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MINI-REVIEW

C_1 Metabolism in *Paracoccus denitrificans*: Genetics of *Paracoccus denitrificans*

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Abstract

Paracoccus denitrificans is able to grow on the C_1 compounds methanol and methylamine. These compounds are oxidized to formaldehyde which is subsequently oxidized via formate to carbon dioxide. Biomass is produced by carbon dioxide fixation via the ribulose biphosphate pathway. The first oxidation reaction is catalyzed by the enzymes methanol dehydrogenase and methylamine dehydrogenase, respectively. Both enzymes contain two different subunits in an $\alpha_2\beta_2$ configuration. The genes encoding the subunits of methanol dehydrogenase (*moxF* and *moxI*) have been isolated and sequenced. They are located in one operon together with two other genes (*moxJ* and *moxG*) in the gene order *moxFJGI*. The function of the *moxJ* gene product is not yet known. *MoxG* codes for a cytochrome c_{551i}, which functions as the electron acceptor of methanol dehydrogenase. Both methanol dehydrogenase and methylamine dehydrogenase contain PQQ as a cofactor. These so-called quinoproteins are able to catalyze redox reactions by one-electron steps. The reaction mechanism of this oxidation will be described. Electrons from the oxidation reaction are donated to the electron transport chain at the level of cytochrome *c*. *P. denitrificans* is able to synthesize at least 10 different *c*-type cytochromes. Five could be detected in the periplasm and five have been found in the cytoplasmic membrane. The membrane-bound cytochrome c_1 and cytochrome c_{552} and the periplasmic-located cytochrome c_{550} are present under all tested growth conditions. The cytochromes c_{551i} and c_{553i}, present in the periplasm, are only induced in cells grown on methanol, methylamine, or choline. The other *c*-type cytochromes are mainly detected either under oxygen limited conditions or under anaerobic conditions with nitrate as electron acceptor or under both conditions. An overview including the induction pattern of all *P. denitrificans c*-type cytochromes will be given. The genes encoding cytochrome c_1, cytochrome c_{550}, cytochrome c_{551i}, and cytochrome c_{553i} have been isolated and sequenced. By using site-directed mutagenesis these genes were mutated in the genome. The mutants thus obtained were used to study electron transport during growth on C_1 compounds. This electron

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transport has also been studied by determining electron transfer rates in *in vitro* experiments. The exact pathways, however, are not yet fully understood. Electrons from methanol dehydrogenase are donated to cytochrome c_{551i}. Further electron transport is either via cytochrome c_{550} or cytochrome c_{553i} to cytochrome aa_3. However, direct electron transport from cytochrome c_{551i} to the terminal oxidase might be possible as well. Electrons from methylamine dehydrogenase are donated to amicyanin and then via cytochrome c_{550} to cytochrome aa_3, but other routes are used also. *P. denitrificans* is studied by several groups by using a genetic approach. Several genes have already been cloned and sequenced and a lot of mutants have been isolated. The development of a host/vector system and several techniques for mutation induction that are used in *P. denitrificans* genetics will be described.

Key Words: *P. denitrificans*; methanol dehydrogenase; methylamine dehydrogenase; PQQ; amicyanin; cytochrome *c*; electron transport; restriction/modification; gene replacement; *mox* genes, *cyc* genes.

Introduction

Methylotrophic organisms are able to oxidize reduced carbon compounds containing one or more carbon atoms but containing no carbon–carbon bonds. A large variety of microorganisms, both gram-positive and gram-negative bacteria as well as yeasts, were found to grow on these substrates. Methylotrophic bacteria can be classified according to their mode of carbon assimilation. Three different pathways exist: the ribulose monophosphate (RuMP) pathway, the serine pathway, and the ribulose biphosphate (RuBP) pathway. All obligate methylotrophic bacteria use the RuMP pathway. In this pathway cell carbon is assimilated at the level of formaldehyde produced by the oxidation of methanol or methylamine. Formaldehyde is fixed via a cyclic route which results in the net production of a C_3 compound as the primary product. In the serine pathway both formaldehyde and carbon dioxide are assimilated via a cyclic pathway to yield also a C_3 compound as the primary product. This route differs from the RuMP pathway in the nature of its intermediates which are carboxylic acids and amino acids instead of carbohydrates. Most serine-type bacteria are facultative methylotrophs. In the ribulose bisphosphate pathway all carbon is assimilated at the level of carbon dioxide, which predominantly arises from the oxidation of C_1 compounds. Relatively few methylotrophs use this mode of carbon assimilation, possibly because this pathway is energetically a costly one. The bacteria using the RuBP pathway are chemolithotrophic or facultative autotrophs using either light, inorganic compounds, or hydrogen as energy source. It has been suggested for these "methanol autotrophs" that acquisition of extra enzymes capable of oxidizing methanol to carbon dioxide by these organisms is a great gain in nutritional versatility and outweighs the disadvantage using this energetically more costly pathway instead of formaldehyde fixation

via the RuMP or the serine pathway (Sahm *et al.*, 1976). Details of the various pathways have been reviewed by Anthony (1982) and Hou (1984). The molecular biology of methylotrophic bacteria has recently been reviewed by De Vries (1986) and De Vries *et al.* (1990).

Methylotrophic Growth of *Paracoccus denitrificans*

P. denitrificans is able to grow on methanol, methylamine, and formate as sole sources of carbon and energy. These compounds are oxidized to carbon dioxide that is subsequently fixed by ribulose bisphosphate carboxylase, one of the key enzymes of the RuBP pathway (Marison and Attwood, 1980; Cox and Quayle, 1975; Bamforth and Quayle, 1978). Several enzymes are involved in these oxidation routes.

Methanol Oxidation

Methanol Dehydrogenase. The oxidation of methanol to formaldehyde is catalyzed by methanol dehydrogenase (MDH) (Anthony and Zatman, 1967). This enzyme is located in the periplasm (Alefounder and Ferguson, 1981) and contains a novel cofactor pyrrolo-quinoline-quinone (PQQ) (Duine *et al.* 1980; Salisbury *et al.*, 1979). As far as has been investigated, all gram-negative bacteria use a similar enzyme (Anthony, 1982). Gram-positive organisms use either a novel type of methanol dehydrogenase in which, apart from PQQ, NAD is required for activity (Duine *et al.*, 1984), or a classical NAD-dependent alcohol dehydrogenase with high affinity for methanol (Arfman *et al.*, 1989).

MDH from *P. denitrificans* has a wide but well-defined substrate specificity and in this characteristic it shows large similarities with MDH from other bacteria. Primary alcohols are oxidized but the efficiency decreases with increasing chain length (De Vries *et al.*, 1988). Secondary alcohols are not oxidized (Bamforth and Quayle, 1978), but formaldehyde (in the hydrated form) is oxidized although with somewhat lower efficiency as methanol (De Vries *et al.*, 1988). It is, however, improbable that this substrate is oxidized by MDH *in vivo* since mutants lacking MDH are still able to grow on methylamine and choline, substrates that both generate formaldehyde during metabolism (Harms *et al.*, 1985). In addition, oxidation of formaldehyde by MDH is energetically unfavorable. Therefore, in some methylotrophs a "modifier" protein is synthesized that decreases the affinity of MDH for formaldehyde, sufficient to stop formaldehyde oxidation (Ford *et al.*, 1985; Page and Anthony, 1986).

Methanol dehydrogenase from *P. denitrificans* was found to have a molecular weight of about 150,000 (Bamforth and Quayle, 1978). Until recently it was believed that the enzyme was a dimer with two identical

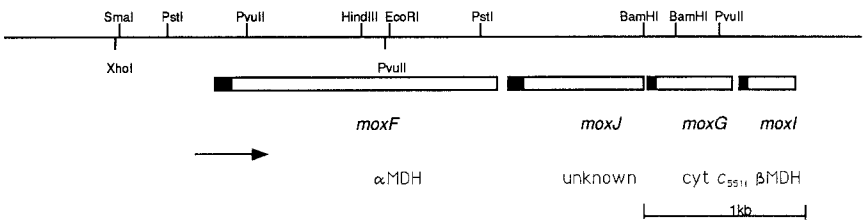

Fig. 1. Scheme of the gene organization of the *mox* genes in *P. denitrificans*. Black boxes indicate signal sequences in the gene products. The arrow indicates the transcriptional direction.

subunits, with a molecular weight of 66,000. Analysis of genes involved in methanol oxidation from *Methylobacterium extorquens* AM1 revealed downstream from the gene coding for this subunit (*moxF*) three additional genes (*moxJ*, *moxG*, and *moxI*) (Anderson and Lidström, 1988). One of these genes (*moxI*) encodes a protein with a molecular weight of 8,500 and it was suggested that this protein is associated to MDH. A protein of similar molecular weight was often co-purified with native MDH (Ohta and Tobari, 1981; Janssen *et al.*, 1987; Elliott and Anthony, 1988). Initially, it has been assumed that this small polypeptide represents an artifact of the purification procedure. But later it has been suggested that it might be a subunit of the enzyme, only dissociating under extreme conditions. Nunn *et al.* (1989) have proven that this small protein is in fact a second subunit of MDH, and that the enzyme consists of two large subunits and two small ones. Since in *P. denitrificans* a similar gene organization (*moxFJGI*) was found (Fig. 1, Van Spanning *et al.*, unpublished results), MDH in this organism might consist of four subunits as well. Indeed antibodies raised against purified MDH reacted with a protein of 66,000 as well as with a protein of 9,400 (this laboratory, unpublished results). The nucleotide sequence of the four *mox* genes of *P. denitrificans* has been determined (Harms *et al.*, 1987, 1989; Van Spanning *et al.*, unpublished results). The *moxF* and *moxI* genes encode the two subunits of MDH. The *moxJ* gene codes for a protein with a molecular weight of 26,500, of which the function is still unknown. The *moxG* gene encodes a cytochrome *c* of 17,700 Da. The deduced amino acid sequence reveals a typical heme binding site, found in all *c*-type cytochromes sequenced thus far. This cytochrome *c* is the one that was isolated from methanol-, methylamine-, or choline-grown cells as the periplasmic-located, soluble cytochrome c_{551i} with a molecular weight of 22,000 (Husain and Davidson, 1986; Bosma *et al.*, 1987a). All four gene products contain at their N-terminus a stretch of amino acids that shows characteristics of a signal sequence, which indicates the periplasmic location of the proteins.

MoxF genes have been isolated from eight different methylotrophic bacteria (Nunn and Lidström, 1986a; Harms *et al.*, 1987; Stephens *et al.*,

1988; Machlin *et al.*, 1988; Dispirito *et al.*, 1989; Bastien *et al.*, 1989; Al-Taho *et al.*, 1990). At the DNA level these genes are highly conserved. The *P. denitrificans moxF* gene hybridizes with DNA fragments containing the *moxF* gene from *M. organophilum* XX and *M. extorquens* AM1, and the *moxF* gene from *M. extorquens* AM1 was used as a probe to isolate the genes from the other five organisms. Comparison of DNA sequences of the *moxF* genes from *P. denitrificans* and *M. organophilum* XX revealed a similarity of 82% (Harms *et al.*, 1987; Machlin and Hanson, 1988). An even higher similarity was found in the deduced amino acid sequence. Comparable homologies were found for the sequences of the genes coding for cytochrome *c* (*moxG* genes) and for the small subunit (*moxI* genes) from *P. denitrificans* and *M. extorquens* AM1 (Nunn and Anthony, 1988; Nunn *et al.*, 1989; Van Spanning, unpublished results). However, antibodies raised against *P. denitrificans* MDH show no cross-reactivity with the MDH proteins of *M. organophilum* XX and *M. extorquens* AM1 (Harms *et al.*, 1985). Furthermore, the enzyme of *P. denitrificans* has a low isoelectric point (3.7) (Bamforth and Quayle, 1978) compared to that of MDH's from other methylotrophs which are usually high. So, although the different MDH's are highly conserved at the DNA and amino acid level, considerable differences still exist.

By using a T7 expression system, Anderson and Lidström (1988) demonstrated that the *moxFJGI* genes from *M. extorquens* AM1 are coordinately expressed. In *M. organophilum* XX, however, mRNA analysis revealed the presence of two different transcripts, which indicates that the *moxF* gene and the *moxG* gene in this organism are not cotranscribed (Bastien *et al.*, 1989; Machlin and Hanson, 1988). By using a promoter probe vector, promoter activity could be demonstrated in *P. denitrificans* upstream of the *moxF* gene. No promoter activity could be found upstream of either the *moxJ* or the *moxG* gene, which suggests that the *moxFJGI* genes in this organism are coordinately expressed as well (Harms *et al.*, 1989; Harms, unpublished results).

Apart from the structural genes encoding MDH and cytochrome *c*, several other genes involved in methanol oxidation have been isolated from *M. extorquens* AM1 and *M. organophilum* XX (Nunn and Lidström, 1986a, Allen and Hanson, 1985; Machlin *et al.*, 1988; Bastien *et al.*, 1989). These genes are located on three separate chromosomal loci. If all genes required for active MDH synthesis have iso-functional counterparts in either species, as many as 16 *mox* genes are required for the synthesis of active MDH. Three genes seem to be involved in the assemblage of apoMDH and PQQ; one gene encodes a regulatory protein, four genes have an up till now unknown function, and four genes are involved in PQQ synthesis. This latter number was extended to six by Biville *et al.* (1989) in their study on the biosynthesis

of PQQ in *M. organophilum* DSM760. The large amount of genes that have been found to be involved in methanol oxidation indicates the complexity of the system. Whether a comparable amount of genes is involved in *P. denitrificans* is not yet known.

Redox Properties of Methanol Dehydrogenase. Methanol dehydrogenase contains PQQ as a prosthetic group and, therefore, belongs to the quinoproteins. PQQ is noncovalently bound to the enzyme and per enzyme two PQQ molecules are present. A PQQ molecule can accommodate an unimpaired electron, giving a free radical that is stable when bound to the protein. Quinoproteins are thus able to catalyze redox reactions by one-electron steps. In this respect, it is understandable that quinoprotein dehydrogenases are well suited for direct transfer of the redox equivalents from the substrates to the one-electron carriers of the respiratory chain. It is well accepted that MDH donates its electrons to the respiratory chain at the level of cytochrome *c* (Willison and John, 1979; Van Verseveld and Stouthamer, 1978a; Anthony, 1982). The *c*-type cytochromes and the electron routes to the electron transport chain will be discussed below. Here it is appropriate to mention that the natural electron acceptor for MDH is the periplasmic located cytochrome c_L (in *P. denitrificans* known as cytochrome c_{551i}).

MDH is routinely assayed at high pH (pH 9.0) using an artificial electron acceptor such as phenazine methosulfate or Wursters blue, ammonia as an activator, and cyanide ions (Anthony and Zatman, 1964). *In vitro*, cytochrome c_L has been shown to be a poor electron acceptor in the MDH assay. Using isolated MDH and cytochrome c_L, one could, however, demonstrate methanol-dependent reduction of the cytochrome catalyzed by MDH in *Methylophilus methylotrophus*, *M. extorquens* AM1, and *P. denitrificans* (Beardmore-Gray *et al.*, 1983). The reaction rates, however, were very low compared to methanol oxidation rates of whole cells or to the rates in the assay using nonphysiological electron acceptors (Ohta and Tobari, 1981). The reaction mechanism for the oxidation of methanol was investigated by using nonphysiological conditions therefore.

Frank *et al.* (1988) proposed a scheme for the reaction mechanism of MDH isolated from *Hyphomicrobium* X (Fig. 2). Isolated MDH contains the stable free radical of PQQ, PQQH · (MDH_{sem}). This compound can be oxidized to MDH_{ox} in the presence of the electron acceptor. The fully oxidized MDH is not stable and reacts in the absence of substrate to an inactive form of MDH. To avoid inactivation by the electron acceptor, the assay mixture is supplemented with cyanide, which is a competitive inhibitor of the substrate and which can react with the $C_5=O$ group of PQQ. This MDH cyanide complex is stable and the addition of cyanide is reversible. In the presence of

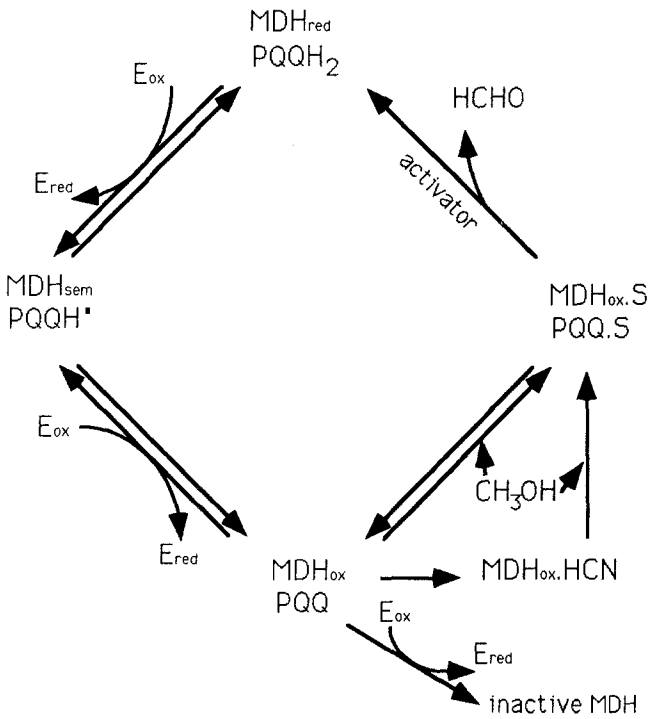

Fig. 2. Reaction mechanism of methanol dehydrogenase (MDH). MDH_{sem}, semiquinone form of MDH; MDH_{ox}, oxidized MDH; $MDH_{ox} \cdot S$, MDH–substrate complex; $MDH_{ox} \cdot HCN$, MDH–HCN complex; MDH_{red}, reduced MDH; S, substrate; P, product; E_{ox}, E_{red}, oxidized and reduced forms of a one-electron acceptor.

methanol, cyanide is rapidly replaced, and the $MDH_{ox} \cdot S$ complex is formed, after which the substrate oxidation takes place. Ammonia acts as an activator in this reaction. The fully reduced MDH (MDH_{red}) is formed and this compound is oxidized to MDH_{sem}. From the results obtained with stopped-flow as well as steady-state kinetics, combined with the isotope effect found for C^2H_3OH, it appeared that oxidation of MDH_{red} to MDH_{sem} and MDH_{ox} with an artificial electron acceptor occurs much faster at pH 9 than at pH 7. The substrate oxidation step was very slow at pH 9 and activation of this reaction by ammonia is needed therefore.

With the knowledge of this reaction mechanism, Dijkstra *et al.* (1988a) investigated the mechanism in *Hyphomicrobium* X with cytochrome *c* as electron acceptor. The intermediates of MDH, seen during the reaction, are identical to those observed with the artificial dyes, indicating that the reaction cycles are not essentially different. The assay showed a pH optimum of approximately 7 and scarcely any stimulation by ammonia.

In *Hyphomicrobium* X cytochrome c_L^{ox} is an excellent oxidizer of the reduced MDH forms at pH 7, but as in the case with the artificial dyes at pH 9, the substrate oxidation step is very slow. Since activation by ammonia at pH 7 is poor, no activation of this reaction was observed. The difference in the rate-limiting steps at the two pH's with the different electron acceptors explains why cytochrome c_L is a poor electron acceptor in the MDH assay. Although ammonia can influence substrate oxidation *in vitro*, it does not seem plausible that this occurs *in vivo* as well. It was found that when permeabilized whole cells are used in the MDH assay, no activation by ammonia is needed. The same holds for MDH that was isolated under anaerobic conditions (Duine *et al.*, 1979). After exposure of this preparation to oxygen, the activity became activator dependent. Recently an oxygen-labile, low-molecular-weight factor was described that was able to act as activator in the MDH assay (Dijkstra *et al.*, 1988b). Therefore, this compound might be the activator of the substrate oxidation step in the *in vivo* situation.

Knowledge of the reaction cycle in *P. denitrificans* is not available. However, some observations indicate that a similar situation may exist in this organism. Reaction of reduced MDH with cytochrome c_{551i} was not observed (Davidson and Kumar, 1989) or the reaction rates were very slow (Beardmore-Gray *et al.*, 1983). For MDH activity measurements in toluene-treated whole cells no absolute requirement for ammonia was observed (De Vries *et al.*, 1988). In addition, these cells displayed 5 to 10-fold higher MDH activities.

Regulation. During growth on methanol, MDH is a major protein in methylotrophs. This enzyme may be synthesized in an amount up to 15% of the total cell protein. The regulation of MDH synthesis has been investigated in a variety of organisms. In *P. denitrificans* an immunological detection technique and a whole-cell activity assay were used to detect MDH with high sensitivity (De Vries *et al.*, 1988). With these techniques it could be demonstrated that MDH was not induced by its natural substrate, methanol. MDH could never be found in cells grown on multicarbon sources that permit relatively fast growth or on mixtures of one of these multicarbon sources or both methanol and methylamine. The expression of MDH and, therefore, the oxidation of methanol is under tight control of catabolite repression. In an other autotrophic methylotroph *Xanthobacter* sp. strain H4-14 a similar strict regulation mechanism was found (Weaver and Lidström, 1985; Meyer, 1990). It seems that since autotrophic methylotrophs utilize methanol solely as energy source to drive the costly RuBP pathway, these organisms will only induce MDH for this purpose if multicarbon sources are absent. In obligate and restricted facultative methylotrophs MDH is produced constitutively, although enzyme activity can be induced to higher levels under specific conditions (Greenwood and Jones, 1986; Jones *et al.*, 1987). In serine-type

methylotrophs MDH is synthesized constitutively also. Higher levels of MDH are detected when methanol is added to the growth medium (Weaver and Lidström, 1985; O'Connor and Hanson, 1977; McNerney and O'Connor, 1980). In several serine-type methylotrophs high levels of MDH could be detected even in the absence of methanol. Expression of MDH activity was shown to be inversely related to the growth rate of the organism (Roitsch and Stolp, 1986).

Carbon limitation in the chemostat was found to derepress catabolite repression in *P. denitrificans*. Low levels of MDH were detected in cells grown under these conditions. A further increase of MDH synthesis was observed in cells grown on methanol, methylamine, or choline. A common feature of these substrates is that they generate formaldehyde during metabolism. A product-induction mechanism with formaldehyde as possible effector was postulated, therefore (De Vries *et al.*, 1988). A similar product-induction mechanism might also exists in other methylotrophs. An increase in MDH was observed in several organisms by adding either methanol or methylamine to the growth medium. Since MDH is always present in these organisms, it is well conceivable that not methanol itself but formaldehyde is the true effector of induction. A mechanism of product induction could feasibly result in over expression of MDH leading to accumulation of the toxic compound formaldehyde. It seems that *P. denitrificans* has overcome this problem by a third posttransitional control mechanism that regulates the amount of MDH synthesized and its activity. This regulation mechanism may involve PQQ biosynthesis, PQQ association with MDH apoprotein, or regulation of expression of the different subunit genes resulting in non-equimolar quantities of the two subunits and consequently in a partly inactive enzyme pool. Since structural as well as regulatory genes involved in methanol oxidation have now been isolated from various methylotrophs (Nunn and Lidström, 1986a; Machlin *et al.*, 1988; Bastien *et al.*, 1989), more detailed studies on the regulation of methanol oxidation have now become possible.

Methylamine Oxidation

Methylamine Dehydrogenase. The oxidation of methylamine to formaldehyde is catalyzed by methylamine dehydrogenase (MADH). Like methanol dehydrogenase, this enzyme is a quinoprotein and is located in the periplasm (Husain and Davidson, 1985). Many methylotrophic bacteria use MADH, but in some methylotrophic bacteria methylamine is oxidized by a flavin dependent methylglutamate dehydrogenase and further dissimilated via the methylglutamate cycle (Anthony, 1982; Biville *et al.*, 1988).

MADH from *P. denitrificans* has a molecular weight of 124,400 (Husain and Davidson, 1987a). The enzyme contains two different subunits in an $\alpha_2\beta_2$ configuration. The large subunit has a molecular weight of 46,700 and the small subunit one of 15,500. The molecular weights and the amino acid compositions of the two subunits are very similar to those of MADH's which have been isolated from other bacteria. The enzyme exhibited a p*I* of 4.3. A similar value was also found in enzymes from other autotrophic and facultative methylotrophs. Higher values for the p*I* were found in obligate and restricted methylotrophs (Lawton and Anthony, 1985; Matsumoto 1978). This variation in p*I* was confered upon each enzyme by its large subunit (Davidson and Neher, 1987). Immunological cross reactivity was observed between the small subunits of the *P. denitrificans* enzyme and MADH isolated from Bacterium W3A1, a restricted facultative methylotroph. No cross-reactivity was observed between the large subunits. Differences between MADH's from facultative and restricted facultative or autotrophic methylotrophs were also observed in the form of substrate specificity (Matsumoto, 1978) and redox properties (Shirai *et al.*, 1978). The enzyme of *P. denitrificans* is able to catalyze the oxidation of a wide variety of primary aliphatic amines and diamines. Secondary, tertiary, or aromatic amines are not oxidized by this enzyme (Husain and Davidson, 1987a). Thus far no data are available about the genes involved in oxidation of methylamine.

Redox Properties of Methylamine Dehydrogenase. The exact nature of the cofactor in MADH is a matter of discussion. The isolated enzyme contains a free radical showing an ESR signal comparable to that found for the semiquinone in MDH_{sem} (De Beer *et al.*, 1980). However, the absorption spectrum is largely different from that of MDH and, furthermore, the cofactor cannot be removed from the enzyme (Husain and Davidson, 1987a). On the other hand, the midpoint potential value for MADH isolated from *P. denitrificans* (100 mV) (Husain and Davidson, 1987b) is very close to the value found for free PQQ at neutral pH (90 mV), and reactivity with hydrazines also point to PQQ (Van der Meer *et al.*, 1987). McIntire and Stults (1986), however, proposed that the actual cofactor was a PQQ-like structure which lacked the carboxylic acid groups of PQQ, and which was attached to the protein with Cys–thio ether and Ser–oxygen ether linkages. Recently the three-dimensional structure of MADH, isolated from *Thiobacillus versutus*, has been determined (Vellieux *et al.*, 1989). It was suggested that the cofactor in this enzyme was not PQQ itself, but a precursor of PQQ in which the pyridine ring was not closed. PQQ, or "pro-PQQ", was found to be covalently attached to the small subunit at two different sites on the protein chain (Ishii *et al.*, 1983).

MADH is routinely assayed at its optimum pH of 7.5 using an artificial electron acceptor such as phenazine ethosulfate. Ammonia and cyanide are

not present in the assay mixture. The natural electron acceptor for MADH is the blue copper protein amicyanin (Husain and Davidson, 1985) that can replace the artificial dye in the assay. By using isolated MADH and amicyanin, methylamine-dependent reduction of the copper protein, catalyzed by MADH at rates comparable to the *in vivo* situation, could be demonstrated (Lawton and Anthony, 1985; Husain and Davidson, 1986). The electrons are subsequently donated to the respiratory chain at the level of cytochrome *c* (Gray *et al.*, 1988; Husain and Davidson, 1985).

The mechanism of methylamine oxidation is largely comparable to the mechanism of methanol oxidation (Davidson, 1989; McWhirter and Klapper, 1989; Van Wielink *et al.*, 1989). Fully oxidized MADH reacts with methylamine to the $MADH_{ox} \cdot S$ complex. In a rate-limiting α-proton abstraction step, substrate oxidation takes place and the 5-amino substituted MADH is formed. During this reaction formaldehyde is formed, but no ammonia is released. In two one-electron oxidation steps with amicyanin as electron acceptor, fully oxidized MADH is formed. In the first of these reactions ammonia is released and the semiquinone is formed.

Regulation. Regulation of MADH synthesis differs from that of MDH synthesis. Enzyme activity and immunological detection of each subunit were observed in cells which had been grown on methylamine as sole carbon source (Husain and Davidson, 1987a). In cells grown on a mixture of a multicarbon source and methylamine, MADH activity could be detected (Harms, unpublished results). So, as soon as the cell senses methylamine, MADH is synthesized.

Growth on Choline

P. denitrificans is able to grow on choline as sole carbon and energy source. This substrate is oxidized in several oxidation steps to glycine. During this oxidation three molecules of formaldehyde are formed. Therefore, choline might be considered as a multicarbon C_1 compound. Growth on choline shows indeed many features of methylotrophic growth. MDH is induced and several *c*-type cytochromes that are found in cells grown on C_1 compounds are synthesized as well.

Oxidation of Formaldehyde and Formate

Formaldehyde formed by the oxidation of methanol and methylamine is transported to the cytoplasm by a transport mechanism in which a transport protein is involved (Köstler and Kleiner, 1989). Once it is present in the cytoplasm, formaldehyde is oxidized to formate by a formaldehyde dehydrogenase (FyDH). In *P. denitrificans* NAD-dependent as well as dye-linked enzyme activities have been demonstrated. Since in many organisms

in vitro-measured activities were low and no specific increase in enzyme activities were noted during growth on methanol, the role of the dye-linked FyDH in methanol metabolism is unclear. Marison and Attwood (1980) have postulated that the dye-linked FyDH in methylotrophs is a general aldehyde dehydrogenase, not directly involved in the dissimilation of C_1 compounds. In contrast, it has been reported for *Pseudomonas aminovorans* that this enzyme is induced during growth on C_1 compounds (Bamforth and O'Connor, 1979). In *P. denitrificans* mutants that were unable to grow on methanol, methylamine, or choline, NAD-dependent FyDH activity could be found. This might indicate that the dye-linked FyDH is also involved in C_1 metabolism in this organism (Harms *et al.*, 1985).

Formate formed by the oxidation of formaldehyde is oxidized to carbon dioxide by a formate dehydrogenase. In the majority of the methylotrophic bacteria, formate is oxidized by a soluble NAD-dependent formate dehydrogenase, and in many of these bacteria this dehydrogenase is the only enzyme that provides NADH for biosynthesis during growth on C_1 compounds (Anthony, 1982). However, in a few bacteria [*Pseudomonas oxalaticus* (Dijkhuizen *et al.*, 1979; Quayle, 1961), *Alcaligenes eutrophus* (Friedrich *et al.*, 1979), *Alcaligenes* LOXI (Chandra and Shetna, 1975), *Archromobacter parvulus* (Rodinov and Zakharova, 1980), and *P. denitrificans* (Harms, 1988)] a second type of formate dehydrogenase has been detected, which is membrane bound and NAD-independent.

Electron Transport during C_1 Metabolism

Electron Carriers

Amicyanin. When grown on methylamine as sole carbon source, *P. denitrificans* synthesizes a blue copper protein (Husain and Davidson, 1985). This protein, called amicyanin, contains a single polypeptide chain with a molecular weight of 15,000 and one copper atom. The EPR spectrum of amicyanin is typical of that observed for Type I copper proteins. Physical and physiological properties indicate that it is different from azurin, the other blue copper protein that has been isolated from *P. denitrificans* (Husain *et al.*, 1986; Martinkus *et al.*, 1980). Amicyanin is a one-electron carrier and can mediate electron transfer between MADH and mammalian cytochrome *c* and the soluble cytochromes c_{550}, c_{551i}, and c_{553i}, cytochromes that are present in *P. denitrificans* cells grown on C_1 compounds (Husain and Davidson, 1985, 1986). Amicyanin is not able to serve as electron acceptor for MDH. The copper protein has therefore exclusively a role in methylamine oxidation, although this protein was not observed in all methylotrophs growing on

methylamine by way of MADH (Anthony, 1988). It is striking, however, that amine oxidase, a protein that is observed in several organisms and that catalyzes the oxidation of amines, is a copper-containing protein (Duine *et al.*, 1987).

Cytochrome c. Electrons originating from the oxidation of methanol and methylamine are transported to the respiratory chain at the level of cytochrome *c*. A large amount of *c*-type cytochromes has been found in *P. denitrificans*.

A complete picture on all *c*-type cytochromes present in the periplasm as well as in the cytoplasmic membrane of *P. denitrificans* cells grown under various culture conditions was given by Bosma (Bosma *et al.*, 1987a; Bosma, 1989) (Table I). At least 10 different *c*-type cytochromes could be detected. Five were present in the periplasm and five were detected in the membrane. The cytochromes could be distinguished by their absorption maximum, their molecular weight, and their midpoint potential. The induction pattern of the different cytochromes might indicate that the cytochromes are all different despite the similarity in molecular weight of some of them.

Cytochrome c_1, being part of the bc_1 complex, and the periplasmic cytochrome c_{550} are present under all growth conditions tested. The gene

Table I. Relative quantities of *c*-type Cytochromes in *P. denitrificans* Cells Grown in Aerobic Carbon-Limited (C-lim), Oxygen-Limited (O_2-lim), or Anaerobic Nitrate-Limited (NO_3-lim) Continuous Cultures on Succinate, Methanol, Methylamine, and Choline[a]

A		15.0 c_{550}	(22) 17.7 c_{551i}	(30) 23.1 c_{553i}	45	60
Succinate	C-lim	++	−−	−−	−−	−−
Succinate	O_2-lim	++	−−	−−	++	−−
Succinate	NO_3-lim	++	−−	+−	+−	++
MeOH	C-lim	++	++	++	−−	−−
$MeNH_2$	C-lim	++	++	++	−−	−−
Choline	C-lim	++	++	++	−−	−−
B		14	22 c_{552}	30	45	(62) 45.2 c_1
Succinate	C-lim	−−	++	+−	−−	++
Succinate	O_2-lim	+−	++	++	++	++
Succinate	NO_3-lim	++	++	++	++	++
MeOH	C-lim	+	++	+	+	++
$MeNH_2$	C-lim	+	++	+	+	++
Choline	C-lim	+	++	+	+	++

[a]The *c*-type cytochromes are characterized by their apparent molecular masses in kDa, and their notation as used in the text. A, periplasmic located cytochromes; B, membrane-bound cytochromes. *i*, indicated inducible; −−, absent, +−, just detectable; +, present; ++, abundantly present.

encoding cytochrome c_1 (*fbcC*) has been isolated and sequenced (Kurowski and Ludwig, 1987; see also Ludwig *et al.*, this issue). Cytochrome c_{550} is the well-known soluble *c*-type cytochrome having a molecular weight of 15,000. The amino acid sequence of this protein has been elucidated (Timkovich *et al.*, 1976; Ambler *et al.*, 1981), and recently the gene encoding this protein (*cycA*) was isolated and sequenced (Van Spanning *et al.*, 1990; Raitio *et al.*, 1990). This cytochrome shows similarity with cytochrome *c* from mitochondria and with the so-called cytochrome c_2 found in the purple nonsulfur bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* (Timkovich *et al.*, 1976). In mitochondria and the *Rhodobacter* strains this cytochrome facilitates electron flow between the bc_1 complex and the cytochrome aa_3 oxidase (Armitage *et al.*, 1985; Baccarini-Melandri *et al.*, 1978; Gennis *et al.*, 1982). Recently it could be demonstrated that the cytochrome c_{550} in *P. denitrificans* may fulfill a similar function (Van Spanning *et al.*, 1990). There are, however, indications that the membrane-bound cytochrome *c* with a molecular weight of 22,000, cytochrome c_{552}, facilitates electron transport between complex III and complex IV also (Berry and Trumpower, 1985; Kuo *et al.*, 1985; Bosma *et al.*, 1987a,b). This cytochrome was isolated as part of a quinol oxidase super complex containing the bc_1 complex and cytochrome aa_3 (Berry and Trumpower, 1985; Bosma *et al.*, 1987b). Cytochrome c_{552} was detected under all growth conditions tested, although lower amounts were found in cells grown under oxygen limitation or under anaerobic conditions with nitrate as electron acceptor.

In cells grown under oxygen-limited conditions three additional *c*-type cytochromes were found: a soluble cytochrome *c* with a molecular weight of 45,000 and two membrane-bound cytochromes with molecular weights of 45,000 and 30,000, respectively. These membrane-bound cytochromes were isolated as part of a super complex which contained the bc_1 complex and showed oxidase activity (Bosma, 1989). Since cytochrome aa_3 is not present, it might indicate that this complex is an alternative oxidase and that the *c*-type cytochromes are involved in electron transport to this cytochrome *c* oxidase (see also Stouthamer, this issue).

In cells grown under anaerobic conditions with nitrate as electron acceptor, the above-mentioned *c*-type cytochromes were also detected, except the soluble one of 45 kDa, which was only present in low amounts. In addition, two other heme *c*-containing proteins are present: nitrite reductase, which contains *c*-type and d_1-type hemes (see also Stouthamer, this issue), and a membrane bound *c*-type cytochrome with a molecular weight of 14,000. This cytochrome may have a role in electron transport to nitrite reductase or nitrous oxide reductase (Bosma, 1989), but it is also possible that it is a subunit of nitric oxide reductase. This enzyme was isolated from *Pseudomonas stutzeri* and in this organism it consisted of two subunits with

molecular weights of 38,000 and 17,000 containing a *b*-type and a *c*-type heme, respectively (Heiss *et al.*, 1989).

In *P. denitrificans* cells grown on methanol, methylamine, or choline, three periplasmic located *c*-type cytochromes could be found (Husain and Davidson, 1986; Bosma *et al.*, 1987a; Bosma, 1989). Two of these, cytochrome c_{551i} and cytochrome c_{553i} with molecular weights of 22,000 and 30,000, are induced only in cells grown on C_1 compounds. The genes encoding cytochrome c_{551i} and c_{553i} have recently been cloned and sequenced (Van Spanning, unpublished results; Ras, unpublished results). The gene encoding cytochrome c_{551i} (*moxG*) is part of the mox operon and its sequence shows homology with the gene encoding cytochrome c_L of *M. extorquens* AM1. The latter cytochrome was found to be the primary electron acceptor of MDH (O'Keeffe and Anthony, 1980; Beardmore-Gray *et al.*, 1983; Nunn and Lidström, 1986b) and recently this was also proven for *P. denitrificans* cytochrome c_{551i} (Van Spanning, unpublished results). The molecular weight of this cytochrome was recalculated on the basis of the deduced amino acid sequence and was found to be 17,700. The gene encoding cytochrome c_{553i} (*cycB*) shows that this cytochrome was synthesized as a pro-cytochrome, since the gene product contains, just as was found for the genes encoding cytochrome c_{550} and cytochrome c_{551i}, a signal sequence necessary to accomplish transport over the cytoplasmic membrane. Cytochrome c_{553i} is able to bind carbon monoxide (Husain and Davidson, 1986).

In all methylotrophs tested so far, at least two *c*-type cytochromes have been detected in the periplasm, cytochrome c_L and cytochrome c_H. Cytochrome c_H corresponds to the typical soluble *c*-type cytochrome found in mitochrondria and in many bacteria. In *P. denitrificans* this cytochrome corresponds to cytochrome c_{550}. Cytochrome c_L belongs to a novel class of *c*-type cytochromes. It is relatively large, it reacts with carbon monoxide, it is rapidly autoreduced at high pH, its redox potential respond to changes in pH, and it contains an unusual environment for the heme (Nunn and Anthony, 1988). The large size and the unusual environment was also observed in cytochrome c_{551i} of *P. denitrificans*. However, this cytochrome does not react with CO and is not autooxidizable (Husain and Davidson, 1986). A cytochrome with characteristics of the *P. denitrificans* cytochrome c_{553} was not reported in other methylotrophic bacteria. However, in a mutant of *M. extorquens* AM1, which was unable to grow on methanol (*moxD* mutant), high amounts of a cytochrome c_{553}, which was hardly detectable in the wild type strain, were found (Day *et al.*, 1990). This cytochrome was able to react with CO and was not auto-oxidizable. Its function is still unknown, but it is able to accept electrons from cytochrome c_L and to donate electrons to cytochrome c_H. In size this cytochrome is smaller than the cytochrome c_{553i} from *P. denitrificans*.

In membranes of cells grown on C_1 compounds five membrane bound *c*-type cytochromes could be found. Cytochrome c_1 and cytochrome c_{552} are present in similar amounts compared to the amounts found in heterotrophically grown cells. The 14,000, 30,000 and 45,000 *c*-type cytochromes were detected in lower amounts during C_1 growth than found in cells grown under anaerobic conditions in the presence of nitrate or under oxygen limitation. Since these *c*-type cytochromes might have a role in electron transport to an alternative cytochrome *c* oxidase or to reductases, it is well conceivable that these oxido-reductases are present under C_1 conditions as well. It has been found that cytochrome aa_3 is the main oxidase in methanol grown cells (Van Verseveld and Stouthamer, 1978a,b; Van Verseveld *et al.*, 1979, 1981). In addition to both cytochrome *c* oxidases, a quinol oxidase must be present in *P. denitrificans* also, since a mutant lacking the bc_1 complex is still able to grow on mannitol or succinate with specific growth rates comparable to the wild type (Van Spanning, unpublished results). Since both cytochrome *c* oxidases are present during growth on C_1 compounds, it is conceivable that this quinol oxidase plays a minor role under these conditions. An interesting observation in this respect is that the maximum specific growth rate of the cytochrome c_1 mutant on methylamine or choline shows a decrease of 30–40% compared to the wild type. This might indicate a shortage in capacity of electron flow to the quinol oxidase under C_1 conditions, which can be explained by assuming that a lower amount of this oxidase is present. Regulation of the various oxidases in *P. denitrificans* is largely unknown. Since growth on C_1 compounds seemed to influence synthesis of all oxidases, this growth condition might be interesting for studying this regulation.

Electron Transport

Information about electron transport routes during methylotrophic growth in *P. denitrificans* has been studied by determining molar growth yields, specific growth rates, and H^+/O ratios from wild type and different mutants grown under a variety of growth conditions (Van Verseveld 1978a,b; Van Verseveld *et al.*, 1979; Bosma, 1989; Van Spanning *et al.*, 1990), or by measuring electron transfer rates in spheroplasts (Davidson and Kumar, 1989) and between different purified electron carriers (Husain and Davidson, 1985, 1986; Gray *et al.*, 1988). The exact pathways, however, are not yet fully understood. In Fig. 3 our current knowledge about the electron pathway from MDH and MADH to the terminal oxidases is shown.

It is generally accepted that cytochrome c_L is the primary electron acceptor of MDH. Since this cytochrome is largely similar to *P. denitrificans* cytochrome c_{551i}, the latter *c*-type cytochrome was thought to be the electron acceptor for MDH in this organism. Electron transfer from MDH to this

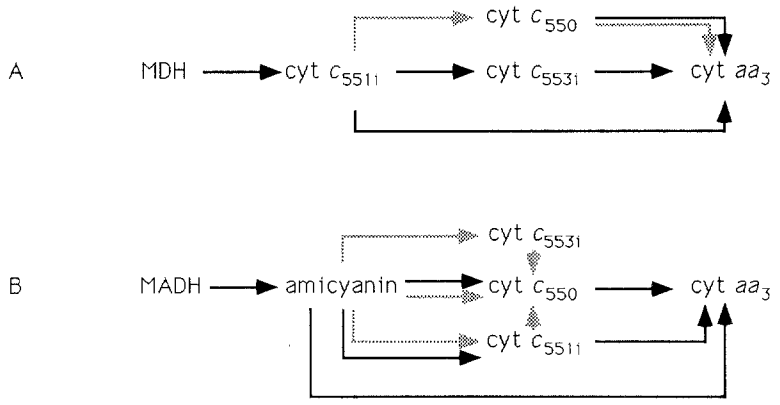

Fig. 3. Scheme of the electron flow from MDH (A) and MADH (B). Solid arrows denote reactions suggested by *in vivo* experiments. Dotted arrows denote reactions suggested by *in vitro* experiments. MDH, methanol dehydrogenase; MADH, methylamine dehydrogenase.

cytochrome could not be demonstrated *in vitro* (Davidson and Kumar, 1989), but as already pointed out, this reaction lacked the correct activator. A mutant in the gene encoding this cytochrome (*moxG* mutant) was found to be unable to grow on methanol, which favors the hypothesized function of the cytochrome (Van Spanning, unpublished results). This observation discounts the role for cytochrome c_{553i} as the primary electron acceptor for MDH, as was suggested by Davidson and Kumar (1989). However, a mutant in the gene encoding this cytochrome (*cycB* mutant) showed a decrease in the specific growth rate on methanol. This might indicate that cytochrome c_{553i} is somehow involved in electron transport from MDH to the terminal oxidase. Possibly this cytochrome acts as an electron acceptor of cytochrome c_{551i} and donates its electrons via cytochrome c_{550} to the terminal oxidase. The latter cytochrome was found to mediate electron transfer *in vitro* from cytochrome c_{551i} and c_{553i} to the cytoplasmic membrane (Davidson and Kumar, 1989). However, a mutant in the gene encoding cytochrome c_{550} (*cycA* mutant) showed unimpaired growth rates on methanol (Van Spanning, unpublished results), which indicates that other routes must exist.

Electrons originating from MADH are donated to amicyanin and then further to a *c*-type cytochrome. Cytochrome c_{550}, cytochrome c_{551i}, as well as cytochrome c_{553i} were able to accept electrons from amicyanin, but cytochrome c_{551i} was the most efficient electron acceptor of the copper protein (Husain and Davidson, 1986). Although rapid amicyanin-mediated transfer from MADH to cytochrome c_{551i} was observed, reduced amicyanin did not reduce oxidized cytochrome c_{551i} in the absence of MADH, which was consistent with the redox properties of these two electron carriers (Gray *et al.*, 1986). Gray *et al.* (1988) demonstrated that MADH and amicyanin could

form a complex in which the midpoint potential of the copper protein was decreased, making electron transfer from this compound to cytochrome c_{551i} possible. These data, therefore, suggests that cytochrome c_{551i} is the natural electron acceptor for amicyanin. However, the *moxG* mutant that lacks this cytochrome showed almost unimpaired growth on methylamine (Van Spanning, unpublished results), which indicates that electrons from MADH do not follow predominantly an electron route via cytochrome c_{551i}. Whether the electrons from amicyanin are donated to cytochrome c_{553i} or cytochrome c_{550} *in vivo* is not known. However, since growth of the *cycB* mutant on methylamine was not different from that of the wild type, while the *cycA* mutant showed a 40% decrease in the specific growth rate on this substrate (Van Spanning *et al.*, 1990), it seems that electron transport during growth on methylamine predominantly follows the pathway via cytochrome c_{550}.

These data indicate that electrons from the dehydrogenases MDH and MADH are not donated to the terminal oxidases via fixed electron routes. It seems that the organism is very flexible and as soon as a *c*-type cytochrome is knocked out by a mutation, another cytochrome *c* is able to replace it. Although one can predict electron routes on the basis of *in vitro* electron transfer rates, one should be careful with conclusions since *in vivo* experiments with specific mutants indicate that other routes are possible as well.

Genetics of *P. denitrificans*

General

Although *P. denitrificans* has already been recognized and studied for 80 years (Beyerinck and Minkman, 1910), it is only during the last 5 years that genetic techniques have become available for this organism. Up till then little was known about the genetics of *P. denitrificans*. The molecular weight of the genome had been reported to be about 3×10^9, and G + C content of the DNA about 66% (Nokhal and Schlegel, 1983). A large plasmid with a molecular weight of more than 300×10^6 was detected (Gerstenberg *et al.*, 1982). A streptomycin-resistant and a leucine–cysteine double auxotrophic mutant had been reported (Paraskeva, 1979). And two reports had appeared on the introduction of DNA into the organism, but the frequencies of transfer were too low to be useful for genetic manipulation (Paraskeva, 1979; Spence and Barr, 1981). Since several groups started to study *P. denitrificans* by using a genetic approach, the organism has now become genetically accessible. Several genes have been isolated and sequenced thus far: the *mox*FJGI genes (Harms *et al.*, 1987, 1989; Van Spanning *et al.*, unpublished results), the genes encoding the bc_1 complex (Kurowski and Ludwig, 1987),

the genes encoding subunits of cytochrome aa_3 with additional open reading frames (Raitio *et al.*, 1987, 1990; Steinrücke *et al.*, 1987), and the genes encoding cytochrome c_{550} (Van Spanning *et al.*, 1990; Riatio *et al.*, 1990) and cytochrome c_{553} (Ras, unpublished results). A common feature of these genes is the high G + C content (around 65%), which is similar to the G + C content of the genome (Nokhal and Schlegel, 1983), and a strong preference (around 90%) for a G or a C at the third position in the codons observed.

Host/Vector Systems

A prerequisite for performing genetic manipulations is a suitable host vector system. An important requirement for the host is the ability to efficiently accept DNA and to stably maintain this DNA in the cell. The vector that is being used for introducing DNA into the cell must contain the capability to replicate and to stably maintain itself in the host. Furthermore the vector needs a suitable selection marker and unique sequences for restriction endonucleases.

P. denitrificans wild type is not a suitable host since plasmid conjugation from *E coli* into *P. denitrificans* occurs invariably at low frequencies (Paraskeva, 1979; De Vries *et al.*, 1989). Low conjugation frequencies and plasmid instability have been frequently reported and were thought to be caused by plasmid-encoded functions (Meyer *et al.*, 1982; Schmidhauser and Helinski, 1985). However, the results obtained with *P. denitrificans* point to host-encoded functions since a variety of both RP4 (IncP1) and pRSF1010 (IncQ) derivatives are conjugated at low frequencies toward this organism (De Vries *et al.*, 1989). A host-specific restriction mechanism operating in *P. denitrificans* is one possible explanation. An interesting observation in this respect was the fact that DNA isolated from this organism was found to be resistant to restriction by the restriction endonucleases *Bam*HI, *Bgl*II, and *Dpn*I and partly resistant to restriction by the enzymes *Sau*3A and *Mbo*I. Knowing the characteristics of inhibition by methylation of these restriction enzymes, it was postulated that a less common nGATCn-dependent modification may occur in *P. denitrificans*. A possible candidate in this respect is N^4-methylcytosine (De Vries *et al.*, 1989).

To overcome the problem of DNA transfer, mutants with enhanced conjugation frequencies for IncP1 and IncQ plasmids have been isolated (De Vries *et al.*, 1989). Some of these mutants were also mutated in their ability to modify its DNA, which indicates that *P. denitrificans* possesses at least one potent host-dependent restriction/modification system which affects conjugation. In addition to the class of restriction-defective mutants, at least one other class of enhanced transfer mutants with unknown defect(s) has been isolated. The mutants have been shown to be well suited for experiments

like complementation analysis (Harms, 1988, Harms *et al.*, 1989), analysis of promoter regions (Harms *et al.*, 1989; this laboratory, unpublished results), transposon mutagenesis (this laboratory, unpublished results), and gene replacement by homologous recombination (Haltia *et al.*, 1989, Van Spanning *et al.*, 1990; Raitio *et al.*, 1990; Gerhus *et al.*, 1990).

With mutants described above, it is not a problem anymore to introduce DNA into *P. denitrificans*. However, special attention should be given to the choice of the cloning vector. Broad host-range plasmids that are generally used in other organisms have been shown to be well suited for gene cloning in *P. denitrificans* as well. These vectors are derived from either RP4 (incompatibility group P1) (Ditta *et al.*, 1980) or RSF1010 (incompatibility group Q) (Barth and Grinter, 1974) and a large amount of derivatives like cosmid vectors, expression vectors, and promoter probe vectors have been constructed. Unfortunately the *tac* promoter or the p_L promoter used in the expression vectors have been shown to be not functional in *P. denitrificans* (this laboratory, unpublished results). On the contrary, the promoter probe vector has been used with success to investigate the promoter of the *mox* operon (Harms *et al.*, 1989; Harms, unpublished results). A disadvantage is the limited amount of antibiotic-resistant genes that can be expressed in this organism. Kanamycin, streptomycin, and spectinomycin resistance markers could be well expressed, but the ampicilline and chloramphenicol markers could not be expressed, while the tetracycline marker could only be used in the range 0.1–0.3 μg per ml (De Vries *et al.*, 1989). This limitation of usable markers severely narrows down the range of available broad host-range plasmids that can be used.

Mutation Induction

Studies on metabolic pathways and metabolic regulation are often performed by using mutants. Mutants might be selected after spontaneous mutation events or after generation of mutations by treatment with mutagens. Chemical mutagens as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and ethyl methane sulfonate (EMS) have been used to isolate mutants with defects in C_1 metabolism and with lesions in the electron carriers cytochrome *c* and cytochrome aa_3 (Harms *et al.*, 1985; Willison and John, 1979; Van Verseveld *et al.*, 1981; Willison *et al.*, 1981). Treatment of *P. denitrificans* with sodium nitrate, hydroxylamine, sodium bisulfite, or UV irradiation did not result in detectable enhancement of mutation induction (Harms *et al.*, 1985). Inherent disadvantage of conventional mutagenesis procedures are the variable stability, possible occurrence of multiple lesions, and the absolute requirement for complementation analysis to classify the obtained mutants genetically. Transposon insertion is a powerful alternative

method for the isolation of stable mutants. It is possible to isolate Tn5-induced mutations in *P. denitrificans* at frequencies around 10^{-4} in plate conjugation experiments (this laboratory, unpublished results) by using commonly used suicide vectors.

A disadvantage of all the above techniques is the poor specificity of the mutation event. A more sophisticated method in this respect is introduction of a mutation by site-directed mutagenesis. A gene of interest is mutated *in vitro* by insertion of an antibiotic resistance gene. This mutated gene is cloned on a suicide vector and the construct is transferred to *P. denitrificans*. By selection for the antibiotic resistance, marker mutants are selected that have received this resistance by means of homologous recombination. The wild type gene has been exchanged by the mutated gene. Several genes (*cycA*, *moxG*, *cycB*, and *fbcC*) encoding *c*-type cytochromes as well as genes encoding subunits of the cytochrome aa_3 oxidase have been mutated by using this strategy (Van Spanning *et al.*, 1989, 1990; Ras *et al.*, unpublished results; Gerhus *et al.*, 1990; Haltia *et al.*, 1989; Raitio *et al.*, 1990).

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