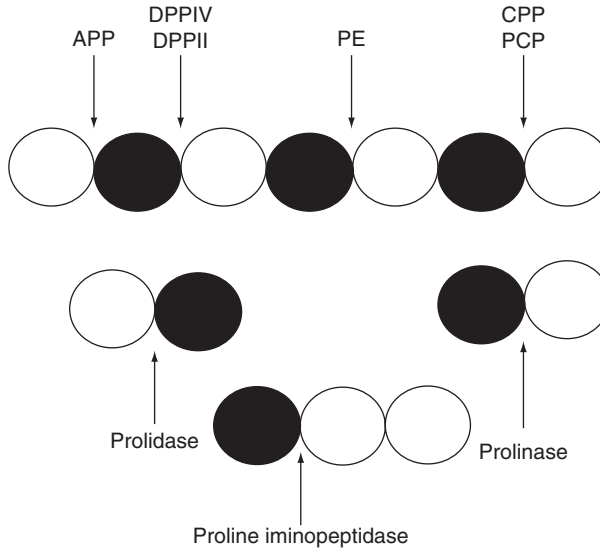

FIGURE 3.2 Characterized proline-specific peptidases: APP, aminopeptidase P; DPPIV, dipeptidyl peptidase IV; DPPII, dipeptidyl peptidase II; PE, prolyl endopeptidase; CPP, carboxypeptidase P; PCP, prolyl carboxypeptidase. These enzymes cleave peptides and proteins (open circles) with a proline residue (filled circles) at specific locations within the protein (Cunningham and O'Connor, 1997). The point of cleavage is indicated by the arrow.

Prolidases have many biotechnological applications. One important application is that it has been shown to be active against organophosphorus (OP) nerve agents. OP nerve agents act by inhibiting acetylcholinesterase (AChE), which leads to a buildup of acetylcholine in the body and can result in hypersecretion, convulsions, respiratory problems, coma and finally death. Previously, enzymes that catalyze the hydrolysis of OPs from the species *Alteromonas* were known as organophosphorus acid anhydrolases (OPAAs). OPAAs were shown to be capable of cleaving the P–F, P–O, P–CN and P–S bonds of the nerve agents, sarin and soman (Cheng *et al.*, 1998). However, OPAA has been reclassified as a prolidase because it is able to efficiently hydrolyze specific X-Pro dipeptides, which is characteristic of prolidases. OP compounds appear to mimic X-Pro substrates in shape, size and surface charges (Cheng and DeFrank, 2000). Based on the activity that prolidases have against some OP agents, prolidases are being studied for use as biodecontaminants for detoxification of OP nerve agents in the field.

Prolidase is also important to the food and dairy industry because it can be used in ripening processes to reduce bitterness of cheese. The reduction in bitterness is due to the release of proline when prolidase is added in the cheese-ripening process (Bockelmann, 1995). It can also be a

critical enzyme for degrading proline-containing peptides generated in fermentation processes, which is important for creating desired flavor and texture attributes for fermented foods (Sullivan and Jago, 1972; Yang and Tanaka, 2008).

In addition, prolidase has been linked to human health disorders such as the syndrome PD. Prolidase deficiencies in humans have been linked to skin disorders and even mental retardation (Endo *et al.*, 1982; Royce and Steinmann, 2002). Prolidase is essential for collagen breakdown and the lack of this enzyme results in serious skin abnormalities. While an increase in prolidase activity and a decrease in collagen in breast cancer tissue may cause increased cancer risk (Cechowska-Pasko *et al.*, 2006), the use of prolidase as a potential biomarker for melanoma is currently being considered, as is its use as a potential therapeutic (Lupi *et al.*, 2006; Mittal *et al.*, 2005, 2007).

Although there have been a number of important biotechnological applications that prolidases have been identified as being potentially suitable for, there are currently limitations preventing the wide-spread use of prolidases in all of these applications. However, by using appropriate bioengineering techniques, candidate prolidases can be tailored to each specific application. This review will provide an update on experimentally defined properties of a number of native and recombinant prolidases, as well a discussion of current and future applications of prolidase enzymes.

II. PROLIDASE

A. Mechanism of substrate specificity and catalysis

Prolidases belong to a group of enzymes called metallopeptidases, enzymes that require a metal for activity. A review by Lowther and Matthews (2002), which compared metallopeptidases functionally and structurally, classified prolidase into a subclass of metallopeptidases that contain a dinuclear active-site metal cluster. Other members of this subclass that have been studied with solved structures include *Escherichia coli* methionine aminopeptidase (MetAP) (Roderick and Matthews, 1993), *E. coli* proline aminopeptidase (APPro) (Wilce *et al.*, 1998), bovine lens leucine aminopeptidase (*b*LeuAP) (Strater and Lipscomb, 1995), *Aeromonas proteolytica* aminopeptidase (*Ap*AP) (Chevrier *et al.*, 1994), *Streptomyces griseus* aminopeptidase (*Sg*AP) (Gilboa *et al.*, 2001), human methionine aminopeptidase-2 (*Hs*MetAP) (Liu *et al.*, 1998), *Pyrococcus furiosus* methionine aminopeptidase-1 (*Pf*MetAP) (Tahirov *et al.*, 1998) and carboxypeptidase G_2 from *Pseudomonas* sp. strain RS-16 (Rowsell *et al.*, 1997). These enzymes share a dinuclear metal center bridged by a water molecule or

hydroxide ion. The metal cluster is essential for the activation of catalysis. It functions to activate a nucleophile for the reaction, as well as participating in substrate binding and stabilizing the transition state (Maher *et al.*, 2004). Some enzymes require two metals in the active site to activate catalysis and others only need one (Maher *et al.*, 2004).

Based upon structural homologies of these enzymes, prolidases can be further categorized into a smaller class of metalloenzymes known as the ''pita-bread enzymes.'' Other enzymes within this class include methionine aminopeptidase (MetAP) (Roderick and Matthews, 1993), aminopeptidase P (APP) (Taylor, 1993) and creatinase (Coll *et al.*, 1990), each of which have slightly different substrate specificity (Table 3.1), but the same conserved metal-binding pocket suggesting they might have a conserved catalytic mechanism (Lowther and Matthews, 2002). These enzymes all contain a characteristic pita-bread fold that encompasses a highly conserved metal center and substrate-binding pocket that is located in the enzyme's C-terminal domain. The substrate specificity of individual prolidases is dependent on the nature of the metal occupying the metal centers (Table 3.1).

The first pita-bread enzyme structures solved were *E. coli*, *Hs*MetAP, *Pf*MetAP and *E. coli* APP (Lowther and Matthews, 2002). These structures confirmed the pita-bread fold and the conserved metal center with metal-binding residues (Aspartate-97, Aspartate-108, Histidine-172, Glutamate-204 and Glutamate-235) (*E. coli* MetAP numbering) (Bazan *et al.*, 1994; Chang *et al.*, 1992; Tsunasawa *et al.*, 1997). *P. furiosus* prolidase (*Pf*prol) has a similar metal-binding center to MetAP and APP. *Pf*prol also has similar N-terminal domains to those seen for APPro and creatinase, whereas *E. coli* MetAP lacks this N-terminal domain. MetAP is active as a monomer, while *Pf*prol and creatinase are dimers and APPro functions as a tetramer.

TABLE 3.1 Pita-bread enzymes and their substrates

Enzyme	Substrate	Reference
Methionine aminopeptidase	H-Met*Xaa-Yaa	Roderick and Matthews (1993)
APP	H-Xaa*Pro-Yaa	Taylor (1993)
Prolidase	H-Xaa*Pro-OH	Yaron and Naider (1993)
Creatinase	H_2N–C(=NH)*N(CH_3)–CH_2–COOH	Coll *et al.* (1990)

* Indicates where the hydrolytic cleavage occurs.

MetAP is specific for dipeptides with N-terminal methionine in the P_1 position and a small uncharged residue such as Gly, Ala, Ser, Thr, Pro, Val, or Cys in the P_1' position (Graham *et al.*, 2006; Hirel *et al.*, 1989). Prolidases are specific for dipeptides with proline in the *trans* configuration in the P_1' position and nonpolar residues in the P_1 position (Ghosh *et al.*, 1998; Grunden *et al.*, 2001; King *et al.*, 1986; Lin and Brandts, 1979), whereas APPro has affinity for substrates with a hydrophobic or basic residue in the P_1 position and a *trans* Pro at P_1' (Lowther and Matthews, 2002). The reaction centers of APP and prolidase require the occupancy of two divalent ions such as Co^{2+} and Mn^{2+} in order to catalyze their reactions. Although two cations must be bound in the metal sites, the relative binding affinity of the metals differs, with there being one tightly bound metal atom and one more loosely bound (Lowther and Matthews, 2002).

E. coli MetAP has been shown to be maximally active when bound with two atoms of Co^{2+} per monomer under aerobic assay conditions or when bound with one Fe^{2+} atom per monomer under anaerobic assay conditions, suggesting that it could have a mononuclear iron center *in vivo* (D'Souza *et al.*, 2000). It is not currently well understood why these enzymes exhibit different reaction efficiencies depending on the cations bound in their metal centers. APP and prolidase demonstrate the highest activities when bound with Co^{2+} and Mn^{2+}, whereas MetAP's highest activities are observed when it is loaded with Fe^{2+} (Lowther and Matthews, 2002). Although initially *E. coli* and *P. furiosus* MetAPs were described as requiring the occupancy of two metals for activity, more recent studies have indicated that they actually function more efficiently as Fe-containing monometallic hydrolases under anaerobic assay conditions (Copik *et al.*, 2005; Cosper *et al.*, 2001; D'Souza and Holz, 1999). A study by Du *et al.* (2005) demonstrated that *P. furiosus* prolidase also showed the highest activity when assayed anaerobically with Fe^{2+} and the second highest activity when Co^{2+} was bound to the enzyme under aerobic assay conditions. This suggests that both *Pf*prol and *E. coli* and *P. furiosus* MetAPs could preferentially use an iron mononuclear metal center *in vivo* under anaerobic conditions but switch to the use of a dimetal Co^{2+} reaction center under iron limitation or aerobic/oxidizing conditions.

B. Proposed reaction mechanism

From comparisons of inhibited and native forms of *E. coli* MetAP, kinetic analyses of MetAP mutants and spectral analyses of MetAP metal centers in response to substrate binding, a reaction mechanism for the cleavage of N-terminal methionine residues by *E. coli* MetAP has been proposed (Lowther and Matthews, 2000, 2002; Lowther *et al.*, 1999). Given the structural correspondence that exists between the *E. coli* MetAP and

*Pf*prol metal centers as well as the similarity in MetAP and prolidase activities, with both involving hydrolysis of peptide bonds, it is reasonable to presume that an analogous reaction mechanism can be ascribed to prolidase. Thus, from analogy, it is predicted that cleavage of the X-Pro peptide bond occurs as follows: (1) substrate binds to the active site and is thought to activate the nucleophile (O_N) and facilitate proton transfer to glutamate residue 313 (E-313); (2) the carboxy anion of the resultant tetrahedral intermediate, originating from the oxygen of the scissile bond (O_C) is stabilized by the expanded coordination sphere of Co1 and interactions with histidine-192 (H-192) and histidine-291 (H-291); (3) resolution of the intermediate to products returns the coordination of Co1 to five, while the metal bridging and H-291 interactions are maintained; and (4) the active site is fully regenerated upon release of the proline and deprotonation of solvent molecules. Note that the amino acid residues refer to *P. furiosus* prolidase numbering (Fig. 3.3).

C. Structure–function information provided by the solved *Pyrococcus furiosus* prolidase structure

P. furiosus prolidase is currently the only solved crystal structure for this enzyme. *P. furiosus* is a hyperthermophilic organism which grows optimally at 100 °C. While there are many biochemically characterized mesophilic prolidases, at this time, there are no solved structures. From the crystal structure of *Pf*prol, it is clear that the enzyme is composed of two domains. It has an N-terminal domain (domain I, residues 1–112), an α-helical linker region (113–123) and a C-terminal domain (domain II, residues 124–348), which contains the traditional ''pita-bread'' type fold (Maher *et al.*, 2004). The N-terminal domain contains a six-stranded mixed β-sheet flanked by five α-helices. The C-terminal region contains the active site where metal binding occurs and is made up of a six-stranded β-sheet with four α-helices on the outer surface (Fig. 3.4). The strongly curved conformation in which the β-sheet exists in *Pf*prol gives rise to the enzyme's inclusion in the ''pita-bread'' family of proteins. There are a number of hydrogen bonds between the two domains, possibly enhancing stability. Locations where hydrogen bonds are present include the end of small helix domain I (residues 24–32) and β-turn domain II (residues 284–294) (Maher *et al.*, 2004). The proposed determinants for substrate specificity are thought to be localized to a region containing the amino acid residues 113–123 in *Pf*prol. These residues link the two domains and the angle is more acute with respect to the C-terminal domain when overlaid with the APPro domain. The active site of *Pf*prol is further crowded by the N-terminal domain of the subunits (residues 36B–39B). This suggests that the substrates coming in to the active site will be discriminated primarily based on size, where greater specificity will

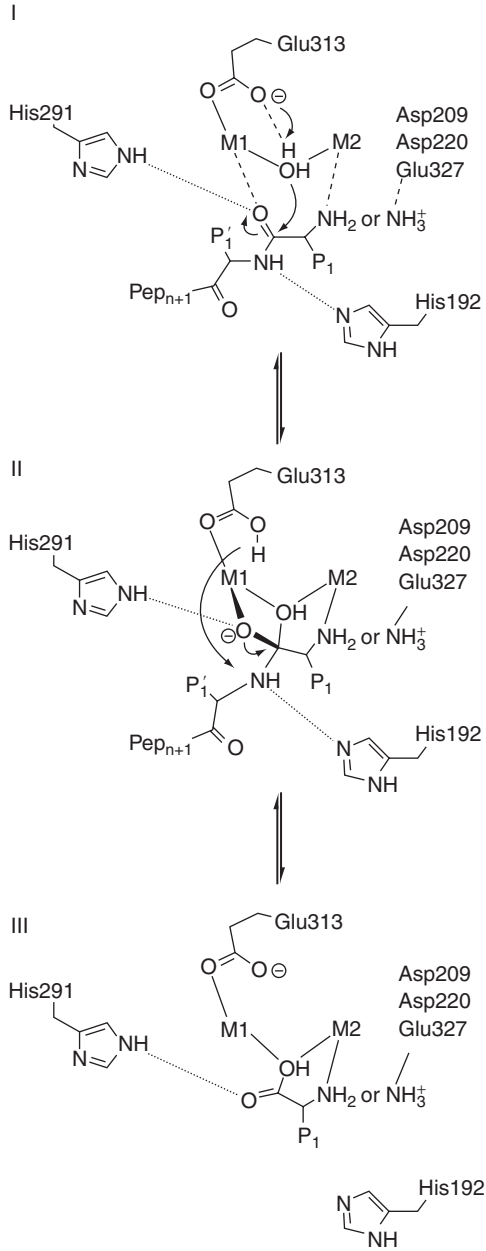

FIGURE 3.3 Proposed reaction mechanism for prolidase based on *P. furiosus* prolidase numbering, modified from Lowther and Matthews, 2002.

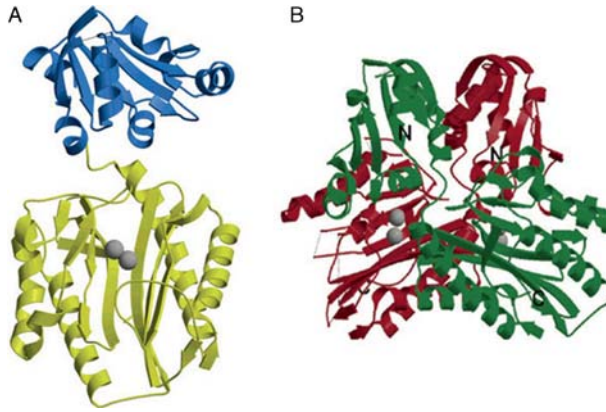

FIGURE 3.4 The structure of a monomer and dimer of *Pf*prol. (A) Ribbon drawing of *Pf*prol monomer showing the N-terminal domain in blue and the C-terminal domain in yellow. The gray spheres indicate the location of the metal center or Zn atoms in the C-terminal region. (B) Ribbon drawing of the dimer of *Pf*prol, the two subunits A and B are in green and red, respectively. From Maher *et al.* (2004).

occur for smaller proline-containing peptides such as dipeptides versus polypeptides (Maher *et al.*, 2004).

The active site of *Pf*prol is located in an oval depression formed on the inner surface of the curved β-sheet of the C-terminal domain of the protein. The primary feature of the active site is the presence of a dinuclear cobalt cluster and as predicted by analogy to MetAP and aminopeptidase metal centers, the amino acids Asp-209, Asp-220, His-284, Glu-313 and Glu-327 were shown to coordinate the metal ions (Maher *et al.*, 2004; Willingham *et al.*, 2001). Specifically, it was shown that His-284 and Glu-313 are monodentate ligands binding solely to the first Co center (Co1), Asp-209 is a monodentate ligand binding solely to the second Co (Co2) and Asp-220 and Glu-327 serve as bidentate ligands of both metal sites. The structure analysis also indicated that a water molecule identified as W176 functions as a bridging molecule for the metal center (Fig. 3.5).

When isolated as either a native or recombinant form, *Pf*prol contains one Co(II) atom per monomer (Ghosh *et al.*, 1998). During the crystallization process, Zn(II) replaced Co(II) in the prolidase active site as a consequence of the crystallization method used (Maher *et al.*, 2004). However, Zn could be replaced with Co in the crystallized prolidase and it restored enzyme activity.

Although the X-ray crystal structure analysis of *Pf*prol was able to definitely establish the structure of the enzyme's metal center-containing active site, the question as to which of the metal sites (Co1 or Co2) is the

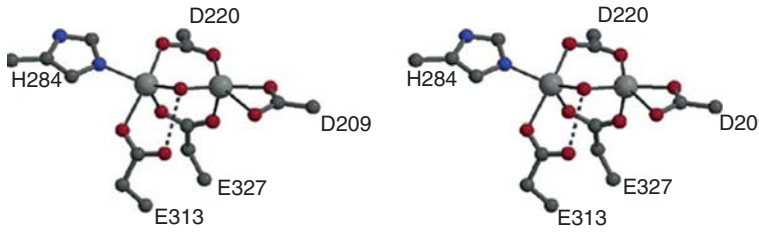

FIGURE 3.5 Stereoview of the active site of *Pf*prol. Gray spheres indicate zinc atoms where residues D209, D220, H284, E313 and E327 are interacting. The dotted line shows the hydrogen bond between E313 and the bridging hydroxide ion. From Maher *et al.* (2004).

tightly bound and which is the loosely bound [K_d (dissociation constant) 0.24 mM] site remained unresolved. Therefore, further studies were undertaken which analyzed key site-directed *Pf*prol mutants to differentiate the binding affinities between the two Co atoms (Du *et al.*, 2005). To look at different affinities of the metal-binding sites, targeted mutations were made in the following locations: Asp209Ala, His284Ala, His284Leu and Glu327Leu within *Pf*prol, where the three letter amino acid code preceding the indicated amino acid position indicates the original amino acid and the three letter amino acid code following the number indicates the mutated residue (Du *et al.*, 2005). Results showed that Co1 is the tightly bound metal and Co2 is the loosely bound metal (K_d 0.24 mM) (Du *et al.*, 2005). Similar findings were observed for *E. coli* MetAP where the Co1 site is the tight binding site and the K_d values of Co1 and Co2 were estimated to be 0.3 $\pm$ 0.2 and 2.5 $\pm$ 0.5 mM, respectively (D'Souza and Holz, 1999). For *P. furiosus* MetAP, the reported K_d values are 0.05 $\pm$ 0.015 and 0.35 $\pm$ 0.02 mM (Meng *et al.*, 2002).

D. Molecular and catalytic properties of recombinant prolidases

The first prolidase both structurally and biochemically characterized was isolated from the hyperthermophilic archaeon *P. furiosus* (Ghosh *et al.*, 1998; Grunden *et al.*, 2001; Maher *et al.*, 2004). It is a homodimer, with a molecular mass of 39.4 kDa per subunit and as purified, it was determined to contain one bound Co^{2+} per subunit (Ghosh *et al.*, 1998). With the addition of cobalt, it shows maximum activity at 100 °C and pH 7.0 with Met-Pro as the substrate (Ghosh *et al.*, 1998; Grunden *et al.*, 2001). *Pf*prol has a narrow substrate specificity, only hydrolyzing dipeptides with a proline in the C-terminus and nonpolar amino acids (Leu, Met, Val,

Phe, or Ala) in the N-terminal position (Ghosh *et al.*, 1998). The dipeptidase is maximally active with the addition of the divalent cations Co^{2+} and Mn^{2+} and it cannot be substituted with other divalent cations (Mg^{2+}, Ca^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}) under aerobic conditions (Ghosh *et al.*, 1998).

*Pf*prol is the most thermostable and thermoactive of all the prolidases isolated to date (Adams and Kletzin, 1996). Other isolated mesophilic prolidases have shown activity up to 55 °C (Adams and Kletzin, 1996; Browne and O'Cuinn, 1983; Endo *et al.*, 1989; Fernandez-Espla *et al.*, 1997; Suga *et al.*, 1995). Among the prolidases that have been characterized, there are variations in subunit numbers and metal requirements. For instance, *Pf*prol is active as a dimer as are the human and *Xanthomonas maltophilia* prolidases (Endo *et al.*, 1989; Suga *et al.*, 1995). Yet *Lactococcus lactis* and *Lactococcus casei* prolidases function as monomers (Fernandez-Espla *et al.*, 1997). Although many prolidases function most efficiently when bound with cobalt, *L. casei*, *X. maltophilia* and human prolidases are most active with manganese, while *Lactobacillus delbrueckii* prolidase requires zinc (Morel *et al.*, 1999; Stucky *et al.*, 1995).

Besides the differences in metal requirements, prolidases also demonstrate differences in substrate specificities. *Pf*prol has the greatest affinity for dipeptides with proline in the C-terminus and cannot cleave dipeptides with proline in the N-terminus. Likewise, *L. delbrueckii pepQ* prolidase can also only cleave X-Pro dipeptides (Stucky *et al.*, 1995). However, *L. lactis* prolidase can cleave dipeptides with proline in either the C- or N-terminal positions (Cheng *et al.*, 1996). On the other hand, *L. casei* and guinea pig brain prolidase can cleave substrates without a prolyl residue.

OPAA-2, previously listed as an organophosphorus acid anhydrolase, from the species *Alteromonas* shows 44% similarity to *P. furiosus* prolidase (Ghosh *et al.*, 1998). OPAAs can hydrolyze OP nerve agents, such as sarin and soman. OPAA has been reclassified as a prolidase because it can also efficiently hydrolyze X-Pro dipeptides (Cheng *et al.*, 1996, 1997, 1999). Like *Lactococcus* prolidases, OPAA is a monomer and requires Mn^{2+} for activity. OPAA, like other prolidases, has a conserved binuclear metal center and shows activity with P–F, P–C and P–O bonds (Cheng *et al.*, 1993). It can also preferentially cleave the dipeptides Leu-Pro and Ala-Pro and is specific for dipeptides with proline in the C-terminal position (Cheng *et al.*, 1996). No activity was observed when OPAA was assayed with the substrates Pro-Leu and Pro-Gly (Cheng *et al.*, 1997). Figure 3.6 shows an alignment of previously characterized prolidases from *Alteromonas*, *L. delbrueckii*, Human and *E. coli*, newly characterized prolidase from *Pyrococcus horikoshii* and *E. coli* MetAP. Percent similarities compared to *Pf*prol for all listed prolidases are included.

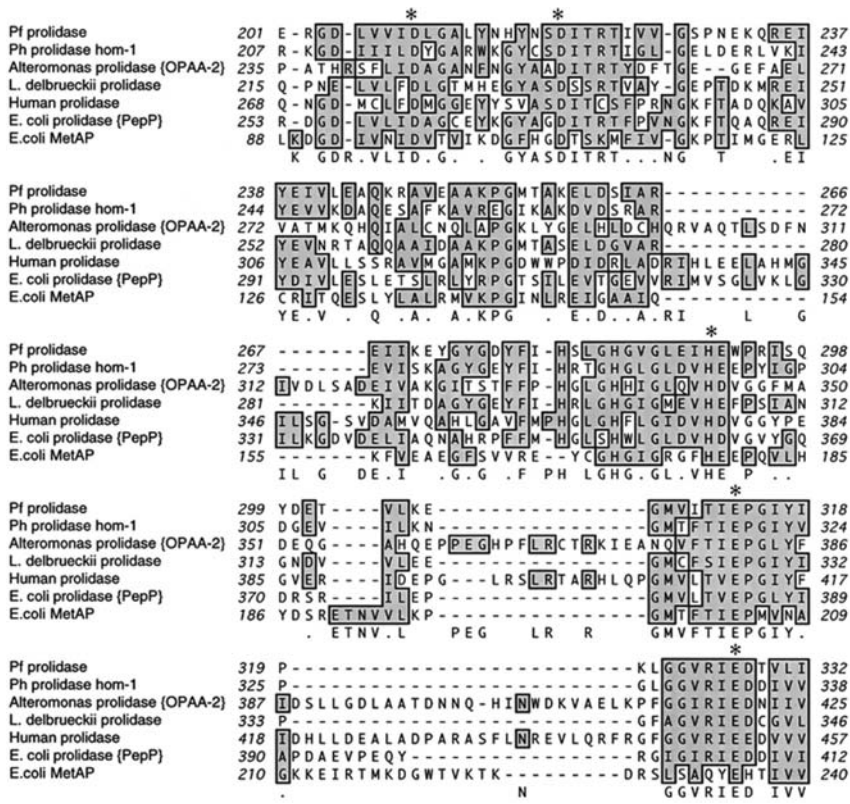

FIGURE 3.6 Clustal alignment generated using MacVector software showing the homology that exists between *P. furiosus* prolidase and other prolidases including *P. horikoshii* homolog 1 (57%), *Alteromonas* (OPAA-2) (44%), *L. delbrueckii* (59%), Human (49%), *E. coli* PepP (52%) and *E. coli* MetAP (32%). The values in parentheses indicate the percent similarity between *Pf*prol and the respective prolidases or MetAP. Asterisks mark the conserved residues in the prolidase and MetAP identifying the dinuclear cobalt metal-binding site.

III. APPLICATIONS OF PROLIDASES

A. Detoxification of OP compounds

OP nerve agents act by covalently binding and inhibiting acetylcholinesterase (AChE), preventing the breakdown of the neurotransmitter acetylcholine (Ach) to choline. This leads to a buildup of acetylcholine in the body and as a result causes continuous nerve impulses and muscle contractions (Grimsley *et al.*, 2000). An OP exposed victim can suffer from convulsions, brain seizures and eventually die from neuronal death. The U.S. Army has a stockpile of 32,000 tons of chemical agents consisting of

the nerve agents GB (sarin or *O*-isopropyl methylphosphonofluoridate), VX and blister agent HD (sulfur mustard) (DeFrank *et al.*, 2000). Under the International Chemical Weapons Treaty, the U.S. was slated to destroy the stockpile by 2007. OP nerve agents were initially employed during World War II and are still being used by various organizations. Sarin gas was used by the Aum Shinrikyo cult in the Tokyo subway attack of 1995. The U.S. soldiers deployed in Iraq during the first Iraq conflict were exposed to nerve agents. Such exposure incidents underscore the need for effective nerve agent detoxification methods to protect civilians and soldiers. Other OP compounds being used in the U.S. include OP pesticides of which over 40 million kilograms are land applied and 20 million kilograms are produced for export each year (Chen *et al.*, 2000). There is concern that these pesticides could leak into ground water and pollute surrounding environments.

Previous forms of disposal have consisted of chemical treatment, open-pit burning, evaporative burial and deep ocean dumping and presently, the EPA has approved incineration (Chen *et al.*, 2000). Incineration is an expensive process and it has raised environmental concerns. As a result, other environment-friendly technologies are now being considered to degrade the stockpiles, including enzyme-based decontamination systems (Cheng and DeFrank, 2000).

In 1946, Mazur described the first work investigating hydrolysis of DFP (diisopropylfluorophosphate), an analog of G-type nerve agents, by enzymes found in rabbit and human tissue extracts (Mazur, 1946). Most of these enzymes were first labeled DFPases and sarinases specific to the nerve agents they degraded. In 1992, they were listed in the category of phosphoric triester hydrolases, named by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology. These enzymes were further separated into two subgroups based on their substrate specificities. The first subgroup is the organophosphate hydrolases (OPHs) (also referred to as paraoxonase and phosphotriesterase), which prefer the substrates paraoxon and P-esters, which have a P–O bond. The second subgroup is diisopropylfluorophosphatases (also including OPAA), which are most active against OP compounds with P–F or P–CN bonds (Cheng and DeFrank, 2000).

OPH, encoded by the *opd* (organophosphate degrading) gene, was isolated first from *Pseudomonas diminuta* MG and *Flavobacterium* (Mulbry *et al.*, 1986). It has been shown to degrade organophosphate pesticides with P–O bonds and is the only enzyme known to cleave the P–S bond (Cheng and DeFrank, 2000; Lai *et al.*, 1995). It can also cleave the P–F and P–CN bonds and the hydrolysis rates are 40–2450 times faster than chemical hydrolysis at temperatures up to 50 °C (Munnecke, 1979). OPH is able to degrade a broad list of substrates including organophosphate pesticides (paraoxon and coumaphos) and OP nerve agents (DFP and sarin)

(Cheng and DeFrank, 2000; Chen *et al.*, 2000; Dumas *et al.*, 1989, 1990). Of its substrates, OPH can hydrolyze paraoxon the fastest with a rate of 10^4 s^{-1} (Grimsley *et al.*, 1997). OPH mutants have been constructed in order to increase the substrate specificity with nerve agents. The following mutations were made in the metal-binding center area of OPH: His257Leu, His257Val and His254Arg, resulting in even higher activity with soman and VX (Lai *et al.*, 1996; Vanhooke *et al.*, 1996).

OPH enzymes have been used in various applications to cleanup and/or detect OP nerve agents. For large scale cleanup of OP compounds, the OPH enzyme has been incorporated into fire-fighting foams (LeJeune *et al.*, 1998). Large scale use of OPH for decontamination of nerve agents has been limited by high cost and poor enzyme stability (Chen *et al.*, 2000). OPH has also been used in detection methods. Biosensors with immobilized recombinant *E. coli* cells expressing OPH are being used for recognizing OP nerve agents (Mulchandani *et al.*, 1998a,b; Rainina *et al.*, 1996). New technology for biosensing and detoxification of OPs is focusing on immobilized cells expressing OPH on the cell surface (Mulchandani *et al.*, 1999). To date, studies involving immobilized *E. coli* (Richins *et al.*, 1997), *Moraxella* sp. (Shimazu *et al.*, 2001), *Saccharomyces cerevisiae* (Takayama *et al.*, 2006) and Cyanobacteria (Chungjatupornchai and Fa-Aroonsawat, 2008) expressing OPH enzymes have been conducted. Both native and recombinant OPH can also be immobilized onto surfaces such as nylon (Caldwell and Raushel, 1991a), porous glass and silica beads (Caldwell and Raushel, 1991b) as well as added to enzyme reactors, but this method still requires pure OPH enzyme which is very costly (Mulchandani *et al.*, 1998b, 1999). In recombinant *E. coli* with active OPH on the cell surface, the enzyme was stable and remained 100% active for more than a month (Chen and Mulchandani, 1998). Immobilized cells expressing OPH were used in batch reactors to test against many OP chemicals. It showed 100% hydrolysis of OP pesticides paraoxon and diazinon in less than 3.5 h (Cheng and DeFrank, 2000; Chen *et al.*, 2000). OPH is being used in medical applications as well. It can be used as an antidote or a therapeutic in preventing OP poisoning (Grimsley *et al.*, 2000). Mice treated with OPH intravenously prevented cholinesterase inhibition when exposed to DFP, sarin, or soman (Tuovinen *et al.*, 1994, 1996). When mice were pretreated with OPH, they were able to resist even higher doses of nerve agents.

Structural data show that organophosphorus hydrolase is a homodimer (35 kDa per monomer) with active sites in the C-terminus (Benning *et al.*, 1994, 1995; Vanhooke *et al.*, 1996). It is a characterized metalloenzyme that contains one or two metal ions needed for catalysis (either zinc or cobalt) (Dumas *et al.*, 1989; Omburo *et al.*, 1993). Zinc is the native metal present in purified OPHs and provides full activity. However, activity can also be supported by Co^{2+}, Cd^{2+}, Ni^{2+} and Mn^{2+}

when these metals are substituted into the enzyme in place of Zn (Omburo *et al.*, 1992). Unlike prolidases, OPH does not hydrolyze dipeptides with proline at the C-terminus (Cheng *et al.*, 1997).

OPAAs have been isolated from squid (Hoskin and Roush, 1982), protozoa (Landis *et al.*, 1987), clams (Anderson *et al.*, 1988), mammals (Little *et al.*, 1989) and soil bacteria (Attaway *et al.*, 1987). OPAAs have been shown to hydrolyze a variety of OP agents including, soman (GD; *O*-pinacolylmethylphosphonofluoridate), sarin (GB; *O*-isopropylmethylphosphonofluoridate), GF (*O*-cyclohexylmethylphosphonofluoridate) and cyanide containing tabun (GA; ethyl-*N*,*N*-dimethylphosphoramidocyanidate) (Cheng *et al.*, 1999). OPAAs isolated from *Alteromonas* species: *Alteromonas haloplanktis*, *Altermonas* sp. JD6.5 and *Alteromonas undina* have been the most extensively studied (Cheng and DeFrank, 2000; Cheng *et al.*, 1993, 1996, 1997, 1998, 1999; DeFrank and Cheng, 1991). The OPAAs from these species are structurally and functionally similar to each other. They share molecular weights between 50 and 60 kDa, an optimum pH between 7.5 and 8.5, a temperature optimum between 40 and 55 °C, a requirement for the metal Mn^{2+} and they are inhibited by the DFP analog, mipafox (Cheng *et al.*, 1997). These enzymes are highly active against the OP nerve agents, soman and sarin. OPAAs show higher soman activities and OPHs show higher activity against the OP pesticide paraoxon (Cheng *et al.*, 1993; DeFrank *et al.*, 1993; Dumas *et al.*, 1990). Comparisons of their activities with nerve agents DFP, GB, GD and GF can be seen in Table 3.2. OPAAs from *Alteromonas* sp. JD6.5 and *A. undina* show the highest activity against GD and lowest against GB, while *A. haloplanktis* OPAA showed lower activity against DFP, GB, GD and GF (Cheng *et al.*, 1997).

OPAA from *A. haloplanktis* and OPAA-2 from *Alteromonas* sp. JD6.5 are very similar with 81% amino acid sequence identity and 91% similarity (Cheng *et al.*, 1997). Both OPH and OPAA enzymes can hydrolyze many of the same substrates; however, there is no significant sequence homology found between any of the known OPH and OPAA enzymes (Cheng and DeFrank, 2000; Cheng *et al.*, 1996), suggesting they are not the same enzyme.

The amino acid sequence from *A. haloplanktis* OPAA and *Alteromonas* sp. JD6.5 OPAA-2 showed high sequence similarity, 51% and 49%, to *E. coli* (X-Pro) dipeptidase or prolidase (Cheng and DeFrank, 2000). *Alteromonas* OPAA has now been classified as a prolidase due to similarities in amino acid sequence and biochemical properties (Cheng and DeFrank, 2000; Cheng *et al.*, 1997). *Alteromonas* OPAAs or prolidases are able to hydrolyze OP nerve agents and dipeptides with proline in the C-terminus, but not dipeptides with proline in the N-terminus (Cheng *et al.*, 1997). Like prolidase, OPAA from *Alteromonas* sp. JD6.5 has the conserved metal cluster center featuring amino acid residues: Asp244,

TABLE 3.2 Specific activity of purified OPAAs/prolidases when DFP, other G-type nerve agents and the proline dipeptides, Leu-Pro and Ala-Pro, are used as substrates

Substrate	*A. undina*[a]	*A. haloplanktis*[a]	*Alteromonas* sp. JD6.5[a]	*P. furiosus*	Human
DFP	1403 ± 49	691 ± 11	1820 ± 74	30[b]	10–75[b]
GB (sarin)	426 ± 36	308 ± 24	611 ± 39	ND	ND
GD (soman)	2826 ± 127	1667 ± 74	3145 ± 95	ND	ND
GF (cyclosarin)	1775 ± 115	323 ± 22	1654 ± 125	ND	ND
Leu-Pro	810	988	636	1066.5[c]	0.28 ±0.158[d]
Ala-Pro	658	725	510	229.5[c]	33.16 ±1.798[d]

OPAA specific activity for nerve agent substrates was calculated based on one unit (U) of OPAA activity being defined as hydrolyzing the release of 1.0 μmol of F^- min^{-1}. For dipeptides, specific activity is calculated as μmol of amino acids released min^{-1} mg^{-1} protein (U mg^{-1}). Specific activities were reported in the following studies:

[a] Cheng *et al.* (1997).

[b] data provided from Dr. Joseph DeFrank of the U.S. Army Edgewood Research, Development and Engineering Center.

[c] Ghosh *et al.* (1998).

[d] Lupi *et al.* (2006).

Asp255, His336, Glu381 and Glu420 (Cheng and DeFrank, 2000). OPAAs from *A. undina*, *A. haloplanktis* and *Alteromonas* sp. JD6.5 can all use the dipeptide Leu-Pro as a substrate and activities of 810, 988 and 636 U mg^{-1}, respectively, have been reported for the cleavage of Leu-Pro by these OPAAs (Table 3.2; Fig. 3.7) (Cheng and DeFrank, 2000). While the substrate Leu-Pro and the G-type nerve agent soman may seem to be very different based on their chemical formulas, they are actually very similar in relation to their three-dimensional structure and electrostatic density maps (Cheng and DeFrank, 2000). The structural similarities in the proline dipeptide and OP substrates used by OPAAs and prolidases suggests that *Alteromonas* OPAAs and prolidases may have evolved from the same ancestral gene (Cheng *et al.*, 1997).

In order to effectively incorporate prolidases into an acceptable decontamination formulation, the enzyme has to be stable over time and not inhibited by the water-based system employed. Table 3.3 shows the current systems including fire-fighting foams or sprays, degreasers, laundry detergent and aircraft deicing solutions (Cheng and DeFrank, 2000). Foams appear to be the best delivery option because they have surface-active agents that help with the solubilization of the substrate and they are able to adhere to vertical surfaces, enabling the enzyme to have significant contact time with substrates over a large surface area.

Currently, to detoxify nerve agent exposed environments, a decontamination solution known as DS2 is being used in conjunction with bleach (Cheng *et al.*, 1999). DS2 is environmentally harmful because it is

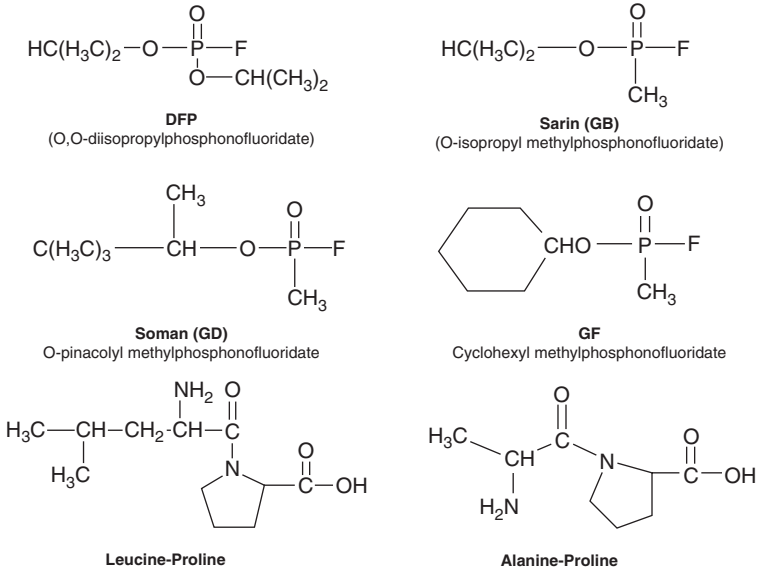

FIGURE 3.7 The chemical structure of G-type nerve agents and proline dipeptides, Leu-Pro and Ala-Pro. Specific activities of OPAA and other prolidases with these compounds as substrates are reported in Table 3.2.

corrosive and contributes additional hazardous waste to the environment. Since the use of current decontamination solution formulations is not a good long-term decontamination strategy, there is a perceived need to optimize an enzyme-based decontamination system. However, limitations of the enzymes that have thus far been examined for use in this process include poor activity at low pH and over a broad temperature range and instability of the enzymes in the presence of harsh solvents, metals, detergents and/or denaturants.

B. Uses in the food industry

During fermentation, food undergoes many chemical changes, which contribute to the taste and nutritional quality. The cheese-making and ripening process relies heavily on microbial metabolism. Cheese is made by the coagulation of milk using starter culture bacteria, usually lactic acid bacteria (LAB) and the enzyme rennet. LAB acidifies milk by converting lactose into lactic acid and the rennet coagulates the mixture. Ripening is driven by the microbial proteolysis process and results in casein protein being broken down into many different peptides and amino acids (Stucky *et al.*, 1995). Some amino acids are known to produce

TABLE 3.3 Effects of *Alteromonas* sp. JD6.5 enzyme in the presence or absence of various biodegradeable and water-soluble wetting agents, degreasers, or foams (amended from Cheng *et al.*, 1999)

Wetting agent, foam, lotion and source	Characteristics	Concentration used	Specific activity (U mg^{-1})
Control (reaction solution only)	–	–	1950
Cold fire (Firefreeze, Rockaway, New Jersey)	Fire-suppressing agent	10	2340
Odor seal (Firefreeze, Rockaway, New Jersey)	Odor removing agent	10	1980
Tide free (Procter and Gamble, Cincinnati, Ohio)	Laundry detergent	0.05	2220
Protectall (J. G. Worldwide Medical, Rockaway, New Jersey)	Skin-care lotion	100	1960
Sta-put (Wilbur Ellis, Fresno, California)	Deposition aid	0.1	1910
Silvex (Ansul, Marinette, Wisconsin)	Fire-fighting foam	1.2	320

a bitter taste. Hydrophobic peptides ranging from 2 to 23 residues play a significant role in the bitterness of cheddar cheese (Sullivan and Jago, 1972). Peptides isolated from casein hydrolysate and cheese result in high hydrophobicity and a high number of aromatic amino acids, causing bitterness (Agboola *et al.*, 2004). In the study by Agboola *et al.*, the role that a number of hydrophobic peptides play in determining the bitterness of ovine milk cheese was examined. To reduce the overall bitterness of the cheese, particular peptides need to be removed by proteolysis. Because of their unique structure, most of the remaining dipeptides are Xaa-Pro- and/or Pro-Xaa-type dipeptides. These proline-containing dipeptides have been shown to significantly contribute to bitterness, especially the Xaa-Pro class of dipeptides (Yang and Tanaka, 2008).

A study by Ishibashi *et al.*, examined how the proline structure contributes to the bitterness of cheese. Pure amino acid L-proline exhibited a sweet flavor, whereas proline-containing peptides were bitter (Ishibashi *et al.*, 1988). They suggested that the imino ring of proline creates a hydrophobic feature that plays a role in bitterness. Prolidases or

proline-specific dipeptidases play a critical role in cheese ripening. In cheese, the proteolytic process described earlier works to degrade casein, resulting in amino acids essential for growth and metabolism. This process is essential for ripening and formation of flavor as well as texture (El Soda, 1993). LAB are used in the fermentation of foods, especially dairy products and they contain many peptidases specific for proline including: proline iminopeptidase (PepI), prolinase (PepR), X-prolyl dipeptidyl aminopeptidase (PepX) and prolidase (PepQ) (Christensen *et al.*, 1999; Sousa *et al.*, 2001). By using prolidase to hydrolyze the bond of Xaa-Pro dipeptides, bitterness in cheese and other fermented foods can be reduced. In a study by Courtin *et al.*, a *L. lactis* proteolytic system was investigated for its ability to increase the ripening process of cheese. By adding excess proline-specific peptidases from lactobacilli, including prolidases, they were able to increase the total amount of free amino acids threefold, in turn speeding up cheese ripening (Courtin *et al.*, 2002). The role of proline-specific dipeptides in the flavor of cheese has been explored, but the role that the single amino acid proline plays is still unknown and requires further examination.

C. Impact on human health

1. Prolidase deficiency

Prolidase deficiency is a rare autosomal recessive disorder of the connective tissue that gives rise to skin lesions, mental retardation and recurrent respiratory infections (Kokturk *et al.*, 2002; Rao *et al.*, 1993; Royce and Steinmann, 2002). Most cases go misdiagnosed, but it is estimated that 1–2 cases per million births are diagnosed with PD (Lupi *et al.*, 2006; Royce and Steinmann, 2002). The human prolidase gene (Peptidase D, PEPD, AC008744) is located on chromosome 19p13.2 and is made up of 15 exons (Tanoue *et al.*, 1990) and contains a polypeptide spanning 493 amino acids (Endo *et al.*, 1989). Point mutations, exon splicing, deletions and duplications of key amino acids in the human prolidase gene are linked to this deficiency (Ledoux *et al.*, 1996; Lupi *et al.*, 2006). Key point mutations Arg184Gln (Arg122 in *Pf*prol), Asp276Asn (Asp209 in *Pf*prol), Gly278Asp (Gly211 in *Pf*prol) and Gly448Arg (Gly323 in *Pf*prol) have been found in patients with this disorder (Maher *et al.*, 2004). These mutations were carefully compared to the *Pf*prol enzyme to evaluate what impact they have on the enzyme's structure and function. The same point mutations in *Pf*prol result in disruption of function and structure of the enzyme (Maher *et al.*, 2004). Diagnosis of PD in the past has been difficult and has resulted in significant numbers of misdiagnosed cases (Lupi *et al.*, 2006; Viglio *et al.*, 2006). Presently, detection methods include screening for prolidase activity in erythrocytes, leukocytes and skin fibroblast cultures and also screening urine for excess X-Pro imidodipeptides (Viglio *et al.*, 2006).

Therapeutic approaches have been explored for PD in the past. Topical treatments with glycine and proline have been used with minimal effectiveness on leg ulcers (Arata *et al.*, 1986; Jemec and Moe, 1996). Oral administration of L-proline was also tried; however, this treatment failed to prevent the ulcerations (Isemura *et al.*, 1979; Ogata *et al.*, 1981; Sheffield *et al.*, 1977). Other methods in preventing PD have included blood transfusions and aphaeresis (Endo *et al.*, 1982; Lupi *et al.*, 2002), the use of corticosteroids (Shrinath *et al.*, 1997; Yasuda *et al.*, 1999), application of growth hormone (Monafo *et al.*, 2000) and an antibiotic topical treatment (Ogata *et al.*, 1981).

Enzyme replacement therapy is currently under investigation with delivery of active prolidase into fibroblasts of PD patients being specifically examined. In a study by Ikeda *et al.* (1997), an adenovirus-mediated gene transfer with human prolidase cDNA was used to provide enzyme replacement therapy in the fibroblasts. This gene transfer resulted in 7.5 times the normal activity of prolidase in the fibroblasts. The main consideration in enzyme replacement therapy is in the type of enzyme delivery system and the stability of the enzyme once it is delivered to the target location. Prolidase enzyme delivery using micro- and nanoparticulate systems has been done and the delivery efficiency of the enzyme into fibroblasts was poor (Colonna *et al.*, 2007; Genta *et al.*, 2001; Lupi *et al.*, 2004). More recently, enzyme replacement studies have been done using liposomes to deliver native prolidase trancellularly into fibroblasts in PD patients (Perugini *et al.*, 2005). Although the delivery of the enzyme to fibroblasts was efficient, the enzyme was only active for 6 days (Perugini *et al.*, 2005). A study by Colonna *et al*., addressed the issue of enzyme stability and efficient enzyme delivery, using PEGylated prolidase loaded in chitosan nanoparticles to restore the normal prolidase activity in PD patient cells (Colonna *et al.*, 2008).

Generating enough recombinant human prolidase for enzyme replacement therapy and structural studies has been a significant challenge. The human prolidase enzyme has been expressed and purified in both eukaryotic and prokaryotic systems, including *S. cerevisiae*, *Pichia pastoris*, chinese hamster ovary (CHO) cells and *E. coli* (Lupi *et al.*, 2006, 2008; Wang *et al.*, 2005, 2006). The recombinant prolidase purified from *E. coli* showed the most promise due to its low production cost, high yield and structural and catalytic properties (Lupi *et al.*, 2006). In enzyme replacement therapy, the stability of the enzyme is critical. Based on current studies, the best candidate recombinant human prolidase generated using an *E. coli* expression system, showed stability and activity at 37 °C for up to 6 days (Lupi *et al.*, 2006). While these findings represent progress in enzyme replacement therapy, further advances in enzyme stability need to occur for human prolidase replacement therapy to be a viable therapeutic option in the treatment of PD.

2. Collagen catabolism

Primarily produced in fibroblasts, collagen is the main fibrous structural protein that makes up connective tissue in vertebrates (Bornstein and Sage, 1989). Up to 25% of total body protein is collagen (Di Lullo *et al.*, 2002). Extracellular matrix (ECM) is made up of 80% collagen and connective tissue and the organic part of bone are made of 90–95% collagen (Dixit *et al.*, 1977). Hydroxyproline and proline make up over 25% of collagen amino acids (Dixit *et al.*, 1977). Collagen has also been recognized as a ligand for integrin (β_1-integrin) cell surface receptors, which are important for regulating ion transport, lipid metabolism, kinase activation and gene expression (Akiyama *et al.*, 1990; Bissell *et al.*, 1982; Carey, 1991; Donjacour and Cunha, 1991; Surazynski *et al.*, 2008). When collagen structure is affected, it can have an impact on cell signaling, metabolism and function, which can lead to tumorigenicity and invasiveness (Surazynski *et al.*, 2008). The secretion of matrix metalloproteinases (MMPs), which break down ECM or collagen, is also an important event in the progression and metastasis of cancer (Cechowska-Pasko *et al.*, 2006).

Intracellular prolidase plays a major role, which might be a step-limiting factor, in the final stage of degradation of collagen into free amino acids (Jackson *et al.*, 1975). Extracellular collagenases break down the initial collagen, which is then followed by prolidase facilitated degradation. Prolidase is able to hydrolyze the most abundant substrate Gly-Pro from degraded procollagen and collagen (Surazynski *et al.*, 2008). It is suggested that prolidase plays a role in metabolism of collagen and recycling of proline for collagen resynthesis (Jackson *et al.*, 1975; Palka, 1996; Yaron and Naider, 1993). The mechanism to explain this is currently being investigated but at this time is unclear.

The absence of prolidase, or PD, has been associated with slow wound healing, due to an abnormal nitric oxide (NO) signaling pathway. NO is associated with collagen metabolism and matrix degradation because it shows high expression when tissues need repair (Lupi *et al.*, 2008; Surazynski *et al.*, 2005). Overexpression of prolidase has been linked to increased levels of nuclear hypoxia inducible factor (HIF-1α), which plays an important role in stress responsive gene expression (Jaakkola *et al.*, 2001; Semenza, 2001; Surazynski *et al.*, 2008). Prolidase activity has also been implicated as a factor in other diseases such as osteogenesis imperfecta (Galicka *et al.*, 2001, 2005), pancreatic diseases (Palka *et al.*, 2002), lung carcinoma planoepitheliale (Karna *et al.*, 2000) and metastasis of breast cancer MCF-7 cells (Miltyk *et al.*, 1999; Palka and Phang, 1998).

In a study by Cechowski-Pasko *et al.* (2006), it was observed that increased prolidase activity in breast cancer tissue correlated with deficiencies in collagen and β_1-integrin receptors and it was suggested that alteration in collagen metabolism in breast cancer tissue may be causing

tissue remodeling and therefore, leading to invasiveness and progression of cancer. Increased prolidase activity may be the cause of the ECM breakdown and thus, it is likely that new drugs will be designed to target prolidase. In a study by Mittal *et al.* (2005), high expression of prolidase activity in melanoma cell lines was also observed. Prolidase was selected as a drug target due to its consistently high expression in melanoma cell lines and its high substrate specificity (Mittal *et al.*, 2005). Prodrugs are being used to decrease the toxicity and side effects of chemotherapeutic agents in cancer patients. Currently, using prolidase as a drug target for selective activation of the prodrug, melphalan, for specific drug delivery to the tumor is being evaluated (Mittal *et al.*, 2005, 2007).

IV. ADVANCES IN AND LIMITATIONS OF THE USE OF PROLIDASE FOR BIOTECHNOLOGICAL APPLICATIONS

There are advantages and disadvantages of using certain prolidases in all of the applications previously discussed. The advantages of using *Alteromonas* recombinant prolidase in biodecontamination foams are due to its high activity against G-type nerve agents, such as soman and sarin. The limitations in using a mesophilic prolidase in the DS2 foam formulation owe to its limited stability under harsh conditions. The formulation includes solvents and other denaturing solutions that reduce the enzyme's ability to function and hydrolyze the target nerve agents optimally. The enzyme also requires the addition of a metal for maximum activity.

The advantage in using the *P. furiosus* prolidase in the DS2 foam composition is in its thermostability. Like the *Alteromonas* prolidase, it too requires the addition of a metal for maximum activity. The native *Pf*prol shows no loss of activity after incubation for 12 h at 100 °C (Ghosh *et al.*, 1998). The recombinant prolidase produced in *E. coli* exhibits a 50% loss of activity after incubation for 6 h at 100 °C (Ghosh *et al.*, 1998). The disadvantages in using *Pf*prol enzyme for application purposes are due to its thermoactivity. At 80 °C there is a 50% loss of activity of *Pf*prol and there is little activity detected below 50 °C (Ghosh *et al.*, 1998). Although the enzyme's thermoactivity currently limits its use at low temperatures, this enzyme is of particular interest because of its stability at high temperatures and ablity to remain active in a decontamination formulation containing organic solvents and/or other denaturants. Purified *Pf*prol was tested against DFP, a G-series OP nerve agent and it exhibited a specific activity of 30 U mg^{-1} at 55 °C. This is comparable to human and squid prolidases, which have been evaluated and were shown to have specific activities averaging between 10 and 75 U mg^{-1} at 30 °C (data provided by Dr. Joseph DeFrank of the U.S. Army Edgewood Research, Development and Engineering Center).

Enzymes isolated from hyperthermophilic organisms have become important in industrial applications in the past decade due to their extreme thermostability. Thermophilic enzymes are proving to be more active and efficient than previously used enzymes isolated from mesophiles and psychrophiles. Their appeal comes from their ability to function in the most extreme environments that include high temperature, high/low salt concentrations and extreme pHs (Niehaus *et al.*, 1999). By using thermostable enzymes in industrial processes, reaction rates are increased, contamination is minimal and the enzyme is very hard to degrade, which enables the enzymes to withstand some of the most extreme conditions encountered in industrial processes.

The limitations of using *P. furiosus* prolidase as a potential biodecontaminant include its low activity at temperatures below 50 °C and its need for cobalt metal for activity. Using the structural information provided from *Pf*prol, there are bioengineering strategies that could address the enzyme's negligible activity at temperatures below 50 °C. The *Pf*prol gene has been altered using both random and targeted mutation strategies. Random mutagenesis strategies that have been used include error-prone PCR, hydroxylamine mutagenesis, serial passage of the *Pf*prol expression plasmid in the *E. coli* XL1-Red mutator strain and the Genemorph II mutagenesis method (Stratagene, La Jolla, California) (Theriot *et al.*, 2008). The mutated prolidase genes were transformed into *E. coli* host strain, JD1(λDE3), which is auxotrophic for proline and does not express *E. coli* encoded prolidases. This strain was used to select and screen mutants at 30 °C. Targeted site-directed mutagenesis was also performed, using the solved crystal structure of *P. furiosus* prolidase as a model. Mutants were screened to determine if key amino acid changes affected catalytic activity, metal dependency and substrate specificity. The goal is to generate a prolidase with increased ability to hydrolyze OP nerve agents at lower temperatures (35–55 °C).

OPH has been genetically modified using the solved crystal structure as a model to target key amino acids in the metal-binding site. Changing specific amino acid residues that are located in the metal-binding pocket enhances the hydrolysis of certain chemical nerve agents and their analogs. OPH metal-binding pocket mutations include His254Arg and His257Leu (Grimsley *et al.*, 2000; Lai *et al.*, 1994). They have shown a 2–30-fold increase in substrate specificity for demeton (P–S bond), which is a VX analog and a decrease in hydrolysis of DFP (P–F bond). Both His257Leu and the double mutant His254Arg/His257Leu demonstrated 11- and 18-fold increased activity for p-nitrophenyl-o-pinacolyl-methyl phosphonate (NPPMP), an analog of soman (P–S bond), respectively (diSioudi *et al.*, 1999; Grimsley *et al.*, 2000). By changing the amino acid residues, hydrogen bonds are disrupted along with electrostatic interactions with side chains (Vanhooke *et al.*, 1996). It has been suggested that this could add flexibility for larger

substrates entering the binding pocket and decrease the affinity for smaller substrates such as DFP (Grimsley *et al.*, 2000).

Genetic engineering of OPH is a good example of how changing one or two amino acids can produce a new enzyme that is altered in its metal-binding and substrate-specificity properties. Enzyme engineering could be the solution to generating an optimum prolidase suitable for each application. For the detoxification of OPs, a prolidase that shows increased hydrolytic cleavage of OP nerve agents can be made. It would also be useful to consider generating a highly expressed prolidase in LAB for reducing bitterness and enhancing flavor during the cheese-making process. For enzyme replacement therapy studies, a more stable recombinant human prolidase is needed for treatment of patients suffering from PD.

V. CONCLUSIONS

Prolidase, a proline dipeptidase, is a metalloenzyme that hydrolyzes the peptide bond between a nonpolar amino acid and a prolyl residue. It has also been shown to hydrolyze the P–F, P–O, P–CN and P–S bond in sarin and soman OP nerve agents. Other applications that rely on prolidase include the degradation of larger peptides to create texture and flavor and aid in the overall cheese-ripening process. Prolidase is also being investigated as a potential therapeutic for PD and currently is being studied for its role in tumorigenesis. Each application that uses prolidase requires an enzyme with particular properties for optimum performance. Genetic engineering of prolidase is being conducted in order to tailor each enzyme for each application. Currently, the only solved crystal structure model of prolidase is from the hyperthermophile *P. furiosus* and as such, it is being used as the model for directed mutation studies for the improvement of prolidases for a variety of applications. Furthermore, studies designed to alter the structure of prolidases will not only provide better optimized enzymes but will also provide critical information about metalloenzymes, hyperthermophilic enzymes and enzyme catalysis that can be applied to other important technologies.

ACKNOWLEDGMENTS

The authors thank Tatiana Quintero-Varca, Zeltina O'Neal, Prashant Joshi and Eli Tiller for their help in analyzing *Pyrococcus* prolidase homologs. We also thank Dr. Joseph DeFrank and Saumil Shah for their assistance in evaluating DFP degradation by *Pyrococcal* prolidases. Support for the studies conducted in the Grunden laboratory that are discussed in this review was provided by the Army Research Office (contract number 44258LSSR).

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CHAPTER 4

The Capsule of the Fungal Pathogen *Cryptococcus neoformans*

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Advances in Applied Microbiology, Volume 68
ISSN 0065-2164, DOI: 10.1016/S0065-2164(09)01204-0

Abstract

The capsule of the fungal pathogen *Cryptococcus neoformans* has been studied extensively in recent decades and a large body of information is now available to the scientific community. Well-known aspects of the capsule include its structure, antigenic properties and its function as a virulence factor. The capsule is composed primarily of two polysaccharides, glucuronoxylomannan (GXM) and galactoxylomannan (GalXM), in addition to a smaller proportion of mannoproteins (MPs). Most of the studies on the composition of the capsule have focused on GXM, which comprises more than 90% of the capsule's polysaccharide mass. It is GalXM, however, that is of particular scientific interest because of its immunological properties. The molecular structure of these polysaccharides is very complex and has not yet been fully elucidated. Both GXM and GalXM are high molecular mass polymers with the mass of GXM equaling roughly 10 times that of GalXM. Recent findings suggest, however, that the actual molecular weight might be different to what it has traditionally been thought to be. In addition to their structural roles in the polysaccharide capsule, these molecules have been associated with many deleterious effects on the immune response. Capsular components are therefore considered key virulence determinants in *C. neoformans*, which has motivated their use in vaccines and made them targets for monoclonal antibody treatments. In this review, we will provide an update on the current knowledge of the *C. neoformans* capsule, covering aspects related to its structure, synthesis and particularly, its role as a virulence factor.

I. INTRODUCTION

The adaptation of microorganisms to their environment is often associated with the acquisition of certain attributes that help improve survival in specific ecological niches. Such adaptations include signal transduction pathways that optimize metabolism to respond to the nutritional environment, stress conditions and interaction with other biological systems, such as other microbes, environmental predators and symbiotic hosts.

In addition, it is common to find morphological changes and the development of specialized structures that provide the microbe with survival benefits during its life cycle. Among these structures, many microbes possess capsules surrounding their cell body. Microbial capsules are usually composed of polysaccharides although some organisms, like *Bacillus anthracis*, have capsules composed of polymerized D-glutamic acids. Microbial capsules play important roles in the lives of these microorganisms, providing resistance to stressful conditions (such as dehydration) and playing a key role in the interaction with the environment.

Although capsules are commonly found among bacteria, there are a few encapsulated fungal species. The best characterized fungal capsule belongs to *Cryptococcus neoformans*. The capsule of this microorganism has been extensively studied because it is the main virulence factor of this pathogenic organism (McClelland *et al.*, 2006). In the environment, the capsule plays a role in the protection of the organism against some stress conditions, such as dehydration (Aksenov *et al.*, 1973). The *C. neoformans* capsule has some functional similarities to those of encapsulated bacteria such as, *Streptococcus pneumoniae* and *Haemophilus influenzae* (De Jesus *et al.*, 2008; Kang *et al.*, 2004). In fact, the cryptococcal polysaccharide is known to share some antigenic determinants with certain pneumococcal polysaccharides (Maitta *et al.*, 2004b; Pirofski and Casadevall, 1996). The capsule is important for virulence, since acapsular mutants do not produce disease in murine models (Fromtling *et al.*, 1982). The definitive experiment establishing the capsule as a virulence factor was accomplished when acapsular mutants were created and shown to be significantly less virulent than wild-type or capsule-reconstituted strains (Chang and Kwon-Chung, 1994). These mutants can survive and replicate in normal laboratory conditions but exhibit a markedly reduced virulence during infection in murine models. Interestingly, acapsular strains can be pathogenic for severely immunocompromised hosts implying a residual pathogenic potential for nonencapsulated yeast cells (Salkowski and Balish, 1991). These studies established that the capsule plays a predominant role in the interaction with the host. Consequently, this structure has been the main focus of attention in many experimental studies. Furthermore, studies have also shown that the capsular polysaccharide has strong immunomodulatory properties and promotes immune evasion and survival within the host (Monari *et al.*, 2006a; Vecchiarelli, 2000). Besides mammalian hosts, studies focused on the capsule have also been extended to include environmental predators such as amoebae, since *C. neoformans* is both a pathogen and an environmental yeast and therefore interacts with multiple types of hosts.

A vast amount of knowledge has been accumulated on the biology, structure and role of the capsule during infection. The purpose of this review is to give an overview on the main aspects of the capsule, including its structure, synthesis and in particular, its role as a virulence factor.

II. CAPSULE COMPONENTS AND STRUCTURE

The most characteristic feature of *C. neoformans* is a polysaccharide capsule that surrounds the cell body. The capsule is not visible by regular microscopy because it is highly hydrophilic and due to its high water content it has the same refraction index as the medium. However, it can be easily made visible by several techniques. The classic image of the capsule is that of a halo surrounding the cell made visible by suspending the yeast in India ink preparations. The halo effect is a consequence of the fact that the capsule does not stain with India ink, visible only by a translucent area. It can also be nicely observed by other microscopic techniques, such as scanning electron and fluorescence microscopy. In Fig. 4.1, we have collected a series of images in which the capsule is made visible by means of these techniques. The cryptococcal capsule is composed of polysaccharide, causing it to be highly hydrophilic with an extremely high-water content of 99% of the total weight of the capsule (Maxson *et al.*, 2007a). The high hydration of the capsule makes it difficult to study. The polysaccharide capsule confers a strong negative charge by virtue of the glucuronic acid residues on its main polysaccharide component (Nosanchuk and Casadevall, 1997). The polysaccharides attached to the capsule are physically organized in fibers that can be observed by electronic microscopy (Cleare and Casadevall, 1999; Frases *et al.*, 2009; Maxson *et al.*, 2007b; Pierini and Doering, 2001). The density of the fibers and polysaccharide molecules varies according to its spatial location, being denser in the inner regions (as will be discussed further) and recently, three different spatial regions have been defined based on this difference (Frases *et al.*, 2009). Topooptical reaction methods have provided information on the physical organization of the capsular molecules in different regions of the capsule (Gahrs *et al.*, 2009). This technique showed that the orientation of the fibers varied through the capsule, being tangentially oriented in the outer layers.

The polysaccharides that constitute the capsule are found in two different locations. The first location is attached to the cell wall, forming the physical structure defined as the capsule. These polysaccharides are also constitutively released by the cell into the surrounding medium and environment and they can be isolated as exopolysaccharides after certain purification protocols. It is not known whether the capsule's release into the medium is an active phenomenon regulated by the cell, or if it is just an unspecific capsule shedding. It is noteworthy that practically all our information about *C. neoformans* capsular polysaccharides originates from studies of exopolysaccharide components released from cells and recovered from culture supernatants. The field has operated under the notion that exopolysaccharide material shed capsular polysaccharide and

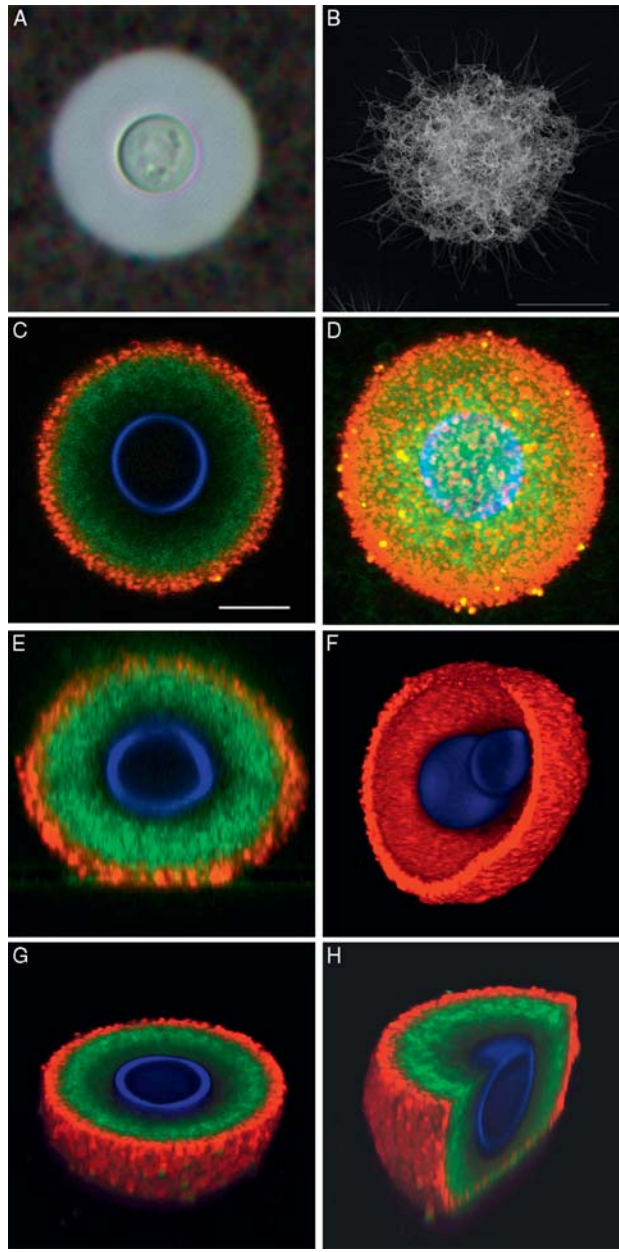

FIGURE 4.1 Different micrographs and compositions showing the polysaccharide capsule of *C. neoformans*: (A) suspension of the cells in India ink; (B) scanning electron microscopy; (C–H) immunofluorescence using specific mAbs to the capsule (green and red fluorescence) also showing the cell wall localization (blue flurorescence);

extrapolated results obtained with this material to infer capsular characteristics. Recent evidence, however, suggests that this assumption may be incorrect and that capsular and exopolysaccharide materials originate from different pools (Frases *et al.*, 2008). The exopolysaccharide material contains two major types of polysaccharides, glucuronoxylomannan (GXM) and galactoxylomannan (GalXM). GXM comprises around 90–95% of the mass and GalXM around 5–8%. In addition, a small proportion of mannoproteins (MPs) have been identified (<1%), but this component has not been studied in detail and its role in capsule architecture, if indeed it has any, remains unknown. Also, traces of sialic acid have been described, but its role in the capsule is also unknown (Gahrs *et al.*, 2009). The polysaccharides have a complex structure that has not been completely characterized yet. An intriguing finding is that capsule structure is very variable depending on the strain (and its environment). It also changes during the course of an infection (Cherniak *et al.*, 1995). Depending on these criteria, five different serotypes (A, B, C, D and AD) have been defined to classify *C. neoformans* strains, depending on the reactivity of the capsule with different rabbit polyclonal sera. These classified serotypes include *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *gattii* (serotypes B and C) and *C. neoformans* var. *neoformans* (serotypes D and AD). Recently, serotypes B and C have been proposed as a new species, named *Cryptococcus gattii*, because they present significant genetic and biological differences with serotypes A and D (Kwon-Chung and Varma, 2006). The capsular structures of the different serotypes have been studied using a variety of techniques, providing insights on the composition and organization of the capsular polysaccharides. In the next sections, we will briefly review the main features of the capsular components.

A. Structure of capsular components

1. GXM structure

Based on the analysis of GXM purified from culture supernatants, this component has a high molecular mass with a complex structure. The average molecular weight can range from 1700 to 7000 kDa, depending on the strain (McFadden *et al.*, 2006) and it comprises about 90% of the capsule's mass. There is evidence that the size distribution of the GXM molecules is more complex than initially observed (McFadden *et al.*, 2006, 2007).

(D) three-dimensional image composition of a *C. neoformans* cell labeled with two different mAbs to the capsule (in blue is the cell wall); (E) side view of a section of cell shown in (D); (F–H) sections showing the three dimensions of the capsule, visualized after staining with mAbs (green and red). Pictures by Oscar Zaragoza and from Maxson *et al.* (2007b).

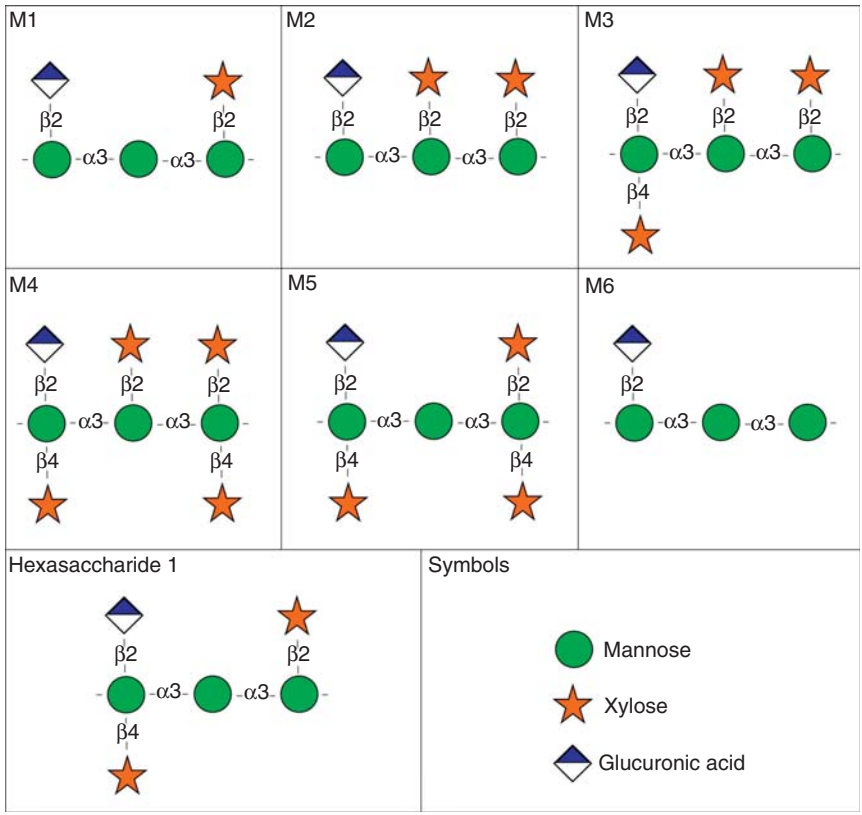

FIGURE 4.2 Repeating polysaccharide motifs of GXM. Motifs M1–M6, which were described by Cherniak *et al.* (1998) as the major structure reporter groups (SRG) of GXM, are shown. Hexasaccharide 1, an additional GXM substituted triad, is also presented. Symbol nomenclature for glycans followed the format available in http://grtc.ucsd.edu/symbol.html.

Structurally, GXM consists of a linear α-(1,3)-mannan main chain with β-(1,2)-glucuronic acid residues attached to every first mannose forming the basic core, with this unit repeated for all serotypes (Fig. 4.2). Mannosyl residues can also be 6-*O*-acetylated and substituted with xylosyl units in β-(1,2)- or β-(1,4)-linkages depending on the serotype (Cherniak and Sundstrom, 1994; Cherniak *et al.*, 1988; McFadden *et al.*, 2006, 2007). Molar ratios of xylose, mannose and glucuronic acid residues vary depending on the serotype with ratios 1:3:1, 2:3:1, 3:3:1 and 4:3:1 for serotypes D, A, B and C, respectively (Cherniak and Sundstrom, 1994).

In contrast to bacterial polysaccharides that have a single oligosaccharide repeating unit, GXM has at least six different structure reported

groups (SRGs) identified to date (Cherniak *et al.*, 1998), based on the shift of the anomeric protons and mannosyl residues by ^{1}H NMR (Cherniak *et al.*, 1998). These SRGs, classified as motifs 1–6 (M1–M6) of GXM, are shown in Fig. 4.2. ^{1}H NMR analysis from GXM of 106 *C. neoformans* isolates showed six different chemotypes (Cherniak *et al.*, 1998). A seventh GXM repeating unit was recently described by Nimrichter and co-workers (Nimrichter *et al.*, 2007), who characterized a substituted triad in GXM from encapsulated cells that had only been described in polysaccharide fractions from a hypocapsular mutant (Bacon *et al.*, 1996). This structure, called hexasaccharide 1 (Bacon *et al.*, 1996), is also shown in Fig. 4.2. Later, McFadden *et al.* found evidence of copolymerization of different GXM repeating units in one polysaccharide using mass spectrometry (McFadden *et al.*, 2007). In some *C. neoformans* strains, GXM is composed of a single repeating unit whereas in other strains the polysaccharide contains multiple units. Furthermore, those strains that utilize the same set of repeating units often differ from one another by the ratio of those units within the GXM molecule. These differences in ratios result in GXM's compositional similarity while allowing for slight differences in structures that translate into antigenic differences (McFadden *et al.*, 2007).

All the structural studies available to date use exopolysaccharides extracted by cetyl trimethylammonium bromide (CTAB) precipitation as described by Cherniak *et al.* (1998). More recently, a new method of exopolysaccharide extraction based on filtration and the tendency of polysaccharide to self-aggregate has been described (Nimrichter *et al.*, 2007). Using this method, concentration of *C. neoformans* supernatants resulted in the formation of a jellified polysaccharide film providing a one-step procedure for its isolation (Nimrichter *et al.*, 2007). Polysaccharides obtained from the ultrafiltration display differences in the reactivity with GXM-specific monoclonal antibodies (mAbs) when compared with polysaccharides obtained by differential precipitation with CTAB. Viscosity analysis associated with inductively coupled plasma mass spectrometry and measurements of zeta potential in the presence of different ions, strongly suggested that polysaccharide aggregation was a consequence of the interaction between the carboxyl groups of glucuronic acid (GlcA) and divalent cations. The length of a GXM fiber from solution supernatants is shorter than the capsule diameter (McFadden *et al.*, 2006). All these results suggest that capsular assembly in *C. neoformans* results from divalent cation-mediated self-aggregation of GXM molecules (Nimrichter *et al.*, 2007).

Polysaccharides recovered from culture supernatants have historically provided an ample and convenient source of material for structural and immunological studies. A major assumption in such studies is that the structural features of the exopolysaccharide material faithfully mirror

those of the capsular polysaccharide. This remains a mere assumption and the exact reasons why the polysaccharide is released are still unknown. In fact, it is conceivable that exopolysaccharide differs from capsular polysaccharide in that the former has structural features intended for extracellular release and thus, the two materials differ in fundamental ways. Suspension of acapsular cells in soluble capsular polysaccharide resulted in the presence of a capsule forming around the cell as was visualized by fluorescence, but the degree of encapsulation was much lower than is commonly seen in regular encapsulated strains. The binding was dependent on the polysaccharide concentration, with high concentrations resulting in nonspecific polysaccharide binding to the cell (Kozel, 1977; Kozel and Hermerath, 1984). These findings suggest that a proportion of exopolysacharides retain their ability to form a capsule. The finding that this capsule is much smaller than what is generally found in encapsulated strains suggests that a significant proportion of exopolysaccharides have different structural features than the polysaccharide that make up the normal capsule in wild-type strains. Structural analysis of the capsular polysaccharides (not exopolysaccharide) has been limited for a long time because of a lack of efficient protocols for separating the capsule from the cell. However, in the last years, different groups have described that exposure of the cells to γ-radiation or suspension in DMSO results in the release of the capsule from the cells (Bryan *et al.*, 2005; Dembitzer *et al.*, 1972; Gates *et al.*, 2004). This has led to the possibility of studying the structural features of the polysaccharide that remain attached to the cell, forming a visible capsule. This approach allowed for the detailed study of different regions of the capsule (inner capsule, medium capsule and outer edge). An additional advantage of stripping the capsule with γ-radiation versus DMSO is that the cells remain viable after radiation treatment (Bryan *et al.*, 2005).

Comparison of exopolysaccharide obtained by two isolation techniques with capsular polysaccharide stripped from cells exposed to γ−radiation or treated to DMSO revealed significant differences in glycosyl composition, mass, size, charge, viscosity, circular dichroism spectra and reactivity with mAbs. This implies that exopolysaccharides and capsular polysaccharides are structurally different and originate from different synthetic pools. Hence, the method used to isolate polysaccharides can significantly influence the structural and antigenic properties of the product (Frases *et al.*, 2008).

a. Structural information provided by antibodies to GXM A large number of mAbs that bind to the *C. neoformans* capsule have been obtained (Casadevall *et al.*, 1992, 1994; Dromer *et al.*, 1987b; Eckert and Kozel, 1987; Pirofski *et al.*, 1995; Todaro-Luck *et al.*, 1989; van de Moer *et al.*, 1990). Most of these antibodies were obtained after mouse immunization

with purified GXM. At present, antibodies differing in affinity, specificity and isotype are available for the study of the capsule. In addition to immunological studies examining their protective effect during infection, these antibodies have proven to be a useful tool to study the structure and properties of the *C. neoformans* capsule. Studies with mAbs have revealed that the capsule has a very heterogeneous structure and that several different epitopes are likely to be present in the same GXM molecule (Belay *et al.*, 1997; Todaro-Luck *et al.*, 1989). They have shown that this heterogeneity can be found between different strains, as well as within a population of the same strain (McFadden *et al.*, 2007). Several factors determine the specificity and affinity of the mAbs to GXM, such as acetylation and xylosylation (Kozel *et al.*, 2003; Todaro-Luck *et al.*, 1989). Several genetic screenings have been performed with the aim of obtaining mutants with altered binding to specific mAbs. These mutants were not acapsular, but deficient in acetylation and xylosylation (Kozel *et al.*, 2003), demonstrating the importance of this process for some of the functions associated with GXM. mAbs to the capsule have also been used in immunoblotting experiments, which have shown that the size distribution of the GXM molecules is very complex (McFadden *et al.*, 2006). It has also been shown that the binding affinity of these antibodies varies according to the different capsule regions (Maxson *et al.*, 2007b). This confirms that capsule structure is very heterogeneous, not only at the level of a single GXM molecule, but also in terms of the spatial and structural organization of the capsule.

2. GalXM structure and localization in the capsule

GalXM constitutes about 8% of the capsular mass (Bose *et al.*, 2003; Vaishnav *et al.*, 1998) and has an α-(1→6)-galactan backbone containing four potential short oligosaccharide branch structures (Fig. 4.3). The branches are 3-*O*-linked to the backbone and consist of an α-(1→3)-Man, α-(1→4)-Man, β-galactosidase trisaccharide with variable amounts of β-(1→2)- or β-(1→3)-xylose side groups (Bose *et al.*, 2003; McFadden *et al.*, 2006; Vaishnav *et al.*, 1998). The GalXM backbone consists of galactopyranose and a small amount of galactofuranose (Vaishnav *et al.*, 1998), unlike GXM, which contains only mannopyranose (Bose *et al.*, 2003). Compositional analysis of GalXM was confirmed by gas chromatography/mass spectrometry of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. The analysis revealed that the molar percentage for GalXM were as follows: xylose, 22%; mannose, 29%; and galactose, 50% (De Jesus *et al.*, 2008; Vaishnav *et al.*, 1998). GalXM is very sensitive to oxidation with sodium periodate, which destroys the integrity of most of the carbohydrats. Xylose residues are completely eliminated and two-thirds of galactose and mannose residues oxidized (Cherniak *et al.*, 1982). To elucidate

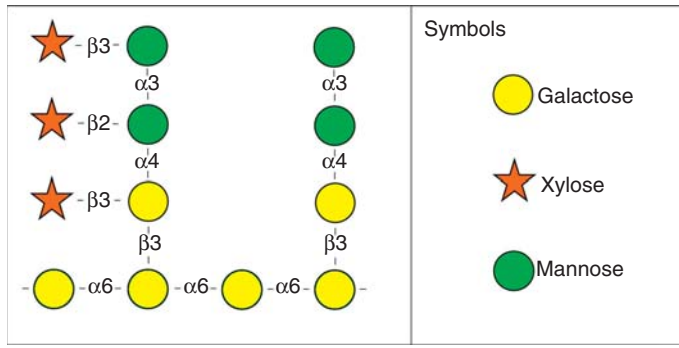

FIGURE 4.3 Basic GalXM structure. A GalXM motif has a α-(1→6) galactan backbone. The branches are β-3-*O*-linked to the backbone and consist of an α-(1→3)-Man, α-(1→4)-Man, β-galactosidase trisaccharide with variable amounts of β-(1→2)- or β-(1→3)-xylose side groups. Symbol nomenclature for glycans followed the format available in http://grtc.ucsd.edu/symbol.html.

the proposed structure of GalXM repeats, proton nuclear magnetic resonance spectrum analysis was used to determine the anomeric region of the galactan building unit. The results showed the anomeric region to be between 5.4 and 4.3 ppm in a one-dimensional ^{1}H spectrum recorded at 600 MHz and 56 °C (De Jesus *et al.*, 2008; Vaishnav *et al.*, 1998). Although the GalXM from serotypes A, C and D all contained galactose, mannose and xylose, it has been shown that the molar ratios are not identical, suggesting that there may be more than one GalXM entity. GalXMs are thought to be a group of complex closely related polysaccharides (James and Cherniak, 1992; Vaishnav *et al.*, 1998).

A recent study showed that GalXM is significantly smaller than GXM (1.7×10^6 kDa), with an average mass of 1×10^5 kDa (McFadden *et al.*, 2006). Additionally, the radius of gyration (R_g), defined by the average distance between the center to the outer edge of the molecule, was measured for both GXM and GalXM. The results revealed that for GXM different strains showed differences of 68–208 nm while GalXM isolated from the acapsular strain cap67 was 1.4-fold smaller than GXM with an R_g of 95 nm (McFadden *et al.*, 2006). Attempts to image GalXM by scanning transmission electron microscopy (STEM) were unsuccessful, possibly because the molecule was too narrow to be detected (McFadden *et al.*, 2006). GalXM is the most abundant polysaccharide in the capsule based on its molar mass, since GalXM has a comparatively smaller molecular weight, with 2–3.5 mol of GalXM for each mole of GXM. The widespread assumption that GXM was the most abundant molecule was based on the total amount of material that was recovered from the culture supernatant (McFadden *et al.*, 2006). It is noteworthy that all studies of GalXM used

material obtained from nonencapsulated strains that failed to secrete GXM. This is due to an absence of adequate methods available that are able to separate GalXM from GXM in exopolysaccharide preparations to yield GalXM of sufficient purity for structural studies. However, given that nonencapsulated strains often have secretion defects (Garcia-Rivera *et al.*, 2004), this introduces the conundrum that the GalXM so obtained may not be in its native state.

While GXM is distributed throughout the capsule, the exact location of GalXM is not known as there is no visualization method available to help determine this (such as an antibody to GalXM). Experiments aimed at purifying GalXM revealed that supernatant fractions following homogenization of cell walls derived from the acapsular strain *cap67* contained both GalXM and MPs. It was demonstrated that GalXM and MPs do not form a part of the cell wall matrix since they are not covalently bound to glucans within the cell wall (James and Cherniak, 1992). GalXM and MPs can be separated using Concanavalin A affinity chromatography which binds MPs, with the eluate containing GalXM (James and Cherniak, 1992; Vaishnav *et al.*, 1998).

Capsular studies by Bryan *et al.* noted the absence of galactose in the fraction removed by radiation alone, which represents the outer most section of the capsule (Bryan *et al.*, 2005). This was interpreted to indicate an absence of GalXM in the outer layers of the capsule. The compositions of the outer layer of the capsule removed by either DMSO or radiation alone were similar. This result was consistent with the observation that both DMSO and radiation removes the same part of the capsule. Neither radiation nor DMSO removed the portion of the capsule in the region closest to the cell wall. This can be explained by the fact that the inner radiation- and DMSO-resistant capsule and the outer radiation- and DMSO-susceptible regions have differences in terms of their chemical compositions, branching, or the density of molecules packed. In these studies, it was also noted that GXM protects against radiation with a possibility that GalXM in the inner capsule also contributed to the effect of radioresistance observed by that layer (Bryan *et al.*, 2005).

Another studies utilized different doses of γ-radiation to investigate the structure of different *C. neoformans* capsular regions (Maxson *et al.*, 2007b). The authors prepared two different fractions of the capsule by irradiating *C. neoformans* H99 strain for 20 min, enough time to allow for the lower density part of the capsule to be stripped. The second part of the experiment included washing and irradiating for another 20 min, which stripped away the higher density region of the capsule. The results revealed no differences in terms of the carbohydrate composition. However, the occurrence of galactose was found with both strippings, which was interpreted to signify that GalXM is present throughout the capsule (Maxson *et al.*, 2007b).

a. Structural information provided by antibodies to GalXM In the early 1990s, van de Moer *et al.* generated six mAbs against spheroplast lysate of *C. neoformans* serotype A. mAb CN6 of the IgM isotype was reactive with GalXM. The CN6 antibody binding was blocked by the presence of GXM but the acapsular mutants were able to bind the antibody (James and Cherniak, 1992; van de Moer *et al.*, 1990). Immunogold transmission electron microscopy and a microagglutination assay demonstrated through epitope recognition by the mAb CN6 that GalXM was located within the cytoplasm and the cell wall in acapsular strains (Vaishnav *et al.*, 1998). Unfortunately, the antibodies were not used again for any subsequent studies and are apparently no longer available.

Recently, GalXM localization was studied using mouse immune serum obtained from mice injected with GalXM conjugated to the protective antigen from *B. anthracis*, which is highly immunogenic (De Jesus *et al.*, 2009). Using this reagent, only low amounts of GalXM were detected as a capsular component and it was associated with vesicles found in the capsule, which suggests that GalXM plays a role as exopolysaccharide and not in the architecture of the capsule. Accordingly, it is important to highlight that GalXM is a powerful immunomodulatory molecule, as will be discussed further.

b. Mannoproteins Classically, MPs have been considered as minor components of the capsule, but their role in capsule architecture has never been established. Their presence was identified in culture filtrates by ^{13}C NMR analysis of the GXM-free portion of the GalXM-enriched fraction (Murphy, 1988; Reiss *et al.*, 1985). Very little is known, however, about their function and the role they play in the capsule. Some articles have investigated the location of MPs on cryptococcal cells and concluded that they are mainly found in the inner cell wall and not associated with GXM or GalXM (Vartivarian *et al.*, 1989). It has been shown that MPs are highly immunogenic during infection, which will be discussed in a different section.

B. Capsule dynamics

In this section, we will review an important aspect of the capsule that has deep implications for the virulence of the organism, namely the ability of *C. neoformans* to modify the size and structure of its capsule in response to various stimuli. Since the capsule is presumably the first fungal structure that interacts with host cells, the capability for multiple combinations of capsule size and structure makes this a very versatile structure. Changes in capsule size are typical features among *C. neoformans* strains during the interaction with the host and are considered an early morphologic response during infection. Capsule enlargement occurs after a few

hours of infection in murine models (Feldmesser *et al.*, 2001). Moreover, it is also observed to occur during *C. neoformans* intracellular parasitism. This phenomenon has been elegantly illustrated using live imaging techniques (see Supplemental movie 1 in Ma *et al.*, 2006). In addition, during the course of infection, the immunogenic properties of the capsule can change, a finding that is also considered as an adaptation phenomenon to survive in the host.

1. Capsular enlargement

The size of the capsule is very variable, not only among strains, but also between individual cells of the same strain, to such an extent that a given strain can exhibit large differences in capsule size depending on the specific environmental conditions. There are many different conditions that lead to capsule enlargement in natural environments. In laboratory conditions, there are several factors that are known to influence capsule size.

In the late 1950s, it was Littman who described for the first time that capsule size in *C. neoformans* could be modulated *in vitro* by placing the cells in different growth conditions. He described several minimal media in which he observed a significant increase in capsule size (Littman, 1958). The inducing factors in that media still remain unknown. In 1985, carbon dioxide was described as a factor that had an influence on capsule size after Granger *et al.*'s finding that when the cells were placed in a CO_2-enriched atmosphere (5%) the capsule manifested a significant enlargement (Granger *et al.*, 1985). These authors also isolated mutants defective in capsule growth in the presence of CO_2 and demonstrated that it had reduced virulence in murine models, which provided the first evidence that the phenomenon of capsular enlargement played a role in the pathogenesis of the microorganism. Consistent with these findings it was found that strains which differed in terms of their capsule size also differed in their virulence and in their effects on macrophages. The strain with reduced virulence was not able to increase capsule size in the presence of CO_2. During infection, the strain unable to induce capsule size elicited a rapid inflammatory response in the host and was efficiently cleared from the lungs (Blackstock and Murphy, 1997; Blackstock *et al.*, 1999). These reports provided strong evidence that capsule enlargement is a process that plays an important role during infection. Several studies have found, however, that virulence is not correlated with *in vitro* capsule size (Clancy *et al.*, 2006; Dykstra *et al.*, 1977; Littman and Tsubura, 1959). This lack of correlation is most probably due to the fact that *in vitro* capsule size does not predict the degree of encapsulation of a strain during infection seen *in vivo*. Furthermore, *C. neoformans* has multiple virulence factors that contribute to the overall virulence phenotypes and it is possible that differences in noncapsular virulence factor expression affect the overall

virulence potential to obscure a correlation with capsule size (McClelland *et al.*, 2006). In 1993, iron limitation was shown to result in capsule enlargement (Vartivarian *et al.*, 1993). The fact that both CO_2 and iron limitation influenced capsule size is particularly interesting because these conditions mimic some situations that the microorganism encounters during lung colonization. In addition, capsule size during infection varies according to the organ infected, the lung being the place where higher capsule size is found, which indicates that microenvironments during infections have a great impact on capsule size (Rivera *et al.*, 1998). Iron is an essential nutrient for the life of the yeast in which its concentration in the host is normally very low, so the yeast must compete with iron binding proteins from the host such as transferrin. It is not known why *C. neoformans* responds to iron limitation by enlarging its capsule, but it has been suggested that the capsule might contribute to iron uptake (Vartivarian *et al.*, 1993). Therefore, capsule growth could increase the iron concentration available to the yeast.

More recently, other media that induce capsule growth have been described. In the context of media that mimic infection situations, mammalian serum was found to induce *in vitro* capsule growth in the absence of CO_2 (Zaragoza *et al.*, 2003a). Those studies also established that the incubation media had a major determinant effect on the efficacy of inducing factors, such as serum or CO_2. Furthermore, results showed that the behavior of different strains varies and that the optimal conditions to obtain capsule growth should be standardized for every strain.

The signals that induce capsule growth are very heterogeneous (iron concentration, CO_2, serum) and it is not clear if they induce capsule enlargement through different pathways and whether there is a common element to all of these factors that the cell senses as a capsule-inducing factor. In all the media described that are known to induce capsule growth, a significant decrease in the growth rate of the yeast has been observed. It is therefore believed that capsule growth occurs under certain conditions where yeast grows very poorly. Accordingly, media which contain low nutrient concentrations, such as diluted Sabouraud buffered at a neutral pH, induced a significant increase in capsule size (Zaragoza and Casadevall, 2004), supporting the hypothesis that capsule growth is probably inversely related to the growth rate of yeast. While a slow as opposed to a rapid growth rate of yeast appears to be a requirement for induction of capsule growth, this is apparently not sufficient, since conditions have been observed in which the yeast grows slowly that do not lead to capsule enlargement. Capsule enlargement is a controlled event and does not occur over a certain limit, since it has been shown that when the cells are serially transferred to fresh-inducing medium, the capsule does not exceed a certain size (Zaragoza *et al.*, 2006b). Interestingly, this limitation depends on the size of the cell body (delimited by the wall),

since a strong positive correlation between capsule size and cell body size has been found during capsule enlargement (Zaragoza *et al.*, 2006b).

2. Physical factors that affect capsule size

Capsule size also changes in the presence of certain factors that affect its structure or its packing without affecting the amount of polysaccharide accumulated in the capsule. One such factor is the effect of the osmotic pressure of the medium. Addition of a high NaCl concentration produces a reduction in capsule size (Dykstra *et al.*, 1977). The same effect is produced by glucose concentrations over 10%, a finding that has been correlated to an increase in osmotic pressure of the medium. There are several explanations for these findings. It is possible that a high osmotic pressure produces an effect whereby a significant part of the water content of the capsule is lost in these conditions, which in turn, results in an increase in the packing of the polysaccharide fibers and thereby a decrease in capsule size. Concerning glucose effect, it has also been described that growth of the cells in high glucose concentration produces a higher polysaccharide release to the growth medium (Cleare and Casadevall, 1999), which suggests that carbon metabolism might be an important factor in capsule synthesis. Another element that has been reported to influence capsule size is the binding of mAbs. Cleare and Casadevall reported that appearance of the cells by scanning electron microscopy was affected by the presence of mAb 2H1 (Cleare and Casadevall, 1999). Later on, it has been described that binding of a high amount of mAb 18B7 to the capsule produces a drastic decrease in capsule size (Zaragoza and Casadevall, 2006). mAbs tend to bind to each other through interactions of the constant regions, which could feasibly produce a force within the capsule resulting in a compaction of the polysaccharide fibers toward the cell body. Moreover, capsule size decreases in the presence of mAbs can result not only in a diminished capsular size, but also in one with irregular edges, in which the morphology changes and the capsule loses its characteristic spherical shape (Zaragoza and Casadevall, 2006).

However, recent findings suggest new explanations for the different packing of the polysaccharide fibers in different conditions. Capsule enlargement is facilitated under slight alkaline conditions (Zaragoza and Casadevall, 2004), which is consistent with the view that capsule growth requires the acidic groups of glucuronic acid (GlcA) residues to be ionized, possibly so that they can react with divalent cations for capsule assembly. It is noteworthy that mammalian physiological fluids are usually slightly alkaline. In addition, since the finding that high NaCl concentration blocks capsule growth does not generally apply to other

solutes, it has been proposed that capsule growth could be inhibited in the presence of Na^+ by univalent neutralization of negatively charged GlcA residues (Nimrichter *et al.*, 2007).

In plant cell walls, galacturonans can be cross-linked by insertion of Ca^{2+} ions between the unesterified carboxyl groups of the uronosyl residues (Vincken *et al.*, 2003). The same property was recently described for GXM which self aggregates through a divalent metal-mediated process (Frases *et al.*, 2008; McFadden *et al.*, 2006; Nimrichter *et al.*, 2007). The efficacy of this association depended on water removal and increased polysaccharide concentration (Nimrichter *et al.*, 2007), which allowed an efficient interaction between GXM fibers and divalent ions at neutral and alkaline pH (pK of glucuronides $\geq$3). Accordingly, carboxyl reduction of GXM, as well as its treatment with EDTA monovalent ions and excessive concentrations of Ca^{2+} impaired self-aggregation (Nimrichter *et al.*, 2007). These observations suggested that divalent metals could regulate the aggregation of GXM fibers.

The cultivation of cryptococci in the absence of divalent ions was associated with smaller capsules and a concomitant increase in the concentration of GXM in supernatants, presumably as a result of incomplete assembly (Nimrichter *et al.*, 2007). In contrast, the addition of increasing concentrations of Ca^{2+} to the culture medium resulted in augmented capsule expression that was associated with decreased levels of soluble GXM in fungal cultures. Finally, high concentrations of Ca^{2+} caused a decrease in capsule sizes (Nimrichter *et al.*, 2007). Interestingly, dead *C. neoformans* cells incorporated exogenous GXM in the presence of varying Ca^{2+} concentrations. A possible explanation for these findings is based on the stoichiometric ratio between divalent cations and glucuronic acid residues. According to this model, divalent ions would be expected to positively modulate capsule assembly by forming ionic bridges between glucuronic acid residues in different GXM fibers. In this context, metal ion concentrations compatible with a 1:2 metal ion-GlcA ratio in cryptococcal cultures would promote capsule growth, while the capsule would be reduced when the metals were provided in excess. These results are consistent with and support the notion that divalent cation-mediated aggregation of GXM is involved in capsule enlargement.

Recent findings suggest that capsule size and packing depends on the interaction of its basic components, GXM and GalXM. Mutants that cannot synthesize GalXM manifest larger capsules than the isogenic wild-type strains, suggesting that GalXM plays an important structural role in maintaining a certain capsule size and in turn, structure and density. It could also suggest that in the absence of GalXM the cell responds by overproducing GXM molecules, resulting in a higher capsule size (Moyrand *et al.*, 2007).

3. Physical changes associated with capsule growth

Capsule growth is achieved by addition of polysaccharide to the capsule, which implies that capsule enlargement is a high energy consuming process. Addition of new polysaccharide to the capsule leads to an increase in the density of the polysaccharide, especially in the regions closer to the cell wall. This has been proven by different groups using different approaches, based on microscopy, immunogenic properties and direct polysaccharide measurement in different capsule regions (Gates *et al.*, 2004; Maxson *et al.*, 2007b; Pierini and Doering, 2001). A characteristic feature of the encapsulated cells is a very high negative zeta potential value due to the high number of glucuronic acid residues in the capsular polysaccharide of cryptococcal cells. Different studies suggest that capsule enlargement is associated with a decrease in the zeta potential value consistent with larger negative surface charges (Maxson *et al.*, 2007b; Nosanchuk and Casadevall, 1997). It has been reported that the binding of mAbs to the capsule changes its permeability, a phenomenon that is correlated with a phenomenon known as quellung reaction (MacGill *et al.*, 2000; Mukherjee *et al.*, 1995a). Yoneda and Doering observed in immunoblotting experiments that the size distribution of exopolysaccharide GXM varied when the capsule enlargement was induced (Yoneda and Doering, 2008), which suggests that in these conditions, the GXM fibers that are produced and incorporated in the capsule are different, at least in size. Consistent with this idea, Frases *et al.* (2009) have recently found that capsule enlargement is achieved by addition of polysaccharide fibers of a higher molecular weight.

4. Capsule growth model

The mode of polysaccharide accumulation on the capsule has been the subject of many different studies. For this purpose, different methods have been used to label the capsule prior to induction and trace the label once the cell triggers the signals to increase capsule size. The first study used a mAb and radioactive xylose (Pierini and Doering, 2001). This approach revealed that the marker moved to the edge of the capsule after induction, an observation that was interpreted as indicating that the capsule grows by accumulation in the inner part of the capsule, displacing the old capsule to the edge. However, since antibody binding is not covalent and antibody–antigen interactions are prone to dissociation and association, there was some concern that the antibody was not a stable capsule geographical marker. More recently, a similar approach was employed using a marker that covalently binds to the capsule (Zaragoza *et al.*, 2006b). This marker was provided by proteins from the complement systems, which bind to the polysaccharide capsule fibers through covalent bonds. Using this approach, it was shown that the old

polysaccharide fibers remained in a position close to the cell wall after capsule induction, suggesting that capsule growth was achieved by addition of the polysaccharide fibers at the capsule edge (Fig. 4.4A). The authors confirmed this result by giving a pulse of radioactive mannose prior to capsule induction and then establishing the localization of the radioactive label in different regions of the capsule obtained after γ-radiation treatment of the cells. Consistent with this last model,

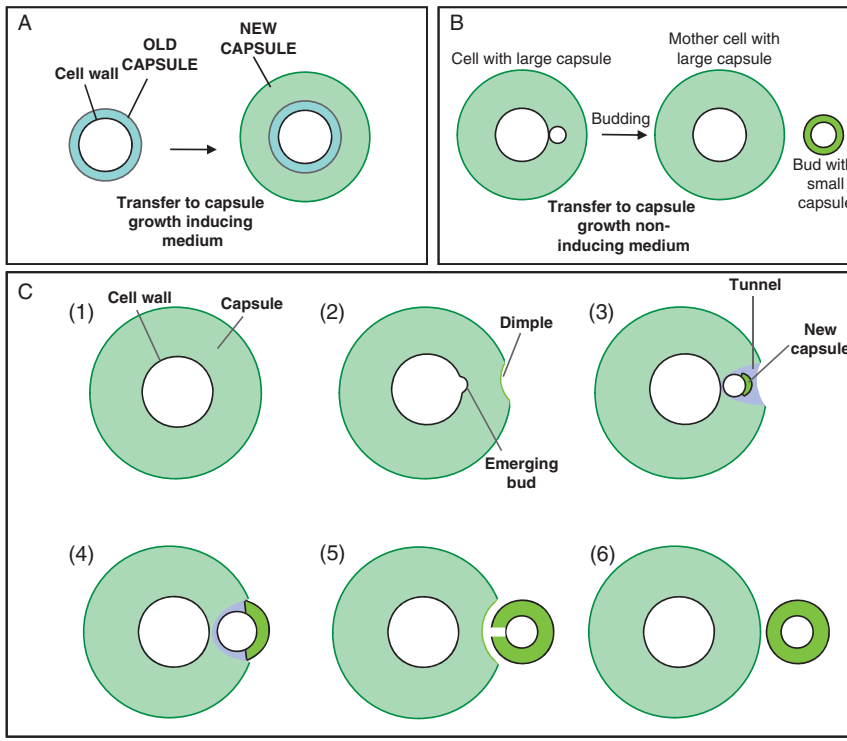

FIGURE 4.4 Model of capsule growth and capsule rearrangements during budding in *C. neoformans* (from Zaragoza *et al.*, 2006b). (A) Model of capsule growth. After capsule enlarges, a newly synthesized capsule (light grey) accumulates at the edge of the capsule, with the old capsule (dark grey) remaining close to the cell wall. (B) Model of adaptation to noncapsule growth-inducing conditions. Cells with large capsules, when transferred to a medium that does not induce capsule growth, cannot degrade the capsule, but the new emerging buds have a small capsule. (C) Rearrangements of the capsule during budding. Panels 1–6 illustrate schematically different stages of budding. When bud arises, a dimple and a tunnel are formed (2, 3), which allows the separation of the bud. Bud growth is accompanied by capsule growth in the bud (3, 4). Capsule of the mother cell closes as the bud separates from the mother cell, at the same time that the bud completes the capsule without taking any polysaccharide of the mother cell (5), which will allow for the complete separation of the bud (6).

Charlier *et al.* found that during infection, there is a difference in the binding pattern of specific antibodies to the capsule after capsular enlargement during infection (Charlier *et al.*, 2005). These investigators infected mice with a *C. neoformans* strain that reacted to mAb E1 which is specific for serotype A. After 6 h of infection, they observed that a new area surrounding the E1-binding region appeared which was not reactive to this antibody anymore, but to another mAb named CRND-8, raised against serotype D strains. These findings suggest that new polysaccharide is added at the capsule edge such that capsular enlargement proceeds by apical growth.

5. Capsule rearrangements during budding

Capsule growth poses a problem for the cell because during budding, the new cell has to traverse the width of the parental capsule to separate from the mother cell. Theoretically, the presence of a dense net of polysaccharide fibers around the cell wall could interfere with bud migration and separation. However, the fact that a cell buds successfully implies the existence of mechanisms to ensure bud separation. Several lines of evidence have suggested the existence of a mechanism for this phenomenon. (1) When a *C. neoformans* cell is coated with a mAb and then placed in a medium that induces budding, the mAb does not migrate to the bud (Pierini and Doering, 2001; Zaragoza *et al.*, 2006b). (2) The binding of mAbs to the capsule over the bud is different from the binding to the capsule of the mother cell, which suggests that the capsules do not mix during budding (Zaragoza *et al.*, 2006b). (3) When budding cells with large capsule are incubated with complement, immunoflourescence staining for complement proteins is not observed in the regions that separate the bud and the mother cell (Zaragoza *et al.*, 2006b). (4) When cells with large capsule start budding, it is possible to observe some rearrangement at the regions of the capsule of the mother cell where the bud is forming. Furthermore, it is possible to observe a dimple on encapsulated budding cells which suggests that the capsule disappears at the region where the bud will appear. In cells coated with complement, it is also possible to observe some rearrangements in the capsular region of bud emergence. This suggests that during budding, the capsule of the mother cell opens, leaving an empty space for the bud to migrate through. (5) The region of the capsule where the bud is going to emerge becomes highly permeable to India ink (Pierini and Doering, 2001). (6) When budding cells with large capsule are placed in the presence of serum for a very short time period, thus allowing complement deposition, complement proteins accumulate in the region between the bud and the mother cell, suggesting that in the capsule region where the capsule is going to pass through are not fibers, which would facilitate the migration of the bud. (7) Scanning electron micrographs have shown a physical separation between the capsules of the mother cells and the bud (Zaragoza *et al.*, 2006b).

All these observations led us to propose a model to explain how the bud crosses the capsule of the mother cell without being retained, or entangled, within the polysaccharide fibers of the mother cell (see Fig. 4.4C). We hypothesized that when the bud emerged from the mother cell, there are some rearrangements in the region where the bud has to pass, which produce the formation of a tunnel in the capsule, creating a physical space that allows the bud to migrate through the capsule. The description of bud scars in the capsule supports this idea (Cleare and Casadevall, 1999). The presence of chitin-like structures in the cell wall which are projected into the capsule have been recently described (Rodrigues *et al.*, 2008a). Staining for this structure is particularly abundant in the neck around the bud, which suggests that these structures could be involved in the migration of the bud through the mother's cell capsule.

6. Capsule degradation

An interesting feature that has hardly been addressed is how the capsule is degraded and how the cells diminish their capsule size in adaptation to the environment. For example, after passage through hosts, the capsule of the *C. neoformans* cells reverts to a small size. During budding, the creation of the tunnel described above could imply the degradation of the polysaccharide fibers in the budding area of the capsule. However, despite these situations in which a reduction or degradation of capsule size is required, no capsule degrading enzymes have been described so far in *C. neoformans*. Moreover, preliminary findings indicate that when cells with a large capsule are shifted to a medium where the capsule is small, the cells do not degrade the capsule and they retain a considerable capsule size (Zaragoza *et al.*, 2006b). Although the cells initially placed in the new medium remain with an enlarged caspule, the buds that emerge in the new medium have a small capsule, which suggests that when *C. neoformans* is shifted to a medium which does not require a large capsule, the adaptation mechanism cause the cells to invest their cellular energy in producing buds with the proper capsule size and not in degrading the capsule (Fig. 4.4B). It also suggests that during budding, a mechanism of capsule release occurs in the budding area without actual capsule degradation. Since budding implies cell wall degradation and the capsule is linked to the cell wall, it is believed that the capsule fibers are released by rearrangements in cell wall, rather than through direct capsular polysaccharide degradation. This issue remains a key point in the cryptococcal biology that needs to be further examined.

7. Capsule structural changes *in vitro* and during infection

In addition to capsule growth, several other phenomena indicate that the capsule is dynamic, which is related to an inherent ability of *C. neoformans* cells to alter their capsular structure. As mentioned above, not only are

physical changes observed in terms of density, but also alterations with regards to the structure of the polysaccharide fibers. Turner *et al.* found that capsule structure from different strains from within the same serotype (serotype A) differed in their structure as shown by chemical analysis and ^{13}C NMR spectroscopy (Turner *et al.*, 1992). Different groups have found that even within the same population, there are significant changes in the immunoreactivity of *C. neoformans* cells to specific mAbs (Garcia-Hermoso *et al.*, 2004; McFadden *et al.*, 2007). This heterogeneity is also a characteristic feature of these cells seen during infection (Garcia-Hermoso *et al.*, 2004).

Structural changes are relevant during the course of infection, especially during the interaction with phagocytic cells. Addition of purified capsular polysaccharide isolated from different serotype A strains to acapsular mutants produces different effects on phagocytosis, for example (Small and Mitchell, 1989). Furthermore, *C. neoformans* phagocytosis by amoeba is influenced by the incubation medium, a phenomenon that has been related to differences in capsule structure in accordance with the surrounding environment (Zaragoza *et al.*, 2008b). Finally, capsule structure changes have been implicated in dissemination (Charlier *et al.*, 2005). These implications will be discussed in more detail in the following sections.

8. Capsule and phenotypic switching

As is seen in other fungi (such as *Candida albicans*), *C. neoformans* undergoes macroscopic changes, determining different phenotypic morphologies, through a process known as phenotypic switching (Goldman *et al.*, 1998). Two different variants have been defined, smooth and mucoid. These variants manifested different virulence and it was later shown that they differed in terms of their capsular structure (Fries *et al.*, 1999; McFadden *et al.*, 2007). Similar findings have recently been reported for *C. gattii* (Jain *et al.*, 2006). Although it has not been demonstrated that capsule changes are responsible for phenotypic switching, this phenomenon and its correlation with capsule variation is a clear example of how *C. neoformans* can produce dynamic capsule changes in correlation to variations in phenotypes and related to virulence.

III. CAPSULE SYNTHESIS IN *CRYPTOCOCCUS*

A. Genes, enzymes and signaling pathways

1. The *CAP* genes

During the last decade, different genes involved in capsule biosynthesis have been identified. The first one, *CAP59*, was cloned by complementation of an acapsular mutant isolated by UV random mutagenesis (Chang

and Kwon-Chung, 1994). The gene was deleted by homologous recombination and resulted in an acapsular phenotype associated with loss of virulence in an animal model of cryptococcal infection. The restoration of virulence by complementation of the acapsular phenotype represented the first molecular evidence for the capsule as a virulence factor of *C. neoformans*. At the time of the study by Chang and Kwon-Chung (Chang and Kwon-Chung, 1994), no sequences similar to that found in the *CAP59* gene had been reported. More recently, *CAP59* sequence presents homology with a cryptococcal α-1,3-mannosyltransferase (Sommer *et al.*, 2003). Cap59p has a putative transmembrane domain and it was further suggested to play a role in capsule secretion (Garcia-Rivera *et al.*, 2004). Interestingly, *CAP59* was considered to be related to the *L27* gene (Chang and Kwon-Chung, 1994), which encodes for a ribosomal protein that is present in secretory vesicles carrying capsular polysaccharides in *C. neoformans* (Rodrigues *et al.*, 2007, 2008b).

Following the molecular characterization of *CAP59*, three other *CAP* genes were identified. *CAP64* was the second capsule-related gene described in *C. neoformans* (Chang *et al.*, 1996). Deletion of *CAP64* resulted in an acapsular strain that was avirulent in mice. As with *CAP59*, complementation of the *cap64* mutation restored both capsule expression and virulence. Interestingly, the *CAP64* gene from a serotype D isolate complemented the acapsular phenotype of a serotype A strain, indicating that the gene product of *CAP64* did not contribute to serotype specificity of *C. neoformans* polysaccharides (Chang *et al.*, 1996).

The third gene required for capsule formation was *CAP60* (Chang and Kwon-Chung, 1998), which is closely linked to a cellulose growth-specific gene from *Agaricus bisporus*, *CEL1*. *CAP60* is located on the same chromosome as *CAP59* but, although similarity exists between Cap59p and Cap60p, the gene products cannot functionally substitute each other by direct complementation or by domain swap experiments. Immunogold labeling localized Cap60p at the nuclear membrane, although antibody reactivity was also detected in both the plasma membrane and the cell wall (Chang and Kwon-Chung, 1998). Deletion of *CAP60* resulted in acapsular cells that were moderately virulent in mice and complementation of the mutation restored capsule expression and virulence (Chang and Kwon-Chung, 1998).

CAP10 was the fourth characterized gene required for capsule formation and virulence in *C. neoformans* (Chang and Kwon-Chung, 1999). The gene sequence shows homology with a gene encoding a cryptococcal xylosyltransferase (Klutts *et al.*, 2007). *Cap10* mutants were unable to produce fatal infection in mice, as demonstrated with other acapsular strains such as *cap59*, *cap60* and *cap64* (Chang and Kwon-Chung, 1994, 1998, 1999; Chang *et al.*, 1996). Complementation of the *cap10* mutation restored capsule and virulence (Chang and Kwon-Chung, 1999).

The construction of Cap10p as a green fluorescence protein-conjugate revealed that the product of *CAP10* is distributed in patches within the cytoplasm of yeast cells, in cluster formations resembling vesicles.

In addition to Cap proteins, new genes necessary for capsule construction have been identified during the screen of a mutant library created by *Agrobacterium tumefaciens*-mediated transformation (Walton *et al.*, 2006), but their biochemical function and role in capsule production remain unknown.

2. Cryptococcal enzymes involved in GXM biosynthesis

The connections between the building units of GXM require the activity of different glycosyl transferases. The activity of glycosyltransferases involves the transfer of a monosaccharide from an activated sugar phosphate to an acceptor molecule. Most commonly, sugar nucleotide derivatives are used as glycosyl donors and, in the case of GXM, these basic building units consist of UDP-glucuronic acid, UDP-xylose and GDP-mannose. Some of the genes encoding for the enzymes responsible for the synthesis of these activated sugar donors have been characterized in *C. neoformans* and shown to have key roles in capsule biosynthesis and function, as will be detailed below.

Mannose is the most abundant sugar unit in GXM and its incorporation into the polysaccharide depends on the synthesis of GDP-mannose. The GDP-mannose biosynthesis pathway is initiated by the cytosolic conversion of fructose-6-phosphate into mannose-6-phosphate by phosphomannose isomerase (PMI). Mannose-6-phosphate, the product of the reaction catalyzed by PMI, is then sequentially converted into mannose-1-phosphate and GDP-mannose by the enzymes phosphomannomutase and GDP-mannose pyrophosphorylase, respectively. The *C. neoformans* gene encoding for PMI, the enzyme responsible for the first step of GDP-mannose biosynthesis, was characterized (Wills *et al.*, 2001). This gene, denominated *MAN1*, was disrupted and the resulting mutant had a poor ability to form a capsule. The mutant also produced reduced levels of exopolysaccharide and, in comparison with wild-type and reconstituted cells, had attenuated virulence in both rabbit and mouse models of cryptococcosis (Wills *et al.*, 2001).

GDP-mannose is used as a mannose donor for polysaccharides, but also for glycolipids and glycoproteins. As mentioned above, this activated sugar is produced in the cytosol, although the process of mannosylation generally occurs within different organelles. GXM synthesis, for example, is thought to occur in Golgi-related structures (Yoneda and Doering, 2006). The activity of GDP-mannose transporters is therefore required for the transfer of nucleotide-activated mannose from the cytosol to the organelles in which mannosyl transfer to acceptor molecules will occur. The occurrence of two functional GDP-mannose transporters (Gmt1 and

Gmt2) has been recently reported in *C. neoformans* (Cottrell *et al.*, 2007). Interestingly, this study demonstrates that deletion of *GMT1* generates poorly encapsulated cells that are not responsive to capsule induction conditions. In contrast, deletion of *GMT2* does not affect capsule expression. This observation clearly indicates that the transporters have distinct roles, some of them apparently relevant for capsule biosynthesis, while others are probably related to mannosylation of other glycoconjugates.

Glucuronic acid is the second sugar unit of GXM. Its addition to the polysaccharide requires the synthesis of UDP-glucuronic acid, which occurs via oxidation of UDP-glucose by UDP-glucose dehydrogenase in the cytoplasm. The gene encoding for the enzyme (*UGD1*) was cloned and expressed in *Escherichia coli* (Bar-Peled *et al.*, 2004), revealing that the cryptococcal enzyme is a dimer whose activity is regulated by its NAD^+- and UDP-glucose-binding sites. Igd1p showed membrane localization. The result was unexpected since there is no signal in the polypeptide sequence typical of membrane association or anchorage, which suggested the indirect association with the membrane via interactions with other proteins. Two independent studies revealed that disruption of *UGD1* results in acapsular cells with alterations in cell integrity, morphological defects at the bud neck, lack of growth in an animal model of cryptococcosis and enhanced sensitivity to temperature, detergent, NaCl and sorbitol (Griffith *et al.*, 2004; Moyrand *et al.*, 2004).

Xylose is the third monosaccharide component of GXM. It is used by fungal glycosyltransferases in the form of UDP-xylose, which is synthesized from the decarboxylation of UDP-glucuronic acid by UDP-glucuronic acid decarboxylase. The subcellular location of UDP-glucuronic acid decarboxylase is uncertain. In plants, UDP-xylose synthesis occurs both in the cytosol and in membranes (Reiter, 2002) and it is currently not known which source of UDP-xylose the different Golgi-localized xylosyltransferases (described later in this section) are utilizing. UDP-xylose and UDP-glucuronic acid transporters, supposedly present at the Golgi apparatus membrane, are also probably required for GXM synthesis in *C. neoformans*. As expected, mutant cells that had *UGD1* disrupted and, consequently, lacked UDP-glucuronic acid synthesis, failed to produce UDP-xylose (Griffith *et al.*, 2004; Moyrand *et al.*, 2004). The cryptococcal gene encoding for UDP-xylose synthase (*UXS1*) was also characterized and deleted, but *uxs1*Δ strains were encapsulated and grew at 37 °C (Kozel *et al.*, 2003). These results suggest that xylose incorporation into GXM is not essential for capsule formation, although it is clearly relevant for biological functions (Klutts and Doering, 2008).

Nucleotide-activated sugar donors are used by cryptococcal glycosyltransferases to form GXM. Considering that the polysaccharide consists of an α-1,3-mannan backbone containing *O*-acetyl, β-1,2/β-1,4-xylosyl- and β-1,2-glucuronyl substitutions, proteins like α-1,3-mannosyltransferase,

O-acetyltransferase, β-1,2-glucuronyltransferase and β-1,2- and β-1,4-xylosyltransferase are expected to occur in *C. neoformans*. In fact, both early and more recent studies describe characterization of some of these enzymatic activities (Jacobson, 1987; Klutts and Doering, 2008; Klutts *et al.*, 2006, 2007; Moyrand *et al.*, 2004; Sommer *et al.*, 2003; White and Jacobson, 1993; White *et al.*, 1990). In several cases, the genes encoding these enzymes were characterized, as will be described below.

An α-1,3-mannosyltransferase that catalyzes the transfer of mannose from GDP-mannose to α-1,3-linked mannose disaccharides has been described (Sommer *et al.*, 2003). The related gene, *CMT1*, was shown to be homologous to *CAP59*, a cryptococcal gene involved in capsule synthesis by participating in the process of GXM export (Garcia-Rivera *et al.*, 2004). *C. neoformans* cells in which *CMT1* expression was affected by RNA interference or genetic deletion had no 1,3-mannosyltransferase activity (Sommer *et al.*, 2003). Interestingly, the product of *CMT1*, Cmt1p, copurifies with a protein (Cas31p) that is homologous to the *CAP64* gene. Cas31p is required for xylosylation and *O*-acetylation of GXM (Moyrand *et al.*, 2004). As discussed by Sommer and coworkers (Sommer *et al.*, 2003), the copurification of two *CAP* protein homologs suggests that these polypeptides might normally be associated. In addition, the hypothesis that Cmt1p is a mannosyltransferase could suggest the product of *CAP59* also acts in the synthesis of different glycans, since mannosylation is a common feature of different cryptococcal glycoconjugates (Gutierrez *et al.*, 2007; Heise *et al.*, 2002).

The analysis of capsular phenotypes revealed that approximately 50% of the cells in which expression of the putative mannosyltransferase (Cmt1p) was blocked were acapsular (Sommer *et al.*, 2003). The remaining population, however, presented modified capsules, with lower density and shorter fibers than wild-type cells. Therefore, paradoxically, at least some of the *cmt1* mutant cells had no mannosyltransferase, but were encapsulated. A hypothesis raised by the authors is that, since fungi have a propensity to have multiple enzymes of overlapping specificity involved in glycan synthesis, other enzymes could compensate for the loss of Cmt1p. However, the hypothesis that Cmt1p is not directly related to GXM synthesis cannot be ruled out.

Once the α-1,3-mannan is synthesized, *O*-acetyl, glucuronyl and xylosyl residues are added to the growing polysaccharide. Cryptococcal protein fractions containing xylosyltransferase and glucuronyltransferase activities were described by White and coworkers (White *et al.*, 1990). Potential acceptor molecules were investigated and, interestingly, *O*-acetylated mannan served as an acceptor for glucuronyl residues but xylomannan did not. In addition, glucuronomannan served as an acceptor for xylosyl residues, although acetylated mannan did not. These observations led to the conclusion that, during GXM synthesis, mannan

is first assembled and then *O*-acetylated. Glucuronate is then added to the polysaccharide, followed by xylosyl residues.

Janbon and coworkers described for the first time a capsule synthesis gene, *CAS1*, coding for an enzyme that is apparently required for GXM *O*-acetylation (Janbon *et al.*, 2001). The gene product, Cas1p, had no homology with any known protein putatively involved in protein or polysaccharide *O*-acetylation, but sequence analysis using a program designed to find putative protein families when querying a new sequence that has failed identification using alignment methods classified Cas1p as a putative glycosyltransferase. Mutant cells in which *CAS1* had been deleted synthesized regular GXM, except that it lacked *O*-acetyl groups. Strikingly, *cas1* mutant cells were more virulent than the parental strain (Janbon *et al.*, 2001). Further studies revealed that *O*-acetylation is important for recognition of GXM by mAbs, complement activation, tissue accumulation of GXM and interference with neutrophil migration, although it had no relevance on phagocytosis by neutrophils (Kozel *et al.*, 2003). The relationship between the hypervirulent phenotype of *cas1* mutants and the properties described above remain unclear. It is noteworthy that antibody selection was used to isolate a spontaneous *O*-acetylation deficient variant that was hypovirulent (Cleare *et al.*, 1999b). Another gene (*CAS3*) necessary to complete *O*-acetylation of the capsule was described by Moyrand *et al.* (2004). The product of *CAS3*, Cas3p, shares homology with five other putative proteins, named Cas31p, Cas32p, Cas33p, Cas34p and Cas35p. Mutational analysis of the related genes demonstrated that a *cas31* mutant produces GXM with the same *O*-acetylation as that of the parental strain, while the polysaccharide from a *cas3* mutant strain had a 70% loss of *O*-acetylation. Interestingly, GXM from a *cas3*–*cas31* double mutant was completely de-*O*-acetylated. Cas31p and Cas3p were demonstrated to have very similar sequences, suggesting that they correspond to proteins that have similar functions and different regulatory mechanisms (Moyrand *et al.*, 2004).

Klutts and coworkers (Klutts *et al.*, 2007) have recently described a β-1,2-xylosyltransferase activity that transfers xylose to an α-1,3-mannose dimer. The corresponding gene, *CXT1*, was cloned and successfully expressed in *Saccharomyces cerevisiae*, which normally lacks endogenous xylosyltransferase activity. Interestingly, the product of *CXT1*, Cxt1p, is homologous to Cap10p, a product of the capsule-related gene *CAP10*. The characterization of Cxt1p represented the first *CAP* gene homolog whose product had a defined biochemical function and this homology suggests that *CAP10* and its homologs (*CAP1*, *-2*, *-4* and *-5*) encode glycosyltransferasaes that are likely to be involved in capsule synthesis. The cryptococcal xylosyltransferase is not homologous to any related enzyme in mammalian or plant cells, so its characterization defined a novel family of glycosyltransferases. This family includes *C. neoformans* Cap10p and its

homologs and is exclusive to fungi. Cas31p, described above to be involved in capsule *O*-acetylation, is also required for xylosylation (Moyrand *et al.*, 2004), suggesting that at least some of the proteins involved in capsule assembly are multifunctional.

CXT1 deletion did not affect *in vitro* growth of the mutant cells or the general morphology of their capsules, as concluded from India ink staining and immunofluorescence with mAbs to GXM (Klutts and Doering, 2008). This result was in agreement with findings by Moyrand *et al.* (2004), showing that mutant strains that lost the ability to form UDP-xylose are still encapsulated. Structural analysis of GXM and GalXM measured by NMR from *cxt1* mutants prepared on encapsulated or acapsular backgrounds revealed that the enzyme is involved in the synthesis of both polysaccharides (Klutts and Doering, 2008). However, NMR structural analysis of GXM and GalXM revealed that both polysaccharides were missing β-1,2-xylose residues. A 30% reduction of β-1,2-xylose was noted in the abundance of this residue in GXM as compared to wild-type strains. In the mutant strains, GalXM was almost completely devoid of β-1,2-linked xylose and β-1,3-linked xylose residues. Interestingly, fungal burden in the lungs of mice infected with the *cxt1* strain was significantly reduced compared to wild-type strains, indicating that deletion of *CXT1* results in an attenuated virulence (Klutts and Doering, 2008). Recently, Cxt1p has been involved in the addition of xylose residues to glycosphingolipids (Castle *et al.*, 2008), a fact which could contribute to the reduced virulence observed.

According to Doering and colleagues, at least 12 glycosyltransferases would be required for GXM synthesis (Doering, 2000; Klutts *et al.*, 2006). It is clear, therefore, that several of these enzymes remain yet to be characterized, including key molecules such as xylosyltransferases forming β-1,4 linkages with mannose, as well as glucuronyltransferase.

3. Cryptococcal enzymes involved in GalXM synthesis

Recently, GalXM mutant strains were generated for a putative UDP-glucose epimerase (Uge-1) and a putative UDP-galactose transporter (Ugt1p). The study revealed that galactose metabolism plays a central role on the virulence of *C. neoformans* (Moyrand *et al.*, 2007). The *UGE1* gene encodes a putative UDP-glucose epimerase, an enzyme that converts UDP-glucose and UDP-galactose. The *UGT1* gene encodes a putative Golgi UDP-galactose transporter. In infection studies, *uge1* mutants are cleared from the brain and lungs. The *ugt1* mutant is found in a significantly lower burden in the brain, lung and spleen. Over time *C. neoformans* is completely eliminated in the brain and lung with diminished levels in the spleen. Carbohydrate composition analysis revealed that the mutants do not secrete GalXM although their capsule size is larger than the isogenic wild-type strain. Both mutants had a growth

defect at 37 °C that was suppressed by 1% galactose addition to the medium, which suggested that absence of these enzymes also produced a cell wall defect. To date, the reason why GalXM deficiency results in disminished brain dissemination is unknown and it is unclear if the effect is due to a reduction in fungal replication in the brain, or because of impairment in crossing through the blood–brain barrier. Further studies are required to clarify the role of GalXM in dissemination.

4. Signaling pathways involved in capsule synthesis

Caspsule synthesis is a complex process that responds to the environment. For this reason, this process is highly regulated by different signal transduction pathways. In *C. neoformans*, several signal transduction pathways and their functions in *C. neoformans* have been identified (for review, see Kozubowski *et al.*, 2009). Components of the cyclic AMP signal transduction pathway, as well as mechanisms that regulate pH and CO_2 sensing, utilization of carbon, nitrogen, serum components and iron, are also relevant to capsule expression in *C. neoformans*, as summarized in Table 4.1 and discussed below.

The mechanisms by which environmental signals regulate capsule synthesis are not known, but the regulation of *CAP* genes in *C. neoformans* suggests that capsule formation is a complex and finely coordinated biosynthetic pathway. In fact, different compounds and environmental conditions have been shown to regulate capsule size by affecting the expression of *CAP* and other genes. Analyses of capsule size and quantitative real-time PCR indicated that, under capsule-inducing conditions, the expression of *CAP* genes is increased (Okabayashi *et al.*, 2005). In contrast, capsule-repressing conditions, such as supplementation of culture media with high glucose concentrations, results in the concomitant decrease of capsule size and *CAP* gene expression (Okabayashi *et al.*, 2005). The functions of these genes have not been clearly described, however. Since homologs of the *CAP* genes have been identified in fungal species that do not produce capsules (Janbon, 2004), their roles are probably not exclusively related to capsule production.

4.1. Iron sensing and capsule synthesis in C. neoformans

As stated above, iron concentration is one of the main environmental factors that influence capsule synthesis. Recently, genes encoding high affinity iron permeases (CFT1 and CFT2) were shown not to be involved in capsule size regulation. SAGE and Northern blot analyses demonstrated that iron limitation results in elevated transcripts of the *CAP60* gene (Lian *et al.*, 2005). The iron-responsive transcription factor Cir1 was also demonstrated to be a requirement for elaboration of the capsule, since *cir1* mutants of both the serotype D and serotype A strains were acapsular (Jung *et al.*, 2006). The *cir1* mutants also failed to form polysaccharide capsules in the

TABLE 4.1 Genes involved in capsule synthesis in *C. neoformans*

Gene	Putative biochemical functions	References
CAP59	Similar to CMT1, a gene encoding for mannosyltransferase; regulation of polysaccharide secretion	Chang and Kwon-Chung (1994), Garcia-Rivera *et al.* (2004), Sommer *et al.* (2003)
CAP64	Displays similarity to PRE1, a gene encoding the yeast proteasome subunit	Chang *et al.* (1996)
CAP60	Displays similarity to CEL1, a cellulose growth-specific gene of *A. bisporus*	Chang and Kwon-Chung (1998)
CAP10	Similar to CXT1, a gene enconding xylosyltransferase	Chang and Kwon-Chung (1999), Klutts *et al.* (2007)
CIR1	Iron sensing	Lian *et al.* (2005)
GPA1	Protein G alpha subunit 1, signal transduction pathways	Alspaugh *et al.* (1997)
GPR4	G protein-coupled receptor, signal transduction pathways	Xue *et al.* (2006)
PKA1	cAMP-dependent protein kinase catalytic subunit, signal transduction pathways	D'Souza *et al.* (2001)
PKR1	Protein kinase regulatory subunit, signal transduction pathways	D'Souza *et al.* (2001)
PDE1	Low-affinity phosphodiesterases; regulation of cAMP levels	Hicks *et al.* (2005)
CAC1	Adenylyl cyclase, signal transduction pathways	Alspaugh *et al.* (2002)
ACA1	Adenylyl cyclase-associated protein 1, signal transduction pathways	Bahn *et al.* (2004)
CAN2	Carbonic anhydrase 2, CO_2 sensing	Mogensen *et al.* (2006)
NRG1	Carbohydrate-regulated transcription factor	Cramer *et al.* (2006)
MAN1	GDP-mannose biosynthesis	Wills *et al.* (2001)
GMT1	Mannose transporter	Cottrell *et al.* (2007)

(*continued*)

TABLE 4.1 (*continued*)

Gene	Putative biochemical functions	References
UGD1	UDP-glucuronic acid biosynthesis	Bar-Peled *et al.* (2004), Griffith *et al.* (2004), Jacobson (1987), Moyrand and Janbon (2004)
UXS1	UDP-xylose biosynthesis	Bar-Peled *et al.* (2001), Moyrand *et al.* (2004)
CMT1	Mannosyltransferase	Sommer *et al.* (2003)
CAS1	GXM *O*-acetylation	Janbon *et al.* (2001)
CAS3	GXM *O*-acetylation	Moyrand *et al.* (2004)
CAS31	GXM *O*-acetylation	Moyrand *et al.* (2004)
CXT1	Xylosyltransferase	Klutts and Doering (2008), Klutts *et al.* (2007)

presence of elevated CO_2 concentrations. Microarray analysis allowed the evaluation of the potential targets of Cir1 regulation resulting in the loss of capsule (Jung *et al.*, 2006). The only gene that had its regulation altered in the *cir1* mutants was *CAS32*, which belongs to a family (CAS) of genes that are homologs of *CAP64* (Moyrand *et al.*, 2004). The *CAS* genes are involved in insertion of xyloxyl substitutions and/or *O*-acetylation of GXM (Moyrand *et al.*, 2004). Therefore, it was not clear whether transcriptional changes in capsule-related genes caused the capsule defect in the *cir1* mutants. It has therefore been speculated that Cir1 could be necessary for capsule formation through the regulation of genes needed for sensing iron and CO_2 levels (Lian *et al.*, 2005).

4.2. Role of cAMP and other signal transuction pathways in capsule synthesis in C. neoformans Elements of the cyclic AMP (cAMP)-dependent pathway control melanin and capsule production in *C. neoformans* (D'Souza and Heitman, 2001). Strains in which the cryptococcal gene encoding for adenylyl cyclase (*CAC1*) was disrupted lost the ability to synthesize capsule and melanin (Alspaugh *et al.*, 2002). Consequently, the mutants were unable to cause disease in animal models of cryptococcosis. Exogenously added cAMP suppressed the capsule defect of adenylyl cyclase mutant strains (Alspaugh *et al.*, 2002). The reintroduction of the *CAC1* gene in the mutant strains reverted the *cac1* phenotype to wild-type phenotypes. An upstream regulatory element of adenylyl cyclase, Aca1 (for adenylyl cyclase-associated protein 1) was further demonstrated to

regulate capsule production via the Cac1–cAMP–protein kinase A (PKA) pathway (Bahn *et al.*, 2004). In addition, the activity of Cac1 is stimulated by HCO_3^-, produced through hydration of CO_2 by carbonic anhydrase 2 (Can2) (Mogensen *et al.*, 2006), which is consistent with the well-described ability of *C. neoformans* to increase capsule size in response to CO_2 (Granger *et al.*, 1985; Zaragoza *et al.*, 2003a).

The gene encoding the major cAMP-dependent protein kinase catalytic subunit in *C. neoformans* (*PKA1*) was identified and disrupted (D'Souza *et al.*, 2001). *Pka1* strains failed to produce melanin or capsule and were avirulent as a result. ''Interestingly, disruption of the gene encoding the regulatory subunit Pkr1 yielded mutant strains which overproduced capsule'' due to a constitutively activated cAMP pathway. These mutant strains were hypervirulent in animal models (D'Souza *et al.*, 2001). Pka1 catalytic subunit acted downstream of the Pkr1 regulatory subunit, since the phenotype of a *pkr1–pka1* double mutant was similar to that observed in single *pka1* mutants (D'Souza *et al.*, 2001).

The cAMP pathway is highly regulated by the cell and genes involved in this regulation have been identified. An early study (Alspaugh *et al.*, 1997) revealed that the α-subunit 1 of the cryptococcal G protein (Gpa1), which in other species is necessary to activate adenylate cyclase, regulates capsule expression in *C. neoformans* in response to environmental signals. The impaired ability of mutant cells lacking Gpa1 to produce capsule and melanin led to an attenuated virulence in animal models of cryptococcosis. Exogenous cAMP restored mating, melanin and capsule production in *gpa1* mutant strains (Alspaugh *et al.*, 1997). This observation supported the hypothesis that cAMP-dependent signaling proteins were involved in capsule formation. Indeed, it has been further demonstrated that Gpa1 transcriptionally regulates at least nine genes that are known or presumed to function in capsule synthesis or assembly, including *CAP10*, *CAP59* and *CAP64* and genes encoding for *O*-acetyltransferase and UDP-xylose synthase (Pukkila-Worley *et al.*, 2005). Gpa1 is apparently under the control of the G protein-coupled receptor Gpr4, which engages amino acids in the cAMP–PKA pathway involved in the regulation of capsule formation (Xue *et al.*, 2006). Recently, it has been shown that disruption of *CRG2*, encoding a G-protein, results in increased cAMP levels and capsule enlargement, which suggests that Crg2 negatively regulates the Gpa1–cAMP pathway (Shen *et al.*, 2008).

Using a gene microarray approach, Cramer and coworkers (Cramer *et al.*, 2006) demonstrated that the expression of *NRG1*, a gene encoding for a homolog of the *C. albicans* Nrg1 and *S. cerevisiae* Nrg1/Nrg2 family of transcription factors regulated in response to carbohydrate availability, is altered in a *gpa1* mutant of *C. neoformans*. Nrg1 is under transcriptional control of the cAMP pathway (Liu, 2001). In fact, glucose and intact cAMP are required for wild-type levels of transcription of the cryptococcal

NRG1 gene (Cramer *et al.*, 2006). Disruption of the *C. neoformans NRG1* gene by homologous recombination resulted in hypocapsular mutants with attenuated virulence. The reduced ability of *nrg1* mutants to form a capsule was probably associated with an effect on the expression of *UGD1*, which encodes a UDP-glucose dehydrogenase that is required for capsule formation (Moyrand *et al.*, 2004). Interestingly, the Nrg1 protein contains a consensus sequence for phosphorylation by the cAMP-dependent PKA that is required for its full function (Cramer *et al.*, 2006).

Intracellular cAMP concentrations depend on the activity of the low- and high-affinity phosphodiesterases (PDEases), Pde1 and Pde2, respectively. In *C. neoformans*, mutational analysis revealed that Pde2 has no apparent role in regulating intracellular cAMP levels. In contrast, Pde1 is clearly relevant to cAMP-dependent signaling pathways regulating capsule formation (Hicks *et al.*, 2005). Intracellular cAMP levels are elevated in a *pde1* mutant. In addition, deletion of the *PDE1* gene restored the ability of *gpa1* mutants to form capsule (Hicks *et al.*, 2005). Measurements of cAMP intracellular content revealed that the levels of this second messenger in a double *pde1–pka1* mutant strain is approximately 15-fold higher than the cAMP content in a *pka1* single mutant strain. In addition, a mutation in a consensus PKA phosphorylation site reduces Pde1 function. These results clearly indicate the occurrence of a feedback regulation system controlling cAMP levels, via its degradation by Pde1 (Hicks *et al.*, 2005).

The role of other signal transduction pathways in capsule synthesis has not been so extensively studied as in the case of the cAMP pathway. During capsule enlargement, the signal transduction pathways involved in capsule enlargement can vary depending on the inducing signal (Zaragoza *et al.*, 2003a). Using mutants affected in cAMP accumulation or Ras1, capsule enlargement was shown to be different when capsule growth was induced by serum or CO_2. Recent findings suggest that protein kinase C, which is activated by diacylglycerol, influences in capsule structure (Gerik *et al.*, 2008). Concerning MAPK, several pathways have been identified in *C. neoformans* (pheromone response pathway, dependent on Ste12 and Cpk1 and stress-activated pathways, dependent on Hog1). Disruption of *STE12α* in serotype D affects capsule production during infection, but not during *in vitro* growth (Chang *et al.*, 2000). Disruption on the same gene in serotype A background resulted in a 50% reduction in capsule size (Yue *et al.*, 1999), although virulence in murine models was not affected. Concerning Hog1, it was found that this pathway negatively regulated capsule synthesis in serotype A background, since *hog1* mutants had larger capsules than isogenic wild-type strains (Bahn *et al.*, 2005). However, this effect disappeared when elements of the cAMP were disrupted in conjunction with *HOG1*,

suggesting that Hog1 could negatively regulate elements from the cAMP pathway. Curiosly, disruption of Hog1 did not have any effect on serotype D background. These findings indicate that capsule synthesis is a complex process that is regulated at multiple levels, such as environmental signals, induction of different transduction pathways and serotype-linked elements.

B. GXM traffic in *C. neoformans*

Microbial polysaccharides are usually polymerized at the outer layer of the plasma membrane or even at the extracellular space. Bacterial capsular polysaccharides, for instance, are synthesized by plasma membrane glycosyltransferases whose active sites face the extracytoplasmic environment (Raetz and Whitfield, 2002; Whitfield, 2006; Whitfield *et al.*, 2003). In fungal cells, glucan and chitin synthases, enzymes responsible for the synthesis of cell wall components, also localize to the plasma membrane (Leal-Morales *et al.*, 1994; Ortiz and Novick, 2006; Valdivia and Schekman, 2003), which supports the idea that chitin and glucan are polymerized at extracellular sites. In contrast to other microbial polysaccharides, GXM is synthesized intracellularly (Feldmesser *et al.*, 2001; Garcia-Rivera *et al.*, 2004), more specifically inside the Golgi apparatus (Yoneda and Doering, 2006). GXM traffic to the cell surface apparently involves transport by secretion of polysaccharide-containing vesicles (Feldmesser *et al.*, 2001; Garcia-Rivera *et al.*, 2004; Rodrigues *et al.*, 2007), which somehow cross the cell wall, releasing their content into the extracellular space. The released polysaccharide is then connected to the cell wall or incorporated into the growing capsule by putative mechanisms that will be discussed below.

The first evidence that GXM is synthesized intracellularly was presented in a study by Feldmesser *et al.* (2001), who noted that mAbs to the polysaccharide reacted with cytoplasmic structures of about 100–200 nm in diameter consistent with vesicles. These results were further supported by subsequent studies using immunogold labeling. Four different mAbs to GXM reacted with cytoplasmic vesicles in four strains of *C. neoformans* (Garcia-Rivera *et al.*, 2004), suggesting that the capsular polysaccharide is synthesized in the cytoplasm and exported to the exterior of the cell in secretory vesicles.

Different genes related to secretion mechanisms in yeast have been described in the last three decades (Lyman and Schekman, 1996). In yeast cells, highly conserved small GTPases are fundamental to exocytic processes (Spang and Schekman, 1998). One of the GTPases regulating exocytosis is Sec4p, a highly conserved molecule that belongs to the Sec4/Rab8 subfamily of Rab GTPases (Lipschutz and Mostov, 2002).

This protein has homologs in several eukaryotic systems and, in fact, a Sec4p cryptococcal homolog was recently described (Yoneda and Doering, 2006). A gene encoding for a Sec4p homolog in *C. neoformans*, *SAV1*, has been cloned and disruption of the gene resulted in decreased acid phosphatase secretion, suggesting a role in protein secretion. The most prominent cellular feature of the *sav1* mutant, however, was a marked accumulation of post-Golgi exocytic vesicles in the cytoplasm (Yoneda and Doering, 2006). These vesicles were recognized by an antibody to GXM, confirming that the synthesis of the polysaccharide occurs at intracellular sites. This study also supported the hypothesis that GXM traffic involves secretory vesicles derived from the Golgi apparatus.

The fact that treatment of *C. neoformans* with brefeldin A, an inhibitor of the Golgi-derived transport of molecules, resulted in a significant inhibition of capsule formation (Hu *et al.*, 2007) supports the notion of capsular polysaccharide synthesis in Golgi-derived vesicles. However, Golgi-derived vesicles, in the classic secretory pathway, are expected to fuse with the plasma membrane and release their internal content to the extracellular space (Mallabiabarrena and Malhotra, 1995). Therefore, such a secretion mechanism could explain how GXM reaches the periplasmic space, but not how the polysaccharide reaches the outer layer of the cell wall to be incorporated into the growing capsule. Late endosomes, the cellular precursors of multivesicular bodies, can be connected with structures from the Golgi and the *trans*-Golgi network (Fevrier and Raposo, 2004). Therefore, one possibility is that the GXM-containing, post-Golgi vesicles of *C. neoformans* connect to the endosomal pathway, resulting in the release of exosome-like structures. In this case, the production of GXM-containing extracellular vesicles by *C. neoformans* would be expected. In fact, different studies supported the hypothesis that *C. neoformans* produce exosome-like vesicles containing the polysaccharide (Rodrigues *et al.*, 2000, 2007, 2008b). An insertional mutagenesis screen identified *ARF1* as a gene necesary to produce capsule (Walton *et al.*, 2006). This gene encodes an ADP-ribosylating factor involved in vesicle formation, which supports the notion that vesicles are key mediators in capsule formation.

Membrane vesicles that migrate from the plasma membrane to the cell wall of *C. neoformans*, as well as in the region between the cell wall and the capsule, have been described in different studies (Rodrigues *et al.*, 2000; Takeo *et al.*, 1973a,b). The occurrence of vesicles within the cell wall of cryptococci or in its adjacent extracellular microenvironment was also evidenced after *in vitro* growth and vesicle-like structures near the edge of the capsule or in the cryptococcal cell wall were also observed in lung sections of mice infected with *C. neoformans* (Rodrigues *et al.*, 2007). These vesicles were isolated from *C. neoformans* cultures by differential

centrifugation and analyzed by transmission electron microscopy (Rodrigues *et al.*, 2007). They corresponded to spheres within the diameter range of 20–400 nm presenting as bilayered membranes. Using morphological criteria, four main vesicle groups were identified, including (i) electron dense and (ii) electron lucid vesicles, (iii) vesicular structures with membrane-associated electron dense regions and (iv) vesicles containing hyperdense structures resembling a dark pigment. Vesicle production required cell viability and mass spectrometry analysis of vesicular components revealed the presence of different lipids (Rodrigues *et al.*, 2007) and proteins (Rodrigues *et al.*, 2008b) associated with virulence mechanisms of *C. neoformans*. The association of vesicular secretion and virulence was recently confirmed (Panepinto *et al.*, 2009). These authors showed that mutant strains unable to express the exocytosis-related protein Sec6p were defective in a number of virulence factors including laccase, urease and soluble polysaccharide and demonstrated attenuated virulence in mice.

Vesicle secretion is apparently not exclusive to encapsulated cells. Although immunogold labeling of vesicles purified from encapsulated cells with an antibody to GXM followed by transmission electron microscopy revealed that the polysaccharide is present in the vesicular matrix, extracellular vesicles are also detectable in culture supernatants of acapsular mutants (Rodrigues *et al.*, 2007, 2008b). In addition, a recent study revealed the existence of discrete GalXM-containing lipid bodies in the surface of *C. neoformans*, suggesting that this polysaccharide may also be delivered in vesicles (De Jesus *et al.*, 2009). In fact, chromatographic analysis of vesicle preparations of acapsular cells revealed the presence of galactose, xylose and mannose.The mechanism of vesicular secretion of capsular components is putatively relevant for capsule assembly, since induction of capsule expression is accompanied by an enhanced detection of GXM in vesicular fractions (Rodrigues *et al.*, 2007). Acapsular cells were able to extract GXM from vesicular fractions and incorporate the polysaccharide into their cell surfaces, indicating that the fungus has the metabolic apparatus to extract GXM from membrane vesicles. The mechanisms potentially involved in GXM release from vesicles and incorporation into the growing capsule are still not clear. According to Siafakas and coworkers (Siafakas *et al.*, 2007), the cell wall-associated enzyme phospholipase B could be involved in the release of the molecules transported across cell walls inside membrane-bound vesicles.

The biogenesis of the *C. neoformans* secretory vesicles is still obscure, but several different lines of evidence lead to the hypothesis that they are -exosome-like structures. Early studies by Takeo and coworkers described in *C. neoformans* unusual plasma membrane invaginations containing small vesicles, which would be associated with capsule synthesis (Takeo *et al.*, 1973a,b). Multivesicular compartments in close association

with the cell wall were also recently described in *C. neoformans* (Rodrigues *et al.*, 2008b). Finally, proteomic analysis of the *C. neoformans* vesicles revealed a complex protein composition that included several proteins described in mammalian exosomes (Rodrigues *et al.*, 2008b). These observations, together with morphological data, support the idea that extracellular vesicles produced by *C. neoformans* are exosome-like structures. A scheme comprising vesicle formation and GXM traffic in *C. neoformans* is proposed in Fig. 4.5.

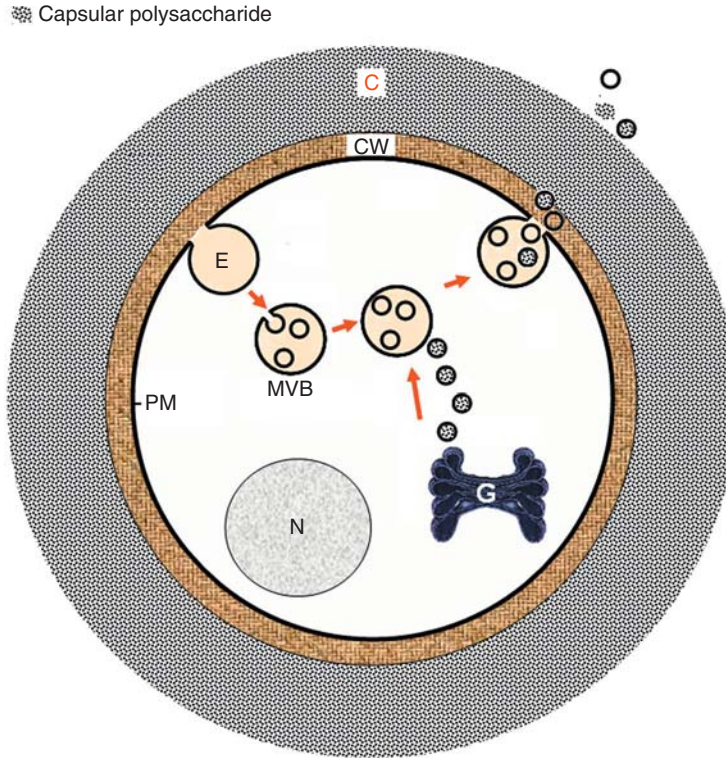

FIGURE 4.5 Proposed model for GXM traffic in *C. neoformans*. GXM is synthesized in the Golgi apparatus and released in post-Golgi vesicles. These vesicles would be integrated into endocytic compartments/multivesicular bodies that are further addressed to the plasma membrane. After fusion of endosome-derived structures with the plasma membrane, small vesicles are released into the periplasmic space to be translocated to the extracellular space. Extracellular vesicles would then release their content, which includes capsular polysaccharides. Abbreviations: N, nucleus; G, Golgi apparatus; PM, plasma membrane; E, endosome; MVB, multivesicular body; CW, cell wall; C, capsule.

C. Polysaccharide connections at the *C. neoformans* surface

The cell wall of *C. neoformans* is a complex molecular network that includes, chitin and MPs, lipids, pigments and bioactive enzymes besides well-characterized structural components such as glucans (Nimrichter *et al.*, 2005). This heterogeneous structure is surrounded by multiple layers of capsular polysaccharides, which implies that capsule assembly in *C. neoformans* involves the connection of capsular components to cell wall elements, as well as molecular interactions within the capsule.

Proteins are apparently not involved in the connection of cell wall components to the capsule (Reese and Doering, 2003; Rodrigues *et al.*, 2008a). In contrast, polysaccharides play a key role in capsule attachment to the cell wall. It has been demonstrated that GXM anchoring to the cryptococcal wall requires α-1,3-glucan (Reese and Doering, 2003; Reese *et al.*, 2007). Cryptococcal cells with a disrupted α-1,3-glucan synthase gene lost the ability to synthesize α-1,3-glucan (Reese *et al.*, 2007). These cells had the ability to secrete capsular material, although they lacked surface capsule (Reese and Doering, 2003). Interestingly, acapsular mutants of *C. neoformans* treated with glucanase, but not protease, still bound to GXM, but in a defective manner (Reese and Doering, 2003). This result indicated that cell wall molecules other than glucan are required for capsule attachment to the cell wall.

The presence of cell wall chitin-derived molecules in *C. neoformans* has been demonstrated by microscopic and molecular approaches (Baker *et al.*, 2007; Banks *et al.*, 2005; Rodrigues *et al.*, 2008a). Chitosan, the de-*O*-acetylated form of chitin, was demonstrated to be necessary for the correct architecture of the cell wall, melanin and capsule complex (Baker *et al.*, 2007). In addition, chitin-derived oligomers, whose presence at the surface of *C. neoformans* had been suggested a few years ago (Woyke *et al.*, 2002), have been recently shown to be involved in capsule anchoring to the cell wall (Rodrigues *et al.*, 2008a).

The wheat germ agglutinin (WGA) is a lectin that binds to sialic acids and β-1,4-*N*-acetylglucosamine (GlcNAc) oligomers. In *C. neoformans*, different techniques revealed that chitin-bound oligomers of GlcNAc, but not sialic acids or intact chitin, are the molecules recognized by the lectin (Rodrigues *et al.*, 2008a). Confocal microscopy demonstrated that the chitin-like oligomers recognized by WGA form projections linking the capsule to the cell wall (Fig. 4.6). Interestingly, some of the molecules recognized by WGA formed unique ring-like structures connecting the capsule to the cell wall during yeast budding (Rodrigues *et al.*, 2008a). The projection of chitin-like oligomers into the capsule, combined with the study of Zaragoza and colleagues showing the occurrence of capsule channels (Zaragoza *et al.*, 2006a) strongly supports the concept that the

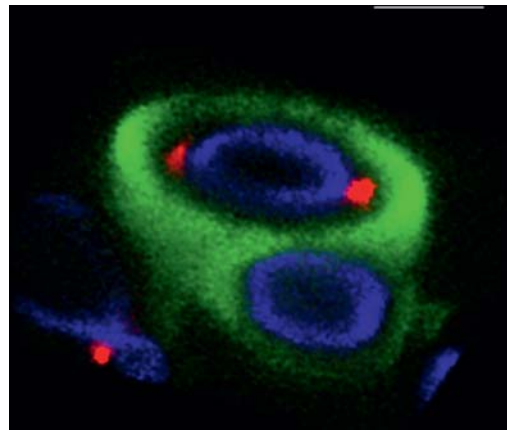

FIGURE 4.6 Cell wall–capsule connections in *C. neoformans*. Staining of capsular structures with a monoclonal antibody to GXM (green), cell wall chitin with calcofluor white (blue) and chitin-derived oligomers with WGA (red) followed by confocal analysis reveals that projected structures recognized by WGA connect the cell wall and the capsule. For experimental details, see Rodrigues *et al.* (2008a).

capsule contains heterogeneous microenvironments despite a relatively homogenous appearance when visualized by India ink suspension and light microscopy. The mechanisms by which this singular architecture is maintained are still not understood.

Treatment of encapsulated *C. neoformans* cells with chitinase released GXM from the cell surface and reduced capsule size (Rodrigues *et al.*, 2008a). The fact that chitinase treatment did not fully remove the cryptococcal capsule may be explained by the ability of cell wall glucans to anchor capsular polysaccharides (Reese and Doering, 2003; Reese *et al.*, 2007). Based on the well documented ability of acapsular mutants to bind GXM to the cell surface (Kozel, 1977), it was demonstrated that chitinase-treated acapsular cells still bound soluble GXM (Rodrigues *et al.*, 2008a). However, in contrast to untreated cells, yeast cells that were preincubated with the enzyme-bound GXM in a manner that formed a loose polysaccharide coat at the surface of *C. neoformans*, as previously described for glucanase-treated acapsular mutants (Reese and Doering, 2003). In combination, these results indicate an association of chitin-derived structures and α-1,3-glucan with GXM. However, the hypothesis that other components connect glucans and chitin-derived structures to capsular components cannot be discarded, since loss of cell wall polysaccharides would disturb cell wall assembly and, consequently, capsule anchoring.

IV. CAPSULE FUNCTIONS IN *C. NEOFORMANS*: THE CAPSULE AS A VIRULENCE FACTOR: FUNCTION DURING THE INTERACTION WITH THE HOST

C. neoformans virulence is a complex process and there is evidence that both fungal components and the immune response to infection combine to damage the host (Casadevall and Pirofski, 2003). Concerning fungal factors, the capsule is the major virulence factor involved in the pathogenesis. As stated previously, the polysaccharide from the capsule can be found attached to the cell forming the physical structure and released to the medium, as exopolysaccharide. Multiple studies indicate that both types of polysaccharide confer beneficial effects for the fungus during its interaction with the host. In general, the capsule virulent function is conveyed by mediation of a protective effect for cryptococcal cells, such as interfering with phagocytosis by macrophages and ameboid environmental predators. However, the capsular polysaccharide also contributes to virulence through multiple deleterious effects on the immune response, which in turn facilitates fungal propagation in tissues and ultimately kills the host.

A. Role of the capsule during interaction with the host

The presence of the capsule confers beneficial effects to *C. neoformans* during infection, especially during its interaction with phagocytic cells and environmental predators.

1. Phagocytosis

Phagocytosis is an important process required for the clearance of a large number of pathogens. From the first studies in the early 1970s, it was shown that the capsule had strong antiphagocytic properties, which was the first evidence of its role as virulence factor. As a consequence, the capsule is involved not only in evading killing by phagocytic cells, but also by means of inhibiting a subset of several phenomena which are dependent of phagocytosis. For example, a reduced phagocytosis translates into reduced T cell proliferation (Syme *et al.*, 1999) and antigen processing and presentation by macrophages, all resulting in an interference with the T cell response (Vecchiarelli *et al.*, 1994a,b). Despite the inhibitory role of the capsule, phagocytosis does occur during infection. During the first hours of infection, a significant proportion of the yeast cells injected in the lungs are found inside phagocytic cells (Feldmesser *et al.*, 2000). This result indicates that during infection, there are mechanisms that overcome the antiphagocytic effect of the capsule. So far two mechanisms for this have been described: (1) the presence of opsonins, (2) direct interaction of the polysaccharide fibers with phagocytic receptors that occur after capsule structure rearrangements.

a. Opsonins required for phagocytosis Classically, there have been two types of opsonins that induce *C. neoformans* phagocytosis, which are antibodies and proteins from the complement system. These opsonins bind to the capsular polysaccharide. We will briefly review the interaction that is formed between these opsonins and the polysaccharide fibers.

(a) *Antibody-mediated phagocytosis*. A large number of mAbs that recognize GXM have been obtained (Casadevall *et al.*, 1992; Dromer *et al.*, 1987b; Eckert and Kozel, 1987; Kozel *et al.*, 1998; Pirofski *et al.*, 1995; Todaro-Luck *et al.*, 1989). In addition to the protective effect of these antibodies, their binding to the capsule and function as phagocytic opsonins have also been tested. IgG antibodies bind the *C. neoformans* capsule and they induce phagocytosis through binding to the Fc receptors (FcRI and FcRIII). Interestingly, IgM to capsular polysaccharide is opsonic in the absence of complement. IgM opsonic efficacy correlates with the binding pattern of the antibodies to the capsule. IgM to GXM can bind in two different patterns to the capsule as shown by immunofluorescence, punctuate and annular (Cleare and Casadevall, 1998; Cleare *et al.*, 1999a; Nussbaum *et al.*, 1997). Annular pattern correlates with a protective effect in mice and with a good opsonic efficacy. In contrast, mAbs that bind to the capsule in a punctuate pattern are not protective and do not induce phagocytosis. Since no FcR has been identified for IgM antibodies, the mechanism by which this isotype induces *C. neoformans* phagocytosis posed a challenging question. Analysis of this phenomenon revealed that phagocytosis of *C. neoformans* in the presence of IgM antibodies occurred through binding of the polysaccharide fibers to the complement receptors (CR) and specifically to CD18. It was hypothesized that the binding of the antibody to the capsule produced some structural rearrangements that resulted in the exposure of CD18 binding sites in the polysaccharide, which in turn produced phagocytosis through the CR (Taborda and Casadevall, 2002). A similar effect was demonstrated for IgG antibodies and $F(ab)_2$ fragments (Netski and Kozel, 2002; Zaragoza *et al.*, 2003b). In addition to mAbs to the capsule, other opsonic antibodies have been described. In this sense, Merkel and Scofield described the isolation of an opsonic mAb whose epitope was abundantly found, not in the capsule, but at the level of the cell wall (Merkel and Scofield, 1999).

(b) *Complement-mediated phagocytosis*. The other opsonin whose function has been extensively studied in *C. neoformans* is complement. The capsule of *C. neoformans* is a strong inducer of complement system activation through the alternative pathway, resulting in a large deposition of iC3b on the capsule (Kozel and Pfrommer, 1986; Kozel *et al.*, 1989). Encapsulated cells and purified GXM-induced C3 release from

peritoneal cells and the amount of C3 released correlated with the degree of encapsulation of the cell (Blackstock and Murphy, 1997). However, binding of C3 to the capsule is not sufficient to induce phagocytosis, because there are many *C. neoformans* strains that are not phagocytosed in the presence of complement. It was later found that there was a negative correlation between the phagocytic index of the strains and the size of the capsule (Yasuoka *et al.*, 1994), suggesting that complement-mediated phagocytosis inhibition correlated with the molecular size of the GXM molecules produced by different strains. Earlier studies had suggested that cells with a large capsule bound a lower amount of C3, also suggesting a mechanism by which cells with a larger capsule were less efficiently phagocytosed (Kozel *et al.*, 1996). More recently, studies using confocal microscopy revealed that C3 deposition was different in cells with small and large capsules. In cells with large capsule, C3 was located close to the cell wall, away from the capsule edge. This differential localization suggested a mechanism by which capsule enlargement interfered with the interaction of C3 in the capsule with its corresponding phagocytic receptor (Zaragoza *et al.*, 2003b). Interestingly, C3 proteins from different sources (human, mouse, rat) bound differentially to the capsule, suggesting that the complement pathway from different hosts may have different functions in the defense against *C. neoformans* (Gates and Kozel, 2006), which could explain the differences in susceptibility of the host to *C. neoformans* infection (see comment in Pirofski, 2006).

(c) *Effect of the interaction between the different opsonins with the capsule.* During the course of an infection, both complement and antibodies are available and it has been shown that the presence of both opsonins can result in different outcomes. For example, the presence of antibodies to the capsule produces strong complement activation through the classical pathway, leading to a fast deposition of complement on the capsule (Kozel *et al.*, 1998). This effect is largely dependent on the isotype of the antibody. While IgG mAbs have a strong effect, IgM produces a moderate activation. In addition, it has also been shown that addition of mAbs to the capsule not only produces C activation through the classical pathway, but, when added in a sufficient amount, allows complement deposition at the capsule edge, as opposed to deep inside the capsule, resulting in an increase in C-mediated phagocytosis (Zaragoza and Casadevall, 2006).

2. GXM receptors

Several receptors have been described that interact with the capsular polysaccharide, triggering different responses in phagocytic cells (Ellerbroek *et al.*, 2004c; Levitz, 2002; Monari *et al.*, 2005a; Yauch *et al.*, 2004).

These receptors are involved in phagocytosis, either because they directly induce or prevent pathogen engulfment, or because they belong to pattern recognition receptors, which are involved in phagocytosis regulation, such as Toll-like receptors. In the first group, GXM can interact with CD18, which forms part of the complement receptor and this interaction can trigger CR-mediated phagocytosis in the absence of opsonin. GXM can also directly interact with FcγRIIB (Monari *et al.*, 2005a), which has been involved in *C. neoformans* uptake by phagocytic cells (Monari *et al.*, 2005a; Syme *et al.*, 2002). However, FcRγII engagement produces inhibitory signals that contribute to immune unresponsiveness, as will be reviewed later on. In the second group, it has been described that GXM can interact with CD14 and Toll-like receptors 2 and 4. These receptors are involved in macrophage stimulation and their functions have been characterized.

The role of other receptors that are known to bind mannose and other polysaccharides has been studied, since the capsular polysaccharide is particularly rich in mannose. These receptors are the mannose receptor and SIGN-R1. In the case of the mannose receptor, it has been shown that it plays a role in *C. neoformans* uptake by dendritic cells (Syme *et al.*, 2002). In addition, *C. neoformans* produces immunomodulation through secretion of MPs which interact with the mannose receptor (Levitz and Specht, 2006). The SIGN-R1 receptor binds bacterial polysaccharides, but in the case of *C. neoformans*, it has been shown that it does not bind GXM (De Jesus *et al.*, 2008).

The role of various cellular receptors in phagocytocis and GXM localization has been studied using knock-out mouse strains. None of the receptors were completely required for serum clearance or hepatosplenic polysaccharide degradation (Yauch *et al.*, 2005). In another study, Yauch *et al.* (2004) sought to study the role of the CD14, TLR2 and TLR4 receptors and the function of MyD88, in cryptococcal infection *in vitro*. The results revealed that MyD88 plays a critical role during cryptococcosis while the TLR2 and CD14 receptors play minor roles. This study demonstrated that MyD88$^{-/-}$ mice succumbed to *C. neoformans* infection significantly earlier than wild-type mice when infected either i.n. or i.v. MyD88$^{-/-}$ mice infected intranasally had increased numbers of CFU in the lungs and elevated GXM levels in the lungs and sera, which correlated with the reduced survival of these mice. Additionally, mice lacking TLR2 had significantly reduced survival only after pulmonary challenge, while CD14$^{-/-}$ mice displayed a trend towards reduced survival after intravenous infection. Since the MyD88$^{-/-}$ mice were more susceptible to cryptococcosis than the TLR2 and TLR4 mutant mice, the authors suggested that MyD88 besides serving as an adaptor protein for TLR SIGN-R1 there are other yet-to-be identified receptors for GXM on red pulp macrophages

(De Jesus *et al.*, 2008). They also suggested that it does not appear to be involved in macrophage uptake of GXM.

3. Capsule role during macrophage intracellular parasitism and intracellular GXM accumulation

A key feature of cryptococcal pathogenesis is its ability to evade killing inside phagocytic cells. *C. neoformans* is considered a facultative intracellular pathogen. This finding was first observed in the early 1070s (Diamond and Bennett, 1973) and it has been more carefully studied in the last years in macrophages, microglial cells and endothelial cells (Chretien *et al.*, 2002; Coenjaerts *et al.*, 2006; Feldmesser *et al.*, 2000; Lee *et al.*, 1995b). *C. neoformans* can survive inside macrophages and exploits the intracellular macrophage environment for its replication. Furthermore, there are several mechanisms described that allow *C. neoformans* extrusion from infected macrophages (Alvarez and Casadevall, 2006; Ma *et al.*, 2006) or even cell-to-cell spread (Alvarez and Casadevall, 2007; Ma *et al.*, 2007). Concerning the role of the capsule in this process, it has been shown that acapsular mutants cannot replicate inside phagocytic cells (Feldmesser *et al.*, 2000), which indicates that the capsule plays a key role in intracellular parasitism. Along these lines, acapsular mutants are not extruded by macrophages and this phenotype is reverted by precoating of acapsular cells with GXM (Alvarez and Casadevall, 2006). A typical feature after *C. neoformans* engulfment is that there is an important accumulation of GXM-containing vesicles (Lee *et al.*, 1995b; Tucker and Casadevall, 2002). These vesicles became permeable during the course of intracellular infection, which suggested a new mechanism by which *C. neoformans* interfered with macrophage functioning (Tucker and Casadevall, 2002). Since phagocytic cells have several receptors for GXM, they can directly engulf this capsular polysaccharide component (Monari *et al.*, 2003). However, binding and internalization differ between phagocytic cells. GXM accumulated constitutively in macrophages, in contrast to neutrophils, in which it was rapidly expelled (Monari *et al.*, 2003). Accumulation of GXM by macrophages was dependent on the cytoskeleton and several macrophage intracellular pathways (Chang *et al.*, 2006b). The presence of intracellular polysaccharide altered the function of the macrophages at multiple levels. For example, once phagocytosed, encapsulated cells do not induce nitric oxide synthase, a phenomenon that does occur when acapsular mutants are ingested (Naslund *et al.*, 1995). Other mechanisms by which capsular components can interfere with the activity of immune cells will be reviewed below. Finally, it has recently been shown that the capsule confers protection against reactive oxygen species, which are important antifungal molecules produced by macrophages during phagocytosis. In addition, capsule enlargement also increased the survival of the cells in the presence of

these radicals and antifungal molecules, such as defensins and Amphotericin B (Zaragoza *et al.*, 2008b). These results suggest mechanisms for how *C. neoformans* evades killing inside the phagolysosome and are in agreement with published data in *Klebsiella pneumoniae* (Campos *et al.*, 2004), which demonstrate that the bacterial capsule confers protection against defensins.

4. Capsule and dissemination

Extrapulmonary dissemination is a key process for disease causation during fungal pathogenesis (reviewed in Eisenman *et al.*, 2007; Filler and Sheppard, 2006). Several microbial factors, such as laccase, phospholipase, and the capsule are involved in the dissemination process. The capsule plays an important role in this process, since acapsular mutants have impaired dissemination to the brain (Wilder *et al.*, 2002). Although this effect could be due to the lack of virulence of the strain and rapid clearance by the immune system, there are reports that the polysaccharide capsule plays an active role in this process. For example, a seminal report indicates that during dissemination through the organism, *C. neoformans* manifests important capsule structure changes that may be involved in tissue invasion (Charlier *et al.*, 2005). These authors found that a serotype A strain (H99) that was exclusively reactive with a serotype A-specific mAb *in vitro*, became reactive to a serotype D-specific mAb during endothelial crossing of the blood–brain barrier. These findings suggest that *C. neoformans* is able to modulate its capsule structure during dissemination, allowing for adaptation to different environments, thereby enhancing dissemination.

During the course of infection and dissemination, *C. neoformans* must cross epithelial and endothelial barriers. *C. neoformans* can bind to epithelial cells (Merkel and Cunningham, 1992). In this way, the capsule contributes to the binding of *C. neoformans* cells to human alveolar epithelial cells (Barbosa *et al.*, 2006). This interaction resulted in a decreased viability of epithelial cells. In a different report the same authors demonstrated that epithelial cells did bind GXM through interaction with CD14 and that this binding resulted in an increased IL-8 production (Barbosa *et al.*, 2007). Other reports, however, also suggest that acapsular mutants can efficiently bind to human lung epithelial cell lines (Merkel and Scofield, 1997), implying that binding to epithelia can also occur through selective binding of yeast adhesins present in the cell wall.

Concerning endothelial cells, capsular and acapsular *C. neoformans* cells cross brain microvascular endothelial cells through transcytosis (Chen *et al.*, 2003). Transcytosis was accompanied by disruption of tight junctions and alterations in the cytoskeleton. The role of the capsule in binding endothelial cells is not clear. Binding to endothelial cells occurs for both encapsulated and acapsular strains, although binding and

transcytosis, was more efficient for encapsulated strains (Chen *et al.*, 2003). In agreement with this idea, a mutant defective in CPS1 (encoding a putative glycosyltranferase) manifested a slight decrease in capsule size and presented a marked reduced adherence to brain endothelial cells (Chang *et al.*, 2006a). However, although this mutant has a smaller capsule size, the structure is still present, which indicates that the capsule is not a complete requirement for *C. neoformans* to bind to endothelial cells. In this sense, Ibrahim *et al.* found that acapsular strains produced damage in umbilical vein endothelial cells and were more efficiently phagocytosed than encapsulated strains, suggesting that the escape from the vasculature and dissemination occurred through acapsular or poorly encapsulated cells (Ibrahim *et al.*, 1995) and that the invasive mechanisms in the BBB are different from other blood vessels. Accordingly, acapsular mutants were recently shown to manifest *in vitro* invasive growth, a phenomenon not observed in encapsulated strains (Zaragoza *et al.*, 2008a). Mutants defective in inositol phosphosphingolipid–phospholipase C1 are hyperencapsulated and do not disseminate to the brain (Shea *et al.*, 2006). Similar findings have been found with mutants that cannot synthesize GalXM, which are hyperencapsulated and fail to disseminate to the brain during infection (Moyrand *et al.*, 2007). Such mutants presumably have a capsule with a different structure and lack GalXM, which might play an independent role in dissemination. Thus, the attribution that larger capsules inhibit dissemination must be considered tentative until validated by subsequent studies.

Once the fungal cell has been internalized by the endothelial cell, transcytosis to the abluminal size of the cell occurs. During this process, endothelial cells have antimicrobial mechanisms based on the procuction of free radicals. Coenjaerts *et al.* found that endothelial cells have the ability to kill *skn7 C. neoformans* mutant cells (lacking a transcription factor that regulates oxidative stress response) through production of free radicals (Coenjaerts *et al.*, 2006). The fact that wild-type *C. neoformans* cells can evade this antimicrobial activity suggests that there are mechanisms that protect the cell from killing, enhancing survival, transcytosis and dissemination. We recently described that the capsule and capsular enlargement plays a key role in the protection against free radicals (Zaragoza *et al.*, 2008b) and it is reasonable to think that encapsulated strains are more likely to survive and disseminate than acapsular strains.

B. Role of the exopolysaccharides during infection

The capsular components are released into the surrounding environments and are found in serum and tissues during infection. In fact, noncryptococcal cell-associated exopolysaccharides have been found freely in various types of tissues (De Jesus *et al.*, 2008; Goldman *et al.*,

1995; Lee *et al.*, 1996; Lendvai *et al.*, 1998) (see also Lee and Casadevall, 1996). Various approaches have been carried out to study the role of capsular polysaccharides on the immune response. They included investigation of the role of encapsulated and acapsular strains, as well as *in vitro* or *in vivo* studies using purified capsular fractions, the latter based on exposure in host models. These approaches have shown that these exopolysaccharides exert a large number of effects inside the host, which will be reviewed in the following sections.

1. GXM unresponsiveness

Early studies using total polysaccharide from *C. neoformans* revealed that this antigen had a propensity for inducing immunological paralysis (Blackstock and Hall, 1984; Gadebusch, 1958a,b; Robinson *et al.*, 1982). Several reports indicate that the capsular polysaccharide plays an important role in *C. neoformans*-induced immunological unresponsiveness. Murphy *et al.* showed that the polysaccharide caused a dose-dependent primary immunological response in mice using the hemolytic plaque assay as indicated by the increase in antibody producing cells. However, after a subsequent challenge with capsular polysaccharide, a state of immunological unresponsiveness was induced. There was a diminution of antibody response and the abolition of spleen plaque forming cells rather than neutralization of antibody by excess antigen (Murphy and Cozad, 1972). The results supported the view that tolerance was due to terminal differentiation without proliferation of immunocompetent cells. A second study showed that the induction of unresponsiveness in the host appears to be dependent upon the total amount of polysaccharide to which the host is exposed rather than the duration of exposure. The study suggested that cryptococcal polysaccharide is sequestered until a sufficient amount of polysaccharide is accumulated for the induction of paralysis (Kozel *et al.*, 1977).

In addition to inhibition of antibody production, *C. neoformans* and capsular polysaccharides interfere with proper inflammatory responses. Mody and Syme found that *C. neoformans* GXM diminished human lymphocyte proliferation (Mody and Syme, 1993). This process was mainly due to the antiphagocytic effect elicited by the capsular polysaccharides (Syme *et al.*, 1999). Blackstock *et al.* compared the immune response elicited by two different *C. neoformans* strains, which differed in their ability to induce *in vitro* capsule enlargement and virulence (Blackstock *et al.*, 1999). The authors found that after infection the strain was unable to induce capsule size, was not associated with GXM accumulation in serum and was cleared from infected lungs after an early inflammatory response. In contrast, the virulent strain, which was heavily encapsulated, produced a delayed DTH response (after 15 days of infection) and a significant accumulation of polysaccharide in serum. This delayed

response correlated with a difference in the cytokine expression pattern. Mice infected with heavily encapsulated strain failed to upregulate Th1 cytokines, such as IL-2 and IFN-γ and induced a significant accumulation of IL-10, which was not found in mice infected with the hypocapsular mutant. These results suggested that *C. neoformans* and its capsular components, produced anergy through IL10 upregulation and Th1 cytokines downregulation (Blackstock *et al.*, 1999).

Older studies suggest that *C. neoformans* GXM polysaccharide produces some of its immunosuppressive effect by activation of a specific subset of T-supressive cells which secrete T-supressive factors (TsF) (Blackstock, 1996). This factor induced macrophage unresponsiveness and, in turn, inhibited phagocytosis (Blackstock *et al.*, 1987; Morgan *et al.*, 1983; Rubinstein *et al.*, 1989). GXM is a Type 2 T cell-independent antigen (Sundstrom and Cherniak, 1992). GXM tolerance was T cell-mediated at a low dose, but T cell independent at high doses. Moreover, the effect seemed to be mediated through $CD4^+$ T cells. In rat experimental models, it was also shown that *C. neoformans* produced an immunosuppressive state after 2 and 3 weeks of infection, which was associated with function of accessory cells (such as macrophages) and inhibition of spleen cell proliferative response to mitogens (Rossi *et al.*, 1998). This unresponsiveness was also induced by purified GXM and was associated with a decrease in the number of viable spleen mononuclear cells, increased production of IL-10 and IL-4, reduced levels of IL-2 and decreased CD11a surface expression (Chiapello *et al.*, 2001). Addition of GXM to dendritic cells can inhibit T cell proliferation. Recently, Yauch *et al.* described that GXM inhibited T cell proliferation when exposed to dendritic cells. This inhibition occurred not only in the presence of dendritic cells, but also when T cells were activated by anti-CD3 antibody, concanavalin A, or ionomycin (Yauch *et al.*, 2006).

In addition to modulation of acquired immune system (B and T cells), capsular polysaccharide also interferes with some elements of the innate immune system. For example, the capsule inhibits aggregation of *C. neoformans* cells by surfactant protein D, which is an important component of the lung's innate defense system (van de Wetering *et al.*, 2004). Recently, it has been demonstrated that direct engagement of GXM to FcRγII expressed in monocytes, macrophages and dendritic cells, leads to an inhibitory signal that contributes to immune suppression (Monari *et al.*, 2006b).

The immune unresponsiveness resulting from GXM does convey some beneficial effects on the host during other types of inflammatory pathologies. For example, GXM acts as a potent immunosupressor during induced arthritis (Mirshafiey *et al.*, 2004).

2. GXM immunomodulation

During *C. neoformans* infection, the expression of a large number of immune mediators is modulated by GXM (reviewed in Monari *et al.*, 2006a; Vecchiarelli, 2000). In addition, these immunomodulatory effects vary depending on cell type.

The outcome of the interaction between *C. neoformans* and monocytes differs depending on whether the yeast cell has a capsule. Acapsular strains elicited higher levels of some proinflammatory cytokines from monocytes, such as TNF-α, IL-1-β and IFN-γ (Vecchiarelli *et al.*, 1995; Walenkamp *et al.*, 1999). This effect of the capsular polysaccharide is largely mediated through GXM, which can influence the expression of many molecules with important functions in the immune response. GXM also induces IL-10 and IL-8 secretion by human monocytes (Walenkamp *et al.*, 1999). Since IL-10 is a potent downregulator of proinflammatory cytokines (such as TNF-α and IL1-β), this finding suggests a mechanism by which *C. neoformans* polysaccharide interferes with cell-mediated immunity (Vecchiarelli *et al.*, 1996). *C. neoformans* encapsulated cells induced IL-12 poorly when compared to acapsular mutants. It was later described that GXM reduced IL-12 through the increase in IL-10 expression, since neutralization of IL-10 with antibody reversed the GXM suppressive effect (Retini *et al.*, 2001). It has recently been shown in murine macrophage cell lines that GXM induces TNF-β accumulation (Villena *et al.*, 2008). Cytokine regulation by *C. neoformans* and GXM is also mediated through the complement pathway (Chaka *et al.*, 1997b). GXM also induces IL-6 in monocytes (Delfino *et al.*, 1997). The same effect was found in rat alveolar macrophages. In this case, the effect was mediated after phagocytosis of encapsulated cells opsonized with specific antibodies (Li and Mitchell, 1997). The expression of CD4 on monocytes was different when challenged with encapsulated and acapsular strains. Acapsular strains induced higher CD4 levels and this effect was downregulated by addition of exogenous GXM (Pietrella *et al.*, 1998).

In contrast, the capsule and purified GXM can have different effects on PMN cells. GXM causes L-selectin shedding from neutrophils, a phenomenon which is accompanied with reduced TNF-α receptor expression, but increased CD15 and CD11b expression (Dong and Murphy, 1996). Furthermore, *C. neoformans* and purified GXM induce the expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) in neutrophils. Interestingly, in the case of encapsulated cells, the effect was dependent on the size of the capsule (Retini *et al.*, 1996). However, this effect was not directly mediated through GXM, but through the complement activation pathway by capsule components, especially C3a and C5a, which stimulate PMN to produce proinflammatory molecules (Vecchiarelli *et al.*, 1998). Some studies showed higher IL-6 expression in

peripheral blood mononuclear cells (PBMC) after prolonged stimulation with acapsular strains (Siddiqui *et al.*, 2006). The capsule also modulates the expression of C5aR (Monari *et al.*, 2002). Exposure of PMN cells to encapsulated cells or acapsular mutants treated with GXM resulted in a drastic decrease of C5aR on the PMN cells membrane, suggesting a mechanism by which GXM interferes with neutrophil function.

The reason why GXM produces different effects on macrophages and neutrophils is not known, but these cell types interact differently with GXM (Monari *et al.*, 2003). While GXM is expulsed from the cells in neutrophils, macrophages show a continuous intracellular accumulation of GXM. This difference may, in turn, produce different effects in cytokine production.

Consistent with the idea of GXM producing different effects in different cell types are findings that GXM from certain serotypes induces IL-8 production in human fetal microglial cells, but not in astrocyte cultures (Lipovsky *et al.*, 1998a). Barluzzi *et al.* studied the effect of encapsulated and acapsular strains in microglial cell cultures. These authors did not find any difference in the expression of several cytokines, such as TNF-α, IL-1β, IL-6, IL-12p40 and granulocyte macrophage colony stimulating factor. They did find, however, that lipopolysaccharide-induced TNF-α expression was blocked by encapsulated or acapsular yeasts or exposure to purified GXM or GalXM (Barluzzi *et al.*, 1998).

GXM also regulates cytokine expression by $CD4^+$ T cells (Mariano Andrade *et al.*, 2003). GXM does not induce CD4 cell proliferation, but interfered with the immune system, resulting in increased fungal replication through a mechanism that involved enhancement of IL-10 and IL-4 production. These findings were interpreted as indicative that GXM contributes to virulence by interfering with the development of an effective cell-mediated immune response that was unable to inhibit fungal replication.

3. GalXM immunomodulation

Although most of the immunomodulatory effects of the capsular polysaccharide studies have focused on GXM, GalXM can also contribute to these effects. GXM, GalXM and MPs can each elicit an increase in TNF-α in whole blood cultures (Delfino *et al.*, 1996). Another study demonstrated that GXM-induced TNF-α accumulation was dependent on the presence of serum. In the case of GalXM, however, TNF-α was serum independent (Chaka *et al.*, 1997b). GalXM induces IL-6 in monocytes (Delfino *et al.*, 1997). In another study, the cytokine expression profile of human monocytes in the presence of different capsular components was investigated, demonstrating that GalXM and GXM exerted similar effects (Walenkamp *et al.*, 1999). Recent findings in murine macrophages (RAW 264.7 cell line) showed that GalXM and GXM exert different effects on cytokine

production, with GalXM having a profound effect on TNF-α induction in addition to NO production through an iNOS expression (Villena *et al.*, 2008). In microglial cells, neither the addition of GalXM or GXM was associated with an increase in TNF-α accumulation, but both GXM and GalXM blocked this induction in the presence of LPS (Barluzzi *et al.*, 1998). These results might suggest that GXM and GalXM share immunomodulatory properties.

4. Effect of GXM on antigen presentation

C. neoformans encapsulation is associated with reduced antigen presentation by macrophages (Collins and Bancroft, 1991). Purified GXM inhibited antigen presentation in monocytes exerted by acapsular cells (Retini *et al.*, 1998). This effect was dependent on phagocytosis and was mediated through an increase in IL-10, resulting in a significant reduction in MHCII. In addition, the capsule was a poor stimulator of costimulatory molecule expression required for antigen presentation, such as B7-1 (CD80) and B7-2 (CD86), although encapsulated cells increased expression of ligand CTLA-4 when compared to acapsular cells (Pietrella *et al.*, 2001b). This result is relevant for the host's defense since B7 costimulatory molecules are necessary to elicit an efficient T cell response against *C. neoformans* (Monari *et al.*, 1999b). Despite the negative effects of GXM on the expression of costimulatory and MHC molecules, injection into mice of antigen-presenting cells (APCs) previously exposed *in vitro* to GXM inhibited T-suppressive cell responses, a phenomenon that led to enhanced survival of infected mice (Blackstock and Casadevall, 1997). This protective effect of GXM-APC stimulated cells was associated with an early DTH response development and was mediated through increased production of some cytokines, such as IL-2 and IFN-γ (Blackstock *et al.*, 2000). The authors concluded that during this process, GXM-dependent and GXM-independent effects occurred. GXM-specific effects were dependent on MHC class II, while GXM-independent effects required B7-1 and B7-2 molecules (Blackstock, 2003).

The capsular polysaccharide also interferes with the normal function of dendritic cells, which are professional APCs necessary to initiate T cell-mediated responses. Acapsular *C. neoformans* mutants are rapidly ingested by dendritic cells, which produce upregulation of maturation markers, such as MHCI and II, CD40 and CD83 (Monari *et al.*, 2002). This maturation was not observed when dendritic cells were exposed to encapsulated cells or purified GXM and suggested another mechanism by which the capsule interfered with T cell-mediated responses. These findings have been recently confirmed by analyzing the gene expression pattern of dendritic cells exposed to encapsulated and acapsular cells (Lupo *et al.*, 2008).

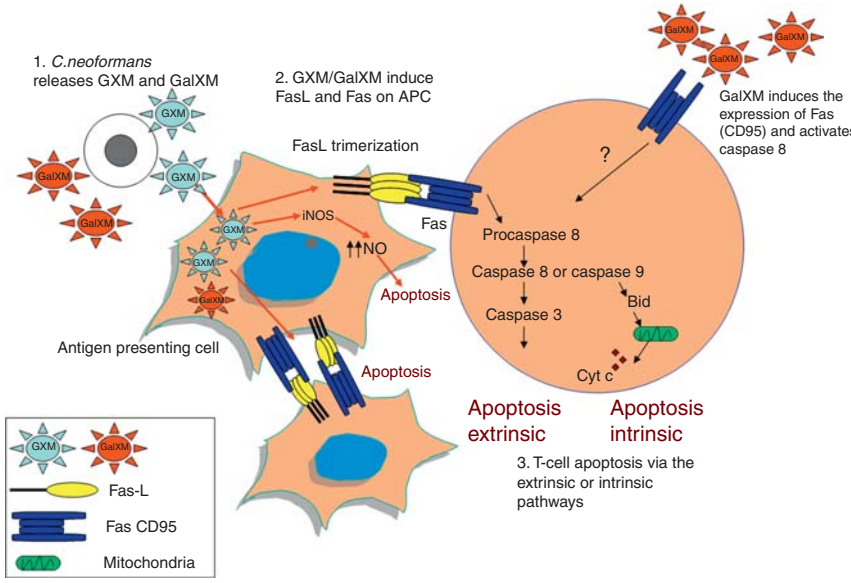

FIGURE 4.7 Scheme summarizing all the levels at which capsular polysaccharides induce apoptosis on murine APCs and T cells. GXM and GalXM induce apoptosis on APCs, such as macrophages and T cells. In macrophages, GXM and GalXM induce accumulation of molecules involved in apoptosis induction, such as FasL and Fas. These molecules induce apoptosis in both macrophages and T cells. In addition, in T cells, GalXM can directly induce apoptosis through Fas molecules. Apoptosis occurs through caspase pathway.

5. GXM-induced apoptosis

Several groups have demonstrated that soluble GXM can induce apoptosis in different systems (see scheme in Fig. 4.7). This phenomenon was first described in rat lymphocytes (Chiapello *et al.*, 2003). GXM-mediated apoptosis was first observed *in vitro* and the results were corroborated in animal models, which proved that there were a high proportion of apoptotic cells in rat lungs and spleens during *C. neoformans* infection. A similar finding was observed when rats where treated with purified GXM (Chiapello *et al.*, 2004). This process correlated with the modulation of some cytokines by GXM, such as IL-10, IL-2, IFN-γ and TNF-α. In rat macrophages, GXM-induced apoptosis has been recently correlated with iNOS induction and NO accumulation (Chiapello *et al.*, 2008). GXM was also shown to induce apoptosis in murine cells, which was associated with expression of the Fas ligand. This induction required GXM binding to TLR4 and correlated with GXM accumulation in the macrophages. Moreover, these macrophages induced apoptosis of T cells or jurkat

cells that expressed Fas (Monari *et al.*, 2005b). Recently, the mechanism of GXM-induced apoptosis of T cells was further elucidated. It was found that both *in vitro* and *in vivo* systems, the binding of FasL produced by APCs, by Fas on T cells triggers caspase cascades that cause T cell death (Monari *et al.*, 2008). Caspase 8 was identified to play a central role in apoptosis since it cleaves caspase 3 via the extrinsic and intrinsic apoptotic pathways. It was also found that caspase 9 cleavage can also activate caspase 3 independent of caspase 8. Therefore, the caspase 8 and caspase 9 pathways cooperate in an amplification loop for efficient cell death. The authors provide evidence that both capsase 8 and 9 are activated in one single cell. Collectively, the data indicate that GXM-induced apoptosis involves, a cross talk between pathways within a single cell. Recently, GXM was also shown to induce apoptosis in macrophages through a mechanism that involves an increase in both Fas and FasL in the phagocytic cells (Villena *et al.*, 2008).

6. GalXM-induced apoptosis

GalXM inhibited PBMC and T cell proliferation, increased IFN-γ and IL-10 production and induced apoptosis of T lymphocytes by DNA fragmentation through the activation of caspase 8 (Pericolini *et al.*, 2006). GalXM induces death receptors such as Fas and through subsequent activation of caspase-8, it is most likely that the Type I apoptotic pathway is involved in this process. These results suggested the possibility of a putative receptor for GalXM on T cells that may be involved in triggering apoptotic signals. GXM- and GalXM-mediated apoptosis appear to have fundamentally different mechanisms. GXM does not interact directly with T cells, but rather mediates apoptosis by inducing FasL on antigen-producing cells such as macrophages (Monari *et al.*, 2005b). In contrast, GalXM appears to directly interact with galectins on T cells to induce apoptosis. However, this mechanism needs to be further elucidated (Pericolini *et al.*, 2006). GalXM is also more potent than GXM at the induction of Fas/FasL expression and apoptosis on macrophages (Villena *et al.*, 2008).

7. Regulation of chemotaxis and cellular migration by capsular components

Leukocyte migration to infected tissues is a complex process that involves chemotaxis of immune cells, binding to endothelial surfaces and movement across the endothelium. The capsular components can influence chemotaxis (see review in Ellerbroek *et al.*, 2004d). The first evidence suggesting that the capsule interfered with cell migration was provided by the finding that capsular components induced PMN migration through the activation of the complement system by the alternative pathway (Diamond and Erickson, 1982; Laxalt and Kozel, 1979). Consistent

with this finding, CSF from rabbits with chronic cryptococcal meningitis contained a host-derived factor with chemotactic activity toward PMN and monocytes (Perfect and Durack, 1985). In addition, encapsulated cells induced chemotaxis of human neutrophils through a direct mechanism (Dong and Murphy, 1993). Acapsular strains also exerted this effect, but in an indirect manner. The direct effect was mediated by GXM, while the other components of the capsule (GalXM and MPs) played an indirect role through complement pathway modulation. Interestingly, capsular components from *C. gattii* did not have any effect on neutrophil migration (Dong and Murphy, 1995a), which suggests a mechanism that *C. gattii* evades the interaction with this type of phagocytic cells.

The interplay of these chemotactic activities *in vivo* is very complex. Despite the chemotactic properties of GXM, there is a characteristic inhibition of leukocytes infiltration into infected tissues in cryptococcosis (Baker and Haugen, 1955). Dong and Murphy also showed that elements from the capsule inhibited leukocyte migration (neutrophils, lymphocytes and monocytes) to infected areas (Dong and Murphy, 1995b). Furthermore, GXM-induced shedding of L-selectins from neutrophil membranes as well as reducing TNF receptor expression, but increased CD15 and CD11b (Dong and Murphy, 1996). L-selectins are surface molecules necessary for neutrophil migration into tissues. GalXM and MPs did not affect L-selectin shedding by comparison. These findings suggest a mechanism by which GXM may reduce neutrophil migration into infected tissues by preventing neutrophil adhesion to endothelial surfaces. A similar effect was found on T cells (CD4 and CD8) through a mechanism that required tyrosine kinase (Dong *et al.*, 1999). Some reports have suggested that L-selectin shedding was not the determinant factor that inhibits migration and the same authors involved E-selectin in the migration process (Ellerbroek *et al.*, 2002). The same authors reported that GXM interfered with rolling and fixed binding of neutrophils on the endothelium using a flow model (not static) and that this interference was mediated through TLR4, CD14 and E-selectin (Ellerbroek *et al.*, 2004c). The effect was dependent on the 6-*O*-acetylation of mannose residues in GXM (Ellerbroek *et al.*, 2004a). In addition, GXM can bind to CD18, interfering with its role in neutrophil migration (Dong and Murphy, 1997). GXM also interfered with PMN migration in response to IL-8 (Lipovsky *et al.*, 1998a) and in agreement with this finding, it was found that the CSF leukocyte cell count was inversely correlated with the amount of GXM present in the serum (expressed as the GXM proportion in serum related to the amount present in CSF) (Lipovsky *et al.*, 1998b). GXM also inhibited leukocyte migration into CSF in rabbit experimental models of bacterial meningitis (Lipovsky *et al.*, 2000), as well as in some models which induce nonrelated neutrophil migration, such as rat model of myocardial ischaemia (Ellerbroek *et al.*, 2004b).

8. Mannoproteins

Although it has not been demonstrated that these immunogenic proteins are in fact capsular components, we will briefly review the effect of MPs on the host.

C. neoformans MPs are highly immunogenic (Levitz and Specht, 2006). Most immunological responses to MPs in *C. neoformans* are proinflammatory (Murphy, 1988) and MPs in general elicit stronger effects than GXM or GalXM. The MP fraction CneF stimulated delayed-type hypersensitivity whereas the fraction MP4 can desensitize neutrophils toward chemoattractant challenges. MPs produce a strong TNF-α accumulation (Delfino *et al.*, 1996), dependent on serum-labile components (Chaka *et al.*, 1997b). MPs also regulated the expression of other cytokines, such as IL-12, IL-6, IL-10, IFN-γ and IL-8 in monocytes (Delfino *et al.*, 1997; Pitzurra *et al.*, 2000; Walenkamp *et al.*, 1999). The effect of MPs has also been tested *in vivo* and these studies have shown that treatment of mice with MP-induced IL-12 and IFN-γ expression, which was associated with a protective response to *C. neoformans* that included enhanced antifungal activity, early inflammatory responses and clearance of fungal burden from the brain (Pietrella *et al.*, 2001a). *In vivo* impairment of IL-12 induction interfered with the protective effect elicited by MPs (Pietrella *et al.*, 2004). Furthermore, these MPs elicit a protective response, not only against *C. neoformans*, but also against other fungal pathogens such as *C. albicans* (Pietrella *et al.*, 2002). In addition, MPs-induced proliferation of human PBMC (Orendi *et al.*, 1997), a phenomenon not observed with GalXM. This induction resulted in an enhancement of HIV replication in infected PBMC, which suggested a mechanism by which *C. neoformans* enhanced the viral infection. Later, a specific MP of a molecular size of 105 kDa was demonstrated to be involved in monocyte proliferation induction (Pitzurra *et al.*, 1997).

MPs also promote maturation and activation of dendritic cells (Pietrella *et al.*, 2005). MP-induced expression of mature DC markers and IL-12 and MP-activated DC efficiently activated both CD4 and CD8 T cells. MPs also exert a cooperative stimulation of DC with CpG-oligodeoxynucleotides, which induces proinflammatory cytokines (Dan *et al.*, 2008b).

Mannosylation is required for MP-mediated T cell stimulation (Specht *et al.*, 2007), consistent with the finding that MP stimulatory effect are the result of interactions with human mannose binding protein (Chaka *et al.*, 1997a) or the mannose receptor (Dan *et al.*, 2008b; Mansour *et al.*, 2002). More recently, MP uptake was shown to be similar by dendritic cells from wild-type and mannose receptor KO mice, although MPs only induced CD4 lymphoproliferation in cells from wild-type mice, but not in KO mice (Dan *et al.*, 2008a). This finding indicated that the mannose receptor is

required for some of the effects mediated by MPs. There are other mechanisms by which DC can bind MPs, although these other mechanisms were not effective in inducing a protective T cell response.

Several *C. neoformans* MPs have been isolated. Mansour *et al.* identified a specific 98 kDa MP (MP98) involved in the stimulation of T cell responses (Levitz *et al.*, 2001). The gene for MP98 was cloned (CDA2) and was found to contain a domain with similarity to chitin deacetylase. The role of chitin deacetylases is to convert chitin to chitosan, the deacetylated form of chitin. Using a collection of putative chitin deacetylase deletion strains cda1, cda2, cda3 and a polysaccharide deacetylase fpd1, Baker *et al.* characterized the deacetylases and their biological roles (Baker *et al.*, 2007). The study revealed that a single chitin deacetylase does not adversely affect the ability of *C. neoformans* to produce chitosan. Strains with a *cda1*Δ*cda2*Δ mutation had increased capsular diameters. The results imply that chitosan produced by Cda1 and Cda2 is sufficient to retain normal capsule integrity. However, a quadruple mutation *cda1cda2cda3 fpd1* abolished chitosan production. Strains lacking the chitosan had incomplete mother daughter cell separation and were sensitive to cell wall inhibitors. Additionally, mutants lacking chitosan were unable to completely retain melanin, another major virulence factor in *C. neoformans* (Baker *et al.*, 2007).

In 2002, the same group identified a new MP involved in T cell activation. It had an apparent molecular weight of 88 kDa and was therefore named MP88 (Huang *et al.*, 2002). This protein contained a putative glycosyl phosphatidyl inositol anchor site and had homology to 11 other *C. neoformans* proteins, including MP98 (Huang *et al.*, 2002).

Recently new immunogenic MPs have been identified, with molecular weights of 250, 125, 115 and 84 kDa. The genes encoding MP84 and MP115 were cloned and had homology with polysaccharide deacetylases and carboxylesterases, respectively (Biondo *et al.*, 2005).

C. Origin of the capsule as virulence factor

An intriguing issue in the *C. neoformans* capsule field is how this structure has evolved as a virulence factor in mammals. It is difficult to understand how an environmental yeast that produces infection after inhalation can possess such a specialized structure for virulence in animals while not really requiring an animal host for any aspect of its life cycle.

To properly answer this question, it is important to emphasize that although *C. neoformans* is an environmental organism, it frequently interacts with a large number of nonconventional hosts widely found throughout nature (Casadevall and Perfect, 1998). For example, *C. neoformans* is known to interact with amoebas, such as *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* and the slime mould *Dictyostelim discoideum*

(Bunting *et al.*, 1979; Castellani, 1955; Steenbergen *et al.*, 2003). Some amoebas can phagocytose and kill *C. neoformans* while this fungus can survive ingestion by others (Steenbergen *et al.*, 2001). In addition, *C. neoformans* has developed some mechanisms that allow phagocytotic inhibition, intracellular fungal replication and killing evasion (Steenbergen *et al.*, 2001; Zaragoza *et al.*, 2008b).

C. neoformans intracellular pathogenesis in mammalian cells is associated with GXM accumulation in specific vesicles (Tucker and Casadevall, 2002). Remarkably, the interaction of *C. neoformans* with ameboid protozoal cells results in cytopathic changes in the host cell that are strikingly similar to those observed with mammalian cells. In both amoeba and macrophages, the capsular polysaccharide appears to function as an intracellular aggressin whereby polysaccharide-filled vacuoles from phagosomal membrane blebbing and accumulate in the cytoplasm (Steenbergen *et al.*, 2001). In addition, *C. neoformans* can infect nematodes (Mylonakis *et al.*, 2002), insects (Apidianakis *et al.*, 2004; Mylonakis *et al.*, 2005), toads (Seixas *et al.*, 2008) and goats (Chapman *et al.*, 1990).

In most of these interactions, the capsule plays a critical protective role for *C. neoformans*. Acapsular mutants are more efficiently engulfed by amoebas and killed to a higher degree than encapsulated fungal cells (Steenbergen *et al.*, 2001). Moreover, capsule enlargement and structural changes induced by starvation can also inhibit *C. neoformans* phagocytosis by amoebas (Zaragoza *et al.*, 2008b). In the case of *Caenorhabditis elegans*, the capsule is also required for virulence and killing of the nematode, since acapsular mutants were cleared by the worm (Mylonakis *et al.*, 2002). A similar situation was found with the caterpillar *Galleria mellonella* (the greater wax moth), a model in which acapsular mutants showed a markedly reduced virulence (Mylonakis *et al.*, 2005). However, the role of the capsule in the virulence in *Drosophila melanogaster* was different. In this model, acapsular mutants showed a slightly reduced virulence in comparison to encapsulated cells, but were still able to kill the insect (Apidianakis *et al.*, 2004), indicating that the capsule is dispensible for virulence during *D. melanogaster* infection.

The fact that the capsule plays a protective role during interaction of *C. neoformans* with environmental factors such as dehydration (Aksenov *et al.*, 1973) and in the interaction with environmental predators suggests a mechanism by which the *C. neoformans* capsule has adapted to become a virulence factor in multiple hosts. It is hypothesized that during evolution, *C. neoformans* has adapted the capsule from a protective structure to a feature that confers selective advantage during the interaction with the different hosts that it interacts with. As a consequence, it acquired the ability to produce a large number of deleterious effects on mammalian hosts (Casadevall *et al.*, 2003). This hypothesis provides an explanation for

the origin of virulence factors in enviromental microbes that are pathogenic for animals and allows us to propose how virulence is a feature specifically acquired after numerous exposures and interactions with different types of hosts by natural selection (Casadevall and Pirofski, 2007).

V. USE OF CAPSULAR COMPONENTS AS ANTIFUNGAL TARGETS AND VACCINE

Given the critical role of the capsule in virulence, considerable efforts have been undertaken to develop antibodies and vaccines that target this structure. Two general approaches have been taken. First, many mAbs have been developed with the purpose of targeting the capsular components, as a passive immunization. Studies with these mAbs have shown that antibody function against *C. neoformans* is extremely complex in the sense that some immunoglobulins confer protection, while others do not and still others may even result in enhancement of disease. In a second approach, the use of the capsular polysaccharide or capsule-mimetic structures as a protective active vaccine has been proposed. In the following section, we will briefly review the main advances in these two fields.

A. Capsule as an antifungal target: mAbs to the capsule as therapeutic alternative

Although the protective role of the humoral response during *C. neoformans* infection has historically been questioned and remains uncertain, there have been two main reasons for developing these mAbs: (1) the capsule is the main virulence factor of this fungal pathogen, so blocking it with mAb theoretically could produce some protective effects; and (2) it was observed that although the capsule is poorly immunogenic, there are some epitopes that can elicit protective antibodies (Cleare *et al.*, 1994).

The binding interactions between mAbs and the capsule and the interactions between *C. neoformans* and the host have been extensively studied. Although the capsule is dispensable for the life of the yeast, binding of mAbs to this structure produces a large number of effects in *in vitro* and *in vivo* models. The protective effect of antibodies binding to GXM has been extensively tested in murine models (Dromer *et al.*, 1987a; Feldmesser and Casadevall, 1997; Fleuridor *et al.*, 1998; Mukherjee *et al.*, 1992, 1995c) and the ability to change the outcome of disease has been observed in various infection models (Mukherjee *et al.*, 1993, 1994b). Numerous studies have been performed and a specific mAb (18B7) was evaluated in a Phase I/II trial in patients with HIV-associated

cryptococcosis as an adjunct to antifungal therapy for cryptococcal meningitis (Larsen *et al.*, 2005). To improve the potential efficacy of antibody therapy in humans, mouse–human chimeric and human antibodies have been created. These antibodies prolong mouse survival and this effect is dependent on the characteristics of the binding of the mAbs, such as specificity and isotype (Fleuridor *et al.*, 1998; Maitta *et al.*, 2004a; McLean *et al.*, 2002). Moreover, mAbs to GXM enhance antimicrobial effects of some antifungal drugs, such as amphotericin B, fluconazole and flucytosine (Dromer and Charreire, 1991; Feldmesser *et al.*, 1996; Mukherjee *et al.*, 1994a, 1995b).

Passive antibody administration is not always associated with a protective effect. For example, mAbs vary in efficacy depending on the infecting *C. neoformans* strain (Mukherjee *et al.*, 1995d), which suggests that strain differences could be a key determinant in the protective effect of the antibodies. Another key factor that determines the effect of mAbs to *C. neoformans* is the variable region of the mAb. Several studies have demonstrated that antibody efficacy depends on the isotype (Mukherjee *et al.*, 1992; Sanford *et al.*, 1990). IgG1 isotype is generally considered protective, whereas IgM is either protective or neutral and IgG3 is found to be disease-enhancing in some strains of mice. There are numerous explanations for the different effects according to the antibody isotype, including differences in distribution in the body, half-life, opsonic capacity and complement activation ability. However, antibodies expressing the same variable regions can differ in terms of their affinity, specifity and protective efficacy (Torres *et al.*, 2005, 2007). For some antibodies, differences in the binding to the capsule correlate with different *in vivo* effects (Beenhouwer *et al.*, 2007). There is evidence that the binding pattern of the antibodies to the capsule influences the final effect of the antibodies. For example, as stated above, IgM antibodies, which bind to the capsule in a punctuate pattern, are not protective, while IgM mAbs which bind in an annular pattern prolong survival in mice (Cleare and Casadevall, 1998; Nussbaum *et al.*, 1997).

In a recent study, computer modeling to predict pore-hindered diffusion and binding of the GXM-specific antibody within the *C. neoformans* capsule was modelized (Rakesh *et al.*, 2008). Using the finite element method (FEM), they created a model which represents the *in vivo* binding of GXM-specific antibody to a *C. neoformans* cell taking into account the intravenous infusion time of antibody, antibody diffusion through capsular pores and Michaelis–Menten kinetics of antibody binding to capsular GXM (see Fig. 4.8). The model predicted rapid diffusion of antibody to all regions of the capsule where pore size was greater than the Stokes diameter of the antibody. Binding occurred primarily at intermediate regions of the capsule. The GXM concentration in each capsular region was the

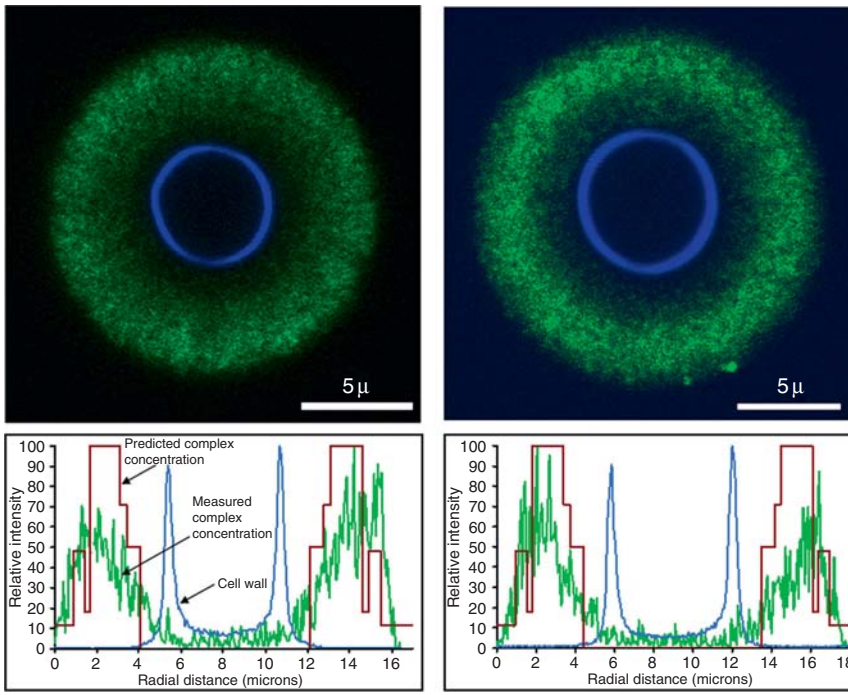

FIGURE 4.8 Comparison of model prediction with experimental results obtained for *C. neoformans* cells using immunofluorescence. Green shows the concentration of complex. The boundaries of the cell wall are shown in blue, detected using calcoflour. Shown are data for two cell sizes (adapted from Rakesh *et al.*, 2008).

principal determinant of the steady-state antibody–GXM complex concentration, while the forward binding rate constant influenced the rate of complex formation in each region. The concentration profiles predicted by the model closely matched experimental immunofluorescence data (Fig. 4.8). Inclusion of different antibody isotypes (IgG, IgA and IgM) into the modeling algorithm resulted in similar complex formation in outer capsular regions, but different depth of binding at inner regions. These results have implications for the development of new antibody-based therapies (Rakesh *et al.*, 2008).

Several studies have established that dose is a critical parameter in determing the efficacy of passive antibody administration. A single mAb can behave as protective, not protective, or disease-enhancing depending on the antibody dose and/or the number of infecting pathogens (Taborda and Casadevall, 2001; Taborda *et al.*, 2003). At high antibody doses, some mAbs lose protective efficacy. This feature, the so-called prozone effect,

is an important issue that must be considered when interpreting passive antibody administration results.

mAbs to the capsule play an important role as opsonins during phagocytosis (see Section IV.A) and also in regulating complement activation (Kozel *et al.*, 1998). In addition, mAbs to the capsule can enhance the antifungal activity of macrophages and microglia (Lee *et al.*, 1995a; Mukherjee *et al.*, 1995e, 1996) and also of neutrophils from HIV patients (Monari *et al.*, 1999a). However, the function of mAbs is critically dependent on the host's cell-mediated immunity (Yuan *et al.*, 1997), B cells (Rivera *et al.*, 2005) and Th1 and Th2 cytokines (Beenhouwer *et al.*, 2001). These studies indicate that mAbs to the capsule exert their *in vitro* action by modulating the inflammatory response, rather than through a direct antifungal effect. The mechanism by which mAbs binding to the capsule exert their action is therefore very complex and binding to the epitope is not sufficient to achieve protection. mAbs to the capsule can regulate the inflammatory response elicited during infection, possibly by altering the release of mediators of inflammation such as cytokines (Feldmesser *et al.*, 1998, 2002). Engagement of Fc receptors appears to be a requirement for efficacy, since mAbs are not protective in $FcRI^{-/-}$ or $FcRIII^{-/-}$ KO mice (Yuan *et al.*, 1998). Interestingly, mAbs can be protective in some cases of immunodeficiency, such as the absence of complement component 3 (Shapiro *et al.*, 2002). In addition, the mouse genetic background is an important determinant for the effect of the antibodies, which confirms that host elements are important to determine whether a mAb binding to the capsule will have a protective effect or not (Rivera and Casadevall, 2005).

In addition to passive mAb administration, new strategies based on enhancing the activity of mAbs to the capsule have been developed to improve their efficacy. For example, radiolabeling of these mAbs converted an antibody into a microbicidal molecule that causes reduction in capsule size, changes in cellular metabolism and an apoptosis-like death in fungal cells (Dadachova *et al.*, 2006). This effect is highly dependent on the antibody isotype and the affinity constant of the mAb binding to the polysaccharide capsule (Dadachova *et al.*, 2007). The same dependence of the fungicidal ability of radiolabeled antibodies on the isotype was observed for *C. neoformans* biofilms which are characterized by massive deposits of exopolysaccharide (Martinez *et al.*, 2006). Administration of radiolabeled GXM-specific mAb 18B7 prolonged survival of AJ/Cr mice lethally infected with *C. neoformans* (Dadachova *et al.*, 2003). The success of these studies has led to the emergence of the whole new field of radioimmunotherapy (RIT) of infectious diseases which has been subsequently expanded to treatment of bacterial and viral infections (reviewed in Dadachova and Casadevall, 2008).

B. Use of capsular components as vaccine

GXM synthesis is different from that of other capsular polysaccharides in that it is a heteropolymer that, in theory, could display an almost infinite number of structural combinations, which could translate into antigenic changes and enhance its structural complexity (McFadden *et al.*, 2007). Fortunately, mAbs that recognize all strains have been identified, suggesting that certain immunudominant epitopes may be conserved across strains. The rationale for GXM-based conjugate vaccines is that they elicit antibodies that are opsonic and bind soluble polysaccharide, promoting its clearance.

The first immunization studies with GXM were done by injecting the polysaccharide with an ion exchange resin or in an emulsion with Freund's adjuvant. This strategy, however, only elicited a weak antibody response mostly of the IgM isotype (Cauley and Murphy, 1979; Gadebusch, 1958a; Kozel *et al.*, 1977). In the late 1960s, Goren and Middlebrook generated the first GXM (or cryptococcal polysaccharide)-based glycoconjugate vaccine by coupling unfractionated capsular polysaccharide (which most likely contained all three capsular components) to a bovine gamma globulin by nitrocarbanilation and diazotization (Goren and Middlebrook, 1967). This vaccine was highly immunogenic but did not elicit a protective antibody response. In the 1990s, a second glycoconjugate vaccine was developed based on fractionating the GXM component and linking it to tetanus toxoid (TT) (Devi *et al.*, 1991). Boosting immunization yielded antibodies' of the IgG isotype. mAb 18B7 was generated using the GXM–TT conjugate.

An alternative approach in using *C. neoformans* as a vaccine antigen that is conceptually related to glycoconjugate vaccines in terms of the type of immunity that is elicited is the development of a peptide that mimics a GXM epitope. A mimetic is a peptide epitope that inhibits the binding of an antibody to its native antigen (Pirofski, 2001). The peptide can elicit a protective antibody response by stimulating the production of protective antibodies that bind to polysaccharide (Pirofski, 2001). The approach used to develop the peptide mimetope vaccine employed a decapeptide phage display library in which peptide epitopes were expressed on the surface protein of the M13 bacteriophage which were then screened by using human IgM mAb to *C. neoformans* GXM, 2E9. This mAb was produced from immortalized lymphocytes from a volunteer who received the GXM–TT vaccine (Valadon *et al.*, 1996). A group of these phage peptide epitopes were isolated and were considered GXM mimetics.

The peptide mimetic P13 was conjugated to TT or BSA and used to vaccinate mice. The vaccine elicited both IgM and IgG antibodies to both GXM and P13, suggesting that P13 is a GXM mimetope. The P13–TT conjugate induced protection in mice that is probably antibody mediated.

Despite promising results, mimetope-mediated protection and vaccine efficacy is not fully understood.

There have been several attempts to synthesize oligosaccharides that reproduce the structural repeat of GXM (Garegg *et al.*, 1996; Oscarson *et al.*, 2005; Vesely *et al.*, 2008), including a heptasaccharide representing the putative immunodominant motif of serotype A GXM (Oscarson *et al.*, 2005). The heptasaccharide consists of two M2 repeats (see Fig. 4.2 for structural interpretation) and one mannose residue. The M2 repeat was selected because it is commonly found in GXM of serotype A strains, although this repeat is also found in other serotypes. The synthetic heptasaccharide was strongly recognized by two different IgMs (Oscarson *et al.*, 2005). Conjugation of the heptasaccharide to human serum albumin resulted in an immunogenic compound that elicited high-titer IgG responses in mice when given with complete Freund's adjuvant. The antibody response elicited by the oligosaccharide conjugate vaccine had characteristics of a T cell-dependent response (Oscarson *et al.*, 2005). The immunogenic oligosaccharide with the structural motif of GXM therefore represents a potential synthetic vaccine against this fungal pathogen.

VI. FUTURE PERSPECTIVES

After 50 years of intensive study that has included serological, biochemical, immunological, microscopic, genetic and physical investigations, much is known about the *C. neoformans* capsule. The capsule is widely acknowledged to be an indispensable virulence factor and continues to occupy much of the investigative attentions of the cryptococcal field. However, despite hundreds of publications detailing capsule-related studies, the capsule remains an enigmatic structure that continues to defy complete understanding. At this time there is probably a reasonably good understanding of the various mechanisms by which the capsule contributes to virulence. While the chemical composition of capsular polysaccharide has been well described, we still do not know the answer to some basic questions concerning capsular biochemistry such as whether some fibrils include branches. There is no current evidence for branched polysaccharides but the biochemical methods do not rule out occasional branched molecules. The areas in which the least is known include the architecture, organization and synthesis of the capsule. No comprehensive model has yet been proposed for molecular mechanisms responsible for capsular enlargement.

The major problem with regards to studying the architecture of the capsule is a dearth of techniques that yield structural information for a highly hydrated, fragile and variable polysaccharide fibrilar mesh. Therefore, much of what we know about the capsule has been inferred

from studies of soluble polysaccharide. A better understanding of how the capsule is assembled may require the development of new approaches that combine approaches from various disciplines.

On the immunological front several important open questions remain. The role of the capsule during intracellular pathogenesis provides the paradoxical finding that a structure that inhibits phagocytosis also functions to undermine the host cell after being ingested. The origin of polysaccharide-containing vesicles that fill the cytoplasm of infected macrophages has not been solved. Similarly, the mechanism by which the capsule contributes to the phenomenon of cryptococcal exocytosis is unknown. Capsular polysaccharides are powerful immunomodulators but their cellular receptors have not been fully characterized.

C. neoformans has the largest, most distinctive and best characterized capsule in the field of medical microbiology and studies of capsular structure, synthesis and function will continue to occupy the field for many years to come.

ACKNOWLEDGMENTS

We are indebted to Karen Dente for the outstanding review and editing of the manuscript. O.Z. is a ''Ramón y Cajal'' fellow and is supported by grants 1025/06 and 1142/08 from MICINN and 1181/06 from Instituto de Salud Carlos III. A.C. is supported by NIH grants AI033774, HL059842 and 5R37AI033142. E.D. is supported by NIH grant AI60507.

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CHAPTER 5

Baculovirus Interactions *In Vitro* and *In Vivo*

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Contents

Abstract

Baculoviruses are promising viral insecticides and are safe for the environment. Interaction of baculoviruses *in vitro* and *in vivo* is a basic molecular and ecological question that has practical applications in agriculture. Cellular secretion is also a fundamental property in cell–cell communication. Here, we review recent investigations on how baculoviruses interact with insect cells and insect hosts. We focus particularly on a new interaction mechanism in which a secretion from cells infected with one virus enhances

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Advances in Applied Microbiology, Volume 68
ISSN 0065-2164, DOI: 10.1016/S0065-2164(09)01205-2

infection by a second virus. We also discuss a hypothesis that the secreted signals may serve as ligands that bind to the receptors on the surface of the cells that harbor the suppressed genomes of *Thysanoplusia orichalcea* MNPV (ThorMNPV) in Sf21 and *Spodoptera exigua* MNPV (SeMNPV) in High 5 to initiate signal transduction leading to the activation of genome replication of ThorMNPV in Sf21 and SeMNPV in High 5. We also discuss how the enhanced replication of SeMNPV replication by *Autographa californica* MNPV (AcMNPV) in nonpermissive insect cells depends on the types of cells. Interaction of baculoviruses in insects focused on mutualism and antagonism, even though the mechanism is not clear on mutualism. The antagonism of a *Nucleopolyhedrovirus* (NPV) with a *Granulovirus* (GV) has been extensively studied by a metalloprotein in the capsule of GV that disrupts the peritrophic membrane, a physical barrier to NPV entry to the midgut of larvae, to facilitate NPV infection.

I. INTRODUCTION

Baculoviruses are a family of insect-specific viruses that have been used to control insect pests in agriculture and forestry (Cunningham and Entwistle, 1981; Moscardi, 1999). These viruses have also been used in genetic studies and for protein production in research and pharmaceutical applications (Luckow, 1991; Tani *et al.*, 2008). Baculoviruses are large, enveloped DNA viruses with a circular genome ranged from 90 to 240 kbp and characterized by the inclusion of virions in protein matrices (Theilmann *et al.*, 2005). These viruses are categorized into two genera, *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV), depending upon the nature of the occlusions—several virions in polyhedra or occlusion bodies (OBs) for NPVs and a single virion in a capsule or granule in GV (Funk *et al.*, 1997). NPVs have been further divided into two groups based on the genomic DNA sequence homology, Group I and Group II (Jehle, 2004). Additionally, nucleocapsids may be enveloped in bundles in some NPVs (MNPV) or as single nucleocapsid in a virion (SNPV and GV) (Federici, 1997).

The number of insect species that a specific baculovirus can infect varies, but most can only infect one or a few species or replicate efficiently in a few cell lines from the same or related insect species. For example, the *Spodoptera exigua* MNPV (SeMNPV) can only replicate well in cells derived from *S. exigua* and can only kill the larvae of *S. exigua*. In contrast, *Autographa californica* MNPV (AcMNPV), the type species of the family Baculoviridae, has a wide host-range, infecting many insect species and cells from different tissues and different species (Cheng and Carner, 2000; Cheng *et al.*, 2005; Groner, 1986; McIntosh and Grasela, 1994). Two or

more viruses have also been isolated from a single larva during a mixed infection (Cheng and Carner 2000; Cheng *et al.*, 2005; Lauzon *et al.*, 2005; Tanada, 1959a,b). How these viruses interact within a cell, a population of cells, or a population of insects is not well studied. In this review, we will concentrate on how a wide host-range NPV interact with narrow host-range NPVs in different insect cell lines. Interaction of viruses can also be seen as an ecological question but for this aspect the readers are encouraged to consult a more comprehensive review (Cory *et al.*, 1997). Interactions between viruses other than baculoviruses have also been previously reviewed (Sherman, 1985; Waner, 1994). Prior to discussing baculovirus interactions in cells, we will review the general infection process of baculovirus in cells as an aid to understanding how the viruses can interact. We will also briefly discuss the current knowledge of virus–virus interaction in the insect host.

II. BACULOVIRUS INFECTION PROCESS

Natural infection of baculoviruses starts when an insect larva ingests virion-containing polyhedra scattered on the vegetation from a previous infection cycle. The polyhedra dissolve in the alkaline environment (pH 9–11) of the insect midgut and the virions (occlusion-derived virus or ODV) enter and replicate in the midgut. Progeny viruses bud through the basal lamina to form budded viruses (BV) in the hemocoel. The BVs then infect tracheal, fat body and other tissue cells, or are transmitted through the tracheal matrix to other parts of the body (Federici, 1997). The infection normally results in the death of the host (Cheng and Carner, 2000; Cheng *et al.*, 2005; Federici, 1997).

When the BVs from the midgut invade the hemocoel, they replicate in susceptible cells of specific tissues and produce progeny BVs that subsequently infect other cells. Unlike many mammalian viruses, where specific receptors must be present on the cell surface to negotiate with the invading virus to determine the entry, NPV BVs apparently do not require specific receptors to enter the cells (Miller and Lu, 1997). BVs can enter many cell types in the insect host and even mammalian cells, but only certain tissue or cell types support the completion of an infection cycle. Most investigations of viral entry to different cell lines were conducted with AcMNPV, which can infect many host insects and replicates in many cell lines (Groner, 1986; McIntosh and Grasela, 1994).

For an NPV to complete an infection cycle, the BV must first attach to the cell surface. Viral entry of NPVs occurs by absorptive endocytosis (Volkman and Goldsmith, 1985). Once the BV contacts the cell surface, the plasma membrane envaginates to engulf the BV and form an endocytic vesicle (endosome) in the cytoplasm (Hefferon *et al.*, 1999; Wang *et al.*,

1997; Wickham *et al.*, 1992). The endosome carrying the BV is transported to the vicinity of the nucleus. During the transportation, the pH in the endosome becomes acidic from the action of proton pump on the membrane and this acidic environment triggers fusion of the BV envelope and the endosomal membrane, thereby releasing the nucleocapsids into the cytoplasm. The GP64 protein involved in cell attachment also plays roles in envelope fusion in the endosome (Blissard and Wenz, 1992). The released nucleocapsids containing the viral genome and viral capsid protein are transported into the nucleus with the assistance of filamentous actin cables formed in the cytoplasm (Charlton and Volkman, 1991, 1993; Lanier and Volkman, 1998). Once the nucleocapsids are in the nucleus, they uncoat and present the viral DNA in the expressible form. The host RNA polymerase II transcribes early genes such as *IE1* and *p143* (helicase). These early genes products are required for viral DNA replication and subsequent gene transcription (Friesen and Miller, 1986). Genes transcribed after the viral DNA replication are called late genes and include many of the virus structural proteins (Friesen and Miller, 1986). GP64 is secreted and transported to the cell surface and integrated into the plasma membrane (Blissard and Wenz, 1992). Viral replication produces multiple copies of the viral genome and these are assembled with DNA binding proteins and capsid proteins to form progeny virions in the nucleus. Some of the virions receive *de novo* synthesized envelopes and are embedded in the polyhedrin (POLH) protein matrix that remains in the cells. Others are budded out of the cells as BV to initiate another infection cycle. During the budding, GP64 on the plasma membrane becomes part of the BV envelope (Blissard and Wenz, 1992).

III. BACULOVIRUS INTERACTIONS

Six categories of interactions exist between different species: antagonism, mutualism, commensalisms, amensalism, competition and neutralism (Begon *et al.*, 1996) and these can be applied to virus interaction in cells and host insects, but we will particularly focus on three of them, antagonism, mutualism and neutralism (Table 5.1). In antagonism between two species, one species benefits at the expense of the other and this is typified by predation and parasitism in cellular organisms. These terms are generally unsuitable with respect to viruses; so in this review, antagonism will refer to when the replication of one virus benefits the replication of another virus at its own expense (Table 5.1). In neutralism, two species interact without causing harm or benefit. Neutralism in virus interactions means the two species of viruses can infect cells, but infection of one virus in certain cells does not interfere with the infection of another virus to the other cells (Table 5.1). Neutralism is also called independent

TABLE 5.1 Types of virus interactions

Type of interaction	Effect on X	Effect on Y
Antagonism	+	−
Mutualism	+	+
Neutralism	0	0
Commensalism	+	0
Competition	−	−
Amensalism	−	0

+, The effect is beneficial; −, the effect is detrimental; 0, there is no effect.

proliferation (Lowe and Paschke, 1968). In mutualistic interactions, both viruses benefit from the presence of the other virus (Table 5.1). This review will focus mostly on antagonism with brief discussions on neutralism and mutualism.

Interactions are required for the evolution of species and viruses are not an exception since they require a cell to replicate and thus must coevolve with the host (Cory and Myers, 2003). In baculoviruses, many NPVs replicate only in cell lines from the host insect or closely related insects from which the NPV was originally isolated. One exception to this generalization is the well-studied AcMNPV that can infect many cell lines from different species (McIntosh and Grasela, 1994). The features that allow AcMNPV to infect many cell lines are not well understood and are difficult to study. Furthermore, lack of cytopathic effect in infection of NPVs in nonpermissive cells also makes the interaction of two NPVs *in vitro* difficult to detect and study. Historically, DNA–DNA hybridization has been used to study viral DNA replications of each NPV in coinfection to detect viral interactions (Palli *et al.*, 1996; Yanase *et al.*, 1998).

IV. BACULOVIRUS INTERACTIONS *IN VITRO*

A. Antagonistic interactions

Since most NPVs replicate well only in a few cell lines, coinfection of a cell line with two NPVs, one that replicates well and one that replicates poorly, has been used to elucidate mechanisms in host–virus interaction and host-range factors of baculoviruses (Kondo and Maeda, 1991; Maeda *et al.*, 1993; McClintock and Dougherty, 1987; Mori *et al.*, 1992; Wang *et al.*, 2008; Yanase *et al.*, 1998). If the two NPVs are closely related at the genomic DNA levels, recombination may take place and this should not be considered as antagonistic interaction. For example, the *Bombyx mori* NPV (BmNPV) and AcMNPV share high DNA homology. However,

BmNPV replicates well in BmN cells that is nonpermissive for AcMNPV replication. Coinfection of BmN cells with BmNPV and AcMNPV results in recombinant virus production. The DNA helicase (*p143*) gene between the two viruses was recombined, leading to AcMNPV receiving a portion of the helicase gene of BmNPV. This allowed AcMNPV replication in the otherwise nonpermissive BmN cells (Kondo and Maeda, 1991; Mori *et al.*, 1992). We will focus on experiments that did not produce recombinant viruses but instead on experiments that one virus provides a *trans*-acting factor that activates another NPV within a cell and viral-induced secretion that leads to improved DNA replication of otherwise nonpermissive cells.

1. Interaction between LdMNPV and AcMNPV in Ld652Y cells

Lymantria dispar MNPV (LdMNPV) belongs to the Group II NPV whereas AcMNPV belongs to Group I NPV due to low levels of DNA homology (Jehle, 2004). Ld652Y cells are permissive for LdMNPV replications but nonpermissive for AcMNPV infection (McClintock and Dougherty, 1987). To understand why Ld652Y cells are nonpermissive for AcMNPV infection, coinfection of Ld652Y cells with LdMNPV and AcMNPV was performed (McClintock and Dougherty, 1987). Superinfection of Ld652Y cells previously infected with LdMNPV at 8-h postinfection (p.i.) with AcMNPV resulted in maximal BV production of AcMNPV but significant reduction of LdMNPV BV, suggesting an antagonistic interaction between LdMNPV and AcMNPV in Ld652Y cells (McClintock and Dougherty, 1987). In this antagonistic interaction, AcMNPV replication in nonpermissive Ld652Y cells benefited at the expense of LdMNPV. LdMNPV replication in Ld652Y cells probably supplied a *trans*-acting factor that enhanced AcMNPV replication in nonpermissive Ld652Y cells.

To further scrutinize the roadblocks that prevent AcMNPV infection in Ld652Y cells, viral entry was tested as the restriction point, but results showed that AcMNPV entered Ld652Y cell lines (McClintock and Dougherty, 1987). Subsequently, the extremely low levels of AcMNPV viral and host cellular protein synthesis at 16–20-h p.i. in AcMNPV-infected Ld652Y cells is the reason for the poor replication (Guzo *et al.*, 1992; McClintock *et al.*, 1986; Morris and Miller, 1992). NPV gene transcription follows a temporal cascade scheme that proteins of early (E) gene transcripts initiated by host RNA polymerase can *trans*-activate late (L) gene transcription and the L genes are transcribed after viral DNA replication (Friesen and Miller, 1985). The AcMNPV genome replicated and low levels of both E and L AcMNPV genes were transcribed and exported to the cytoplasm and these cytoplasmic AcMNPV transcripts could be translated in an *in vitro* system (Guzo *et al.*, 1992; Morris and Miller, 1993). This indicates that the restriction point of AcMNPV infection in Ld652Y cells is at the level of translation (Du and Thiem, 1997a,b; Miller and Lu, 1997; Thiem *et al.*, 1996).

The *trans*-acting factor of LdMNPV that enhanced AcMNPV gene translation in nonpermissive Ld652Y cells was identified by screening an LdMNPV overlapping cosmid library in a cosmid/AcMNPV cotransfection experiment (Thiem *et al.*, 1996). This LdMNPV factor was a 25.7-kDa acidic polypeptide named host-range factor-1 (HRF-1). The antagonistic interaction between LdMNPV and AcMNPV in Ld652Y cells can, therefore, be described as follows: in an Ld652Y cell containing both genomes of LdMNPV and AcMNPV, mRNA of both viruses are synthesized and exported to the cytoplasm. The HRF-1 from LdMNPV elevated viral protein synthesis of AcMNPV that promoted AcMNPV DNA replication and viral infection. However, replication of AcMNPV in Ld652Y cells then inhibited LdMNPV DNA replication and viral infection by an undefined mechanism.

2. Interaction between CfMNPV and AcMNPV in CF-203 cells

The *Choristoneura fumiferana* MNPV (CfMNPV) replicates well in CF-203 cells derived from the midgut of *C. fumiferana* (Palli *et al.*, 1996; Sohi *et al.*, 1993) but AcMNPV cannot replicate in CF-203 cells due to viral-induced apoptosis (Palli *et al.*, 1996; Sohi *et al.*, 1993). In coinfection of CF-203 cells with CfMNPV and AcMNPV, CfMNPV reduced AcMNPV-induced apoptosis by 50% to allow AcMNPV replication at an elevated level compared to CF-203 cells infected only with AcMNPV. However, when CF-203 cells were preinoculated with CfMNPV at least 12 h prior to inoculation with AcMNPV, apoptosis was prevented and a full infection of AcMNPV occurred, showing expression of early and late genes as well as OB production (Palli *et al.*, 1996). This suggests that an antiapoptosis product from CfMNPV in conjunction with host cellular factors prevented the AcMNPV-induced apoptosis and, thus, allowing AcMNPV to complete the infection cycle (Palli *et al.*, 1996).

The interaction between CfMNPV and AcMNPV in CF-203 cells may only loosely fit into antagonistism since, while AcMNPV replication in CF-203 cells benefited from CfMNPV, the researchers did not study the affect on CfMNPV replication (Palli *et al.*, 1996). If AcMNPV in CF-203 cells does not inhibit CfMNPV, the interaction between the viruses would be considered commensalism, where one species benefits at no expense of the other (Table 5.1).

3. Interaction between AcMNPV and SeMNPV in Sf21 cells

SeMNPV is highly virulent to *S. exigua* larvae but it cannot kill the closely related species *Spodoptera frugiperda*, even though all classes of genes of SeMNPV were detected in *S. frugiperda* larvae after inoculation with SeMNPV OBs *per os* (Simon *et al.*, 2004). The high potency of SeMNPV against *S. exigua* has led to its development as a viral insecticide for *S. exigua* control in several countries. The narrow host-range of SeMNPV

also exists *in vitro* where it can only replicate efficiently in cell lines derived from *S. exigua*, such as Se301 (Hara *et al.*, 1993, 1994). SeMNPV replicates very poorly in Sf21 cell line derived from *S. frugiperda* while AcMNPV replicates well in both Se301 and Sf21 (Heldens *et al.*, 1997; Yanase *et al.*, 1998). Coinfection of SeMNPV and an AcMNPV OB-negative recombinant virus with the β-gal gene under a late polh promoter lead to identify AcMNPV genes that can help SeMNPV replication in Sf21 (Yanase *et al.*, 1998). When a multiplicity of infection (MOI) of 1 plaque-forming unit (pfu)/cell for AcMNPV and 5 pfu/cell for SeMNPV was used to coinfect Sf21 cells, AcMNPV significantly enhanced SeMNPV DNA replication compared to DNA replication of SeMNPV single infection in Sf21 cells at an MOI of 5 pfu/cell (Yanase *et al.*, 1998). Enhanced DNA replication of SeMNPV in coinfection of both viruses in Sf21 inhibited AcMNPV DNA replication based on DNA–DNA hybridization studies (Yanase *et al.*, 1998). At an MOI of 1 pfu/cell infection of Sf21 by SeMNPV, no OB could be formed at 72-h p.i. In the coinfection, 7.2% Sf21 cells showed OB formation. Therefore, AcMNPV not only helped DNA replication of SeMNPV in Sf21 but also helped production of OBs by SeMNPV in Sf21 (Yanase *et al.*, 1998). The enhanced DNA replication might be due to recombination between the two viruses but plaque assay did not find any recombinant virus possibly due to low genome sequence homology between SeMNPV and AcMNPV (Jehle, 2004; Jehle *et al.*, 2006; Yanase *et al.*, 1998). AcMNPV likely provided *trans*-activating factors that helped SeMNPV replication in nonpermissive Sf21 cells. However, these *trans*-acting factors have not yet been identified (Yanase *et al.*, 1998). This is a typical antagonistic interaction between AcMNPV and SeMNPV in Sf21 cells where SeMNPV benefits in replication from AcMNPV at the expense of the later.

4. Interaction between AcMNPV and SeMNPV in High 5 cells

AcMNPV replicates well in High 5 cells (BTI-TN5B1–4) derived from *Trichoplusia ni* (Granados *et al.*, 1994). SeMNPV infection in High 5 cells is poor and cannot be easily detected by microscopy by looking for OB formation in the nucleus. To facilitate the detection of viral infection in cells, a green-fluorescent protein (GFP) gene expression cassette with an AcMNPV *polh* promoter was inserted at the *p10* locus of SeMNPV to generate vSeGFP by the homologous recombination method (O'Reilly *et al.*, 1992). A red-fluorescent protein (RFP) gene expression cassette with an AcMNPV *polh* promoter was inserted at the *polh* locus of AcMNPV by the bacmid system to construct vAcRed (Wang *et al.*, 2008). Using fluorescence microscopy, coinfection of cells with two NPVs independently expressing GFP and RFP allows examination of live interactions of viruses within a cell or a population of cells. This method, called

dual color tag system (DCTS), has been demonstrated useful in understanding virus interaction in cells (unreported data).

Single infection of High 5 cells (about 70% confluency) with vSeGFP at an MOI of 0.1 pfu/cell shows delayed marker gene expression (GFP expression) compared to permissive SeI cell infection. The time of GFP detection (late gene expression after viral DNA replication) by fluorescence microscopy is day 3 p.i. Most of the High 5 cells infected with vSeGFP at 12-h p.i. showed spindle or fibroblast shapes similar to the mock-infected High 5 cells, suggesting active cell growth. However, infected cells showed morphological changes compared to mock-infected High 5 cells at 24-h p.i. In mock-infected High 5 cells, majority of the High 5 cells are sphere shaped at 24-h p.i. since cells reached almost 100% confluency, but about 50% High 5 cells infected with vSeGFP maintained spindle or fibroblast-like shape—typical of these cells at low density (unreported data). One possible explanation for the morphological differences between High 5 cells inoculated with vSeGFP and High 5 cells mock-infected within the first 48-h p.i. is that the High 5 cells that absorbed vSeGFP stopped cell growth and maintained the spindle or fibroblast shapes but the uninfected High 5 cells in the mock-infection or High 5 cells without vSeGFP continued growth, reached confluency and then become rounded.

In High 5 cells coinfected with vSeGFP and vAcRed at an MOI of 0.1 pfu/cell each, some High 5 cells showed GFP expression at 24-h p.i. but no RFP expression could be detected at this time. The High 5 cells showing GFP expression (vSeGFP infection or replication) continued to increase so that by 72-h p.i., a 90-fold more GFP expression were obtained in High 5 cells coinfected with vSeGFP and vAcRed compared to High 5 cells infected with vSeGFP alone (unreported data). A DNA replication assay using quantitative real-time PCR confirmed the fluorescent protein expression is representative of viral DNA replication (unreported data). At day 6 p.i., no further increase of GFP expression occurred in the High 5 cells and the cells started dying. When infection rates were compared at day 6 p.i., the vSeGFP infection rate in single infection of High 5 reached about 0.4% whereas the vSeGFP infection rate reached 18% in the coinfection of High 5 cells, representing a 48-fold increase of infection rate compared to the vSeGFP single infection rate in High 5 (Fig. 5.1A (a, b), B (a, b) and –C). The vAcRed infection rate reached about 100% in High 5 cells in single infection but reduced to about 3% in the coinfection, representing a 35-fold reduction (Fig. 5.1A (c, d), B (a, c) and D). This suggests that the interaction between vSeGFP and vAcRed in High 5 cell infection is antagonistic, since vAcRed helped vSeGFP replication in High 5 at the expense of itself. This is shown in the merged picture of the GFP and RFP channels in the coinfection at day 6 p.i. Cells expressing GFP showed no RFP expression (Fig. 5.1B (d)).

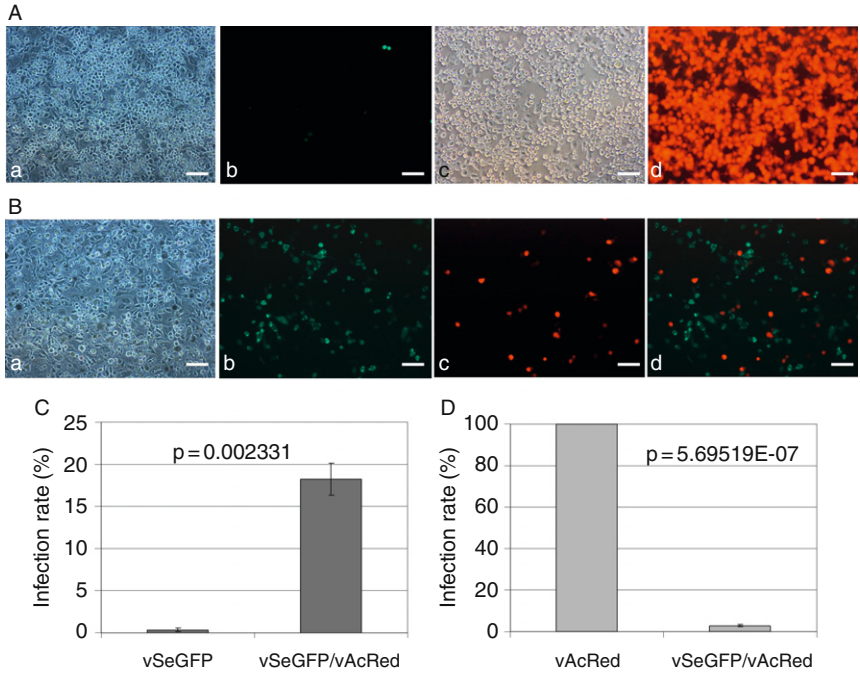

FIGURE 5.1 Antagonistic interaction between vSeGFP and vAcRed in High 5 cells. (A) Single infection of vSeGFP (0.1 MOI) and vAcRed (0.1 MOI) in High 5 cells. Images were taken at day 6 h p.i.: (a, b) infection of vSeGFP in High 5 cells imaged in the bright and GFP channel, respectively; (c, d) infection of vAcRed in High 5 cells imaged in bright and GFP channel, respectively. (B) Coinfection of vSeGFP and vAcRed in High 5 cells to show enhancement of vSeGFP infection by vAcRed and inhibition of vAcRed infection by vSeGFP. High 5 cells were coinfected by vSeGFP/vAcRed and imaged at day 6 h p.i. in the bright field (a), in the GFP channel (b) and the RFP channel (c); (d) merge of (b) and (c). Scale bar = 100 μm. (C) Quantitative analysis of enhancement of vSeGFP infection by vAcRed in Hi5 by GFP. Infection rates were calculated by four random images in bright field and GFP or RFP channels based on ratios between the numbers of cells showing fluorescence and total cells. (D) Quantitative analysis of inhibition of vAcRed infection by vSeGFP in High 5 cells by infection rate analysis as in (C). Vertical bars represent standard error of the mean. *P* values were calculated by *t*-test.

Since the infection rate of vSeGFP in High 5 cells is extremely low, one of the problems for vSeGFP to infect High 5 might be at the viral entry level. This was not the case found as centrifuged media from Sf21cells infected with vAcRed can activate vSeGFP replication in vSeGFP in High 5 (unreported data). After High 5 cells were absorbed with vThGFP and the cells were washed to remove vSeGFP and centrifuged media of Sf21 cells infected with vAcRed was added to the High 5 that had been absorbed

with vSeGFP, GFP expression by vSeGFP in High 5 was detected in 24-h p.i. and the GFP expression High 5 cells continued to increase to reach about 15% similar to coinfection of vSeGFP and vAcRed (Fig. 5.1B (b, d) and C). This suggests that the media of Sf21 cells infected with vAcRed contained factors that enhanced vSeGFP replication. This also suggests that in the coinfection of High 5 cells with vSeGFP and vAcRed, the vSeGFP entered the cells but had a hard time to replicate probably due to host cell suppression of viral replication. Since SeMNPV and AcMNPV share low DNA sequence homology and the chances of recombination between the two viruses are low. Moreover, coinfection of Sf21 cell with SeMNPV and AcMNPV did not produce recombinants (Yanase *et al.*, 1998). It therefore appears the secreted molecules of High 5 cells infected with vAcRed in the coinfection reversed the replication block for vSeGFP. Replication of vSeGFP in High 5 then in return inhibited vAcRed replication—a typical antagonistic interaction between the vSeGFP and vAcRed in High 5 cells.

5. Interaction between AcMNPV and ThorMNPV in Sf21 cells

ThorMNPV was originally isolated from *Thysanoplusia orichalcea* and can infect and kill *Pseudoplusia includens* and *T. ni*, but cannot infect or kill *S. frugiperda*, *S. exigua*, *Anticarsia gemmatalis*, or *Helicoverpa zea* by OB *per os* (Cheng *et al.*, 2005; Wang *et al.*, 2008). ThorMNPV replicates well in High 5 cells but poorly in Sf21 cells (Cheng *et al.*, 2005; Wang *et al.*, 2008). Alternatively, AcMNPV can replicate well in both Sf21 and High 5 cells. As discussed earlier, coinfection of two NPVs with certain insect cell lines has been successfully used to isolate recombinants of narrow host-range NPV with improved infection in nonpermissive cells (Kondo and Maeda, 1991; Mori *et al.*, 1992). Since ThorMNPV and AcMNPV are both Group I NPV, they share about 80% DNA sequence homology (Cheng *et al.*, 2005). To understand if AcMNPV is able to help ThorMNPV replication in nonpermissive Sf21 cells by either recombination or *trans*-activation (McClintock and Dougherty, 1987; Palli *et al.*, 1996; Yanase *et al.*, 1998), a GFP expression cassette using an AcMNPV *polh* late promoter was inserted at a nonessential gene locus, *gp37*, to generate vThGFP (Cheng *et al.*, 2001; Wang *et al.*, 2008).

Coinfection of Sf21 cells with vThGFP and vAcRed showed earlier (day 1 p.i.) late gene promoter (*polh*) activities for GFP expression compared to single infection of Sf21 cells with vThGFP (day 3 p.i.). Sf21 cells infected with vAcRed showed an almost 100% infection by day 3 p.i. Infection kinetics comparison between vThGFP/AcBacmid and vThGFP showed the vThGFP infection rate in coinfection is 25-fold higher than vThGFP infection alone in Sf21 and the infection rate increase in coinfection is closely related to vThGFP DNA replication by quantitative real-time PCR (Wang *et al.*, 2008). The increase of genome replication of vThGFP in coinfection compared to the single infection is probably not

due to the transfer of GFP gene to vAcRed by recombination (Wang *et al.*, 2008). When fluorescence images for single- and coinfection were analyzed at day 4 p.i., an infection rate of 7.5% was obtained in vThGFP single infection in Sf21 cells, whereas a 95% infection rate by vThGFP in the coinfection was obtained suggesting a 13-fold increase (Fig. 5.2A (a, b), B (a, b) and C). vAcRed produced a 96% infection rate in Sf21 by single infection but the infection rate dropped to 21% in the coinfection, which represents a 4.5-fold reduction (Fig. 5.2A (b, c), B (c) and D). The interaction between vAcRed and vThGFP is antagonistic because

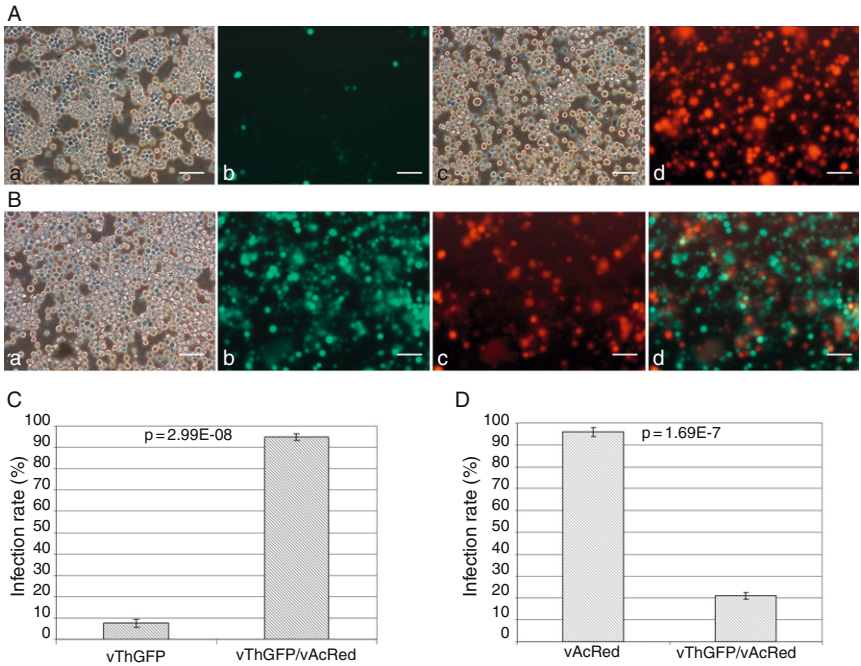

FIGURE 5.2 Antagonistic interaction between vThGFP and vAcRed in Sf21 cells. (A) Single infection of vThGFP (10 MOI) and vAcRed (10 MOI) in Sf21. Images were taken at 96-h p.i.: (a, b) infection of vThGFP in Sf21 cells imaged in the bright and GFP channel, respectively; (c, d) infection of vAcRed in Sf21 cells imaged in bright and RFP channel, respectively. (B) Coinfection of Sf21 with vThGFP/vAcRed (10 MOI each) showing enhanced infection of vThGFP by vAcRed. Sf21 cells were coinfected with vThGFP/vAcRed and photographed in the bright channel (a), GFP channel (b) and RFP channel (c) at 96-h p.i.; (c) merge of (b) and (c). Scale bar = 100 μm. (C) Quantitative analysis of enhancement of vThGFP infection by vAcRed in coinfection compared with vThGFP infection alone in Sf21 by infection rates. (D) Quantitative analysis of inhibition of vAcRed infection by vThGFP in coinfection compared with vAcRed infection alone in Sf21. Vertical bars represent standard error of the mean ($n = 4$). *P* values were calculated by *t*-test.

vAcRed helped vThGFP infection at the expense of itself. Most of the Sf21 cells expressing GFP did not express RFP as no yellow is seen when the GFP and RFP images of coinfection were merged (Fig. 5.2B (d)).

To understand the mechanisms of enhanced infection of vThGFP in coinfection with vAcRed in Sf21, two hypotheses were tested, recombination and *trans*-activation (Wang *et al.*, 2008). Recombination hypothesis has been tested by plaque assay using BV generated from coinfection of Sf21 cells (O'Reilly *et al.*, 1992). BVs infection of vThGFP did not form a GFP plaque that required a cluster of cells infected by viruses, but individual cells were infected by vThGFP where RFP plaques formed by vAcRed were not close to the cells infected by vThGFP. (Fig. 5.3A (a–c)). BVs from coinfection formed GFP plaques surrounded in close proximity to vAcRed plaques (Fig. 5.3B (a–c)). When four of these GFP plaques (vThGFP1) were purified and compared for infection with the parent vThGFP in High 5 and Sf21 cells, both vThGFP and vThGFP1 showed an almost 100% infection rate in High 5 at day 3 p.i. but poor in infecting Sf21 cells (Wang *et al.*, 2008). Thus, the enhanced infection of vThGFP in coinfection with vAcRed in Sf21 is not due to recombination (Wang *et al.*, 2008).

Since both enhanced infection of AcMNPV and SeMNPV in nonpermissive cell lines in coinfection studies suggested *trans*-activation, this

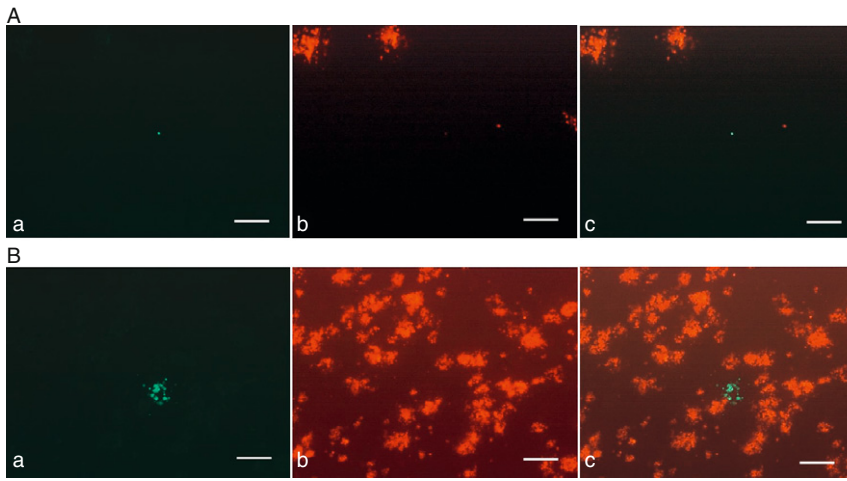

FIGURE 5.3 Plaque assay showing interaction between vThGFP and vAcRed by viral-induced cellular secretion. (A) Budded viruses from Sf21 cells coinfected with vThGFP/vAcRed could not form a GFP plaque when it is far way from the RFP plaques formed by vAcRed in Sf21 cells at day 5 p.i.: (a) Sf21 cells infected with vThGFP imaged in the GFP channel, (b) RFP channel, (c) merge of (a) and (b). (B) Formation of a vThGFP plaque in Sf21 cell in close proximity to RFP plaques formed by vAcRed at day 5 p.i.: (a) Sf21 cells infected with vThGFP imaged in the GFP channel, (b) RFP channel, (c) merge of (a) and (b). Scale bar = 200 μm.

hypothesis was tested for the enhanced vThGFP infection in semipermissive Sf21 cells (McClintock and Dougherty, 1987; Palli *et al.*, 1996; Wang *et al.*, 2008; Yanase *et al.*, 1998). Sf21 cells were cotransfected with vThGFP genomic DNA and an AcMNPV cosmid library (five clones) with all five cosmids or by omitting one at a time and GFP expression was compared among the transfected cells (Li *et al.*, 1999; Wang *et al.*, 2008). No difference of GFP expression were detected among these cotransfection (Wang *et al.*, 2008). Therefore, the conventional mechanism of *trans*-activation cannot explain the enhanced infection of vThGFP in coinfection with vAcRed in Sf21 cells. Since very few Sf21 cells in the coinfection showed yellow (Fig. 5.2B (d)), the hypothesis that vAcRed-induced cellular secretion in Sf21 cells that enhanced vThGFP infection in Sf21 was tested (Wang *et al.*, 2008). Also, High 5 cells coinfected with vSeGFP and vAcRed did not show coexpression of GFP and RFP in the same cells suggesting no coreplication. To test the secretion enhancement hypothesis, BVs were removed from media of Sf21 cells infected with vAcRed at day 4 p.i. and mixed with Grace's media (1:1) to perform vThGFP infection assay in Sf21 cells. Enhanced infection vThGFP in Sf21 cells was observed compared to normal vThGFP infection, indicating some factor(s) is secreted from the vAcRed-infected cells (Wang *et al.*, 2008). Furthermore, vThGFP showed enhanced replication and formed GFP plaques in close proximity to the RFP plaques formed by vAcRed in plaque assay (Fig. 5.3). Since agarose overlay on cells is more limiting to BVs mobility than proteins, a concentration gradient of secreted factors would occur (Pluen *et al.*, 1999). Therefore, it is highly likely that the enhanced infection or DNA replication of vThGFP in coinfection of Sf21 cells with vAcRed is due to the secretion of Sf21 cells induced by vAcRed.

Viral-induced cellular secretion has been well studied in mammalian systems, especially when mammalian cells infected by RNA viruses. For example, human cells infected by viruses secrete interferon that can bind to receptors on cells to initiate signal-transduction pathway leading to upregulation of gene expression to inhibit viral replication (Sen, 2001; Watanabe, 2004). In invertebrates, a mammalian interferon homolog has been reported in *Drosophila* that regulates gene expression upon bacterial challenge (Georgel *et al.*, 1995). It is plausible that the AcMNPV-induced cellular secretion molecules also bind to receptors on the cell surface to initiate signal transduction leading to activation of vThGFP replication in Sf21 and vSeGFP replication in High 5 cells.

B. Neutralistic interactions

Even though our main emphasis in the review is on the antagonistic interaction of viruses, a neutralistic interaction review will contrast with the antagonistic interaction. In fact, all species interact at a certain level in

nature. The neutralistic interaction with no effect on the two partners is difficult to prove due to sensitivities of our detection tools.

To visualize neutralistic interactions of baculoviruses in cells, the DCTS has been used. Coinfection of High 5 cells with vSeGFP and vAcRed showed an antagonistic interaction (Section IV.A.4) while a neutralistic interaction was observed in Sf21 cells coinfected with the same viruses. Sf21 cells are highly permissive for AcRed infection but nonpermissive for vSeGFP (SeMNPV) (Hara *et al.*, 1994; Yanase *et al.*, 1998). Inoculation of Sf21cells with vSeGFP alone at an MOI of 0.1 pfu/cell showed a <0.5% infection rate at day 3 p.i. (Fig. 5.4A (a, b) and C).

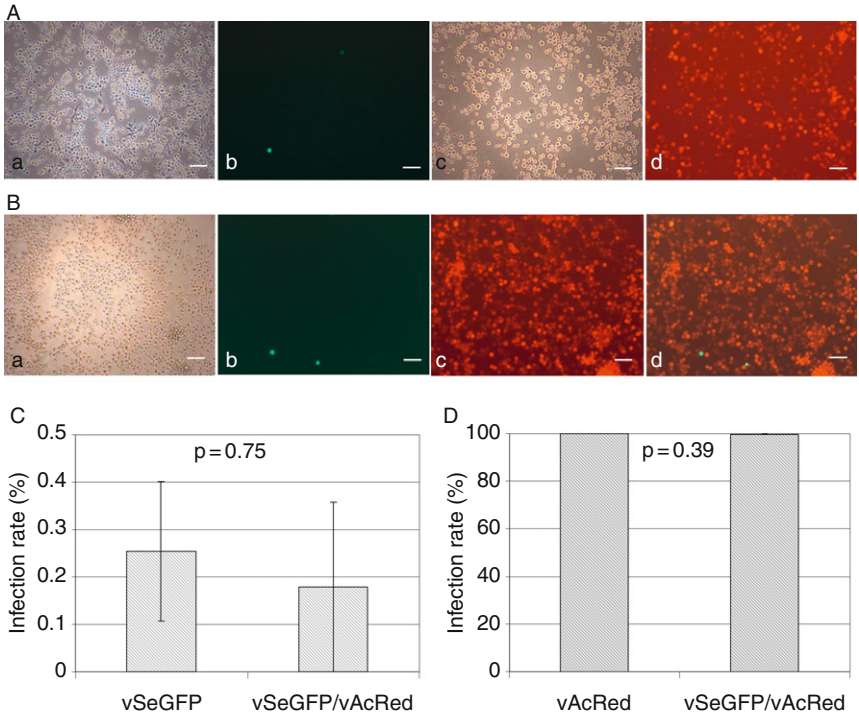

FIGURE 5.4 Neutralistic interaction between vSeGFP and vAcRed in Sf21 cells. (A) Single infection of vSeGFP (0.1 MOI) and vAcRed (0.1 MOI) in Sf21. Sf21 cells were infected by either vSeGFP or vAcRed and imaged at 72-h p.i.: (a, b) infection of vSeGFP in Sf21 cells imaged in the bright and GFP channel, respectively; (c, d) infection of vAcRed2 in Sf21 cells imaged in the bright and the GFP channel, respectively. (B) Coinfection of vSeGFP and vAcRed in Sf21 to show no enhancement of vSeGFP infection by vAcDsRed2 and no inhibition of vAcRed infection by vSeGFP. Sf21 cells were coinfected by vSeGFP/vAcRed and imaged in bright field (a), in the GFP channel (b) and the RFP channel (c); (d) merge of (b) and (c). Scale bar = 100 μm. (C) Quantitative infection rate analysis of no enhancement of vSeGFP infection by vAcRed in Sf21. (D) Quantitative infection rate analysis of no inhibition of vAcRed infection by vSeGFP in Sf21. Vertical bars represent standard error of mean ($n = 4$).

Inoculation of Sf21 cells with vAcRed showed an almost 100% infection rate (Fig. 5.4A (c, d) and D). In coinfection of Sf21 cells with vSeGFP and vAcRed, the low infection rate for vSeGFP and high infection rate for vAcRed is similar to the single infection of Sf21 cells with either vSeGFP or vAcRed (Fig. 5.4A–D). Therefore, vAcRed did not help vSeGFP replication in nonpermissive Sf21 cell and vSeGFP did not inhibit vAcRed infection in Sf21 cells, which is consistent to a neutralistic interaction. The antagonistic interaction between AcMNPV and SeMNPV has been demonstrated by DNA–DNA hybridization (Yanase *et al.*, 1998). One possible reason for the different results could be that different strains of SeMNPV and different passage numbers of Sf21 cell line were used in these experiments.

Neutralistic interactions of baculoviruses *in vitro* also exist in cell lines derived from *S. exigua* infected with vAcRed and vSeGFP. Five *S. exigua* cells were tested for the interactions of vAcRed and vSeGFP by coinfection. These cell lines included SeE1, SeE4, SeE5, Spex-II A and UCR (Gelernter and Federici, 1986; Goodman *et al.*, 2001; Zhang *et al.*, 2006). Neither detrimental nor beneficial effects were detected by the DCTS. None of the tested *S. exigua* cell lines are highly permissive for either vSeGFP or vAcRed infection (unreported data).

V. BACULOVIRUSES INTERACTIONS *IN VIVO*

Different baculoviruses can infect a single larva resulting in a mixed infection in nature. Several NPVs have been isolated from mixed infections of larvae. For example, a single-nucleocapsid NPV (ThorSNPV) and a multinucleocapsid NPV (ThorMNPV) have been isolated from *T. orichalcea* larvae (Cheng and Carner, 2000; Cheng *et al.*, 2005). NPVs isolated from *C. fumiferana* actually were also a mixture of two NPVs, CfMNPV and CfDEFNPV (*C. funiferana* defective nucleopolyhedrovirus) (Lauzon *et al.*, 2005). Two types of baculovirues (NPV and GV) were isolated from a mixed infection of *Pseudaletia unipuncta* (Tanada, 1956, 1959a,b, 1961). As with *in vitro* infections, at least three types of interactions *in vivo* exist between or among NPVs: neutralism, mutualism and antagonism (Table 5.1). We will discuss each type of the interactions using examples.

A. Neutralism

Neutralistic interaction of baculoviruses is probably rare in nature as all species interact with each other to a certain extent. The levels of interaction and sensitivities of our detection may affect the interpretation of this

neutralistic phenomenon. The ThorSNPV and ThorMNPV were originally present in the natural population of *T. orichalcea* larvae in West Java, Indonesia (Cheng and Carner, 2000; Cheng *et al.*, 2005). The original sample of NPVs consists of majority of the ThorSNPV and a minimal amount of ThorMNPV. The shape of ThorSNPV OBs is tetrahedral but the shape of ThorMNPV OBs is polyhedral (Cheng and Carner, 2000; Cheng *et al.*, 2005). Initial propagation of the mixture of the two NPVs was carried out in soybean looper, *P. includens*. Successive passage of the original inoculum in *P. includens* resulted in progressive reduction of the ThorSNPV and increase of the ThorMNPV (Cheng and Carner, 2000; Cheng *et al.*, 2005). When ThorSNPV and ThorMNPV were separated and purified to perform bioassays in third instar *P. includens*, ThorSNPV showed a LD_{50} of 65,636 OBs/larva, while ThorMNPV showed a LD_{50} of 242 OBs/larva (Cheng and Carner, 2000; Cheng *et al.*, 2005). ThorMNPV has 271-fold higher potency than ThorSNPV to the third instar *P. includens*. Bioassay using the initial inoculum consisting majority of ThorSNPV and minimal ThorMNPV also showed very low infectivity to *P. includens* similar to ThorSNPV. In the late passage where ThorMNPV was the major proportion and ThorSNPV was the minimal proportion, the infectivity of the mixture was similar to that of ThorMNPV (unreported data). The two NPVs replicate in the fat body and trachea of *P. includens* but the two types of OBs were not found in the same cells (Cheng *et al.*, 2005). Therefore, it is highly likely, the interaction between ThorSNPV and ThorMNPV in *P. includens* larvae is neutralistic.

B. Mutualism

Mutualism may be favored in evolution since both species benefit in the interaction. The wild-type NPV isolated from *C. funiferana* was a mixture of at least two types of NPVs, the CfMNPV and the CfDEFNPV (Arif *et al.*, 1984; Lauzon *et al.*, 2005). CfDEFNPV is defective in infecting *C. funiferana* larvae *per os* because the virus cannot pass the midgut to enter the hemoceol to replicate in other tissues, whereas CfMNPV can infect *C. funiferana* larvae *per os* (Lauzon *et al.*, 2005). In a mixed infection with CfMNPV, the CfDEFNPV is able to pass the gut of *C. funiferana* larvae and replicate in other tissues in the hemoceol. Therefore, the CfMNPV assists CfDEFNPV in an undefined mechanism in passing the midgut of *C. funiferana* larvae. Once both CfMNPV and CfDEFNPV are in the hemoceol, CfDEFNPV synergizes CfMNPV infection in *C. funiferana* larvae (Lauzon *et al.*, 2005). Therefore, both viruses get benefits from each other, a typical mutualistic interaction.

Mutualistic interactions also exist in various variants of *S. frugiperda* multicapsid nucleopolyhedrovirus (SfMNPV) (Lopez-Ferber *et al.*, 2003).

Natural populations of SfMNPV are present as mixtures of different genotypes. Some genotypes have deletions up to 15 kb compared to the complete genome of SfMNPV (Lopez-Ferber *et al.*, 2003). These mutants are unable to infect *S. frugiperda* larvae because the *per os* infectivity gene (*pif*) that is required for *per os* infectivity of OBs in larvae is in the 15-kb deletion (Kikhno *et al.*, 2002). Replication of the defective genome viruses in cell lines is fine (Lopez-Ferber *et al.*, 2003). These genotypes with *pif* gene deletions occur at about 35% frequency in the natural population of SfMNPV (Lopez-Ferber *et al.*, 2003). The wild type that contains both complete genome and genomes with *pif* deletion showed higher pathogenecity than the complete genome type, suggesting a mutualistic interaction between these virus genotypes (Lopez-Ferber *et al.*, 2003). In this interaction, the genotypes with *pif* gene deletion could not produce *per os* infection in larvae but benefit from being co-occluded within the OBs of the complete genotype. Once the deletion genotypes are in the hemoceol, they probably replicate faster than the complete genotype due to short genome length (Barrett and Dimmock, 1986; Lopez-Ferber *et al.*, 2003). In fact, natural populations of baculoviruses are always mixtures of different genotypes (Erlandson *et al.*, 2006; Maeda *et al.*, 1990; Smith and Crook, 1988). Plaque-purified LdMNPV showed different infectivity in cell lines (Lynn *et al.*, 1993) but the interactions of these genotypes in the wild-type baculoviruses are not well understood.

C. Antagonism

The antagonistic interactions between baculoviruses are exemplified in a mixed infection of *P. unipuncta* larvae with an NPV and GV (Tanada, 1959a,b; Tanada and Hukuhara, 1971). In the interaction between the NPV and GV in *P. unipuncta* larvae, the GV helped the NPV infection in the larvae but the infectivity of the mixed infection is less than that by the GV alone. Since OBs of baculoviruses are dissolved in the gut juice to release the ODVs that must pass the peritrophic membrane (PM) to reach the midgut cells to initiate primary infection, the PM, in fact, is a physical barrier to the ODVs. In the coinfection, a metalloprotein called enhancin in granules of GV can disrupt the PM by digesting a major protein constituent of the PM. This facilitates the entry of the ODVs of NPV into the midgut cells (Roelvink *et al.*, 1995; Wang and Granados, 1997; Wang *et al.*, 1997). However, when both the NPV and GV are in the hemoceol, the replication of the NPV apparently interferes with the replication of GV, leading to the reduced mortality of larvae inoculated with both viruses when compared to single GV inoculation (Tanada and Hukuhara, 1971).

VI. CONCLUSIONS

Both *in vivo* and *in vitro* interactions of baculoviruses have molecular, cellular and ecological significances. Molecular and cellular interactions of baculoviruses in different insect cells in some cases allow the viruses to hijack the cells to secrete signals that change the permissibility of cells for viral infection. Since secreted molecules from cells often bind to receptors to initiate signal transduction leading to either activating or inactivating gene transcription, the enhancement of replication of vThGFP in Sf21 and vSeGFP in High 5 cells by vAcRed is probably through ligand–receptor binding that leads to activation of the suppressed viral replication in these cells. This should be an additional mechanism of interaction of viruses *in vitro* other than the mechanism of *trans*-activation. *In vitro* infection of baculoviruses in larvae is a natural ecological phenomenon that has potential practical applications that should be further explored for the development of viral insecticides in integrated pest management program in agriculture and forestry.

VII. FUTURE PROSPECTS

Since the virus interaction through the potential ligand–receptor interaction is a new area that has not been well addressed, this should be analyzed further by identifying the secreted molecules by either mass spectrometry or peptide sequencing. Once the secreted molecules have been identified, the receptors on the cell surface can be identified by immuno-pull-down assay using antibodies generated from secreted peptides. Further studies will be carried out on identifying the signal-transduction pathway. The reasons why vThGFP and vSeGFP could not replicate well are not known and should be investigated. This will lead to a better understanding of the virus interaction *in vitro*. In the *in vivo* interaction of baculoviruses in larvae, the mechanisms that the defective viruses (CfDEFNPV) cannot infect larvae *per os* should be studied that will lead to understanding how the CfMNPV assisting CfDEFNPV in passing the gut of *C. funiferana* larvae.

ACKNOWLEDGMENTS

The authors thank Drs. Lihua Wang and Tamer Salem who provided the initial investigations of the virus interaction work. Mr. Xin-Hua Cheng is credited for providing further evidence to support the secretion enhancement hypothesis in the virus interaction. This work is partially supported by a USDA grant (58-3148-7-164) to XWC.

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CHAPTER 6

Posttranscriptional Gene Regulation in Kaposi's Sarcoma-Associated Herpesvirus

Nicholas K. Conrad

Contents

Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi's sarcoma, primary effusion lymphoma and some cases of multicentric Castleman's disease. To understand the pathogenesis and life cycle of KSHV, significant focus has been

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Advances in Applied Microbiology, Volume 68
ISSN 0065-2164, DOI: 10.1016/S0065-2164(09)01206-4

placed on determining how KSHV factors influence viral and cellular gene expression. The importance of transcriptional regulation by KSHV is well documented, but several KSHV posttranscriptional regulators are also essential for KSHV replication and pathogenesis. KSHV miRNAs regulate translation and stability of cellular mRNAs that may be important for tumorigenesis. The ORF57 protein has been reported to enhance several posttranscriptional processes including viral mRNA export, RNA stability and pre-mRNA splicing. SOX, Kaposin B and the PAN-ENE regulate the stability of viral or cellular transcripts. Together, these observations point to the importance of posttranscriptional regulation in KSHV. With the growing appreciation of posttranscriptional regulation in cellular gene expression, it seems likely that the list of viral posttranscriptional regulatory schemes will expand as new details of KSHV gene regulation are uncovered.

I. INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV; HHV-8) is the causative agent of Kaposi's sarcoma (KS) and at least two lymphoproliferative disorders (Du *et al.*, 2007; Ganem, 2006; Laurent *et al.*, 2008; Sullivan *et al.*, 2008). KS is an angiogenic polyclonal tumor composed of elongated spindle cells that are likely of endothelial origin. In the U.S., KS is most commonly associated with immunocompromised AIDS patients and therapeutically immunosuppressed transplant recipients. However, in specific regions of the Mediterranean and sub-Saharan Africa, KS is observed in individuals with no apparent immune deficiency. KSHV also causes primary effusion lymphoma (PEL), a rare clonal B-cell tumor and some cases of multicentric Castleman's disease (MCD). Both MCD and PEL are predominantly found in immunocompromised patients.

KSHV is a member of the herpesviridae, a family of enveloped viruses with linear dsDNA genomes ranging in size from ~125 to 230 kb (Pellet and Roizman, 2007). Herpesviruses can be divided into three subfamilies, α, β and γ and each subfamily is represented among the eight known human herpesviruses. Human α-herpesviruses include the herpes simplex viruses (HSV1 and HSV2) and varicella zoster virus (VZV), while the β subfamily includes cytomegalovirus (CMV), HHV-6 and HHV-7. KSHV and Epstein–Barr virus (EBV), another oncogenic herpesvirus (Cohen *et al.*, 2008; Thorley-Lawson and Allday, 2008), belong to the γ-herpesviruses subfamily. Like all herpesviruses, KSHV establishes a lifelong latent infection during which the genome circularizes and is maintained as a stable episome in the absence of viral replication. KSHV-infected cells in KS, PEL and MCD samples are predominantly latently infected with a small percentage of infected cells undergoing lytic replication. Therefore, latent

gene expression is likely to be essential for pathogenesis, but specific lytic genes may contribute to pathogenicity by a nonautonomous mechanism (Du *et al.*, 2007; Ganem, 2006; Laurent *et al.*, 2008; Sullivan *et al.*, 2008).

Transcriptional control of viral and cellular genes by KSHV proteins is essential for viral replication and maintenance of latency. Upon lytic reactivation of latently infected PEL cells in culture, a sophisticated gene expression cascade ensues resulting in production of progeny virions (Jenner *et al.*, 2001; Sun *et al.*, 1999). Lytic reactivation is induced by the KSHV transcription activator, Rta (ORF50), which is both necessary and sufficient to induce lytic reactivation of latently infected cells (Deng *et al.*, 2007; Hayward *et al.*, 2006; Staudt and Dittmer, 2007; West and Wood, 2003). Rta activates transcription either by binding to promoters directly or it can be recruited by cellular factors, including RBP-Jκ, a downstream effector in the Notch pathway. In addition to reactivation of the lytic cycle, KSHV transcription factors regulate gene expression in latently infected cells. The latency-associated nuclear antigen (LANA) is a multifunctional protein that activates and represses viral and cellular gene transcription (Nicholas, 2007; Verma *et al.*, 2007). The Rta promoter is repressed by LANA, suggesting that one mechanism used by KSHV to maintain a latent infection is by repression of Rta transcription.

The functions of Rta and LANA demonstrate the importance of transcriptional control in the KSHV life cycle, but the significance of posttranscriptional regulation of viral and cellular gene expression should not be overlooked. Recent evidence demonstrates that as many as 92–94% of cellular genes undergo alternative spicing and that alternative polyadenylation is also relatively common (Licatalosi *et al.*, 2008; Pan *et al.*, 2008; Wang *et al.*, 2008). In addition, miRNAs regulate translation and RNA stability in many organisms to modulate a variety of cell functions (see Section II). Not surprisingly, KSHV has adopted posttranscriptional mechanisms to control viral and host gene expression. This review focuses on posttranscriptional regulation of viral and cellular gene expression by KSHV. In particular, the reported roles of KSHV miRNAs, ORF57, SOX, Kaposin B and the PAN-ENE on viral and cellular gene expression are explored.

II. KSHV-ENCODED miRNAs

The field of gene regulation has been revolutionized by the discovery of miRNAs and ~22 nucleotide (nt) RNAs posttranscriptionally regulate gene expression (Ambros, 2004; Bartel, 2004; He and Hannon, 2004; Lee *et al.*, 2006). The importance of miRNAs is underscored by their wide-ranging roles in biology. All multicellular organisms encode miRNAs and hundreds of miRNAs have been identified in the human genome. Recent estimates

indicate that over 60% of human mRNAs are regulated by miRNAs (Friedman *et al.*, 2008). Moreover, miRNA-mediated gene regulation is involved in many important biological processes including, but not limited to, development (Yekta *et al.*, 2008), aging (Boehm and Slack, 2006), oncogenesis (Croce, 2008; Deng *et al.*, 2008; Esquela-Kerscher and Slack, 2006) and immune response (Baltimore *et al.*, 2008; Tili *et al.*, 2008).

miRNAs are endogenously expressed noncoding RNAs that are processed from RNA polymerase II (pol II) primary transcripts (pri-miRNAs) (Ambros, 2004; Bartel, 2004; He and Hannon, 2004; Lee *et al.*, 2006). An ~80 nt hairpin within the primary transcript is cleaved on both strands by the Drosha-DGCR8 microprocessor complex and the resulting ~60 nt hairpin (pre-miRNA) is subsequently exported from the nucleus via the karyopherin family export factor, exportin-5. Once in the cytoplasm, Dicer cleaves the pre-miRNA and the miRNA strand is incorporated into the multiprotein complex RISC, while the non-miRNA strand is degraded. In some cases, both strands are incorporated into RISC and function as miRNAs. Within the RISC complex, miRNAs target specific genes by hybridizing to the 3′ UTRs of targeted mRNAs. The complementarity between miRNA and mRNA is imperfect, but perfect complementarity in a 6–8 nt seed sequence at the 5′ end of the miRNA is usually required (Lewis *et al.*, 2003). The RISC–miRNA complex then regulates gene expression by inhibiting translation and degrading the mRNA (Behm-Ansmant *et al.*, 2006a,b; Giraldez *et al.*, 2006; Wu *et al.*, 2006).

A. Discovery and conservation of KSHV-encoded miRNAs

Given the ubiquitous nature of miRNAs and their compact size, it is not surprising that some viruses use miRNAs to regulate both viral and host mRNAs (Gottwein and Cullen, 2008). KSHV miRNAs were identified by several groups using a variety of small RNA cloning and computational methods (Cai *et al.*, 2005; Grundhoff *et al.*, 2006; Pfeffer *et al.*, 2005; Samols *et al.*, 2005). KSHV encodes 12 pre-miRNAs that are processed into as many as 17 mature miRNAs. The pri-miRNAs are transcribed from the latency-associated region of the KSHV genome, which includes several protein-coding genes (LANA, v-cyclin, v-FLIP, Kaposin) and multiple overlapping transcripts (Cai and Cullen, 2006; Pearce *et al.*, 2005). Consistent with their genomic locus, KSHV miRNAs are expressed during latency but they remain detectable throughout the lytic cycle (Cai *et al.*, 2005; Grundhoff *et al.*, 2006; Pfeffer *et al.*, 2005; Samols *et al.*, 2005). Conservation of KSHV miRNAs further suggests that they provide an important function for KSHV. Clinical isolates from 14 KS and two MCD patients as well as five PEL cell lines showed that KSHV-encoded miRNAs are highly conserved (Marshall *et al.*, 2007). The latent

expression and high conservation of KSHV miRNAs imply that their function is critical to the viral life cycle and/or pathogenicity.

B. Targets of KSHV miRNAs

While detailed functional information for each of the KSHV miRNAs remains unknown, some progress is being made toward identifying the targets of KSHV miRNAs. The most experimentally accessible way of revealing miRNA targets is to express the miRNA in cells and use microarray profiling to identify downregulated mRNAs. One problem associated with this approach is that the miRNA may primarily inhibit translation and leave mRNA target levels only slightly altered. As a result, small miRNA-mediated decreases in mRNA levels may be detectable but difficult to distinguish from stochastic variations in gene expression or experimental error. To address this issue, Ganem and colleagues profiled host gene expression under several distinct experimental conditions that maintain or lack KSHV miRNA activity and only those mRNAs regulated under every condition tested were considered candidate miRNA targets (Ziegelbauer *et al.*, 2009). This stringent approach led to the discovery that KSHV miR-K5 represses BCLAF1, a key apoptosis-inducing factor. In an independent study, Renne and colleagues identified thrombospondin 1, a tumor suppressor and antiangiogenic factor as a KSHV miRNA target (Samols *et al.*, 2007). Bioinformatic methods predict that some KSHV miRNAs may target Rta, which would lead to maintenance of latent infection by repressing the viral lytic induction (Murphy *et al.*, 2008). This prediction awaits experimental corroboration; however, HSV miRNAs have been reported to control reactivation from latency (Umbach *et al.*, 2008). In addition, KSHV miR-K12-11 miRNA targets overlap significantly with those of the cellular miR-155 (see Section II.C). These predicted and experimentally verified targets implicate KSHV miRNAs as regulators of apoptosis, angiogenesis and viral reactivation.

C. KSHV miR-K12-11 is an ortholog of miR-155

KSHV is a master thief of cellular genes; that is, the KSHV genome encodes cytokines, antiapoptotic factors, etc., are evolutionarily derived from cellular transcripts (Moore and Chang, 1998). Recent results from the Renne and Cullen laboratories demonstrate that this molecular thievery (or at least mimicry) may not be limited to protein-coding genes (Gottwein *et al.*, 2007; Skalsky *et al.*, 2007). Specifically, both groups independently reported that KSHV miR-K12-11 is a viral ortholog of miR-155. miR-155 and miR-K12-11 have identical seed sequences and miR-K12-11 and miR-155 can downregulate identical targets in reporter assays. To look for endogenous targets, miR-K12-11 was expressed in

heterologous cells and microarray analysis was used to identify genes downregulated in the presence of miR-K12-11 expression. A subset of the candidate mRNA targets was validated using a variety of approaches. First, a significant fraction of the genes identified contain seed matches to miR-K12-11 in their 3′ UTRs. Second, many of the same transcripts were also downregulated upon miR-155 expression. Third, fusion of the 3′ UTR of the candidate mRNAs to reporter transcripts was sufficient to confer miR-K12-11 responsiveness to the reporters. Fourth, endogenous protein production was decreased in the presence of miR-K12-11. Finally, protein levels of one predicted target, Fos, were rescued upon introduction of antagomirs to miR-K12-11 in KSHV-infected cells (Gottwein *et al.*, 2007). These data show that miR-155 and KSHV miR-K12-11 have overlapping targets and likely share similar functions.

The known functions of miR-155 suggest a connection between KSHV miRNAs and pathogenesis. Prior to the discovery of miR-155, the noncoding polyadenylated *BIC* RNA was observed to be upregulated in lymphomas and leukemias and was associated with oncogenesis (Clurman and Hayward, 1989; Tam *et al.*, 1997, 2002). Later it was shown that the *BIC* transcript included miR-155 and that miR-155 was expressed in some lymphomas (Eis *et al.*, 2005; Kluiver *et al.*, 2005; Metzler *et al.*, 2004). Moreover, B-cell specific expression of miR-155 in transgenic mice is sufficient to induce B-cell lymphoproliferation (Costinean *et al.*, 2006). Consistent with its role as a putative oncomir, miRNA-155 expression is tightly regulated during B-cell development (Thai *et al.*, 2007) and, as expected, it is not expressed in PEL-derived cell lines (Gottwein *et al.*, 2007; Skalsky *et al.*, 2007). Thus, it is reasonable to speculate that the progrowth properties of miR-155 expression, as supplied by miR-K12-11, link KSHV miRNA expression with pathogenesis. Interestingly, other oncogenic herpesviruses upregulate miR-155 activity. The Marek's disease virus (MDV), which causes lymphomas in chickens, encodes a miR-155 ortholog (Zhao *et al.*, 2009). In contrast, EBV-encoded miRNAs do not include a miR-155 ortholog, but EBV induces the expression of cellular miR-155 (Jiang *et al.*, 2006; Rahadiani *et al.*, 2008; Yin *et al.*, 2008). These observations provide a link between the misregulation of cellular miR-155 targets and KSHV-, MDV- and EBV-associated disease.

III. ORF57, A MULTIFUNCTIONAL REGULATOR OF GENE EXPRESSION

KSHV ORF57 (Mta) is a posttranscriptional regulatory protein that is conserved among herpesviridae, but no homologs have been identified in other organisms. The founding member of this family is the HSV ICP27 (IE63) protein that enhances several transcriptional and

posttranscriptional processes. In addition, ICP27 inhibits host cell expression by acting as a splicing inhibitor (Sandri-Goldin, 2008). KSHV ORF57 is a 51-kDa protein that is expressed in the nucleus of lytically infected cells where it is enriched in nuclear subdomains called speckles (Bello *et al.*, 1999; Gupta *et al.*, 2000; Kirshner *et al.*, 2000; Lamond and Spector, 2003). However, ORF57 shuttles between the nucleus and cytoplasm (Bello *et al.*, 1999), supporting possible cytoplasmic and/or transport functions. Most importantly, using an ORF57-deleted KSHV bacmid (Zhou *et al.*, 2002), two groups have shown that ORF57 is essential for viral replication and the expression of specific lytic phase genes (Han and Swaminathan, 2006; Majerciak *et al.*, 2007). While we are only beginning to understand ORF57 mechanisms, the emerging picture is that ORF57 is involved in many facets of KSHV gene expression including mRNA export, transcription, splicing and RNA stability (Boyne and Whitehouse, 2006; Malik and Schirmer, 2006; Swaminathan, 2005).

A. ORF57 stimulates the export of intronless viral mRNAs

It was observed over 20 years ago that genes interrupted by introns are often expressed at higher levels than their intron-lacking, cDNA counterparts (Le Hir *et al.*, 2003; Maniatis and Reed, 2002). Indeed, the presence of an intron in a pre-mRNA and/or pre-mRNA splicing have been reported to enhance transcription, 3′-end formation, mRNA stability, mRNA export, translation and even protein stability (Le Hir *et al.*, 2003; Maniatis and Reed, 2002). These effects can be attributed to the extensive coupling among RNA synthesis and processing events in the mammalian cell. Herpesviruses use the host cell expression machinery, but most of their genes are not interrupted by introns. To compensate for the effects of introns on gene expression, the herpesviridae have evolved mechanisms utilizing ICP27 family members to stimulate the expression of intronless transcripts.

One mechanism to couple pre-mRNA splicing with mRNA export involves the recruitment of the transcription and export (TREX) complex to spliced transcripts. The TREX complex includes the mRNA export factors Aly/REF and the helicase UAP56, as well as components of the human THO complex (Strasser *et al.*, 2002). Upon splicing, the messenger ribonucleoprotein particle (mRNP) is remodeled and TREX is deposited near the 5′ end of the transcript (Cheng *et al.*, 2006; Custodio *et al.*, 2004; Masuda *et al.*, 2005; Reed and Cheng, 2005). *In vitro*, splicing of the 5′ proximal intron, but not downstream introns, leads to deposition of the TREX complex on a substrate mRNA (Cheng *et al.*, 2006; Masuda *et al.*, 2005). Aly/REF then recruits the mRNA export factor TAP, which subsequently binds to the nuclear pore, leading to translocation to the cytoplasm (Fontoura *et al.*, 2005; Weis, 2002). Additional splicing-coupled

mechanisms may lead to more efficient mRNA export. For example, some splicing-associated serine/arginine-rich (SR) proteins are dephosphorylated coincident with pre-mRNA splicing and the resulting hypophosphorylated isoforms selectively bind TAP (Gilbert and Guthrie, 2004; Huang *et al.*, 2003). Thus, splicing modifies mRNP composition and mRNP phosphorylation status to enhance TAP recruitment and mRNA export.

Because splicing enhances mRNA export and most herpesvirus genes are intronless, herpesviridae have evolved mechanisms to stimulate mRNA export in the absence of splicing. For HSV mRNAs, ICP27 enhances mRNA export by recruiting cellular export factors (Sandri-Goldin, 2008). Recent evidence suggests that ORF57 stimulates KSHV mRNA export by a similar mechanism. Several groups have shown that ORF57 binds to Aly/REF *in vitro* and in cell extract and a minimal Aly/REF binding domain has been identified *in vitro* (Boyne *et al.*, 2008; Majerciak *et al.*, 2006a; Malik *et al.*, 2004b; Nekorchuk *et al.*, 2007). ORF57 binds TAP directly *in vitro* (Malik *et al.*, 2004b); however, its binding is enhanced significantly by Aly/REF, suggesting that the physiologically relevant interaction requires an Aly/REF bridge. Similarly, TREX components Hpr1 and UAP56 copurify with ORF57 from extracts in an Aly/REF-dependent manner (Boyne *et al.*, 2008).

The interactions between ORF57, TREX and TAP suggest that ORF57 may stimulate mRNA export by recruiting cellular mRNA export factors and functional assays strengthen this conclusion. Clements and colleagues showed by cell fractionation experiments that coexpression of ORF57 decreases the ratio of nuclear-to-cytoplasmic RNA of an unspliced pre-mRNA reporter (Malik *et al.*, 2004b). More recently, Boyne *et al.* (2008) showed that ORF57 enhances export of the KSHV ORF47 mRNA, as assessed by cell fractionation and by *in situ* hybridization. Moreover, these authors demonstrated an ORF57-dependent association of TREX components on intronless mRNAs in cell lysates. These observations support the model that ORF57 recruits the TREX complex to intronless viral mRNAs, thereby enhancing viral intronless mRNA export.

B. ORF57 affects transcription and RNA stability

While interactions with the mRNA export machinery support a role for ORF57 in nucleocytoplasmic transport, other published data strongly suggest that ORF57 function is not limited to its export activity. First, ORF57-mediated changes in the ratio of nuclear-to-cytoplasmic RNA are variable. In some cases, little or no change is observed, suggesting ORF57 does not enhance the export of these transcripts (Gupta *et al.*, 2000; Majerciak *et al.*, 2006a,b; Nekorchuk *et al.*, 2007), while in other cases a change in this ratio is detected (Boyne *et al.*, 2008; Malik *et al.*, 2004b;

Nekorchuk *et al.*, 2007). Whether these differences are due to the target RNAs being used or differences in experimental procedures is unclear. The effects of differential RNA stability in these subcellular locales and the difficulty in acquiring ''clean'' nuclear and cytoplasmic fractions may further limit the usefulness of this approach. Second, several groups have shown that levels of a nuclear transcript (*p*oly*a*denylated *n*uclear (PAN) RNA, see Section VI) are enhanced by ORF57 expression (Han and Swaminathan, 2006; Kirshner *et al.*, 2000; Majerciak *et al.*, 2007; Nekorchuk *et al.*, 2007). Because this RNA is not exported, the observed ORF57-dependent increase in PAN RNA levels cannot be attributed to enhanced export. Third, an ORF57 mutant with reduced REF-binding maintains activation function (Nekorchuk *et al.*, 2007). Finally, an ~40% knockdown of Aly/REF did not diminish ORF57 enhancement of viral genes in transfected cells (Majerciak *et al.*, 2006a). Taken together, it seems likely that ORF57 affects viral gene expression independent of its effects on mRNA export.

While this review focuses on posttranscriptional mechanisms of gene regulation in KSHV, it is important to point out that ORF57 has also been implicated in transcriptional control. Based on reporter assays, the Ganem lab was the first to propose that ORF57 played a role in transcription (Kirshner *et al.*, 2000) and ORF57 has subsequently been shown to interact with the KSHV transcription factor Rta (Malik *et al.*, 2004a; Palmeri *et al.*, 2007). Recent work demonstrates that ORF57 associates with a target gene *in vivo*, binds DNA and contains an AT-hook DNA-binding domain that is essential for full ORF57-mediated gene activation (Palmeri *et al.*, 2007). However, the relative contributions of ORF57 to posttranscriptional and transcriptional regulation of viral gene expression remain unknown.

Both direct and indirect evidence suggests that ORF57 protects viral transcripts from cellular RNA degradation pathways. Indeed, both ICP27 and the ICP27 family member in EBV, SM, have been shown to stabilize specific mRNAs (Brown *et al.*, 1995; Corcoran *et al.*, 2006; Nicewonger *et al.*, 2004). That ORF57 may affect RNA stability was proposed by Swaminathan and colleagues based upon the observation that ORF57 enhanced RNA expression independent of its effects on mRNA export and that these effects were promoter- and transcription-independent (Gupta *et al.*, 2000; Nekorchuk *et al.*, 2007). In addition, reporter mRNAs were more abundant after actinomycin D treatment in the presence of ORF57 than in its absence, suggesting that ORF57 increases the stability of these transcripts (Palmeri *et al.*, 2007). Finally, our laboratory has observed a difference in the half-life of several ORF57 target mRNAs using a transcription pulse strategy (unpublished observations). Taken together, these data support a model in which ORF57 affects the stability of its targets. However, whether it directly affects stability by inhibiting the activity of RNA decay enzymes, or whether it stabilizes transcripts

indirectly by enhancing other RNA processing steps (e.g., 3′-end formation) remains to be elucidated. It is currently unknown if ORF57 stabilizes nuclear RNAs, cytoplasmic RNAs, or both. Understanding how ORF57 affects RNA stability will lead to insights into its mechanism of enhancing gene expression in virally infected cells.

C. ORF57 enhances viral pre-mRNA splicing

In addition to its other roles, two reports from the Zheng laboratory suggest that ORF57 promotes KSHV pre-mRNA splicing. While most KSHV genes are intronless, as many as 30% of KSHV genes contain introns and are expressed during lytic and latent phases (Zheng, 2003). Some of these intron-containing genes are poorly expressed in ORF57-deleted bacmid genomes, suggesting a role for ORF57 in activation of intron-containing gene expression (Majerciak *et al.*, 2007). More detailed analyses show that ORF57 has several characteristics consistent with its activity as a splicing factor (Majerciak *et al.*, 2008). Deletion of ORF57 increases the ratio of pre-mRNA to mRNA produced from the K8 and Rta genes and ORF57 preferentially interacts with pre-mRNAs in cell extract. Furthermore, ORF57 colocalizes and coimmunoprecipitates with cellular splicing factors. Interestingly, SM can complement these activities (Majerciak *et al.*, 2008) and has been described as an alternative splicing factor (Verma and Swaminathan, 2008).

The mechanism of ORF57-dependent enhancement of splicing remains unclear. Because ORF57 splicing activity is independent of other viral factors, it will be interesting to determine how it interacts with cellular splicing factors and how this leads to the observed changes in pre-mRNA-to-mRNA ratios. Furthermore, ORF57 enhances the export of a reporter pre-mRNA in the absence of its splicing (Malik *et al.*, 2004b), but it remains unclear how ORF57 ''chooses'' between enhancing the export of a particular intron-containing transcript or stimulating its splicing. Zheng and colleagues note that KSHV genes are more likely to have longer first exons and shorter introns than cellular transcripts (Majerciak *et al.*, 2008) and it may be this difference in gene structure that necessitates ORF57 splicing activity. Regardless of whether future studies support this specific model, determining the mechanistic details of ORF57-mediated splicing enhancement may provide insights into splicing regulation in both KSHV and its host cell.

IV. SOX DESTABILIZES CELLULAR mRNAs

Viruses often inhibit host cell gene expression to simultaneously block antiviral responses and favor viral gene expression (Glaunsinger and Ganem, 2006). During lytic replication, KSHV accelerates mRNA turnover

rates of host cell messages (Glaunsinger and Ganem, 2004b). The KSHV *s*hut*o*ff and e*x*onuclease protein (SOX; ORF37) is necessary for host cell shutoff and SOX is sufficient to degrade cellular mRNAs in the absence of other viral factors. Surprisingly, SOX is the homolog of the HSV alkaline exonuclease, a DNase important for viral genome packaging with no role in HSV host shutoff (Wilkinson and Weller, 2003). The alkaline exonuclease DNase domains are conserved in SOX, which suggests that it maintains packaging function but has acquired an additional host shutoff activity. Consistent with this idea, the exonuclease and shutoff functions of SOX are genetically separable (Glaunsinger *et al.*, 2005). Mutations in domains conserved across the herpesviridae are generally associated with DNase activity but not shutoff activity, while mutations in divergent domains inhibited shutoff activity without affecting DNase activity. Recently, the EBV homolog of SOX has been shown to have similar host shutoff function (Rowe *et al.*, 2007; Zuo *et al.*, 2008).

The HSV shutoff protein vhs (UL41) endonucleolytically cleaves cellular mRNAs (Glaunsinger and Ganem, 2006; Matis and Kudelova, 2001; Taddeo *et al.*, 2004). Even though SOX expression leads to host mRNA degradation, SOX appears to have no RNase activity and no additional viral proteins are necessary for shutoff (Glaunsinger and Ganem, 2004b). Thus, it seems likely that SOX functions through the host cell RNA degradation machinery. However, neither the enzymes responsible nor the subcellular locale of SOX-induced RNA degradation has been elucidated. Another mystery regarding SOX function is how viral mRNAs and a subset of cellular transcripts escape the otherwise general shutoff activity (Glaunsinger and Ganem, 2004a). Uncovering these mechanisms may be particularly meaningful because some cellular transcripts that escape shutoff (e.g., IL-6) have implications for development of KSHV-related disease.

V. KAPOSIN B STABILIZES AU-RICH mRNAs

During latency, the KSHV Kaposin B protein employs cellular factors to stabilize mRNAs containing AU-rich elements (AREs) in their 3′ UTRs (McCormick and Ganem, 2005; Wang and Boshoff, 2005). AREs are important *cis*-acting posttranscriptional modulators of gene expression that destabilize transcripts and regulate translation of mRNAs encoding cytokines, proto-oncogenes, interleukins, etc. (Barreau *et al.*, 2005; Brennan and Steitz, 2001; Khabar, 2005). Under specific cellular conditions, activation of the p38/MAPK pathway results in the stabilization of these otherwise labile messages (Dean *et al.*, 2004). The Kaposin B protein is translated from an unusual mRNA containing overlapping reading frames of two 23 amino acid repeat sequences, DR1 and DR2

(Sadler *et al.*, 1999). Kaposin B protein consists of multiple DR2 and DR1 repeats at the N-terminus and C-terminus, respectively. A yeast two-hybrid screen using Kaposin B as bait revealed an interaction with cellular MK2 (the *M*APK-associated *k*inase 2) and *in vitro* binding assays demonstrated that the interaction is direct (McCormick and Ganem, 2005). Expression of Kaposin B activates MK2 as judged by two criteria: (1) Kaposin B increases the levels of phosphorylated MK2 in cells and (2) it increases MK2-dependent kinase activity in cell extract. The authors further show that transient expression of Kaposin B stabilizes ARE-containing reporter mRNAs and leads to increased levels of IL-6 and GM-CSF proteins, both of which are translated from ARE-containing mRNAs. Latent infection of cells with KSHV stabilizes reporter transcripts that contain AREs and Kaposin B is the only latent protein sufficient for this activity. Together, these data suggest that Kaposin B increases the levels of cellular ARE-containing transcripts during latent infection by activating MK2 activity.

VI. THE PAN-ENE IS A *cis*-ACTING RNA STABILITY ELEMENT

In addition to regulating RNA stability in *trans*, KSHV has evolved an elegant mechanism that stabilizes an abundant nuclear transcript called the PAN RNA in *cis*. This lytic phase transcript is pol II-transcribed, 7-methylguanosine-capped, polyadenylated and accumulates in the nucleoplasm and in nuclear speckles (Sun *et al.*, 1996; Zhong and Ganem, 1997; Zhong *et al.*, 1996). There are no homologs of PAN RNA in other herpesviruses, including other closely related γ-herpesviruses. Neither the function of PAN RNA nor the phenotype of a PAN RNA knockout virus is known. However, the unusual abundance of this RNA – as much as 70% of the polyadenylated transcripts in a lytically infected cell are PAN RNA – suggests that PAN RNA is essential for some aspect of KSHV biology (Song *et al.*, 2001; Sun *et al.*, 1996; Zhong *et al.*, 1996).

A 79-nt sequence in PAN RNA is essential for its high levels of nuclear accumulation and this element is sufficient to confer high levels of nuclear accumulation to an intronless β-globin reporter mRNA (Conrad and Steitz, 2005). The element was dubbed the ENE (*e*xpression and *n*uclear retention *e*lement), but more recent observations suggest that the ENE protects unstable nuclear RNAs from degradation (Conrad *et al.*, 2006). The ENE increases the half-life of polyadenylated transcripts both in cultured cells and *in vitro*. Mechanistically, the function of the ENE is driven by hybridization of the poly(A) tail to a U-rich loop in the ENE. The result of this interaction is that the 3′ end of the transcript is sequestered from exonucleases (Conrad *et al.*, 2006, 2007). Indeed, protection of transcripts by the ENE from 3′ to 5′ decay can be observed in the absence

of cellular proteins, further supporting an RNA-driven mechanism. Together, these data show that the ENE is a stabilizing element that works in *cis* to inhibit RNA decay. The ENE may not be unique: a yeast mRNA and a mammalian nuclear noncoding RNA contain elements that may be mechanistically analogous to the ENE (Muhlrad and Parker, 2005; Wilusz *et al.*, 2008).

Several lines of evidence suggest that the ENE stabilizes transcripts from rapid nuclear RNA decay that is part of a cellular RNA quality control system. Detailed kinetic analyses of PAN RNA degradation in cultured cells uncovered two independent pools of PAN RNA, one that degrades rapidly ($t_{1/2}$ ~15 min) and another that degrades more slowly ($t_{1/2}$ ~3 h) (Conrad *et al.*, 2006). Deletion or mutation of the ENE increases the fraction of PAN RNA that is degraded rapidly. Taken with the observation that the ENE inhibits deadenylation *in vitro*, these data suggest that the ENE protects transcripts from a rapid 3′ to 5′ exonucleolytic decay pathway. Moreover, the ENE stabilizes β-globin pre-mRNAs that contain mutated 5′ or 3′ splice sites and intronless β-globin RNAs, but not their intron-containing counterparts. Because of the links between mRNA export and splicing (Cheng *et al.*, 2006; Le Hir *et al.*, 2003; Maniatis and Reed, 2002; Valencia *et al.*, 2008), the ENE may be protecting nuclear transcripts from a quality control pathway that normally serves to degrade ''nonexportable'' mRNAs. If this interpretation is correct, the name ENE is misleading: the nuclear accumulation of ENE-containing transcripts is the result of protection of ''nonexportable'' RNAs from nuclear degradation and not due to retention of those transcripts. This model is further supported by the observation that tethering export factors to intronless β-globin-ENE transcripts or restoration of the intron of these reporters leads to their accumulation in the cytoplasm (Conrad and Steitz, 2005). These studies of ENE function appear to have uncovered a rapid RNA decay pathway involved in nuclear RNA surveillance; however, more rigorous tests are necessary to confirm this model.

VII. SUMMARY

KSHV is an oncogenic virus that causes several malignancies. Extensive knowledge about the processes that control its life cycle and pathogenesis will yield better strategies to combat KSHV-associated disease and may yield insights into human cellular function and general disease mechanisms. KSHV posttranscriptionally regulates viral and cellular gene expression at the level of RNA stability, translation, mRNA export and splicing. The reported activities of KSHV factors involved in posttranscriptional regulation are summarized in Fig. 6.1. At present, the mechanistic details of many of these regulatory pathways remain uncertain and it seems

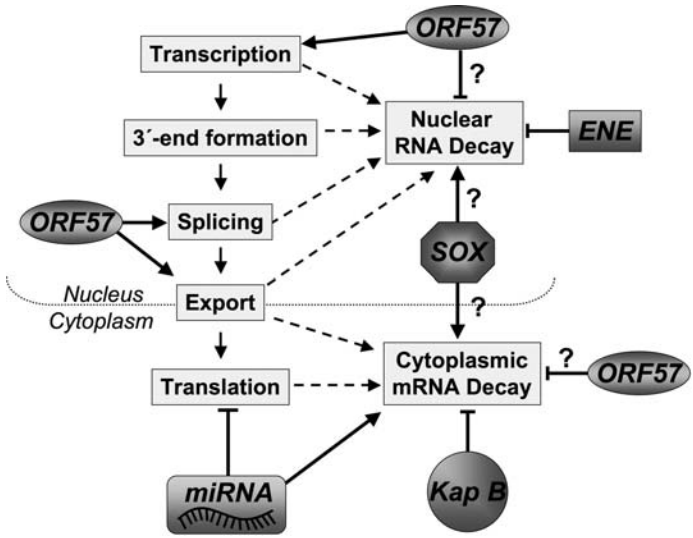

FIGURE 6.1 Summary of KSHV posttranscriptional regulatory processes. Details are described in the text. Light gray text boxes represent key steps in RNA biogenesis, while the dark gray shapes represent KSHV posttranscriptional regulatory factors. Biogenesis steps are presented as a flowchart for ease of representation; however, 3′-end formation and splicing likely occur cotranscriptionally *in vivo*. The dashed arrows pointing to the RNA decay boxes denote that a transcript is subject to degradation at any point in its biogenesis. The question marks indicate that it is unknown whether ORF57 and SOX affect nuclear RNA stability, cytoplasmic RNA stability, or both.

likely that additional KSHV posttranscriptional regulatory mechanisms remain to be recognized. Undoubtedly, interesting insights at the intersection of posttranscriptional regulation, viral replication and pathogenesis await future discovery.

ACKNOWLEDGMENT

The author thanks Sarah Hulsey for critical review of this manuscript.

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INDEX

CONTENTS OF PREVIOUS VOLUMES

Volume 40

Volume 41

Volume 42

Volume 43

Volume 44

Volume 45

Volume 46

Volume 47

Volume 48

Volume 49

Volume 50

Volume 51

Volume 52

Volume 53

Volume 54

Volume 55

Volume 56

Volume 57

Volume 58

Volume 59

Volume 60

Volume 61

Volume 62

Volume 63

Volume 64

Volume 65

Volume 66

Volume 67