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Coping with formaldehyde during C1 metabolism of *Paracoccus denitrificans*

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Abstract

Methylotrophic bacteria are capable of growth using reduced one-carbon (C1) compounds like methanol or methylamine as free energy sources. *Paracoccus denitrificans*, which is a facultative methylotrophic organism, switches to this type of autotrophic metabolism only when it experiences a shortage of available heterotrophic free energy sources. Since the oxidation of C1 substrates is energetically less favourable than that of the heterotrophic ones, a global regulatory circuit ensures that the enzymes involved in methylotrophic growth are repressed during heterotrophic growth. Once the decision is made to switch to methylotrophic growth, additional regulatory proteins ensure the fine-tuned expression of the participating enzymes such that the steady-state concentration of formaldehyde, the oxidation product of C1 substrates, is kept below cytotoxic levels. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Just like many eubacteria of the α-cluster of Gram negative purple bacteria, *Paracoccus denitrificans* [7] is an organism unable to ferment available growth substrates, and is therefore completely dependent on respiration linked oxidative phosphorylation for free energy transduction [27,69]. Its usual habitats include soil, sewage or sludge, environments where the cell is continuously challenged by variations in the availability of suitable carbon and free energy sources as well as of terminal electron acceptors. One important strategy to survive is the proper adaptation of metabolic pathways in response to changes in that environment. *P. denitrificans* has such a nutritional adaptability as judged by the fact that it can grow chemolithoautotrophically with hydrogen, thiosulphate, methanol, formaldehyde, formate, or methylamine as sole source of free energy, or heterotrophically with a variety of sugars, organic compounds and amino acids, and alcohols [70]. During anaerobiosis, N-oxides like nitrate, nitrite, nitric oxide, or nitrous oxide can substitute for oxygen as terminal electron acceptors [59]. Part of its versatility in utilization of electron donors and acceptors resides in its ability to change the make-up of its respiratory network.

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The protein-based signal transduction circuits controlling these adaptations at the DNA-level are rapidly emerging. Just as has been recognized for many other bacterial species, *P. denitrificans* may follow a semi-intelligent strategy in which the extent of expression and operation of cellular metabolic pathways is controlled according to an energetic hierarchy [68]. The bacterium prefers environmental electron sources and sinks that generate the most free energy for growth and maintenance. When *P. denitrificans* encounters organic substrates as carbon and free energy sources and oxygen is present as terminal electron acceptor of respiration, the cell expresses the enzymes required for this type of metabolism and suppresses those engaged with alternative respiratory pathways. The enzymes involved in processes that do not require oxygen are only expressed if the alternative electron sink is available and oxygen is absent [69]. A proper control of this so-called oxic–anoxic switch is extremely useful, since not only is oxygen respiration the respiratory mode that delivers the most free energy per electron, the alternative process of nitrate respiration (denitrification) confronts the cell with the toxic intermediates nitrite and nitric oxide.

The other type of control mechanism well studied in *P. denitrificans* is engaged with the proper choice of the electron donor of the respiratory network. The cell prefers heterotrophic substrates but when these have run out, it can switch to another type of metabolism to respire alternative electron donating substrates. When it encounters methanol or methylamine for example, the enzymes that are required for the oxidation of these so-called C1 substrates are induced [35]. The electrons released during these oxidation steps are transferred from these enzymes via dedicated electron acceptors to the constitutive part of the respiratory network of the cell. Although the harvest of free energy is safeguarded in this way, this mode of respiration compromises cell functions in view of the cytotoxicity of formaldehyde, the oxidation product of both methanol and methylamine. One may thus anticipate that the expression of formaldehyde dehydrogenase (GD-FALDH), which is the enzyme consuming formaldehyde, is coordinately regulated as well. This review describes the organization and function of the gene clusters encoding the key enzymes involved in C1 metabolism as well as the signal transduction pathways that orchestrate their expression.

2. The make-up and bioenergetics of the *P. denitrificans* respiratory network

The constitutive part of the respiratory network of *P. denitrificans* consists of nicotinamide adenine dinucleotide (NADH) dehydrogenase, succinate dehydrogenase, ubiquinone-10, the cytochrome-bc_1 complex, and cytochromes c_{550} (located in the periplasm), and c_{552} (anchored to the membrane). The cell has the potential to synthesize three different types of terminal oxidase: the ba_3-type quinol oxidase and the aa_3-type and cbb_3-type cytochrome *c* oxidases [61]. The aa_3-type oxidase is the structural and functional homologue of the enzyme found in the mitochondrial respiratory chains. The cbb_3-type oxidase is found in bacteria only and has an about 10-fold higher affinity for oxygen than the aa_3- and ba_3-type cytochrome *c* oxidases [50]. Expression of this high affinity oxidase increases at decreasing oxygen concentrations [10,49]. The ba_3-type quinol oxidase of *P. denitrificans* is the counterpart of the well known *Escherichia coli* bo_3-type quinol oxidase [19,55]. This type of oxidase is common to all genera of eubacteria. In *P. denitrificans* it may serve to prevent the respiratory system from getting too reduced [77]. NADH dehydrogenase, cytochrome bc_1 and the terminal oxidases are protein complexes that span the cytoplasmic membrane. The free energy they make available by catalyzing the electron transfer reactions is used for the generation of a proton gradient across that membrane. With respect to its organization and function, the aerobic respiratory network of *P. denitrificans* closely resembles

the one that is operating in mitochondria of eucaryotic cells to a large extent ([1]).

3. Enzymes and their genes required for C1 metabolism

The sequential steps of C1 metabolism in *P. denitrificans* are shown in Fig. 1. A scheme of the corresponding electron transfer network is shown in Fig. 2. Methanol or methylamine are oxidized to formaldehyde in the periplasm by the quinoproteins methanol dehydrogenase (MDH) and methylamine dehydrogenase (MADH), respectively. Formaldehyde, formed in either reaction, is transported to the cytoplasm and is coupled non-enzymatically to reduced glutathione (GSH). The *S*-hydroxy methyl glutathione is converted by NAD-GSH-dependent GD-FALDH to *S*-formylglutathione. The latter compound is hydrolyzed by *S*-formylglutathione hydrolase (FGH) to formate and GSH [32,33].

3.1. MDH

MDH catalyzes the oxidation of methanol to formaldehyde. It is a periplasmic quinoprotein with a molecular mass of about 150 kDa [6]. The enzyme consist of two identical large subunits of 66 kDa [31,47] and two identical small subunits of 9 kDa [66]. Each tetramer contains two molecules of pyrroloquinoline quinone (PQQ) which are non-covalently bound to the large subunit [21]. It has been reported that a Ca^{2+}-ion is essential for maintaining PQQ in its correct configuration [17,54]. The three-dimensional structure of MDH from *P. denitrificans* has been determined at 2.5 Å resolution [74]. The structure was similar to those of *Methylophilus methylotrophus* [75] and *Methylobacterium extorquens* AM1 [25]. The large subunit of MDH has an eight-fold radial symmetry,

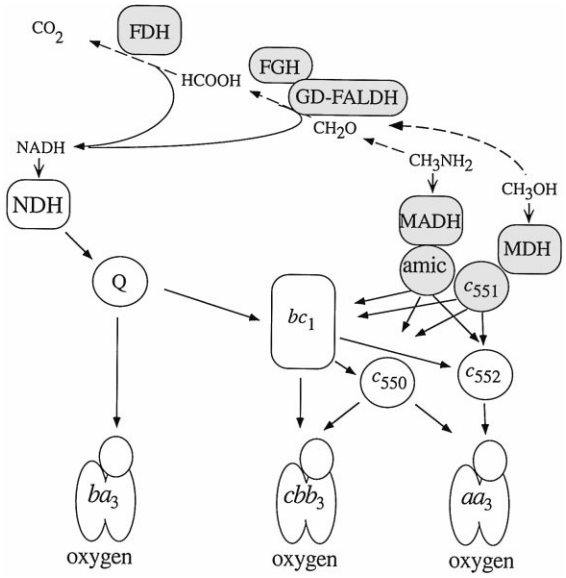

Fig. 2. Scheme of the *P. denitrificans* respiratory chain during C1 metabolism: NDH, NADH dehydrogenase; ubiquinone (Q); MDH, methanol dehydrogenase; MADH, methylamine dehydrogenase; GD-FALDH, NAD-GSH-dependent formaldehyde dehydrogenase; FGH, *S*-formylglutathione hydrolase; FDH, formate dehydrogenase.

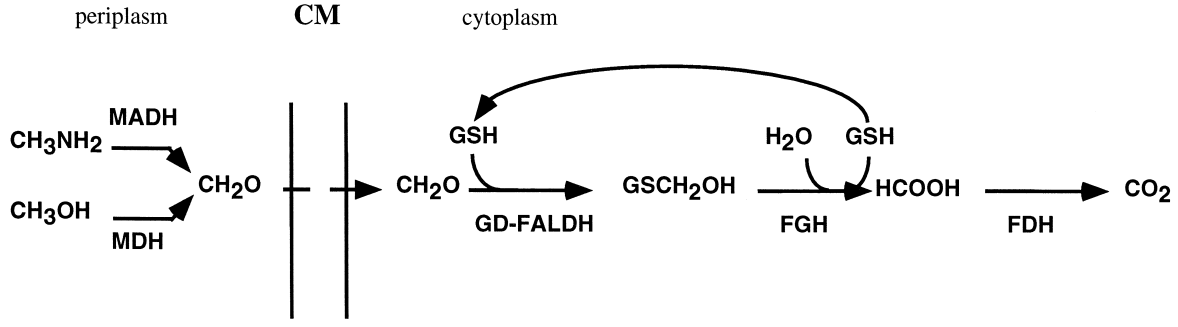

Fig. 1. Initial steps of C1 metabolism. CM, cytoplasmic membrane; GSH, glutathione; GD-FALDH, NAD-GSH dependent formaldehyde dehydrogenase; FGH, *S*-formylglutathione hydrolase; FDH, formate dehydrogenase.

with its eight β-sheets stabilized by a novel tryptophan docking motif. The eight antiparallel twisted β-sheets, or 'blades' each consist of four β-strands. The latter are arranged topologically like the letter 'W' and the eight 'W's are arranged circularly, forming the main disc-shaped body of the subunit. In seven of the eight 'W's, there is a tryptophan located in the middle of the outer strand of each β-sheet. The tryptophans are involved in three different types of interaction which may help to stabilize the eight-fold propeller assembly. Each large subunit contains one PQQ and one Ca^{2+}, which are located in the funnel-shaped central channel at the top of the eight 'W' disc. Two adjacent cysteine residues, Cys-103 and Cys-104, form a disulphide bridge in a trans- and non-planar configuration. This disulphide bridge may hold the prosthetic group in place in the active site. Cleavage of the bridge by reduction causes the PQQ to move to a location away from the active site [4]. In addition, the disulphide bridge may facilitate insertion of PQQ into the protein during assembly in the periplasm [3]. Baker et al. noticed that MDH is structurally related to nitrite reductase (NIR), a bacterial enzyme that reduces nitrite to nitric oxide during denitrification [5]. Despite the lack of overall sequence homology, the β-propellor domain of NIR proved to be closely superimposable on that of MDH. The relevance of such a structure for two functionally distinct types of enzyme is not clear. The obvious features that these two enzymes have in common are (i) their periplasmic location, (ii) their unique types of cofactor (heme d_1 for NIR, PQQ for MDH), and (iii) their production of cytotoxic reaction products (nitric oxide for NIR, formaldehyde for MDH). Whether the funnel of these enzymes is involved in channelling, the toxic compound towards the enzyme degrading it, is a matter of speculation.

The oxidation of methanol is coupled to the reduction of the prosthetic group (PQQ) to the corresponding quinol (PQQH2), followed by two sequential single electron transfers to its dedicated electron acceptor cytochrome c_{551i} (i, inducible). From there, the electrons are passed via the cytochromes c_{550} and c_{552} to the terminal cytochrome *c* oxidases.

Methanol oxidation is found in a wide variety of methylotrophic bacteria [2]. Apart from *P. denitrificans*, much of the current knowledge on the physiology and molecular genetics of this process derives from studies on the facultative methylotrophic bacteria *M. extorquens* AM1, *M. organophilum* XX, and *M. organophilum* DSM 760 (reviewed in Ref. [42]). Like in *P. denitrificans*, the methanol oxidizing branch of these bacteria is composed of MDH, a dedicated cytochrome *c*L (the counterpart of cytochrome $c_{551i)}$), a cytochrome *c*H (the counterpart of cytochromes c_{550}), (L and H refer to low and high iso-electric points, respectively), and a cytochrome *c* oxidase.

3.2. The genes encoding MDH and its cofactor PQQ

The synthesis of a fully active methanol oxidizing pathway in *P. denitrificans* requires at least 25 genes. These include the *pqq* genes involved in cofactor biosynthesis, and a comprehensive set of so-called *mxa* (*m*ethanol o*x*idation group *a*) and *flh* (*f*orma*l*de*h*yde dehydrogenase) genes. Some of the latter encode the enzymes catalyzing reactions in the methanol oxidation pathway, others encode enzymes involved in calcium insertion into MDH, and again others encode proteins involved in regulation of gene expression and protein activity, Refs. [31,66] and Harms, unpublished). An overview of the gene clusters involved in C1 metabolism of *P. denitrificans* is presented in Fig. 3. *mxaF*, encoding the large subunit, and *mxaI* encoding the small subunit, are closely linked with several additional genes in the gene order *mxaFJGIRSACKLD* [32–34,66]. Mutant studies revealed that the gene products of *mxaJ*, *mxaR* and *mxaS* are required for the formation of an active MDH ([30,66]. The *mxaG* gene encodes the 17.7-kDa cytochrome c_{551i} which

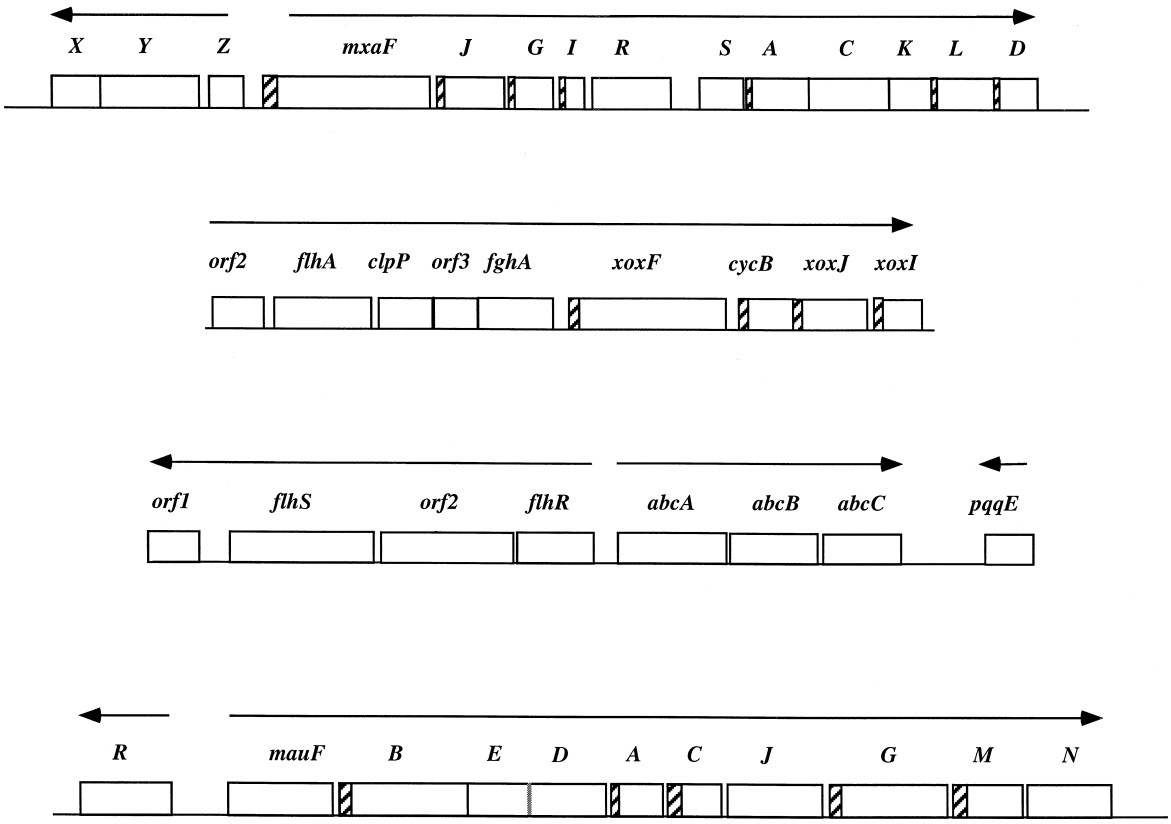

Fig. 3. Four gene clusters involved in C1 metabolism of *P. denitrificans*. Open boxes represent genes, hatched boxes encode signal sequences required for transport of the proteins to the periplasm. The direction of transcription of the genes is represented by the direction of the arrows. For details, see the text.

mediates electron transport between MDH and the electron transport chain [66]. The gene products of *mxaACKLD* are partly identical in sequence with gene products from *M. extorquens* AM1. In the latter organism, these proteins are involved in insertion of the PQQ cofactor and the Ca^{2+} ion into MDH [54].

Genes encoding proteins that are involved in PQQ biosynthesis have been isolated from several methylotrophs, i. e. *M. extorquens* AM1 [45,58,60], *M. organophilum* DSM760 and XX [9,45] and *Methylobacillus flagellatum* [26]. In *M. extorquens* AM1 two gene clusters (*pqqA-E; pqqFG*) are involved in PQQ biosynthesis. The cluster *pqqABCDE* was also found in the non-methylotrophs *Acinetobacter calcoaceticus* and *Klebsiella pneumonia* [28,44]. In the latter strain, the cluster harbours an additional gene, i.e., *pqqF*. These six genes were able to complement an *E. coli* strain that was unable to synthesize PQQ [43]. The product of *pqqA* is a peptide of 23 to 29 amino acids that contains conserved tyrosine and glutamic acid residues and has been proposed to be the peptide precursor from which PQQ is derived [29,44,72]. The gene product of *pqqF* shares identity with proteins that belong to a family of endopeptidases. It has been suggested that this protein is involved in processing of the peptide precursor during the biosynthesis of PQQ [44]. Recently, we isolated a 23-kbp DNA fragment of *P. denitrificans* with additional C1 genes (Harms et al, submitted; Fig. 3). The sequence revealed the *flhRS* genes involved in global regulation of C1 gene expression (see below), genes that belong to the *pqqA-E* gene cluster, and a cluster of three

genes, *abcABC*, encoding subunits of a protein complex that may belong to the family of so-called ABC transporters. These transporters are specialized transmembrane carriers of small to moderate size often hydrophobic molecules and are activated by ATP. The organization of the genes suggests that the AbcABC transporter is involved in C1 metabolism. If so, it may be required for translocation of PQQ from the cytoplasm to the periplasm or of formaldehyde from the periplasm to the cytoplasm.

3.3. MADH

MADH and its electron acceptor amicyanin are synthesized and transferred to the periplasm during growth on methylamine [11,37]. Like MDH, MADH has an $\alpha_2-\beta_2$ subunit configuration made up by a small (16 kDa; β-MADH) and a large (47 kDa; α-MADH) subunit, which is shaped like a superbarrel with radial symmetry just like α-MDH [12,71]. In contrast to MDH, the active site in MADH resides in the small subunit [13]. This site is created after the cross-linking and modification of two tryptophan residues of the peptide chain, resulting in the formation of the prosthetic group tryptophan–tryptophyl quinone (TTQ). Another remarkable feature of the small subunit is that its precursor has an unusually long signal sequence which is required for transport across the cytoplasmic membrane [8,15]. Berks noticed that β-MADH shares this property with distinct sets of periplasmic proteins containing special types of cofactor [8]. The downstream part of these signal sequences resembles the signal peptides that are recognized by the so-called Sec translocon proteins. The upstream part contains a somewhat conserved (S/T)RRxFLK motif. Berks proposed that these proteins share a common specialization in their export pathway. Perhaps TTQ or one of its precursor forms requires a special folding of the apo-enzyme such that transport requires a specialized translocon different from the normal Sec apparatus. At present, it is not known how and to what extent the translocation route and the TTQ biosynthesis route are integrated.

MADH oxidizes methylamine to ammonium and formaldehyde. Electrons derived from that oxidation step are transferred via the blue copper protein amicyanin to the electron transport network at the level of cytochrome *c* [37,65]. Amicyanin is a blue copper protein and has a single type 1 copper in its active site. The structures of amicyanin [16,22] and its complex with MADH [12] have been determined by crystallographic analyses. Mutant studies suggest that the interactions between the two proteins in the crystal are the same as in solution and are stabilized by a combination of ionic and van der Waals interactions [18]. The structure of a ternary complex with MADH, amicyanin and cytochrome c_{551i} [14] is also available, but the physiological significance of this type of interaction is questionable since the cytochrome is not an electron acceptor of MADH or amicyanin in vivo as judged by amicyanin and cytochrome mutant analyses [65,66].

3.4. The mau gene cluster encoding MADH

The *mau* (*m*ethyl*a*mine *u*tilizing) gene cluster of *P. denitrificans* consists of 11 genes (Fig. 3). Ten of these encode the structural proteins and proteins involved in cofactor biosynthesis and are transcribed in one direction in the order *mauFBEDACJGMN*; the 11th, *mauR*, is a regulatory gene located upstream and divergently transcribed from the other *mau* genes [62,63,65,67]). The *mau* gene cluster of *P. versutus* has a similar organization except that its *mauR* gene is transcribed in the same direction as the other *mau* genes [36]. The large and small subunits of MADH are encoded by the *mauB* and *A* genes, respectively [15]. Amicyanin is encoded by *mauC*. The *mauJ* gene may well be involved in processing or stability of amicyanin since absence or presence of the amicyanin gene in other methylamine utilizing organisms goes along with absence or presence

of the *mauJ* gene, respectively [24]. The *mauF* and *mauE* genes encode membrane bound proteins of unknown function but are essential for methylamine oxidation [62]. MauD is a soluble protein with a conserved Cys–Pro–Xaa–Cys motif, which is also found in proteins that are involved in making disulphide linkages. The six disulphide bridges of MADH might be formed by MauD. MauM and MauN are again proteins of unknown function. They contain conserved cysteine motifs resembling those involved in the binding of [2Fe–2S] and [4Fe–4S] clusters of known proteins. Surprisingly, although the genetic organization suggests that the *mauMN* genes are part of the *mau* gene cluster, they play no role in C1 metabolism of *P. denitrificans* as judged by mutant analyses [63]. So far, *Met. flagellatum* is the only organism where MauM is required for synthesis of active MADH [24]. The remaining gene, *mauG*, encodes a protein with two haem *c* binding motifs that resembles cytochrome *c* peroxidase [63]. MauG is involved in TTQ biosynthesis. This conclusion is based on the observation that a MauG mutant synthesizes an inactive apo-MADH, which is present in the periplasm but which lacks spectroscopically detectable TTQ [63]. Mass analyses of this mutant enzyme suggest that only one of the two oxygen atoms of the quinone is present in pre-TTQ (Van der Palen, unpublished). Formation of the other may require MauG.

Our current hypothesis on the TTQ biosynthesis route is that one of the tryptophan residues in the primary amino acid chain of β-MADH is already converted into the quinone form in the cytoplasm, maybe by a tryptophan hydroxylase or by an enzyme dedicated to PQQ biosynthesis. This form of β-MADH is then translocated by the specialized translocon to the periplasm after which the second oxygen is attached onto that tryptophan residue by the MauG protein. Then the connection of the ring structures of the tryptophan residues and the formation of the disulphide bridges may well be final steps in the biosynthesis.

3.5. GD-FALDH and FGH

The oxidation product of both methanol and methylamine is formaldehyde, which is translocated across the membrane to the cytoplasm. This transport process has been suggested to involve a specific formaldehyde carrier [39]. Formaldehyde is oxidized in the cytoplasm to formate via an NAD-linked glutathione-dependent GD-FALDH and FGH [32,33].

GD-FALDH of *P. denitrificans* is a tetrameric protein with four identical subunits and a total molecular mass of 40 kDa. Van Ophem and Duine [64] reported on the enzyme activity of partly purified GD-FALDH of *P. denitrificans*. The enzyme was dependent on NAD and reacted like plant, yeast, mammalian and *E. coli* GD-FALDHs. The enzyme was ineffective in oxidizing ethanol, but it readily catalyzed the oxidation of long-chain primary alcohols and, in the presence of reduced GSH, the oxidation of formaldehyde. GD-FALDH (EC 1.2.1.1) is widely distributed in plants, yeasts, mammals and bacteria. GD-FALDH isolated from a number of mammals is identical to group I subclass III of NAD(P)-dependent alcohol dehydrogenases (ADHs) [53]. The primary structure of GD-FALDH of *P. denitrificans* [52] is 36% identical to the deduced amino acid sequences of isolated GD-FALDH genes of plants and yeasts, and 60% identical to mammalian GD-FALDHs. Several residues are conserved in exactly the same spacing as has been found in the primary structure of group I ADHs of different organisms [38] and they coordinate the zinc atoms and the coenzyme. The sequence data suggest that GD-FALDH of *P. denitrificans* also contains two zinc atoms per subunit. The domain Gly-195-Xaa-Gly-197-Xaa-Xaa-Gly-200 is most likely involved in NAD-binding [73]. GD-FALDH is expressed at basal levels during growth on heterotrophic substrates ensuring that formaldehyde can be oxidized as soon as the cell encounters it. Much higher amounts of the enzyme were found in cells grown on methanol, methylamine and choline. A common

feature of these substrates is the transient formation of formaldehyde. A mutant in GD-FALDH is unable to grow on these substrates, indicating that GD-FALDH is essential for methylotrophic growth [32,33].

S-formylglutathione is hydrolyzed to formate and GSH by FGH. This enzyme has not yet been isolated from *P. denitrificans*. FGH isolated from human liver and *Kloeckera* sp. No2201 is a homodimer with a molecular mass of 58 kDa. The *Candida boidinii* FGH is a heterodimer with subunits of 35 kDa and 25 kDa. Studies on the polymorphism of FGH in human red blood cells revealed that the enzyme is identical to human esterase D [23]. Human esterase D is a member of a group of non-specific esterases. The native enzyme has a molecular weight of 70 kDa, and consists of two identical subunits [41]. Esterase D has been found in most human tissues, but the highest activities were found in placenta, kidney, liver and erythrocytes. The gene encoding this protein has been isolated and sequenced [40].

3.6. The genes involved in formaldehyde oxidation

The *flhA* gene, encoding GD-FALDH, has been isolated and was found to be located on a genomic fragment that contained several other genes involved in C1 metabolism [51,52] (see Fig. 3).

The *fghA* gene encoding FGH has been isolated from *P. denitrificans*. FGH is a homologue of human esterase D. The *fghA* gene was found to be located downstream of *flhA* separated by *clpP*, of which the involvement in C1 metabolism could not be demonstrated [32,33]. A *fghA* mutant failed to grow on methanol and methylamine, while growth on choline was still possible [32,33]. The regulation of expression of *flhA* and *fghA* is comparable since basal levels are attained during heterotrophic growth, while upregulation occurs during growth on methanol, methylamine or choline. The recently identified sensor–regulator pair FlhRS is also responsible for the upregulation of *flhA* (Harms et al., submitted).

3.7. Xox, a homologue of MDH

Downstream of the *flhA* and *fghA* genes, a gene cluster has been identified, *xoxF*, *cycB*, *xoxJ*, the gene products of which showed identity with those of *mxaFGJ*, respectively [32,33,51]. The gene *cycB* encodes a cytochrome c_{553i} that is synthesized in response to formaldehyde, just as are MDH, GD-FALDH and FGH. A mutation in this gene reduces the maximal specific growth rate on methanol [51]. The gene *xoxF* encodes a homologue of the large subunit of PQQ containing dehydrogenases. We speculate that XoxF is responsible for formaldehyde detoxification in the periplasm. Formaldehyde may react with methanol yielding a hemiacetal, which in yeasts is oxidized to methylformate by the enzyme methylformate synthase [46,56]. Perhaps XoxF fulfils a similar role in *P. denitrificans*.

4. Control of expression of C1 genes

4.1. Global control of expression of genes involved in C1 metabolism

Expression of both MADH and MDH is blocked when the cell experiences heterotrophic substrates in addition to the C1 substrate [35,76]. The view that a global transcription activator is responsible for this phenomenon has been corroborated by the analysis of a mutant disturbed in many aspects of C1 metabolism [32,33]. This mutant was unable to grow on methanol, on methylamine, and on choline. Moreover, it lacked the potential to express the *fghA*, *flhA*, *mxa*, *mau*, and *xox* genes. The identification of the mutated gene has been facilitated by the isolation of a genomic locus that fully complemented the mutation (Harms, unpublished). The protein sequences of two of the genes present on this locus, *flhRS*, showed high similarity to proteins of two-component regulatory systems:

FlhS may well be a protein histidine kinase and FlhR a DNA binding response regulator. The general principle for signal transduction via two component regulatory systems is that binding of an effector molecule activates the kinase, which then transfers a phosphate group to the response regulator. Once phosphorylated, the latter has an increased affinity for the promoter region of its target gene. Its binding facilitates transcription of that gene by the RNA polymerase [48]. We speculate that the global regulators FlhRS mediate the onset of expression of all C1 genes in response to intracellular formaldehyde and to depletion of the heterotrophic substrate. If this view is correct then the presence and/or activity of FlhRS should be suppressed during heterotrophic growth.

4.2. Dedicated regulation of the mxa gene cluster

Expression of the *mxa* genes is also controlled by the gene products of the *mxaXYZ* genes, which are located upstream of the *mxaFJGI* gene cluster [34,76]. Their sequences suggest that MxaY and MxaX are a protein histidine kinase and a DNA binding response regulator, respectively, again members of the family of two-component regulators. The effector molecule for the sensor domain of MxaY may well be formaldehyde since MDH and its dedicated cytochrome c_{551i} are expressed not only during growth on methanol but also during growth on methylamine or choline, which are all carbon sources that yield formaldehyde during oxidation. If formaldehyde is indeed the trigger, one may expect a progressive expression of MDH and other genes required for C1 metabolism during the switch from heterotrophic growth to methanol oxidation as a result of that product induction mechanism. MxaY was shown to be dispensable for this regulation, suggesting that an alternative kinase, perhaps FlhS, can take over its role. The role of MxaZ in regulation of *mxa* gene expression is still unclear. The MxaYX regulatory system appears to be specific for expression of the *mxa* genes since *mxaX* mutants had an unimpaired expression of the *mau*, *flhA*, *fghA* and *xox* genes [34].

4.3. Dedicated regulation of the mau gene cluster

The expression of the *mau* gene cluster is regulated by MauR, which is a transcription activator that belongs to the family of one-component LysR-type regulators [68]. The spatial organization of the functional domains involved in DNA binding, signal perception, and dimerization in the members of this family is conserved [57]. Many LysR-type regulators are activated by the molecule that serves as the substrate for the enzyme whose expression it controls. Consequently, methylamine is proposed to be the molecule that triggers the expression of the *mau* gene cluster [68]. The current view with respect to the mode of action of LysR-type regulators is that signal perception (i.e., binding of the activator molecule) induces dimerization of the regulator followed by binding to a target site upstream of the promoter. As a result, the dimer may come into contact with RNA polymerase, after which transcription gets underway. Indeed it has been shown that MauR binds to the *mau* promoter region [20]. Promoter probe studies revealed that *mau* gene expression during growth on methylamine was almost 1600-fold higher than during heterotrophic growth. The expression of the *mauR* gene itself was constitutive and not autoregulated, a feature different from that of most other *lysR*-type genes. Counterparts of MauR are found in *Thiobacillus versutus* [36] but as yet not in *M. extorquens* AM1.

5. Concluding remarks

One of the characteristics of living cells is that a change in their environment may evoke an adaptation of its biochemistry. This adaptive competence of a cell is determined not only by

its genetic potential, but also by the signal transduction pathways that orchestrate the proper expression of that potential and by the functioning of its metabolism which sets the level of the inducers. In the case of *P. denitrificans*, quite a few regulatory genes are integrated in a complex signal transduction network that controls the switch from heterotrophic to C1 metabolism. The tasks of this regulatory network are, first, to keep the C1 genes almost but not completely silent ('standby') at conditions were energetically more favourable substrates are available and, second, to ensure that the concentrations and activities of MDH, MADH (enzymes producing formaldehyde) and GD-FALDH (enzymes consuming formaldehyde) are induced to high levels when heterotrophic substrates run out and C1 substrates remain, and third that the expression levels of these enzymes are balanced to one another in order to keep the steady state concentrations of formaldehyde below cytotoxic levels yet allow for high flux through the pathway. Although this regulatory network is far from understood, the unravelling of some of its features allows a refinement of our view on the sequence of events after the switch. The current data indicate that the FlhRS two component signal transducers are expressed and/or activated by formaldehyde and by a so-far unknown signal diagnostic for depletion of heterotrophic substrates. Once activated, the response regulator binds to the target promoters of the C1 gene clusters. This event promotes the expression of the *fgh* and *flh* gene clusters, while the *mxa* and *mau* promoters are set in the stand-bye operation mode. As a result, only the formaldehyde consuming enzyme is synthesized. The onset of the *mxa* and *mau* genes encoding the formaldehyde producing enzymes MDH and MADH is triggered by the binding of a second and dedicated transcriptional activator (MxaX and MauR for *mxa* and *mau* gene expression, respectively) that acts in synergism with FlhR. This feature that a metabolic pathway is not

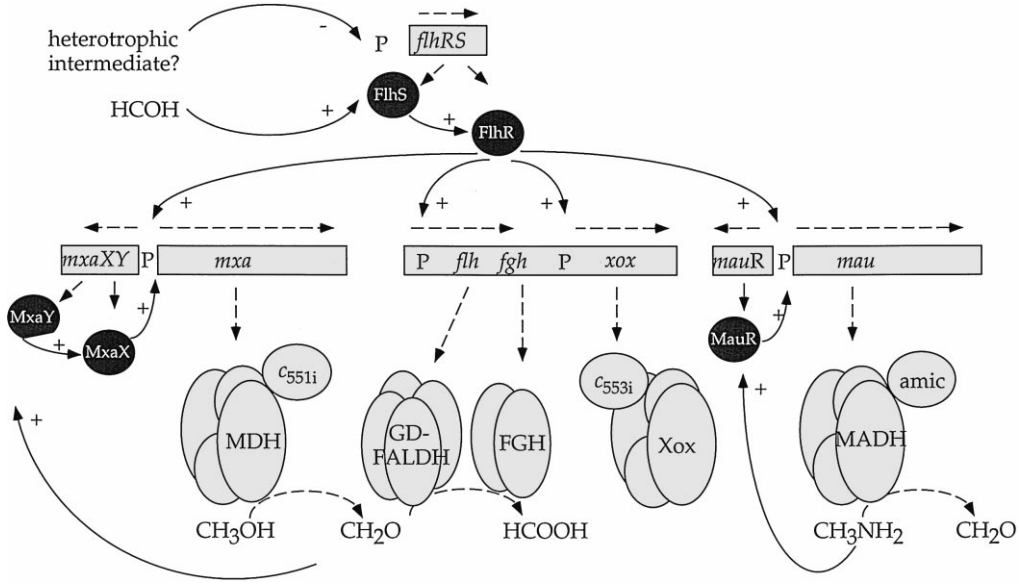

Fig. 4. Model of regulatory pathways involved in C1 metabolism in *P. denitrificans*. Genes and gene clusters are boxed; promoter regions are designated 'P'. Structural proteins are methanol dehydrogenase (MDH), methylamine dehydrogenase (MADH), NAD-GSH-dependent formaldehyde dehydrogenase (GD-FALDH), *S*-formylglutathione hydrolase (FGH), the product of the *xox* genes (Xox), the cytochromes c_{551i} (c_{551i}) and c_{553i} (c_{553i}), and amicyanin (amic). Regulatory proteins are the proposed formaldehyde sensors MxaY and FlhS, and their allocated regulators MxaX and FlhR, respectively, and the LysR-type activator of *mau* gene expression (MauR). Vertical arrows represent the transcription–translation process. Horizontal arrows indicate the direction of transcription. Solid lines indicate the processes involved in signal transduction and regulation (+, positive; −, negative).

induced by its substrate, but by an intermediate, turns the regulatory system into a switch-like one that can make 'decisions'. The presence of C1 substrates will initially lead to the formation of some formaldehyde because pathway expression is at the basal ('standby') level. This will lead to an increase in the level of formaldehyde which will in turn lead to an increase in pathway expression through the FlhR regulation up to a new level, which is determined by the relative induction of the pathway enzymes. These regulatory properties turn the regulation of C1 metabolism into a prime example of how even single cells manage their function in a semi-intelligent way. As molecular and functional properties are being identified, this intelligence is becoming defined by features such as decision making (the on–off switch), association (presence of methanol increases readiness for oxidation of intermediates and other C1 substrates), communication (cross talk between the two component regulatory systems) and collaboration (synergistic connections between the one and two component regulatory systems). A scheme of the regulatory pathways that control gene expression during C1 metabolism in *P. denitrificans* is shown in Fig. 4.

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