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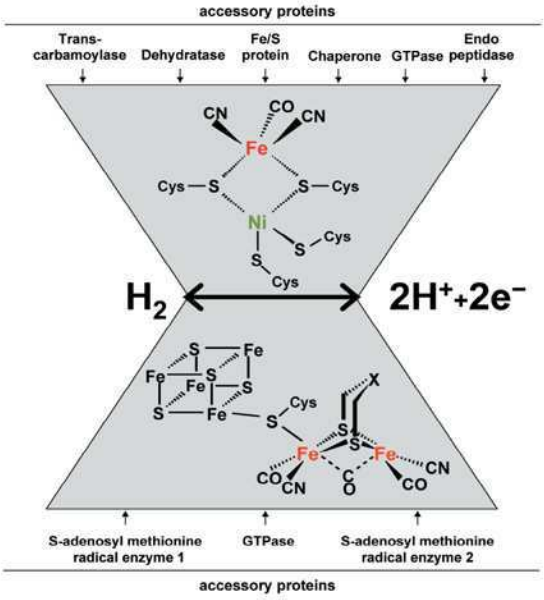

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This volume of *Advances in Microbial Physiology* is dedicated to the memory of my father, M. Keith Poole (1923–2006), who loved words, knowledge and their union.

Robert K. Poole

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Maturation of Hydrogenases

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ABSTRACT

Enzymes possessing the capacity to oxidize molecular hydrogen have developed convergently leading to three classes of enzymes: [FeFe]-, [NiFe]-, and [FeS]-cluster-free hydrogenases. They differ in the composition and the structure of the active site metal centre and the sequence of the constituent structural polypeptides but they show one unifying feature, namely the existence of CN and/or CO ligands at the active site Fe. Recent developments in the analysis of the maturation of [FeFe]- and [NiFe]- hydrogenases have revealed a remarkably complex pattern of mostly novel biochemical reactions. Maturation of [FeFe]-hydrogenases requires a minimum of three auxiliary proteins, two of which belong to the class of Radical-SAM enzymes and the other to the family of GTPases. They are sufficient to generate active enzyme when their genes are co-expressed with the structural genes in a heterologous host, otherwise deficient in [FeFe]-hydrogenase expression. Maturation of the large subunit of [NiFe]-hydrogenases depends on the activity of at least seven core proteins that catalyse the synthesis of the CN ligand, have a function in the coordination of the active site iron, the insertion of nickel and the proteolytic maturation of the large subunit. Whereas this

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core maturation machinery is sufficient to generate active hydrogenase in the cytoplasm, like that of hydrogenase 3 from *Escherichia coli*, additional proteins are involved in the export of the ready-assembled heterodimeric enzyme to the periplasm via the twin-arginine translocation system in the case of membrane-bound hydrogenases. A series of other gene products with intriguing putative functions indicate that the minimal pathway established for *E. coli* [NiFe]-hydrogenase maturation may possess even higher complexity in other organisms.

ABBREVIATIONS

SAM	*S*-adenosyl methionine
EPR	Electron paramagnetic resonance
FTIR	Fourier-transform infrared
IR	Infrared

1. INTRODUCTION

Hydrogenases catalyse the oxidation of elemental hydrogen into protons plus electrons or the reduction of protons into hydrogen. The direction of

the reaction is determined by the redox potential of the individual electron donors or acceptors to which the reactions are coupled and it defines the enzymes as belonging to the ''hydrogen-uptake'' or ''hydrogen-evolving'' classes. In a physiological context, hydrogenases can fulfil one of the following three tasks. First, the majority of the enzymes present in microorganisms have a function in energy conservation. They exploit the high-energy substrate hydrogen that can come from geological or biological sources and they transfer the electrons via some respiratory chain to a terminal acceptor, which is coupled to a generation of membrane potential and adenosine triphosphate (ATP). Second, hydrogenases can provide a sink for electrons derived from the oxidation of some substrate thereby driving metabolic processes kinetically and/or thermodynamically (Thauer *et al.*, 1977). Microorganisms frequently possess the genetic capacity for the formation of hydrogen uptake and hydrogen-evolving enzymes in the same cell. A paradigmatic example is *E. coli*, which is able to synthesize two isoenzymes with a function in hydrogen oxidation, namely hydrogenases 1 and 2 (Ballantine and Boxer, 1985), encoded by the *hya* and *hyb* operons, respectively, and one gas-evolving enzyme, hydrogenase 3 encoded by the *hyc* operon (for review see Sawers *et al.*, 2004). An intricate genetic regulation network ensures that the expression of the individual isoenzyme is appropriately adjusted to the metabolic need. A fourth isoenzyme, designated hydrogenase 4, is encoded by the *hyf* operon (Andrews *et al.*, 1997) and is assumed to constitute an alternative gas-evolving enzyme (formate hydrogenlyase 2). Since its physiological role is not yet clear and no information exists on its maturation, it will not be addressed further in this review. Other organisms like *Ralstonia (R.) eutropha* have the capacity for synthesis of a membrane-bound and of a cytoplasmically located soluble enzyme. Whereas the membrane-bound enzyme feeds the electrons directly into a respiratory chain, the soluble enzyme is coupled to a diaphorase activity and interconverts elemental hydrogen and NADH (for review see Friedrich and Schwartz, 1993). Hydrogen oxidation and evolution is of paramount importance for the metabolism of certain consortia in which hydrogen consumers drive the metabolism of proton reducers via interspecies hydrogen transfer. The biodiversity of hydrogenases has been reviewed recently by Vignais *et al.* (2001) and by Robson (2001a).

In contrast to the metabolic functions of hydrogen uptake and hydrogen-evolving enzymes, a third class of enzymes, the regulatory or H_2-sensing hydrogenases, control hydrogenase gene expression in response to elemental hydrogen as stimulus. Regulatory hydrogenases, of which the paradigm is the enzyme from *R. eutropha*, bind hydrogen and submit the signal to a two-component regulatory system, which controls the expression of hydrogenase

structural and maturation genes (for review see Friedrich *et al.*, 2005). They belong to the [NiFe] class of enzymes and possess only minor catalytic activity.

All known hydrogenases are metalloenzymes and they present one of the most intriguing examples of convergent evolution. On the basis of their active site metal centres, they are classified into [FeFe]-, [NiFe]- and [FeS]- cluster-free hydrogenases. Although the amino acid sequences of the structural proteins of [FeFe]-, [NiFe]- and [FeS]-cluster-free enzymes are completely unrelated and the metal centres have different architecture and composition, they share one unique property, namely the coordination of the active site Fe of the [NiFe]- and [FeFe]-enzymes with the diatomic ligands CO and CN (see below and Fig. 1) and that of the [FeS]-cluster-free enzyme with two CO ligands (Lyon *et al.*, 2004).

The formation of these metal centres and their integration into the apoprotein follow a complex pathway and requires the participation of accessory proteins with novel biochemical roles. As no information is available yet for the maturation of the [FeS] cluster-free enzyme class, this review concentrates only on the knowledge on the formation of [NiFe]- and [FeFe]-hydrogenases. The topic of maturation of [NiFe]-hydrogenases has been reviewed by Maier and Böck (1996b), Blokesch *et al.* (2002), Mulrooney and Hausinger (2003), Kuchar and Hausinger, (2004), Vignais and Colbeau (2004) and Sawers *et al.* (2004).

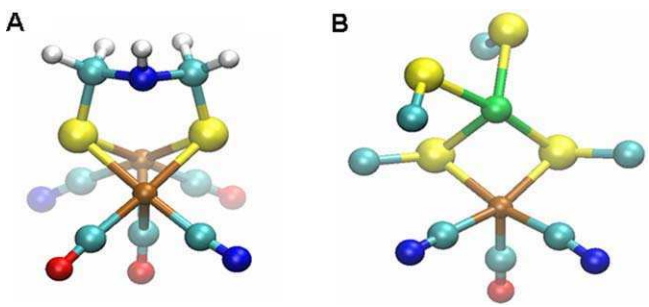

Figure 1 Atomic structure of the [FeFe]-hydrogenase and [NiFe]-hydrogenase catalytic sites. Atom depictions of the [FeFe]-hydrogenase H-cluster, 2Fe-centre (A), and the [NiFe]-hydrogenase [NiFe]-centre (B) as generated from the structures of *Cl. pasteurianum* [FeFe]-hydrogenase I (PDB file 1FEH, Peters *et al.*, 1998) and *D. vulgaris* Miyazaki F [NiFe]-hydrogenase (PDB file 1UBK, Ogata *et al.*, 2002). (See color plate section page 225)

2. MATURATION OF [FeFe]-HYDROGENASES

2.1. The [FeFe]-Hydrogenase Catalytic Domain and H-Cluster

The [FeFe]-hydrogenase family is characterized by a conserved polypeptide that comprises the catalytic domain, which in the mature form contains a unique catalytic [FeS]-cluster, or H-cluster (Fig. 1A). In some instances, the catalytic domain exists in combination with auxiliary [FeS]-cluster domains, forming more complex cluster arrangements. In view of the fact that the H-cluster is a basic requirement for a functional [FeFe]-hydrogenase, this portion of the review will focus on recent developments fundamental to H-cluster biosynthesis. A brief overview of the extensive contributions made by numerous groups regarding the details of the [FeFe]-hydrogenase catalytic site structure will be presented to outline the requirements for [FeFe]-hydrogenase maturation. Amino acid sequence alignments of [FeFe]-hydrogenases isolated from a variety of organisms have identified a conserved catalytic domain (Fig. 2) (Voordouw and Brenner, 1985; Peters *et al.*, 1998; Nicolet *et al.*, 2000; Vignais *et al.*, 2001). This domain is composed of three motifs, L1FeFe, L2FeFe and L3FeFe (reviewed in Vignais *et al.*, 2001) that

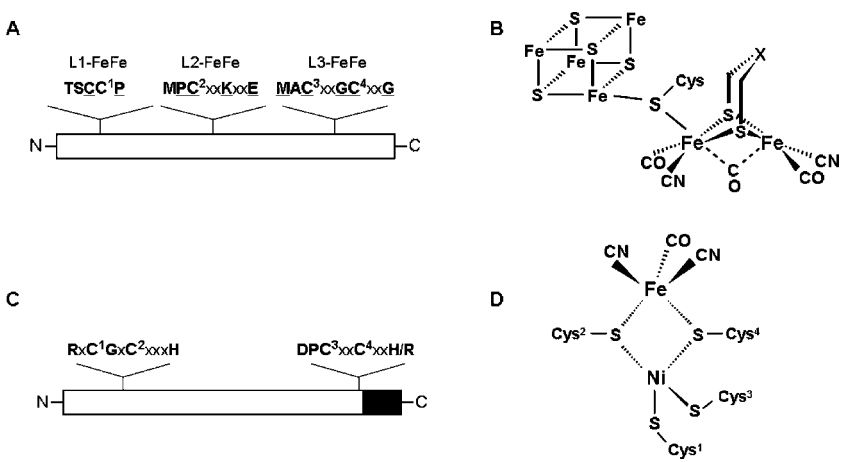

Figure 2 Schematic diagrams of the [FeFe]-hydrogenase and [NiFe]-hydrogenase catalytic domains and active sites. (A) Relative location of the conserved motifs (Vignais *et al.*, 2001) in the catalytic domain responsible for coordinating the H-cluster. (B) In A, the underlined amino acids are conserved and the numbered cysteines function in Fe coordination. (C) Relative location of the conserved motifs in the [NiFe]-hydrogenase catalytic domain responsible for coordinating the active site (D).

coordinate the [FeFe]-hydrogenase H-cluster, forming the catalytic site (Fig. 2A). Motif residues that function in H-cluster coordination have been elucidated from the X-ray crystal structures of the [FeFe]-hydrogenases CpI from *Clostridium (Cl.) pasteurianum* and DdH from *Desulfovibrio (D.) desulfuricans* resolved to 1.8 and 1.6 Å, respectively (Peters *et al.*, 1998; Nicolet *et al.*, 1999; Nicolet *et al.*, 2000). In agreement with extensive biochemical, and spectroscopic studies on purified [FeFe]-hydrogenases (reviewed in Adams, 1990), the structure of the H-cluster is comprised of a 4Fe-centre bridged through a cysteine amino acid to an unusual 2Fe-centre (illustrated in Fig. 2).

The 4Fe-centre is a cubane [4Fe4S] cluster, which is coordinated by three cysteines, one from each of the L1FeFe, L2FeFe and L3FeFe motifs (Fig. 2A). A fourth cysteine in the L3FeFe motif bridges the two centres, and is the only protein ligand coordinating the 2Fe-centre. The 2Fe-centre contains an unusual assortment of ligands, and shares unique features with the NiFe-catalytic site of [NiFe]-hydrogenases (Fig. 1). Through a combination of X-ray and Infrared (IR) spectroscopic investigations, it was discovered that [NiFe]-hydrogenases possess CN and CO ligands bound to the iron atom, which presumably function through backbonding to stabilize the iron in a low valence and low-spin state (Bagley *et al.*, 1994; Bagley *et al.*, 1995; Volbeda *et al.*, 1995). A comparison of the IR spectra of purified [FeFe]-hydrogenase to purified [NiFe]-hydrogenase showed that the two enzymes exhibited similar Fe-cyanyl and Fe-carbonyl signals, and it was the first hint that Fe-ligation in the two enzymes was similar (van der Spek *et al.*, 1996; Pierik *et al.*, 1998). Confirmation of CN and CO ligands to the 2Fe-centre of [FeFe]-hydrogenase came from additional IR studies (Pierik *et al.*, 1998) combined with the CpI and DdH X-ray structures (Peters *et al.*, 1998; Nicolet *et al.*, 2000). As shown in Figs. 1 and 2, the iron atoms possess terminal, asymmetric tandem CN and CO ligands, with a CO bridging the 2Fe-centre (reviewed in Nicolet *et al.*, 2000). The thiolates also bridge the 2Fe-centre and are derived from an unknown organic ligand, tentatively assigned as either di-thiopropane or di-(thiomethyl)amine (Fan and Hall, 2001; Nicolet *et al.*, 2001). A precise determination of the bridging moiety has not been obtained through experimental investigation. However, theoretical investigations of 2Fe-centre models have been used to suggest the di-(thiomethyl)amine assignment, where an amine near the open coordination site of the distal iron atom could participate in an acid-base mechanism of hydrogen catalysis (Fan and Hall, 2001; Nicolet *et al.*, 2001). An organized series of extraordinary biochemical reactions must occur to synthesize the H-cluster. These include the synthesis of the CN, CO and the dithiolate ligand of the 2Fe-centre, ligation of the 2Fe- and 4Fe-centres, and finally

incorporation into the structural protein to achieve complete [FeFe]-hydrogenase maturation.

2.2. Maturation of the [FeFe]-Hydrogenases Catalytic Domain

The [FeFe]-hydrogenases are organized into modular domains, where accessory clusters functioning as inter- and intra-molecular electron-transfer centres are electronically connected to the highly conserved catalytic domain. Modularity of [FeFe]-hydrogenases was first observed from the amino acid sequences of the enzymes isolated from species of *Desulfovibrio* and *Clostridium* (Voordouw and Brenner, 1985; Meyer and Gagnon, 1991; Gorwa *et al.*, 1996; Atta and Meyer, 2000). The *Desulfovibrio* periplasmic [FeFe]-hydrogenases are two-subunit enzymes, consisting of an [FeS]-cluster-free small subunit, and a catalytic large-subunit containing two accessory clusters, or F-clusters, along with the H-cluster (Voordouw and Brenner, 1985; Nicolet *et al.*, 1999). In contrast, the clostridial [FeFe]-hydrogenases are typically single subunit enzymes, with up to four F-clusters in addition to the H-cluster (Chen *et al.*, 1974; Adams, 1990; Meyer and Gagnon, 1991; Kaji *et al.*, 1999). More recently, the NAD(P)H-dependent [FeFe]-hydrogenases from *Thermotoga (T.) maritima* and *Thermoanaerobacter (Th.) tengcongensis* have been isolated and shown to consist of three and four subunits, respectively (Verhagen *et al.*, 1999: Soboh *et al.*, 2004). The purified catalytic subunits of [FeFe]-hydrogenases from these thermophilic organisms gave EPR spectra consistent with the presence of H- and F-clusters.

Sequence composition of the [FeFe]-hydrogenases that have been characterized to date display the F-cluster binding sites as co-linear cysteine-rich motifs, similar to the [FeS]-cluster binding motifs found in ferredoxins. These F-cluster domains are most often N-terminal with respect to the conserved catalytic domain (Vignais *et al.*, 2001). That the F-cluster domains could be biosynthesized as functional proteins in the absence of a catalytic domain was demonstrated through expression of an N-terminal, [2Fe2S]-cluster-binding peptide of [FeFe]-hydrogenase I (CpI) from *Cl. pasteurianum*. The expressed CpI-peptide folded as a fully functional [2Fe2S]-cluster protein (Atta *et al.*, 1998; Kümmerle *et al.*, 1999). Suggestive of a model for separate mechanisms of [FeFe]-hydrogenase accessory (F-cluster) and catalytic (H-cluster) domain maturation, the initial purifications of the [FeFe]-hydrogenases from *Megasphaera elsdenii* (Filipiak *et al.*, 1989) and *D. desulfuricans* (DdH) (Pierik *et al.*, 1992) yielded non-catalytic iso-forms that contained only the F-clusters. The results detailed above suggest that the modular organization of [FeFe]-hydrogenase [FeS]-cluster motifs observed in the primary structure is

also manifested mechanistically during cluster biosynthesis and enzyme maturation. Indeed, there are examples of [FeFe]-hydrogenases that consist of only a catalytic domain, which are found in several species of green algae including *Chlamydomonas (Ch.) reinhardtii*, *Scenedesmus (S.) obliquus* and *Chlorella fusca* (Erbes *et al.*, 1979; Happe and Naber, 1993; Florin *et al.*, 2001; Winkler *et al.*, 2002). The metal content analysis of these isolated algal [FeFe]-hydrogenases show that they represent the simplest forms of [FeFe]-hydrogenases yet identified, consisting of only a catalytic H-cluster. Truncated derivatives of the *Clostridium acetobutylicum* [FeFe]-hydrogenase I have been created that also lack accessory F-clusters and consist of only the catalytic domain. Like their algal counterparts, the bacterial enzyme derivatives undergo maturation into active [FeFe]-hydrogenases (Cohen *et al.*, 2005; King *et al.*, 2006). Altogether these results suggest that biosynthetic pathways for the catalytic H-cluster and accessory [FeS]-clusters are to some extent independent.

2.3. Genetics of H-Cluster Biosynthesis

2.3.1. Initial Discovery and Identification of Maturation Genes/Proteins

The [FeFe]-hydrogenase maturation proteins were initially discovered in the eukaryotic green alga *Ch. reinhardtii*, when it was shown that two novel radical *S*-adenosylmethionine (Radical-SAM) proteins, HydEF and HydG, are required for *Ch. reinhardtii* [FeFe]-hydrogenase enzyme activity (Posewitz *et al.*, 2004). The *Ch. reinhardtii* mutant, *hydEF-1*, was isolated by screening random mutants of *Ch. reinhardtii* for colonies unable to produce hydrogen. Subsequent research demonstrated that the *hydEF* gene is disrupted in this mutant and that complementation of *hydEF-1* with a wild-type copy of the *hydEF* gene restores hydrogenase activity (Posewitz *et al.*, 2004; Posewitz *et al.*, 2005). Remarkably, a second gene required for [FeFe]-hydrogenase assembly, *hydG*, was identified in the *Ch. reinhardtii* genome directly adjacent to the *hydEF* gene (Fig. 3). In wild-type *Ch. reinhardtii* cultures both *hydEF* and *hydG* are anaerobically induced concomitantly with the two *Ch. reinhardtii* [FeFe]-hydrogenase genes, *hydA1* and *hydA2* (Happe and Kaminski, 2002; Forestier *et al.*, 2003), suggesting a mechanism of co-regulation.

In the *hydEF-1* mutant, both hydrogenase genes are induced and full-length hydrogenase protein accumulates; however, hydrogenase activity was not observed using a variety of assay conditions. It was therefore concluded that the *hydEF-1* mutant was unable to synthesize an active [FeFe]-hydrogenase. Additional evidence supporting the conclusion that the HydEF and HydG

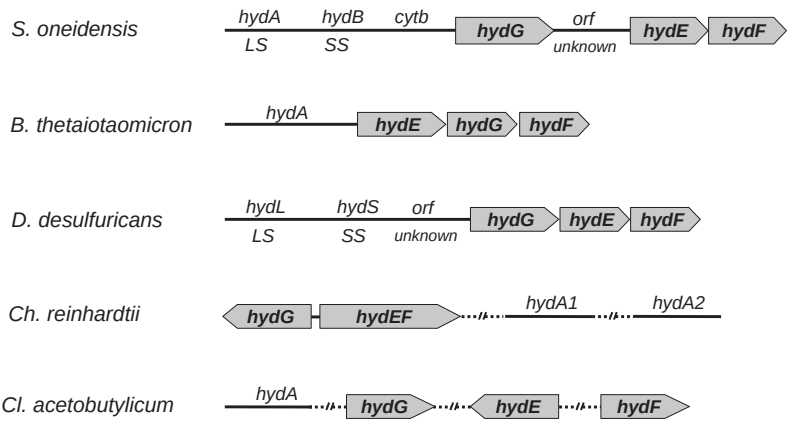

Figure 3 Organization of structural and accessory genes whose products are required for the synthesis and maturation of [FeFe]-hydrogenases. Representative organisms include: *Shewanella (S.) oneidensis*, *Bacteroides (B.) thetaiotaomicron*, *Desulfovibrio (D.) desulfuricans G-20*, *Chlamydomonas (Ch.) reinhardtii* and *Clostridium (Cl.) acetobutylicum*. Members of the *hyd* gene family are printed inside the arrows, with their relative orientations in the genome indicated by the direction of the arrowhead. LS, large subunit; SS, small subunit.

proteins are required for the formation of an active [FeFe]-hydrogenase was shown by the heterologous expression of active *Ch. reinhardtii* HydA1 in *E. coli*, a bacterium that lacks a native [FeFe]-hydrogenase (Posewitz *et al.*, 2004). The expression of the *hydA1* construct alone or co-expression of the *hydA1* and *hydEF*, or *hydA1* and *hydG* genes in *E. coli* all resulted in the expression of non-functional HydA1 protein after purification. However, the co-expression of *Ch. reinhardtii hydA1* along with both *hydEF* and *hydG* in anaerobic *E. coli* cultures yielded an active HydA1 enzyme.

Analysis of the HydEF protein demonstrates that it contains two unique segments, which are homologous to two distinct prokaryotic proteins, HydE and HydF that are found exclusively in the organisms containing [FeFe]-hydrogenase. As discussed below, both HydE and HydG belong to the emerging Radical-SAM (also referred to in the literature as the SAM Radical or AdoMet radical) superfamily of proteins, and HydF contains a putative GTPase domain (Fig. 4).

2.3.2. Genomics and [FeFe]-Hydrogenase Maturation Genes

The advent of whole genome sequencing has enabled the screening of genomic databases for microorganisms with homologues of the [FeFe]-hydrogenase

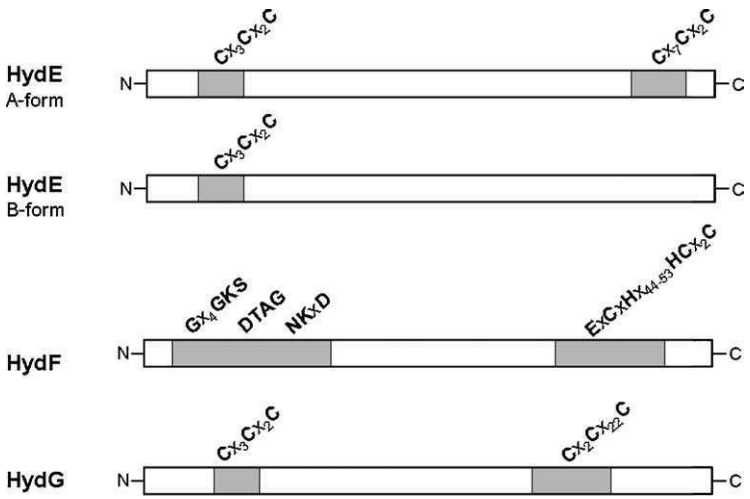

Figure 4 Scheme of the maturation proteins HydE, HydF and HydG. Functionally important motifs including: Radical-SAM domains, putative FeS cluster domains and GTPase motifs as discussed in the text are highlighted. Two forms of HydE are displayed in which a C-terminal C–X_7–C–X_2–C motif is present in the A-form but absent in the B-form.

maturation genes identified in *Ch. reinhardtii* (Posewitz *et al.*, 2004). In every organism identified to possess Open Reading Frames (ORFs) homologous to the *Ch. reinhardtii hyd* maturation genes, there were additional genes or ORFs that encoded proteins with the signature H-cluster motifs characteristic of the [FeFe]-hydrogenase catalytic domain. Among these are organisms that have been demonstrated to synthesize [FeFe]-hydrogenases, including *Cl. acetobutylicum*, *Cl. perfringens*, *D. desulfuricans*, *D. vulgaris*, *T. maritima* and *Th. tengcongensis* among others (Voordouw and Brenner, 1985; Hatchikian *et al.*, 1992; Gorwa *et al.*, 1996; Kaji *et al.*, 1999; Verhagen *et al.*, 1999; Soboh *et al.*, 2004). From homology searches three patterns have emerged for the genomic arrangements of Hyd maturation genes (Fig. 3, Table 1); (1) independent (e.g., *Cl. acetobutylicum*), (2) fusion (e.g., *Ch. reinhardtii*), and (3) operon (e.g., *D. desulfuricans*).

Typically, organisms that possess the maturation and structural genes in an independent arrangement also express and synthesize cytoplasmic [FeFe]-hydrogenases. Examples of independent gene arrangements are found in the genomes of various clostridial species including *Cl. perfringens* (Shimizu *et al.*, 2002) and *Cl. acetobutylicum* (Nolling *et al.*, 2001) (Fig. 3). An underlying requirement of an independent arrangement of Hyd

Table 1 Examples of maturation gene organizations in microbial species synthesizing [FeFe]-hydrogenases

Organism	Gene Arrangement	Cellular Location	Gene	NCBI Annotation
Clostridium acetobutylicum	Independent	Cytoplasmic	*hydG*	HydG, CAC1356
			hydE	HydE, CAC1631
			hydF	HydF, CAC1651
			hydA	HydA, CAC0028
Chlamydomonas reinhardtii	Fusion	Chloroplastic	*hydEF*	HydEF, AY582739
			hydG	HydG, AY582740
			hydA1	HydA1, AY055755
			hydA2	HydA2, AY090770
Desulfovibrio desulfuricans	Operon	Periplasmic	*hydG*	HydG, Dde2278
			hydE	HydE, Dde2277
			hydF	HydF, Dde2276
			hydL	HydL, Dde2281
			hydS	HydS, Dde2280

maturation and structural genes is a mechanism to co-regulate expression under appropriate metabolic conditions. In microbial species with more than one [FeFe]-hydrogenase (i.e., *Cl. acetobutylicum*), changes in growth conditions influence the levels of enzyme activities (Gorwa *et al*., 1996). Whether similar growth conditions also regulate the levels of maturation proteins remains to be determined.

A gene fusion arrangement of *hydE* and *hydF* was initially identified for *hydEF* isolated from *Ch. reinhardtii* (Posewitz *et al*., 2004). As an example of a hydrogen-metabolizing green alga, *Ch. reinhardtii* is able to biosynthesize two soluble, nuclear-encoded [FeFe]-hydrogenases (Happe and Kaminski, 2002; Forestier *et al*., 2003), which localize to the chloroplast stroma (Happe *et al*., 1994). Currently a functional basis to explain the existence of a HydE and HydF fusion for maturation of [FeFe]-hydrogenases in *Ch. reinhardtii* has not been elucidated. Moreover, in the green algae, the temporal relationships underlying maturation, translocation and compartmentalization, as [FeFe]-hydrogenases proceed from precursors to chloroplast-localized, holo-enzymes are undefined. Intriguingly, the divergent orientation of *hydEF* and *hydG* in *Ch. reinhardtii* (Fig. 4) (Posewitz *et al*., 2004), is similar to the divergent transcription of the maturation gene operon *hyp* and the [NiFe]-hydrogenase-3 operon *hyc* in the *E. coli* genome (Fig. 5). In *Ch. reinhardtii*, this gene arrangement may provide a mechanism to divergently

express the eukaryotic *hydEF* and *hydG* genes from the same promoter region, whereby the requisite signal transduction leads to the regulated expression of both maturation genes at the required levels under the appropriate metabolic conditions.

The third type of [FeFe]-hydrogenase maturation and structural gene organization, the operon, is characteristically shared with the organization of genes encoding the [NiFe]-hydrogenase maturation proteins, as well as for the genes encoding the structural and enzyme-specific accessory proteins in *E. coli* (discussed below and shown in Fig. 5). Coincidently, the *E. coli* [NiFe]-hydrogenases, with the exception of the formate-linked, H_2-evolving type (e.g., *E. coli* hydrogenase 3), are localized to the periplasmic space. This is also observed in other [FeFe]-hydrogenases that are operon-encoded (Voordouw and Brenner, 1985; Voordouw *et al.*, 1989; Heidelberg *et al.*, 2002). Examples include the soluble [FeFe]-hydrogenases from *D. vulgaris* and *D. desulfuricans* (Fig. 4), which are translocated to the periplasmic space via recognition of a twin-arginine-translocation (TAT)-dependent signal peptide by the TAT complex (Prickril *et al.*, 1986; Van Dongen *et al.*, 1988; Voordouw, 2000). An exception might exist for localization of the operon-encoded [FeFe]-hydrogenase seen in the *Bacteroides thetaiotaomicron* genome. The *B. thetaiotaomicron* [FeFe]-hydrogenase operon possesses an [FeFe]-hydrogenase structural gene along with a complete set of maturation genes (Fig. 4). However, the expressed [FeFe]-hydrogenase appears to be cytoplasmic (M.C. Posewitz, unpublished results).

2.4. H-Cluster Biosynthetic Proteins: Families and Functions

2.4.1. Radical SAM Enzymes

To date, only three strictly conserved [FeFe]-hydrogenase maturation proteins, HydE, HydF and HydG, have been identified; however additional proteins, such as TM1420 in *T. maritima*, may be involved in the maturation of the more complex [FeFe]-hydrogenase from this organism (Pan *et al.*, 2003). The HydE and HydG [FeFe]-hydrogenase maturation proteins both contain the signature $C–X_3–C–X_2–C$ motif that is characteristic of the Radical-SAM protein superfamily. Radical-SAM enzymes are frequently involved in metabolic pathways, cofactor biosynthesis, production of deoxyribonucleotides, incorporation of sulphur into organic substrates, and the anaerobic synthesis of unique biomolecules (Frey, 2001; Sofia *et al.*, 2001; Frey and Magnusson, 2003; Fontecave *et al.*, 2004; Marsh *et al.*, 2004; Layer *et al.*, 2005; Walsby *et al.*, 2005). Moreover, the nitrogenase accessory

protein, NifB, putatively belongs to the Radical-SAM superfamily and is involved in the biosynthesis of the iron–molybdenum-cofactor (FeMoco) of nitrogenase (Allen *et al.*, 1995), another enzyme capable of hydrogen production. Although the enzymatic activity of NifB has yet to be determined (Dos Santos *et al.*, 2004), iron and sulphur from the metabolic product of NifB, NifB cofactor, become incorporated into the nitrogenase enzyme (Allen *et al.*, 1995). Therefore, in addition to the synthesis of unique organic molecules, including thiolated species, a precedent for the involvement of a putative Radical-SAM protein in the biosynthesis of the catalytic site of a metalloenzyme exists.

Lysine-2,3-aminomutase (LAM) was the first Radical-SAM enzyme to be reported in seminal experiments done by Barker and co-workers in 1970 (Chirpich *et al.*, 1970; Frey and Reed, 2000). Radical-SAM proteins were recognized as a protein superfamily when advanced sequence profiling methods demonstrated that over 600 proteins involved in diverse cellular processes share significant sequence similarity, including the $C–X_3–C–X_2–C$ motif (Sofia *et al.*, 2001). A [4Fe4S] cluster is coordinated by the three cysteines of this motif and the methionine carboxylate and amine of SAM bind the [4Fe4S] cluster at the open iron coordination site in a bidentate fashion (Cosper *et al.*, 2002; Walsby *et al.*, 2002; Chen *et al.*, 2003; Walsby *et al.*, 2005).

The Radical-SAM superfamily may currently be the most intensively studied group of FeS proteins and several excellent reviews regarding the Radical-SAM superfamily have recently appeared (Frey, 2001; Frey and Magnusson, 2003; Fontecave *et al.*, 2004; Marsh *et al.*, 2004; Layer *et al.*, 2005; Walsby *et al.*, 2005). Radical-SAM enzymes are found in all three kingdoms of life. In addition to participating in numerous biosynthetic processes, Radical-SAM proteins also serve as activating enzymes for the generation of protein radicals in anaerobic ribonucleotide reductase, pyruvate formate lyase and benzylsuccinate synthase.

Radical generation is initiated by the one-electron reduction of SAM by the [4Fe4S] cluster coordinated to the $C–X_3–X_2–C$ motif. This results in the cleavage of SAM, generating the highly reactive 5′-deoxyadenosyl radical and methionine (Cosper *et al.*, 2000; Henshaw *et al.*, 2000; Wu *et al.*, 2000). The 5′-deoxyadenosyl radical then abstracts a hydrogen atom from a substrate that is positioned in close proximity to SAM, and is unique to each subclass of Radical-SAM enzymes. The reductive cleavage of SAM *in vitro* has been observed to proceed in the absence of the appropriate substrate; however, the rate of SAM cleavage often increases significantly in the presence of the physiologically relevant substrate.

The structures of four Radical-SAM enzymes, BioB (Berkovitch *et al.*, 2004), coproporphyrinogen(III) oxidase (HemN) (Layer *et al.*, 2003),

molybdopterin (MoaA) (Hanzelmann and Schindelin, 2004) and LAM (Lepore *et al.*, 2005) have been determined. These structures demonstrate that Radical-SAM proteins share a $(\beta/\alpha)_6$ repeat in their structural core, which is described as an incomplete triosephosphate isomerase (TIM) barrel (Nicolet and Drennan, 2004). In the case of BioB, the protein contains the full $(\beta/\alpha)_8$ TIM-barrel fold. Alignments of the four Radical-SAM structures place the [4Fe4S] cluster that coordinates SAM in the same location relative to the $(\beta/\alpha)_6$ core within an extended loop (Lepore *et al.*, 2005). Although the $(\beta/\alpha)_6$ core of the Radical-SAM proteins characterized to date are similar, each protein contains a unique N- and/or C-terminal region(s) that potentially modulates substrate access to the active site. The structural and biophysical characterization of the Radical-SAM superfamily of proteins is providing valuable insights into the properties of these enzymes and additional examination of the Radical-SAM superfamily, particularly HydE and HydG, will provide critical insights into the mechanism of [FeFe]-hydrogenase biosynthesis.

2.4.2. HydE

The HydE proteins are highly homologous to the biotin synthase (BioB) Radical-SAM proteins and are inaccurately annotated as such in several genomic databases. BioB is involved in the insertion of sulphur into dethiobiotin to form biotin (Jarrett, 2005). Interestingly, two additional Radical-SAM proteins, lipoyl synthase (LipA) (Zhao *et al.*, 2003) and MiaB (Pierrel *et al.*, 2004), have also been demonstrated to incorporate sulphur into organic substrates and it is therefore possible that HydE plays a role in the synthesis of the unique dithiolate bridging ligand of the 2Fe-catalytic centre in [FeFe]-hydrogenases. However, this has yet to be demonstrated experimentally and, as discussed below, HydG is also similar to Radical-SAM proteins involved in the biosynthesis of sulphur-containing compounds. The $C–X_3–C–X_2–C$ Radical-SAM motif is conserved in all HydE proteins and three additional cysteine residues that form a $C–X_7–C–X_2–C$ motif in the C-terminal portion of the protein are found in several, but not all HydE proteins (Fig. 4).

The biophysical characterization of *T. maritima* HydE (TmHydE) overproduced in *E. coli* was recently reported (Rubach *et al.*, 2005). Following purification, anaerobic reconstitution of TmHydE with iron was done in the presence of dithiothreitol, sodium sulphide and ferrous salt, followed by treatment with EDTA to remove iron bound nonspecifically. EPR and UV spectroscopy indicate that reconstituted TmHydE most likely contains two distinct [4Fe4S] clusters in preparations containing eight iron atoms and eight sulphur atoms per polypeptide chain (Rubach *et al.*, 2005). However,

the authors note that the spectra are complex and contain some unique features. Therefore, a definitive assignment regarding the precise nature of the physiological [FeS]-clusters will have to await further characterization. The anaerobically reconstituted TmHydE catalytically cleaved SAM into 5′-deoxyadenosine (AdoH) and methionine when reduced with dithionite, confirming that the TmHydE protein has the expected SAM cleavage activity (Rubach *et al.*, 2005).

As observed in all structurally characterized Radical-SAM proteins, one [4Fe4S] cluster is putatively coordinated to the N-terminal Radical-SAM motif in HydE. The second [FeS]-cluster is likely to be coordinated to three cysteine residues in the C-terminal $C–X_7–C–X_2–C$ motif, which is found in several HydE homologues (Fig. 4).

This is consistent with the observation that mutation of all three cysteines in the Radical-SAM $C–X_3–C–X_2–C$ motif to alanine results in a TmHydE triple mutant that coordinates an [FeS]-cluster after anaerobic reconstitution (Rubach *et al.*, 2005). Interestingly, the three C-terminal cysteine residues that putatively bind the second [FeS]-cluster are not found in all HydE homologues. The two forms of HydE are differentiated as A- and B-form in Fig. 4. This difference in HydE primary sequence has led to speculation that HydE homologues lacking the C-terminal cysteine motif may not actually be HydE proteins (Rubach *et al.*, 2005) or that subtle differences may exist in the assembly mechanism in some organisms. The former is unlikely, as several of the HydE homologues that lack the C-terminal $C–X_7–C–X_2–C$ sequence are present within putative hydrogenase operons in several organisms (e.g., *B. thetaiotaomicron*, *D. desulfuricans* and *Shewanella oneidensis*). Moreover, the [FeFe]-hydrogenase operon from *B. thetaiotaomicron*, which encodes a HydE homologue without the three C-terminal cysteine residues, was cloned and expressed in *E. coli* resulting in an active [FeFe]-hydrogenase (M.C. Posewitz, unpublished). Therefore, in some cases the three C-terminal cysteines found in several HydE homologues are not essential for [FeFe]-hydrogenase maturation. However, the three cysteines of the Radical-SAM motif have been mutated to serine in *Cl. acetobutylicum* HydE (CaHydE) and were shown to be critical for [FeFe]-hydrogenase assembly *in vivo*, using an *E. coli* heterologous expression system (King *et al.*, 2006).

2.4.3. HydF

HydF amino acid alignments have divulged that members of this protein family possess two conserved domains (Posewitz *et al.*, 2004, 2005; see Fig. 4). The HydF N-terminal domain shares homology with the large family of NTP-binding proteins (Leipe *et al.*, 2003), whereas the C-terminal

domain has conserved cysteine and histidine amino acids arranged in a C–X–H–$X_{(44–53)}$–HC–X_2–C motif suggestive of [FeS]-cluster coordination (Posewitz *et al.*, 2004; Brazzolotto *et al.*, 2006; King *et al.*, 2006). The GTP-binding domain of the HydF proteins contains sequence motifs homologous to the glycine-rich P-loop, magnesium-binding and GTP-specific distal motifs characteristic of the GTPase protein family (Leipe *et al.*, 2003; Posewitz *et al.*, 2004; Brazzolotto *et al.*, 2006) (Fig. 4). Recently, HydF from *T. maritima* (TmHydF) was purified and reconstituted and was demonstrated to have GTP-hydrolysis activity, and to possess a [4Fe4S] cluster ligated by three cysteines (Brazzolotto *et al.*, 2006). The fourth ligand to the [4Fe4S] cluster in TmHydF was predicted to be non-cysteinyl, based on the Electron Paramagnetic Resonance (EPR) spectrum of the $S = 1/2$ reduced cluster, and the exchangeability with solvent. The Hyperfine Sublevel Correlation spectra of the reconstituted TmHydF excluded N-ligation of the [4Fe4S] cluster, leaving open the possibility for O-ligation, or ligation through a small-molecular-weight exogenous ligand. The observation that all the HydF homologues identified so far contain both the conserved GTPase domain and [FeS]-cluster binding domain suggests that GTPase activity and the [4Fe4S] cluster are required for HydF participation in H-cluster biosynthesis and [FeFe]-hydrogenase maturation. In agreement with the observed sequence conservation, mutational analyses of HydF from *Cl. acetobutylicum* have shown that an intact GTP-binding P-loop motif, and a [4Fe4S] cluster motif are both required to achieve maturation of *Cl. acetobutylicum* [FeFe]-hydrogenase I (King *et al.*, 2006).

2.4.4. HydG

The HydG proteins are highly homologous to the Radical-SAM ThiH family of proteins and are erroneously annotated as such in several genomic databases. ThiH is involved in the biosynthesis of thiamine, although its precise role in thiamine biosynthesis is not fully defined (Martinez-Gomez *et al.*, 2004). All HydG homologues analyzed to date contain the C–X_3–C–X_2–C Radical-SAM motif and three additional conserved cysteine residues that form a C–X_2–C–X_{22}–C motif in the C-terminal portion of the protein, which putatively coordinates another [FeS]-cluster (see Fig. 4). As in the case of CaHydE, the three cysteines of the Radical-SAM motif in *Cl. acetobutylicum* HydG (CaHydG) were mutated to serine and shown to be essential for [FeFe]-hydrogenase assembly *in vivo* (King *et al.*, 2006). Moreover, mutation of the three conserved cysteines in the C-terminal C–X_2–C–X_{22}–C motif of CaHydG indicates that these amino acids are also

critical for maturation of an active [FeFe]-hydrogenase *in vivo* (King *et al.*, 2006).

Rubach *et al.* reported the first purification and biophysical characterization of a HydG enzyme (Rubach *et al.*, 2005). The *T. maritima* HydG (TmHydG) protein was overproduced in *E. coli*, and anaerobically reconstituted as described above for the TmHydE protein. Similar to TmHydE, EPR and UV spectroscopy indicate that anaerobically reconstituted TmHydG most likely contains two distinct [FeS]-clusters. In addition to the [4Fe4S] cluster coordinated to the Radical-SAM motif, the second [FeS]-cluster is putatively coordinated by the C–X_2–C–X_{22}–C motif. However, the TmHydG protein is reported to be difficult to isolate and manipulate (Rubach *et al.*, 2005), and additional research is required to fully characterize the coordinated [FeS]-clusters. When reduced with dithionite, the reconstituted TmHydG contains the expected SAM cleavage activity (Rubach *et al.*, 2005).

Similar to HydG, the Radical-SAM proteins BioB and LipA both coordinate a second distinct [FeS]-cluster, which is coordinated by three C-terminal cysteines (Ugulava *et al.*, 2001; Cicchillo *et al.*, 2004). BioB and LipA are involved in the incorporation of sulphur into organic substrates and in the case of both BioB and LipA, the sulphur has been proposed to originate from the second C-terminal [FeS]-cluster (Ugulava *et al.*, 2001; Tse Sum Bui *et al.*, 2004; Cosper *et al.*, 2004; Cicchillo and Booker, 2005; Jarrett, 2005). However, mobilization of sulphur from the second [FeS]-cluster for incorporation into biotin is still the subject of debate (Ollagnier-de-Choudens *et al.*, 2002). The Radical-SAM protein, MiaB, is also involved in thiolation chemistry and facilitates the incorporation of sulphur into 2-methylthio-N^6-isopentenyl-adenosine (ms^2i^6A) (Pierrel *et al.*, 2004). Interestingly, MiaB contains a second distinct [FeS]-cluster (Rubach *et al.*, 2005); however, the source of the sulphur atom required for ms^2i^6A biosynthesis is yet to be determined (Pierrel *et al.*, 2004). In the case of LipA, two sulphur atoms are inserted into the C-6 and C-8 positions of protein-bound derivatives of octanoic acid. If propane or dimethylamine were substrates for either HydE or HydG, incorporation of the two sulphur atoms into these substrates to form the [FeFe]-hydrogenase bridging dithiolate ligand would be highly analogous to the LipA facilitated reaction.

2.5. Heterologous Expression of [FeFe]-Hydrogenases

Upon identification of [FeFe]-hydrogenase structural genes, several attempts were made to heterologously express the proteins in *E. coli* to obtain

enzymatically active forms. The first reported attempt was the expression of the periplasmic [FeFe]-hydrogenase cloned from *D. vulgaris* (Voordouw and Brenner, 1985; Voordouw *et al.*, 1987). This resulted in synthesis of an inactive form of the [FeFe]-hydrogenase with two redox titratable F-clusters, but no catalytic H-cluster. Moreover, the inactive enzyme failed to localize to the periplasmic space, and was primarily localized in the cytoplasm (Voordouw *et al.*, 1987; Van Dongen *et al.*, 1988). Similar results were obtained for expression of *Cl. pasteurianum* and *M. elsdenii* [FeFe]-hydrogenase clones expressed in *E. coli* under anaerobic conditions (Atta and Meyer, 2000). The results are supported by the fact that the [NiFe]-hydrogenases and [FeFe]-hydrogenases are phylogenetically distinct enzyme families (Vignais *et al.*, 2001), and that *E. coli* lacks a native [FeFe]-hydrogenase. Thus, it was proposed that the maturation pathways of each enzyme family are also distinct, functionally unable to cross-react with the structural proteins from the other family. An exception to this proposed model has been reported for heterologous expression of an active *Cl. pasteurianum* [FeFe]-hydrogenase I (CpI) in the cyanobacterium *Synechococcus* PCC7942 (Asada *et al.*, 2000). Native gel chromatography and H_2-evolution experiments *in vivo* showed the presence of additional hydrogenase activity in transformed *Synechococcus* cells that expressed the CpI gene. At this time, it is difficult to reconcile a mechanism for CpI [FeFe]-hydrogenase maturation in the absence of *hydE*, *hydF or hydG* homologues in this organism, with the observation for the implicit requirement of a functional HydEF protein to achieve maturation of [FeFe]-hydrogenases in *Ch. reinhardtii* (Posewitz *et al.*, 2004). Moreover, in recombinant *E. coli* systems under conditions where [NiFe]-hydrogenase maturation proteins are expressed, there is no evidence of [FeFe]-hydrogenase maturation without the co-expression of functional HydE, HydF and HydG proteins (King *et al.*, 2006). Nonetheless, it is an intriguing result with potential biotechnological interest for engineering of more efficient biological hydrogen production systems.

More recently, successful heterologous systems for [FeFe]-hydrogenase expression have been developed from the use of either [FeFe]-hydrogenase synthesizing organisms (i.e., *Cl. acetobutylicum*) as host for native and non-native structural gene expression (Girbal *et al.*, 2005), or from the use of *E. coli* as a host for expression of [FeFe]-hydrogenase structural and maturation proteins (King *et al.*, 2006). Co-expression of the *hydEF* and *hydG* genes cloned from *Ch. reinhardtii* with the *hydA1* gene in *E. coli*, discussed above, was the first demonstration of the capacity of the Hyd proteins to biosynthesize the H-cluster during maturation of [FeFe]-hydrogenase catalytic domains (Posewitz *et al.*, 2004). The successful expression of both native and algal [FeFe]-hydrogenases was later demonstrated in

Cl. acetobutylicum using plasmid-based expression of the structural genes *hydA* from *Cl. acetobutylicum*, and *hydA1* genes from the green alga *Ch. reinhardtii* and *S. obliquus*. Complementing these results was the cloning and expression of *hydE*, *hydF* and *hydG* from *Cl. acetobutylicum* in *E. coli*, which were shown to maturate both clostridial and algal [FeFe]-hydrogenases (King *et al.*, 2006). The ability to express [FeFe]-hydrogenases in non-native hosts such as *E. coli*, which is easily manipulated genetically, offers great potential to facilitate investigation of the [FeFe]-hydrogenase maturation process.

2.6. Model for Catalytic Site Maturation

Although the precise mechanism of [FeFe]-hydrogenase assembly is currently unknown, the [FeFe]-hydrogenase maturation proteins belong to well-studied protein superfamilies, which provides a foundation for elucidating the roles of HydE, HydF and HydG in [FeFe]-hydogenase maturation. Characterization of the Radical-SAM superfamily is rapidly providing new insights into the functionality of this highly versatile group of enzymes and several characteristics exhibited by these proteins are consistent with the requirements for assembly of the [FeFe]-hydrogenase catalytic site. First, three different Radical-SAM enzymes are known to incorporate sulphur into novel substrates and it is likely that either HydE and/or HydG is required to synthesize the di-(thiomethyl)amine (or dithiopropane) ligand that bridges the 2Fe-centre of the [FeFe]-hydrogenase active site. Because a single Radical-SAM enzyme, LipA, is capable of providing two sulphur atoms to protein-bound derivatives of octanoic acid (Cicchillo and Booker, 2005), an analogous reaction by either HydE or HydG would require only one of the two Radical SAM enzymes involved in [FeFe]-hydrogenase assembly. This assumes that the synthesis of the bridging ligand is similar to the LipA-facilitated reaction, which is premature. Nevertheless, precedent in the Radical-SAM literature suggests that either HydE or HydG via a radical mechanism synthesizes the bridging dithiolate ligand.

Second, iron and sulphur originating from the NifB cofactor ultimately become incorporated into nitrogenase. Although the precise role of NifB in nitrogenase maturation is unclear (Dos Santos *et al.*, 2004), the involvement of this putative Radical-SAM protein in the assembly of an iron metalloenzyme may have parallels to the biosynthesis of the [FeFe]-hydrogenase active site. Lastly, members of the Radical-SAM superfamily facilitate a number of difficult synthetic reactions, often under anaerobic conditions. In addition to the bridging dithiolate ligand, the 2Fe-catalytic centre also has

CN and CO ligands. It is conceivable that either HydE and/or HydG are responsible for the biosynthesis of these ligands from metabolic precursors. Since CN and CO can have toxic effects within the cell, it is likely necessary to synthesize these ligands at the site of H-cluster assembly. A common feature of Radical-SAM enzymes is the coordination of a unique substrate in close proximity to the site of radical formation within the TIM-barrel fold. These ligands would therefore be synthesized in a controlled environment within the Radial-SAM protein. Moreover, the presence of distinct [FeS]-clusters in the other [FeFe]-hydrogenase maturation proteins may initially provide the iron coordinated to these ligands and serve as a scaffold in assembly of the [FeFe]-hydrogenase catalytic site.

Insight into the functional role of HydF in H-cluster biosynthesis and [FeFe]-hydrogenase maturation may also be elucidated from the examples of biochemically similar proteins known to participate in maturation of other metalloenzymes. The Ni-containing enzymes CO-dehydrogenase (CODH) (Jeon *et al*., 2001), urease (Moncrief and Hausinger, 1997) and [NiFe]-hydrogenase (reviewed here) each require either GTP (urease, [NiFe]-hydrogenase) or ATP hydrolysis (CODH-dehydrogenase) by a system-specific maturation protein during the Ni-insertion step. A more complex example of NTP-dependent chaperone function in metallocentre biosynthesis appears to exist for the role of Fe-protein (NifH) in nitrogenase maturation. NifH has been proposed to fulfill a dual function through participation in maturation of both the [8Fe8S] P-cluster and FeMoco (reviewed in Rubio and Ludden, 2005). Whereas NifH-dependent steps in P-cluster biosynthesis are independent of the presence of MgATP, the maturation of FeMoco is known to require NifH-catalysed MgATP-hydrolysis (Hu *et al*., 2005). In a more general role, NTPase proteins are also known to function less specifically in the biosynthesis of [FeS]-cluster proteins. Examples include the ApbC/Mrp-family originally identified in *Salmonella* mutants defective for a variety of [FeS]-dependent biochemical pathways (Skovran and Downs, 2003). In the budding yeast, members of this protein family, like Cfd1 (Roy *et al*., 2003) and Npb35p (Hausmann *et al*., 2005), are found to be required for the biosynthesis of cytosolic (Cfd1, Nbp35p) and nuclear (Nbp35p) [FeS]-cluster proteins, respectively. A common theme shared by these examples is the use of nucleotide-hydrolysis to promote and/or regulate metalloprotein biosynthesis. In the case of [FeFe]-hydrogenase maturation, mutational analysis of *Cl. acetobutylicum* HydF has shown that both the NTP-binding P-loop and [FeS]-cluster binding motifs are essential to achieve biosynthesis of active [FeFe]-hydrogenase (King *et al*., 2006). The manner in which HydF contributes to maturation of [FeFe]-hydrogenase catalytic domain together with HydE and HydG, and what role

NTP-binding/hydrolysis or [FeS]-cluster(s) have in H-cluster biosynthesis and [FeFe]-hydrogenase maturation remains an open question.

The HydE, HydF and HydG proteins required for [FeFe]-hydrogenase maturation were only recently identified (Posewitz *et al*., 2004, 2005) and research into the mechanism of [FeFe]-hydrogenase maturation is in its infancy. However, the high level of interest in both [FeS]-cluster biogenesis (Frazzon and Dean, 2003; Fontecave *et al*., 2005; Johnson *et al*., 2005; Rubio and Ludden, 2005) and hydrogenase assembly (Maier and Böck, 1996b; Blokesch *et al*., 2002; Mulrooney and Hausinger, 2003; Kuchar and Hausinger, 2004; Sawers *et al*., 2004; Vignais and Colbeau, 2004) bodes well for rapid progress in understanding the mechanism of [FeFe]-hydrogenase maturation.

Recently, a potential mechanism for H-cluster biosynthesis was proposed (Peters *et al*., 2006). This hypothesis suggests roles for HydE, HydF and HydG and identifies possible precursors to the dithiolate, CO and CN ligands of the 2Fe-center. The model is derived in part from biochemical themes such as sulphur mobilization and glycyl radical formation that are common to other SAM-dependent pathways (e.g., LipA and lipoic acid, BioB and biotin, pyruvate formate lyase (PFL) activating enzyme and PFL). Moreover, noting the diversity of organisms that possess the capacity to biosynthesize [FeFe]-hydrogenases and the apparent absence of additional genes required for [FeFe]-hydrogenase maturation, the authors suggest that ordinary metabolic intermediates, possibly amino acids such as glycine and aspartate, provide the building blocks for ligand biosynthesis. The proposed mechanism first involves the formation of a [2Fe2S]-cluster precursor bound to either HydE or HydG. The sulphides of this cluster are then alkylated by an organic radical generated by one of the Radical-SAM enzymes to form the bridging dithiolate ligand. Alkylation of the sulphides would protect these sulphur atoms from further modification making the Fe atoms more reactive in subsequent radical reactions. The authors suggest that addition of the CO that bridges the two iron atoms might occur during this initial step of dithiolate ligand synthesis. Once formed, the dithiolate bridged, 2Fe-precursor could be transferred to the second SAM enzyme, or alternatively, to the apo-enzyme itself, in a step requiring HydF-dependent GTP hydrolysis. The second Radical-SAM enzyme, either HydE or HydG, is then proposed to generate glycyl radicals that react with the Fe atoms of the dithiolate bridged [2Fe2S] cluster. Glycyl radical decomposition at these Fe atoms would then generate CO and CN at an equivalent stoichiometry. Two successive rounds of decomposition are required for the addition of a CO and CN to each iron atom. This hypothetical glycyl-radical-decomposition pathway to CO and CN biosynthesis is supported by DFT calculations,

which show that the high-energy requirement for coordination of glycine to a reduced iron can be overcome through the generation of the radical intermediate. Reduction of the iron atoms in the 2Fe subcluster to Fe(I) appears to be critical for the formation of the Fe–CO bond and is putatively achieved by the transfer of electrons to the 2Fe-precursor from one of any of the additional [FeS]-clusters present in HydE, HydF or HydG. Biosynthesis of the 4Fe-center is presumed to occur by means of a standard FeS-cluster biosynthetic pathway (e.g., the ISC pathway), and inserted prior to the 2Fe-center. Completion of the bonding arrangement of the H-cluster to the catalytic site and dissociation of a maturation protein(s) enzyme-complex would possibly be facilitated by HydF-dependent GTP-hydrolysis.

3. MATURATION OF [NIFE]-HYDROGENASES

3.1. The [NiFe]-Cluster

3.1.1. Structure and Coordination

[NiFe]-hydrogenases are heterodimeric enzymes with a large subunit of an average molecular mass ranging between 60 and 65 kDa and a small subunit of a size between 30 and 35 kDa (Przybyla *et al.*, 1992; Friedrich and Schwartz, 1993; Wu and Mandrand, 1993). The large subunit coordinates the dinuclear active site (Fig. 1B), whereas the small subunit harbours between one and three Fe–S clusters that lead the electrons to or from the active site. Fig. 2 (Panels C and D) gives a scheme of the structure of the [NiFe]-centre and its coordination by the large subunit. Only features relevant for the maturation process are indicated and discussed. Since the conclusions on the composition of the active site drawn from the first X-ray structure of a [NiFe]-hydrogenase (Volbeda *et al.*, 1995) were largely supported by the results from structures of enzymes from other organisms (Fontecilla-Camps *et al.*, 2001; Frey *et al.*, 2001), it is safe to conclude that the basic features reflect a general theme. Nevertheless, it needs to be recalled that most of the information gathered for the maturation process was obtained from [NiFe]-hydrogenases of *E. coli*, *R. eutropha*, *Bradyrhizobium (B.) japonicum*, *Rhizobium (Rh.) leguminosarum* and *Azotobacter (A.)* species for which three-dimensional structures are not available. The conclusions relating maturation steps with structural details, therefore rest on analogy assumptions.

The nickel of the active site is coordinated by the thiolates of four cysteine residues that are contributed by two CxxC motifs whereby cysteine residue 3

(see Fig. 2D) in the enzyme of certain organisms is replaced by a selenocysteine residue (He *et al*., 1989; Sorgenfrei *et al*., 1993a). Their involvement in the coordination of the centre has been predicted by thorough work conducted by Przybyla *et al*. (1992). One motif is located in the N-terminal part of the large subunit sequence at varying distances relative to the N-terminus; the other one is positioned invariably three amino acids from the C-terminus of the mature subunit. The iron of the active site that has been detected by X-ray crystallography is directly connected to the nickel atom via two of the thiolates that also coordinate the nickel. As predicted by Fourier Transform Infrared Spectroscopy (FTIR) (Bagley *et al*., 1994; Bagley *et al*., 1995) and later proven by the same method, the iron atom carries three diatomic ligands, namely two cyanyl and one carbonyl moieties (Happe *et al*.,1997; Pierik *et al*., 1999). Modelling of the ligands into the X-ray structure indicated that the CO is located in a hydrophobic pocket, whereas the CN groups undergo hydrogen bonding with side chain groups of the protein (Volbeda *et al*., 1996; Fontecilla-Camps *et al*., 2001). The metal centre is located in the interior of the heterodimer close to the large interface between the two subunits. The scheme of Fig. 2C also depicts the C-terminal extension present in the precursor of the large subunit, which is removed during the maturation process.

3.1.2. Consideration of Functions Required for Cluster Biosynthesis and Maturation

The balanced synthesis of enzymes with complex metal clusters like those of hydrogenases requires the coordination of three-basic processes, namely the formation of the apoprotein, the uptake and provision of the metal(s), and the assembly of the centre and its incorporation to generate the catalytically active end product. Considerable information is available on the synthesis and the regulation of the apoprotein part of [NiFe]-hydrogenases under different physiological regimes. It is summarized in several recent reviews (Friedrich *et al*., 2001; Vignais and Colbeau, 2004; Sawers *et al*., 2004). On the other hand, comparatively less is known on the adjustment of the rate of metal uptake to its requirement and on the biochemical partial reactions involved in the formation and insertion of the cluster. The cellular accumulation of iron is not specifically coupled to hydrogenase formation, since it serves a housekeeping function for microorganisms because of its role as a building block of many and diverse cofactors and coenzymes. In contrast, hydrogenases are often the only, or one of a few, nickel-dependent enzymes in microbial cells and, accordingly, the uptake of nickel and its regulation are intimately connected with [NiFe]-hydrogenase formation. The rates and

the level of nickel accumulation and its specific donation to the maturation machinery are thus crucial processes.

Maturation of the [NiFe]-hydrogenases itself is a complex process, which requires the activities of a considerable number of accessory proteins with novel functions as already predictable from the chemical structure of the centre and its location within the mature protein. They include enzymes for the synthesis of the CN and CO ligands and mechanisms for their attachment to the active site iron at a precise stoichiometry. Because the metal centre is located in the interior of the heterodimer, a further mechanism must warrant that the apoprotein is accessible for the incoming metal or centre and that some conformational event buries it inside after the insertion is completed. Furthermore, the first crystal structure of a [NiFe]-hydrogenase, showed that the mature enzyme lacks a C-terminal segment that is encoded by the reading frame (Volbeda *et al.*, 1995). Its removal and the consequence for the maturation process had to be elucidated. Finally, since nickel and iron are directly bonded and coordinated by four thiolates, the existence of some control must be postulated so that the correct metal is coordinated by the cognate site.

On the basis of the present, still limited, information integrated from several biological systems and taking the gaps in our knowledge into account, the maturation of [NiFe]-hydrogenases implicates the following steps: (i) After active transport of the nickel across the cytoplasmic membrane, it is assumed that a nickel-binding protein accepts the metal and transfers it to the maturation site. (ii) It is unknown at present whether Fe insertion into the large subunit precedes the coordination with the CO and CN ligands or whether the already fully coordinated metal is incorporated. Irrespective of this open issue, however, biosynthesis of CO and CN and coordination of Fe with the ligands are the unique steps in the assembly of the active site metal centre. (iii) Both *in vivo* and *in vitro* evidence support the contention that Fe insertion precedes the insertion of nickel or at least is a prerequisite for it. The incorporation of both metals results in an apoprotein form in which the metal centre is not yet closed by one or both of the thiolate bridges that link the Fe and Ni sites. The activity of an endopeptidase removes the C-terminal extension from the precursor protein which is followed by the closure of this bridge(s). (iv) Maturation of the small subunit of [NiFe]-hydrogenases occurs in parallel to, and independently from, large subunit maturation. After relieving an existing blockade of premature assembly of the two subunits, the heterodimer can be formed and targeted to its cellular site which – depending on the enzyme – can be either the cytosol or the inside or outside of the cytoplasmic membrane.

3.2. Genetics of the Maturation of [NiFe]-Hydrogenases

3.2.1. The hyp Genes

Mutants with lesions in the formation of active hydrogenases have been described by several groups in the pre-genomic era of microbial physiology, mostly for *E. coli* and *Salmonella typhimurium* (Barrett *et al.*, 1984; Krasna, 1984; Yerkes *et al.*, 1984; Lee *et al.*, 1985; Sankar *et al.*, 1985; Waugh and Boxer, 1986; Wu and Mandrand-Berthelot, 1986; Sankar and Shanmugam, 1988a,b; Stoker *et al.*, 1989). Analysis of their phenotypes delivered important information on the physiological role and on electron donor/receptor coupling properties. Because of the multiplicity of hydrogenases in these organisms and the overlapping functions of many gene products in the maturation of all isoenzymes, it was difficult at that time to correlate the genetic defect with lesions in a structural or auxiliary gene. There were two exceptions, however. First the report that a mutation in *pyrA*, the gene for carbamoylphosphate (CP) synthetase, abolishes all hydrogenase activity in *Salmonella* (Barrett *et al.*, 1984). This heuristic finding came more than 10 years before the discovery of the CO and CN ligands in the active site metal centre (Bagley *et al.*, 1994; Bagley *et al.*, 1995; Volbeda *et al.*, 1996; Happe *et al.*, 1997; Pierik *et al.*, 1999) and before the finding that this metabolite is the source of the CN ligands (Paschos *et al.*, 2001). The second one is the observation that certain mutants of *E. coli* (designated *hydB*, now *hypB*) lacking hydrogenase activity can be rescued by supplementing the medium with high-nickel concentrations (Waugh and Boxer, 1986). Although this property did not strictly rule out a mutation in the structural genes, it was suggestive of a lesion in some nickel delivery system.

The determination of the nucleotide sequence of the chromosomal region (min 58–59), in which most of the mutations from *E. coli* resulting in a hydrogenase-deficient phenotype mapped, delivered information on an operon (*hyc*) coding for components of hydrogenase 3 from *E. coli* (Böhm *et al.*, 1990; Sauter *et al.*, 1992) and of genes (*hyp*) located at both sides of the *hyc* operon (see Fig. 5). Their inactivation in most cases resulted in the loss of the activity of all three hydrogenases (Lutz *et al.*, 1990; Yamamoto *et al.*, 1990; Lutz *et al.*, 1991; Jacobi *et al.*, 1992; Maier *et al.*, 1996). The introduction of in-frame deletions into the *hyp* genes and the biochemical and physiological analysis showed that the resulting mutants share the following common properties: they accumulate precursor forms of the large subunits of all three hydrogenases that still possess the C-terminal extension and these precursor forms do not contain nickel (Jacobi *et al.*, 1992; Maier *et al.*, 1996). The inactivation of two of the genes (*hypA* and *hypC*) leads only to

the loss of hydrogenase 3 activity. Their functions in the maturation of isoenzymes 1 and 2 are taken over by two homologues (*hybF* and *hybG*, respectively) encoded in the operon (*hyb*) coding for hydrogenase 2 (Menon *et al.*, 1990; Menon *et al.*, 1991; Blokesch *et al.*, 2001; Hube *et al.*, 2002; Blokesch *et al.*, 2004a) (see Fig. 5).

Homologues of the *hyp* genes are present in all organisms capable of forming [NiFe]-hydrogenases. Fig. 5 gives a few examples for their chromosomal organization. In bacteria, most, or at least subgroups, of the *hyp* genes are clustered in transcriptional units. In view of the fact that gene clustering can be taken frequently as an indication for the physical or functional interaction of the gene products, it is interesting to note that *hypA* is almost exclusively clustered with the *hypB* gene and that *hypC*, *hypD* and (in most organisms also) *hypE* are co-organized in apparent transcriptional units. This mirrors well the joint function of HypA and HypB in nickel

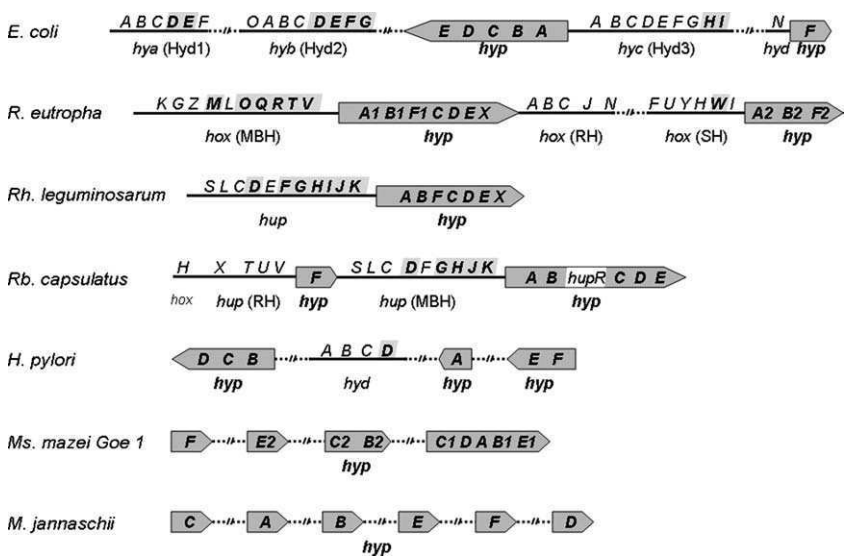

Figure 5 Organization of structural and accessory genes whose products are required for the synthesis and maturation of [NiFe]-hydrogenases. Organisms: *Escherichia (E.) coli*, *Ralstonia (R.) eutropha*, *Rhizobium (Rh.) leguminosarum*, *Rhodobacter (Rb.) capsulatus*, *Helicobacter (H.) pylori*, *Methanosarcina (Ms.) mazei* and *Methanocaldococcus (M.) jannaschii*. Members of the *hyp* gene family are printed inside the arrows, their direction of transcription is given by the arrow. Putative and proven maturation genes not belonging to the *hyp* gene family are shaded and printed in bold letters. The organization of only the *hyp* genes are given for the two archaea listed. (For further details see text.)

insertion and the formation of a ternary complex between HypC, HypD and HypE in the biosynthesis and coordination of the CN ligand (see below). *hypF*, on the other hand, is genetically separated in many organisms, although its product interacts physically and functionally with the HypE protein. In archaea, genes for auxiliary proteins in [NiFe]-hydrogenase maturation are less frequently clustered in transcriptional units as the two selected organisms listed in Fig. 5 show.

The *hyp* gene operons from *Rh. leguminosarum* and *B. japonicum* and one of the *hyp* operons from *R. eutropha* also contain a gene designated *hypX* (Rey *et al*., 1996; Buhrke and Friedrich, 1998) that is the promoter most distal gene of the operon and does not have an apparent homologue in organisms like *E. coli* or *Helicobacter (H.) pylori* (Robson, 2001b). HypX does not appear to be essential for maturation of the hydrogenases from *R. eutropha* (Buhrke and Friedrich, 1998), but may be required for changing the biochemical properties of the mature enzyme (Bleijlevens *et al*., 2004; see also below). On the other hand, inactivation of *hypX* from *Rh. leguminosarum* severely restricts hydrogenase maturation (Rey *et al*., 1996).

A complication in the analysis of biochemical functions of the *hyp* gene products arises from the fact that they may be present in multiple copies either on the chromosome or, in addition, on a plasmid. Thus, three copies of most of the *hyp* genes are present in *R. eutropha* (Wolf *et al*., 1998; Schwartz *et al*., 2003; Lenz *et al*., 2005). It is still open whether this multiplicity represents a genetic redundancy with no physiological relevance or whether it reflects the fact that this organism has the task of maturing three different hydrogenases. One set of the *hyp* gene copies of this organism appears to be silent, as judged from the fact that it does not support maturation in the absence of the other two copies. However, although the cluster as an entity may be non-functional, this must not necessarily hold for all of its individual genes.

3.2.2. Accessory Genes Co-Expressed with the Hydrogenase Structural Genes

The Hyp proteins constitute the core machinery for insertion of the [NiFe] metallocentre into the large subunit apoprotein, i.e., they may act in the maturation of more than one hydrogenase in the cell. Accordingly, in the majority of instances their respective genes are expressed separately from those located in the structural gene operons. A second set of maturation genes, however, is normally co-expressed with the structural genes within the same transcriptional unit, as their products are involved in the maturation of the specific isoenzyme with which they are co-synthesized. On the basis of their function, two classes can be differentiated presently. The first one encompasses endopeptidases like HycI, HyaD and HybD from *E. coli*,

which proteolytically process the precursors of the large subunit from isoenzymes 3, 1 and 2, respectively, after metal incorporation (for review, see Theodoratou *et al.*, 2005). The same situation holds for *R. eutropha* in which HoxW and HoxM proteolytically cleave the precursors of the large subunits from the soluble and membrane-bound hydrogenases, respectively (Thiemermann *et al.*, 1996; Massanz *et al.*, 1997). The second family comprises chaperone-like proteins, which coordinate the assembly and export of periplasmic hydrogenases via the TAT-export system. Representatives in *E. coli* are the *hyaE* and *hybE* gene products that are required for the export of the cofactor-containing heterodimeric hydrogenases 1 and 2 (for review, see Palmer *et al.*, 2005) and, possibly also *hycH*, whose product appears to fulfil an anti-assembly function in hydrogenase 3 synthesis in the cytoplasm (Ekaterini Theodoratou and August Böck, unpublished results). Finally, in exceptional cases, *hyp* genes may also be located in a common transcriptional unit with structural genes, apparently when their usual maturation function cannot be performed because the isoenzymes are very unalike in their sequence. Examples are the *hybF* and *hybG* gene products (see above).

3.2.3. Genes Coding for Additional Accessory Proteins

Accessory genes belonging to the two classes discussed above are the only ones characterized until now for the maturation system of the *E. coli* hydrogenases. In other organisms, maturation seems to be more complex involving the function of additional gene products. As Fig. 5 displays, a set of 5 genes with a function in hydrogenase synthesis is located between the structural gene and the *hyp* operons on the genome of several organisms. These are the *hupGHIJK* genes in *Rh. leguminosarum* and their homologues *hoxOQRTV* in *R. eutropha. In Rhodobacter (Rb.) capsulatus* these genes are present in the same order with the exception that the *hupI* and *hupJ* genes are fused into a *hupJ* single reading frame (Vignais *et al.*, 2001). Recent evidence indicates that the gene products of the *hupGHIJ* operon are involved in the maturation of the small subunit of the *Rh. leguminosarum* hydrogenase (Manyani *et al.*, 2005), whereas a function in large subunit formation has been postulated for HupK (Imperial *et al.*, 1993; see also below). As already noted, *R. eutropha* and *Rh. leguminosarum* possess an extra *hyp* gene (*hypX*) which is absent in the genome of *Rb. capsulatus*.

3.2.4. Genes for Nickel Uptake Systems

One class of mutants pleiotropically deficient in the synthesis of all hydrogenases from *E. coli* (original designation *hydC* in *E. coli*) was identified as

possessing a lesion in nickel transport (Wu and Mandrand-Berthelot, 1986; Wu *et al*., 1989; Wu *et al*., 1991). The genes of the operon *nikABCDE-nikR* code for the components of an ATP-binding cassette transporter in which NikA is the periplasmic-binding protein (Navarro *et al*., 1993), NikB and C are the membrane integral proteins forming the uptake channel, and NikD and E are the proteins located on the inside of the cytoplasmic membrane, coupling transport to ATP hydrolysis. NikR is a DNA-binding protein, which accepts nickel as ligand and regulates transcription of the operon in a complex fashion. Homologues of the *nik* transporter genes are present in other organisms (for review, see Eitinger and Mandrand-Berthelot, 2000; Mulrooney and Hausinger, 2003).

A nickel-specific permease has been identified in *R. eutropha* as being responsible for nickel uptake. It is the product of the *hoxN* gene, which is located downstream of the gene cluster coding for components of the regulatory hydrogenase of this organism (Fig. 5) (Eberz *et al*., 1989; Eitinger and Friedrich, 1991). Similar to *nik* mutants of *E. coli*, interruption of *hoxN* greatly reduces the level of active hydrogenases formed. HoxN is a single membrane integral protein with an 8 transmembrane-domain architecture, which is selective for nickel over cobalt. HoxN homologues have been found in many other organisms, like *B. japonicum* (Fu *et al*., 1994) or *H. pylori* (Mobley *et al*., 1995).

3.3. Functions of Accessory Proteins in the Maturation of [NiFe]-Hydrogenases

3.3.1. Synthesis of the CN and CO Ligands

As stated above, those organisms possessing solely the genetic capacity for the formation of [FeFe]-hydrogenases do not carry homologues of the *hyp* genes on their chromosome. Assuming that at least some of the *hyp* gene products have a function in CO and/or CN synthesis, the path of formation of these ligands should be different in the maturation systems of the [FeFe] and [NiFe] enzymes.

3.3.1.1. CN Biosynthesis

The capacity for the synthesis of cyanide is widely distributed among organisms, ranging from bacteria to plants. The pathways involved and described until now for bacteria are functional only under aerobic or microaerobic conditions, since molecular oxygen is either required as a substrate or as a terminal electron acceptor (Knowles, 1976; Blumer and Haas, 2000).

Pathways that operate in the complete absence of oxygen had not been described hitherto.

Information on how cyanide may be formed by *E. coli* came first from the derived amino acid sequence of the *hypF* gene (Paschos *et al.*, 2001). It codes for a monomeric protein of 82.1 kDa molecular mass with a complex domain structure (Fig. 6). The amino terminal part of the polypeptide shares significant sequence (Wolf *et al.*, 1998) and 3D structural similarity (Stefani *et al.*, 1997; Rosano *et al.*, 2002) with acylphosphatases from eukaryotic organisms. It is followed by two classical zinc finger motifs (Yamamoto *et al.*, 1990), which are identical in their amino acid sequences. In the C-terminal portion there is a sequence signature similar to that present in *O*-carbamoyltransferases, which are involved in nodulation factor or antibiotic biosynthesis (see Paschos *et al.*, 2001).

The purification and characterization of the HypF protein from *E. coli* indeed showed that the protein accepts CP as a substrate (Paschos *et al.*, 2002). HypF hydrolyses CP in the absence of other substrates and it cleaves ATP into adenosine monophosphate (AMP) and pyrophosphate when CP is present. The latter reaction can be reversed as visualized by the incorporation of radioactive pyrophosphate into ATP in the presence of CP. The actual physiological reaction, however, consists in the transfer of the carbamoyl group to the C-terminal thiolate of a second protein which is HypE (Figs. 6 and 7) (Reissmann *et al.*, 2003). The chemical nature of the intermediate formed during the carbamoylation reaction by the interaction of CP and ATP is still unknown. Two possibilities have been discussed, namely the formation of carbamoyl-adenylate as postulated by Reissmann *et al.* (2003) or, more probable, the formation of carbamoyl-ADP (NH_2–CO–O–PO_3–AMP), which

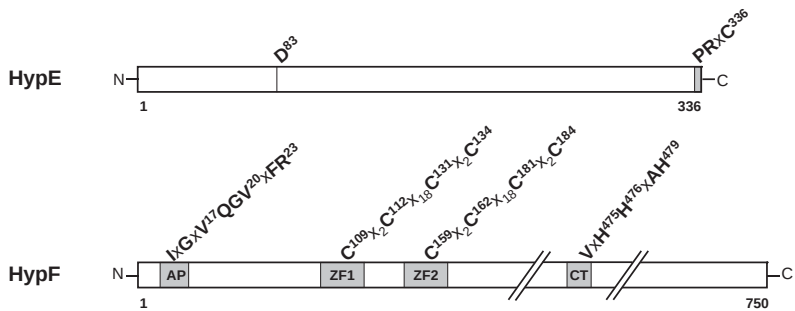

Figure 6 Schematic diagrams of the sequences of maturation proteins HypE and HypF from *E. coli*. The sequences of functionally important motifs discussed in the text are highlighted. Abbreviations in the HypF sequence: AP: acylphosphatase motif; ZN1 and ZN2: zinc finger motifs; and CT: carbamoyltransferase motif.

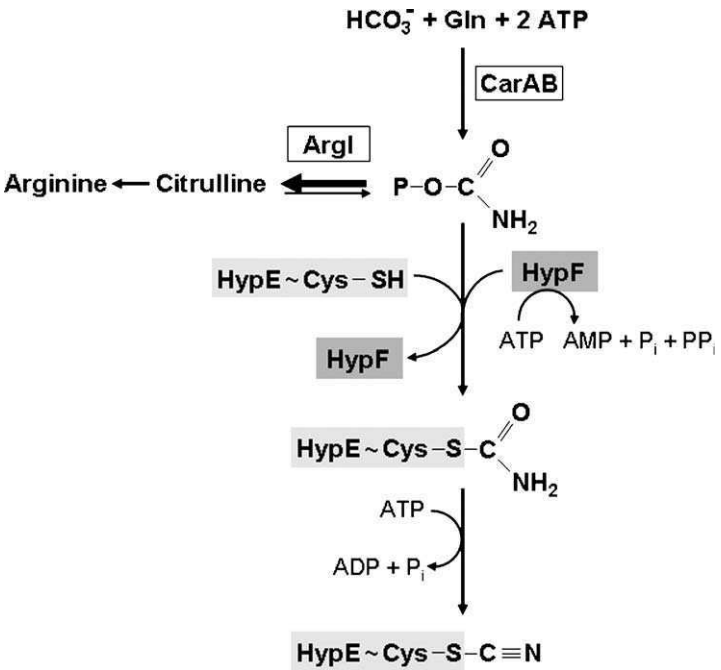

Figure 7 Biosynthesis of the CN ligand of the active site iron by maturation proteins HypF and HypE. The synthesis of carbamoylphosphate from bicarbonate and glutamine by carbamoylphosphate synthetase (CarAB) and from citrulline via the reaction of ornithine transcarbamoylase (ArgI) is indicated.

may release AMP and inorganic phosphate after transfer of the carbamoyl group to HypE. The hydrolytic reactions described above are assumed to constitute side reactions in the absence of the physiological carbamoyl acceptor HypE. This assumption has been proven by the replacement of the active site arginine (R23) of HypF (Rosano *et al.*, 2002) by chemically similar (R23Q) or dissimilar (R23E) residues (Fig. 6); the alterations lead to lower activity in the former and complete loss of activity in the latter case (Blokesch *et al.*, 2004b).

The role of CP as an educt for the synthesis of the cyanide ligands of [NiFe]-hydrogenases *in vivo* has been proven by the phenotype of *carAB* mutants (genes for CP synthetase), since they are defective in the generation of active hydrogenases in *E. coli* (Paschos *et al.*, 2001). The lesion could be suppressed by inclusion of citrulline as an alternative carbamoyl group donor into the medium. Citrulline is cleaved by ornithine carbamoyltransferase (OTCase) into CP and ornithine. The level of suppression by citrulline could

be augmented by overexpression of the gene for OTCase (*argI*) to overcome the unfavourable equilibrium of the OTCase reaction or by blockage of its conversion into arginine (Blokesch and Böck, 2002). It should be recalled again that about a decade before the chemical structure of the metal centre of [NiFe]-hydrogenases was resolved, Barrett *et al.* (1984) had reported that mutants of *Salmonella enterica* serovar typhimurium with a defective *pyrA* gene (previous designation for *carAB*) were devoid of hydrogenase activity. At that time, this was assigned to some regulatory influence of the CP synthetase protein itself or of one of its reaction products. In summary, both the phenotype of *carAB* strains and the chemical reaction catalysed by the HypF protein show that CP, in addition to serving as substrate for the biosynthesis of arginine and pyrimidines, also has a function in hydrogenase maturation.

Although the overall reaction catalysed by HypF is known, a number of issues are still open apart from the identity of the unknown reaction intermediate. In *R. eutropha* and some other organisms a truncated HypF variant has been detected, which lacks the acylphosphatase and both zinc finger domains. Intriguingly, this variant is functional in the synthesis of active hydrogenases, but only of the membrane-associated isoenzyme (Wolf *et al.*, 1998; Lenz *et al.*, 2005). It will be important to see whether this enzyme also accepts CP as a substrate *in vitro*. From the lack of the acylphosphatase domain one would conclude that it uses a different substrate. Moreover, the role of the zinc finger domains is unresolved. Replacements of several cysteine residues (C109A, C109A/C112A, C159A, C159A/C162A) alone or in combination yielded variants with properties indicating a structural role of these domains, possibly in the interaction with HypE, but this needs further proof. It is also open whether a metal is coordinated by the zinc fingers. Atomic-absorption spectroscopy of purified HypF indicated the presence of both iron and zinc as possible cofactors, but at greatly substoichiometric ratios (Paschos *et al.*, 2002).

During carbamoyltransfer the HypF protein has to interact with the substrate protein HypE. The formation of such a complex has been suggested by means of the yeast 2-hybrid system expressing genes coding for proteins from *H. pylori* (Rain *et al.*, 2001) and proven for the purified HypF and HypE proteins from *E. coli* during the course of the carbamoyl transfer reaction (Blokesch *et al.*, 2004b); its formation has also been demonstrated in *R. eutropha* (Jones *et al.*, 2004). HypE is a monomeric protein of molecular mass of 35.1 kDa sharing sequence similarity with the PurM protein (aminoimidazole ribonucleotide synthetase) and the SelD (selenophosphate synthetase) protein (Li *et al.*, 1999). PurM catalyses an ATP dependent dehydration of the substrate and possesses a novel ATP-binding domain,

whose characteristic residues are conserved in HypE (Li *et al.*, 1999). In addition to the presence of this ATP-binding site, the members of the HypE family contain the conserved tetrapeptide PR(I/V)C at their C-terminal ends (Fig. 6) (Reissmann *et al.*, 2003).

The partial reactions catalysed by HypF and HypE were delineated via mass spectrometric analysis of the *in vitro* reaction products (Reissmann *et al.*, 2003). First, the carbamoyl group is transferred by HypF from CP to the C-terminal cysteine residue (C336) of HypE resulting in HypE-thiocarboxamide. In a second reaction, which requires ATP the thiocarboxamide is dehydrated to the HypE-thiocyanate with release of ADP and inorganic phosphate (Fig. 7). By analogy to other ATP-dependent dehydration reactions, it is assumed that phosphorylation of the hydroxyl of the tautomeric form of the thiocarboxamide is followed by its removal upon subsequent dephosphorylation (Reissmann *et al.*, 2003).

The postulated pathway of CN synthesis by HypF and HypE and the mechanisms involved are supported by the following experimental results: purified HypE protein catalyses a low-intrinsic hydrolysis of ATP into ADP and phosphate (Reissmann *et al.*, 2003), which is absent in a variant containing the amino acid replacement D83N (Blokesch *et al.*, 2004b), a residue shown for the PurM protein to be essential for ATP binding (Li *et al.*, 1999). Whereas in the presence of ATP the thiocarboxamide is immediately processed to the thiocyanate, this does not take place with the D83N variant (Blokesch *et al.*, 2004b, Roseboom *et al.*, 2005). This variant, however, is still fully active as carbamoyl acceptor. Deletion or oxidation of the C-terminal cysteine C336 destroys the acceptor activity (Reissmann *et al.*, 2003; Blokesch *et al.*, 2004b).

3.3.1.2. CO Biosynthesis

Reactions in metal complex chemistry have been described in which metal carbamoyl complexes are converted into metal-cyano or metal-carbonyl complexes (see Paschos *et al.*, 2001). It was initially postulated, therefore, that CP may also be the biosynthetic precursor of the CO ligand. Possible routes could consist of the transfer of the carbamoyl group from HypE-thiocarboxamide to the metal on a further protein (see below), followed by deamination or, alternatively, in the initial coordination of the active site iron by three CN groups where one of it via ligand chemistry is hydrated and deaminated to the CO moiety. The latter mechanism would also explain the strictly maintained ratio of two cyanides to one carbon monoxide at the active site iron.

Recent experimental evidence seems to contradict these possibilities. The D83N variant of HypE, which cannot dehydrate the thiocarboxamide to the

thiocyanate does not transfer the carbamoyl group to the HypCxHypD complex (Roseboom *et al.*, 2005; see below), whereas the cyanyl group is readily transferred (Blokesch *et al.*, 2004c). Consistent with this result obtained for *E. coli*, $^{13}CO_2$ labelling experiments with *Allochromatium vinosum* indicated that, whereas CN is exclusively derived from CO_2, consistent with CP as the precursor, CO appears to be provided by a different educt. Because the C1 of acetate is incorporated to a significant extent into CO whereas the C2 carbon is not at all incorporated, the precursor could be related to acetate or a follow-up product of it (Roseboom *et al.*, 2005). In this connection, Rey *et al.*, (1996) reported that one of the hydrogenase maturation proteins (HypX) from *R. leguminosarum* shares sequence similarity with N^{10}-formyl-tetrahydrofolate dependent enzymes. In a second segment the protein displays sequence motifs characteristic of enoyl-coenzyme A hydratases/isomerases. HypX is also present in *R. eutropha* but not in *E. coli* (Buhrke and Friedrich, 1998). Although a role for HypX has been proposed for the attachment of additional ligands to the nickel of the metal centre (Bleijlevens *et al.*, 2004), THF would be also an attractive cofactor for the donation of C1-moieties.

In summary, the available information indicates that the biosynthesis of the CO and CN ligands in the maturation of [NiFe]-hydrogenases takes place via different paths. Translating this knowledge to the formation of active [FeFe]-hydrogenases, it suggests that the cyano ligand should be synthesized by a different route, since these organisms do not contain homologues for HypF and HypE. On the other hand, it cannot be excluded that CO is formed via identical pathways in both systems, which also would suggest that heterologous expression in *E. coli* of the operon coding for the structural and accessory proteins of [FeFe]-hydrogenases may use the CO biosynthesis capacity of this host. Further information at the biochemical level, however, is required in order to draw firm conclusions.

3.3.2. Possible Functions of Proteins HypC and HypD in Fe Coordination

Since CN is formed in a protein-bound state, the next question was how the CN group of the HypE-thiocyanate is transferred to the hydrogenase active site iron, and which accessory proteins are involved in this step. Experimental evidence was gained during a search for complexes between maturation proteins; it was found that protein HypC tightly interacts with pre-HycE (the precursor of the large subunit of hydrogenase 3 from *E. coli*) and also with the accessory protein HypD and that the intracellular amounts of these complexes are greatly elevated in the cells starved for CP as a consequence of a *carAB* mutation (Drapal and Böck, 1998; Blokesch and Böck, 2002). As the

HypCxHypD complex was resolved upon supplementation of the cells with citrulline, the complex was assumed to be an intermediate "downstream" in the maturation process relative to CN synthesis at protein HypE.

3.3.2.1. Properties of the HypC and HypD Proteins

HypC from *E. coli* contains 90 amino acid residues and possesses a molecular mass of 9.6 kDa; its size in other organisms ranges between 75 and 108 amino acids. A conspicuous property of all members of this family is the presence of the MCxxxP N-terminal sequence motif in which x represent apolar residues (Fig. 8). The N-terminal methionine in HypC from *E. coli* is removed; thus the mature protein contains a cysteine residue at the N-terminus, which has been shown to be of functional importance (Magalon and Böck, 2000a).

HypD from *E. coli* contains 373 amino acids and has a molecular mass of 41.4 kDa; its size in other organisms lies in the range between 347 and 385 amino acids. HypD from *E. coli* is monomeric and contains approximately 4 mol of Fe per mol of protein. Electron paramagnetic resonance and Mössbauer spectroscopy revealed the presence of a $[4Fe4S]^{2+}$ cluster that is reducible (Blokesch *et al.*, 2004c; Roseboom *et al.*, 2005). The invariant sequence motifs of protein HypD are highlighted in Fig. 8; there are a CxxHxH signature sequence at position 41–46 (motif I) that resembles a metal-binding motif, a thioredoxin-like motif (CPVC) between positions 69 and 72 (motif II) and a $C–X_{12}–C–X_6–C–X_{16}–C$ motif in the C-terminal segment of the protein. To assess the function of these conserved amino acid residues they were replaced mainly by alanine or serine. Exchanges of all conserved cysteines were detrimental for the function of HypD in hydrogenase maturation (Melanie Blokesch and August Böck, unpublished

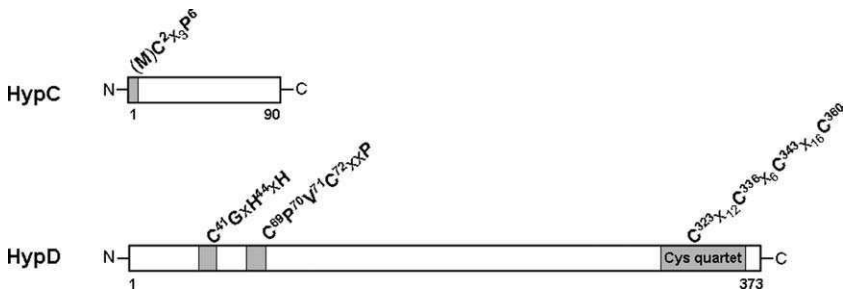

Figure 8 Schematic diagrams of the sequences of maturation proteins HypC and HypD from *E. coli*. The N-terminal methionine residue of HypC is posttranslationally removed. Functionally important motifs are highlighted and discussed in the text.

results). The C-terminal cysteine quartet emerged to be essential for the stability of the protein providing convincing evidence for an important structural role, like coordination of the [4Fe-4S] cluster (Melanie Blokesch and August Böck, unpublished results).

3.3.2.2. The HypCxHypD Complex is an Intermediate in Fe Coordination with the CN Ligand

The complex between HypC and HypD, which accumulates upon CP starvation in a *carAB* strain disappears when the cells are supplied with citrulline as an alternate source of CP (Blokesch and Böck, 2002); on the other hand, in the absence of an adequate supply of nickel, HypC can be detected in a complex with the precursor of the large subunit, pre-HycE. From the fact that the HypCxHypD complex is not resolved after citrulline supplementation in a mutant, which is devoid of the large subunit, but migrates to a different position in non-denaturing polyacrylamide gels, it was concluded that HypCxHypD receives the ligand(s) and transfers it to the pre-HycE polypeptide (Blokesch and Böck, 2002). Knowing that HypE-SCN is the source of the cyano ligand it was possible to transfer it to purified HypCxHypD complex *in vitro*, provided that the complex had been isolated from anaerobically grown cells (Blokesch *et al.*, 2004c). The requirement for docking of HypE-SCN to the HypCxHypD complex during the transfer reaction was then documented by the isolation of a ternary complex consisting of all three maturation proteins that accepts the carbamoyl-group from HypF and donates it to the HypCxHypD part of the complex (see scheme of Fig. 10).

The chemical bond between the HypCxHypD complex and the CN group has not been characterized yet; however, the following properties are relevant: (i) Upon mild denaturation the complex loses the radioactivity of CN indicating that it is no longer present as thiocyanate, (ii) transfer neither takes place to either free HypC or free HypD, nor to a mixture of them or to complex isolated from aerobically grown cells, (iii) incubation of the complex with ferricyanate, but not with ferrocyanate abolishes transfer activity irreversibly, and (iv) oxidation of the complex with 5,5'-dithiobis(2-nitrobenzoic-acid) (DTNB) reversibly inhibits transfer (Melanie Blokesch and August Böck, unpublished results). These facts can be integrated into the working model depicted in Fig. 10 (part A). Chemical model reactions have shown that the transfer of the CN moiety from a thiocyanate to an iron can follow either a nucleophilic or electrophilic mechanism (Reissmann *et al.*, 2003). In the model of Fig. 10, the transfer of an electrophilic CN to a nucleophilic iron is depicted. The model also assumes that an iron atom is coordinated by thiolates from HypC and HypD and that the [FeS]-centre of

HypD provides an electron for coordination of the CN. Since HypCxHypD is isolated from anaerobic cells, it is pre-reduced and can perform one such transfer cycle. To accept a second CN^+ moiety, both Fe centres (the postulated additional Fe and the FeS cluster) need to be reduced to regenerate the active state (not shown by the scheme). When substitution by three ligands is completed it is assumed that the oxidized form of HypD is displaced (Fig. 10, Part B) and after acquisition of Fe^{2+} and HypC, it is reduced to the initial form (Fig. 10, Part E).

Several issues, however, are unresolved: there is no proof that the CN^+ moiety actually is transferred to a metal of the HypCxHypD complex although the chemical properties of the complex and of the product after transfer support this notion. If this holds true, it needs to be determined whether this is an ''extra'' iron as depicted in the scheme or whether transfer occurs to a putative solvent-exposed iron of the [4Fe4S] cluster of maturation protein HypD. In the latter situation, full substitution of the iron with all three ligands and transfer of the Fe-L_3 moiety to the precursor of the large subunit (Fig. 10, Part B) would leave HypD with an [3Fe4S] cluster, similar to the iron cycling cluster of aconitase (Brown *et al.*, 2002). The acquisition of the next Fe^{2+} could follow the aconitase precedent. Although highly speculative, this model sets the stage for experimental analysis.

Transfer of the putative, fully coordinated active-site iron to the precursor of the large hydrogenase subunit is another open issue. This function must not necessarily involve a free state for the putative HypCxFeL_3 intermediate, but could consist in the displacement of the HypD interaction partner from the HypCxHypD–FeL_3 complex with the thiols of the large subunit apoprotein (Fig. 10, Part B). One possibility is that one or both of the two thiols of the N-terminal active site motif remove(s) HypD from the complex resulting in the HypC–pre-HycE complex, which predominantly can be detected in extracts from cells in which nickel insertion or proteolytic processing is blocked (Drapal and Böck, 1998; Magalon and Böck, 2000a). The stability of the complex in the presence of reducing agents and its sensitivity to alkylating agents support the chemical bonding suggested in Fig. 10 (Magalon and Böck, 2000a). Such a function of HypC is also supported by the fact that the N-terminal cysteine residue of HypC is important both for entering a stable complex with HypD and also with the precursor of the large subunit (Magalon and Böck, 2000a). Equally, replacement of the cysteine residue from the N-terminus of HybG also abolishes the capacity for entering a stable complex with HypD and the precursor of the large subunit of hydrogenase 2, HybC (Blokesch *et al.*, 2001), but leaves the capacity for interaction and therefore competition with the binding of the homologue HypC to HypD.

An apparent contradiction of the model is that the complex between HypC and the large subunit of hydrogenase 3 can be detected in all *hyp* mutants, including *hypD*, although the level in strains with lesions in nickel insertion or proteolytic processing is much higher (Drapal and Böck, 1998; Blokesch and Böck, 2002). It may suggest that HypC itself may be able to form a basal level of complex with the precursor of the large subunit, even in the absence of ligands or the HypD protein. It cannot be excluded either that it constitutes an unproductive form due to some oxidation of cysteine motifs involved in metal coordination. This would be in accordance with the finding that the amount of pre-HycE not caught in the interaction with HypC is much higher.

A number of organisms possess the genetic capacity to synthesise two HypC species, both of them essential. It has been postulated that one of them solely reacts with HypD and the other one with the precursor of the large subunit (Maroti *et al.*, 2003). This would be unlike the *E. coli* system, where HypC is dedicated only to the formation of hydrogenase 3 and HybG to the synthesis of isoenzymes 1 and 2 and both proteins interact with protein HypD. Moreover, organisms like *H. pylori* possess only one *hypC* gene (and only one hydrogenase), so its product has to undergo both interactions. To assess the functions of two HypC forms, as present for example in *Thiocapsa roseopersicina*, *Rh. leguminosarum* or *R. eutropha*, biochemical experiments are essential to scrutinize the interactions of these multiple HypC forms with HypD on one side and all large hydrogenase subunits formed by the organism on the other side.

3.3.3. Nickel Insertion

3.3.3.1. Nickel Transport

Nickel uptake precedes metalloenzyme synthesis and can be considered as the first maturation step since the rate of uptake needs to be adjusted to the rate of apoprotein synthesis, and must not exceed it in order to prevent toxicity. A functional nickel uptake system is required not only for acquisition from the environment, where it may be present in limiting concentrations (Ureta *et al.*, 2005), but also essential for controlling the fidelity of metal incorporation: thus, a *nikA* mutant of *E. coli* accumulates a precursor of the large subunit of hydrogenase 3 in the presence of zinc in the medium, which can no longer be proteolytically processed, indicating that zinc had been incorporated into the precursor (Magalon *et al.*, 2001). Since most of the information is available on nickel transport and on hydrogenase maturation for *E. coli*, we will focus the discussion mainly on this biological system. The *nikABCDE* operon is under control of two major signals acting

at the transcriptional level, namely oxygen, mediated by the FNR protein as an activator (Wu *et al*., 1989), and nickel concentration mediated by the NikR protein as a repressor of expression (de Pina *et al*., 1999). Both proteins bind to the promoter region upstream of *nikA* and affect expression in opposite ways. Under anaerobiosis, the condition under which hydrogenases are synthesised in *E. coli* (for review see Sawers, 1994; Sawers *et al*., 2004), plus adequate nickel supply, FNR upregulates transcription of the *nik* operon. In the presence of high nickel concentrations, repression by NikR overrides activation by FNR and reduces expression. However, the situation is complicated by the extremely high affinity of NikR for nickel which lies in the pM range equivalent to less than a single molecule per cell. Moreover, depending on the concentration of nickel, NikR forms two different NikR–promoter–DNA complexes (Chivers and Sauer, 2000; Chivers and Sauer, 2002). These data imply, first, that the hydrogenase maturation system would not be able to scavenge sufficient metal at such low concentrations and, second that some maturation component, which sequesters nickel for maturation (such as a nickel chaperone) has to compete with NikR for nickel. This dilemma is addressed in recent work by Rowe *et al*. (2005). These authors provide information that NikR functions under a broad range of nickel concentrations and that its activity as a repressor is also controlled by some as yet unidentified component of the hydrogenase maturation machinery. Modulation of NikR function in this way adjusts the expression level of the *nik* operon to the metabolic need, namely incorporation into hydrogenases. A similar coordination between uptake and metal enzyme synthesis has been observed in the synthesis and maturation of urease by *H. pylori* (Ernst *et al*., 2005). NikR of this organism, an ortholog of NikR from *E. coli*, represses the expression of *nixA* (a homologue of *hoxN*) in a nickel-dependent manner, whereas it activates the expression of the *ureA* structural gene. The differential effect is mediated by the position of the binding site on the DNA. Thus, depending on the different numbers of nickel-dependent enzymes synthesised by an organism, different regimes may be used for coordination of metal influx and incorporation.

3.3.3.2. Functions of Proteins HypA, HypB and SlyD in Nickel Insertion

The existence of (a) protein(s) with a function in nickel–incorporation into hydrogenases was indicated initially by the phenotype of the *hypB* mutant from *E. coli*, whose deficiency in the formation of active enzymes could be rescued by fortifying the medium with high nickel concentrations (Waugh and Boxer, 1986; Jacobi *et al*., 1992). A similar phenotype was demonstrated for strains with a lesion in the *hypA* gene, first for urease and hydrogenase synthesis by *H. pylori* (Olson *et al*., 2001; Mehta *et al*., 2003a) and subsequently

for the synthesis of hydrogenase 3 from *E. coli* (Hube *et al.*, 2002; Blokesch *et al.*, 2004a). The formation of hydrogenases 1 and 2 from *E. coli* was unaffected by *hypA* mutations; the reason is that the *hybF* gene, which is closely related in sequence to *hypA* (Menon *et al.*, 1994) from the operon coding for the components responsible for the generation of active hydrogenase 2, fulfils the same function as *hypA* during the maturation of hydrogenases 1 and 2 (Hube *et al.*, 2002). The hydrogenase deficiency of *hypA hypB* double mutants and *hypA hypB hybF* triple mutants also could be phenotypically suppressed by high nickel concentrations. The concentration range of the metal active in *in vivo* or *in vitro* complementation was identical for the single *hypA*, *hypB* and *hybF* mutants and did not differ from those observed for the *hypA hypB* and *hybF hypB* double and *hypA hypB hybF* triple mutants, which provided circumstantial evidence that the same biochemical process is affected and that HypB cooperates with HypA in the formation of hydrogenase 3 and with HybF in the generation of isoenzymes 1 and 2 (Blokesch *et al.*, 2004a).

HypB. The HypB protein has been purified from *E. coli* (Maier *et al.*, 1993), *Rh. leguminosarum* (Rey *et al.*, 1994), *B. japonicum* (Fu *et al.*, 1995) and *H. pylori* (Mehta *et al.*, 2003b). HypB from *E. coli* is a homodimer made up of 31.6 kDa monomers and it does not contain any cofactor detectable by absorption in the UV–Vis light range. As suggested by the existence of guanine nucleotide-binding motifs in the sequence (Fig. 9), purified *E. coli* HypB protein binds GDP and GTP and possesses a low intrinsic GTPase activity ($0.17\,min^{-1}$) with an apparent affinity for GTP of 4 μM (Maier *et al.*, 1993). Similar properties were also reported for HypB from

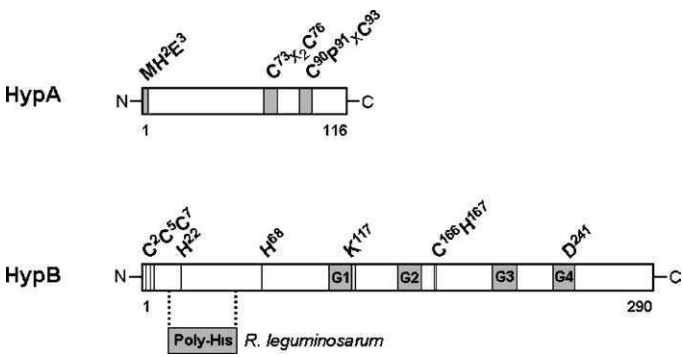

Figure 9 Schematic diagrams of the maturation proteins HypA and HypB. Functionally important sequence motifs are highlighted and discussed in the text. G1, G2, G3 and G4 denote the motifs conserved in GTPases. Poly-his gives the position of the poly-histidine segment present in the HypB species from *Rh. leguminosarum* or *B. japonicum*. This segment is not present in the HypB protein from *E. coli*.

B. japonicum and *H. pylori* (Fu *et al*., 1995; Mehta *et al*., 2003b), whereas no GTPase activity could be detected *in vitro* for the purified HypB protein from *Rh. leguminosarum* (Rey *et al*., 1994) The exchange of amino acid residues (K117, D241) known to interact with the substrate (Maier *et al*., 1995; Mehta *et al*., 2003) or to determine the substrate specificity (Maier *et al*., 1995) (Fig. 9) demonstrated that there is an absolute requirement for GTP hydrolysis in order that HypB functions in hydrogenase maturation. Intriguingly, NTPases similar to HypB are also required for assembly of other nickel-containing metal centres like those of urease and carbon monoxide dehydrogenase (Lee *et al*., 1992; Kerby *et al*., 1997; see also above).

The phenotypic suppression of *hypB* mutations by high nickel concentrations prompted studies by several groups on the nickel-binding properties of the protein. Strong binding of Ni^{2+} was demonstrated for the enzymes from *Rh. leguminosarum* (4 Ni per monomer) (Rey *et al*., 1994) and *B. japonicum* (9 Ni per monomer) (Fu *et al*., 1995; Olson and Maier, 2000), which even facilitated isolation of the protein by nickel chelate affinity chromatography. Nickel binding by these HypB species was correlated with the existence of an N-terminally located segment of the protein enriched with histidine residues (Fig. 9). Deletion of 23 of the 24 histidines from this region of the protein from *B. japonicum* abolished nickel sequestering, but the resulting protein retained hydrogenase maturation activity. Only 1 Ni^{2+} was still bound by the mutant protein. It was concluded that HypB from these organisms has a dual function, namely nickel storage mediated by the polyhistidine stretch and GTP hydrolysis for maturation (Olson and Maier, 2000). It needs to be resolved whether this remaining capacity of the mutant variant to bind the metal is connected with some nickel-donor function of the protein and, if so, by which residues of the protein it is coordinated.

HypB from *E. coli* is an exception, since apart from histidine residues in the positions 22 and 68, it lacks the polyhistidine stretch. Replacement of H22 or H68 by alanine residues did not affect the maturation activity of the protein, supporting the notion that this sequence stretch of the protein has no function in nickel insertion into hydrogenases (Christina Oertli, Gabriele Morlock and August Böck, unpublished results).

It was reported initially that purified HypB from *E. coli* grown in a medium fortified with nickel did not contain statistically significant amounts of the metal (Maier *et al*., 1993), but a reinvestigation by Leach *et al*. (2005) revealed the presence of the metal and indicated the existence of two Ni-binding sites at the purified protein. The analysis of an N-terminally truncated form of HypB and the replacement of the cysteine residues in positions 2, 5 and 7 of the protein (Fig. 9) allowed the correlation of the high-affinity-binding site with the presence of these cysteines residues. High-affinity

binding was specific for nickel over zinc. In contrast, low-affinity nickel binding was not specific for nickel. It was present in the N-terminally truncated protein and amino acid replacements allocated it to the C166H167 doublet, which is conserved only in the HypB family of GTPases and the invariant C198 residue within the GTPase domain of HypB (Leach *et al.*, 2005).

Amino acid exchanges were introduced to probe the physiological relevance of putative metal coordination sites of HypB from *E. coli*. The replacement of the three Cys residues located close to the N-terminus (see Fig. 9) singly or in several combinations only quantitatively reduced maturation activity *in vivo*; concomitantly, the cellular amounts of the mutant gene products were reduced (Christina Oertli, Gabriele Morlock and August Böck, unpublished results). The conclusion that the N-terminal Cys motif may contribute to, but is not essential for nickel insertion *in vivo* is also supported by the fact that the HypB species from archaea and from bacteria like *H. pylori* do not contain this cysteine-rich motif (Robson, 2001b).

In contrast, replacement of C166 by alanine was detrimental for maturation whereas exchange by serine allowed some residual activity to develop. Replacement of H167 by alanine, glycine or glutamine led to a blockade of maturation; exchange by cysteine only reduced the activity quantitatively. Intriguingly, alteration of the C166 H167 doublet into H166 C167 also allowed the development of a reduced level of activity (Sabine Rode and August Böck, unpublished results). The C166A, H167A and C198A and C166S variants of HypB displayed GTPase activities equal or close to that of the wild-type protein demonstrating that the effect on the maturation activity of these variants is not a consequence of an interference with GTP hydrolysis (Leach *et al.*, 2005; Sabine Rode and August Böck, unpublished results). It appears, therefore, that another essential function of the protein, which could consist of metal binding and/or delivery, is affected.

HypA. The phenotype of *hypA* mutants described above indicates a function of the gene product in nickel insertion into hydrogenases and also ureases. Since HypA from *E. coli* is difficult to purify, most of the biochemical studies have been conducted initially with HybF from this organism, which is a homologue of HypA (Menon *et al.*, 1994; Hube *et al.*, 2002; Blokesch *et al.*, 2004a). Detailed investigations have also been performed with the readily available HypA protein from *H. pylori* (Mehta *et al.*, 2003a). HypA from this organism is a homodimer whereas HybF has been isolated as a monomer (Blokesch *et al.*, 2004a). Both proteins bind nickel in a 1:1 ratio (per monomer) and with a half-saturation concentration in the low-micromolar range. HybF from *E. coli* was shown also to contain stoichiometric amounts of zinc (Blokesch *et al.*, 2004a). Amino acid

replacement studies of HypA from *H. pylori* pointed to the involvement of the conserved histidine-2 residue in the binding of nickel (Mehta *et al.*, 2003a), a result confirmed for the purified HybF protein from *E. coli* (Blokesch *et al.*, 2004a). A second residue putatively involved in nickel binding is glutamate-3 since a replacement by the chemically similar glutamine yielded a variant still functional in nickel binding and hydrogenase maturation, but the exchange by a leucine residue was detrimental. Evidence was gathered by amino acid exchanges that zinc is coordinated by a conserved quartet of cysteine residues; zinc and nickel binding appeared to be independent processes (Blokesch *et al.*, 2004a).

Recently, the purification of HypA from *E. coli* was reported; the characterization of the protein revealed properties identical to those described for HybF from *E. coli* supporting the notion that HypA and HybF are true homologues (Atanassova and Zamble, 2005). In addition, these authors presented evidence for the existence of a homodimeric state of HypA although the major amount of the purified preparation still migrated in the form of a monomer. Indications that HypA interacts with HypB were also given, thus raising the possibility that, like in *H. pylori*, a heteromeric complex made up of two HypB and two HypA (or HybF) polypeptides might constitute the assembly active in nickel insertion also *in E. coli*.

SlyD. During a search for polypeptides from *E. coli* interacting with HypB, the SlyD protein, which is a metal-containing proline *cis–trans* isomerase (Hottenrott *et al.*, 1997), was identified as interaction partner (Zhang *et al.*, 2005). The mutational inactivation of SlyD impaired hydrogenase activity and the decrease could be rescued by the inclusion of high nickel concentrations in the medium. Whereas *slyD* mutants possessed lower nickel concentrations in the cytoplasm, overproduction of the protein increased the content about two-fold. Thus, although there is no absolute requirement for the activity of SlyD, it appears to fulfil an important role in the sequestering of nickel or the optimization of the maturation process via an as yet unknown mechanism.

3.3.3.3. On a Possible Mechanism for Nickel Incorporation

The functions of HypA, HypB and SlyD in the nickel incorporation steps are dispensable since they may be compensated by providing increased concentrations of the metal both *in vivo* and *in vitro*. However, *in vivo* close to toxic concentrations of nickel are required to achieve complementation and the yield of active enzyme generated is much lower than that obtained in the presence of these accessory proteins (Jacobi *et al.*, 1992). Consequently, their function may reside in the stimulation of the rate and/or the direction of incorporation. It is clear that the present information is too scarce and

also controversial to allow the postulation of a detailed mechanism. For example, all three proteins, HypA, HypB and SlyD have now been reported to bind nickel and the physiological relevance of this binding for the maturation process has not been clarified yet. Also, removal of the high-affinity nickel-binding site from *E. coli* HypB does not abolish its maturation activity *in vivo* and the low-affinity site is not selective for nickel. There is some genetic evidence, however, that the HypA protein might be the nickel donor itself or guide the donor to the cognate large hydrogenase subunit. In the *E. coli* system HypA is required for maturation of hydrogenase 3, whereas its homologue HybF provides the same function in the maturation of both hydrogenase 1 and 2. The sequences of the large subunits of isoenzymes 1 and 2 are very similar but, apart from the active site motifs, not closely related to that of isoenzyme 3 (Böhm *et al.*, 1990; Menon *et al.*, 1994). This may have necessitated the development of two different metal donors for the interaction with the target protein.

The necessity for GTP hydrolysis as a prerequisite for nickel insertion is easier to visualize. According to the general biological role as switch proteins (for review, see Vetter and Wittinghofer, 2001), GTP hydrolysis and the connected conformational switch may be required for the release of nickel from the donor protein that may be either HypB or HypA itself or a common complex; alternatively, GTP hydrolysis may be involved in the resolution of the donor protein from the target once the metal has been transferred. The recent demonstration that a complex between HypB and the large subunit of the *R. eutropha* hydrogenase can be detected may indicate that the whole insertion process takes place within a supramolecular complex between HypA, HypB and the target protein (Winter *et al.*, 2005). An interesting example for such a docking-release role of GTP binding or hydrolysis in protein–protein interaction has recently been reported for the TorD protein, which is the chaperone controlling the coordination of the assembly and the TAT-mediated export of the trimethylamine-N-oxide reductase from *E. coli* (Hatzixanthis *et al.*, 2005).

3.3.4. Proteolytic Cleavage and Closure of the [NiFe] Centre

Incorporation of the metals takes place into the precursor of the large subunit that differs from the mature protein by the presence of a C-terminal extension (Gollin *et al.*, 1992; Volbeda *et al.*, 1995). The removal of this extension, which varies both in sequence and in length among the different hydrogenases, constitutes the last step in the maturation of the large subunit of [NiFe]-hydrogenases. The cleavage site is located three amino acids C-terminal to the cysteine-4 residue of the C-terminal motif DPCxxCxxH/R

coordinating the active site metal cluster (see Fig. 2C) (Gollin *et al*., 1992; Sorgenfrei *et al*., 1993b; Menon *et al*., 1993; Rossmann *et al*., 1994). The gene responsible for synthesis of the endopeptidase that catalyses processing of the precursor of the large subunit of hydrogenase 3 from *E. coli* has been identified as *hycI*, the promoter-distal gene of the *hyc* operon (Böhm *et al*., 1990; Rossmann *et al*., 1995). By sequence comparison, other members of the maturation endopeptidase family have been identified. The products, ranging in size between 130 and 209 amino acid residues, are highly specific for their substrates, which explains their co-expression with the structural genes. Thus, *E. coli* has the capacity for the formation of three of these enzymes, cleaving the precursors of the large subunit from hydrogenase 1 (HyaD), hydrogenase 2 (HybD) and hydrogenase 3 (HycI) (Rossmann *et al*., 1995). Similarly, *R. eutropha* uses two endopeptidases, HoxM and HoxW, to proteolytically mature the precursors of the large subunits of the membrane-bound and soluble hydrogenases (Thiemermann *et al*., 1996; Massanz *et al*., 1997).

The endopeptidases HybD and HycI have been purified and the crystal structure of the monomeric HybD protein (17.5 kDa) could be solved (Fritsche *et al*., 1999). HybD possesses an α/β structure consisting of a twisted five-stranded β-sheet surrounded on one side by three, and on the other side by two, helices. The structure contains a cadmium ion from the crystallization buffer that is positioned in a cleft of the monomeric protein and is penta-coordinated by the oxygens of a glutamyl (E16) and aspartyl (D62) residue, the imidazole nitrogen of a histidyl (H93) side chain and by a water molecule. Although the purified HybD and HycI proteins do not contain a metal (Rossmann *et al*., 1995; Fritsche *et al*., 1999), they cleave their substrates only when nickel has been incorporated. From this and the fact that standard protease inhibitors do not block the activity (Menon and Robson, 1994; Theodoratou *et al*., 2000a), it was concluded that the enzyme recognises nickel as a binding motif of the large subunit precursor and that the cadmium site mirrors the nickel-binding site (Theodoratou *et al*., 2000a).

Maturation endopeptidases cleave their substrates at a remarkably conserved position, between a basic residue (H or R) and a nonpolar one (M, I, V or A). Extensive amino acid replacement studies revealed, however, that this is not a strict requirement. Most of the exchanges are tolerated without blocking proteolysis. This allows the conclusion that the nickel in the large subunit precursor constitutes a major-binding motif and determines the cleavage site in a regiospecific manner (Theodoratou *et al*., 2000a,b; Theodoratou *et al*., 2005). Moreover, almost two-thirds of the C-terminal extension can be removed without affecting cleavage and subunit maturation. Truncations beyond this critical size strongly reduce precursor stability.

A conclusion drawn from these observations is that the extension may serve as an intramolecular chaperone keeping the protein in a "ready" conformation for metal addition (Theodoratou *et al.*, 2005). Such a function is also consistent with the fact that the extension must not be covalently linked to the large subunit, but can be part of a separate polypeptide (Sorgenfrei *et al.*, 1993b).

In a search for the mechanism of catalysis, amino acid exchanges were introduced into all conserved positions of the HycI endopeptidase. Only residues involved in the coordination of the cadmium (corresponding to D16, D62, H90 in HycI) were found to be essential for activity. Replacement of D62, besides blocking activity, leads to the accumulation of a tight complex between the endopeptidase and its substrate, the precursor of the large hydrogenase subunit HycE (Theodoratou *et al.*, 2005), which suggests that D62 actually does not constitute a nickel-binding ligand but, rather, may serve as the acidic residue required for activating the water molecule involved in peptide-bond hydrolysis. Therefore, D62 may have been forced into the binding site in the crystal structure because the nickel-containing substrate was absent. Nickel coordination may thus involve either residue H90 *and* D16 or *only* H90 (HycI nomenclature). In the former case (metal-based catalysis), nickel would polarize the carbonyl oxygen bond and D62 would constitute the acidic residue required for activating the water molecule involved in hydrolysis. In the latter case (acid catalysis), D16 would function as the second acidic residue required for the positioning and polarization of the R-carbonyl oxygen bond (Theodoratou *et al.*, 2005).

Pre-HycE, the substrate of the HycI endopeptidase, accumulates in *hycI* mutants in two different forms, first as a defined band also containing HypC and second as a "cloud" of much slower migrating conformers (Magalon and Böck, 2000b). Both forms contain nickel but only the HypC-free form is a substrate for the endopeptidase. Cleavage results in a dramatic shift of the electrophoretic migration position in the gel into that of the mature large subunit and the diffuse distribution of the conformers is condensed into a precise and sharp migration band. This is compelling evidence that the removal of the short peptide from the C-terminus triggers a conformational switch that folds the protein into the defined form of the mature subunit. Equally relevant, change of the conformation is also thought to "close" the metal centre and to internalize it (Fig. 10, Parts C and D). This closure of the centre most plausibly involves the movement of the entire C-terminus containing the nickel coordinated by cysteine-3 and the thiolate of cysteine-4 that is near the newly generated C-terminus into the incomplete centre, thereby bridging the Fe and Ni atoms (Fig. 10, Part D). The evidence is that the replacement of cysteine-4 by another residue still allows Ni insertion and

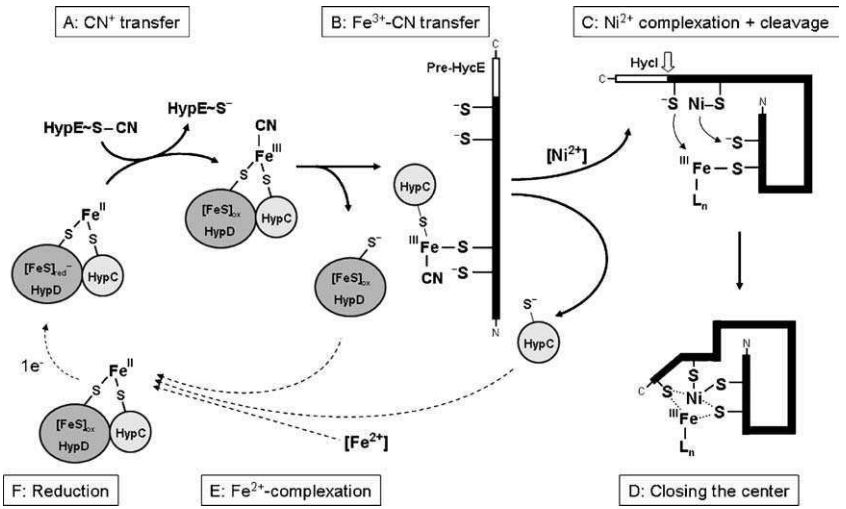

Figure 10 Working model for the transfer of the CN group from HypE-thiocyanate to the postulated iron from of the HypCxHypD complex and its insertion. [FeS] denotes the [4Fe4S] cluster present in the HypD protein, [Ni^{2+}] indicates the donor for nickel and [Fe^{2+}] the donor for iron. Pre-HycE: precursor of the large subunit of hydrogenase 3 from *E. coli*. HycI: maturation endopeptidase for pre-HycE. The cartoon shows the virtual transfer route of only one CN ligand to the HypCxHypD complex and from there to pre-HycE. The ligand-transfer cycle has to be repeated three times. The reduction of the HypCxHypD complex after the first two cycles to the ground state requires the input of two electrons and is not shown.

endoproteolytic cleavage, but results in an inactive enzyme probably because of the lack of the second bridging ligand (Massanz and Friedrich, 1999; Magalon and Böck, 2000a,b; Theodoratou *et al*., 2005). When cysteines 1, 2 and 3 are exchanged, neither nickel insertion nor cleavage can take place.

3.3.5. Maturation of the Small Subunit

The small subunits of most [NiFe]-hydrogenases possess three [FeS] clusters which lead the electrons to the [NiFe] site from an external electron donor or vice versa. Surprisingly, its maturation has not received particular attention yet. Recent reports now highlight that at least four gene products encoded within the *hup* (*hupGHIJ*) and *hox* gene (*hoxOQRT*) clusters downstream of the hydrogenase structural genes from *Rh. leguminosarum* and *R. eutropha*, respectively, are required for appropriate maturation. In-frame deletions

introduced into each of the genes from *Rh. leguminosarum* differentially affected the processing of the small subunit without interfering with maturation of the large subunit. The effect was more pronounced in free-living bacteria cultivated under microaerobic conditions than in bacteroids (Manyani *et al.*, 2005). At least one of the proteins (HupH) was found to enter complex formation with the precursor of the small subunit. Similarly, HoxO and HoxQ from *R. eutropha*, which are the homologues of HupG and HupH, undergo interaction with the small subunit of the membrane-bound hydrogenase from *R. eutropha* (T. Schubert, M. Bernhard, O. Lenz and B. Friedrich, Abstracts 7th Int. Hydrogenase Conf. abstr. pp. 3–5, 2004). On the basis of the observation that mutations in these genes result in a severe reduction of the cellular level of the small subunit, Manyani *et al.*, (2005) speculate on a possible role in the incorporation of [FeS]-clusters. In addition, involvement of one or more of these proteins in the coordinated assembly and export of the small subunit via the TAT system should be considered in view of the fact that HupG, HupH and HupJ display sequence similarity with HyaE, HyaF and HybE from *E. coli* and that HyaE and HybE have a function in the TAT-mediated export of hydrogenases 1 and 2 of this organism (see below). No information exists on the involvement of the housekeeping [FeS] cluster assembly and insertion system in small subunit maturation (Frazzon and Dean, 2003)

3.3.6. Events after Maturation of the Subunits

The maturation of the subunits both of cytoplasmically and periplasmically located hydrogenases takes place in the cytoplasm, which correlates with the exclusive cytoplasmic location of all accessory proteins and with the requirements for metabolites like CP, ATP and GTP. The maturation paths of the two subunits are not interdependent as visualized by the observation that prevention of the maturation of the large subunit by a *hypB* mutation still permits the formation of the [FeS] cluster-containing form of the small subunit of the hydrogenase 2 from *E. coli* albeit in a soluble state within the cytoplasm (Sargent *et al.*, 1998). Further evidence comes from the observations that deletion of the structural gene from one of the subunits does not interfere with the cytoplasmic maturation of the counterpart (Magalon and Böck, 2000b; Jack *et al.*, 2004), and that interference with the maturation of the small subunit allows the formation of a matured large subunit (Manyani *et al.*, 2005). A coordination mechanism, however, must exist to ensure that the heterodimeric structure reaches the correct cellular target. For example, hydrogenase 3 from *E. coli* is loosely attached to the inside of the cytoplasmic membrane and no attachment of either subunit takes place when the

other one is missing (Magalon and Böck, 2000b), thus supporting the observations by Sargent *et al.* (1998). A protein involved in this control may be HycH, the product of the penultimate gene of the *hyc* operon. HycH stays bound to all intermediates of pre-HycE maturation and leaves it only when mature small subunits are present (Ekaterini Theodoratou and August Böck, unpublished results).

Hydrogenases of the majority of organisms, on the other hand, are membrane-bound facing the periplasmic side of the membrane and they need to be exported accordingly. The export is mediated by the TAT system (Rodrigue *et al.*, 1999; Sargent *et al.*, 1999; Bernhard *et al.*, 2000; Meloni *et al.*, 2003), whose function lies in the export of cofactor-containing, predominantly redox-active enzymes (for review see Palmer and Berks, 2003). The isoenzymes 1 and 2 from *E. coli*, encoded by the *hya* and *hyb* operons, belong to this category. Either operon contains a gene, *hyaE* and *hybE*, respectively, whose product coordinates the assembly of the heterodimeric active enzyme with its export. For HybE it has been shown that it does so by interacting both with the signal peptide of the small subunit precursor and with the large subunit (Dubini and Sargent, 2003). Deletion of *hybE* leads to correct targeting of the small subunit whereas the large one remains in the cytoplasm.

3.3.7. An Integrated Model for the [NiFe]-Hydrogenase Maturation Process

Although important pieces of information on the generation of active [NiFe]-hydrogenases are still lacking, it is attractive to derive a sequential model of the process (see Fig. 10). It is based predominantly on the information gained from genetic and biochemical studies with the *E. coli* system, in particular, the maturation of hydrogenase 3. It must be stressed, however, that the formation of this isoenzyme may involve specific features since it is synthesised only under anoxic growth conditions and it is one of the few hydrogenases to be located at the inside of the cytoplasmic membrane. Deviations in the maturation process followed by other organisms can be expected.

There is convincing evidence that the synthesis of the CO and CN ligands followed by Fe coordination and insertion into the precursor of the large subunit of the hydrogenase 3 from *E. coli*, whether nascent or completely synthesised, constitutes the first event in the maturation process. Similar conclusions were drawn by Winter *et al.* (2005) for the maturation of the regulatory hydrogenase from *R. eutropha*. The evidence gained from the *E. coli* system is that mutants with lesions in the steps of ligand synthesis and Fe coordination (*hypC*, *hypD*, *hypE*, *hypF*) possess a large subunit precursor that does not contain nickel and cannot be cleaved proteolytically. The same conclusion holds for the precursor of the hydrogenase 3 large subunit from a

carAB strain starved of citrulline (Blokesch and Böck, 2002). The precursors present in cells with a mutation in either the *hypA* or *hypB* genes, however, are amenable to endoproteolysis by the maturation endopeptidase when nickel is added (Maier and Böck, 1996a). Strains with a defective gene for the endopeptidase, in contrast, do contain a precursor with tightly bound nickel and it can be matured *in vitro* by purified endopeptidase (Maier and Böck, 1996a; Theodoratou *et al.*, 2000a). Conversely, there is no nickel incorporated into the large subunit of hydrogenase 3 from *E. coli* and *R. eutropha* from which the C-terminal extension has been deleted genetically (Binder *et al.*, 1996; Massanz *et al.*, 1997).

Further supportive evidence for the model of Fig. 10 comes from the analysis of complexes accumulating in the cells under certain physiological regimes or from interactions of maturation components studied *in vitro*. The interaction between HypF and HypE, also during the reaction cycle, has been documented now (Rain *et al.*, 2001; Blokesch *et al.*, 2004b; Jones *et al.*, 2004) as well as complex formation between HypC, HypD and HypE (Blokesch *et al.*, 2004c; Jones *et al.*, 2004). The existence of a supramolecular complex between these four proteins as a kinetic intermediate during the formation of the CN ligand and its transfer to iron can be taken for granted, therefore (Blokesch *et al.*, 2004c). Furthermore, HypC interacts tightly with the precursors of the large subunit of hydrogenase 3 (Drapal and Böck, 1998), whereas the homologue of HypC, HybG, undergoes complex formation with pre-HyaB and pre-HybC, the precursors of the large subunits of hydrogenases 1 and 2, respectively (Blokesch *et al.*, 2001; Butland *et al.*, 2006).

The two proteins with a function in nickel insertion (HypA/HybF and HypB), have been demonstrated to form a complex *in vitro* (Mehta *et al.*, 2003a; Atanassova and Zamble, 2005) and the interaction of HypB, with the large subunit of the sensory hydrogenase from *R. eutropha* has been demonstrated as well (Winter *et al.*, 2005). The final interaction in the maturation cycle then involves the complex of the endopeptidase with its substrate, the precursor of the large subunit, as demonstrated both *in vitro* and *in vivo* (Magalon *et al.*, 2001; Theodoratou *et al.*, 2005). Thus, the phenotypes of the individual *hyp* mutants as well as the biochemical interactions at the level of the gene products support the sequence of the maturation events of the working model (Fig. 10).

In vitro maturation systems have been developed already for the nickel insertion step of the assembly line (Menon and Robson, 1994; Maier and Böck 1996a). Nickel incorporation could be assed *in vitro* via proteolytic processing of the large subunit which requires that the metal has been inserted before cleavage. In such maturation setups active enzyme could be obtained with a yield of 10–15% (Maier and Böck, 1996a). However, the

process was independent from the proteins involved in the *in vivo* system; thus their function could be by-passed not only *in vivo* (Waugh and Boxer, 1986, Olson *et al*., 2001; Mehta *et al*., 2003a, Blokesch *et al*., 2004a) but also chemically *in vitro*. A highly speculative argument for the failure is that the whole maturation process involving the function of all proteins occurs at a supramolecular complex together with the large subunit precursor. This would explain early results that hydrogenase apoprotein formed in the absence of iron and nickel cannot be recycled into active enzyme *in vivo* when the metals are provided later on (Zinoni *et al*., 1984).

3.4. Phylogenetic Considerations

3.4.1. Rationales for the Requirement of Auxiliary Proteins in Hydrogenase Maturation

Organometallic chemistry has shown that the synthesis of both the CO and the CN ligands of metal cyano and metal carbonyl complexes can be derived from metal carbamoyl complexes as educts, albeit at somewhat extreme conditions (for summary see Paschos *et al*., 2001). It was therefore unexpected that the synthesis of the CN ligand of the active site iron does not follow the established chemical way but, rather, uses sulphur chemistry at a macromolecular adaptor. In the search for a rationale, it is attractive to relate this mode of biosynthesis to the evolution of the active site metal centre that in all types of hydrogenases may have originated from more simple [FeS]-clusters (Rees and Howard, 2003). Formation of such clusters and their coordination with the ligands that were abundant in the archaic sulphide-containing biosphere may have resulted in catalysts with low activities. The subsequent change in the chemical environment then provided the selective pressure for their step-wise development into the present-day highly efficient, but also much more complex, structures. Synthesis of the CN ligand (and it is plausible to predict adaptor-based formation to be the case also for the CO ligand) at the HypE protein afforded the advantage that this highly reactive moiety cannot confer toxicity. More important, however, may have been the constraint that the coordination of the active-site iron must occur stoichiometrically, in the classical case of [NiFe] enzymes with CN to CO in the ratio of 2 to 1 at the iron and without coordination of the nickel. Such specific ligand transfer can only be accomplished with the stereospecific property of a protein.

The [NiFe] cluster of hydrogenases represents one of the few active sites in which two different metals are directly bonded. Since the centre is coordinated

by identical chemical groups, namely thiolates, the complex insertion process may also reflect a necessity for separate insertion of each metal to ensure the fidelity of incorporation. Along this line of argument, the precursor of the large subunit does not accept nickel when CN ligand synthesis is blocked (Jacobi *et al.*, 1992; Maier *et al.*, 1996), so the nickel-binding domain is not yet structured appropriately. It must be assumed therefore that the coordination of the protein with the $Fe(CO)(CN)_2$ moiety precedes either the formation of the nickel-binding domain during protein synthesis or, after accepting the coordinated iron, induces a conformational change of the precursor of the large subunit to shape the nickel-binding domain.

Once the two metals together with the ligands have been incorporated, a mechanism must achieve the internalization of the metal centre. Proteolytic cleavage of proteins has long been known as a general principle to induce gross conformational changes into proteins. The function of the maturation endopeptidase, however, is unique, resembling in some way only the incorporation of manganese into the metalloproteins of the photosynthesis system (Merchant and Dreyfuss, 1998). First, cleavage is dependent on the presence of nickel in the precursor, so the enzyme also controls the fidelity of metal insertion (Magalon *et al.*, 2001). Second, a drastic conformational switch is induced causing the new C-terminus to move into the $Fe(CO)(CN)_2$ domain of the large subunit with the thiolate providing a ligand bridging the Fe and the Ni and thereby "closing" the centre. Thus, the complex maturation process required for the generation of active [NiFe]-hydrogenases can be seen as the consequence of a co-evolution between the development of metal clusters possessing higher catalytic rates and of the structural protein providing the suitable chemical environment for stability and function of the cluster.

3.4.2. Conservation of the Maturation System

As pointed out earlier, the sequences of the six core *hyp* system genes in general are highly conserved, from archaea to bacteria (Maier and Böck, 1996b; Robson, 2001a). The only exceptions are the truncated *hypF* variant present in *R. eutropha* and a few other organisms (Lenz *et al.*, 2005) and the presence of the *hypX* gene in several (but not all) organisms whose biochemical implications still need to be resolved. The strict conservation is intriguing in view of the fact that this holds much less for the sequences of the structural genes *et al.* The conservation also highlights the core role of the Hyp proteins in the formation and shaping of the active site.

There are, however, a number of deviations which may reflect important functional differences. The first one to be discussed is *hupK*, which is present in organisms like *Rh. leguminosarum* or *R. eutropha*. As pointed out by

Imperial *et al.* (1993), HupK displays fascinating sequence similarity within and also bordering the sides of the metal cluster-binding motif within the large subunit. A scaffold role for the synthesis of the cluster has been postulated by these authors and it will be interesting to see whether this can be substantiated in biochemical experiments. It would indicate an important difference to the maturation system of *E. coli*, which lacks this gene. The existence of the *hupK* gene in the genome of several organisms is frequently paralleled by the presence of two homologues of the *hypC* gene. Deletion of each of them restricts the generation of active hydrogenase as demonstrated for *Thiocapsa roseopersina* (Maroti *et al.*, 2003) and *Rh. leguminosarum* (Tomas Ruis-Argüeso, Jose Palacios, Juan Imperial, personal communication). For *Rh. leguminosarum*, it was shown that the HypC homologue encoded outside the *hyp* operon (HupF) interacts with the large subunit, HupL (Tomas Ruis-Argüeso, Jose Palacios and Juan Imperial, personal communication). An intriguing possibility could be that HupK indeed functions as a scaffold for [NiFe]-cluster formation and requires the function of an additional HypC homologue. Binding experiments of HupF and HypC to the large subunit HupL and to HupK on one side, and to the maturation protein HypD on the other, should resolve this issue.

Intriguing and puzzling is also the fact that several [NiFe]-hydrogenases exist whose large subunits do not possess the C-terminal extension that is normally cleaved off by the maturation endopeptidase. Examples are the sensory (or regulatory) hydrogenase from *R. eutropha* and *Rb. capsulatus* (for review, see Vignais and Colbeau, 2004; Friedrich *et al.*, 2005) and the energy-converting hydrogenases (Ech) from extreme thermophilic bacteria and methanogenic archaea (Hedderich, 2004; Soboh *et al.*, 2004). The role of the *hyp* gene products in the formation of the regulatory hydrogenase from *R. eutropha* has been investigated and they were found to be essential (Buhrke *et al.*, 2001). This indicates that the basic reactions of metal-cluster synthesis and assembly are identical, but that these particular enzymes do not need the chaperone-like function of the C-terminal extension and its cleavage. A possible explanation could reside in the extremely low activity of this enzyme, which might indicate some difference in the elaborate structuring and the chemical environment of the centre (Winter *et al.*, 2004; Löscher *et al.*, 2005). On the other hand, this explanation does not hold for the highly active enzymes of the Ech class. It can also be excluded that this represents a different evolutionary line of maturation since the hydrogenase 3 from *E. coli* is a member of the Ech family, but uses the standard system including proteolytic processing. An answer to this intriguing difference may come from the analysis of the chemistry of the metal centre and its location within the mature enzyme.

3.4.3. Biotechnological Implications

Biological hydrogen production as an energy source requires the coupling of an electron delivery machinery like photosystem II with the proton reduction activity of hydrogenases. In most instances, this necessitates the heterologous expression of the hydrogenase genes in a suitable genetic background. The reason for the initial failure to achieve this aim (Mura *et al.*, 1996) can now be seen in the complex maturation pathway that depends on multiple interactions of the components, like those of HypF, HypE, HypC and HypD in ligand synthesis and coordination, or on specific actions on the structural polypeptides like the specific cleavage reaction of the precursor of the large subunit by the maturation endoprotease. For heterologous expression, the interdependency of the maturation proteins therefore requires that the organisms involved are reasonably well related so that sequence divergences do not lead to a blockade of these interactions, as demonstrated for the formation of active *D. gigas* hydrogenase by expression of the structural genes in *D. fructosovorans* (Rousset *et al.*, 1998). Alternatively, the co-transfer *en bloc* of all the structural and maturation genes located on transposable elements (Bascones *et al.*, 2000) or on entire plasmids (Friedrich *et al.*, 1984) can lead to successful heterologous generation of active enzyme. The minimal requirement for such a system has recently been highlighted by Lenz *et al.* (2005). These authors achieved high-level and regulated formation of the membrane-bound hydrogenase from *R. eutropha* in *Pseudomonas stutzeri* by transforming this organism with a plasmid containing the operons for the structural genes, the *hyp* genes, the regulatory genes and a set of genes putatively involved in maturation of the small subunit and export of the heterodimeric complex.

This requirement of the maturation genes holds also for attempts to overproduce [NiFe]-hydrogenases. Their balanced and concomitant overexpression must be met, since overproduction of single components may titrate interaction partners into the formation of unproductive complexes. Such effects are apparently involved in the initially inexplicable observation that many of the maturation genes located on plasmids do not fully complement the phenotype of the respective mutations (Jacobi *et al.*, 1992). Another example is the competition between HybG and HypC in complex formation with HypD (Blokesch and Böck, 2002).

4. OUTLOOK

The processes of maturation of [FeFe]-and [NiFe]-hydrogenases are new and exciting areas of bioinorganic chemistry. Because the [FeFe]-hydrogenase

maturation proteins were only recently identified, the process of H-cluster biosynthesis and enzyme maturation is not yet experimentally well defined and it is currently possible only to speculate on the maturation mechanism. Critical questions remain regarding the nature of the substrates utilized by the two Radical-SAM proteins HydE and HydG. It is also presently unclear whether the structural hydrogenase protein or one of the assembly proteins is a scaffold for assembly of the [FeFe]-hydrogenase catalytic site or if intermediates in the assembly process are transferred between assembly proteins prior to incorporation of the catalytic site into the structural enzyme. HydE and HydF exist as a single fusion protein in *Ch. reinhardtii*. It is therefore likely that these two proteins form a complex during the assembly process in other organisms. Whether the other proteins involved in [FeFe]-hydrogenase assembly also form stable complexes during [FeFe]-hydrogenase maturation also remains unresolved.

In comparison to the information available for the assembly and maturation of [FeFe]-hydrogenases, knowledge on the maturation of the [NiFe]-hydrogenses is somewhat more developed. The reasons are that the three-dimensional structure of a [NiFe]-hydrogenase was available much earlier and that the maturation process could be studied in organisms for which elaborate and powerful genetic and molecular biological tools were at hand. However, although most of the fundamentals are known, a vast number of important issues are open. They include, for example, the identity of the substrate and the path of synthesis of the CO ligand, the question of whether the metal centre or intermediates of it are formed on a scaffold protein or the details of the insertion of nickel. It must be stressed also that the studies of [NiFe]-hydrogenase formation are somewhat biased, since the emphasis up to now has been preferentially devoted to the analysis of the maturation of the large subunit. Moreover, the functions of those gene products not present in *E. coli*, but in most other organisms capable of the synthesis of [NiFe]-hydrogenases have been neglected. This fact, and also the deviations in the maturation process exhibited by the sensory hydrogenases, leave it open that several pathways have developed in parallel. However, the coming years will certainly provide exciting experimental insights into the complex processes involved in the maturation of all evolutionary lines of hydrogenases.

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Physiology of *Zymomonas mobilis*: Some Unanswered Questions

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ABSTRACT

The ethanol-producing bacterium *Zymomonas mobilis* can serve as a model organism for the study of rapid catabolism and inefficient energy conversion in bacteria. Some basic aspects of its physiology still remain poorly understood. Here, the energy-spilling pathways during uncoupled growth, the structure and function of electron transport chain, and the possible reasons for the inefficient oxidative phosphorylation are analysed. Also, the interaction between ethanol synthesis and respiration is considered. The search for mechanisms of futile transmembrane proton cycling, as well as identification of respiratory electron transport complexes, like the energy-coupling NAD(P)H:quinone oxidoreductase and the cyanide-sensitive terminal oxidase(s), are outlined as the key problems for further research of *Z. mobilis* energy metabolism.

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ABBREVIATIONS

9A	9-aminoacridine
ADH	alcohol dehydrogenase
ANS^-	1;8-anilinenaphtalenesulphonate
CCCP	carbonyl cyanide-*m*-chlorophenylhydrazone
DCCD	dicyclohexylcarbodiimide
ED	Entner–Doudoroff pathway
EMP	Embden–Meyerhof–Parnas pathway
Y_{ATP}	molar growth yield for ATP (g dry wt. per mol ATP)
$Y_{X/S^{max}}$	molar growth yield for ATP Corrected for energy of maintenance (g dry wt. per mol ATP)
$Y_{X/S}$	molar growth yield for glucose (g dry wt. per mol glucose)
$Y_{X/S^{max}}$	molar growth yield for glucose; Corrected for energy of maintenance (g dry wt. per mol glucose)

1. INTRODUCTION

Zymomonas mobilis is an unusual facultatively anaerobic Gram-negative bacterium, with a very efficient and rapidly operating homoethanol fermentation pathway. It belongs to the family of Sphingomonadaceae (White *et al.*, 1996; Kosako *et al.*, 2000), Group 4 of the alpha-subclass of the class Proteobacteria. Its remarkable ethanol productivity, exceeding by 3–5 fold that of yeast (Rogers *et al.*, 1982), in combination with tolerance to high ethanol and sugar concentrations, have kept *Z. mobilis* in the focus of biotechnological interest over several decades. The complete genome sequence of *Z. mobilis* ZM4, consisting of a single circular chromosome of

2,056,416 bp with 1998 predicted ORFs has been reported recently (Seo *et al*., 2005). Although the microorganism was originally discovered in fermenting tropical plant saps, e.g., in the traditional pulque drink (from *Agave mexicana* sap) of Mexico (Swings and DeLey, 1977), its potential application is not in alcoholic beverages, but rather in biosynthesis of fuel ethanol. Recombinant *Z. mobilis* capable of fermenting pentose sugars is now regarded as a major future promise for fuel ethanol production from wood hydrolysates (Dien *et al*., 2003). Ethanol biosynthesis might be the central, yet not the sole, use of this bacterium. Other end products of *Z. mobilis* metabolism, for example, sorbitol and fructose polymer levan (Viikari, 1988; Sprenger, 1996), also represent interest for the food industry and healthcare.

Not surprisingly, the biotechnological capacities of *Z. mobilis*, in particular, operation of its catabolic pathway, methods of fermentation and genetical modification, have been extensively reviewed. After the classical paper of Swings and DeLey (1977) covering all aspects of the biology of *Z. mobilis* known at that time, a few other general reviews (Montenecourt, 1985; Sahm *et al*., 1992; Doelle *et al*., 1993) and a number of specialised ones, focussed on metabolism or (later) on genetic engineering, have appeared during the following two decades (see, for example, Rogers *et al*., 1982; Baratti and Bu'Lock, 1986; Bringer-Meyer and Sahm, 1988; Viikari, 1988; Ingram *et al*., 1989; Johns *et al*., 1992; Sprenger, 1993, 1996; Sprenger *et al*., 1993). The latest reviews tend to concentrate on metabolic engineering of *Z. mobilis* as well as on modifications of other bacteria by means of introducing genes of the *Z. mobilis* ethanologenic pathway (e.g., Zaldivar *et al*., 2001; Dien *et al*., 2003).

In spite of the recent progress in the molecular biology and biotechnology of *Z. mobilis*, some intriguing basic aspects of its physiology have been left behind and still remain poorly understood. Thus, *Z. mobilis* is considered as a classical example of the ''uncoupled growth'' phenomenon, showing an extremely rapid catabolism, which is quite loosely matched to the needs of cellular biosynthesis (Belaich and Senez, 1965; Lawford and Stevnsborg, 1986; Jones and Doelle, 1991). It is largely the mode of uncoupled growth that makes *Z. mobilis* an outstanding ethanol-producer. In order to explain the constantly high catabolic rate, operation of a growth-independent, constitutive ATP-wasting reaction has been postulated (Jones and Doelle, 1991). Although it must be playing a key role in the uncoupled growth, so far the exact nature of this reaction has not been unraveled.

Aerobic metabolism of *Z. mobilis* represents another controversial issue. The reputation of this bacterium as an ''anaerobic ethanol producer'' appears to be so strong that, in a recent work on quantification of intracellular

carbon fluxes from ^{13}C tracer experiments in seven bacterial species (Fuhrer *et al*., 2005), *Z. mobilis* has been treated as a microorganism "without a respiratory chain". In reality, the *Z. mobilis* cell membrane carries a constitutive and highly active respiratory chain (Strohdeicher *et al*., 1990; Kalnenieks *et al*., 1998), ensuring an oxygen uptake rate that exceeds that of *Escherichia coli*. Notably, neither the exact composition nor the physiological function of the *Z. mobilis* respiratory chain is known. It is clear that respiration in *Z. mobilis* does not serve as an energy source for aerobic growth in the way that respiration does in most facultatively anaerobic and aerobic bacteria (Belaich and Senez, 1965; Bringer *et al*., 1984; Pankova *et al*., 1985; Kalnenieks *et al*., 1993). Moreover, inhibition of respiration causes an unexpected, counterintuitive stimulation of *Z. mobilis* aerobic growth (Kalnenieks *et al*., 2000), difficult to explain on the basis of our present knowledge. Ethanol synthesis and respiration are the two major alternative "sinks" of NADH in *Z. mobilis* catabolism, competing between themselves for reducing equivalents. The problem of competition between the respiratory chain and alcohol dehydrogenase (ADH) reaction, at first glance a simple one, nevertheless is one more topic of respiratory metabolism that needs consideration (Kalnenieks *et al*., 2002).

The aim of the present review is to analyse the problems of *Z. mobilis* physiology, which, in my opinion, have not received enough attention. Some of them, in particular, the structure and function of the respiratory chain, need a fresh look, underpinned by *Z. mobilis* genome sequence data. Although a closely related topic, the physiology and regulation of metabolically engineered *Z. mobilis* strains lie beyond the scope of the review.

2. BASIS FOR THE RAPID CARBOHYDRATE CATABOLISM

2.1. Central Metabolic Routes

Z. mobilis is an obligately fermentative microorganism. It ferments glucose, fructose and sucrose via the Entner–Doudoroff (ED) pathway in conjunction with the enzymes of pyruvate decarboxylase and ADH, producing ethanol and carbon dioxide in equimolar amounts (Gibbs and DeMoss, 1954; Dawes *et al*., 1966). The Embden–Meyerhof–Parnas (EMP) pathway is not operating in this bacterium. The absence of the EMP pathway recently has been confirmed by [1-^{13}C]glucose experiments, in which no ^{13}C label could be detected at the C-3 position of pyruvate (Fuhrer *et al*., 2005). Although a weak phosphofructokinase activity has been reported (Viikari, 1988), it was

probably an artefact, because the gene for phosphofructokinase is lacking in *Z. mobilis* (Seo *et al*., 2005). Likewise, most enzymes of the pentose phosphate pathway are missing (De Graaf *et al*., 1999; Seo *et al*., 2005).

Early studies of the central metabolism of *Z. mobilis* were made by Dawes *et al*. (1970). They found that the tricarboxylic acid cycle in this bacterium is truncated and apparently functions only to provide precursors for biosynthesis. The enzyme activities of α-ketoglutarate dehydrogenase, succinyl thiokinase, succinate dehydrogenase and fumarase (Dawes *et al*., 1970) as well as that of malate dehydrogenase (Bringer-Meyer and Sahm, 1989) are lacking. Accordingly, the genes for α-ketoglutarate dehydrogenase complex and malate dehydrogenase have not been found in the genome sequence (Seo *et al*., 2005). A pyruvate dehydrogenase complex has been purified and characterised, and the sequence and localisation of the corresponding genes have been analysed (Neveling *et al*., 1998). Two anaplerotic enzyme activities, those of PEP carboxylase and malic enzyme, have been found in cell-free extracts (Bringer-Meyer and Sahm, 1989). The *Z. mobilis* genome also contains genes for PEP carboxylase, citrate lyase, malic enzyme and fumarate dehydratase (Seo *et al*., 2005).

The established set of reactions of central metabolism seems sufficient to explain the ability of *Z. mobilis* to grow on glucose in a mineral salts medium supplemented with pantothenate and biotin (Montenecourt, 1985) in the absence of amino acids. However, the enzymatic reactions producing building blocks for biosynthesis are extremely weak in comparison to the mainstream catabolic reactions of pyruvate kinase and pyruvate decarboxylase (Bringer-Meyer and Sahm, 1989). In part, that explains the tiny percentage of the substrate carbon converted into biomass, and, at the same time, the very efficient conversion of glucose into ethanol. *Z. mobilis* rapidly catabolises up to 95–98% of the substrate carbon to ethanol and carbon dioxide, while only 3–5% of substrate carbon is converted into biomass (Swings and DeLey, 1977; Rogers *et al*., 1982).

2.2. Entner–Doudoroff Pathway

The high ethanol yield and productivity observed in *Z. mobilis* result from its unique physiology, particularly, from the properties and regulation of its catabolic route (Fig. 1), the ED, or 2-keto-3deoxy-6-phosphogluconate (KDPG) pathway (for detailed reviews, see Viikari 1988; Sprenger, 1996). All the fermentative and ethanologenic enzymes of *Z. mobilis* have been isolated and characterised, and the corresponding genes were cloned and sequenced in the 1980s and 1990s (for reviews and references, see Viikari,

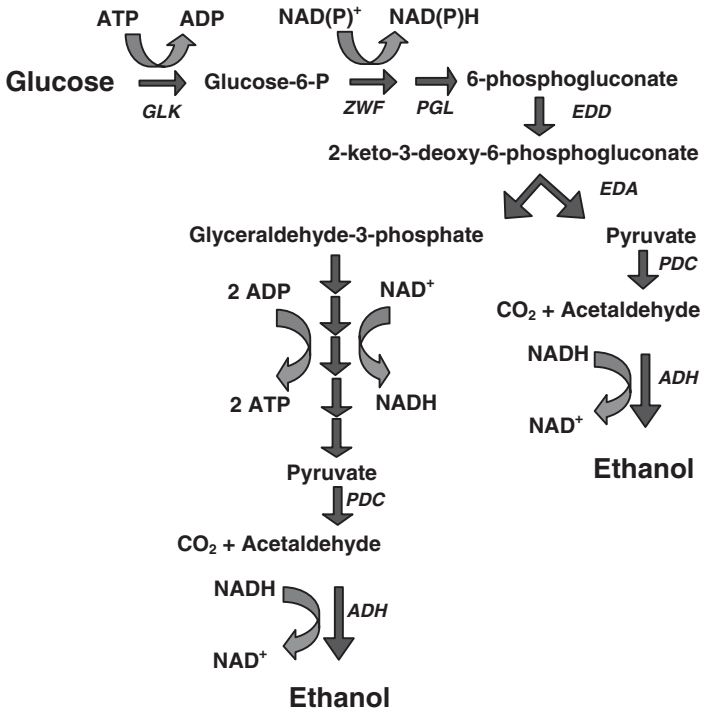

Figure 1 The Entner–Doudoroff pathway and ethanologenesis. The branch from glyceraldehyde-3-phosphate to pyruvate is identical to the Embden–Meyerhof–Parnas pathway. Abbreviations: *GLK*, glucokinase; *ZWF*, glucose-6-phosphate dehydrogenase; *PGL*, phosphogluconolactonase; *EDD*, 6-phosphogluconate dehydratase; *EDA*, 2-keto-3-deoxy-gluconate aldolase; *PDC*, pyruvate decarboxylase; *ADH*, alcohol dehydrogenase.

1988; Mejia *et al*., 1992; Sahm *et al*., 1992). The genes of pyruvate decarboxylase (*pdc*) and ADH II (*adhB*) have been introduced into several other bacteria, for example, *E. coli* (Ingram *et al*., 1987; Tao *et al*., 2001), *Klebsiella oxytoca* (Ohta *et al*., 1991) and photoautotrophic cyanobacteria *Synechococcus* sp. (Deng and Coleman, 1999), to promote ethanol synthesis.

Z. mobilis is the only known microorganism that uses the ED pathway anaerobically, in place of the EMP glycolytic pathway. The ED pathway, probably, is the oldest and energetically the least efficient fermentative pathway (Conway, 1992; Romano and Conway, 1996). It produces only 1 mole of ATP per mole of consumed glucose. Furthermore, in contrast to most other bacteria, *Z. mobilis* use a facilitated diffusion system with a glucose facilitator protein (GLF) for intracellular glucose transport

(uniport), which does not utilise metabolic energy, and is suited for growth in sugar-rich media (DiMarco and Romano, 1985; Snoep *et al.*, 1994; Weisser *et al.*, 1995). The glycolytic pathway in this organism appears to function with minimal allosteric control. Unlike yeast, it lacks allosterically regulated pyruvate kinase and phosphofructokinase, typical of EMP glycolysis (Barrow *et al.*, 1984; Strohhacker *et al.*, 1993; Snoep *et al.*, 1996). Allosteric inhibition by phosphoenolpyruvate has been demonstrated for the second enzyme of the ED pathway, glucose-6-phosphate dehydrogenase (Scopes, 1997). Notably, the same enzyme appears to exert a considerable control over the glycolytic flux. The flux control coefficient of glucose-6-phosphate dehydrogenase for early stages of batch growth was found to be 0.4, or even higher (Snoep *et al.*, 1996). Glucokinase and the glucose transporter also might contribute to the flux control under these conditions. On the other hand, when ethanol is present at high concentration (around 10% w/w), which is typical for the late fermentation stages on a sugar-rich medium, the flux control is shifted to enolase and phosphoglycerate mutase (Barrow *et al.*, 1984; Strohhacker *et al.*, 1993; Snoep *et al.*, 1996). Significantly, during the flux-control studies it was seen that changes in glycolytic flux are not accompanied by changes in growth rate, thus demonstrating that ATP production is excessive (saturating) for the needs of biosynthesis (Snoep *et al.*, 1996), in full agreement with the concept of catabolism not coupled to growth.

2.3. Uncoupled Growth

2.3.1. Why is Glucose Catabolism in Z. mobilis So Rapid?

Carbohydrate metabolism in *Z. mobilis* operates as a true "catabolic highway" (Sprenger, 1996). The rate at which cells of *Z. mobilis* convert glucose into ethanol plus CO_2 in an exponentially growing culture under anaerobic conditions reaches 0.75–1.0 μmol glucose mg dry wt.$^{-1}$ min^{-1} (Rogers *et al.*, 1982; Viikari, 1988; Jones and Doelle, 1991; Arfman *et al.*, 1992; Fuhrer *et al.*, 2005). That is three to five times faster than observed in yeast (Rogers *et al.*, 1982), and approximately 1.2–1.5 times faster than in the Gram-positive obligately fermentative *Streptococcus bovis*, which serves as another example of the uncoupled growth phenomenon among bacteria (Cook and Russell, 1994). High activity of the glycolytic enzymes, of course, is a prerequisite of such high catabolic rates. The enzymes involved in fermentation are expressed constitutively, and comprise as much as 50% of *Z. mobilis* total protein (Algar and Scopes, 1985; An *et al.*, 1991). Furthermore, the high

cytoplasmic protein levels of the glycolytic enzymes in *Z. mobilis* correlate with an increased stability of their transcripts (Mejia *et al.*, 1992). In combination, the low ATP yield of the ED pathway and the abundant expression of fermentative enzymes certainly help to explain the high catabolic rate in *Z. mobilis* (Dien *et al.*, 2003). Although in recent papers on metabolic engineering of *Z. mobilis*, such an explanation often is taken as sufficient, it should be stressed however that it is incomplete from the stoichiometric point of view. Statements about the low energetic efficiency of the ED pathway picture *Z. mobilis* as a bacterium, suffering from the lack of ATP. That may be misleading: a simple calculation shows that, in comparison to yeast, catabolism of *Z. mobilis* generates ATP with a considerably higher specific rate. Yeast produce 2 moles of ATP per mole of glucose in the EMP pathway, at the same time having a three to five (but not just two) times lower catabolic rate (Rogers *et al.*, 1982). Hence, in comparison to yeast, the ATP production rate in *Z. mobilis* is excessive.

Under steady-state conditions, the high specific rate of ATP synthesis must be balanced by an equally rapid utilisation. Obviously, cell-biomass synthesis is by far not the main consumer of ATP. As already mentioned, *Z. mobilis* produces little cell mass (Bauchop and Elsden, 1960; Belaich and Senez, 1965; Rogers *et al.*, 1982), growing with a low energetic efficiency. The relatively low growth yield values, ranking between 2.3 and 10.5 g dry wt. $(\text{mol glucose})^{-1}$ (Bauchop and Elsden, 1960; Belaich and Senez, 1965; Stouthamer, 1977; Lawford and Stevnsborg, 1986; Sahm and Bringer-Meyer, 1987; Kim *et al.*, 2000; Lawford and Rousseau, 2000), together with the high catabolic rate, point to the presence of some ATP-spilling reaction in *Z. mobilis*, possibly in the form of a futile cycle or a bypass reaction. Apparently, ATP spilling would permit glycolysis to proceed without a concomitant biomass synthesis under conditions when essential growth factors are absent, as in the classical case of pantothenate limitation in *Z. mobilis* culture (Belaich and Senez, 1965). ATP spilling in *Z. mobilis* is activated at elevated glucose concentrations. Decoupling of growth from ethanol production in batch cultures at high (up to 20%) glucose concentrations was observed by Veeramallu and Agrawal (1986). Lawford and Stevnsborg (1986) reported that increasing the concentration of glucose from 3% to 6% in the defined minimal medium feed to the chemostat results in a decrease of $Y_{X/S^{max}}$ $Y_{X/S^{max}}$, from 9.0 to 7.2 g dry wt. $(\text{mol glucose})^{-1}$. Knowing that for *Z. mobilis* $Y_{X/S^{max}}$ is equal to $Y_{X/S^{max}}$, due to the stoichiometry of 1 mole ATP generated per mole of glucose consumed (Bauchop and Elsden, 1960), the value of 7.2 g dry wt. $(\text{mol glucose})^{-1}$ must be regarded as low in comparison to other microorganisms (Stouthamer, 1977). The growth yield markedly decreases, while the catabolic rate is further accelerated at acidic

medium pH (4.0 versus 6.5), and under nutrient limitations other than glucose (nitrogen, phosphate or potassium) (Lawford and Stevnsborg, 1986; Jones and Doelle, 1991). Furthermore, addition of glucose to a washed, non-growing cell suspension elicits glucose consumption at a high specific rate. The ability of non-growing *Z. mobilis* cells to catabolise glucose depends little on the conditions of their preceding cultivation (the type of nutrient limitation, medium pH, etc.); therefore, the putative ATP-spilling reaction, activated in the presence of excess glucose, must be largely growth-independent and constitutive (Jones and Doelle, 1991).

2.3.2. The Nature of the ATP-Spilling Reaction

The membrane F_0F_1-type H^+-ATPase has been considered as the most likely candidate for the recycling of excess ATP in *Z. mobilis* (Lazdunski and Belaich, 1972; Reyes and Scopes, 1991). Significant activity of other types of energy-consuming futile cycles at present seems less plausible. Experiments by Jones and Doelle (1991) failed to support the presence of any kinase/phosphatase-type futile cycle in the ED pathway. Operation of futile cycles of potassium or ammonium transport under special growth conditions, like those demonstrated in *E. coli* (Mulder *et al.*, 1986; Buurman *et al.*, 1991), could not be a priori excluded. However, our present knowledge about the properties of ion transport systems in *Z. mobilis* is too poor for speculations.

Lazdunski and Belaich (1972) suggested that *Z. mobilis* has two ATPase activities: a high-affinity system, possibly pumping protons across the cytoplasmic membrane, and a low-affinity system that functions only as an ATPase. The high-affinity proton-pumping ATPase, most probably, is the same enzyme as that later purified from the cell membrane and characterised by Reyes and Scopes (1991). Among the other ATP-hydrolysing activities in *Z. mobilis*, which also include acid and alkaline phosphatases and a periplasmic 5′-nucleotidase, the proton-pumping ATPase might have the highest contribution to the intracellular ATP hydrolysis. According to calculations based on the proton-pumping ATPase activity in membrane preparations, its contribution might reach over 20% of the total intracellular ATP turnover (Reyes and Scopes, 1991; Zikmanis *et al.*, 1999). The key role of the membrane proton-pumping ATPase in cellular energy dissipation of *Z. mobilis* is further supported by experiments with the F_0F_1-ATPase inhibitor dicyclohexylcarodiimide (DCCD) (Kalnenieks *et al.*, 1987b). A marked increase of the growth yield on glucose was achieved when cells were grown in the presence of 0.5 mM DCCD. A similar effect of DCCD was reported also for *S. bovis* by Russell and Strobel (1990). As in *Z. mobilis*, in this obligately fermentative bacterium F_0F_1-ATPase has been identified as the

major free energy-spilling reaction under conditions of excess glucose (Russell and Cook, 1995). Glucose consumption by non-growing cells of *S. bovis* could be completely inhibited by the F_0F_1-ATPase inhibitor.

However, finding the key role of the proton-pumping ATPase does not solve the problem of ATP spilling. It is clear that the H^+-ATPase itself does not dissipate energy but, instead, converts it into the form of transmembrane proton-motive force (Δp). The problem of ATP spilling by the H^+-ATPase, therefore, transforms into an equivalent problem of Δp dissipation. To put it another way, the fact that the proton-pumping ATPase of *Z. mobilis* might be playing a major role in the ATP-spilling implies also an active pathway for a futile dissipation of the generated transmembrane Δp (a futile transmembrane proton cycle). The summary of hypothetical pathways of energy spilling in *Z. mobilis* is depicted in Fig. 2. *S. bovis* could serve as an example, in which it has been established that ATP spilling by F_0F_1-ATPase is coupled to a futile cycling of protons. A variable proton leak across the membrane, dramatically increasing at elevated intracellular ATP concentration (above 3 mM) under conditions of glucose excess in the medium, has been shown to be the major energy-spilling reaction in this bacterium (Cook and Russell, 1994).

2.3.3. A Futile Cycle of Protons?

In order to see if the same principle works in *Z. mobilis*, one should be able to monitor the membrane proton conductance and the magnitude of the proton-motive force, particularly under conditions of uncoupled growth. Quantitative study of the proton-motive force in *Z. mobilis* has proven to be complicated, because of the low permeability of its outer membrane. It was possible to measure pH gradient (ΔpH) by means of ^{31}P NMR (Barrow *et al.*, 1984) as well as by transmembrane distribution of radioactively labelled benzoic acid (Kalnenieks *et al.*, 1987a,b; Osman *et al.*, 1987). The intracellular pH in *Z. mobilis* with both methods was found to be comparatively low. Its value at the external pH close to 6 does not exceed 6.4 (Barrow *et al.*, 1984; Kalnenieks *et al.*, 1987a). In an exponentially growing culture, change of the medium pH values from 5.6 to 3.5 causes a shift of intracellular pH from 6.4 to 5.75, showing the ability of *Z. mobilis* to maintain ΔpH of more than 2 units (Kalnenieks *et al.*, 1987a). However, in a batch culture at late stages of fermentation the intracellular pH may fall as low as 5.3 (Osman *et al.*, 1987) and, possibly, limit the rate of metabolism. Unfortunately, no suitable probe has been found for quantitative determination of the transmembrane electric potential ($\Delta\Psi$). Only fluorescent probes have been successfully applied as semiquantitative or qualitative indicators of $\Delta\Psi$ change in the study of membrane energisation/deenergisation (see Section 3.3). Several attempts to

permeabilise the outer membrane of *Z. mobilis* for the commonly used quantitative $\Delta\Psi$ probe, the lipophilic cation tetraphenylphosphonium (TPP^+), have failed (Kalnenieks *et al.*, 1987a; Ruhrmann and Krämer, 1992). So far, the only report on $\Delta\Psi$ values in *Z. mobilis* is that of Ruhrmann and Krämer (1992) based on measurements of SCN^- extrusion from energised cells. Using ^{14}C-labelled SCN^- and benzoic acid, they found that the proton-motive force is maintained between −132 and −138 mV over the medium pH range from 4 to 5.5. $\Delta\Psi$ reaches –110 mV at pH 5.5, but, as in most other bacteria (Padan *et al.*, 1981), decreases its absolute value (becomes more positive) at more acidic external pH, as the contribution of the ΔpH component grows. However, due to its negative charge, the cytosolic concentration of SCN^- is decreased according to the Nernst equation with increasing $\Delta\Psi$, positive outside. That significantly limits the accuracy of measurement of $\Delta\Psi$, when its absolute value is above 100 mV. Apparently, a different method needs to be applied for the study of $\Delta\Psi$ in the pH range between 5.5 and 6.5, which is mostly used for cultivation of *Z. mobilis*.

In contrast to *S. bovis*, neither the intrinsic membrane proton conductance nor the magnitude of the transmembrane proton flux (calculated from calorimetric measurements), have been measured for *Z. mobilis*. Taking into account the similarity of metabolic behaviour, as well as the similar role of F_0F_1-ATPase for the energy-spilling pathway in both microorganisms, the presence of a futile proton cycle in *Z. mobilis* a priori seems realistic, and deserves further examination. Perhaps impaired maintenance of the proton-motive force due to high proton leakage contributes also to the apparent inefficiency of the oxidative phosphorylation in aerobically growing *Z. mobilis* (see Section 3.3).

Studies of membrane permeability in *Z. mobilis* so far have focussed mainly on the non-specific, membrane-disrupting effects of ethanol. The detrimental action of ethanol at high concentrations on the permeability of *Z. mobilis* plasma membrane was extensively studied in the 1980s (Osman and Ingram, 1985; Osman *et al.*, 1987). It was shown that ethanol causes an increase in the rate of leakage of small molecules and ions, including protons. The accumulation of ethanol during fermentation may be responsible for the gradual collapse in ΔpH seen in batch cultures grown on media with high (20%) glucose concentration during the stationary growth phase (Osman *et al.*, 1987). Although ethanol decreases the barrier function and resistance of the plasma membrane, and thus probably adds to the energetic uncoupling at some stages of growth, it still cannot be regarded as the clue to the uncoupled growth phenomenon. That is because the energy spilling in *Z. mobilis* occurs also in the absence of ethanol in the medium (at early stages of growth or in a washed cell suspension with glucose).

Paradoxically, however, the possible Δp-dissipating effect of carbon dioxide, the second major end product of *Z. mobilis* catabolism, has not been analysed. Several papers about the effect of CO_2 on fermentation performance of *Z. mobilis* (see e.g., Nipkow *et al.*, 1985; Veeramallu and Agrawal, 1986), as well as on that of yeast, *E. coli* and some other bacteria (Janda and Kotyk, 1985; Lacoursiere *et al.*, 1986) were published in the 1980s. They describe complex inhibitory and uncoupling effects of carbon dioxide on the culture growth and product synthesis, yet do not consider the putative mechanisms at the membrane level. *Z. mobilis* is one of the most rapid producers of CO_2 among microorganisms. Apparently, the major part of CO_2 leaves the cell by passive diffusion in the form of a neutral molecule. Measurements with erythrocytes suggest that the lipid bilayer of the cell membrane does not represent a serious diffusion barrier for CO_2 (Forster *et al.*, 1998). At the same time, part of the generated CO_2 in the cytoplasm might undergo hydration in the reaction, catalysed by carbonic anhydrase (Merlin *et al.*, 2003), with subsequent dissociation of carbonic acid into a proton and bicarbonate anion (Fig. 2). Knowing the respective equilibrium constants (Mills and Urey, 1940; Merlin *et al.*, 2003) (Fig. 2), and taking 6.4 for the intracellular pH, we can estimate that, under equilibrium conditions, approximately 10% of carbon dioxide in *Z. mobilis* should be present in the form of bicarbonate anion.

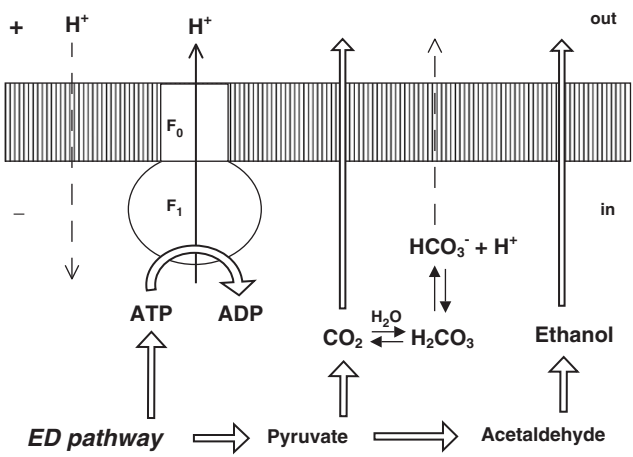

Figure 2 The putative energy-spilling pathways in *Z. mobilis*. Hypothetical transmembrane fluxes, causing dissipation of the proton-motive force, are shown with dashed arrows.

Export of bicarbonate anions from the cell would represent an efficient pathway of Δp dissipation, equivalent to import of protons: a unit negative charge would be translocated into the external medium, decreasing ΔΨ, while a proton would be left behind in the cytoplasm, diminishing the transmembrane pH gradient (Fig. 1). We may speculate that for this mechanism to work in *Z. mobilis*, at least following conditions should be met: (1) a high intracellular concentration of CO_2; (2) equilibrium between the intracellular pools of CO_2 and HCO_3^-; (3) sufficient membrane permeability to HCO_3^-; and (4) an inside alkaline transmembrane gradient of pH. While, obviously, points (1) and (4) are fulfilled, points (2) and (3) need further examination. For efficient conversion of carbon dioxide into bicarbonate anion, the presence of carbonic anhydrase is essential (Merlin *et al.*, 2003). To our knowledge, there is no published evidence for carbonic anhydrase activity in *Z. mobilis* cells, yet the corresponding gene appears to be present in the genome (Seo *et al.*, 2005). As the permeability of the lipid bilayer for charged species is very much lower than for neutral molecules, most probably a specific electrogenic transport system must be operating for bicarbonate to create a measurable depolarising effect. Recently, active transport of bicarbonate ions has been extensively studied in cyanobacteria (Badger and Price, 2003). It has been demonstrated that several energy-dependent uptake systems serve to accumulate HCO_3^- in the cytosol, which is subsequently used to elevate CO_2 concentration around Rubisco. While photosynthetic microorganisms need mechanisms to accumulate carbon dioxide, the rapid ethanologens, like *Z. mobilis*, apparently need to solve the opposite problem, which could, in principle, be related to the uncoupled growth.

3. STRUCTURE AND FUNCTION OF THE RESPIRATORY CHAIN

3.1. Membrane Electron Carriers

3.1.1. Cytochromes

Long ago it was established that *Z. mobilis* possesses a constitutive respiratory chain (Belaich and Senez, 1965; Pankova *et al.*, 1985; Strohdeicher *et al.*, 1990). Today it is still one of the least well-understood bacterial respiratory chains. In particular, data about its cytochrome composition are scarce and contradictory. Belaich and Senez (1965) in their pioneering work, using microspectroscopy at liquid nitrogen temperature, detected two

distinct and sharp absorption bands at 550 and 620–621 nm in spectra of aerobically cultivated cells, which they ascribed to α-bands of cytochromes *c* and a_2 (now cytochrome *d*), respectively. In anaerobic cells they found an additional band at 560 nm, presumably corresponding to cytochrome *b*. Later, Pankova *et al.* (1985) reported spectral features of cytochromes c_{552}, b_{558} and d_{629} in room-temperature spectra of membrane vesicle preparations. More recent spectroscopic studies of *Z. mobilis* membrane preparations (Kalnenieks *et al.*, 1998, 2000) verified the presence of *b*- and *d*-type cytochromes with absorbances at 557–558 and 629–631 nm, respectively as well as revealed a minor spectral feature of a putative *a*-type cytochrome around 602–605 nm. In addition, a peak at 415–416 nm and a trough at 429–435 nm were found in CO + reduced *minus* reduced difference spectra (Kalnenieks *et al.*, 1998). The latter features are characteristic for cytochrome *o* (Poole, 1994). However, in low-temperature photodissociation spectra, recorded under anoxic conditions at −102 °C, no characteristic signals of cytochrome *o* could be seen. Assuming a very fast recombination of CO taking place after transient illumination, which is not typical for the binuclear centre of cytochrome *o*, authors referred to this component as 'the cytochrome *o*-like component' (Kalnenieks *et al.*, 1998). No further attempts to identify the cytochrome *o*-like component have been undertaken since. A peak at 439 nm and a trough at 420 nm in the low-temperature photodissociation spectra (Kalnenieks *et al.*, 1998) indicated presence of the high-spin cytochrome b_{595}, apparently associated with cytochrome *d*, as shown previously for *E. coli* (Poole, 1994).

Spectral signals of cytochrome *d* and cytochrome b_{595} led researchers to assume that a *bd*-type quinol oxidase (Jünemann, 1997) is present in the respiratory chain of *Z. mobilis* (Kalnenieks *et al.*, 1998). Some confusion, however, was caused by the recent discussion of the genome data. Seo *et al.* (2005) stated that *Z. mobilis* lacks genes for cytochrome *o*-and *d*-type terminal oxidases, hence posing difficult questions about the pathway of electrons to oxygen. Nevertheless, sequences homologous to *cyd*A and *cyd*B (encoding subunits I and II of the cytochrome *bd* quinol oxidase, respectively) can be found in the genome sequence (GenBank Accession number AE008692) deposited by these authors as well as in the unfinished genome sequence of the closely related bacterium *Novosphingobium aromaticovorans*. Therefore, presence of the cytochrome *bd* terminal oxidase in *Z. mobilis* may be regarded as firmly established. At the same time, the genome data do not support presence of cytochromes *a* and *o*, so the source of the corresponding spectral features (Kalnenieks *et al.*, 1998) remains unclear.

In contrast to what has been demonstrated for *E. coli* (Knowles, 1976; Kita *et al.*, 1984a,b; Poole and Cook, 2000), the cytochrome content of

Z. mobilis respiratory chain does not change much in response to aeration or to cyanide addition (Kalnenieks *et al.*, 2000). An aeration-dependent alteration of the α-peak around 556–560 nm, corresponding to cytochrome(s) *b*, has been reported in several publications (Belaich and Senez, 1965; Kalnenieks *et al.*, 1996, 2000), although the observations appear to be somewhat contradictory. Kalnenieks *et al.* (1996, 2000) have observed the α-peak of the *b*-type cytochromes at 557–558 nm for the aerobic culture to reach about double the height of the α-peak in membranes of anaerobic cultures. In contrast, Belaich and Senez (1965) detected the feature of cytochrome *b* only in anaerobically grown cells, as mentioned above. Pankova *et al.* (1985) did not find any difference between cytochrome *b* absorbances of aerobically and anaerobically cultivated cells. The presence of cyanide in the culture medium seems to have no effect upon cytochrome *b* content of *Z. mobilis*. The α-peak in the membranes of cells, grown aerobically in the presence of cyanide, is similar to that of aerobic control cells (Kalnenieks *et al.*, 2000).

The absorbance of cytochrome *d* at 629–631 nm is even less affected by external conditions – it responds neither to aeration, nor to the presence of cyanide (Kalnenieks *et al.*, 2000). This looks particularly strange, because in such well-explored model organisms of bacterial bioenergetics like *E. coli* and *Azotobacter vinelandii* (Knowles, 1976; Kita *et al.*, 1984a,b; D'mello *et al.*, 1996; Poole and Hill, 1997), the *bd*-type terminal oxidase plays the central role in adaptation to various oxygen supply, and also to cyanide. *E. coli* cytochrome *bd* oxidase has a very high affinity for oxygen (D'mello *et al.*, 1996) and is synthesised maximally under conditions of oxygen limitation. Owing to its higher resistance to cyanide, as compared to the *bo'* oxidase, its expression in *E. coli* is stimulated also in the presence of cyanide (Knowles, 1976; Kita *et al.*, 1984b). In *A. vinelandii*, *bd* oxidase has lower oxygen affinity (D'mello *et al.*, 1994), and its synthesis increases with oxygen supply (Poole and Hill, 1997), because it plays a key role in respiratory protection of nitrogenase (Kelly *et al.*, 1990) and in maintenance of viability during stationary phase under aerobic conditions (Edwards *et al.*, 2000). Although data about oxygen affinity of *Z. mobilis* cytochrome *bd* terminal oxidase are lacking, it is clear that this bacterium does not employ its *bd* terminal oxidase for the sake of adaptation to oxygen or cyanide.

3.1.2. Respiratory Dehydrogenases

Early data show that cell-free extracts of *Z. mobilis* oxidise NADH with a stoichiometry of 2 $[NADH + H^+]$:1 O_2 (Dawes *et al.*, 1966; McGill and Dawes, 1971; Bringer *et al.*, 1984), apparently producing water as the end product. This activity could be enriched by separation of cell membrane

vesicles, using ultracentrifugation. Apparently, NADH is being oxidised by membrane-bound NADH:quinone oxidoreductase(s), as in the majority of other bacteria bearing respiratory chain. Strohdeicher *et al.* (1990) demonstrated that the NADH-oxidising activity in *Z. mobilis* membranes strongly depends on the presence of ubiquinone (CoQ_{10}). Extraction of ubiquinone from freeze-dried membranes (without destroying enzyme activities) led to a total loss of respiratory activity with NADH as the substrate while, after reincorporation of ubiqinone, the NADH oxidase activity could be restored to 95% (Strohdeicher *et al.*, 1990).

Both biochemical studies and genome data point to the presence of more than one membrane-bound NADH dehydrogenase in the *Z. mobilis* electron transport chain. Seo *et al.* (2005) have annotated genes for a NADH:ubiquinone oxidoreductase complex as well as for NADH dehydrogenase, homologous to *ndh* (type II NADH dehydrogenase) in the *Z. mobilis* genome sequence. Accordingly, kinetic analysis of NADH oxidation in membrane preparations reveals at least two components with different K_m values for NADH. The apparent K_m for the major NADH oxidase activity in anaerobically grown cells was found to be close to 7 μM (Kalnenieks *et al.*, 1996), resembling the K_m for the energy-coupling NADH dehydrogenase complex I in *E. coli* (Matsushita *et al.*, 1987; Leif *et al.*, 1995). The apparent K_m of the other component, prevailing in aerobically grown cells, is around 60 μM (Kim *et al.*, 1995; Kalnenieks *et al.*, 1996), and most probably could be ascribed to the energy non-generating type II NADH dehydrogenase, encoded by *ndh* (Yagi, 1991).

Although one of the K_m values for NADH oxidation in membranes points to the presence of the NADH dehydrogenase complex I, nevertheless the six genes of the *Z. mobilis* genome, encoding the putative NADH:ubiquinone oxidoreductase complex do not bear homology to those of the *nuo* operon of *E. coli*. Instead, they appear to be closely homologous to the genes of the *rnf* operon, encoding a recently discovered membrane electron transport complex, which is involved in electron transport to nitrogenase in the photosynthetic bacterium *Rhodobacter capsulatus* (Schmehl *et al.*, 1993). Three of the *rnf* gene products, RnfA, RnfD and RnfE, are similar to the membrane components of the Na^+-dependent NADH:ubiquinone oxidoreductase of the bacterium *Vibrio alginolyticus* (Kumagai *et al.*, 1997; Jeong and Jouanneau, 2000). The subunits RnfB and RnfC have been predicted to be hydrophilic and carry iron–sulphur clusters. Notably, RnfC has potential binding sites for NADH and FMN and resembles in this respect the NADH-binding subunit NuoF of the complex I (Kumagai *et al.*, 1997). This novel type of energy-coupling NADH oxidoreductase has been demonstrated to supply electrons for nitrogen fixation, and also for 2,4-dinitrophenol reduction in

R. capsulatus (Saez *et al*., 2001). To the best of our knowledge, there is no evidence concerning nitrogen fixation in *Z. mobilis*, although the whole set of genes, encoding the nitrogen fixation machinery, is present in its genome (Seo *et al*., 2005). We may hypothesise that the whole Rnf complex of *Z. mobilis*, or at least some of its subunits, might be participating in electron transport to oxygen, and thus representing the respiratory chain component with the low K_m for NADH. However, such a possibility, as well as the putative nitrogen-fixating activity of this bacterium, needs extensive further research.

Along with the NADH oxidase activity, *Z. mobilis* cytoplasmic membrane fractions bear also a NADPH oxidase activity, which is slightly lower, yet comparable to that of NADH oxidase (Bringer *et al*., 1984). The known bacterial membrane-bound dehydrogenases are predominantly NADH-specific (Yagi, 1991). The ability to oxidise NADPH in the respiratory chain is a comparably rare feature among bacteria. For *Corynebacterium glutamicum* (Matsushita *et al*., 2001) and for *A. vinelandii* (Bertsova *et al*., 2001) it has been demonstrated that NADPH oxidation in the respiratory chain is accomplished by the type II NADH dehydrogenase (*ndh*). So far, there is no published evidence about the nature of the NADPH-oxidising activity in *Z. mobilis*. Apart from *ndh*, a couple of putative zinc-containing NADPH:quinone oxidoreductase gene sequences have also been annotated in the genome database. On the other hand, using tetrazolium staining of non-denaturating gel, we have recently shown that in membrane extract from aerobically grown cells the band with NADH is positioned in the same place as the band with NADPH (Kalnenieks *et al.*, unpublished). This finding points to the *ndh* gene product as the most likely candidate for the membrane respiratory chain-linked NADPH-oxidising activity in *Z. mobilis*.

NADH and NADPH are the major, yet not the only, *in vivo* electron donors for the *Z. mobilis* respiratory chain. Two other membrane-bound dehydrogenase activities, those of glucose dehydrogenase and D-lactate dehydrogenase, have been reported. Both of them are minor activities, several-fold weaker than the NADH dehydrogenases. Glucose oxidation rates in membrane reach only about 5% of the NADH oxidase activity (Strohdeicher *et al*., 1990). The glucose dehydrogenase has been partially purified and characterised (Strohdeicher *et al*., 1988, 1989). It bears pyrroloquinoline quinone (PQQ) as a tightly bound cofactor, carries out oxidation of glucose to gluconate, and passes electrons into the respiratory chain at the level of ubiquinone (Strohdeicher *et al*., 1990). The rate of D-lactate oxidation in membrane preparations reaches up to 10–20% of the NADH oxidase activity (Kalnenieks *et al*., 1998). The reaction is fairly stereospecific towards the D-stereoisomer of lactate, and is inhibited by oxalate and (less efficiently)

by oxamate, the inhibitors of D-lactate dehydrogenase in *E. coli* (Kohn and Kaback, 1973). Most probably, D-lactate dehydrogenase carries FAD as the cofactor (Kalnenieks *et al.*, unpublished). The gene for succinate dehydrogenase is also present in the genome, yet the corresponding activity is lacking. The physiological role for these minor dehydrogenases in *Z. mobilis* respiration still remains an open question.

3.2. Electron Transport Pathway

3.2.1. Inhibitor Analysis

Thorough inhibitor titrations of *Z. mobilis* respiratory chain have been carried out by Strohdeicher *et al.* (1990) with several quinone analogues: piericidin A, capsaicin, rotenone, HQNO, myxothiazol, antimycin A and stigmatellin. The results obtained give some insights into the putative electron transport complexes, keeping in mind that neither of the quinone-site inhibitors can be regarded as being absolutely specific for one particular respiratory complex. Thus, sensitivity of respiration to the classical inhibitors of the type I NADH dehydrogenase complex, like piericidin A, capsaicin and rotenone (Degli Esposti, 1998; Yagi *et al.*, 1998), indicates presence of an energy-coupling NADH dehydrogenase complex, although the *Z. mobilis* genome does not contain the homologue of the *nuo* operon (see above). Most probably, this is because the NADH-binding subunit of the Rnf complex bears some structural similarity to the NADH-binding subunit of the complex I, as has been found for *R. capsulatus* (Kumagai, *et al.*, 1997; Yagi *et al.*, 1998). Antimycin A and myxothiazol are both known as inhibitors of the cytochrome bc_1 complex (complex III) (Trumpower and Gennis, 1994), although myxothiazol has been shown to inhibit also the cytochrome bo' complex (Meunier *et al.*, 1995) and the type I NADH dehydrogenase complex (Matsushita *et al.*, 1987; Degli Esposti, 1998) of *E. coli*. In the paper of Strohdeicher *et al.* (1990), sensitivity to antimycin A, myxothiazol, HQNO and stigmatellin was taken to indicate that a cytochrome *b* (receiving electrons from ubiquinol and passing them to a *c*-type cytochrome) is localised in the middle part of the electron transport chain of *Z. mobilis*. It should be noted, however, that both antimycin A and HQNO also inhibit cytochrome *bd*, having inhibitor constants in the 10–100 μM range (Jünemann, 1997), and that HQNO is an established inhibitor of the Na^+ translocating NADH:quinone oxidoreductases (Yagi *et al.*, 1998). Recently, support for the presence of the cytochrome bc_1 complex came from *Z. mobilis* genome data (Seo *et al.*, 2005):genes for its key components, the

cytochrome *b* subunit, cytochrome c_1 subunit and Rieske FeS protein, have been identified.

Sensitivity to cyanide is one of the parameters traditionally used to differentiate and characterise the respiratory terminal oxidases (Kita *et al.*, 1984a,b; Poole, 1994). Cyanide titration data of NADH oxidation in the membranes of *Z. mobilis* grown under oxygen-limited conditions (Toh and Doelle, 1997) point to the existence of, at least, two different terminal oxidases. According to these authors, the titration curve is biphasic, with approximately half of the cyanide-sensitive NADH oxidase activity being inhibited at 20 μM cyanide concentration, and the other half at 100–200 μM cyanide. The less cyanide-sensitive part falls in the range of cyanide sensitivities characteristic for cytochrome *d* (Kita *et al.*, 1984b; Jünemann, 1997), while the nature of the remaining, more cyanide-sensitive part is uncertain. Some precaution, however, is necessary, when using cyanide as a tool for analysis of the *Z. mobilis* respiratory chain. Cyanide in the submillimolar concentration range apparently has multiple targets in this bacterium, and some of them remain unidentified.

First, whole cells and membrane preparations differ strikingly in their cyanide-sensitivity (Kalnenieks *et al.*, 2000). With cyanide at 100–500 μM concentration, the initial inhibitory effect upon membrane respiration with NADH is much weaker than upon whole cells respiring ethanol. Kalnenieks *et al.* (2000) hypothesised that some essential component of the cyanide-sensitive (rapidly inhibited by cyanide) respiratory branch is either cytoplasmic, periplasmic or loosely bound to the cell membrane, and hence, easily gets lost in the process of membrane preparation. Spectral features of a *b*-type haem and flavin have indeed been demonstrated in the cytoplasmic fraction, obtained after ultracentrifugation of the cell-free extract and by a subsequent 15-fold concentration of the supernatant by ultrafiltration (Kalnenieks *et al.*, 2000). So far, relation of these components to the whole-cell cyanide-sensitive respiration has not been established. Second, the interpretation of cyanide titrations of *Z. mobilis* respiration is further complicated by the fact that one of the two ADH isoenzymes, the iron-containing ADH II, also appears to be sensitive to submillimolar cyanide concentrations (Kalnenieks *et al.*, 2003; see Section 3.4). This cyanide-sensitive ADH isoenzyme participates in respiration (see Section 4), oxidising ethanol and supplying NADH to the membrane electron transport chain (Kalnenieks *et al.*, 2002). However, ADH II is slowly interacting with cyanide (Kalnenieks *et al.*, 2003), and hence it could hardly be related to the putative rapidly inhibited cytoplasmic (or loosely membrane-bound) respiratory component, lost during membrane preparation. Finally, cyanide at submillimolar concentrations stimulates aerobic growth of *Z. mobilis*

(Kalnenieks *et al.*, 2000, 2003). The mechanism of this paradoxical effect also is not clear (see Section 3.4).

The branched structure of the respiratory chain was supported also by our work on an inhibitor analysis of electron transport with chlorpromazine and myxothiazol (Kalnenieks *et al.*, 1998). It was found that chlorpromazine inhibits the electron transport branch leading to the *bd*-type terminal quinol oxidase. Myxothiazol, on the other hand, inhibits some unidentified alternative pathway(s), but does not affect substantially the branch to cytochrome *bd*. Furthermore, the preferred pathway of electron transport to oxygen was shown to depend on the electron donor. With NADH, the electron flow to cytochrome *bd* seems to prevail, as judged from the high chlorpromazine-sensitivity of oxygen consumption in NADH-oxidising membrane preparations. With D-lactate, electrons are transported mainly to the alternative, less chlorpromazine-sensitive, more cyanide-sensitive branch(es) (Kalnenieks *et al.*, 1998). The mechanism of such an electron donor-specific branching of the electron flux might involve regulation at the level of the redox state of Q_{10}. Thus, in the electron transport chain of *Paracoccus denitrificans*, the reduction state of the quinone pool regulates the branching of the electron flux between a terminal quinol oxidase and the branch with cytochrome bc_1 (Otten *et al.*, 1999). It was shown that membrane fractions of the cytochrome bc_1-negative mutant of *P. denitrificans* (or the wild-type membranes in the presence of antimycin A) consume oxygen at significant rates only at a much higher degree of Q reduction than do the wild-type strain or the quinol oxidase-negative mutant. In *Z. mobilis* membranes, the reduction state of Q_{10} with various electron donors has not been investigated, yet a key regulatory role for Q_{10} also seems plausible. As the NADH dehydrogenases of *Z. mobilis* are much more active than the D-lactate dehydrogenase, they could be expected to sustain a higher degree of Q_{10} reduction, favouring electron transport to cytochrome *bd*. On the other hand, the less active D-lactate dehydrogenase could be expected to reduce the Q_{10} pool to a lower degree, which would be sufficient for the alternative branch, presumably bearing the bc_1-complex with a higher affinity towards ubiquinol.

3.2.2. An Outline of the Electron Transport Chain

In general, the present evidence supports a branched structure of the electron transport chain of *Z. mobilis*, with electrons from several respiratory dehydrogenases passing to Q_{10}, and then travelling further to the terminal oxidases. This is not an unexpected finding, because branching is a common pattern for bacterial electron transport (Poole and Cook, 2000). A scheme of the putative respiratory pathways in *Z. mobilis* is presented in Fig. 3. The

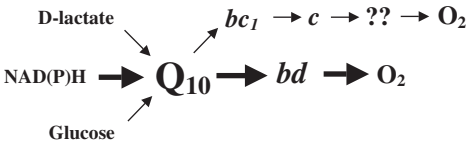

Figure 3 Aerobic electron transport chain of *Z. mobilis*.

electron transport chain carries dehydrogenases for NAD(P)H, glucose and D-lactate, donating electrons to Q_{10}. The presence of at least two terminal oxidases has now become apparent. No doubt remains that a *bd*-type quinol oxidase is terminating one of the electron transport branches. There is a good reason to assume that the bc_1 complex is also present. Most probably, being the main target for myxothiazol, it is localised in another branch, alternative to the *bd* terminal oxidase. The alternative branch might be terminated by some kind of a cyanide-sensitive cytochrome *c* oxidase, because the remaining respiratory activity, after partial inhibition of the cytochrome *bd* branch with chlorpromazine, appears to be much more sensitive to low (below 50 μM) cyanide concentrations (Kalnenieks *et al*., 1998). The genome of *Z. mobilis* contains also a gene for cytochrome *c* peroxidase and, hence, hydrogen peroxide might act as another terminal electron acceptor from the cytochrome *c*-containing branch. It must be noted that *Z. mobilis* is well equipped to cope with the active forms of oxygen: the enzymatic activities (Pankova *et al*., 1985) as well as the corresponding genes (Seo *et al*., 2005) for catalase, an iron-dependent superoxide dismutase and two kinds of peroxidases have been reported. Apart from aerobic terminal oxidases, genome data show the presence of genes for nitroreductase and fumarate reductase (Seo *et al*., 2005), possibly expressed under strictly anaerobic conditions. The corresponding activities in membrane preparations have not been investigated.

Unfortunately, two major uncertainties about the respiratory chain of *Z. mobilis* still persist. First, in spite of the availability of genome sequence, the nature of the alternative terminal oxidase(s) remains a mystery. As stated above, genome data do not support existence of *a*- or *o*-type terminal oxidases in this bacterium, which otherwise could easily account for the cyanide-sensitivity of the alternative pathway(s) (Ashcroft and Haddock, 1975; Kita *et al*., 1984a). A cytochrome cbb_3-type terminal oxidase, like the one found in the nitrogen-fixing *Bradyrhizobium* (Preisig *et al*., 1996), might be a candidate, not conflicting with spectroscopic data. Yet, a BLAST search on the *Z. mobilis* genome with cbb_3 subunit I and II sequences from the taxonomically closely related obligate aerobe *Novosphingobium aromaticovorans* does not reveal the presence of this terminal oxidase. No positive

results were given in a BLAST search using the cyanide-resistant alternative oxidase (AOX), found in higher plants, yeast and trypanosomes, which recently has been detected in *N. aromaticovorans* (Stenmark and Nordlund, 2003). The identity of the cyanide-sensitive respiratory component, which is lost during preparation of membranes (Kalnenieks *et al.*, 2000), is an open question as well. Therefore, the putative *Z. mobilis* terminal oxidases, alternative to the cytochrome *bd*, represent an intriguing area for further research.

Second, the outline structure of the *Z. mobilis* respiratory chain (Fig. 3) does not give any hint for the reason why oxidative phosphorylation in this bacterium should be performing so poorly. Presence of the putative energy-coupling NADH:quinone oxidoreductase complex and the cytochrome bc_1 complex together implies a fairly high energy conversion ratio of the electron transport, even assuming that some of the terminal oxidases are not participating in the generation of the proton-motive force (Trumpower and Gennis, 1994). Clearly, at least one of the terminal oxidases, the *bd*-type terminal quinol oxidase, does participate in generation of the proton-motive force, although with a low efficiency, reaching half of that of ubiquinol-oxidising haem-copper oxidases (Jünemann, 1997; Osborne and Gennis, 1999). Hence, one could expect a fairly efficient oxidative phosphorylation taking place in *Z. mobilis*, which at the physiological level would result in elevated aerobic cell yields, markedly exceeding those of anaerobic culture (Stouthamer, 1977). In reality, however, quite the opposite is observed. The growth yield values of aerobic cultures tend to be even lower than those of anaerobically growing cultures. Low growth yields on glucose under aerobic conditions, together with an inability to grow on non-fermentable substrates, serves as an argument against the operation of oxidative phosphorylation in *Z. mobilis*.

3.3. Oxidative Phosphorylation

3.3.1. Non-Growing Cells and Membrane Vesicles

The *Z. mobilis* cytoplasmic membrane contains all the enzymatic components needed for performing oxidative phosphorylation. Not only a complete, constitutive respiratory chain, but also a proton-dependent F_1F_0-type ATPase is present in the cytoplasmic membrane (see Section 2.3.2). It has been purified and characterised as being a typical bacterial H^+-ATP synthase (Reyes and Scopes, 1991). Notably, Dawes and Large (1970) observed an elevation of the intracellular ATP level in starved aerated *Z. mobilis* after

the addition of ethanol. They assumed oxidative phosphorylation to be the underlying mechanism, yet did not go into further details. Later, we undertook a study of oxidative ATP generation in non-growing cells and membrane vesicles of *Z. mobilis* (Kalnenieks *et al.*, 1993). Strong evidence in favour of oxidative phosphorylation was obtained. A rise of the intracellular ATP concentration was observed when ethanol or acetaldehyde was oxidised by an aerated suspension of starved cells. ATP synthesis appeared to be sensitive to the protonophoric uncoupler CCCP at 10 μM concentration, or to replacement of aeration by gassing with argon. Ethanol-consuming aerated *Z. mobilis* cell suspension generated a transmembrane pH gradient, as monitored by ^{31}P-NMR. On the other hand, ATP synthesis in starved cells could be induced by acidification of external medium of 3.5–4.0 units, thus directly demonstrating the ability of the *Z. mobilis* F_1F_0-ATPase to carry out ATP synthesis at the expense of an artificial proton gradient. The amount of synthesised ATP in this experiment was comparable to what has been previously shown for *E. coli* (Grinius *et al.*, 1975). Membrane vesicle preparations were shown to couple NADH oxidation to ATP synthesis, although, in comparison to *E. coli* membrane vesicles with P/O reaching 0.6–0.7 (Hempfling and Hertzberg, 1979), *Z. mobilis* membrane vesicles were less well coupled, with P/O (measured as ATP/NADH) only about 0.2. Oxidative ATP generation in membranes was sensitive to CCCP, as well as to DCCD, the inhibitor of the F_1F_0-ATP synthase. In addition, membrane energisation (generation of membrane potential) in aerated intact cells with ethanol as the oxidisable substrate was later demonstrated by quenching of ANS^- fluorescence upon ethanol addition (Kalnenieks *et al.*, 1996). Likewise, generation of pH gradient under similar conditions was observed by fluorescence of 9-AA (Kalnenieks *et al.*, 1995), supporting the ^{31}P-NMR data (Kalnenieks *et al.*, 1993). Kim *et al.* (1995) demonstrated generation of a membrane potential and pH gradient in NADH-oxidising membrane vesicles, prepared from aerobically cultivated *Z. mobilis*, using the fluorescent probes oxonol V and quinacrine, respectively.

The accumulating evidence for membrane energy coupling and oxidative phosphorylation in *Z. mobilis* raised the next question as to what might be the energy-coupling sites of the respiratory chain. So far, two approaches have been used to address this problem. Kim *et al.* (1995) applied two different electron acceptors for NADH oxidation by membrane vesicles prepared from aerobically cultivated cells. They were able to demonstrate generation of membrane potential and pH gradient only with oxygen as the terminal electron acceptor, but not with ubiquinone-1 in the presence of cyanide. They concluded that NADH:ubiquinone oxidoreductase does not participate in the generation of proton-motive force. Transmembrane

translocation of protons apparently takes place downstream, in the segment between ubiquinone and oxygen. In full agreement with this observation, they identified the NADH:ubiquinone oxidoreductase as being of type II, or energy non-generating bacterial NADH dehydrogenase, due to its low sensitivity to rotenone and high K_m for NADH (Matsushita *et al.*, 1987; Yagi, 1991; Friedrich *et al.*, 1994; Degli Esposti, 1998).

We chose a different approach (Kalnenieks *et al.*, 1995, 1996), trying to eliminate the putative energy-coupling site I by cultivation of *Z. mobilis* under sulphate-deficient conditions, as previously demonstrated with *E. coli* (Poole and Haddock, 1975). For aerobically cultivated cells, the energy coupling was not affected, indicating that site I might not be functioning, in accordance with findings of Kim *et al.* (1995). On the contrary, for anaerobically cultivated cells sulphate-limitation resulted in a considerable loss of oxidative phosphorylation activity, seen as a decrease of membrane energisation and lowered activity of ATP synthesis in whole cells with ethanol. These results indicate that the site I coupling significantly contributes to the oxidative energy coupling in anaerobically grown cells. According to the genome data, the Rnf-like NADH:quinone oxidoreductase complex (or, at least, some of its subunits) is the most likely candidate for the site I coupling in *Z. mobilis*. Yet, participation of this NADH:quinone oxidoreductase in the electron transport to oxygen and in the aerobic energy coupling in *Z. mobilis* would be a novel function for Rnf-type oxidoreductases, and needs to be confirmed by direct molecular evidence. Functioning of Rnf in the aerobic respiratory chain would raise further questions, concerning the role of sodium-motive force in the energy metabolism of this bacterium.

Putting together the bits of evidence, we can conclude that a shift in energy-coupling downstream in the electron transport chain takes place in *Z. mobilis* under the transition from anaerobic to aerobic growth conditions. A somewhat analogous, aeration-dependent shift of the energy-coupling sites has been well established in *E. coli* (Calhoun *et al.*, 1993; Unden, 1998; Poole and Cook, 2000). Under vigorous aeration, the type II NADH dehydrogenase together with the proton-pumping *bo′* terminal quinol oxidase represents the major electron pathway of *E. coli* respiratory chain. Under oxygen limitation, the energy-coupling type I NADH dehydrogenase complex functions in tandem with the less energetically efficient *bd* terminal oxidase (instead, having a higher affinity for oxygen). In general, these rearrangements ensure prevalence of electron transport pathways with medium (non-maximal) energetic efficiency under all growth conditions. For *E. coli* this finding has been interpreted in terms of irreversible thermodynamics: in order to be able to grow rapidly, the bacteria have to give up some efficiency of energy conversion (Westerhoff *et al.*, 1983). Clearly, in

Z. mobilis, oxidative phosphorylation does not play the same physiological role for rapid growth, as it does in *E. coli*, so the reasons for the aeration-dependent shift in energy coupling might be different. For *Z. mobilis* the aeration-dependent rearrangements in the coupling sites might be a relic from an aerobic ancestor, or, alternatively, they might bear some physiological function, not yet recognised in bacteria.

3.3.2. Does Z. mobilis Use Oxidative Phosphorylation for Aerobic Growth?

To answer this question, one should first try to estimate what contribution to the biomass yield could be expected theoretically from respiratory energetics like that of *Z. mobilis*. Based on the evidence from the previous sections, we can roughly calculate what might be the quantitative effect of oxidative phosphorylation on the biomass yield of *Z. mobilis*, growing under conditions of vigorous aeration. First, we should note that respiring *Z. mobilis* is short of reducing equivalents. Owing to the truncated Krebs cycle (see Section 2) the ED pathway is the only source of NAD(P)H in aerobically growing cells. Let us assume for calculations that under vigorous aeration typically about one-third of the maximum ethanol yield is reached (Viikari, 1988; Zikmanis *et al*., 1999; Kalnenieks *et al*., 2000). In that case, from the 2 moles of NADH, generated per mole of glucose in the ED pathway (which, under anaerobic conditions, are almost entirely used for ethanol synthesis), 1.33 moles would be oxidised in the respiratory chain, and only 0.67 moles would be left for ethanol synthesis (see Fig. 4). We may further assume that the site I coupling is not active in aerated cells (see Section 3.3.1), and that electron flow is divided between the *bd* terminal oxidase branch and the putative bc_1 branch. For the *bd* terminal oxidase the $H^+/2e^-$ ratio is 2 (Jünemann, 1997), while the bc_1 complex together with the unidentified terminal oxidase might have a higher $H^+/2e^-$ value. Taking the value of 3 as a realistic mean $H^+/2e^-$ ratio for the entire aerobic respiratory chain, and also a value of 3 for the H^+/ATP stoichiometry of ATP synthase (Kashket, 1983; Fillingame *et al*., 2003), we come to approximately 1 mole of ATP synthesised per mole of NADH oxidised. Hence, when the aerobic ethanol yield is 33% of its maximum (anaerobic) value, 1.33 moles of ATP per mole of catabolised glucose should arise from oxidative phosphorylation activity, in addition to the 1 mole of ATP coming from the substrate-level phosphorylation. For a very rough estimation, we may assume Y_{ATP} around 10 g dry wt. $(\text{mol ATP})^{-1}$ (Bauchop and Elsden, 1960; Stouthamer, 1977). The expected biomass yield ($Y_{X/S}$) of an aerobic culture of *Z. mobilis* would then be slightly above 20 g dry wt. $(\text{mol glucose})^{-1}$.

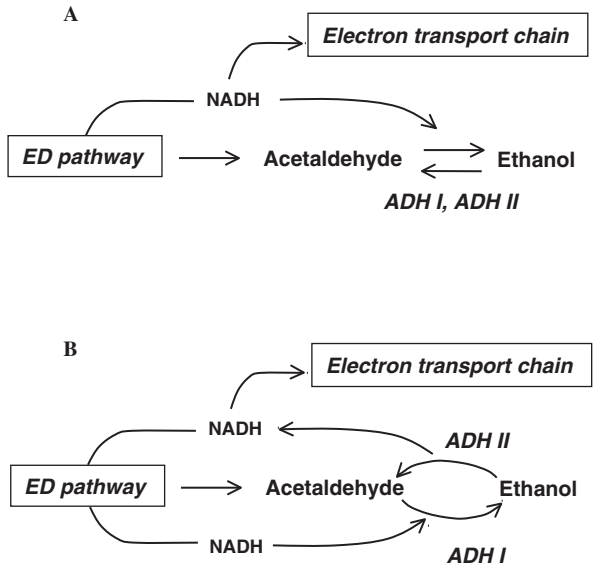

Figure 4 The distribution of the reducing equivalent flux between the respiratory chain and alcohol dehydrogenase reaction. (A) both alcohol dehydrogenase isoenzymes are catalysing ethanol synthesis and competing with the respiratory chain for NADH; (B) two isoenzymes operating in opposite directions – ADH I catalysing ethanol synthesis, with ADH II oxidising ethanol and supplying NADH to the respiratory chain.

Values of $Y_{X/S}$ close to 20 g dry wt. (mol glucose)$^{-1}$ indeed were reported by Zikmanis *et al.* (1997, 1999) under special growth conditions. These involved high growth rate in either exponential phase batch culture ($\mu > 0.4\,h^{-1}$), or continuous cultivation (flow rate $0.4\,h^{-1}$), high intensity of aeration (pO_2 51% of saturation in the fermentor), and low glucose concentrations (6.25–100 mM, corresponding to 1.125–18 gL^{-1}). The authors demonstrated that $Y_{X/S}$ in anaerobically growing control cultures under otherwise identical growth conditions did not exceed 10.0–10.2 g dry wt. (mol glucose)$^{-1}$ (Zikmanis *et al.*, 1997, 1999). Hence, in these experiments the contribution of aerobic energetics to the culture growth was clearly demonstrated, its magnitude being remarkably close to our estimated value.

However, at lower specific growth rate, less intense gassing, or higher glucose concentrations, the contribution of aeration to the culture growth yield drops to zero, or even becomes negative. Over the last three decades, in a number of works devoted to aerobic metabolism of *Z. mobilis*, very low $Y_{X/S}$ values for aerobic cultures have been reported. Belaich and Senez

(1965) found that for aerobic batch cultures, grown on complex medium with 1 gL^{-1} glucose concentration, $Y_{X/S}$ was 8.1 g dry wt. (mol glucose)$^{-1}$, and thus did not differ from $Y_{X/S}$ value of anaerobic culture under similar conditions. Bringer *et al.* (1984) reported 10.1 and 8.8 g dry wt. (mol glucose)$^{-1}$ for exponentially growing (at the specific growth rates $\mu > 0.3\,h^{-1}$) aerobic batch cultures in a complex medium with 20 gL^{-1} glucose concentration at 20% and 13% oxygen saturation, respectively. In these experiments the aerated cultures were found to grow with the same yield or just slightly below the corresponding anaerobic controls, having $Y_{X/S}$ around 9.6–10.9 g dry wt. (mol glucose)$^{-1}$.

In chemostat cultivations the same tendency is seen. A fairly broad range of culture conditions has been covered, particularly at low specific growth rates. At a flow rate (D) of 0.23 h^{-1} in a vigorously aerated chemostat (41% oxygen saturation), fed with complex medium containing 50 gL^{-1} glucose, $Y_{X/S}$ was only 5 g dry wt. (mol glucose)$^{-1}$ (Kalnenieks *et al.*, 2000). Under oxygen-limitation, $D = 0.13\,h^{-1}$, and 100 gL^{-1} glucose in the feed, the growth yield was found to be 6.8 g dry wt. (mol glucose)$^{-1}$, while the corresponding anaerobic control reached 9.5 g dry wt. (mol glucose)$^{-1}$ (Pankova *et al.*, 1985). The same group reported an aerobic growth yield of only 2.69 g dry wt. (mol glucose)$^{-1}$ under more energetic aeration (Pankova *et al.*, 1988). At D of 0.08 h^{-1} and 140 gL^{-1} of glucose in the feed, only 1.4 g dry wt. (mol glucose)$^{-1}$ for aerobic culture and 2.3 g dry wt. (mol glucose)$^{-1}$ for anaerobic culture was obtained (Sahm and Bringer-Meyer, 1987). It is not surprising *per se* that the growth yield of *Z. mobilis* inversely correlates with growth rate and substrate concentration, because the relative impact of maintenance requirements at low growth rate (Pirt, 1965) as well as that of energy-spilling reactions at high glucose concentrations (see Section 2) increase. Yet, what seems most surprising and obviously needs an explanation is the fact that under such conditions the contribution of aerobic energy metabolism to the culture growth is equal to zero, or even negative.

Toh and Doelle (1997) have studied the effects of oxygen-limited growth in more detail. They gradually increased the input oxygen partial pressure from 0 to 290 mmHg in a chemostat (0.85 L working volume, 0.15 $Lmin^{-1}$ gassing, 300 r.p.m. stirring rate), which was operated at $D = 0.137\,h^{-1}$, and fed with complex medium, containing 24 gL^{-1} glucose. They noted that the growth yield gradually increased from about 5 g dry wt. (mol glucose)$^{-1}$ at 0 mmHg O_2 to 6.8 g dry wt. (mol glucose)$^{-1}$ at higher oxygen partial pressures. At 290 mmHg, when O_2 was no longer limiting, and pO_2 in the fermentor raised to 25% of saturation, $Y_{X/S}$ dropped to 3.6 g dry wt. (mol glucose)$^{-1}$. Notably however, the extrapolated $Y_{X/S}^{max}$ value under oxygen-limited growth in this study was 22.8 g dry wt. (mol glucose)$^{-1}$, resembling

the aerobic yield of 20.3 g dry wt. (mol glucose)$^{-1}$, measured by Zikmanis *et al.* (1999).

3.3.3. *Possible Reasons for the Inefficient Aerobic Growth*

Accumulation of acetaldehyde in the aerobic culture is the most widely accepted explanation of the poor aerobic growth of *Z. mobilis*. Acetaldehyde is one of the metabolites (together with dihydrohyacetone, acetoin and acetate), accumulating specifically in aerobic cultures (Viikari, 1986, 1988; Tanaka *et al.*, 1990). Its accumulation is the direct result of NADH withdrawal from the ADH reaction by the respiratory chain (see Fig. 4). Apparently, acetaldehyde is the most reactive of the accumulated compounds. It is reported to inhibit *Z. mobilis* growth at only 0.5 gL^{-1} concentration in the medium (Wecker and Zall, 1987; Ishikawa *et al.*, 1990; Ishikawa and Tanaka, 1992). In shaken flasks at an early stationary phase of aerated batch culture, acetaldehyde concentration may exceed 2 gL^{-1} (Kalnenieks *et al.*, 2000), which is known to cause severe inhibition of growth and metabolism in *Z. mobilis* (Wecker and Zall, 1987). Accumulation of acetaldehyde at high concentrations therefore explains why the growth and glucose consumption of aerobic batch culture become inhibited soon after the beginning of exponential phase.

Yet, inhibition of the culture growth at high acetaldehyde concentrations does not contribute much to an explanation of the low growth yield values in the explored range of the aerobic growth conditions. Thus, variation of acetaldehyde concentration in the range below 1 gL^{-1} hardly affects the growth yield. In the oxygen-limited chemostat experiments of Toh and Doelle (1997) an almost constant $Y_{X/S}$ around 6.8 g dry wt. (mol glucose)$^{-1}$ was observed over the steady-state acetaldehyde concentration range from 0.12 to 0.8 gL^{-1}. On the other hand, at the same acetaldehyde concentration, chemostat cultures with different growth yield values have been obtained. For acetaldehyde concentration around 0.4 gL^{-1}, a culture with a growth yield of 5 g dry wt. (mol glucose)$^{-1}$ (Kalnenieks *et al.*, 2000) as well as one with a growth yield of 20.3 g dry wt. (mol glucose)$^{-1}$ (Zikmanis *et al.*, 1999) have been reported. Zikmanis *et al.* (1999) have even observed a positive correlation between the growth yield and acetaldehyde concentration in the range between 5 and 9 mM (approx. 0.2–0.4 gL^{-1}) at D close to 0.4h^{-1}. This seems to be in agreement with the observations that acetaldehyde at low concentration helps *Saccharomyces cerevisiae* and *Z. mobilis* cells to recover after environmental shock (Stanley *et al.*, 1997).

The inhibitory effect of this metabolite upon ATP generation in oxidative phosphorylation is quite small (Kalnenieks *et al.*, 1993). In starved cells,

acetaldehyde itself can serve as a substrate for ATP synthesis, and only a slight inhibitory effect is noted at concentrations close to 1 gL^{-1}. The inhibition by acetaldehyde of ATP synthesis induced by an artificial pH gradient is negligible (Kalnenieks *et al.*, 1993). Weak inhibition of oxidative phosphorylation in non-growing cells leads us to suppose that acetaldehyde could not be the main reason for the apparent absence of oxidative phosphorylation in growing cultures. Interestingly, Toh and Doelle (1997) reported inhibition of the membrane ATPase activity under aerobic growth conditions, and Zikmanis *et al.* (1999) suggested that acetaldehyde might be the inhibitor. These workers put forward a hypothesis, stating that the rise of $Y_{X/S}$, as seen with *Z. mobilis* under certain aerobic culture conditions, could be the result of redirection of the ATP flux towards cellular biosynthesis, because the main energy-spilling reaction, the membrane H^+-ATPase, is inhibited. This would imply participation of the aerobic respiratory chain in the generation of the transmembrane proton-motive force in place of the inhibited membrane ATPase, yet without additional ATP production via oxidative phosphorylation. Although interesting, this hypothesis seems problematic, because in both works only a very slight inhibition of ATPase was observed under the conditions in which the aerobic metabolism was found to contribute significantly to the increase of the growth yield.

Inefficient energy coupling in the membrane (possibly, the same unidentified mechanism, causing the uncoupled growth under anaerobic conditions; see Section 2.3.3), or activation of an energetically inefficient respiratory pathway under conditions of low specific growth rate/high glucose concentration seem the most realistic alternatives to acetaldehyde. Unfortunately, both are merely speculative at the moment. The only experiment-based conclusion could be drawn from cultivation experiments in the presence of cyanide (Kalnenieks *et al.*, 2000). Namely, the cyanide-sensitive component of respiration almost does not differ from the cyanide-resistant part with respect to its contribution to growth efficiency, because aerobic growth with partially inhibited respiration in the presence of cyanide does not cause any significant change of $Y_{X/S}$.

3.4. The Cyanide Effect

One might speculate that the simplest way to discover the physiological role of respiration in a bacterium like *Z. mobilis* would be to switch it off, and see what happens with an aerobically growing culture. Following this idea, we used cyanide as the respiratory inhibitor. Cyanide was chosen because it is

one of the few water-soluble inhibitors able to cross the membranes of *Z. mobilis*, thus allowing it to be used in growing, intact cells. Unexpectedly, we found that at submillimolar concentrations (20–500 μM) it stimulates culture growth, at the same time inhibiting respiration (Kalnenieks *et al.*, 2000). In an aerobic batch culture, the lag phase in the presence of cyanide is slightly extended, but exponential growth persists longer, resulting in higher final biomass density. The inhibition of respiration is transient, possibly due to degradation and/or evaporation of cyanide (Kalnenieks *et al.*, 2000). Cyanide elevates the aerobic growth yield only to a minor extent, but mostly acts to improve the kinetics of growth and glucose consumption, which otherwise, during aerobic shaken-flask cultivation, rapidly become inhibited.

Rapid cessation of growth and glucose uptake happens largely due to a massive acetaldehyde accumulation during aerobic batch cultivation in shaken flasks (see Section 3.3.3). Obviously, when respiration is partially or fully inhibited by cyanide, more reducing equivalents are directed to ethanol synthesis and, hence, less acetaldehyde is accumulated (Fig. 4). This, in part, explains the observed effect. However, in a continuous culture, in which acetaldehyde has been gassed out of the medium by means of vigorous aeration, the growth-stimulating effect of cyanide still persists (Kalnenieks *et al.*, 2000).

The growth in the presence of cyanide absolutely does not influence the cyanide-resistance of oxygen consumption (Kalnenieks *et al.*, 2003), and also no cyanide-induced changes of the cytochrome content can be seen in the spectra of membrane preparations (see Section 3.1.1). In a further search for intracellular targets for cyanide in *Z. mobilis*, it was found that the iron-containing ADH II also is sensitive to cyanide (Kalnenieks *et al.*, 2003). At submillimolar concentrations, cyanide causes a gradual inhibition of this enzyme. Notably, the apparent cyanide-sensitivity of ADH II varies in response to the intracellular NADH concentration. Recently, it was shown that cyanide acts as a competitive inhibitor of ADH II, competing with nicotinamide nucleotides, and that NADH increases both cyanide-resistance and oxygen-resistance of this enzyme (Kalnenieks *et al.*, 2005). The variable cyanide-sensitivity of ADH II looks even more intriguing in the context of the finding that ADH II is one of the major stress proteins in *Z. mobilis* (An *et al.*, 1991). However, these unexpected properties of ADH II appear not to be directly involved in the mechanism of the stimulating effect of cyanide. Recently, we found that aerobic growth of an ADH II-negative mutant strain is being stimulated by cyanide to a similar extent (Kalnenieks, unpublished results). Apparently, other targets for cyanide in *Z. mobilis* exist, which are essential for the stimulatory effect.

3.5. The Physiological Role of Respiration in *Z. mobilis*

Above all, *Z. mobilis* respires at higher rates than do several well-explored laboratory microorganisms. Pankova *et al.* (1988) have observed that after a transient increase of aeration in an oxygen-limited continuous culture, the specific rate of oxygen consumption reaches 23.45 mmol g dry wt.$^{-1}$ h^{-1}. That closely resembles the exceptionally high respiration rate of a potassium-limited culture of *Klebsiella aerogenes* (Hueting *et al.*, 1979), and markedly exceeds the oxygen consumption rates seen, for example, in continuous cultures of *E. coli* (Buurman *et al.*, 1991) or *S. cerevisiae* (Visser *et al.*, 2004). Paradoxically, it is still an open question, as to whether there is any physiological role for respiration in this organism. Respiration in most cases does not contribute to the growth yield. Cultivation in the presence of cyanide suggests that the respiration rate in *Z. mobilis* might be excessive for the purpose of culture growth, because its partial inhibition stimulates growth and glucose consumption. On the other hand, energy production for growth is by no means the only function in which a bacterial respiratory chain might be involved (Poole and Cook, 2000).

Oxidative phosphorylation might be expected to serve as a source of energy for stationary phase, non-growing cultures, which have run out of sugar and have accumulated ethanol in the medium (Kalnenieks *et al.*, 1993). Slow oxidation of ethanol as a substrate for energy generation in order to maintain cellular integrity and viability in an aerobic environment, which contains ethanol and is depleted of sugar, might be a part of the natural life cycle of *Z. mobilis*. Although this assumption may look reasonable, direct evidence, showing an improved survival of *Z. mobilis* under aerobic or microaerated conditions in media with ethanol, is still lacking.

Alternatively, the production of inhibitory metabolites, like acetaldehyde, might be a competitive growth strategy of aerated *Z. mobilis*. We may speculate that *Z. mobilis* prefers production of substances, inhibitory for other bacteria, at the expense of rapid growth of its own biomass. Indeed, it has been observed that *Z. mobilis* is inhibitory for other bacteria, like *E. coli*, in interspecies conjugation (Pappas *et al.*, 1997) and possibly acetaldehyde production contributes to this strategy. For anaerobic growth of *Z. mobilis*, a similar idea has been put forward by Snoep *et al.* (1996), stating that the rapid, inefficient conservation of energy by glycolysis might be ecologically advantageous for *Z. mobilis*, living in sugar-rich natural environments. High rates of ethanol production in combination with high level of ethanol tolerance might serve to limit the growth of competing organisms.

A respiratory-protective function of oxygen consumption could be one more intriguing possibility. Rapid respiration, not really needed for *Z. mobilis*

under most growth conditions, probably is important for nitrogen fixation taking place under some special circumstances in the natural environment. Previously, a role of respiration for protection of *Z. mobilis* against oxygen and its active forms has been suggested by Pankova *et al.* (1988). Yet, our recent evidence on the stimulatory effect of cyanide makes the idea of oxygen toxicity for *Z. mobilis* under usual growth conditions to look doubtful. Respiratory protection has been thoroughly investigated in *A. vinelandii*, showing the importance of a rapid and energetically inefficient respiration for free-living, nitrogen-fixating bacteria (Poole and Hill, 1997). Apart from the recent genome data (Seo *et al.*, 2005), nothing is known about nitrogen-fixation in *Z. mobilis*, so in this context its rapid and inefficient respiration may well be just a relic, remaining from a diazotrophic ancestor.

In order to understand the structure, function and physiological role of the respiratory chain, construction of various respiratory mutants would be of prime importance. So far, no respiratory mutants of *Z. mobilis* are available. Hopefully, genetic dissection of *Z. mobilis* respiratory chain in the nearest future will provide answers to at least some of the open questions formulated in this review.

4. RESPIRATION VERSUS ETHANOL SYNTHESIS: THE ETHANOL CYCLE

4.1. Kinetic Parameters of Respiration and Ethanologenesis

As already mentioned in the previous sections, under aerobic conditions some of the NADH generated in the ED pathway is oxidised in the respiratory chain. Obviously, the respiratory chain is competing for NADH with the ADH reaction (Fig. 4). That inevitably leads to a decrease in the ethanol yield relative to anaerobic conditions, and to accumulation of acetaldehyde in the growth medium (Sahm and Bringer-Meyer, 1987; Viikari, 1988). Under vigorous aeration, the decrease of ethanol yield appears to be very significant. Anaerobic cultures produce ethanol with a yield close to the theoretical maximum value of 0.51 g of ethanol per g of glucose (Rogers *et al.*, 1982). Much lower yields, like $0.25\,g\,g^{-1}$ (Belaich and Senez, 1965), $0.17\,g\,g^{-1}$ (Viikari, 1988), $0.29\,g\,g^{-1}$ (Zikmanis *et al.*, 1997), $0.16\,g\,g^{-1}$ (Zikmanis *et al.*, 1999), or $0.13\,g\,g^{-1}$ (Kalnenieks *et al.*, 2000) have been reported for aerobic cultures. The low ethanol yield, as well as accumulation of byproducts, more oxidised than ethanol (acetaldehyde, dihydroxyacetone, acetoin, acetate), indicates that in aerated cultures a substantial, and in

many cases even the major, part of NADH is being oxidised in the respiratory chain.

The seemingly simple and straightforward competition between ethanologenesis and respiration in *Z. mobilis* looks less trivial when we compare the activities and kinetic parameters of the respiratory chain to those of the ADH. In *Z. mobilis*, the reaction of ethanol synthesis is catalysed by the ADH isoenzymes ADH I and ADH II (Neale *et al.*, 1986). Although catalysing the same reaction, both isoenzymes are unrelated to each other structurally: ADH I is a member of group I ADHs and contains zinc in its active site, while ADH II belongs to group III ADHs and contains iron (Reid and Fewson, 1994). If both ADH isoenzymes were simultaneously catalysing ethanol synthesis, the activity of the respiratory chain would be insufficient to compete for NADH with the ADH reaction. Both ADH isoenzymes represent the endpoint of the mighty *Z. mobilis* "catabolic highway" (see Section 2), and together make almost 5% of the soluble cell protein (Kinoshita *et al.*, 1985; Neale *et al.*, 1986), ensuring the high rate of ethanol synthesis in anaerobic culture. The total activity of both isoenzymes in anaerobically grown cell extracts in the direction of acetaldehyde reduction at pH 6.5 is close to 2.1 U mg dry wt.$^{-1}$ (roughly, 4 U mg protein^{-1}), with approximately equal contributions from each isoenzyme (Neale *et al.*, 1986). In permeabilised cells and cell extracts, prepared from aerobically growing continuous culture, a total ADH activity of 1.0–1.2 U mg dry wt.$^{-1}$ has been reported (Toh and Doelle, 1997; Kalnenieks *et al.*, 2005). The activity of the NADH oxidase in cell membrane preparations, as well as the respiratory activity of whole cells with glucose, is much lower. For cell-free extracts the reported values fall in the range between 0.05 and 0.2 U mg total protein^{-1} (Bringer *et al.*, 1984; Pankova *et al.*, 1988; Kalnenieks *et al.*, 1995). Similarly, for washed suspensions of whole cells, the oxygen consumption rate with glucose has been found to be close to 0.10–0.13 U mg dry wt.$^{-1}$ (Bringer *et al.*, 1984; Sahm and Bringer-Meyer, 1987), or around 0.085 U mg dry wt.$^{-1}$ (Pankova *et al.*, 1988). As already mentioned before, batch and continuous cultures of *Z. mobilis* respire with high rates, reaching 0.2–0.4 U mg dry wt.$^{-1}$ (Pankova *et al.*, 1988; Kalnenieks *et al.*, 2000), which is still far below the reported ADH activities.

Not only are the maximum velocities of the ADH isoenzymes higher, but also their K_m values for NADH are lower than that of NADH oxidase. As discussed above, in aerobically growing cells the type II NADH dehydrogenase with K_m for NADH around 60 μM prevails. For ADH I and ADH II, the corresponding K_m values (at saturating acetaldehyde concentration) are 27 and 12 μM, respectively (Kinoshita *et al.*, 1985). The acetaldehyde concentration in aerobic cultures usually reaches several

millimolar to several tens of millimolar (Viikari, 1988; Kalnenieks *et al.*, 2000), while the K_m for acetaldehyde is just 86 μM for ADH I and 1.3 mM for ADH II (Kinoshita *et al.*, 1985). Therefore, in aerobic culture both ADH isoenzymes are indeed operating at near-saturating acetaldehyde concentrations, and the apparent K_m values for NADH *in vivo* might be fairy close to the reported *in vitro* data. In contrast, NADH concentrations might not be saturating. In a vigorously aerated chemostat (1.5 l working volume, air flow at 3 $Lmin^{-1}$ and stirring speed of 400 rpm, resulting in stationary pO_2 of 40–50% saturation) the intracellular NADH concentration reaches only about 10 μM (Kalnenieks *et al.*, 2002, 2005). This is close to the K_m values of both ADH, yet well below the K_m of the NADH dehydrogenase, making the respiratory chain even less competitive than it would be at higher intracellular NADH concentrations. Taken together, it is hard to understand how it could happen that the major part of NADH is being oxidised by the respiratory chain, but not scavenged by the ADH.

4.2. Two ADH Isoenzymes Operating in Opposite Directions?

We have attempted to explain this major discrepancy by the "ethanol cycle" (Fig. 4) model (Kalnenieks *et al.*, 2002). The ethanol cycle hypothesis stems from the analysis of aerobic chemostat cultures, in which the steady state has been perturbed with a small ethanol pulse. The ADH reaction and respiration were investigated in a vigorously aerated continuous culture at 50% pO_2 (Kalnenieks *et al.*, 2002). Under steady state, a slow net production of ethanol took place in parallel with oxygen consumption. However, in response to addition of a small dose of ethanol, a transient burst of ethanol oxidation occurred, seen as rapid acetaldehyde synthesis, rise of the intracellular NADH concentration, and a steep decrease of pO_2 in the chemostat. The estimated rate of the transient ethanol oxidation was approximately four times higher than that of the steady-state background ethanol synthesis. The authors postulated that the rapid, transient ethanol oxidation reveals a perturbation of a cycle, consisting of an ethanol-synthesising and ethanol-oxidising reaction, both running several times faster than the net ethanol synthesis. ADH II was regarded as the putative ethanol oxidiser, while ADH I was assumed to catalyse ethanol synthesis. Such distribution of the roles between the isoenzymes is supported by the fact that an ADH II-negative strain (Kalnenieks *et al.*, manuscript in preparation) under identical culture conditions is unable to oxidise the added ethanol.

The ethanol cycle model explains the capacity of respiration to oxidise a large part of NADH, by turning one of the two ADH isoenzymes from the

role of competitor into a participant in the respiratory process. At the same time, the model raises further fundamental questions. Simultaneous catalysis of a reaction in two opposite directions would be thermodynamically impossible, unless the isoenzymes are situated in different microenvironments and exposed to different reactant concentrations. It seems possible that the ADH isoenzymes in *Z. mobilis* cells are indeed differently supplied with NADH. Notably, direct NADH channelling between dehydrogenases has been the subject of debate in general biochemistry for a long time (Srivastava and Bernhard, 1984; Wu *et al.*, 1991; Martinez Arias and Pettersson, 1997; Miles *et al.*, 1999), and is still going on. The discussions about metabolite (and, in particular, NADH) channelling have been focussed upon the data of pre-steady-state kinetic experiments with purified, concentrated enzyme solutions, with little relevance to intracellular conditions. Much less is known about the *in vivo* situation. There exists some interesting electron microscopic evidence, indirectly supporting NADH channelling in *Z. mobilis* cells. Electron microscopy, using gold-labelled antibodies, reveals existence of a supramolecular complex, involving glyceraldehyde-3-phosphate dehydrogenase and ADH I (Aldrich *et al.*, 1992). In the case of NADH channelling taking place inside the putative enzyme complex, the local concentration of NADH at the ADH I active site would be kept much higher than in the cytosol, promoting acetaldehyde reduction even under conditions of vigorous aeration. Alternatively, the driving force of the ethanol cycle might represent some kind of specific interaction between ADH II and respiratory NADH dehydrogenase(s), facilitating a rapid withdrawal of NADH from the active site of ADH II and substituting it by NAD^+. NADH channelling between some mitochondrial dehydrogenases and the complex I has been described (Fukushima *et al.*, 1989). Further research would be necessary to unravel the driving mechanism of the cycle, as well as to establish its role in the coordination between respiration and ethanol synthesis.

5. CONCLUDING REMARKS

Z. mobilis is a bacterium with clearly "extreme" features of energy metabolism, like its very rapid fermentative catabolism and the apparently inefficient oxidative energy metabolism, without an obvious physiological role for its host, yet with high respiration rate and a somewhat strange mode of regulation. These traits of *Z. mobilis* physiology were extensively studied in the 1980s and 1990s, yet, as it follows from our analysis, several fundamental questions were left behind, as the common interest shifted

predominantly to metabolic engineering of this promising biotechnological producer. However, better understanding of *Z. mobilis* physiology and energetics might help to reach today's goals of metabolic engineering, which inevitably pose difficult questions concerning the energy metabolism of the recombinant strains (see e.g., Kim *et al*., 2000; Lawford and Rousseau, 2000). The unsolved problems deserve serious reconsideration, based on the recently available genome information and on a broader spectrum of molecular methods, now applicable to *Z. mobilis*. The hidden reasons for the poor aerobic energy coupling and the pathway(s) of energy dissipation under conditions of uncoupled growth, as well as the exact structure of the respiratory chain need to be established for this bacterium.

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Microbial Degradation of Organophosphorus Xenobiotics: Metabolic Pathways and Molecular Basis

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ABSTRACT

Organophosphorus (OP) xenobiotics are used worldwide as pesticides and petroleum additives. OP compounds share the major portion of the pesticide market globally. Owing to large-scale use of OP compounds, contaminations of soil and water systems have been reported from all parts of the world. OP compounds possess very high mammalian toxicity and therefore early detection and subsequent decontamination and detoxification of the polluted environment is essential. Additionally, about 200,000 tons of extremely toxic OP chemical warfare agents are required to be destroyed by 2007 under Chemical Warfare Convention (1993). Chemical and physical methods of decontamination are not only expensive and time-consuming, but also in most cases they do not provide a complete solution. These approaches convert compounds from toxic into less toxic states, which in some cases can accumulate in the environment and still be toxic to a range of organisms. Bioremediation provides a suitable way to remove contaminants from the environment as, in most of the cases, OP compounds are totally mineralized by the microorganisms. Most OP compounds are degraded by microorganisms

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in the environment as a source of phosphorus or carbon or both. Several soil bacteria have been isolated and characterized, which can degrade OP compounds in laboratory cultures and in the field. The biochemical and genetic basis of microbial degradation has received considerable attention. Several genes/enzymes, which provide microorganisms with the ability to degrade OP compounds, have been identified and characterized. Some of these genes and enzymes have been engineered for better efficacy. Bacteria capable of complete mineralization are constructed by transferring the complete degradation pathway for specific compounds to one bacterium. In the present article, we review microbial degradation and metabolic pathways for some OP compounds. The biochemical and molecular basis of OP degradation by microbes and the evolution and distribution of genes/enzymes are also reviewed. This article also examines applications and future use of OP-degrading microbes and enzymes for bioremediation, treatment of OP poisoning, and as biosensors.

1. INTRODUCTION

Organophosphorus (OP) compounds are widely used worldwide as pesticides and petroleum additives. OP-based pesticides have been in use since

1937 (Dragun *et al.*, 1984). Owing to the long persistence and toxicity of organochlorine pesticides, the use of OP pesticides has been growing, as they were considered environmentally less problematic (due to their biodegradability) and biologically more efficient. At present, OP pesticides constitute the largest group of pesticides used globally (34%). In the USA alone, about 50,000 tons of OP pesticides are used annually (Ballantyne and Marrs, 1992). OP compounds have been used as agricultural (to control crop pests), domestic (to control mosquitoes, etc.), and veterinary (to control mites and flies of cattle) pesticides. Although biodegradable, OP compounds have attracted a lot of attention from toxicologists due to their high mammalian toxicity and acute and chronic toxicity to other non-target organisms. It is estimated that OP pesticides cause around 3 million poisonings and 200,000 human deaths annually, mostly in developing countries (Karalliedde and Senanayake, 1999). Additionally, acute and chronic exposure to OP compounds has been implicated in a range of nerve and muscular disorders (Ragnarsdottir, 2000; Galloway and Handy, 2003). Another major source of OP xenobiotics includes extremely toxic chemical warfare agents (CWAs), also called ''nerve agents'' due to their mode of action. It is estimated that about 30,000 tons in the USA and about 200,000 tons of nerve agents globally are required to be destroyed under the Chemical Weapons Convention (CWC) by 2007.

The OP pesticides are esters or thiols derived from phosphoric, phosphonic or phosphoramidic acid (Sogorb and Vilanova, 2002). The general chemical structure of OP is shown in Fig. 1. R_1 and R_2 are generally aryl or alkyl groups that are bonded to P atom either directly (phosphinates) or through oxygen (phosphates) or sulphur (phosphothioates) atom. In phosphonates, R_1 is directly bonded to the P atom and R_2 is linked either to an oxygen or sulphur atom (phosphonothioates). At least one of the R groups is linked to $-NH_2$ in phosphoramidates. Finally, the X group, which is also called the ''leaving group'' (because this group is released upon hydrolysis of OP compounds), may be a halogen, aliphatic, aromatic or heterocyclic group.

The mode of action of OPs involves inhibition of acetylcholine breakdown. Acetylcholine plays an important role in transmitting nerve impulses

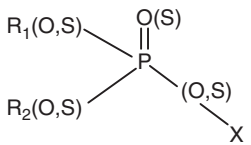


Figure 1 General structure of organophosphorus compounds.

in the brain skeletal and muscular systems. However, after the transmission, acetylcholine must be hydrolysed to avoid overstimulation of the nervous systems. This hydrolysis is brought about by an esterase called acetylcholinesterase (AChE), which results in the formation of choline and acetyl CoA. OP compounds bind to the active site of the AChE, and then the leaving group breaks off from it resulting into phosphorylated AChE. The hydrolysis of phosphorylated AChE is extremely slow and results in the overstimulation of the nervous system which in turn causes agitation, hypersalivation, confusion, convulsion, respiratory failure and ultimately death to insects and mammals (Ragnarsdottir, 2000).

2. MICROBIAL METABOLISM OF ORGANOPHOSPHORUS XENOBIOTICS

Microbial degradation of OP insecticides has been recognized as the most important process controlling their environmental fate (Felsot, 1989). However, the extensive and repeated use of soil-applied OP compounds on certain occasions has led to reduced biological efficacy due to microbial adaptation. This phenomenon was named as enhanced or accelerated biodegradation, and was attributed to the development of a soil microbial population that was able to rapidly mineralize the OP pesticides. The vulnerability of OP compounds to microbial adaptation has been reported for several compounds including the insecticides parathion (Sethunathan, 1973), diazinon (Sethunathan, 1971; Sethunathan and Pathak 1972), isofenphos (Racke and Coats, 1987), chlorfevinphos (Suett *et al*., 1996), phorate (Suett and Jukes, 1997) and the nematicides cadusafos (Karpouzas *et al*., 2004b), ethoprophos (Smelt *et al*., 1987; Karpouzas *et al*., 1999b) and fenamiphos (Anderson and Lafuenza, 1992; Stirling *et al*., 1992; Davis *et al*., 1993; Singh *et al.,* 2003; Karpouzas *et al*., 2004a).

Soils exhibiting enhanced biodegradation of organophosphorus compounds, or soils that were heavily contaminated with high concentrations of such compounds, have commonly been used as sources for the isolation of microorganisms with increased capability to rapidly degrade these compounds. The enrichment culture technique in selective mineral salts media, where the OP pesticides act as the sole carbon, nitrogen or phosphorus source, has been used in the vast majority of cases in order to isolate such pesticide-degrading microorganisms. In most cases, the isolated microorganisms are able to utilize the pesticide as a source of a single element (C, N, P or S). For example, a *Pseudomonas putida* strain was able to use

ethoprophos as a carbon source but not as a phosphorus source (Karpouzas *et al*., 2000). However, other studies have led to the isolation of microorganisms, which could only co-metabolize and were not able to utilize OP compounds as a source of energy. A list of some OP-degrading microorganisms is presented in Table 1.

Studies in pure cultures with the isolated microorganisms revealed that there are four major reactions involved in OPs metabolism: hydrolysis, oxidation, alkylation and dealkylation. Hydrolysis of the phosphoesteric P–O–C or phosphothiesteric P–S–C bonds present in the OP molecules is considered the initial step in their metabolism. The reduced mammalian toxicity of the hydrolysis products is the main reason for the lack of detailed studies on the subsequent transformations of the produced metabolites by the isolated microorganisms. In this paper, we review metabolic pathways of some representative OP compounds (Fig. 2).

2.1. Insecticides

2.1.1. Phenyl-Substituted Organophosphates

Parathion (*O,O-diethyl-O-p-nitrophenylphosphorothioate*): Parathion has been one of the most important OP insecticides worldwide. However, its high mammalian toxicity (LD50 = 10 mg kg^{-1} body weight) has resulted in its recent withdrawal from the European market (Annex I, Directive 91/414/EEC). Several studies have documented the involvement of soil microorganisms in the degradation of parathion in soil under both aerobic (Ferris and Lichtenstein, 1980) and anaerobic conditions (Rebby and Sethunathan, 1983).

Several studies with parathion-enrichment cultures led to the isolation and characterization of a great variety of bacterial species that were able to hydrolyse parathion (Sethunathan and Yoshida, 1973; Siddaramappa *et al*., 1973; Adhya *et al*., 1981). Sethunathan and Yoshida (1973) were the first to report the isolation of a *Flavobacterium* strain ATCC 27551, which was able to rapidly hydrolyse parathion leading to the accumulation of *p*-nitrophenol. In a concurrent study, Siddaramappa *et al*. (1973) isolated two bacteria, a *Bacillus* sp. and a *Pseudomonas* sp. from flooded soil. *Pseudomonas* sp. hydrolysed parathion and then released nitrite from the *p*-nitrophenol. On the contrary, *Bacillus* sp. was unable to hydrolyse parathion but was able to use *p*-nitrophenol as a sole carbon source as soon as it was formed. Munnecke and Hsieh (1974) reported the isolation of a mixed bacterial culture consisting of four *Pseudomonas* sp., a *Xanthomonas* sp., an *Azotomonas* sp.

Table 1 A list of isolated microorganisms that degrade OP pesticides

Compound	Microorganisms	Reference
Parathion	*Bacillus subtilis*	Yasuno *et al.* (1965)
	Rhizobium spp.	Mick and Dahm (1970)
	Chlorella pyrenoidosa	Zuckerman *et al.* (1970)
	Flavobacterium sp. ATCC 27551	Sethunathan and Yoshida (1973)
	Bacillus sp. And *Pseudomonas* sp.	Siddaramapa *et al.* (1973)
	Mixed bacterial culture (*Pseudomonas* spp., *Azotomonas* sp., *Xanthomonas* sp., *Brevibacterium* sp.)	Munnecke and Hsieh (1974)
	Penicillium waksmani	Rao and Sethunathan (1974)
	Pseudomonas stutzeri, *Pseudomonas aeruginosa*	Daughton and Hsieh (1977)
	Unidentified bacteria	Cook *et al* (1978a)
	Pseudomonas spp.	Rosenberg and Alexander (1979)
	Pseudomonas diminuta	Serdar *et al.* (1982)
	Arthrobacter sp., *Bacillus* sp.	Nelson (1982)
	Pseudomonas sp., *Xanthomonas* sp.	Tchelet *et al.* (1993)
Methylparathion	*Bacillus subtilis*	Miyamoto *et al.* (1966)
	Flavobacterium sp. ATCC 27551	Adhya *et al.* (1981)
	Trichoderma viride	Baarschers and Heitland (1986)
	Pseudomonas sp., *Flavobacterium* sp.	Chaudhry *et al.* (1988)
	Bacillus sp.	Sharmila *et al.* (1989)
	Bacillus sp.	Ou and Sharma (1989)
	Unidentified bacteria	Misra *et al.* (1992)
	Pseudomonas putida	Rani and Lalithakumari (1994)
	Burkholderia sp.	Hayatsu *et al.* (2000)
	Burkholderia cepacia, *Bacillus* sp.	Keprasertsup *et al.* (2001)
	Plesiomonas sp.	Zhongli *et al.* (2001)
	Pseudomonas sp.	Zhongli *et al.* (2002)
	Pseudomonas sp.	Yali *et al.* (2002)
Fenitrothion	*Bacillus subtilis*	Miyamoto *et al.* (1966)
	Flavobacterium sp. ATCC 27551	Adhya *et al.* (1981)
	Pseudomonas sp.	Adhya *et al.* (1981)

Table 1 (*continued*)

Compound	Microorganisms	Reference
	Bacillus sp.	Sharmila *et al.* (1989)
Diazinon	*Arthrobacter* sp., *Streptomyces* sp.	Gunner and Zuckerman (1968)
	Flavobacterium ATCC 27551	Sethunathan and Yoshida (1973)
	P. putida	Rosenberg and Alexander (1979)
	Pseudomonas sp.	Adhya *et al.* (1981)
	Arthrobacter sp.	Ohshiro *et al.* (1996)
Chlorpyrifos	*Arthrobacter* sp.	Misra *et al.* (1992)
	Fungi	Bumpus *et al.* (1993)
	Micrococcus sp.	Guha *et al.* (1997)
	Arthrobacter sp.	Ohshiro *et al.* (1996)
	Flavobacterium ATCC 27551	Mallick *et al.* (1999)
	Hypholoma fasciculare, Coriolus versicolor	Bending *et al.* (2002)
	Enterobacter sp.	Singh *et al.* (2004)
	Alcaligenes faecalis	Yang *et al.* (2005)
Malathion	*Thrichoderma viride*	Matsumura and Boush (1966)
	Aspergillus niger, *Penicillium notatum*, *Rhizoctonia solani*	Matsumura and Boush (1966)
	Rhizobium trifolii, R. Leguminosarum	Mostafa *et al.* (1972b)
	Arthrobacter sp.	Walker and Stojanovic (1974)
	Bacterial strains	Paris *et al.* (1975)
	Bacterial strains	Bourquin (1977)
	Pseudomonas sp.	Rosenberg and Alexander (1979)
	Pseudomonas sp.	Singh and Seth (1989)
	Aulosira fertilissima, Nostoc muscorum	Subramanian *et al.* (1994)
	Micrococcus sp.	Guha *et al.* (1997)
	Aspergillus sydowii, *A. Flavus*, *Fusarium oxysporum*	Hasan (1999)
	Pseudomonas sp.	Imran *et al.* (2004)
	Fusarium oxysporum f.sp. Pisi	Kim *et al.* (2005)
Monocrotophos	Bacteria (*Acinetobacter* sp., *Nocardia* sp., *Arthrobacter* sp., *Pseudomonas* sp.)	Stackhouse (1980)
	Fungi (*Alternaria*, *Alusidium*, *Gliocladium*, *Penicillium*, *Sepedonium*)	Stackhouse (1980)

Table 1 (*continued*)

Compound	Microorganisms	Reference
	Chlorella vulgaris, Scenedescus bijugatus, Synechococcus elongates, Nostoc linkia, Phormidium tenue	Megharaj *et al.* (1987)
	Aulosira fertilissima, Nostoc muscorum	Subramaniam *et al.* (1994)
	Pseudomonas aeruginosa, *Clavibacter michiganense*	Subhas and Singh (2003)
Dimethoate	*A. sydowii*	Hasan (1999)
	Pseudomonas aeruginosa	Deshpande *et al.* (2001)
	A. niger	Liu *et al.* (2001)
Phorate	*Pseudomonas fluorescens, Thiobacillus thiooxidans*	Ahmed and Casida (1958)
	Streptomyces sp.	Gauger *et al.* (1986)
	Rhizobium sp., *Pseudomonas* sp., *Proteus* sp.	Bano and Musarrat (2003)
Ethoprophos	*Streptomyces* sp.	Gauger *et al.* (1986)
	P. putida epi and epii	Karpouzas *et al.* (2000)
	Sphingomonas paucimobilis, *Flavobacterium* sp.	Karpouzas *et al.* (2005b)
Cadusafos	*Sphingomonas paucimobilis*, *Flavobacterium* sp.	Karpouzas *et al.* (2005b)
Isofenphos	*Streptomyces* sp.	Gauger *et al.* (1986)
	Pseudomonas sp.	Racke and Coats (1987)
	Arthrobacter sp.	Racke and Coats (1988)
	Arthrobacter sp.	Ohshiro *et al.* (1996)
Phosphinothricin	*Rhodococcus* sp., *P. Paucimobilis*	Tebbe and Reber (1988)
	Agrobacterium tumefaciens, *Alcaligenes* sp., *Pseudomonas* sp., *Serratia* sp., *Enterobacter* sp.	Bartsch and Tebbe (1989)
Glyphosate	*Pseudomonas* sp. PG2982	Moore *et al.* (1983)
	Flavobacterium sp.	Balthazor and Hallas (1986)
	Arthrobacter sp. GLP-1	Pipke *et al.* (1987)
	Arthrobacter atrocyaneas	Pipke and Amrhein (1988a)
	Arthrobacter sp. GLP-1/Nit-1	Pipke and Amrhein (1988b)
	Pseudomonas sp. Lbr	Jacob *et al.* (1988)
	Agrobacterium radiobacter	Mcauliffe *et al.* (1990)
	Rhizobium meliloti, R. Leguminosarum, R. trifolli,	Liu *et al.* (1991)

Table 1 (*continued*)

Compound	Microorganisms	Reference
	R. galega, Agrobacterium rhizogenes, A. tumefaciens	
	Penicillium citrinum	Zboinska *et al.* (1992)
	Pseudomonas pseudomallei	Penaloza-Vazquez *et al.* (1995)
	Bacterial strains	Dick and Quinn (1995)
	Penicillium notatum	Bujacz *et al.* (1995)
	A. niger, *Thrichoderma viride*, *T. harzianum*, *Siopulariopsis* sp., *Alternaria* sp.,	Krzysko-Lupicka *et al.* (1997)
	Streptomyces sp.	Obojska *et al.* (1999)
	Geobacillus carboxylosilyticus	Obojska *et al.* (2002)
	Penicillium chromogenum	Klimek *et al.* (2001)
	Penicillium janthinellum, *P. simplicissimum*, *Mucor* sp., *Alternaria alternata*	Lipok *et al.* (2003)
Edifenphos	*Pyricularia oryzae*	Uesugi and Tomizawa (1971)
Pyrazophos	*P. oryzae*	Dewaard (1974)
	Alternaria sydowii, *A. flavus*, *fusarium oxysporum*	Hasan (1999)

and a *Brevibacterium* sp. which was able to rapidly hydrolyse parathion. Further, metabolic studies revealed that only one of the bacteria was able to metabolize parathion to *p*-nitrophenol and diethylthiophosphoric acid (DETP). Complementary studies by Munnecke and Hsieh (1976) suggested that parathion degradation by the mixed bacterial culture followed different degradation pathways under aerobic and anaerobic conditions. Under aerobic conditions, the primary pathway involved an initial hydrolysis of parathion-yielding DETP and *p*-nitrophenol, which was further metabolized to simple non-aromatic products. Under anaerobic conditions, the aromatic nitro group of parathion was reduced to aminoparathion, which subsequently undergoes hydrolysis to yield *p*-aminophenol and DETP. Serdar *et al.* (1982) isolated from the above-mentioned consortium a *Pseudomonas diminuta* GM strain that possessed a plasmid-mediated hydrolytic mechanism responsible for the hydrolysis of parathion to *p*-nitrophenol and DETP.

Another bacterial consortium isolated from a parathion-adapted soil exhibited a synergistic degrading activity (Daughton and Hsieh, 1977). The bacterial culture was shown to contain a strain of *Pseudomonas stutzeri*

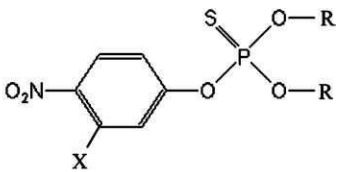
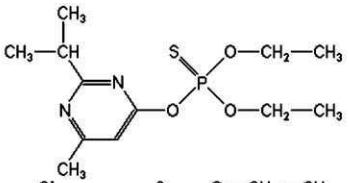
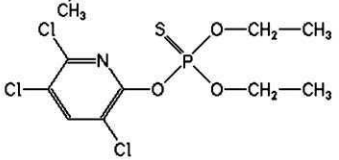
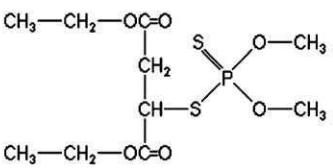
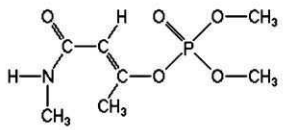
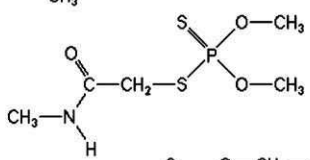
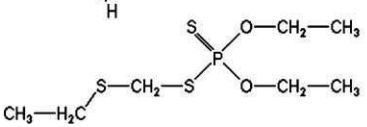
Insecticides	
parathion (R = CH_2CH_3, X= H) methyl parathion (R = CH_3, X= H) fenitrothion (R = CH_3, X= CH_3)	
diazinon	
chlorpyrifos	
malathion	
monocrotophos	
dimethoate	
phorate	

Figure 2 The chemical structures of OP pesticides.

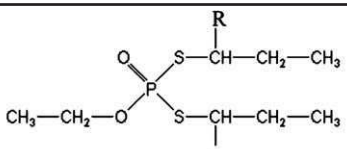
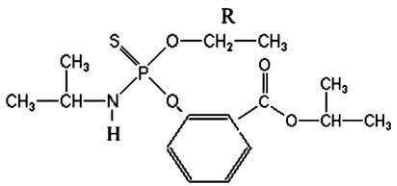
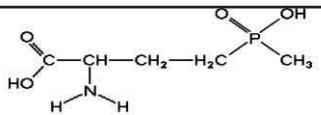
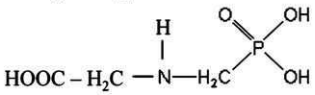
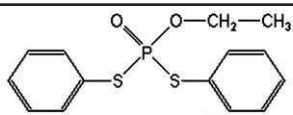
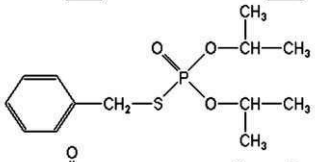
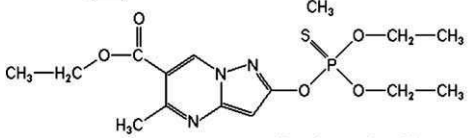
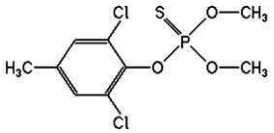
Nematicides	
Ethoprophos (R = H) Cadusafos (R = CH_3)	
Isofenphos	
Herbicides	
Phosphinothricin	
Glyphosate	
Fungicides	
Edifenphos	
Iprobenfos	
Pyrazophos	
Toclofos methyl	

Figure 2 (*Continued*)

capable of rapidly hydrolysing parathion to DETP and *p*-nitrophenol; the resultant *p*-nitrophenol was utilized as a sole carbon and energy source by the other member of the culture, a strain of *Pseudomonas aeruginosa*. A study by Nelson (1982) reported the isolation of an *Arthrobacter* and a *Bacillus* strain from a soil sample collected from Israel. Further studies showed that the *Arthrobacter* strain was able to utilize parathion, but particularly its hydrolytic product, *p*-nitrophenol, as a sole carbon source, unlike the *Bacillus* strain which was capable of hydrolysing parathion only in the presence of an extra carbon source. A *Pseudomonas* sp. and *Xanthomonas* sp. were isolated from a pesticide disposal site in northern Israel (Tchelet *et al*., 1993). The two bacterial strains, although different in the location of their hydrolytic enzymes (intra or extracellular), both degraded parathion in two stages: first *p*-nitrophenol was released by parathion hydrolysis while in the second stage, *p*-nitrophenol was degraded.

The vast majority of the isolates involved in parathion degradation were able to use parathion or their hydrolysis products as a sole carbon source (Sethunathan and Yoshida, 1973; Siddaramappa *et al*., 1973; Daughton and Hsieh, 1977). In addition, bacterial isolates were able to utilize the nitrite released from *p*-nitrophenol hydroxylation as a nitrogen source (Munnecke and Hsieh, 1974). Rosenberg and Alexander (1979) first reported the isolation of two *Pseudomonas* strains that were able to hydrolyse several OPs including parathion, and to use the ionic cleavage products like DETP as a sole source of phosphorus. In a previous study, Cook *et al*. (1978a) isolated an unidentified bacterial strain which utilized dimethyl phosphorothioate, dimethyl phosphorodithioate and their diethyl derivatives as a sole source of phosphorus. These compounds constitute possible primary metabolites produced after hydrolysis of OP compounds.

Most of the studies regarding metabolism of parathion by microorganisms have focused on the primary hydrolytic steps involved in pesticide detoxification, thus overlooking the complete transformation of the resulting metabolites like *p*-nitrophenol and DETP. Munnecke and Hsieh (1974) first investigated the transformation of *p*-nitrophenol by a microbial consortium and identified hydroquinone as an early metabolite. They then proposed that hydroquinone was hydroxylated to 1,2,4-benzenetriol prior to *ortho* ring cleavage. However, Raymond and Alexander (1971) had suggested that a *Flavobacterium* sp. converted *p*-nitrophenol into 4-nitrocatechol as the first step before ring fission. Several bacteria belonging to species of *Pseudomonas*, *Moraxella*, *Brevibacterium* and *Arthrobacter* have been found to metabolize *p*-nitrophenol with a concurrent release of nitrite (Simpson and Evans, 1953; Spain and Nishino, 1987; Spain and Gibson, 1991; Jain *et al*., 1994; Ningthoujam, 2005). Two alternative pathways, for

the conversion of *p*-nitrophenol into a common final product maleylacetate, have been demonstrated. The first pathway was suggested by Spain and Gibson (1991) whereby the formation of *p*-benzoquinone results in the release of nitrite from *p*-nitrophenol. Subsequently, hydroquinone is formed and further oxidized by a ring-cleaving dioxygenase to γ-hydroxymuconic semi-aldehyde. This is transformed to maleylacetate which is futher metabolized to β-ketoadipate. In the second pathway, an *Arthobacter* sp. and a *Bacillus* sp. hydroxylated *p*-nitrophenol to produce 4-nitrocatechol which is further oxidized to 1,2,4-trihydroxybenzene (THB) with concomitant release of nitrite. THB is further oxidized to maleylacetate which is then converted enzymatically into 3-ketoadipate (Jain *et al*., 1994; Kadiyala and Spain, 1998). The complete metabolic pathway of parathion degradation by soil microorganisms is presented in Fig. 3.

In most studies, hydrolysis of parathion and formation of *p*-nitrophenol and DETP are the most common initial steps in parathion-microbial metabolism. However, alternative metabolic pathways have also been reported. Munnecke and Hsieh (1976) reported a secondary metabolic pathway of parathion, which involved the oxidation of parathion to paraoxon that was further hydrolysed to *p*-nitrophenol and diethylphosphoric acid. Yasuno *et al*. (1965) found a *Bacillus subtilis* strain that rapidly converted parathion into aminoparathion. Similarly, Mick and Dahm (1970) isolated two *Rhizobium* spp., which rapidly reduced parathion to aminoparathion. Similar degradation pathways have been reported in fungi and algae. A fungus, *Penicillium waksmani*, isolated from an acid sulphate soil, degraded large amounts of parathion to aminoparathion and two unidentified polar metabolites (Rao and Sethunathan, 1974). Similarly, Zuckerman *et al*. (1970) reported that an alga, *Chlorella pyrenoidosa*, was able to metabolize parathion to aminoparathion.

Methyl parathion (*O,O-dimethyl O-*(*p-nitrophenyl*) *phosphorothioate*) and *Fenitrothion* (*O,O-dimethyl O-4-nitro-m-tolyl-phosphorothioate*): Methyl parathion and fenitrothion are still widely used due to their relatively lower mammalian toxicity compared to their analogue, parathion. Several microorganisms that were isolated from parathion enrichments have also been tested for their ability to metabolize methyl parathion and fenitrothion (Rosenberg and Alexander, 1979; Adhya *et al*., 1981).

Only a few studies have focused on the isolation of methyl parathion degrading microorganisms and the investigation of the associated metabolic pathways. Chaudhry *et al*. (1988) isolated a *Pseudomonas* sp. and a *Flavobacterium* sp. from soil collected from a farmyard previously treated with methyl parathion. *Pseudomonas* sp. hydrolysed the pesticide to *p*-nitrophenol but required glucose or another carbon source for growth, unlike

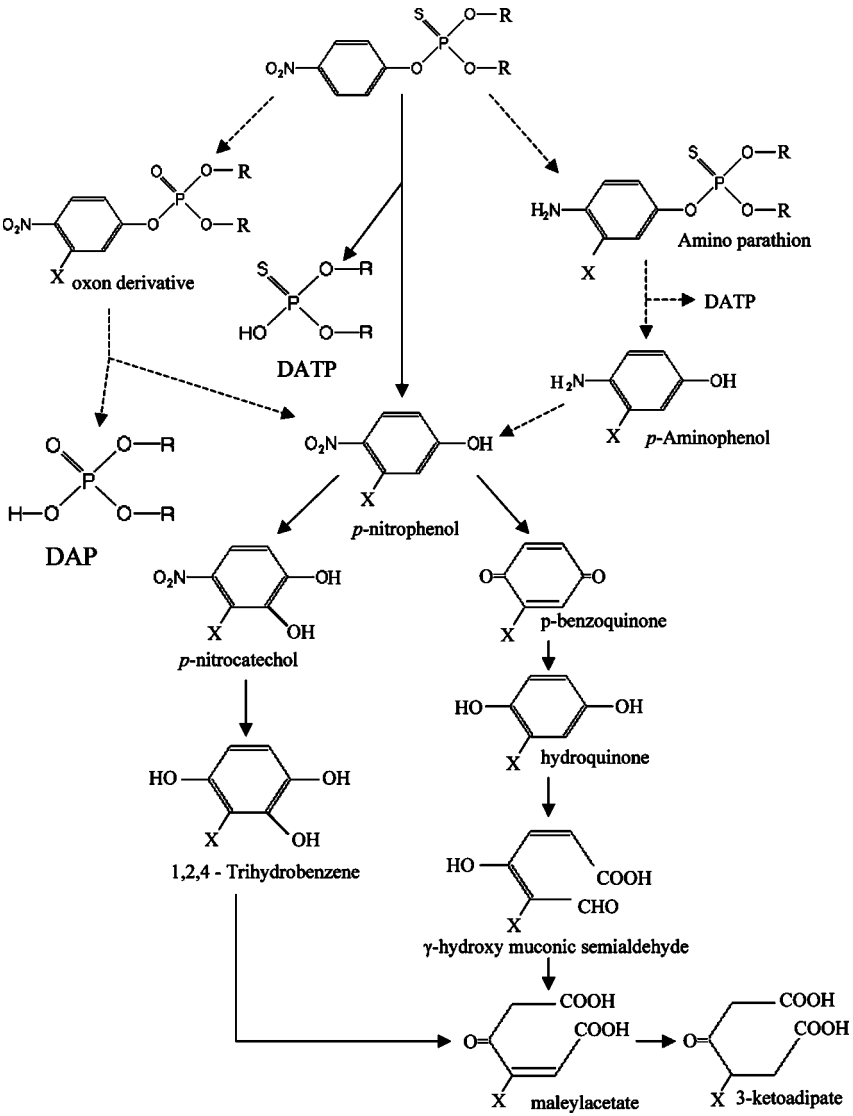


Figure 3 The metabolic pathway of degradation of phenyl-substituted OP insecticides by soil microorganisms. Where $R = CH_3CH_2$, $X = H$ for parathion, $R = CH_3$, $X = H$ for methyl parathion and $R = CH_3$, $X = CH_3$ for fenitrothion, DATP, dialkyl thiophosphate. Metabolic steps designated with dashed lines represent minor metabolic pathways.

Flavobacterium sp., which was able to metabolize *p*-nitrophenol by releasing nitrite which was used by the bacterium as a nitrogen source. *Bacillus* sp. isolated by Sharmila *et al.* (1989) was able to hydrolyse methyl parathion, parathion and fenitrothion in the presence of different concentrations of yeast extract. Particularly noteworthy is the finding that the same bacterium effected both, nitro group reduction and hydrolysis of methyl parathion depending on the concentration of yeast in the liquid medium. Degradation of methyl parathion proceeded via hydrolysis to *p*-nitrophenol and DMTP (dimethyl thiophosphate) in the presence of a concentration (w/v) of yeast extract at 0.5%, by both hydrolysis and nitro group reduction at 0.1 and 0.25%, and exclusively by nitro group reduction at 0.05%. In contrast, degradation of fenitrothion by *Bacillus* sp. proceeded via hydrolysis regardless of the concentration of yeast. Ou and Sharma (1989) isolated another *Bacillus* strain that utilized methyl parathion as a carbon and energy source. However, Rani and Lalithakumari (1994) first reported the isolation of a *P. putida* which utilized methyl parathion as sole carbon and/or phosphorus source. In addition, *P. putida* also utilized the metabolic products derived from the degradation of methyl parathion, such as *p*-nitrophenol, hydroquinone and 1,2,4-benzenetriol, as carbon sources. Subsequently, the later metabolite was further transformed by the *P. putida* strain to maleylacetate following the same metabolic pathways as described before for parathion. In a recent study, a *Burkholderia cepacia* was isolated from a methyl parathion-treated site in Thailand (Keprasertsup *et al.*, 2001). This isolate was able to rapidly degrade methyl parathion and *p*-nitrophenol and utilize them as sole sources of carbon. A *Bacillus* sp. and two unidentified pure cultures which were isolated in the same study were able to degrade commercial grade methyl parathion and not analytical grade methyl parathion. This finding indicates that the pesticide was co-metabolized by these bacteria that were actually grown on other organic compounds present in the pesticide formulation. In a concurrent study, Zhongli *et al.* (2001) isolated a *Plesiomonas* sp., which was able to hydrolyse 200 mg of methyl parathion to *p*-nitrophenol within 15 min, but it was unable to further transform *p*-nitrophenol that was accumulated in the medium. More recently, the same group isolated a *Pseudomonas* sp. strain p3 which was able to utilize methyl parathion as a sole carbon and nitrogen source (Zhongli *et al.*, 2002). In a subsequent study, another *Pseudomonas* sp. was isolated from polluted soils around a Chinese pesticide factory, which effected complete degradation of methyl parathion by using the pesticide as a sole source of carbon and nitrogen (Yali *et al.*, 2002).

Misra *et al.* (1992) reported the isolation of two bacterial isolates which rapidly hydrolysed methyl parathion, parathion and fenitrothion to

p-nitrophenol and which was further metabolized with concomitant release of nitrite. In contrast, 3-methyl-4-nitrophenol, the hydrolysis product of fenitrothion, was not further metabolized by the isolated bacteria. Similar studies by Adhya *et al.* (1981) reported that the parathion-degrading *Flavobacterium* strain ATCC 27551 hydrolysed fenitrothion. However, Hayatsu *et al.* (2000) isolated *Burkholderia* sp. NF100 which utilized both fenitrothion and methyl parathion as carbon sources. The metabolic pathway of methyl parathion and fenitrothion by *Burkholderia* sp. NF100 involved an initial hydrolysis to *p*-nitrophenol and 3-methyl-4-nitrophenol, respectively. These products were further oxidized to hydroquinone and methyl hydroquinone, as has been described before. Evidence for the microbial degradation of fenitrothion and its oxon analogue, fenitrooxon, by fungal isolates was provided by Baarschers and Heitland (1986), who found that the fungus *Trichoderma viride* could hydrolyse both compounds to 3-methyl-4-nitrophenol which was then further degraded by co-metabolic reactions.

Previous studies by Miyamoto *et al.* (1966) observed a different degradation pathway for fenitrothion by a *B. subtilis* strain. The major metabolite, accounting for 65% of the added insecticide, was aminofenitrothion; other minor metabolites detected were DMTP and desmethyl fenitrothion. The aminofenitrothion was then slowly transformed to desmethyl aminofenitrothion. Methyl parathion was metabolized by *B. subtilis* in the same way as fenitrothion but twice as fast. The complete metabolic pathway of methyl parathion and fenitrothion is presented in Fig. 3.

2.1.2. Heterocyclic-substituted Organophosphates

Diazinon (*O,O-diethyl O-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate*): Diazinon is a broad-spectrum OP insecticide mainly used for the control of soil-dwelling insects. Several soil-metabolism studies have documented the strong involvement of soil microflora in the degradation of diazinon (Sethunathan and MacRae, 1969; Sethunathan and Yoshida, 1969). In most studies, diazinon was hydrolysed in non-sterilized soils to DETP and 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMHP). The subsequent breakdown of the pyrimidine ring of IMHP to CO_2 is microbial in nature (Getzin, 1967; Sethunathan and MacRae, 1969; Sethunathan and Yoshida, 1969).

The first report of diazinon-degrading microorganisms was provided by Gunner and Zuckerman (1968) who isolated two bacterial strains, an *Arthrobacter* and a *Streptomyces*, which were able to use diazinon as a sole source of carbon. These bacteria were able to attack the ethyl ester moiety of diazinon when cultured separately, but they were unable to mineralize the

pyrimidinyl ring of diazinon. However, *Arthrobacter* and *Streptomyces* exhibited a synergistic activity against the pyrimidinyl moiety of diazinon when cultured together. Sethunathan and Yoshida (1973) isolated a *Flavobacterium* strain ATCC 27551 from the water of a diazinon-treated rice field. This strain was able to decompose 95% of diazinon within 24 h to produce DETP and large amounts of IMHP. Within 72 h, no IMHP was detected and more than 30% of the added radioactivity as [^{14}C]-ring labelled diazinon was liberated as $^{14}CO_2$, indicating the cleavage of the pyrimidinyl ring. Further studies revealed that this degradation pathway was evident only under aerobic conditions. In a similar manner, a diazinon-enrichment culture from sewage and soil samples resulted in the isolation of a *P. putida* strain, which was able to use diazinon and other OP compounds like malathion, parathion and dimethoate as phosphorus sources (Rosenberg and Alexander, 1979). *P. putida* was able to hydrolyse diazinon to produce DETP and IMHP. The former metabolite was probably further decomposed by phosphomonoesterases and phosphodiesterases to release inorganic P which was assimilated by the bacterium.

A number of bacterial isolates have been isolated from diazinon-enrichment cultures, and, bacteria isolated from enrichment cultures with other OP compounds including parathion or isofenphos were also able to metabolize diazinon. For example, a *Pseudomonas* sp. isolated from a parathion-enrichment culture was capable of hydrolysing diazinon to IMHP, which was only slowly degraded by the bacterium (Adhya *et al*., 1981). Similarly, an isofenphos-degrading *Arthrobacter* strain isolated from a turf green soil was able to rapidly hydrolyse diazinon among other OPs with the presumptive production of DETP and IMHP (Ohshiro *et al*., 1996). The metabolic pathway of diazinon degradation is shown in Fig. 4.

Chlorpyrifos (*O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate*): Chlorpyrifos is a broad-spectrum OP insecticide, displaying insecticidal activity against a wide range of insects and other arthropod pests. It is characterized as moderately toxic compound with acute oral LD50 = 135–165 mg kg^{-1} for rats. Metabolism of chlorpyrifos in soil has been studied extensively and both chemical and microbial activity has been suggested (Racke, 1993). Studies conducted in soil have generally reported significantly longer degradation half-lives under sterilized as opposed to natural conditions, indicating that microbial activities are important in the degradation of chlorpyrifos in soil (Getzin, 1981; Miles *et al*., 1983). The most common metabolic pathway of chlorpyrifos degradation in soil involves an initial hydrolytic cleavage of the P–O–C bond leading to the formation of DETP and 3,5,6-trichloro-2-pyridinol (TCP), which was further mineralized (Somasundaram *et al*., 1987; Racke *et al*., 1988, 1990). It is

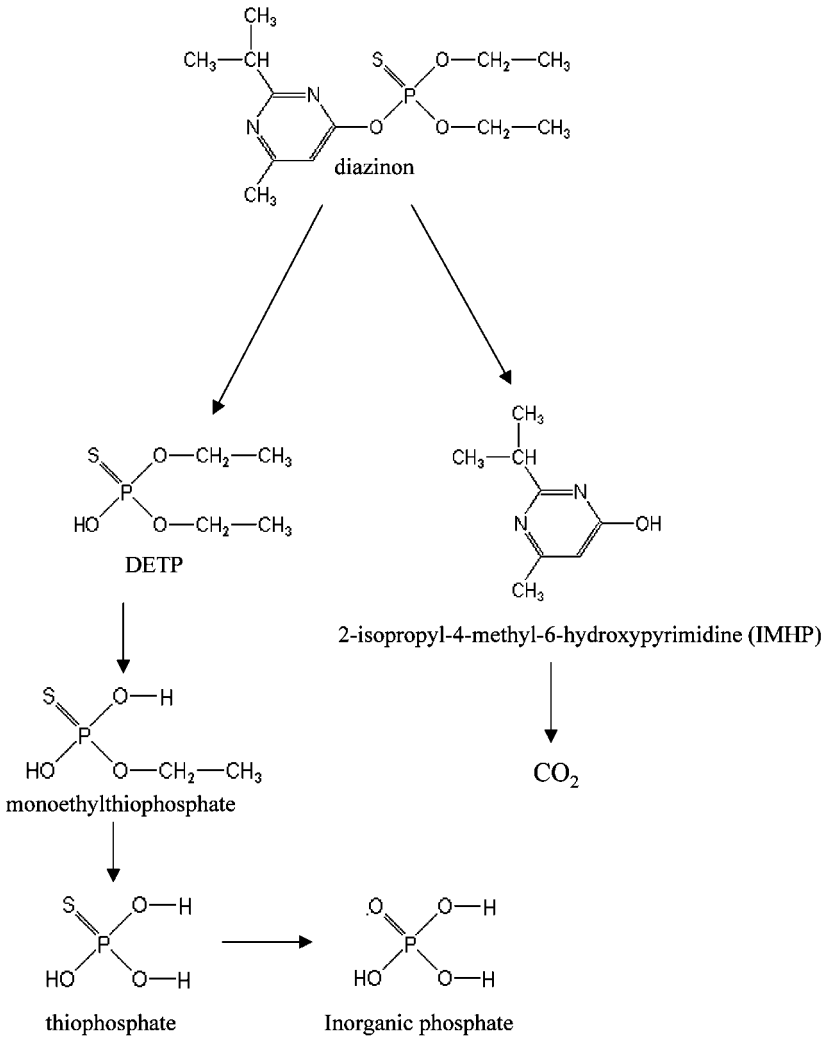


Figure 4 The metabolic pathway of diazinon degradation by soil microorganisms.

documented that the initial hydrolytic step in chlorpyrifos degradation is of a co-metabolic nature, but the cleavage and mineralization of the derived aromatic metabolites (TCP) is employed exclusively by soil microorganisms (Bidlack, 1980; Getzin, 1981; Singh *et al.*, 2003). Other metabolites which

have been detected in soil, but at negligible amounts, included chlorpyrifos oxon, desethyl chlorpyrifos, desethyl chlorpyrifos oxon (Zidan *et al*., 1981), and 3,5,6-trichloro-2 methoxypyrimidine (Bidlack, 1980).

Although chlorpyrifos has been used extensively in a variety of crops, it appears to be resistant to enhanced biodegradation. The most convincing reasoning for the apparent resistance of chlorpyrifos to enhanced microbial degradation was provided by Racke *et al*. (1990) who suggested that the accumulation of TCP, which has anti-microbial activity, hampers the proliferation of chlorpyrifos-degrading microorganisms. However, enhanced biodegradation of chlorpyrifos was observed in Australian soils, where certain soil microorganisms developed the ability to mineralize the toxic TCP, resulting in loss of chlorpyrifos efficacy against termites (Robertson *et al*., 1998).

Chlorpyrifos was degraded co-metabolically in liquid media by *Flavobacterium* ATCC 27551 (Sethunathan and Yoshida, 1973), which was isolated from a diazinon-enriched soil (Mallick *et al*., 1999). On the contrary, in the same study, an *Arthrobacter* sp., which had been isolated previously from a methyl-parathion-enriched soil (Misra *et al*., 1992), utilized chorpyrifos as a sole carbon source. Chlorpyrifos is related to diazinon and methyl parathion in having a common P–O–C linkage and was degraded by hydrolysis of this linkage, as was the case with diazinon and parathion (Sethunathan and Yoshida, 1973). It was further suggested that *Arthrobacter* sp. was probably utilizing DETP, a common hydrolysis product of diazinon, chlorpyrifos and parathion, for its proliferation. Similarly, a *Micrococcus* sp. isolated from a malathion-enriched soil (Guha *et al*., 1997) and an *Arthrobacter* sp. (Ohshiro *et al*., 1996) isolated from a isofenphos-enriched soil were able to hydrolyse chlorpyrifos in liquid media.

Recently, Singh *et al*. (2004) reported the isolation of an *Enterobacter* B-14 strain that could hydrolyse chlorpyrifos to DETP and TCP, but could only utilize DETP as source of carbon and phosphorus. DETP and other related phosphorothionate or phosphorodithioate molecules have been utilized as phosphorus sources by soil bacteria (Cook *et al*., 1978a; Rosenberg and Alexander, 1979). In a similar study, Yang *et al*. (2005) used an enrichment culture from a contaminated soil to isolate an *Alcaligenes faecalis* DSP3 strain which was able to degrade chlorpyrifos and TCA and use them as carbon and phosphorus sources. It was shown that *A. faecalis* DSP3 strain was able to tolerate TCP concentrations as high as 800 mg L^{-1}. This further demonstrates that the key point for the rapid degradation of chlorpyrifos in soil is the presence of a robust TCP-degrading microbial population that can tolerate high concentrations of TCP or mineralize TCP at a rate faster than the rate at which TCP is formed. Feng *et al*. (1997) first

reported the isolation of a *Pseudomonas* strain ATCC 700113, which mineralized TCP in liquid medium with the concurrent evolution of chlorine. Further studies suggested that metabolism of TCP follows two successive dechlorination steps leading to the formation first of chlorodihydro-2-pyridone and then tetra-hydro-2-pyridone (Feng *et al.*, 1998). The ring of the latter metabolite is cleaved to produce maleamide semi-aldehyde, which is finally metabolized to water, carbon dioxide and ammonium.

Metabolism of chlorpyrifos by soil fungi has also been reported (Bumpus *et al.*, 1993). Chlorpyrifos was initially hydrolysed to produce TCP, which was subsequently mineralized by the fungal isolates. *Hypholoma fasciculare*ae and *Coriolus versicolor*, two white-rot fungi, were found to degrade chlorpyrifos in a biomix substrate consisting of soil, wheat and peat (Bending *et al.*, 2002). The ability of white-rot fungi to metabolize a variety of persistent aromatic compounds, including chlorinated compounds like pentachlorophenol and hexachlorocyclohexane, has been well documented (Glaser and Lamar, 1995; Pointing, 2001).

2.1.3. *Aliphatic Organophosphates*

Malathion (*S-(1,2-dicarbethoxyethyl)-O,O-dimethyldithiophosphate*): Malathion is a phosphorodithioate compound that is commonly used as a general purpose insecticide for the control of sucking and chewing insects (Imran *et al.*, 2004). Malathion, unlike other OP insecticides, is characterized by low mammalian toxicity (acute oral LD50 = 1375–2800 mg kg^{-1} for rats) (Tomlin, 2000).

Several microorganisms, including bacteria, fungi and algae, have been found to rapidly metabolize malathion (Laveglia and Dahm, 1977). Carboxyesterase activity, which degrades malathion to its monoacid and diacid derivatives, is the predominant metabolic mechanism (Matsumura and Boush, 1966; Mostafa *et al.*, 1972a,b; Walker and Stojanovic, 1974; Paris *et al.*, 1975; Bourquin, 1977; Singh and Seth, 1989). Monacid and diacid derivatives of malathion were produced by the activity of a fungal cutinase produced by *Fusarium oxysporum* f. sp. pisi (Kim *et al.*, 2005). The role of phosphatase activity in degradation has also been reported (Matsumura and Boush, 1966; Mostafa *et al.*, 1972a,b; Walker and Stojanovic, 1974; Bourquin, 1977). Oxidative desulfuration (Mostafa *et al.*, 1972a,b) and demethylation appeared to be rather minor metabolic pathways (Matsumura and Boush, 1966).

Matsumura and Boush (1966) reported degradation of malathion to monoacid and diacid derivative by a fungal (*Trichoderma viride*) and a bacterial (*Pseudomonas* sp.) isolates. Diethyl malate and desmethyl malathion

were also identified as metabolites but at small amounts suggesting that their production constitutes a minor metabolic pathway. A similar degradation pathway was suggested by Paris *et al.* (1975) for several bacterial isolates, which could degrade malathion to monoacid and diacid with parallel formation of diethyl malate as a minor metabolite. Similarly, an *Arthrobacter* sp. metabolized malathion to its monoacid and diacid derivatives, which were further metabolized to dimethyl phosphorodithioate and dimethyl phosphorothioate, respectively (Walker and Stojanovic, 1974). Bourquin (1977) isolated 11 bacterial isolates from salt-marsh environments after malathion enrichment which utilized malathion as a sole carbon source. The isolated bacteria possessed carboxyesterase activity, which metabolized malathion to its monoacid and diacid derivatives. Small amounts of other metabolites were also produced including desmethyl malathion, phosphorothionates and four carbon dicarboxylic acids which were probably formed as a result of phosphatase activity. Singh and Seth (1989) reported the isolation of a *Pseudomonas* M-3 strain that metabolized 150 mg L^{-1} of malathion within 32 h. *Pseudomonas* M-3 metabolized malathion to its monoacid derivative with the parallel formation of ethanol that was used by the strain as a sole carbon source. Guha *et al.* (1997) isolated a *Micrococcus* sp. from a malathion-enriched soil, which metabolized malathion. Hasan (1999) reported that several fungal species including *Aspergillus sydowii*, *A. flavus* and *Fusarium oxysporum* metabolized malathion as a carbon or phosphorus source. Recently, another malathion-degrading *Pseudomonas* strain was isolated from an agricultural soil (Imran *et al.*, 2004). However, no information on the metabolic pathway of malathion was reported.

The most complete metabolic pathway for malathion was reported by Mostafa *et al.* (1972a,b). In one of the two studies, Mostafa *et al.* (1972a) studied the metabolism of malathion by some fungi including *Aspergillus niger*, *Penicillium notatum* and *Rhizoctonia solani*. The latter fungus was only able to metabolize malathion to its oxon derivative malaoxon, which is a strong AChE inhibitor. However, the other two fungi, *A. niger* and *P. notatum*, initially transformed malathion to malathion monoacid and malathion diacid. Malathion monoacid was subsequently transformed to dimethyl phosphorodithioate, which was not metabolized further. In contrast, malathion diacid was converted by the two fungi into thiophosphate, dimethyl and monomethyl phosphate. In the second study, Mostafa *et al.* (1972b) reported a similar degradation pathway for malathion by two *Rhizobia*: *R. trifolii* and *R. leguminosarum*. The only difference between the metabolic pathways reported in the two concurrent studies was that the *Rhizobium* species finally produced inorganic phosphate. The metabolic pathway of malathion by microorganisms is shown in Fig. 5.

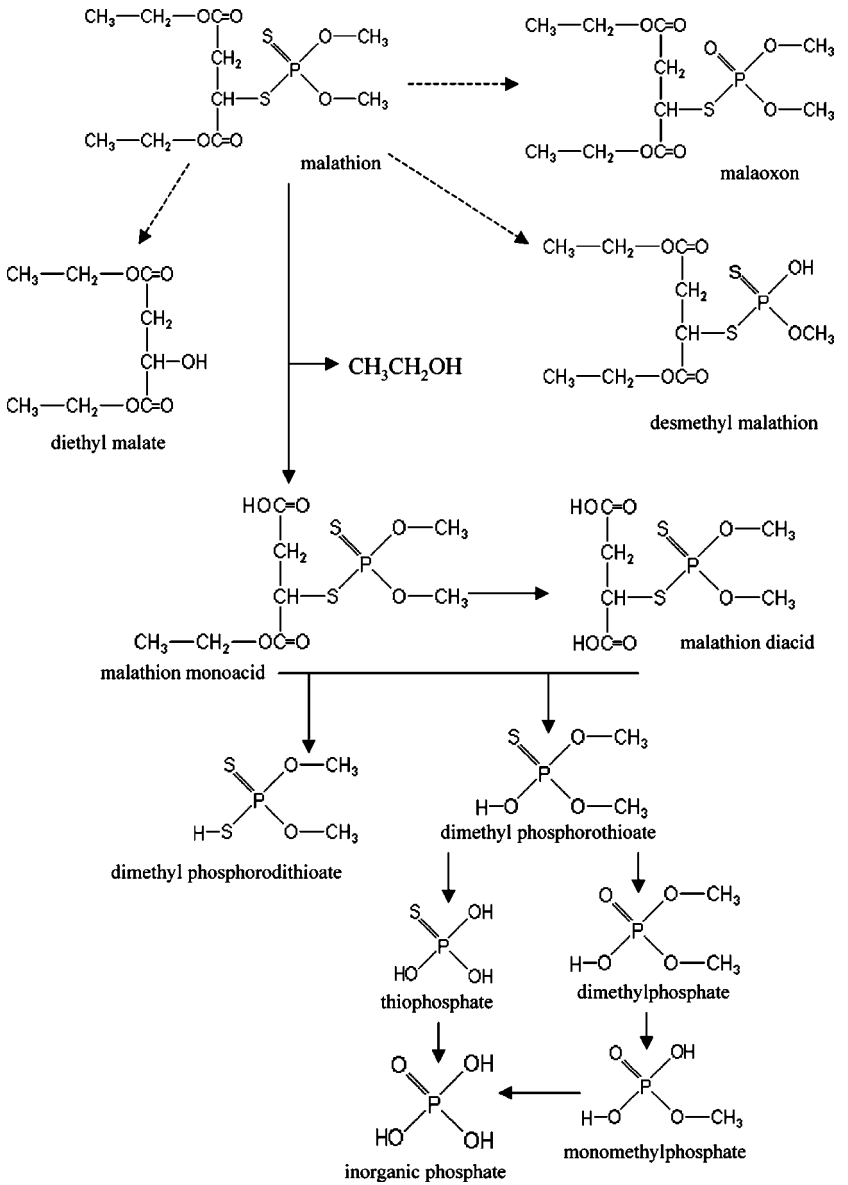


Figure 5 The metabolic pathway of malathion degradation by soil microorganisms. Metabolic steps designated with dashed lines represent minor metabolic pathways.

Most of the microorganisms reported above either co-metabolized malathion or used it as a sole carbon source. However, Rosenberg and Alexander (1979) isolated two *Pseudomonas* sp. that utilized malathion and other OPs as a sole phosphorus source. Similarly, Subramanian *et al.* (1994) observed that two filamentous-heterocystous cyanobacteria, *Aulosira fertilissima* ARM 68 and *Nostoc muscorum* ARM 221, could use malathion and other OPs as a phosphorus source. Cyanobacteria are known to assimilate phosphorus in excess of their requirements (Stewart and Alexander, 1971).

Other non-aromatic organophosphorus insecticides: The microbial degradation of other non-aromatic OP insecticides has attracted little attention. Monocrotophos (*3-hydroxy N-methyl-cis-crotonamide dimethyl phosphate*) has probably been the most studied insecticide among the non-aromatic OPs after malathion. However, there is limited information concerning its microbial metabolic pathways, which is postulated to proceed via an initial hydrolytic step. Stackhouse (1980) reported the isolation of over 100 microorganisms by enrichment techniques from active soil and were screened for their ability to mineralize [^{14}C] monocrotophos. Thirteen bacterial and seven fungal isolates were found to mineralize monocrotophos. Active bacterial species included *Acinetobacter*, *Nocardia*, *Arthrobacter* and *Pseudomonas*, whereas fungi belonged to the genera *Alternaria*, *Alysidium*, *Gliocladium*, *Penicillium* and *Sepedonium*. A study by Rangaswamy and Venkateswarlu (1992) reported the isolation of soil bacteria that rapidly hydrolysed monocrotophos. Four isolates were tentatively identified as *Bacillus* sp., which could completely degrade 40 mg L^{-1} of monocrotophos in less than seven days. Degradation of monocrotophos by the isolated *Bacillus* sp. proceeded via hydrolysis with the production of presumptive metabolites including dimethyl phosphate, *O*-desmethyl monocrotophos and *N*-methyl acetoacetamide. Similarly, Bhadbhade *et al.* (2002) isolated a *Pseudomonas mendocina* MCM B-424 strain that utilized monocrophos as a sole carbon source. *P. mendocina* MCM B-424 possessed a 7.5 kb plasmid whose presence was directly associated with its ability to metabolize monocrotophos. More recently, enrichment cultures from a monocrotophos-treated soil, from a cotton field in India, resulted in the isolation of a *P. aeruginosa* and a *Clavibacter michiganense* subsp. *insidiosum*, which utilized the compound as a phosphorus source (Subhas and Singh, 2003). None of the two isolates utilized monocrotophos as a carbon source and required the presence of glucose to support their growth. Further studies revealed the presence of membrane-associated phosphotriesterase activity whose function was plasmid-mediated.

Degradation of monocrotophos by algae has also been observed. Megharaj *et al.* (1987) reported two green algae, *Chlorella vulgaris* and *Scenedesmus bijugatus*, and three species of cyanobacteria, *Synechococcus elongatus*, *Nostoc linkia* and *Phormidium tenue*, which could metabolize monocrotophos. Metabolic studies revealed the accumulation of four metabolites that were presumed to be hydrolytic products although no metabolite identification was performed. Similarly, Subramanian *et al.* (1994) reported that two cyanobacterial species, *Aulosira fertilissima* and *Nostoc muscorum*, utilized monocrotophos as a phosphorus source even in the presence of alternative inorganic phosphorus.

Another pesticide whose microbial metabolism has received some attention is dimethoate (*O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate*). Deshpande *et al.* (2001) reported the isolation of a *P. aeruginosa* MCMB-427 strain which possessed a plasmid-encoded hydrolytic activity for dimethoate. Fungi have also been reported that were able to degrade dimethoate. Hasan (1999) tested several fungal species for their ability to degrade various OP compounds, and observed that *Aspergillus sydowii* was able to grow on dimethoate when the compound was the sole source of phosphorus. Another fungal isolate, *A. niger* strain ZHY256 was isolated from sewage (Liu *et al.*, 2001), which possessed a hydrolytic enzyme system specific for P–S–C bonds characteristics of dimethoate, malathion and formothion. Hydrolysis of dimethoate by *A. niger* yielded *O,O*-dimethyl phosphorothioate and $HSCH_2C(O)NHCH_3$. No further study on metabolic pathway was carried out.

Phorate (*O,O-diethyl-S-(ethylthio)methyl phosphorodiothioate*) is another non-aromatic OP insecticide whose degradation in soil is microbially triggered. Metabolic studies in soil have revealed that phorate is oxidized to phorate sulfoxide and subsequently to phorate sulfone, which also possess insecticidal activity and persist in soil (Suett, 1971). However, the rapid degradation of phorate sulfoxide and phorate sulfone in soils, previously treated with phorate, indicated that phorate was prone to enhanced biodegradation. Ahmed and Casida (1958) studied the metabolism of phorate by various microorganisms. *Pseudomonas fluorescens* and *Thiobacillus thiooxidans*, hydrolysed significant amounts of phorate in eight days without any formation of sulfoxide or sulfone. In a more recent study, three bacterial isolates, identified as *Rhizobium*, *Pseudomonas* and *Proteous*, were isolated by enrichment culture from an agricultural soil (Bano and Musarrat, 2003). These bacteria showed significant growth in mineral salt medium containing 200 mg L^{-1} of phorate as a sole carbon source. However, no information regarding the metabolic pathway of phorate was elucidated.

2.2. Nematicides

Ethoprophos (O-ethyl S,S-dipropyl phosphorodithioate) and Cadusafos (O-ethyl S,S(1-methyl propyl) phosphorodithioate): Ethoprophos and cadusafos are non-fumigant OP nematicides which are commonly used for the control of plant parasitic nematodes in various crop plantations. These compounds are chemical analogues since they are both phosphorodithioates with an additional methyl group in the *S*-propyl moiety of cadusafos (Fig. 1).

In agreement with most of the other soil-applied OPs, ethoprophos and cadusafos are mainly microbially transformed in the soil environment (Jones and Norris, 1998). Several studies with soil from sites previously treated with ethoprophos have documented its susceptibility to enhanced biodegradation (Smelt *et al*., 1987; Mojtahedi *et al*., 1991; Karpouzas *et al*., 1999a), which was often associated with failure to control nematode infestations (Karpouzas *et al*., 1999b). In contrast to ethoprophos, enhanced biodegradation of cadusafos was only recently reported in banana plantations in Australia (Pattison, 2000), in citrus fields in South Africa (Le Roux *et al*., 2001) and also in potato fields in Greece (Karpouzas *et al*., 2004b). However, the resistance of cadusafos to the development of enhanced biodegradation has also been reported in certain cases (Moens *et al*., 2004; Giannakou *et al*., 2005). Recent reports by Karpouzas *et al*. (2005a) showed that fumigation of an enhanced-cadusafos soil, with the soil fumigants methyl bromide or metham sodium, resulted in a long-lasting inhibition of the microbial degradation of cadusafos.

The microbial degradation of ethoprophos in soil proceeds via cleavage of the P–S–C bonds producing *O*-ethyl-*S*-propylphosphorothioic acid as its major intermediate metabolite, which is further transformed to simple phosphate derivatives with liberation of $CH_3CH_2CH_2SH$ and ethanol (Jones and Norris, 1998). A *Streptomyces* sp. was isolated from an isofenphos-treated soil that could grow on ethoprophos among other OP compounds tested (Gauger *et al*., 1986). Ethoprophos enrichment from an enhanced soil in Greece resulted in the isolation of two *P. putida* strains, epI and epII, which degraded ethoprophos and used it as a sole carbon source (Karpouzas *et al*., 2000). Radiorespirometry studies with [^{14}C]-ethoprophos labelled either in the ethyl- or the propyl moiety suggested that degradation of ethoprophos by *Pseudomonas* strains proceeded via removal of its –*S*-propyl moiety, which was utilized by the bacteria as a sole carbon source. This pathway is further supported by the finding that both *Pseudomonas* strains could degrade cadusafos, whose chemical structure differs only in the –*S*-propyl moiety.

Only recently, Karpouzas *et al.* (2005b) isolated a *Sphingomonas paucimobilis* and a *Flavobacterium* sp. from a potato field which was heavily treated with cadusafos. These isolates could mineralize cadusafos as a sole carbon source. Both isolates rapidly metabolized ethoprophos as well as cadusafos in both liquid culture and in soil, suggesting that the isolated bacteria were actively participating in the degradation of cadusafos in soil in situ. The ability of the cadusafos-degrading bacteria to degrade ethoprophos but not fenamiphos or isazofos, which are characterized by aromatic constituents, indicates that cadusafos metabolism proceeds via removal of the –*S*-propyl moiety (similar for cadusafos and ethoprophos) which is further utilized by bacteria as a carbon source. The identified and postulated metabolic pathways of ethoprophos and cadusafos by microorganisms are illustrated in Fig. 6.

Isofenphos (1-methyl ethyl 3-[[ethoxy[(1-methylethyl)-amino] phosphinothioyl] oxy] benzoate): Isofenphos is a non-fumigant soil-applied OP used for the control of soil-dwelling insects and also at higher dosages for the control of plant parasitic nematodes. Microbial degradation and enhanced biodegradation of isofenphos in agricultural soils have been reported in several studies (Chapman *et al.*, 1986; Niemczyk and Chapman, 1987; Racke and Coats, 1988).

The first report of the degradation of isofenphos by soil microorganisms was established by Gauger *et al.* (1986), who reported the isolation of a *Streptomyces* sp. that could co-metabolize isofenphos and other OPs by alkaline phosphatase activity. In enrichment studies with an isofenphos-treated soil, Racke and Coats (1987) isolated a *Pseudomonas* sp. which metabolized ring-labelled [^{14}C] isofenphos with concurrent evolution of [$^{14}CO_2$]. Metabolic studies with soil bacterial cultures from the same soils suggested that isofenphos is hydrolysed to isopropyl salicylate, which was rapidly transformed to polar metabolites and finally to CO_2. Trace quantities of salicylic acid were also detected, unlike isofenphos oxon that was never detected throughout the study. A complementary study by Racke and Coats (1988) reported the isolation of a more robust isofenphos-degrading bacteria, *Arthrobacter* sp., which completely mineralized 100 mg L^{-1} of [^{14}C] isofenphos in 6 h. This strain also utilized a variety of simple organic substrates as a sole carbon source including salicylic acid and phenyl acetate, which might be produced as intermediate products during degradation of isofenphos. In a later study, Ohshiro *et al.* (1996) isolated an *Arthrobacter* B-5 strain from a turf green soil, which could degrade various OPs but was particularly active against isofenphos. Further studies by the same group revealed that *Arthrobacter* B-5 possessed a hydrolase, which cleaved the aryl phosphoester bond in isofenphos, resulting in the formation of two

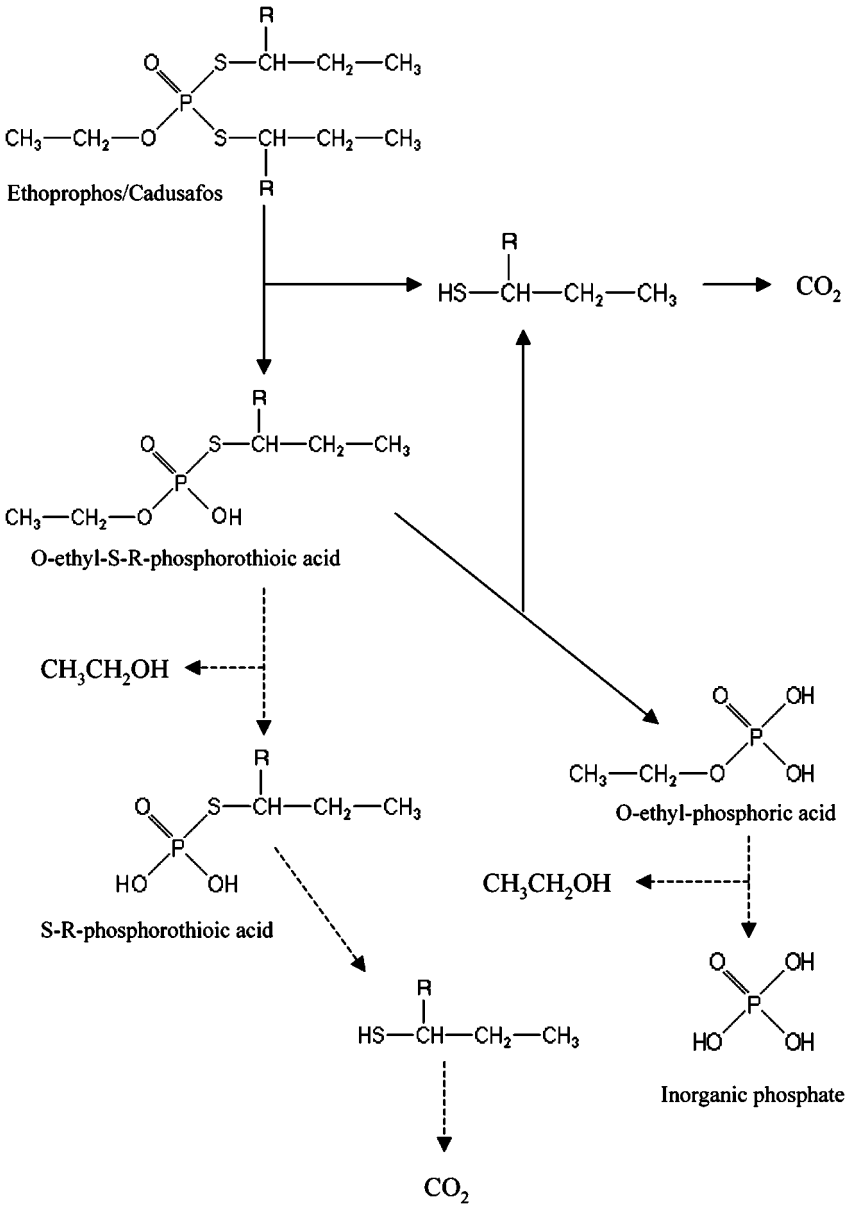


Figure 6 The metabolic pathway of ethoprophos/cadusafos degradation by soil microorganisms. Metabolic steps designated with dashed lines represent minor or postulated metabolic pathways.

metabolites that were identified as *O*-ethyl isopropyl phosphoramidothioate (EIP) and isopropyl salicylate (Ohshiro *et al*., 1997). Later studies showed that *Arthrobacter* B-5 produces two cytoplasmic OPH isozymes that are encoded in the bacterial chromosome (Ohshiro *et al*., 1999)

In general, degradation of isofenphos by the microorganisms isolated so far proceeds via an initial hydrolytic step, which results in the formation of intermediate products like isopropyl salicylate or salicylic acid, which could be further mineralized and used as a carbon source by the soil microflora. The suggested metabolic pathway of isofenphos by microorganisms is shown in Fig. 7.

2.3. Herbicides

Glufosinate – Phosphinothricin (DL-homoalanin-4-ylmethylphosphinic acid):

Glufosinate ammonium is the ammonium salt of an amino acid called glufosinate or phosphinothricin (PPT). PPT is a natural phosphonate which occurs as a constituent of tripeptides produced by the soil actinomycetes *Streptomycetes viridochromogenes* (Bayer *et al*., 1972), *S. hygroscopicus* (Kondo *et al*., 1973) and *Kitasatosporia phosalacinea* (Omura *et al*., 1984), and possesses herbicidal activity. L-phosphinothricin is known to inhibit glutamine synthetase, the enzyme that catalyses the combination of glutamic acid and ammonia (Leason *et al*., 1984). Glufosinate ammonium is used as a non-selective herbicide for the control of annual and perennial weeds in a variety of crop and non-crop situations (Smith, 1988).

Initial degradation studies of PPT in sterilized or dried soil indicated that PPT is degraded by soil-microbial processes (Smith, 1988). Preliminary radiorespirometry studies in soil suggested that PPT was initially transformed to intermediate products that were subsequently mineralized to CO_2. Smith (1989), in a follow-up study, showed that 3-(hydroxymethylphosphinyl)-propionic acid (MPPA), which was formed by oxidative deamination of glufosinate, was the major transformation product of glufosinate in moist non-sterile soils.

Tebbe and Reber (1988) first reported the isolation of several bacteria that were able to use L-PPT as a nitrogen source in pure culture. Two bacterial isolates, a *Rhodococcus* strain DX-35 and *Pseudomonas paucimobilis* FX-90, utilized the amino moiety of L-PPT and released, by the action of an unspecific amino acid oxidase, the corresponding 2-oxo-4[(hydroxyl)-(methyl) phosphinoyl] butyric acid (PPO). This metabolite was transformed by decarboxylation at the late stationary phase of *Rhodococcus* culture to MPPA, the major degradation product of PPT in soil. In a subsequent study, a

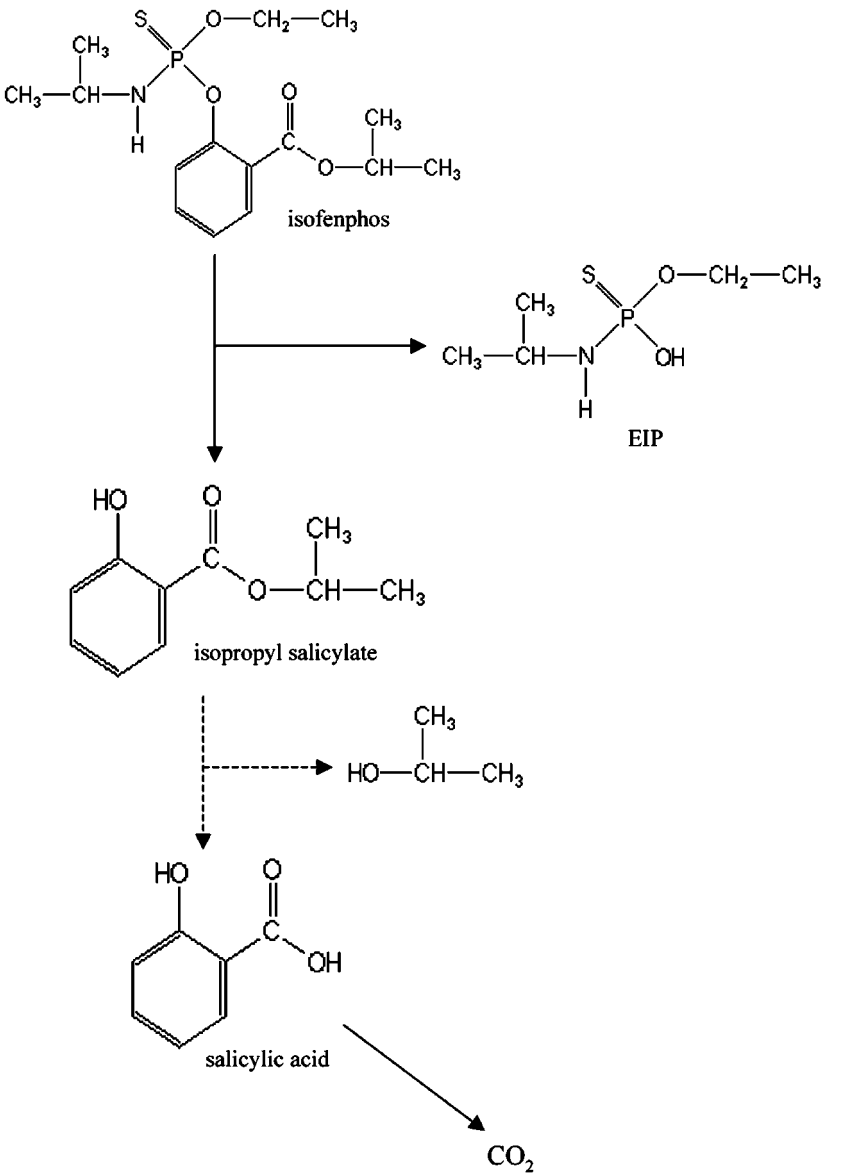


Figure 7 The metabolic pathway of isofenphos degradation by soil microorganisms. Metabolic steps designated with dashed lines represent postulated metabolic pathways.

number of other bacteria including *Agrobacterium tumefaciens*, *Alcaligenes* sp., *Pseudomonas* sp., *Serratia plymuthica* and *Enterobacter* sp., were reported to degrade high concentrations of PPT but were unable to utilize the compound as a nitrogen source (Bartsch and Tebbe, 1989). In cultures containing cell extracts of these strains a different metabolic pathway was observed: PPT, in the presence of acetyl-CoA, was transformed to *N*-acetyl-PPT. All isolates reported to metabolize PPT showed stereo-selectivity towards L-PPT and were not able to utilize D-PPT.

The major metabolites of PPT identified in bacterial cultures, PPO and *N*-acetyl-PPT, were never identified in the soil metabolic studies. On the other hand, MPPA, the major degradation product of PPT in soil, was detected at very low concentration in bacterial cultures. Later studies by Tebbe and Reber (1991) revealed that this discrepancy was attributed to the instability of PPO in the soil environment. Therefore, degradation of PPT in soil is initiated with an oxidative deamination step leading to the formation of PPO that is further biotically or abiotically decarboxylated to MPPA. The latter metabolite is mineralized by soil microorganisms.

In summary, the initial attack on PPT by soil bacteria is at the amino terminus of the molecule rather than at the C–P bond. This is further supported by the finding that no microorganisms have been isolated that could utilize PPT as a sole phosphorus source. Thus the metabolic pathway of PPT seems to differ considerably from that reported for glyphosate, an OP herbicide resembling PPT, where soil bacteria degrade the molecule by cleaving the C–P bond. The pathways of microbial metabolism of PPT are illustrated in Fig. 8.

Glyphosate (*N-phosphonomethylglycine*): Glyphosate is one of the world's leading agrochemicals providing an earning of about 1 billion US dollars per year to its manufacturer, Monsanto Agricultural Products Company. Glyphosate is a non-selective phosphonate herbicide that is used in agricultural and non-agricultural areas for the control of annual and perennial weeds. It acts by interfering with the enzyme that catalyses the sixth step in the shikimate pathway, 5-enol-pyruvyl-shikimate-3-phosphate synthase (EPSPS), thus disrupting the biosynthesis of aromatic amino acids (Jaworski, 1972). The anticipated widespread use of this compound has prompted studies of its environmental fate which demonstrated that microbial degradation is the main process controlling the disappearance of glyphosate in soil (Rueppel *et al*., 1977; Wiren-Lehr *et al*., 1997; Gimsing *et al*., 2004). Several studies have shown a positive correlation between soil microbial biomass, microbial populations, enzymatic activities and glyphosate degradation in soil (Torstensson and Stark, 1979; Lonsjo *et al*., 1980; Araujo *et al*., 2003). For example, Gimsing *et al*. (2004) found a positive correlation

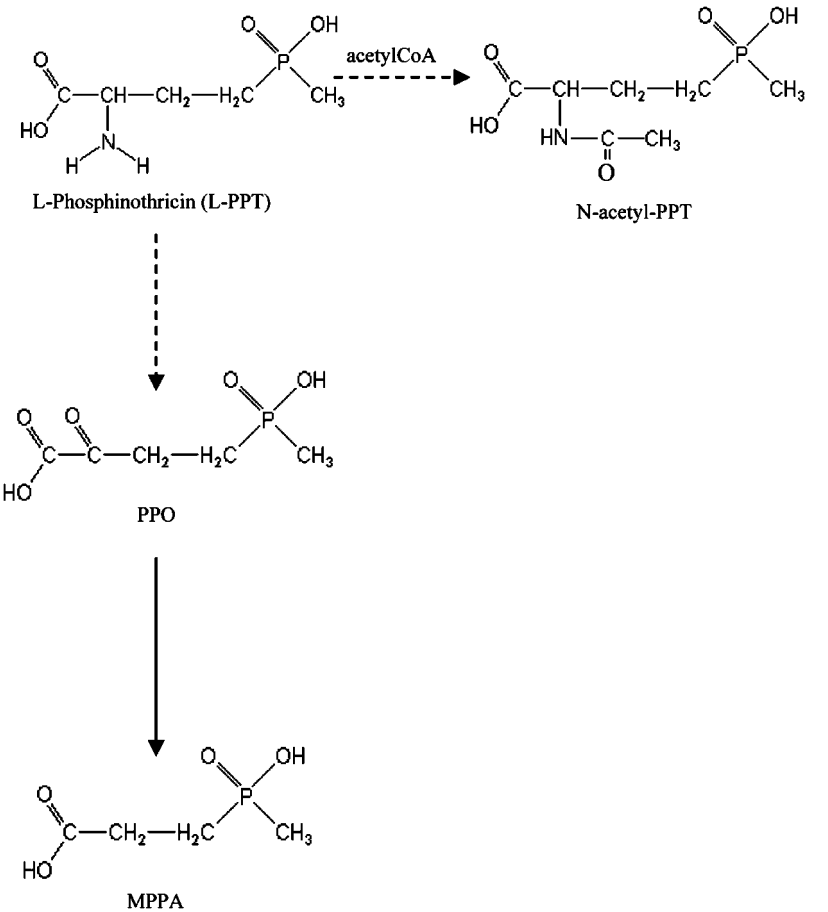


Figure 8 The metabolic pathway of PPT degradation by soil microorganisms. Metabolic steps designated by dashed lines represent reactions that produce transient intermediate metabolites which are only detected in liquid cultures and not in soil.

between the population of *Pseudomonas* sp. in soils and the degradation rates of glyphosate in these soils. Metabolism of glyphosate in soil usually proceeds via cleavage of the C–N bond producing aminomethylphosphonic acid (AMPA), which is then gradually mineralized by soil microflora (Rueppel *et al.*, 1977; Forlani *et al.*, 1999; Araujo *et al.*, 2003). Other metabolites identified included *N*-methylaminomethylphosphonic acid, glycine, *N*,*N*-dimethylaminomethylphosphonic acid and hydroxymethylphosphonic acid (Rueppel *et al.*, 1977).

Glyphosate, as most of the natural or synthetic phosphonates, is characterized by a C–P bond in its molecule that is highly resistant to chemical hydrolysis, thermal decomposition and photolysis (Cook *et al.*, 1978b). Hence, cleavage of the C–P bond in the environment is entirely attributed to microorganisms. Degradation of glyphosate by soil microorganisms proceeds via two different pathways, which have been identified in several bacterial, fungal and actinomycetal isolates (Kertesz *et al.*, 1994). In the first metabolic pathway, the C–P bond of glyphosate is initially cleaved resulting in the release of a phosphate group and a molecule of sacrosine (Shinabarger and Braymer, 1986). The latter is further transformed by a sacrosine-oxidizing enzyme to the amino acid glycine and a C-1 unit. Glycine is used by microorganisms for the biosynthesis of proteins. The single carbon unit is transformed to formaldehyde or formate (single carbon molecules) which either enters the tetrahydrofolate reactions or is released as CO_2. Tetrahydrofolate is a coenzyme responsible for the transfer of single carbon compounds in various cellular additions, such as the incorporation of the carbon atom from formaldehyde to the purine ring of adenine, guanine or the biosynthesis of methionine and serine. This metabolic pathway was first reported in a *Pseudomonas* PG2982 strain that was able to use glyphosate as a sole phosphorus source (Moore *et al.*, 1983; Jacob *et al.*, 1985; Kishore and Jacob, 1987). Subsequently, the pathway was identified in other microorganisms including an *Agrobacterium radiobacter* (McAuliffe *et al.*, 1990), an *Arthrobacter* GLP-1 strain (Pipke *et al.*, 1987), *Rhizobium meliloti* and other *Rhizobium* strains (Liu *et al.*, 1991), and several other strains isolated from soil previously exposed to the herbicide (Dick and Quinn, 1995). All of the above strains were capable of using glyphosate as a sole phosphorus source, but were unable to use the compound as either carbon or nitrogen source. This was attributed to the presence of an uptake regulation system for glyphosate in most phosphonate-degrading microorganisms which limits organophosphonate utilization, since the phosphorus released after cleavage of the C–P bond represses the degradation system (Obojska *et al.*, 1999). However, a mutant of the *Arthrobacter* strain GLP-1, named *Arthrobacter* GLP-1/Nit-1, could utilize glyphosate as its sole nitrogen source as well (Pipke and Amrhein, 1988b). It was shown that the inability of *Arthrobacter* GLP-1 strain to utilize glyphosate as a nitrogen source is due to the stringent control of glyphosate uptake by excess phosphate released during the degradation of the herbicide. In contrast, the mutant strain had developed a reduced uptake affinity for inorganic P, but not for glyphosate, which enabled it to carry on degrading glyphosate and using it as a sole source of nitrogen. A similar ability to utilize glyphosate as both phosphorus and nitrogen source was reported for two *Streptomyces* spp. (Obojska *et al.*, 1999).

In the second metabolic pathway, glyphosate is degraded by cleavage of the C–N bond releasing AMPA and glyoxylate. The former metabolite is subjected to dephosphorylation by enzyme C–P lyases, leading to the formation of methylamine, formaldehyde and is finally mineralized to CO_2. Methylamine is produced by the transformation of several pesticides, including carbofuran and atrazine, and serves as a carbon and/or nitrogen source for microorganisms (Chapalamadugu and Chaudhry, 1992; Kamanavalli and Ninnekar, 2000). This pathway was first reported to occur in a *Flavobacterium* sp., which was isolated from an industrial biosystem processing glyphosate wastes (Balthazor and Hallas, 1986). *Flavobacterium* sp. was able to use glyphosate as a sole source of phosphorus. Later, the same pathway was evident in cultures of a *Pseudomonas* LBr strain, isolated also from a glyphosate waste treatment system, which also used glyphosate as a sole source of phosphorus (Jacob *et al*., 1988). Although the AMPA pathway was identified as the major degradation pathway of glyphosate by this strain, *Pseudomonas* LBr strain was also able to convert about 5% of the initially added glyphosate via formation of sacrosine and glycine. This is the first and only report of a glyphosate-degrading microorganism that could degrade the compound via both metabolic routes. An *Arthrobacter atrocyaneus* (Pipke and Amrhein, 1988a) and a *Pseudomonas pseudomallei* (Penaloza-Vazquez *et al*., 1995) were also reported to metabolize glyphosate via the AMPA pathway. A thermophile *Geobacillus caldoxylosilyticus* T20 strain isolated from a central heating system, was found to utilize glyphosate as a sole source of phosphorus (Obojska *et al*., 2002). Degradation of glyphosate by the thermophilic strain led to the formation of AMPA and glyoxylate. It should be stressed that both *A. atrocyaneus* and *G. caldoxylosilyticus* were isolated from culture deposits or places that had never been exposed to glyphosate, suggesting that the ability to metabolize this compound and probably other organophosphonates is widespread in nature. A detailed illustration of the two pathways of glyphosate microbial metabolism is shown in Fig. 9.

Apart from bacteria and actinomycetes, fungi have been shown to degrade glyphosate. Zboinska *et al*. (1992) first reported the isolation of a fungal strain, *Penicillium citrinum*, which could metabolize glyphosate. Later studies identified a *P. notatum* isolate that metabolized glyphosate via the AMPA pathway (Bujacz *et al*., 1995). Other fungal strains including *Trichoderma viride*, *T. harzianum*, *Scopulariopsis* sp., *Alternaria* sp. and *A. niger*, isolated from soil, showed an enhanced ability to grow on several organophosphonates including glyphosate (Krzysko-Lupicka *et al*., 1997). These fungal strains metabolized glyphosate via the AMPA pathway. All the fungal strains reported above could utilize glyphosate as a source of

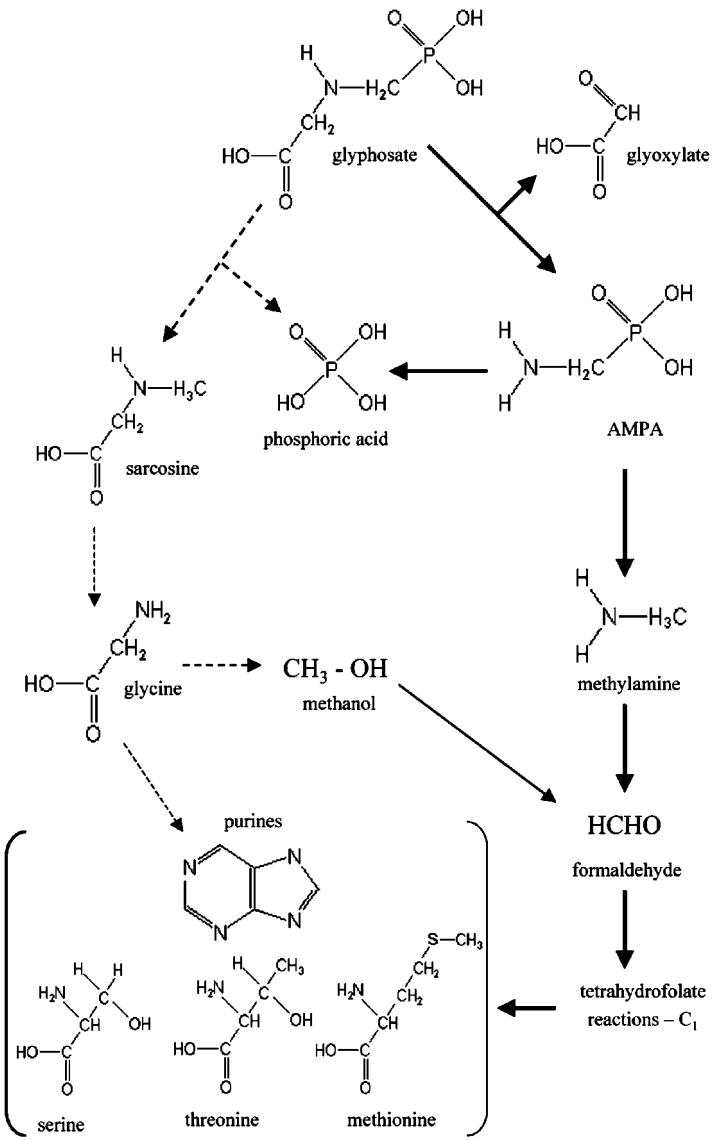


Figure 9 The metabolic pathway of the herbicide glyphosate degradation by soil microorganisms. Metabolic steps of the AMPA-pathway are designated by solid lines, unlike metabolic steps of the sacrosine-pathway, which are designated by dashed lines.

phosphorus. However, Klimek *et al.* (2001) reported for the first time the isolation of a non-nitrate-utilizing strain of *Penicillium chromogenum*, which utilized glyphosate as a sole nitrogen source. Growth of *P. chromogenum* was considerably lower when the herbicide was used as a sole phosphorus source. Recently, Lipok *et al.* (2003) isolated a number of fungi including *Penicillium janthinellum*, *Penicillium. simplicissimum*, *Mucor* sp. and *Alternaria alternata* from non-disinfected carrot seeds, which could utilize glyphosate as a phosphorus source. Interestingly, *A. alternata*, a soil plant pathogen, used glyphosate as a nitrogen source and transformed it via the AMPA pathway.

2.4. Fungicides

OP fungicides comprise a small group of pesticides and include iprobenfos, edifenphos, pyrazophos and toclofos-methyl. Iprobenfos (*S*-benzyl *O*, *O*-diisopropyl phosphorothiolate) and edifenphos (*O*-ethyl *S*,*S*-diphenyl phosphorodithiolate) are systemic fungicides used mainly in Japan for the control of rice blast (*Pyricularia oryzae*). Both phosphorothiolate fungicides inhibit the biosynthesis of phosphatidylcholine, an important component of fungal cell membrane (Kodama *et al.*, 1979, 1980). A few soil metabolic studies have shown that degradation of iprobenfos and edifenphos in soil is mainly microbially mediated (Tomizawa *et al.*, 1976; Tomizawa and Kazano, 1979). In aerobic soils, degradation of edifenphos proceeds initially via removal of the *S*-phenyl or the ethyl moiety of its molecule. The metabolites are further metabolized to diphenyl disulfide, benzensulfonic acid with end products methyl phenyl sulfoxide and sulfone (McNamara and Close, 1976). In anaerobic soils, the main degradation products of edifenphos were thiophenol, diphenyl disulfide and benzenesulfonic acid (Tomizawa, 1975). Parallel soil degradation study for iprobenfos suggested that metabolism of the fungicide proceeded via hydrolysis of the P–S–C bond with production of large amounts of *O*,*O*-diisopropyl hydrogen phosphorothioate (Tomizawa *et al.*, 1976). Uesugi and Tomizawa (1971) studied the metabolism of edifenphos in mycelia of fungus *P. oryzae*, and found that the main metabolic pathway consisted of an initial hydrolysis of one of the P–S bonds, followed by that of the other P–S or the ethyl ester bond finally leading to release of phosphoric acid. Another metabolite which was hydroxylated in one of the phenyl rings was detected in small quantities.

Pyrazophos (ethyl 2-diethoxyphosphinothioyloxy-5-methylpyrazolo[1,5-a] pyrimidine-6-carboxylate), a systemic fungicide, is selectively used for the

control of powdery mildew (*Erysiphe* sp.) in various crops. Pyrazophos has a similar mode of action as edifenphos and iprobenfos. DeWaard (1974) observed that *P. oryzae*, a fungus sensitive to pyrazophos, converted pyrazophos into two fungitoxic products, pyrazophos-oxon and 2-hydroxy-5-methyl-6-ethoxy carbonylpyrazole (1,5-a) pyrimidine. Hasan (1999) reported the isolation of several fungal species for pesticide-treated wheat straw, which utilized pyrazophos as a carbon or phosphorus source. For example, an *A sydowii* strain was able to grow on pyrazophos when the compound was supplied as a carbon or phosphorus source. *A. sydowii*, *A. flavus* and *Fusarium oxysporum* possessed phosphatase activity which hydrolysed 300 mg kg^{-1} of pyrazophos in soil within three weeks.

Toclofos-methyl (*O*-2,6-dichloro-*p*-tolyl *O*,*O*-dimethyl phosphorothioate) is a non-systemic fungicide used in ornamentals and horticultural crops for the control of *Rhizoctonia* sp. It has been suggested that toclofos-methyl acts by peroxidation of lipid components of cell membranes. Degradation of toclofos-methyl in soil proceeds via hydrolysis of the P–O bond leading to 2,5-dichlorocresol. However, dimethylation of the methoxy moieties of toclofos-methyl has also been observed (Tomlin, 2000). No microorganisms have been isolated so far, which can metabolize toclofos-methyl. An isofenphos-degrading *Arthrobacter* strain was unable to degrade toclofos-methyl and other OP compounds (Ohshiro *et al*., 1996).

2.5. Chemical Warfare Agents (CWAS)

The OP CWAs (also known as nerve agents) are a group of extremely toxic compounds and constitute the major proportion of total CWAs worldwide. Nerve agents are generally classified into two main groups: G-agent and V-agent. G-agents are non-persistent and volatile compounds whereas V-agents are persistent, non-volatile and more toxic than G-agents. Munro *et al*. (1994, 1999) have reviewed toxicity and environmental fate of CWAs in detail. In this paper, we review different aspects of microbial degradation of CWAs.

As per CWC (1993), about 175 countries are required to destroy approximately 200,000 tons of nerve agents by 2007. The USA alone will have to destroy approximately 30,000 tons of nerve agents under CWC. Initially, alkaline hydrolysis was used for the destruction of nerve agents but the resistance of some CWAs to this process led to adoption of incineration as an alternative approach. However, because of the growing concern regarding emissions during the incineration and protests by environmentalists

and local communities, destruction of CWAs by incineration has been abandoned. Consequently, there is a need to develop an alternative safe and environmentally friendly method of CWAs destruction.

G-agents: G-agents consist of three main types, commonly known as GA, GB and GD. GA (name given as American denomination; chemical name ethyl *N*,*N*-dimethylphosphoroamidocyanidate), which is also known as "Tabun," contains a cyanide group and is vulnerable to hydrolysis. It enters the body through the respiratory tract and causes death through failure of the respiratory system. It is soluble in water but is also readily soluble in organic solvents and can therefore easily enter the body through skin (Munro *et al*., 1999). Sarin or GB (isopropyl methylphosphonofluoridate) causes immediate death due to complete failure of the respiratory system. It is volatile and completely soluble in water. Soman or GD (pinacolyl methylphosphonofluoridate) is structurally similar to GB. Its volatility is intermediate between GA and GB. It is less water-soluble than the other two G-agents, and consequently can rapidly penetrate through skin, and has greater toxicity (Munro *et al*., 1999)

P. diminuta (which was isolated for degradation of parathion) and *Alteromonas* spp. have been successfully used for degradation of all G-agents (Defrank *et al*., 1993; Mulbry and Rainina, 1998). Complete degradation of G-agents is likely to produce phosphoric acid as with other OP compounds. A simplistic diagram illustrating the degradation pathway of nerve agents is presented in Fig. 10. Tabun (GA) contains several possible microbial degradation sites. Possible initial steps include O-dealkylation, C-dealkylation, nitrile hydrolysis and N-dealkylation (Morrill *et al*., 1985). Studies of the environmental fates of such compounds suggested that dimethyl amine, dimethylphosphoramidate and triethyl phosphates are possible intermediates that are readily biodegradable (Munro *et al*., 1999). The major metabolites identified for GB and GD degradation are isopropylmethylphosphonic acid (IMPA) and pinacolyl methylphosphonic acid (PMPA), respectively. PMPA is then hydrolysed to IMPA, which is an extremely stable compound with a predicted half-life of 1,900 years (Rosenblatt *et al*., 1975). However, several groups of bacteria have been reported to utilize IMPA and PMPA as a source of phosphorus (Cook *et al*., 1978b; Zhang *et al*., 1999) with two bacterial species *Pseudomonas testosterone* and *P. melophthora* being reported to metabolize IMPA to methane and inorganic phosphorus by breaking C–P bonds of methyl phosphonic acid (MPA) (Daughton *et al*., 1979).

V-agents: V-agents are mainly dominated by one compound called VX (*O*-ethyl-S[di-isopropylamino] ethyl-methylphosphonothioate). VX is a moderately persistent nerve agent and is characterized by a P–S bond and

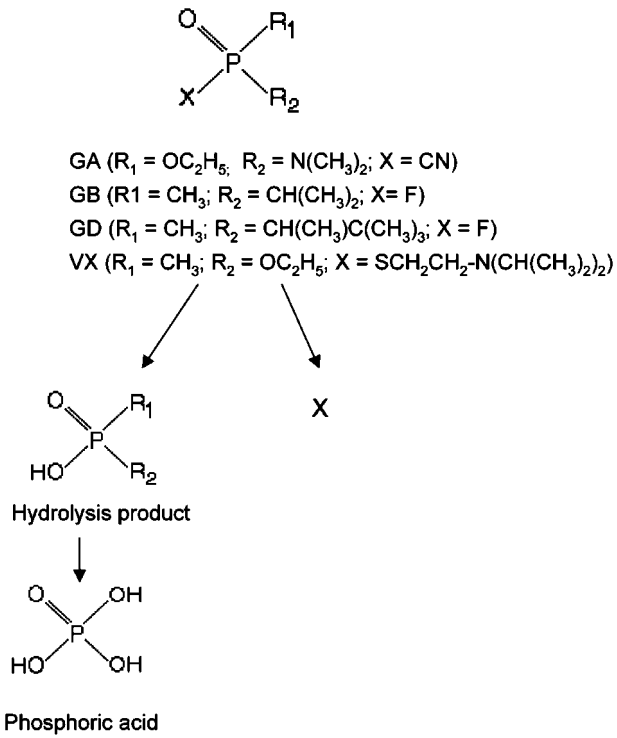


Figure 10 A simplistic diagram showing the metabolic pathway for degradation of OP chemical warfare agents. The hydrolysis products for GA, GB, GD and VX are ethyldimethylamido phosphoric acid, IMPA, PMPA and ethyl methyl phosphoric acid respectively.

therefore belongs to the phosphorothiolates group. It is less volatile than G-agents, water soluble and relatively resistant to hydrolysis (Munro *et al.*, 1999). It is largely resistant to microbial degradation and *Alteromonas* spp. cannot hydrolyse it. However, *P. diminuta* has been shown to degrade it although at a very low rate (about 0.1% towards VX as compared with parathion). However, a mutant generated for OPH of *P. diminuta* has shown up to 33% increase in its activity against VX (Gopal *et al.*, 2000). Oxidative hydrolysis of VX produces ethyl methyl phosphonic acid (EMPA), which can be degraded by *Burkholderia caryophilli* and *P. testosterone* (Elashvili and DeFrank, 2001).

3. BIOCHEMICAL AND MOLECULAR BASIS FOR DEGRADATION OF ORGANOPHOSPHORUS XENOBIOTICS

Several enzymes/genes capable of degrading organophosphorus compounds have been isolated and characterized. Two enzymes, organophosphate hydrolase (OPH) and organophosphorus acid anhydrolase (OPAA) and their encoding genes *opd* and *opaA*, respectively, have received most of the attention due to their capability to degrade a wide range of OP compounds including CWAs.

3.1. OPH/opd

Organophosphate hydrolase (OPH) or phosphotriesterase is the most studied enzyme involved in OP degradation. OPH was initially characterized from a soil bacterium *Flavobacterium* sp. strain ATCC 27551, which was isolated from a paddy field in the Philippines (Sethunathan and Yoshida, 1973). Since then, OPH has been isolated and characterized from a range of taxonomically and geographically distinct bacteria (Raushel, 2002).

OPH exhibits activity against a range of OP compounds including parathion, methyl parathion and fensulfothion, among many others (Dumas *et al.*, 1989) and OP CWAs (Dumas *et al.*, 1990). The purified enzyme is capable of hydrolyzing paraoxon at a rate that approaches the diffusion control limit. The turnover number for zinc-substituted OPH for paraoxon hydrolysis is $2100\,s^{-1}$, while the corresponding value for K_{cat}/K_m is 4×10^7 $M^{-1}\,s^{-1}$(Ghanem and Raushel, 2005). OPH is a homodimeric metalloprotein with a molecular weight of ~72 kDa (Benning *et al.*, 1994). It is a member of the amidohydrolase superfamily (Holm and Sander, 1997) and consists of two identical subunits containing 336 amino acids. High-resolution X-ray structure analysis showed that OPH protein folds into an $(\alpha\beta)_8$-barrel motif with the active site located at the carboxy-terminal end of the central β-sheet core (Raushel, 2002). The active site of OPH contains two zinc ions per subunit. The α-metal which is buried in the active site is linked to two histidine residues (His-55 and His-57) and an aspartic acid (Asp-301), whereas, the more solvent-exposed β-ion is bound to two histidines (His-201 and His-230) and a water molecule. The two metal ions are bridged by the carboxylated Lys-169 and a water/hydroxide molecule (Ghanem and Raushel, 2005). A study by Vanhooke *et al.* (1996) demonstrated that OPH consisted of three subsites for substrate binding: (1) the large subsite which contains His-254, His-257, Leu-271 and Met-317, (2) the small subsite which

is made up of Gly 60, Leu-303, Ser-308 and Ile-106, and (3) the hydrophobic leaving group pocket which consists of Phe-306, Phe-132, Trp-131 and Tyr-309 (Fig. 11). The side chains of these 12 residues determine the substrate specificity and stereoselectivity of OPH.

It is believed that OP compounds bind to the binuclear metal center located in the active site of the OPH via coordination of the phosphoryl oxygen to the β-metal ion which weakens the linkage of the bridging hydroxide to the β-metal. This metal and the non-ester oxygen of the substrate interaction increase the electrophilicity of the phosphorus centre. The bound hydroxide initiates the nucleophilic attack with proton abstraction from Asp-301 of the α-sheet (Efremenko and Sergeeva, 2001). It is hypothesized that His-354 may facilitate the transfer of a proton from the active site to the bulk solvent.

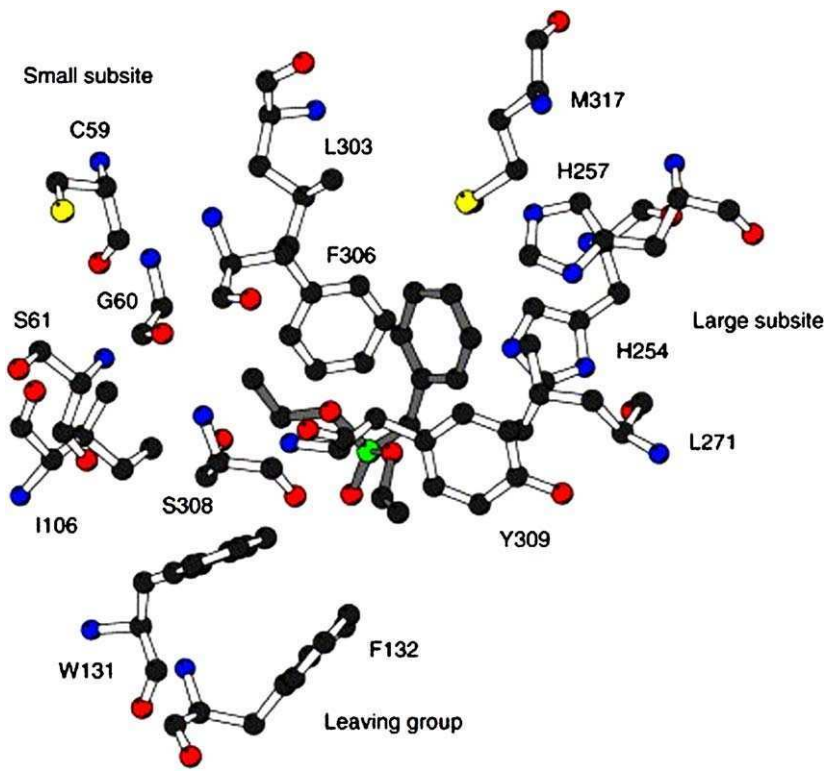


Figure 11 The active site of organophosphorus hydrolase and the relative position of the amino acid residues. Reproduced with permission from Raushel (2002). (See color plate section page 225)

Table 2 Comparative account of OPH and OPAA

Properties	Oph	Opaa
Structure	Dimer	Monomer
Encoding gene	*Opd*	*Opaa*
Initial source	*Pseudomonas diminuta*	*Alteromonas* sp. JD6.5
Catalytic efficiency (K_{cat} s^{-1})		
Paraoxon (p–o)	3170	124
Dfp (p–f)	465	1820
Sarin (p–f)	56	611
Soman (p–f)	5	3145
Vx (p–s)	0.3	0

OPH has a wide range of substrate specificities and has been demonstrated to catalyse hydrolysis of P–O, P–F, P–CN and P–S bonds to different extents. It has the highest activity against the P–O linkage (with K_{cat}/K_m value of 5.5×10^{-7} mol^{-1} L s^{-1} for paraoxon) and least specificity for the P–S bond (with K_{cat}/K_m value of 6.8×10^{-2} mol^{-1} L s^{-1} for VX) (Table 2; Efremenko and Sergeeva, 2001). OPH requires zinc for its activity and it has been demonstrated that replacement of zinc ions with other divalent metals such as cobalt, cadmium, copper, iron, manganese and nickel had effects on its catalytic activity. Enzymatic activity of Co^{2+}-reconstituted OPH has the greatest activity against paraoxon (Omburo *et al*., 1992). Cd^{2+}- and Zn^{2+}-reconstituted enzymes were shown to have from one-half to one-tenth of the maximal activity exhibited by Co^{2+}-reconstituted enzyme, while other metal-substituted enzymes had dramatically less efficacy against tested OP compounds. Recently, Manavathi *et al*. (2005) argued that the increase in specific activity of OPH caused by the Zn^{2+} and Co^{2+} is due to improved folding of expressed protein. It was also observed that a monometal enzyme may be effective for catalysis of P–S bond (Di Sioudi *et al*., 1999).

The substrate specificity of OPH is broad, and the catalytic efficiency and the rate limiting steps depend on the pK_a of the leaving group of substrates. An experiment with analogues of paraxoan revealed that OPH preferentially hydrolysed the *Sp*-enantiomer within a racemic mixture of chiral OP compounds with almost 100-fold higher activity for the *Sp*-enantiomer (Hong and Raushel, 1999). This is an important observation in the sense that toxicity of nerve agents is also stereoselective. But more importantly, most of the nerve agents are racemic mixtures and, therefore, it is essential that detoxifying enzymes should be able to hydrolyse both enantiomers. It was demonstrated that the locations of the binding subsites determine the catalytic properties of OPH. Later, the size and shape of these binding sites

were remodelled through a rational designing via site-directed mutagenesis which revealed that stereoselectivity of the wild type OPH can be enhanced, relaxed and reversed (Raushel, 2002). For increased hydrolysis of racemic mixture, the small subsite of the OPH was expanded by replacing Phe132, Ser308 and Ile106 with glycine and/or alanine residues (Wu *et al*., 2001). In many instances the stereoselectivity for *Sp*- and *Rp*-enantiomers of ethyl phenyl *p*-nitrophenyl phosphate decreased from 21:1 to ~1:1.3 without interfering with the efficacy and rate of catalysis. In another case, an actual reversal of stereoselectivity was demonstrated by simultaneous enlargement of the small subsite and reduction in the large subsite. This was achieved by obtaining a mutant Il106G/F132G/H257Y/S308G of OPH. This mutant had catalytic activity of 1:460 for the *Sp*/*Rp* pair of enantiomers (Wu *et al*., 2000).

The OPH encoding, *opd* (*o*rgano*p*hosphate *d*egrading) gene has received most of the attention among characterized OP degrading genes. It was first isolated and sequenced from *P. diminuta* and was reported to be present on a 66 kb plasmid, pCMS1 (Serdar et al., 1982). The *opd* gene isolated from *Flavobacterium* sp. ATCC25 was also located on a plasmid, pPDL2 (43 kb). Comparative studies of the two plasmids, pPDL2 and pCMS1, by restriction analysis and hybridisation experiments have shown that the *opd* genes were located in a highly conserved region that was extended to 2.6 kb upstream and 1.7 kb downstream of *opd* (Mulbry *et al*., 1986, 1987). The nucleotide sequence for *opd* genes obtained from *Flavobacterium* (isolated in the Philippines) and *P. diminuta* (isolated in the USA) showed 100% sequence similarity, while plasmids on which the genes were based were unrelated (Mulbry *et al*., 1987, 1989b; Harper *et al*., 1988; Serdar *et al*., 1989). A *Flavobacterium balustinum* isolated from an agricultural soil in India was reported to harbour an *opd* gene on a plasmid (Somara and Siddavattam, 1995) that has 98% sequence similarity with *opd* genes obtained from *Flavobacterium* and *P. diminuta* (Somara *et al*., 2002). Since the identical *opd* genes were isolated from non-identical plasmids and from temporally, geographically and taxonomically different groups of bacteria, it was hypothesised that this gene may be part of a mobile genetic element or transposon (Mulbry *et al*., 1987). The first evidence to this end was provided by Siddavattam *et al*. (2003). By sequencing the entire conserved region of pPDL2 of *Flavobacterium*, they confirmed the presence of eight ORFs. ORF243, which is placed adjacent to the *opd* gene and transcribed in the opposite direction, was reported to code for proteins responsible for *p*-nitrophenol (a metabolite of parathion or methyl parathion) degradation. The *opd* gene and ORF243 are flanked by an insertional sequence encoding a complete *istAB* operon and by transposase genes (*tnpA* and *tnpR*)

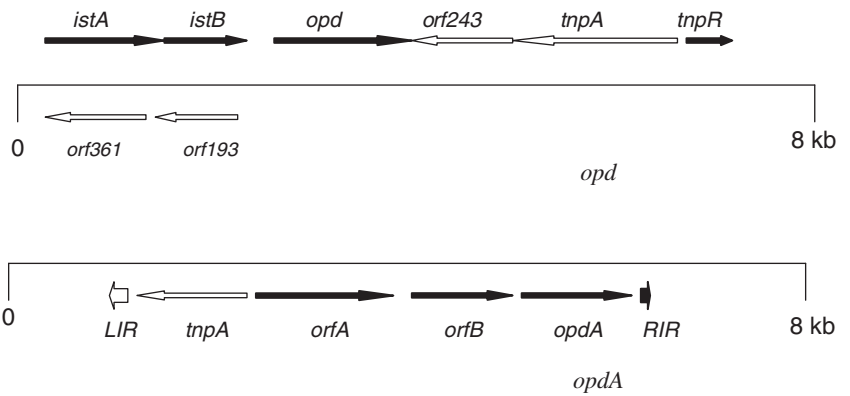


Figure 12 Comparative presentation of the *opd* operon in *Flavobacterium* sp. and the *opdA* operon in *Agrobacterium radiobacter* (adapted with permission from Siddavattam *et al*., 2003 and Horne *et al*., 2003). It shows the position and orientation of eight ORFs identified from the plasmid pPDL2 of *Flavobacterium* sp. and six similar ORFs identifed from the conserved region of the chromosome from *A. radiobacter*.

characteristic of *Tn3* family transposon (Fig. 12). A 2.5 kb region upstream of *opd* gene contains two ORFs transcribed in the same direction with significant homology to the *IstA* and *IstB* proteins encoded by members of the *IS21* family of transposons (Mahillon and Chandler, 1998). Two other ORFS were found on the complementary strand to *istA* and *istB*. The only homologous sequence in database for these two ORFs was reported in *Agrobacterium tumefaciens* C58 at similar position (Siddavattam *et al*., 2003).

3.2. OPDA/opdA

The overall structure of organophosphate-degrading enzyme (OPDA) is very similar to that of OPH. OPDA was isolated and characterized from an *A radiobacter* strain, which was isolated in Australia (Horne *et al*., 2002a). It is also reported that the mechanism of catalysis for both enzymes is almost identical (Yang *et al*., 2003). Just like OPH, OPDA has an $(\alpha\beta)_8$ barrel tertiary structure with a binuclear metal centre at the active site. A hydroxide ion and a carboxylated lysine bridge are the two metal ions in the active site, while the α-metal is further coordinated by two histidine residues (His-55 and His-57) and one aspartic acid (Asp-301) and the β-metals by two histidine (His-201 and His-230). OPH and OPDA differ at position 254 and

257. These sites are occupied by histidine in OPH and arginine residues in OPDA. Just like OPH, OPDA is active with a variety of divalent metals with highest activity in Co^{2+}-reconstituted enzyme (Yang *et al*., 2003). Initially, the OPDA mechanism of catalysis was thought to be similar to OPH. However, Jackson *et al*. (2005) proposed that for OPDA, the substrate preferentially binds at the α-metal when they are sufficiently small, but when the substrates are larger, due to a bulkier leaving group, they are unable to bind α-metal and are therefore forced to bind at the β-metal, which they argue is consistent with that of other binuclear metallophosphoesterases.

Despite having amino acid sequence similarity with OPH, OPDA shows difference in its substrate specificity which suggests a state of evolutionary flux (Yang *et al*., 2003). The amino acid sequence of OPDA and OPH are 90% similar (Horne *et al*., 2002a). The most significant difference between these two proteins is the presence of an additional 20 amino acid residues at the C-terminus of the OPDA. There are a few sequence differences throughout the protein including the active site. It was hypothesised that these sequence differences are responsible for the variations in the substrate specificity. OPDA has demonstrated higher activity (high K_{cat} value) against shorter side chains and can hydrolyse fenthion and phosmet for which OPH has no activity (Horne *et al*., 2002a). It is argued that the differing amino acid residues at 254 and 257 (which are arginine in the case of OPDA and histidine in the case of OPH) result in an overall reduction in the subsite size and that is why OPDA preferentially degrades smaller side chain molecules, such as methyl parathion rather than the diethyl equivalent (Yang *et al*., 2003). Apart from the sequence differences, the water structure in the active site differs in the two proteins.

The gene that encodes OPDA, called *opdA*, was obtained and sequenced from *A. radiobacter* P230. Although, this gene was chromosome based, it has 88% nucleotide sequence similarity with *opd* genes (Horne *et al*., 2002a). Later, the same group found a transposase gene (*tnpA*) upstream of the *opdA* gene (transcribes in opposite direction of *opdA*) of *A. radiobacter* P230 as well as inverted repeats (LIR and RIR), indicative of insertion sequences flanking the two genes. Two additional putative ORFs (ORF-A and ORF-B) lie between *opdA* and *tnpA* and transcribe in the same direction as the *opdA* gene (Fig. 12). The inferred translation for these two ORFs gave amino acid similarity to two proteins encoded on the *Geobacillus stearothermophilus* IS5376 transposon (Horne *et al*., 2003). It is postulated on the basis of sequence similarity between *opd* and *opdA* and the catalytic activities of encoded enzymes that *opdA* has evolved more recently than *opd* (Horne *et al*., 2003).

This observation has an evolutionary angle. The origin and role of *opd* is unknown. Since *opd*-like genes have been reported from several bacteria, the presence of conserved regions with a transposon system indicates that lateral transfer of the gene have played an important role in its wide distribution. Its location on both plasmid (*opd*) and chromosome (*opdA*) suggests that one interspecific transposition occurred prior to the sequence diversity between *opd* and *opdA*, with another more recent horizontal transfer to account for the finding of identical *opd* sequences in *P. diminuta* and *Flavobacterium* (Horne *et al*., 2003). Why and how bacterial *opd* evolves is still not known. Transposition as a mechanism of acquisition of antibiotic resistance genes among bacteria is well established. But this argument does not apply to OP degrading gene because OP compounds are not toxic to bacteria (owing to lack of any AChE). Then why is the *opd* gene transferred so readily in the environment? One hypothesis is that due to the large-scale use of OP pesticides, *opd* gene acquisition provides a nutritional benefit. Most bacteria harbouring the *opd* gene sequences are able to use OP pesticides either as a phosphorus or carbon source. But the origin of the gene is still not clear. It is believed that this gene was present in the environment long before OP xenobiotics were used. The *opd*-like genes were reported from soils and bacteria that were never exposed to OP compounds (Singh *et al*., 2003). This observation received support from the fact that a similar gene was found in *Escherichia coli* and *Mycobacterium tuberculosis* genomes (Philipp *et al*., 1996; Blattner *et al*., 1997). On the basis of its distribution among prokaryotes and eukaryotes, it is hypothesized to be of ancient origin probably long before the divergence of Archaea, Prokaryota and Eukaryota (Horne *et al*., 2003).

3.3. OPAA/opaA

An organophosphorus acid anhydrolase (OPAA) was first isolated and purified from *Alteromonas* strain JD6.5. It is a single peptide with a molecular weight of 60 kDa and possesses high activity against a range of organophosphorus compounds including the G-agents of CWAs (Cheng *et al*., 1999). It has a pH optimum of 8.5 and a temperature optimum of 50 °C. Later, OPAA was isolated from several strains of *Alteromonas*. Among the tested strains, *Alteromonas undina* exhibited the most promising levels of activity and a broad range of substrate specificity (Cheng *et al*., 1993). Maximum activity of OPAA was reported in the presence of Mn^{2+} and Co^{2+} (DeFrank and White, 2002). In comparison with OPH, it has lower catalytic efficiency against paraoxon but higher against G-agents (Table 2). However, it does not have any activity against P-S bond. The

three-dimensional structure of the OPAA has not been determined yet, which would be important to facilitate understanding of the mechanism of catalysis.

The natural function of the OPAA is not known but has been proposed that it is a dipeptidase that catalyses dipeptide with a proline residue at the C-terminus (Cheng *et al*., 1996). It has similar stereoselective properties to OPH. OPAA also preferentially hydrolyse the *Sp*-isomers of OP trimesters, although the overall activity is substantially lower. Similarly, it preferentially acts against the *Rp*-enantiomer of Sarin and Soman in racemic mixtures (Hill *et al*., 2000).

The gene (*opaA*), which encodes OPAA, was isolated from *Alteromonas* sp. JD6.5 (Cheng *et al*., 1996). The nucleotide sequence revealed an ORF of 1551 nucleotides. There was no sequence similarity between *opd* and *opaA* despite having functional similarity. As with *opd*, *opaA* is widely distributed in the prokaryotes and eukaryotes but its environmental role is not clear. The sequence comparison between *opaA*, *E. coli pepP* (encoding aminopeptidaseP) products and human prolidase showed significant similarities (Cheng *et al*., 1996). On this basis, it has been hypothesized that *opaA* and human prolidase have evolved from the same ancestor. But recent findings, that aminopeptidaseP can also degrade several OP compounds (Jao *et al*., 2004), suggest that this gene may also have ancestry linked with *pepP*.

3.4. Other Enzymes and Genes Involved in OP Degradation

Several other enzymes have been isolated from a range of bacterial and fungal species that can degrade OP compounds of different linkage and structures. For example, aminopeptidaseP from *E. coli* was reported to degrade a wide range of OP compounds including various analogues of paraoxon (Jao *et al*., 2004). This finding is interesting in the sense that OPAA was hypothesized to be related or evolved from aminopeptidaseP. The gene encoding aminopeptidaseP (*pepP*) has been isolated, cloned and overexpressed (Jao *et al*., 2004). Mulbry and Karns (1989) isolated three different OP hydrolases from Gram negative isolates. While they have similar temperature optima (40 °C), the substrate specificity and structure of these enzymes were different from one another, and also from the known OPH. HocA (hydrolysis of caroxon) is another OP hydrolysing enzyme isolated from *Pseudomonas monteilli* (Horne *et al*., 2002b). This enzyme is unique in the sense that it is not a metalloenzyme and its activity is controlled by the presence of phosphate in the growth medium. The gene encoding this enzyme, *hocA,* consists of 501 bp and encodes a protein of 19 kb. This protein does not have any sequence similarity to any protein in the database (Horne

et al., 2002b). Recently, methyl parathion degrading genes were cloned and expressed. Zhongli *et al.* (2001) isolated a novel chromosome-based methyl parathion degrading (*mpd*) gene from a *Plesiomonas* sp. Later, another *mpd* gene was isolated from a 70 kb plasmid of *Pseudomonas* sp. strain WBC-3 which had 99.5% sequence similarity with the *mpd* gene from *Plesiomonas* sp. (Liu *et al.*, 2005). Since the *mpd* gene in *Pseudomonas* sp. is flanked by two insertion sequences (*IS600*), it was suggested that strain WBC-3 may have evolved from a *p*-nitrophenol (a methyl parathion metabolite) utilizer by acquiring *mpd* transposon through interspecific transposition (Liu *et al.*, 2005).

While several OP-degrading enzymes have been isolated from bacteria, only a few were reported from fungi. A dimethoate-degrading enzyme was isolated and characterized from *A. niger* ZHY256. This enzyme (60 kDa) can degrade the P–S linkage of dimethoate, formothion and malathion (Liu *et al.*, 2001). Another novel fungal enzyme for OP compound degradation was obtained from *Penicillium lilacinum* BP303. This 60 kDa enzyme is reported to degrade both P–O and P–S bonds (Liu *et al.*, 2004).

4. POTENTIAL APPLICATIONS

4.1. Bioremediation and Detoxification

Bioremediation and detoxification aspects of isolated bacteria are receiving increasing attention due to the high mammalian toxicity of OP compounds and the obligation of all countries under CWC to destroy CWAs by 2007. Pesticide contamination of environments occurs mainly due to excessive use, mishandling, accidental spillage and large volume of waste produced as a result of the bulk use of pesticides. For example, OP compounds are used in large quantities for the control of animal pests. It is estimated that the USA alone generates 15,000 L (active ingredient, 1600 mg L^{-1}) of coumaphos waste every year under the cattle-tick eradication programme (Mulbry *et al.*, 1998). Mexico is estimated to generate the same amount of coumaphos waste.

Bioremediation provides a cheap and environmentally friendly approach for the decontamination and detoxification of contaminated environments. Since, the first reported use of a bacterial isolate for detoxification of OP compounds by Munnecke (1976), a number of studies have been carried out to evaluate bacterial potential to degrade OP compounds in water and soils. However, the first major success at pilot scale was reported by Karns *et al.* (1987) and Kearney *et al.* (1986), where they found complete destruction of coumaphos waste at the pilot scale by using OPH-producing bacteria. Later,

a consortium of microbes was used in a filter bioreactor that can be used for 15,000 L of coumaphos removal at one time. Two such units have been operational in the USA since 1996 (Mulbry *et al*., 1998). Owing to inherent difficulties associated with the use of living cells such as nutritional requirements, delivery of fresh inocula, oxygen demand, etc., the use of purified enzymes was suggested to be an efficient approach. Karns *et al.* (1998) successfully used cell-free OPH for detoxification of coumaphos. A range of carriers were tested for effective use of OPH for decontamination and detoxification of several OP compounds. For example, OPH was immobilized on nylon membrane, powder, silica beads, glass and it was then used for detoxification of OP compounds (Caldwell and Raushel, 1991a,b). OPH was reported to be efficient in degrading OP compounds when incorporated into fire-fighting foam (LeJeune *et al*., 1998).

As mentioned earlier, the efficacy of OPH varies dramatically between different substrates and has also shown preferential stereoselectivity which is dependent on shape and sizes of three subsites. In recent years, two different strategies in biomolecular engineering have been taken to genetically engineer enzymes or microorganisms for addressing this problem; (1) a rational-design approach that involves the engineering of enzymes with desired characteristics using site-directed mutagenesis and (2) a directed evolutions approach which involves random mutagenesis (using error-prone PCR, in vivo DNA shuffling and other gene recombination techniques) followed by screening to obtained enzymes with desired characteristics (Ang *et al*., 2005). A representation of the two approaches is illustrated in Fig. 13. By using site-directed mutagenesis, Chen-Goodspeed *et al.* (2001a, b) enlarged the small subset of OPH by replacing Ile106 with alanine which resulted in elimination of 20–90-fold preferential degradation of *Sp*-enantiomer of some chiral substrates. Similarly, another mutant I106G/F132G, which also enlarged small subsite resulted in a 270-fold increase in *Rp*-enantiomer degradation without sacrificing high turnover rate for the *Sp*-enantiomer. Several other mutants of OPH generated by rational-design approaches produced more efficient enzymes for catalysis of poor substrates such as soman, VX and demons S (Di Sioudi *et al*., 1999). Another rational-design approach for bioremediation of OP compounds involved construction of a single microorganism in which desirable biodegradation pathways or enzymes from different organisms are brought together by genetic engineering to perform specific reactions (Ang *et al*., 2005). To this end, two different groups created bacteria, which degrade OP compounds into CO_2 and H_2O. Shimazu *et al.* (2001) constructed a shuttle vector, pPNCO33, which contained truncated ice nucleation protein (INPNC) gene and the *opd* gene and expressed in *Moraxella* sp. (a soil organism that can grow on *p*-nitrophenol).

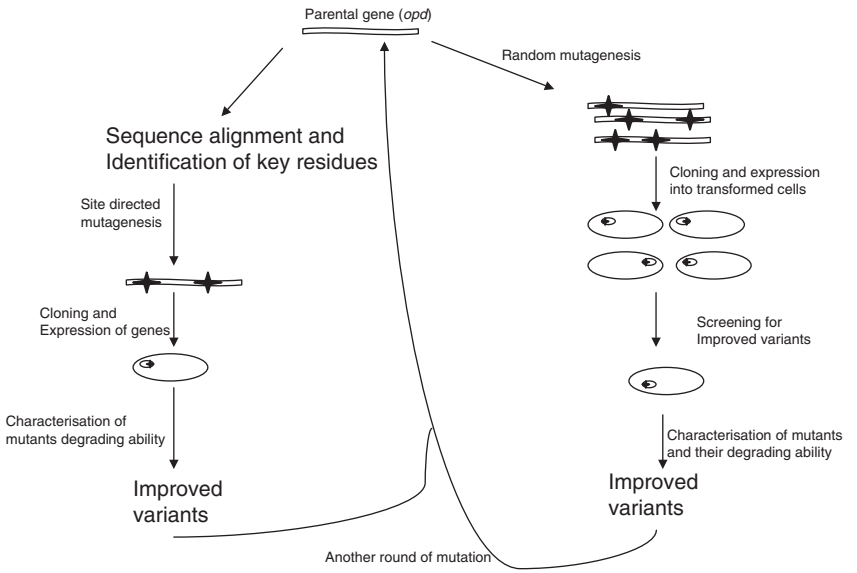


Figure 13 A schematic diagram showing two different biomolecular approaches exploited to improve the catalytical efficiency of OPH.

INPNC provided expression of *opd* on the cell surface thus alleviating the potential substrate uptake limitations. The engineered microbes were able to rapidly utilize paraoxon, parathion and methyl parathion. Later, Walker and Keasling (2002) transformed *P. putida* KT2442 with a plasmid that contained the *opd* gene which allowed it to hydrolyse parathion. Later, they transformed another plasmid harbouring the *p*-nitrophenol degradation operon, which allowed *P. putida* to use nitrophenol generated from parathion as a source of carbon and energy. This approach holds good prospects for disposal of toxic compounds such as CWAs because it leads to complete mineralization of the compounds, and thereby avoiding environmental contamination with intermediate metabolites, which are generally formed during the physical and chemical approaches of waste disposal.

The directed evolution approach was used to increase the efficacy of OPH against poor substrates such as methyl parathion and chlorpyrifos. Following a random mutagenesis by two rounds of DNA shuffling of the *opd* gene, the mutated genes were cloned and screened for improved variants. One variant 22A11 was found to hydrolyse methyl parathion 25-fold faster than the wild OPH (Cho *et al.*, 2002). Similar approaches were taken to improve the catalysis rate of chlorpyrifos. Further analysis of the mutant suggested that two common mutations I274N and H257Y, resulted in this increased

efficacy. Mutant B3561 was found to exhibit a 725-fold increase in the K_{cat}/K_m value for chlorpyrifos, a similar rate at which wild OPH degrades paraoxon. Analysis of the variant suggested that two mutations had the real impact on efficacy of OPH: A80 V and K185R. It was suggested that the latter mutation had an impact on stabilization of the overall structure of the OPH, which resulted in improved variants (Cho *et al.*, 2004). Recently, McLoughlin *et al.* (2005) used the direct evolution approach to isolate mutants with an increased expression for OPDA. In an earlier study they transformed *E. coli* which co-expressed the *opdA* gene (originally isolated from *Agrobacterium radiobacter*) and the glycerophosphodiesterase encoding gene (originally isolated from *Enterobacter aerogenes*) and therefore, can use paraoxon as the sole source of phosphorus (McLoughlin *et al.*, 2004). In the later study, they used random mutagenesis on engineered *E. coli* and isolated an improved variant for OPDA expression (McLoughlin *et al.*, 2005)

4.2. Analytical Applications: Biosensors

A biosensor is a bioanalytical technique, which allows a rapid, cost-effective and **in-field** monitoring of contaminants. OPH-based biosensors take advantage of the enhanced self-life, catalytic stability and reuseability conferred to OPH through immobilisation (Di Sioudi *et al.*, 1999). Rainina *et al.* (1996) constructed a biosensor for the detection of OP contaminants using *opd* gene-harbouring immobilized *E. coli* cells. Simonian *et al.* (1997) developed a multi-enzyme biosensor for OP compounds using OPH, acetylcholinesterase and cholinoxidase in a single system and reported more discriminatory results. Later, two different approaches were used for OPH-based biosensors: potentiometric, measurement of local pH change (Mulchandani *et al.*, 1998, 1999) and amperometric measurement of electro-active enzyme products (Wang *et al.*, 1999; Chough *et al.*, 2002). Further improvement was made by combining the advantage of both, where the amperometric device displays well-defined signals from the oxidized leaving group, and potentiometic detection has been accomplished with silicon-based pH sensitive transducers (Wang *et al.*, 2002, 2003)

4.3. Medical Applications: Use of OPH as a Medicine against OP Poisoning

As previously mentioned, OP pesticide poisoning is a worldwide health problem with about 3 million poisonings and 200,000 deaths annually (WHO, 1990; Karalliedde and Senanayake, 1999). Pre-treatment with

carbamate compounds is used as a prophylaxis against OP poisoning. Like OP compounds, carbamates are AChE inhibitors but carbamate-induced inhibition is reversible. The attack by a water molecule releases AChE from the carbomylated-AChE complex. This approach of prophylaxis is based on the blockage of AChE during the time necessary for the elimination of OP compounds from the body. Another approach is the use of pyridostigmine bromide as a pre-treament. This chemical was used by the USA-led troops in the first Gulf War. Although debatable, the role of pyridostigmine bromide was implicated with neurological disorders (famously known as Gulf War syndrome) in several soldiers after the war (Moss, 2001). All pharmacological treatments are based on the administration of drugs with an intrinsic neurotoxicity that have long-term impacts on health. OPH as a treatment for OP poisoning provides an advantage over chemical treatments because it does not have any toxic effect if administered in suitable carriers. OPH obtained from different isolates was tested for its use as an antidote or a prophylactic measure for OP poisoning. Several studies have demonstrated that the administration of exogenous OPHs to experimental animals confers protection against OP poisoning. The protection was attributed to an increase in the removal of OP molecules by OPH before they reach the target esterase. It was later observed that OPH from *P. diminuta*, delivered along with human paraoxonase, gave the best protection against a wide range of OP compounds (Sogorb *et al.*, 2004). Successful use of OPH as a prophylactic treatment in mice, exposed to high concentrations of paraoxon, has been reported (Ashani *et al.*, 1991). In order to overcome the immunological response to exogenous protein injected directly into blood, the use of suitable carriers has been tested. These carriers must provide permeability to OP compounds and avoid immunological reactions. OPH encapsulated in erythrocytes and liposomes was successfully used to achieve this goal (Sogorb *et al.*, 2004). Because wild OPH has low activity against several OP compound including CWAs, future work should be directed towards animal and clinical trials, with mutant OPH as an antidote for specific OP compounds.

5. CONCLUDING REMARKS

To conclude, OP compounds are biodegradable and can be metabolized by a taxonomically diverse range of microorganisms. A number of microbes have been isolated that can mineralize OP compounds as a source of carbon or phosphorus. Complete metabolic pathways are known for only a few OP compounds such as parathion and glyphosate. Research on this aspect should continue for other OP compounds, as metabolites of several

compounds are pollutants with the potential to have deleterious environmental effects. Several gene/enzyme systems responsible for OP metabolism have been identified and engineered for better efficacy. The search for new microbes and genes/enzymes should continue, as it may lead to the discovery of a more efficient system. New gene/enzyme systems may also shed some light on the origin, evolution and distribution of known OP-degrading gene/enzymes. Genetically engineered microbes, which harbour genes for the complete utilization of OP compounds, hold great promise. So far, only few microbes have been constructed that can metabolize some pesticides (mainly paraoxon). This demonstrates the potential of this approach that can be exploited to achieve complete destruction of extremely toxic compounds.

The major challenge in the next few years will be safe disposal of CWAs. Although all CWAs are required to be destroyed by 2007, the successful completion of this task seems unrealistic. Owing to public opposition to incineration and the time required to gain sufficient scientific knowledge for pilot-scale microbial degradation of CWAs, the best way forward seems to be the hydrolysis of CWAs (which makes them non-toxic) and stockpiling of metabolites until the pilot-scale technology for their destruction is made available. Microbes that can degrade CWAs and their metabolites are known. It will be a major achievement if a bacterial consortium can be constructed using these microbes, which can be used at a pilot scale for CWAs destruction.

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Surface Adhesins of *Staphylococcus aureus*

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ABSTRACT

An important facet in the interaction between *Staphylococcus aureus* and its host is the ability of the bacterium to adhere to human extracellular matrix components and serum proteins. In order to colonise the host and disseminate, it uses a wide range of strategies, the molecular and genetic basis of which are multifactorial, with extensive functional overlap between adhesins. Here, we describe the current knowledge of the molecular features of the adhesive components of *S. aureus*, mechanisms of adhesion and the impact that these have on host-pathogen interaction.

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ABBREVIATIONS

ECM	extracellular matrix
Fg	fibrinogen
Fn	fibronectin
Fnbp	fibronectin-binding protein
Ig	immunoglobulin
MSCRAMM	microbial surface component recognising adhesive matrix molecules
WTA	wall teichoic acid

1. INTRODUCTION

The Gram-positive bacterium *Staphylococcus aureus* is a highly adaptive, versatile pathogen and is a leading cause of a wide range of invasive diseases in humans and animals (Waldvogel, 1995; Lowy, 1998). The ability to adhere to extracellular matrix (ECM) and plasma proteins is a crucial factor in the colonisation and dissemination of *S. aureus* throughout the host. Adherence to the host matrix is the initial step in the infective process and is mediated by bacterial surface adhesins, typically known as MSCRAMMs (*m*icrobial *s*urface *c*omponents *r*ecognising *a*dhesive *m*atrix *m*olecules) (Foster and Höök, 1998). In addition to merely attaching the bacterium to ECM, certain MSCRAMMs are able to interfere with the host immune response or are essential in the process that triggers the internalisation of *S. aureus*. Recently, evidence has accumulated that the ability to exist intracellularly may be important in *S. aureus* pathogenesis (Gresham *et al.*, 2000; Haslinger-Loffler *et al.*, 2005; Sinha and Herrmann, 2005).

An extensive substrate repertoire exists among *S. aureus* adhesins, with many being able to bind multiple ligands. There is also significant functional overlap, with several MSCRAMMs having the capacity to bind the same host components. The purpose of such plasticity is not well understood. However, having such a wide ranging adhesive capability may allow production of specific components under a broad span of environmental conditions and, in particular niches, to allow the organism to tailor its interaction with the host. This seems likely, given that *S. aureus* causes disease in an extensive diversity of tissues and is the aetiological agent of such a wide variety of pathologies.

Most MSCRAMMs described to date are covalently bound to the cell-wall peptidoglycan, although there are several examples which are not (Table 1) (Chhatwal, 2002) (see Fig. 1 for a schematic representation of cell surface components). There are also a number of ligand-binding proteins that are secreted into the extracellular milieu, but these may not affect the adhesion of the bacteria. The roles of these proteins in *S. aureus* pathogenesis are reviewed elsewhere (Chevakis *et al.*, 2005).

Here, we review the current knowledge of the adhesive components of *S. aureus*, paying particular attention to their broad spectrum of activity. In addition to discussing the mechanisms by which the adhesins are attached to the surface of the bacterium, the mechanisms by which they bind to their substrate and the effect to which the interaction has on the host–pathogen dynamics will be described.

2. SORTASE AND THE COVALENT ATTACHMENT OF PROTEINS TO THE CELL WALL

In common with many other species of Gram-positive bacteria, *S. aureus* possesses an array of proteins that are covalently attached to the cell wall peptidoglycan (Navarre and Schneewind, 1999; Mazmanian *et al.*, 2001). In most cases, a C-terminally located LPXTG motif followed by hydrophobic residues and a positively charged tail (which function to retain the protein within the membrane) are essential for covalent attachment (Schneewind *et al.*, 1993). *In silico* analysis of six *S. aureus* genomes revealed the presence of generally 21 genes encoding surface proteins belonging to the LPXTG family (Roche *et al.*, 2003a).

Surface protein containing LPXTG motifs are cleaved by sortase (SrtA), a membrane-bound transpeptidase, between the threonine and glycine of the LPXTG motif (Navarre and Schneewind, 1994), an amide bond is formed

Table 1 List of known adhesins and covalently attached surface proteins of *S. aureus*

Protein	Mode of attachment	Ligand specificity	References
Spa	Covalent (SrtA)	IgG, IgM, von Willebrand Factor, TNFR1	Uhlén *et al.* (1984); Vidal and Conde (1985); Hartleib *et al.* (2000); Gőmez *et al.* (2004).
FnBPA	Covalent (SrtA)	Fibronectin, Fibrinogen, Elastin	Signás *et al.* (1989); Greene *et al.* (1995); Wann *et al.* (2000); Roche *et al.* (2004)
FnBPB	Covalent (SrtA)	Fibroncetin, Elastin	Jönsson *et al.* (1991); Greene *et al.* (1995); Roche *et al.* (2004)
ClfA	Covalent (SrtA)	Fibrinogen	McDevitt *et al.* (1995)
ClfB	Covalent (SrtA)	Fibrinogen, Cytokeratin 10	Ní Eidhin *et al.* (1998); O' Brien *et al.* (2002b)
SdrC	Covalent (SrtA)	Unknown	Josefsson *et al.* (1998a)
SdrD	Covalent (SrtA)	Unknown	Josefsson *et al.* (1998a)
SdrE	Covalent (SrtA)	Unknown	Josefsson *et al.* (1998a)
Pls	Covalent (SrtA)	Cellular lipids including ganglioside M3. Promotes adherence to nasal epithelial cells	Huesca *et al.* (2002); Roche *et al.* (2003b)
Cna	Covalent (SrtA)	Collagen	Patti *et al.* (1992)
IsdA	Covalent (SrtA)	Fibrinogen, Fibronectin, Fetuin, Haemoglobin, Transferrin, Haemin	Taylor and Heinrichs (2002); Mazmanian *et al.* (2003); Clarke *et al.* (2004)
IsdB	Covalent (SrtA)	Haemoglobin, Haemin	Mazmanian *et al.* (2003)

Table 1 (*continued*)

Protein	Mode of attachment	Ligand specificity	References
IsdC	Covalent (SrtB)	Haemin	Mazmanian *et al.* (2003)
IsdH	Covalent (SrtA)	Haptoglobin, Haptoglobin-Haemoglobin complex	Dryla *et al.* (2003)
SraP	Covalent (SrtA)	Unknown, binds platelets	Siboo *et al.* (2005)
SasG	Covalent (SrtA)	Unknown, binds nasal epithelial cells	Roche *et al.* (2003b)
SasB	Covalent (SrtA)	Unknown	Roche *et al.* (2003a)
SasD	Covalent (SrtA)	Unknown	Roche *et al.* (2003a)
SasF	Covalent (SrtA)	Unknown	Roche *et al.* (2003a)
SasK	Covalent (SrtA)	Unknown	Roche *et al.* (2003a)
SasH	Covalent (SrtA)	Unknown	Roche *et al.* (2003a)
Ebh	Ionic	Fibronectin	Clarke *et al.* (2002)
Emp	Ionic	Fibrinogen, Fibronectin, Vitronectin	Hussain *et al.* (2001)
Atl (amidase)	Ionic	Unknown	Foster (1995) Oshida *et al.* (1995)
Atl (glucosaminidase)	Ionic	Fibronectin	Foster (1995) Oshida *et al.* (1995) Clarke and Foster, unpublished
Aaa	Ionic	Fibrinogen, Fibronectin, Vitronectin	Heilmann *et al.* (2005)
Enolase	Ionic	Laminin	Carneiro *et al.* (2004)
EbpS	Transmembrane	Elastin	Downer *et al.* (2002)
Wall teichoic acid	Covelent (to peptidoglycan)	Unknown, binds epithelial and endothelial cells	Weidenmaier *et al.* (2004); Weidenmaier *et al.* (2005)

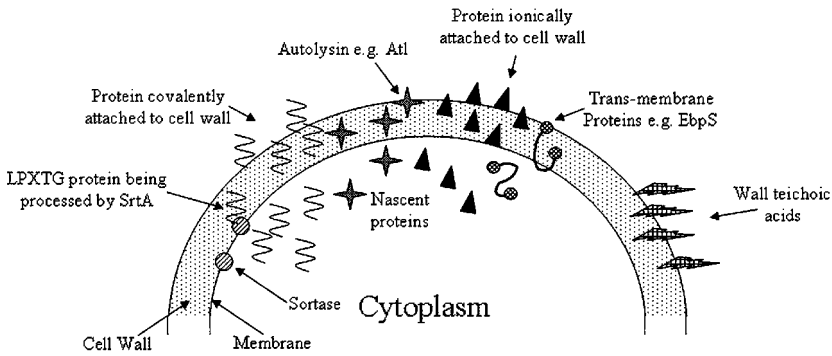

Figure 1 Schematic representation of the cell surface adhesins of *S. aureus*. Nascent proteins with LPXTG motifs are processed by membrane-bound sortase prior to incorporation into the cell wall. Many proteins are ionically bound to the cell wall and include autolysins (involved in peptidoglycan hydrolysis). WTAs and transmembrane proteins such as EbpS are present.

between the carboxyl group of threonine and the amino group of cell wall cross-bridges, and the complex is incorporated into the cell wall (Ton-That *et al.*, 1999; Mazmanian *et al.*, 1999; Perry *et al.*, 2002). A second, iron-regulated sortase (SrtB) exists in *S. aureus*, which cleaves its sole known substrate (IsdC) at an NPQTN motif, anchoring it to the cell wall (Mazmanian *et al.*, 2002).

Isogenic *S. aureus srtA* and *srtB* mutants are significantly less virulent in mouse models of infection (Mazmanian *et al.*, 2000; Mazmanian *et al.*, 2001; Jonsson *et al.*, 2002; Jonsson *et al.*, 2003). In these mutants, the lack of a number of known and putative virulence factors on the surface of the bacteria leads to a significant reduction in their ability to establish an infection and cause disease.

3. THE COVALENTLY ATTACHED CELL WALL PROTEINS

3.1. Protein A (Spa)

As the first surface protein of *S. aureus* to be identified (Jensen, 1958), much of the original work regarding sortase, catalysed attachment to the cell wall, was carried out with Protein A (Spa) as the model (Schneewind *et al.*, 1992; Mazmanian *et al.*, 2001). Classically regarded as an immunoglobulin (Ig) G

binding protein, other research has revealed potential alternative functions. It can bind both von Willebrand factor (vWF), a large multimeric serum glycoprotein that mediates platelet adhesion at sites of endothelial damage (Hartleib *et al*., 2000), and TNFR1, a receptor for tumour-necrosis factor-α (Gómez *et al*., 2004).

The binding of Spa to the Fcγ (the fragment of Igs which is involved in effector function) region of IgG is mediated by the five N-terminal homologous tandem repeats E, D, A, B and C, each approximately 60 amino acid residues long, forming three α-helices (Sjödahl, 1977; Uhlén *et al*., 1984; Starovasnik *et al*., 1996). X-ray crystallography and NMR studies of the B domains in complex with the Fc region of IgG subclass I, have revealed that the binding between the two molecules involves 9 amino acids in the IgG fragment and 11 in the Protein A domain (Deisenhofer, 1981; Gouda *et al*., 1998). The binding of Protein A to the Fc portion of IgG renders it unavailable for recognition by the Fc receptor or polymorphonuclear leukocytes, thus reducing the rate of phagocytosis (Forsgren and Sjoquist, 1966).

In addition to binding the Fc portion of IgG is the less well-characterised binding to the IgG Fab region (the region of Igs responsible for antigen recognition) (Vidal and Conde, 1985). Crystal structure analysis of the Spa domain D-IgM Fab complex shows two of the α-helices interacting with the variable region of the Fab heavy chain (V_H), without the involvement of the hypervariable regions implicated in antigen recognition (Graille *et al*., 2000). Correlation with antibody sequence usage indicates that the Fab binding specificity is restricted to products of the human variable region of the Fab heavy chain V_H3 family that represents nearly half of the inherited V_H genes (Sasso *et al*., 1989, 1991; Hillson *et al*., 1993; Sasano *et al*., 1993). It is presumed that through interactions with surface membrane-associated V_H3-encoded B-cell antigen receptors (Romagnani *et al*., 1982), stimulation with Spa can contribute to the selection of these B-cells and promote their production of antibodies that may include rheumatoid factor antibodies (Kristiansen *et al*., 1994; Kozlowski *et al*., 1995). It is interesting to note that *S. aureus* isolated from patients with Kawasaki disease, where such immune activation is seen (Laxer *et al*., 1987), express relatively high levels of Protein A (Wann *et al*., 1999).

Spa has been proposed to have a role in the pathogenesis of endovascular disease by binding vWF (Hartleib *et al*., 2000), which mediates platelet adhesion at sites of endothelial damage (Ruggeri *et al*., 1983). Upon release by endothelial cells and platelets, vWF multimers are subject to cleavage by plasma proteases, resulting in a heterogeneous array of multimers which then bind to subendothelial components such as collagens, proteoglycans and glucosaminoglycans (Ruggeri and Warre, 1993). Indeed, under physiological

shear-stress conditions, a Spa–vWF-collagen binding mechanism may contribute to the establishment of infected thrombi (Mascari and Ross, 2003).

The finding that Spa can interact with TNFR1 and stimulate an inflammatory response in airway epithelial cells (Gómez *et al.*, 2004), taken with its ability to bind vWF, shows that its interactions with the host are not limited to Igs, and its role in staphylococcal pathogenesis is more complex than previously thought.

3.2. The Fibronectin-Binding Proteins (FnbpA and FnbpB)

S. aureus possesses two related fibronectin (Fn) binding proteins. The encoding genes are in tandem but transcribed separately (Signás *et al.*, 1989; Jönsson *et al.*, 1991; Greene *et al.*, 1995). Although both proteins contribute to the ability of the bacterium to bind Fn (Greene *et al.*, 1995), FnbpA has also been shown to possess Fibrinogen (Fg) binding activity (Wann *et al.*, 2000), and both proteins have been shown to bind elastin (Roche *et al.*, 2004).

Most strains of *S. aureus* express both FnbpA and FnbpB, but a study using a large number of isolates from infected patients showed that there was no difference in the adherence of isolates with one or two *fnb*, but that isolates associated with invasive disease were more likely to have both genes (Peacock *et al.*, 2000). Furthermore, deficient adherence and host-cell invasion of *S. aureus* Newman has been shown to be due to point mutations centrally located within both *fnbA* and *fnbB* of this strain, which in both cases results in a stop codon. Thus, truncated versions of these proteins are secreted into the culture medium and not anchored to the cell wall (Grundmeier *et al.*, 2004).

The structures of FnbpA and FnbpB are similar to each other and to the Fnbps of streptococci (Joh *et al.*, 1994). The ability to bind different ligands resides in different domains (Fig. 2). The Fg and elastin-binding activity of these proteins requires the A domain, which exhibits substantial amino acid sequence homology with other MSCRAMMs, particularly the *S. aureus* Fg-binding protein, ClfA (McDevitt *et al.*, 1994; Wann *et al.*, 2000; Roche *et al.*, 2004). The D domain, which is almost identical in both FnbpA and FnbpB, is located very close to the cell-wall-spanning domain and is generally regarded as the primary domain responsible for the interaction with Fn (Flock *et al.*, 1987; Signäs *et al.*, 1989; Patti *et al.*, 1994a). This domain consists of a tandem repeat of a *c.* 45 amino acid long unit (D1, D2, D3), followed by a single incomplete unit (D4). A fifth unit (Du) is located in *c.* 100 amino acid

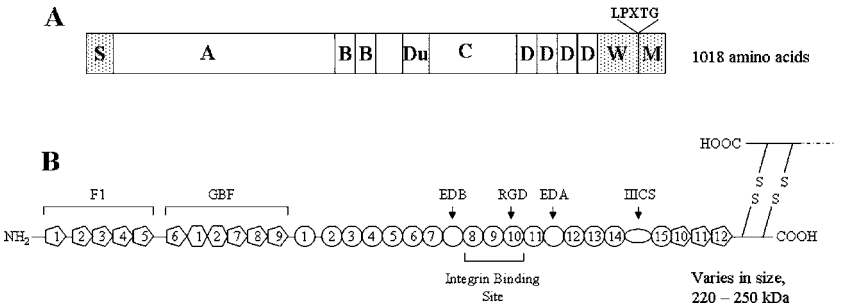

Figure 2 (A) Schematic domain organisation of FnbpA. The signal sequence (S), wall-spanning region (W), membrane-spanning region and positively charged residues (M) and the position of the LPXTG motif are marked. The domains are defined in the text (based on Foster and Höök, 1998). (B) Molecular organisation of Fn (Petersen *et al*., 1989). It is composed almost entirely of three types of modules: F1 (pentagonal), F2 (hexagonal) and F3 (circular). The F1 domain is bound by the D domains of the Fnbps. The RGD integrin binding and GBF gelatin-binding fragment sequence are indicated. EDB, EDA and IIICS represent alternatively spliced regions.

residues N-terminal of D1 that is also capable of binding Fn (Joh *et al*., 1999; Massey *et al*., 2001). The D-domain binds to a 29 kDa N-terminal domain of Fn, which consists of a string of repetitive F1 modules (Fig. 2) (Mosher and Proctor, 1980; Schwarz-Linek and Potts, 2004). NMR and circular dichromism studies of the C-terminal end of the Fnbps have revealed a lack of secondary structure in the absence of Fn (House-Pompeo *et al*., 1996; Penkett *et al*., 1998). The Fnbps join a list of proteins that, under physiological conditions, are intrinsically disordered or have large unstructured regions (Wright and Dyson, 1999; Uversky *et al*., 2000). Under in vivo physiological conditions, unfolded proteins have relatively short life spans, and this may form part of a regulatory strategy (Wright and Dyson, 1999). Furthermore, it has been suggested that in unstructured proteins, the unfolded state seen is less frequently adopted in vivo due to protein–ligand interactions (Uversky *et al*., 2000). In agreement with this is the observation that binding domains of Fnbps undergo transition to an ordered state upon interaction with Fn (House-Pompeo *et al*., 1996; Penkett *et al*., 2000).

The function of Fnbps is more complicated than merely mediating an interaction between *S. aureus* and Fn. Internalisation into non-professional phagocytes, such as keratinocytes (Mempel *et al*., 2002; Kintarak *et al*., 2004), endothelial cells (Peacock *et al*., 1999; Sinha *et al*., 1999; Que *et al*., 2005), epithelial cells (Dziewanowska *et al*., 1999; Lammers *et al*., 1999; Jett

and Gilmore, 2002; McElroy *et al.*, 2002) and osteoblasts (Ahmed *et al.*, 2001) is dependent on Fnbps (Dziewanowska *et al.*, 1999; Sinha *et al.*, 2000). Indeed, these proteins alone can mediate invasion, as latex beads coated with Fnbps are internalised by host cells (Sinha *et al.*, 2000). This phenomenon is shared with *Streptococcus pyogenes*, which requires its Fnbp, known as SfbI/F1, for invasion of non-professional phagocytes (Molinari *et al.*, 1997; Jadoun *et al.*, 1998; Ozeri *et al.*, 1998; Rohde *et al.*, 2003). In both organisms, the process requires the integrin $\alpha_5\beta_1$ to bind the Fn RGD (Arg-Gly Asp) motif (Fowler *et al.*, 2000) to form a bridge between the invading bacterium and the mammalian cell (Fig. 3) (Ozeri *et al.*, 1998; Dziewanowska *et al.*, 1999; Sinha *et al.*, 1999). The process by which phagocytosis is stimulated requires certain host factors. The need for host kinases in integrin-mediated invasion of *S. aureus* is well established (Agerer *et al.*, 2003; Fowler *et al.*, 2003; Wang *et al.*, 2006). Upon Fn-binding *S. aureus*, focal-contact-associated proteins tensin, vinculin, zyxin and focal adhesion kinase (FAK) are recruited to the sites of bacterial attachment. Tyrosine phosphorylation of several host proteins associated with bacterial attachment sites occurs, including FAK and cortactin, an actin-binding protein and substrate of Src kinase (Agerer *et al.*, 2005). The importance of internalisation in vivo is unclear, as *S. aureus* is classically regarded as an extracellular pathogen. However, internalisation could be involved in the

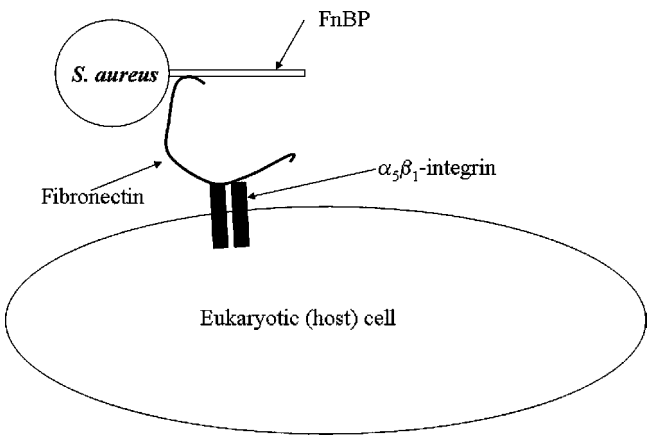

Figure 3 Schematic model (based on Foster, 2002) indicating how binding of Fn modules promotes bacterial attachment to host cells. The N-terminal F1 module of Fn binds the D domains of FnBP. The RGD module of Fn binds to the $\alpha_5\beta_1$-integrin on the host cell, thus stimulating tyrosine phosphorylation, rearrangement of the actin cytoskeleton and bacterial internalisation.

entrance or exit from the vasculature, as localised infections frequently metastasise and may become systemic by disseminating through the vascular system (Gottlieb *et al*., 2000; Petti *et al*., 2002). Furthermore, recent research has shown that internalisation of *S. aureus* into endothelial cells was necessary for the induction of apoptosis (Haslinger-Loffler *et al*., 2005).

In addition to mediating this interaction with non-professional phagocytes, Fn or Fg binding by FnbpA, but not by FnbpB, has been shown to induce aggregation of platelets (Heilmann *et al*., 2004). A model has been proposed whereby FnbpA binds to soluble Fn and/or Fg in blood, binding them to the bacterium. The integrin GPIIb/IIIa on resting platelets can recognise these ligands and bind to them, creating a bacteria-ligand-platelet cross-bridge (Fitzgerald *et al*., 2006).

It should also be noted that the protective environment afforded to internalised bacteria, which would be hidden away from host defences and antibiotics, could account for the high frequency of relapsing *S. aureus* infections (Lowy, 1998).

Much research effort has been spent examining the role of Fnbps in pathogenesis. There is contradictory data regarding their importance, with some data showing that mutation of Fnbps causes attenuation in the ability of *S. aureus* to adhere to damaged heart valve tissue (Kuypers and Proctor, 1989). This activity is probably due to the Fg-binding activity of FnbpA, as deletion of the domain responsible for such interactions has been shown to completely abrogate infectivity in vivo, without compromising the Fn-binding or cell internalisation in vitro (Que *et al*., 2005). Other studies show no such phenomenon (Flock *et al*., 1996). However, when carrying out such work, care needs to be taken to use the appropriate model of infection and bacterial strains. Interestingly, other workers have found that mutation of Fnbps actually increases the virulence of *S. aureus* in a rat model of pneumonia (McElroy *et al*., 2002). This suggests that Fnbp-mediated internalisation into alveolar epithelial cells is not a virulence mechanism, at least in this model of infection, but rather that it decreases virulence of *S. aureus*. In support of this, work done in *S. pyogenes* showed that bacteria missing Sfb/F1 Fnbp were less virulent in mice, but that this virulence was partially restored when these bacteria were used to infect mice lacking plasma Fn. Furthermore, dissemination of bacteria was more efficient in mice lacking Fn, demonstrating that plasma Fn bound to the bacterial surface downregulates virulence by limiting bacterial spread (Nyberg *et al*., 2004). Indeed, growth of *S. aureus* in *ex vivo* medium (used peritoneal dialysate), containing a complex mixture of human proteins, showed that the bacterium may become saturated with target proteins (including Fn) prior to contact with solid surfaces (Massey *et al*., 2002).

3.3. The Sdr Family of Proteins

A family of covalently attached surface proteins exist in *S. aureus* which are characterised by the presence of a domain containing extensive Ser-Asp dipeptide repeats (Josefsson *et al.*, 1998a) and similar structural arrangement (Fig. 4) (Foster and Höök, 1998). Two proteins from this family, ClfA and ClfB are among the most intensively studied members of the *S. aureus* proteome and as such will be discussed separately from the other, less well-understood, members.

3.3.1. The Fibrinogen-Binding Proteins (ClfA and ClfB)

Two structurally similar Fg-binding proteins are expressed by *S. aureus* (McDevitt *et al.*, 1994; Ní Eidhin *et al.*, 1998). Unlike the Fnbps, the genes are not closely linked and are distinct rather than allelic variants (Foster and Höök, 1998). Both proteins consist of a Fg-binding domain, known as the A domain, which is ~500 amino acid residues long. This is linked to the cell

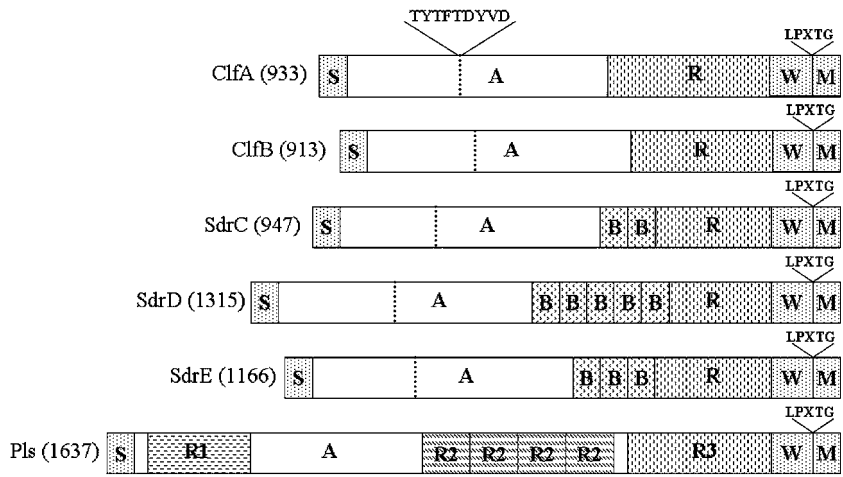

Figure 4 Schematic domain organisation of members of the Sdr family of proteins (based on Foster, 2002). Size in amino acid residues is given in brackets. The signal sequence (S), wall-spanning region (W), membrane-spanning region and positively charged residues (M) and the position of the LPXTG motif are marked. The *c.* 500 amino acid residue A domains of ClfA, ClfB, SdrC, SdrD and SdrE show 25–30% sequence identity and all possess a conserved TYTFTDYVD sequence. The A domain of Pls is not homologous to the A domain of the other proteins shown here, but R3, like R, is composed mainly of SD dipeptide repeats.

wall by a flexible R domain, which allows presentation of the A domain on the surface and is ~300 residues long, consisting mainly of Ser-Asp repeats (Fig. 4) (McDevitt *et al*., 1995; Hartford *et al*., 1997; Ní Eidhin *et al*., 1998). The A domain is similar to that found in Fnbps. In FnbpA, the A domain is also responsible for binding Fg (Wann *et al*., 2000). There are extensive differences in the amino acid sequences of ClfA and ClfB, with only 27% identity between them (Foster, 2002). The two proteins bind different parts of the Fg molecule. Fg is a large (*c*. 340 kDa) protein comprising three polypeptide chains (Aα, Bβ, γ), with two copies of each chain present per molecule. ClfA binds the C-terminal of the γ-chain (McDevitt *et al*., 1997) and ClfB the Aα- and Bβ-chains (Ní Eidhin *et al*., 1998).

The regions of both A domains responsible for binding Fg have been mapped, and amino acids responsible for the binding determined. The C-terminal end of the A domain in ClfA is responsible for Fg binding, with the adjacent Glu^{526} and Val^{527} important for the reaction (Hartford *et al*., 2001a). Analysis of the primary structure of ClfA showed the presence of a Ca^{2+}-binding EF-hand motif at residues 310–321 (O'Connell *et al*., 1998). Ca^{2+} bound to ClfA within the EF-hand motif induces a change in the secondary structure such that binding to Fg is inhibited. The inhibition occurs in the range of 1–10 mM Ca^{2+} and the concentration of free Ca^{2+} in the blood plasma is 1.3 mM, although this can vary more widely in extracellular spaces (Brown *et al*., 1995). It has thus been hypothesised that at platelet-rich thrombi, and possibly on the surface of freshly implanted biomaterial, the Ca^{2+} concentration is considerably lower and may allow ClfA to bind Fg. Thus as bacteria circulate in plasma, they tend to adhere to Fg/platelet-containing coagulation sites (Foster, 2002). The crystal structure of part of the A domain (residues 221–559) has been resolved, and the protein was shown to consist of two domains of a novel variant of the IgG fold (Deivanayagam *et al*., 2002). From structural predictions, it also appears likely that the C-terminal, two-thirds of the Fg-binding A regions of ClfB, FnbpA and FnbpB, also contains two such subdomains. Thus it is proposed that the A regions of these MSCRAMMs are mosaic proteins containing several IgG-like domains (Deivanayagam *et al*., 2002).

The ClfB A domain is composed of three subdomains called N1, N2 and N3. N1 has an elongated structure, while N2, N3 and N23 are globular (Perkins *et al*., 2001). Between the N1 and N2 subdomains, the SLAVA motif is sensitive to the actions of aureolysin, removing residues 44–197 or 44–199 from the N-terminus (McAleese *et al*., 2001). Removal of N1 at Ser^{197} serves to activate Fg-binding activity, however proteolytic cleavage at Ala^{199} results in the loss of that activity (Perkins *et al*., 2001). The proportion of truncated ClfB increases as a culture of *S. aureus* grows, and its

cleavage is responsible for the decrease in Fg binding of stationary phase cells (McAleese *et al.*, 2001). It is thus hypothesised that a regulated processing of N1 greatly affects Fg-binding activity of ClfB (Perkins *et al.*, 2001).

It has been proposed that the mechanism for ClfA and ClfB binding to Fg is identical to that of SdrG (Ponnuraj *et al.*, 2003), a Fg-binding protein of *Staphylococcus epidermidis* (Hartford *et al.*, 2001b). The proposed ''dock, lock and latch'' mechanism describes the structural changes that stabilise the overall MSCRAMM A domain–ligand complex. Structural comparison of the apo-protein, open conformation of the binding domain with the closed conformation observed in the protein–ligand complex, reveals that the C-terminal segment of the protein ''locks'' on to the ''docked'' peptide by causing a cover and sequesters it by ''latching'' on to the neighbouring N2 domain. Thus, ligand binding to the MSCRAMM appears to involve multiple steps: initially, the peptide docks into an IgG cleft (of which there are either two or three in this class of adhesin), which is followed by a structural rearrangement at the C-terminal of the MSCRAMM, where it crosses over the binding cleft and locks the ligand in place. The crossover results in the formation of backbone hydrogen bonds between the bound ligand and the covering segment of the adhesin, securing the ligand in the binding cleft. Finally, complementation of a β sheet in the N2 domain results in insertion of the C-terminal strand between two other strands, which constitutes a ''latching'' event and stabilises the overall structure (Ponnuraj *et al.*, 2003).

Both clumping factor proteins have been shown to be virulence factors in *S. aureus*. ClfA and to a lesser extent ClfB are involved in causing endocarditis in an experimental rat model (Moreillon *et al.*, 1995; Entenza *et al.*, 2000). Infective endocarditis is a serious condition in humans, characterised by bacteria colonising and invading previously undamaged heart valves (Moreillon and Que, 2004). The formation of platelet–bacteria thrombi on the surface of heart valves is essential for the development of this disease (Sullam *et al.*, 1996), since platelets attached to a damaged valve serve as foci for attachment of organisms circulating in the blood (Sullam *et al.*, 1990; Yeaman *et al.*, 1992; Gong *et al.*, 1995). Several studies have shown that *S. aureus* can bind platelets in vitro (Hawiger *et al.*, 1979; Bayer *et al.*, 1995; Sullam *et al.*, 1996). Moreover, addition of *S. aureus* to platelet-rich plasma has been shown to induce platelet aggregation, which was not seen when the bacteria were treated with trypsin (Hawiger *et al.*, 1979), suggesting a role for proteins on the bacterial cell surface.

Both ClfA and ClfB have been shown to mediate binding to, and activation of, platelets by *S. aureus*, with ClfA having a more potent pro-aggregatory activity than ClfB (Siboo *et al.*, 2001; O'Brien *et al.*, 2002a).

ClfA promotes activation of platelets by Fg-dependent and Fg-independent processes, and both mechanisms require specific IgG antibodies bound to the A domain of ClfA (Loughman *et al.*, 2005). In Fg-dependent platelet activation, the model proposes that bacterial cells armed with sufficient surface-bound Fg can engage resting platelet glycoprotein GPIIb/IIIa, aided by bound IgG molecules (which encourages clustering of FcγRIIa receptors), triggering activation of signal transduction pathways that lead to aggregation of platelets. Alternatively, IgG and complement deposition can interact with FcγRIIa and a complement receptor, respectively, resulting in platelet activation and aggregation (Loughman *et al.*, 2005).

ClfA has also been implicated as a virulence factor in staphylococcal arthritis (Josefsson *et al.*, 2001; Palmqvist *et al.*, 2005). However, free Fg does not appear to be required for induction of this disease. Wild-type *S. aureus* have been shown to be much more arthrogenic than an isogenic *clfA* mutant, but depletion of Fg from experimentally infected animals also significantly aggravated the infection, when compared to control treatment (Palmqvist *et al.*, 2004a). Interestingly, ClfA has also been shown to impede macrophage phagocytosis of *S. aureus*, a phenomenon that also does not require the presence of intact Fg. Additionally, the same study showed that the *clfA*-mutant strain caused more release of proinflammatory mediators by macrophages than the wild-type strain (Palmqvist *et al.*, 2004b). Clearly ClfA possesses the ability to cause arthritis and inhibit macrophage phagocytosis and enhanced immunostimulatory activity, but the host factors involved in these processes remain to be determined.

ClfB enhances adherence of *S. aureus* to desquamated nasal epithelial cells (O'Brien *et al.*, 2002b). Such a process is likely to be crucial in successful nasal colonisation and appears to be mediated by ClfB binding to cytokeratin 10 that is present on the surface of squamous cells (O'Brien *et al.*, 2002b; Walsh *et al.*, 2004).

3.3.2. SdrC, SdrD and SdrE

These proteins are predicted to have a similar structural organisation to ClfA and ClfB (Fig. 4), with the exception of an additional B repeat domain of unknown function, containing 110–113 amino acid residues located between the A and R domain (Josefsson *et al.*, 1998a). The B domain contains a consensus EF-hand loop in each repeat for binding Ca^{2+} at a higher affinity that is seen in the case of ClfA ($K_d = 4\,\mu M$) (Josefsson *et al.*, 1998b). Bound Ca^{2+} induces a rigid rod-like structure of the B domain. It is not known if the B domain has ligand-binding activity or if its role is purely structural.

The ligand-binding activities of these proteins have yet to be elucidated, although a variant of SdrD (called Bsp) is, unlike SdrD itself, able to bind bone sialoprotein (Tung *et al.*, 2000). SdrE, when expressed heterologously in *Lactococcus lactis*, is able to promote platelet aggregation, most likely mediated by binding to a plasma protein that acts as a bridge between the bacteria and a platelet receptor (O'Brien *et al.*, 2002a; Foster, 2002).

3.3.3. Pls

Some strains of MRSA express a high-molecular mass (*c.* 175 kDa) plasmin-sensitive cell wall protein, called Pls (Hildén *et al.*, 1996). Pls has been shown to attenuate bacterial binding to immobilized Fn and IgG (Juuti *et al.*, 2004). Conversely, Pls is a virulence factor in septic arthritis (Josefsson *et al.*, 2005), promotes cell–cell interactions and mediates adherence to cellular lipids, including ganglioside GM3 (Huesca *et al.*, 2002), which constitutes 65% of the total gangliosides in keratinocyte membranes (Paller *et al.*, 1992, 1993). Furthermore, the binding specificity encompasses the recognition of sphingolipids (Huesca *et al.*, 2002), which with cholesterol-enriched vesicles called caveolae are implicated in internalisation of bacteria and viruses (Anderson, 1998; Comolli *et al.*, 1999; Shin *et al.*, 2000; Shin and Abraham, 2001). Pls has also been shown to promote bacterial attachment to nasal epithelial cells, but not to buccal epithelial cells or cultured keratinocytes (Roche *et al.*, 2003b). The specific ligand(s) involved in this interaction remain unknown. The structure of Pls is shown in Fig. 4. It consists of three distinct repeat regions, one of which (R3) is characteristic of the Sdr family, being composed of dipeptide SD repeats (Savolainen *et al.*, 2001).

3.4. The Collagen Adhesin (Cna)

In order for *S. aureus* to adhere to collagenous tissues, a specific receptor is necessary (Speziale *et al.*, 1986; Switalski *et al.*, 1989). The protein Cna (Patti *et al.*, 1992) has been shown to mediate adherence of *S. aureus* and artificially coated latex beads to cartilage (Switalski *et al.*, 1993; Gillaspy *et al.*, 1998).

Cna consists of a large non-repetitive A domain that possesses the protein's collagen-binding activity (Patti *et al.*, 1993), followed by 1–4 copies, depending upon the strain, of consecutively repeating B domains (Fig. 5) (Gillaspy *et al.*, 1997). Within the A domain, a truncated (19 kDa) region has been shown to be sufficient for binding to collagen (Patti *et al.*, 1993). The crystal structure of the recombinant polypeptide has been resolved,

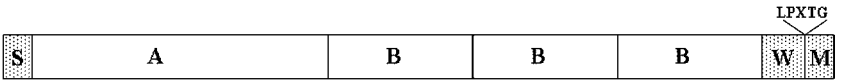

Figure 5 Schematic domain organisation of Cna. The signal sequence (S), wall-spanning region (W), membrane-spanning region and positively charged residues (M) and the position of the LPXTG motif are marked. The A domain possesses the ligand-binding activity. Three repetitive B domains are shown, but between 1 and 4 can be present depending on the strain.

showing the protein to fold as a jelly roll composed of two β-sheets connected by two short α-helices. One β-sheet has a noticeable trench transversing it. Molecular modelling studies have shown that it could accommodate a collagen triple helix (Symersky *et al.*, 1997). Mutational analysis in this trench has shown that it does indeed represent the collagen-binding site (Patti *et al.*, 1995). This binding scheme has been suggested for other collagen-binding proteins (Emsley *et al.*, 1997; Perona *et al.*, 1997). The function of the B domain remains unclear, as there is no evidence that it plays any role in the interaction with collagen (Rich *et al.*, 1998) or to act as a stalk, extending the ligand-binding domain out into the extracellular milieu from beneath any capsule that may be present (Snodgrass *et al.*, 1999).

The collagen adhesin has been shown to be a virulence factor in experimental models of septic arthritis (Patti *et al.*, 1994b; Xu *et al.*, 2004) and osteomyelitis (Elasri *et al.*, 2002). In both diseases, Cna is involved in development of the pathology, but other components are also important. It has also been shown that Cna plays a role in the maintenance of endocarditis, but not generally in the establishment of the infection (Hienz *et al.*, 1996).

3.5. The Iron-Regulated Surface Determinants (IsdA, IsdB, IsdC and IsdH)

S. aureus possesses four covalently attached, iron-regulated surface proteins (Mazmanian *et al.*, 2002; Morrissey *et al.*, 2002; Dryla *et al.*, 2003). It has been proposed that all four of these proteins are involved in the uptake of haem–iron across the cellular envelope (Mazmanian *et al.*, 2003). Certainly, all four proteins have been shown to bind one or more iron-containing proteins, such as transferrin, haemin or haemoglobin (Taylor and Heinrichs, 2002; Dryla *et al.*, 2003; Mazmanian *et al.*, 2003; Clarke *et al.*, 2004; Mack *et al.*, 2004), but there remains no clear evidence of a role in iron uptake.

Three of these proteins, IsdA, IsdB and IsdC, are encoded by genes at the same locus, with IsdH separate (Mazmanian *et al.*, 2003). All are

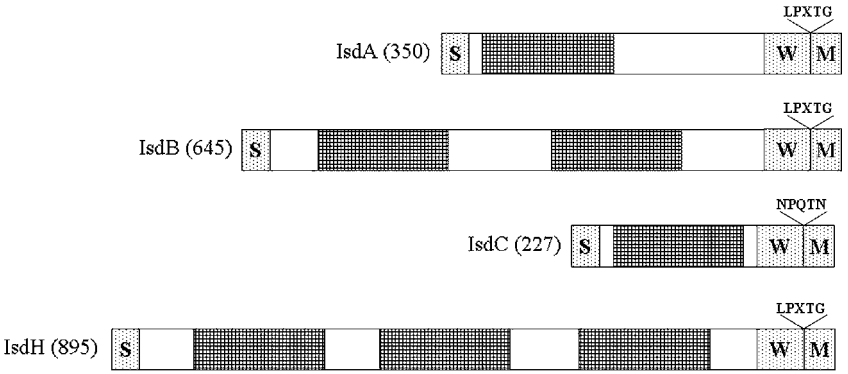

Figure 6 Schematic domain organisation of members of the Isd family of proteins. Size in amino acid residues is given in brackets. The signal sequence (S), wall-spanning region (W), membrane-spanning region and positively charged residues (M) and the position of the LPXTG or NPQTN motif are marked. NEAT domains are shown as hatched areas on each protein.

transcriptionally controlled by the iron-responsive regulator Fur (Horsburgh *et al.*, 2001; Dryla *et al.*, 2003; Mazmanian *et al.*, 2003; Clarke *et al.*, 2004). IsdC is the sole known substrate of sortase B (SrtB), with which it is co-transcribed (Mazmanian *et al.*, 2002). Each protein contains one to three NEAT domains (Fig. 6), which are found in a range of proteins from Gram-positive bacteria (Andrade *et al.*, 2002). These domains have been shown to be the active sites for ligand binding in both IsdA and IsdH (Dryla *et al.*, 2003; Clarke *et al.*, 2004). Originally designated on the basis of putative structure (Andrade *et al.*, 2002), the NEAT domains of IsdA and IsdH show significant sequence differences, which may account for differences in substrate specificity (Clarke *et al.*, 2004).

To date, IsdA is the most intensively studied of this set of covalently attached surface proteins. It first came to light for its ability to bind transferrin (Taylor and Heinrichs, 2002). However, mutation in *isdA* does not abolish the ability of *S. aureus* to use transferrin, haemoglobin, haemin or $FeSO_4$ as an iron source (Clarke *et al.*, 2004). Furthermore, it has been shown that IsdA does not possess the ability to remove iron directly from transferrin, but that the siderophore-mediated iron-acquisition system plays a dominant, and more importantly, essential role in this process (Park *et al.*, 2005).

Studies analysing the growth of *S. aureus* under conditions modelled on those in vivo, have shown that IsdA is expressed during growth in serum and human dialysate (Wiltshire and Foster, 2001; Morrissey *et al.*, 2002). Under such conditions, the ability of *S. aureus* to bind Fn and Fg increases

significantly, a phenomenon that is due to the expression of IsdA (Clarke *et al*., 2004). Indeed, IsdA has been shown to bind a wide selection of human serum and ECM proteins in vitro, although the reason for such a broad spectrum of activity remains unclear. The capacity cannot be explained by a general glycosylation of human proteins (Clarke *et al*., 2004).

IsdH has been shown to bind human haptoglobin, a protein that sequesters free haemoglobin in serum. Moreover, it binds the haptoglobin–haemoglobin complex with even greater affinity. Importantly, FACS analysis using biotin-labelled haptoglobin showed that IsdH was the sole protein responsible for this activity when *S. aureus* was grown in standard laboratory media (Dryla *et al*., 2003).

While IsdB and IsdC have been shown to bind various iron-containing proteins, no phenotypic evidence for their role in iron uptake has been reported (Mazmanian *et al*., 2003).

3.6. Serine-Rich Adhesin for Platelets (SraP)

Identified as a homologue of a large platelet-binding surface glycoprotein, GspB of *Staphylococcus gordonii* (Bensing and Sullam, 2002; Siboo *et al*., 2005), SraP, otherwise known as SasA (Roche *et al*., 2003a), is a large (*c*. 227 kDa) covalently attached surface glycoprotein with platelet-binding activity (Siboo *et al*., 2005). SraP was found to be present in a number of *S. aureus* clinical isolates, and is a virulence factor in infective endocarditis (Siboo *et al*., 2005).

3.7. Other Covalently Attached Surface Proteins

There are 22 proteins that are known to be covalently attached to the cell wall of *S. aureus* by sortase A or B (Mazmanian *et al*., 2002; Roche *et al*., 2003a). Here we have discussed 15 of these proteins, and the roles of the remaining seven are yet to be determined, although SasG has been shown to promote adherence to nasal epithelial cells and is presumably a factor in nasal colonisation (Roche *et al*., 2003b).

4. THE NON-COVALENTLY ATTACHED ADHESINS

Associated with the cell wall are a number of ionically bound proteins. These include, in many species, multiple peptidoglycan hydrolases, proteases

and other proteins, including adhesins. Gram-positive bacteria employ several different mechanisms to attach proteins ionically to the cell wall. *Listeria monocytogenes* binds InlB directly to the lipoteichoic acid via carboxy-terminal tandem repeats (Jonquieres *et al*., 1999; Braun *et al*., 2001). The LytA protein of *Streptococcus pneumoniae* contains a 20-amino-acid repeat that binds to choline-substituted teichoic acid or lipoteichoic acid (Holtje and Tomasz, 1975). The WapA protein of *Bacillus subtilis* contains a repeat region that binds the protein ionically to the cell wall peptidoglycan (Foster, 1993). All of these mechanisms result in wall-anchored proteins that may interact with the external environment.

4.1. Ebh

The extracellular matrix-binding protein homologue (Ebh) is encoded by the largest gene in *S. aureus*. The *ebh* gene is 28,605 and 31,494 bp in *S. aureus* strains 8325 and COL, respectively (Clarke *et al*., 2002). In some strains, two genes are present, *ebhA* and *ebhB*, which encoded proteins homologous to the C- and N-terminal parts of Ebh respectively (Kuroda *et al*., 2001).

Analysis of the sequence of Ebh reveals that the protein consists of several domains, including a large central region with 44 imperfect repeats of 126 amino acids. A fragment of this domain was cloned and overexpressed and found to bind Fn (Clarke *et al*., 2002). Furthermore, it has been suggested that Ebh may be involved in adhesion to endothelial cells (Sinha and Herrmann, 2005), which would be consistent with Fn-binding activity.

Ebh contains a putative C-terminal membrane-spanning domain that may attach it to the cell membrane, and also a putative peptidoglycan-binding repeat region that may bind it ionically to the cell wall peptidoglycan. Ebh is present at its apparent full size (*c.* 1.1 MDa) bound ionically to the cell wall of *S. aureus* (Clarke *et al*., 2002).

4.2. Emp (Extracellular Matrix Protein-Binding Protein)

Like IsdA, Emp displays a broad-binding specificity for ECM and plasma proteins (Hussain *et al*., 2001). Most notable was the very high affinity for vitronectin ($K_D = 122$ pM). It has been suggested that this high affinity may enable the bacterium to locally recruit a number of factors involved in various biological processes, such as complement activation, homeostasis and tissue remodelling (Sinha and Herrmann, 2005).

4.3. Autolysins as Ligand-Binding Proteins

Atl, the major autolysin of *S. aureus* (Foster, 1995; Oshida *et al.*, 1995) has the same overall organisation as three other staphylococcal autolysins AtlE (Heilmann *et al.*, 1997), Aas (Hell *et al.*, 1998) and AtlC (Allignet *et al.*, 2001). In addition to the bacteriolytic activities of these proteins, they have all been shown to confer adhesive properties to cells and are able to bind human ligands in vitro (Heilmann *et al.*, 1997; Hell *et al.*, 1998; Allignet *et al.*, 2001). Experiments in our laboratory have shown that recombinant glucosaminidase domain of Atl is able to bind Fn (Clarke and Foster, unpublished).

Aaa is a multifunctional protein (also known as Sle1) acting as both an autolysin and adhesin, with the ability to bind Fn, Fg and vitronectin (Heilmann *et al.*, 2005). It is an amidase, which is involved in cell separation (Kajimura *et al.*, 2005). Mutation of *aaa* reduced the capacity of *S. aureus* to cause disease in a mouse model of infection (Kajimura *et al.*, 2005).

4.4. Enolase, the Laminin-Binding Protein

It has been proposed that the ability of *S. aureus* to cross the vasculature, correlates to its affinity for laminin (Lopes *et al.*, 1985), an abundant ECM protein. α-Enolase has been identified as being the surface protein responsible for this activity (Carneiro *et al.*, 2004).

5. EBPS, THE ELASTIN-BINDING PROTEIN

Elastin is a major component of the ECM that plays a crucial role in maintaining the structural integrity and function of tissues in which reversible extensibility and deformability are required (Sandberg *et al.*, 1981). The ability of *S. aureus* to bind elastin is mainly due to EbpS (Downer *et al.*, 2002), although it has been shown that FnbpA and FnbpB are both able to bind elastin as discussed above (Roche *et al.*, 2004) Originally described as a surface-associated protein (Park *et al.*, 1996, 1999), EbpS is unique among the adhesins of *S. aureus*, as the only adhesive transmembrane protein described thus far (Downer *et al.*, 2002). Digestion of cell wall peptidoglycan with lysostaphin or mechanical breakage produced protoplasts and membrane fractions respectively, which still retained EbpS. Use of PhoA and LacZ fusions to EbpS between hydrophobic and hydrophilic

domains showed that it was indeed an integral membrane protein with two membrane-spanning domains. The N-terminal, which contains the elastin-binding domain, is exposed to the extracellular milieu (Downer *et al*., 2002).

6. WALL TEICHOIC ACIDS AS NON-PROTEINACIOUS ADHESINS

The cell envelope of Gram-positive bacteria generally contains wall teichoic acid (WTA), which is a complex surface-exposed polymer. The WTA produced by *S. aureus* is composed of *c*. 40 ribitol phosphate repeating units modified with *N*-acetylglucosamine and D-alanine (Endl *et al*., 1983). The role of WTA in adhesion to human nasal epithelial cells was first reported by Aly *et al*. (1980), who showed that treatment of human cells with teichoic acid extracted from *S. aureus* significantly reduced the binding of the bacterium.

Deletion of *tagO*, a gene involved in the biosynthesis of WTA in *S. aureus*, abolished synthesis and reduced the ability of the bacteria to bind primary human nasal and airway epithelial cells (Weidenmaier *et al*., 2004). Moreover, the mutation caused a reduction of *S. aureus* nasal carriage using the cotton rat model (Weidenmaier *et al*., 2004). Further studies showed WTA to be involved in adhesion to vascular endothelial cells. The Δ*tagO* mutant showed no significant difference in its susceptibility to opsonophagocytosis, killing by a prototypic platelet microbicidal protein, or binding to platelets, fibronectin or fibrinogen. However, it bound endothelial cells significantly less well than the parental strain, and beads coated with WTA also bound endothelial cells in a dose-dependent manner (Weidenmaier *et al*., 2005). In a rabbit model of infectious endocarditis, the mutant strain showed a significant reduction in its ability both to colonise sterile cardiac vegetations and to proliferate within such vegetations, the kidney and spleen (Weidenmaier *et al*., 2005).

7. OTHER NON-COVALENTLY ATTACHED ADHESINS

There remain many more *S. aureus* surface components that may play a role in the host–pathogen interaction. One such protein, IsaB (Lorenz *et al*., 2000) is homologous to a heparin-binding protein of *S. epidermidis* (Fallgren *et al*., 2001) and it may be through discoveries in other organisms,

such as this, that we gain further insight into the potential role of adhesins in *S. aureus*.

8. CONCLUSIONS

The interaction between *S. aureus* and its human host is multifactorial, requiring many differentially expressed components. The array of host ligand-binding proteins possessed by *S. aureus* poses interesting questions as to their functions. This organism can inhabit a variety of different environments, even within the same individual host. Therefore, there may be a requirement for different adhesins, which bind either a broad or narrow spectrum of ligands. The biological relevance of environmental regulation for most adhesins is unknown. Exceptions are the Isd proteins, which are specifically produced under conditions of iron limitation, which is indicative of host association (Clarke *et al*., 2004). It is also important to remember that in vitro observations may not be representative of in vivo function. The true in vivo ligands for many adhesins cannot be firmly concluded.

Given the wide range of novel putative surface components found in the genome of *S. aureus* (Kuroda *et al*., 2001), and the increasing repertoire of ligands for many known adhesins, there are many likely complex interaction mechanisms to be unravelled. For example, the newly discovered mechanism for adhesion of *S. aureus* binding to red blood cells via their sialoglycoprotein(s) has been shown to be dependent on plasma proteins other than Fg or IgG, and does not involve *S. aureus* ClfA or Spa (Shin *et al*., 2005). Such novel mechanisms provide new and interesting challenges in the study of *S. aureus* pathogenesis.

The study of MSCRAMMs has led to the development of several potentially efficacious immunological therapeutic and prophylactic strategies, to control *S. aureus* (Flock, 1999). The use of donor serum with a high anti-ClfA IgG titre has been shown to have the potential to reduce sepsis caused by *S. aureus* and mortality in very low birth weight infants (Vernachio *et al*., 2003; Bloom *et al*., 2005). There is currently much research effort into the development of humanised anti-ClfA monoclonal antibodies for treatment of *S. aureus* infections (Hall *et al*., 2003; Patti, 2004; Domanski *et al*., 2005), which could be used for passive immunotherapy. The collagen adhesin, Cna, has been proposed as a target for both anti-adhesive antibody therapy (Visai *et al*., 2000) and as a vaccine component (Nilsson *et al*., 1998). FnbpA and FnbpB have both also been suggested as targets for passive immunotherapy (Rozalska and Wadstrom, 1993; Flock and Brennan, 1999).

Thus the continued investigation of *S. aureus* adhesins is not only revealing novel insights into host–pathogen interaction but also opening avenues for the control of this important pathogen.

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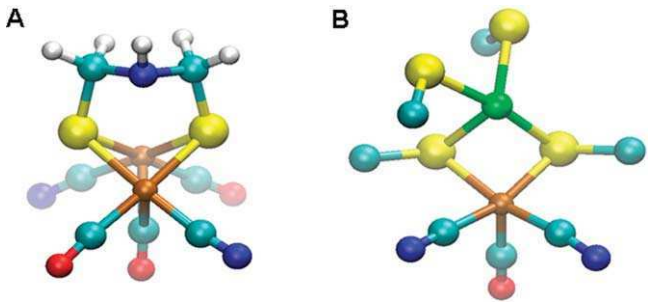

Plate 1 Atomic structure of the [FeFe]-hydrogenase and [NiFe]-hydrogenase catalytic sites. Atom depictions of the [FeFe]-hydrogenase H-cluster, 2Fe-centre (A), and the [NiFe]-hydrogenase [NiFe]-centre (B) as generated from the structures of *Cl. pasteurianum* [FeFe]-hydrogenase I (PDB file 1FEH, Peters *et al*., 1998) and *D. vulgaris* Miyazaki F [NiFe]-hydrogenase (PDB file 1UBK, Ogata *et al*., 2002). Colors identify positions of Fe (brown), Ni (green), C (cyan), S (yellow), N (blue), O (red) and H (white) atoms in the respective structures. (See page 4, this volume)

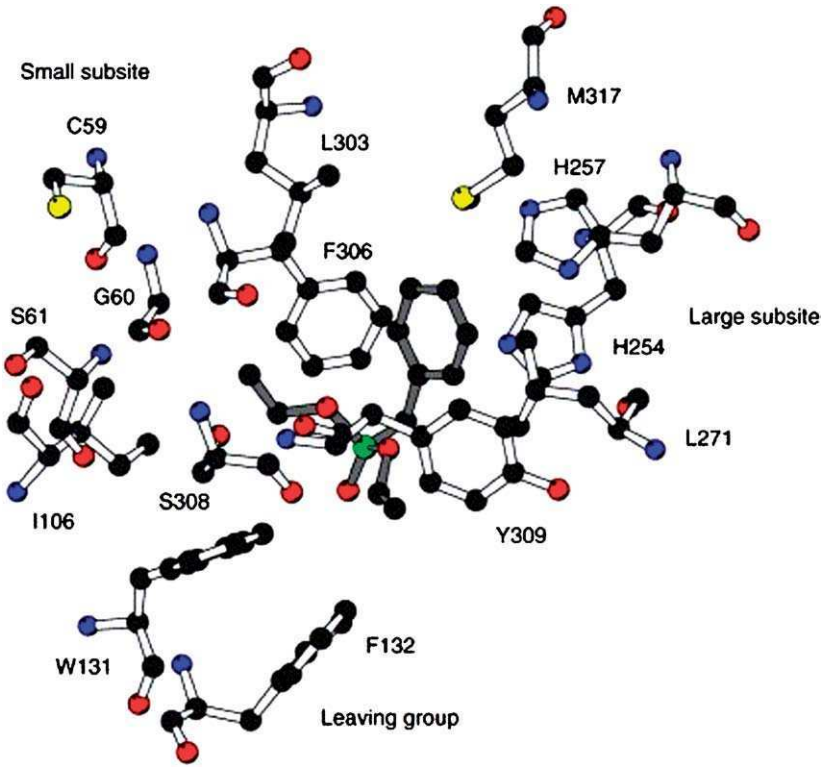

Plate 2 The active site of organophosphorus hydrolase and the relative position of the amino acid residues. Reproduced with permission from Raushel (2002). (See page 158, this volume)

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