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Isolation, Sequencing, and Mutagenesis of the Gene Encoding NAD- and Glutathione-Dependent Formaldehyde Dehydrogenase (GD-FALDH) from *Paracoccus denitrificans*, in Which GD-FALDH Is Essential for Methylotrophic Growth

JOYCE RAS,[1] PETER W. VAN OPHEM,[2] WILLEM N. M. REIJNDERS,[1] ROB J. M. VAN SPANNING,[1] JOHANNIS A. DUINE,[2] ADRIAAN H. STOUTHAMER,[1] AND NELLIE HARMS[1]*

Department of Microbiology, Biological Laboratory, Vrije Universiteit, 1081 HV Amsterdam,[1] and Department of Microbiology and Enzymology, Delft University of Technology, 2628 BC Delft,[2] The Netherlands

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NAD- and glutathione-dependent formaldehyde dehydrogenase (GD-FALDH) of *Paracoccus denitrificans* has been purified as a tetramer with a relative molecular mass of 150 kDa. The gene encoding GD-FALDH (*flhA*) has been isolated, sequenced, and mutated by insertion of a kanamycin resistance gene. The mutant strain is not able to grow on methanol, methylamine, or choline, while heterotrophic growth is not influenced by the mutation. This finding indicates that GD-FALDH of *P. denitrificans* is essential for the oxidation of formaldehyde produced during methylotrophic growth.

Paracoccus denitrificans is a gram-negative, aerobic soil bacterium which is able to grow on methanol, methylamine, and choline. The oxidation of methanol and methylamine to formaldehyde is catalyzed by the periplasmically located enzymes methanol dehydrogenase and methylamine dehydrogenase, respectively. During growth on methylamine, formaldehyde is transported to the cytoplasm by a transport mechanism in which a transport protein is involved (16). During the oxidation of choline to glycine, several molecules of formaldehyde are produced. In the presence of formaldehyde and reduced glutathione, the compound *S*-hydroxymethylglutathione is nonenzymatically formed. NAD- and glutathione-dependent formaldehyde dehydrogenase (GD-FALDH) oxidizes *S*-hydroxymethylglutathione to *S*-formylglutathione (14, 19, 20, 24, 27), which is oxidized further via formate to carbon dioxide. In this report, we describe the purification of GD-FALDH of *P. denitrificans* and the isolation and mutagenesis of the gene encoding this enzyme. From growth characteristics of the mutant strain, it can be concluded that GD-FALDH of *P. denitrificans* is essential for methylotrophic growth.

Purification and biochemical analysis of GD-FALDH. GD-FALDH was purified approximately 50-fold from methylamine-grown *P. denitrificans* cells, as shown in Table 1. By this method, 65 g (wet weight) of cells was harvested and washed with 50 mM Tris hydrochloride (pH 7.5). The cells were disrupted with a French pressure cell, yielding the cell extract. Enzyme activity was measured routinely by determining the rate of NADH formation at 340 nm at room temperature. The assay mixture contained (final concentrations) 0.1 M $Na_4P_2O_7$-HCl (pH 9.0), 2 mM (reduced) glutathione, and 2.5 mM NAD. After 30 s of incubation with enzyme solution, the reaction was started by adding formaldehyde to a final concentration of 5 mM. Activities were calculated by using a molar absorption coefficient for NADH at 340 nm of 6,220 M^{-1} cm^{-1} (4). $(NH_4)SO_4$ was added to the cell extract. The enzyme precipitated between 25 and 60% saturation. The precipitate was dissolved in 10 mM potassium phosphate buffer (KPB; pH 7.0) and applied to a Phenyl-Sepharose HP column (12.4 by 2.6 cm) equilibrated with 1.5 M $(NH_4)SO_4$ in 10 mM KPB (pH 7.0). After washing of the column with the same buffer, elution occurred with a gradient of 1.5 to 0 M $(NH_4)SO_4$ in 10 mM KPB in 3 h at a flow rate 3 ml/min. GD-FALDH eluted when the $(NH_4)SO_4$ concentration was almost 0 M. The active fractions were pooled and concentrated by using an Amicon membrane filter with a cutoff of 30 kDa and subsequently dialyzed for 16 h against 1 liter of 20 mM KPB (pH 7.2). The preparation was applied to a Mono Q 10/10 column equilibrated with 20 mM KPB (pH 7.2). Elution was carried out with a gradient of 0.2 to 0.5 M KCl in 20 mM KPB (pH 7.2) in 30 min at a flow rate of 2 ml/min. The enzyme eluted at 0.3 M KCl. After pooling and concentration, GD-FALDH was adsorbed to a 5′-AMP–Sepharose 4B (Pharmacia) column (5.0 by 2.2 cm) equilibrated with 0.1 M KPB (pH 7.0). Elution occurred with a gradient of 0 to 4 mM NAD in 0.1 M KPB (pH 7.0) in 1 h at a flow rate 1 ml/min. Polyacrylamide gel electrophoresis (PAGE) under denaturating conditions using Pharmacia Phast system equipment showed always only one protein band. Therefore, the enzyme preparation is most likely homogeneous. Native PAGE showed sometimes several protein bands, possibly as a result of aggregation. Gel filtration on Superdex-200 indicated a relative molecular mass of 150 kDa, while PAGE under denaturating conditions revealed a protein with a relative molecular mass of 40 kDa. This finding indicates that GD-FALDH of *P. denitrificans* is a tetramer. The purified GD-FALDHs of acetate-grown *Escherichia coli* (8), formaldehyde-grown *Pseudomonas putida* F61 (13), several yeasts (13, 24), and mammals (11, 28) also consist of subunits with a molecular mass of 40 kDa, but these enzymes are dimers. However, recently the glutathione-independent FALDH of *Pseudomonas putida* has been isolated as a tetramer with subunits with a molecular mass of 42 kDa (9). The N-terminal amino acid sequence of the purified GD-FALDH of *P. denitrificans* was determined by Edman degradation with a model

* Corresponding author. Mailing address: Department of Microbiology, Biological Laboratory, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands. Fax: 31.20.4447123. Electronic mail address: harms@bio.vu.nl.

TABLE 1. Purification of GD-FALDH of *P. denitrificans*

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)[a]	Recovery (%)	Purification factor
Cell extract	4,100	3,360	0.82	100	1.0
$(NH_4)_2SO_4$ precipitation	3,300	3,100	0.94	92	1.1
Phenyl-Sepharose	1,090	1,420	1.3	42	1.6
Mono Q	210	1,095	5.2	33	6.3
5′-AMP–Sepharose	18	738	41.0	22	50.0

[a] One unit = 1 μmol of NADH converted min^{-1}.

473A gas-phase amino acid Sequenator (Applied Biosystems, Foster City, Calif.).

Recently, Van Ophem and Duine (29) reported on the enzyme activity of the partly purified GD-FALDH of *P. denitrificans*. From these data, it was clear that the enzyme 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 glutathione, the oxidation of formaldehyde (8, 11, 28, 29, 33).

Cloning and sequencing of the gene encoding GD-FALDH. Recently, we described the cloning and mutagenesis of a gene, *cycB*, encoding cytochrome c_{553i} (21). The mutant Pd6121, containing a kanamycin resistance gene in *cycB*, was used to clone DNA upstream of *cycB*. Chromosomal DNA of the mutant was isolated as described previously (2), digested with *Sal*I, and cloned in the *Sal*I site of pUC18. Kanamycin-resistant colonies were isolated, and plasmid DNA isolated from these clones was shown to contain a chromosomal fragment of 7 kb with 0.8 kb upstream of *cycB*. By using the same method, we isolated an *Eco*RV fragment that contains an additional 6.4 kb upstream of the *cycB* gene. This fragment was subcloned, and the nucleotide sequence of an 1.25-kb fragment, located 5.4 kb upstream of the *cycB* gene, was determined by using the dideoxy-chain termination method of Sanger et al. (23) combined with the M13 cloning system. A Sequenator (Applied Biosystems) was used to determine the sequences. Computer analysis of the sequences was done with the DNA-Strider software. A restriction map of the *Eco*RV fragment and the sequencing strategy are shown in Fig. 1.

The sequence reveals an open reading frame encoding a protein of 376 amino acids and a calculated molecular weight of 39,890. This putative protein starts with a stretch of 18 amino acids that is identical to the 18 N-terminal amino acids sequenced from the purified GD-FALDH protein. This result clearly demonstrates that the gene (*flhA*) encoding GD-FALDH has been cloned. A putative Shine-Dalgarno sequence (GAAGGAGAAGA), indicating a ribosome-binding site, was present 4 bp upstream from the initiation codon. The codon usage of the gene is typical for *P. denitrificans* genes. The G+C content of the *flhA* gene is 64.9%, and there is a 92.1% preference for a G or C at the third position in the codons.

For homology studies on amino acid sequences, the international protein and DNA data banks were screened on-line by using GenBank (1, 7). GD-FALDH (EC 1.2.1.1) is widely distributed in plants, yeasts, mammals, and bacteria. Recently, it was established that GD-FALDH isolated from a number of mammals is identical to group I subclass III of NAD(P)-dependent alcohol dehydrogenases (ADHs) (6, 11, 12, 15, 22, 25). The primary structure of GD-FALDH of *P. denitrificans* is 36% identical to the deduced amino acid sequences of GD-FALDH genes of plants and yeasts, and 60% identical to mammalian GD-FALDHs. From the enzyme isolated from *E. coli*, 47 N-terminal amino acids were sequenced (8); of these, 47% are identical to the N terminus of *P. denitrificans* GD-FALDH. Recently, the gene encoding the glutathione-independent FALDH (*fdhA*) isolated from *Pseudomonas putida* was cloned (9); during the revision of this report, the gene coding for FALDH from *Methylobacter marinus* A45 was published (26). The deduced amino acid sequences of these genes are 14 and 19%, respectively, identical to the *P. denitrificans* GD-FALDH sequence. In Fig. 2, the deduced amino acid sequences of the GD-FALDHs of human liver and *P. denitrificans* are aligned with the FALDHs of *Pseudomonas putida* and *M. marinus* A45. Several residues are conserved in exactly

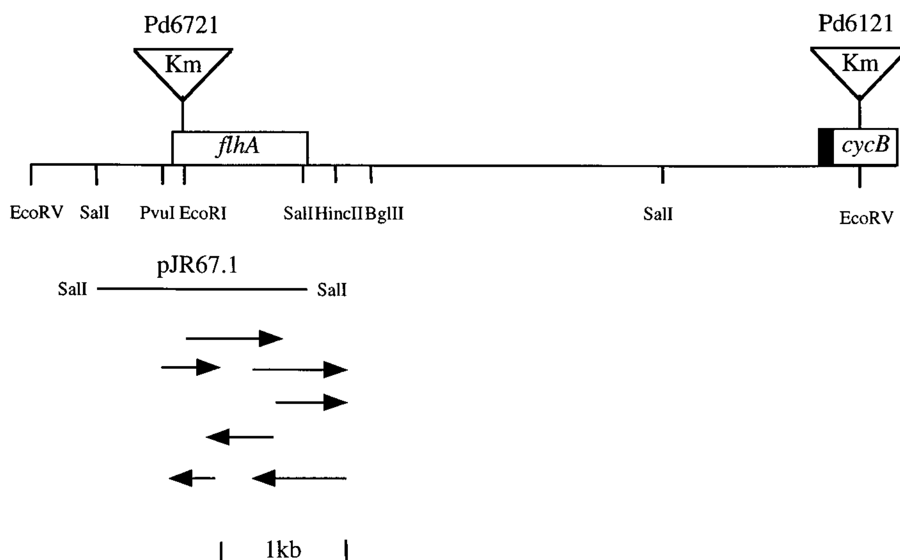

FIG. 1. Physical map and strategy for sequencing the *P. denitrificans* chromosomal fragment containing *cycB*, the gene encoding cytochrome c_{553i} (21), and *flhA*, the gene encoding GD-FALDH. Open bars indicate open reading frames, and the signal sequence of *cycB* is shown by a black box. Triangles indicate positions of insertion of a kanamycin resistance gene.

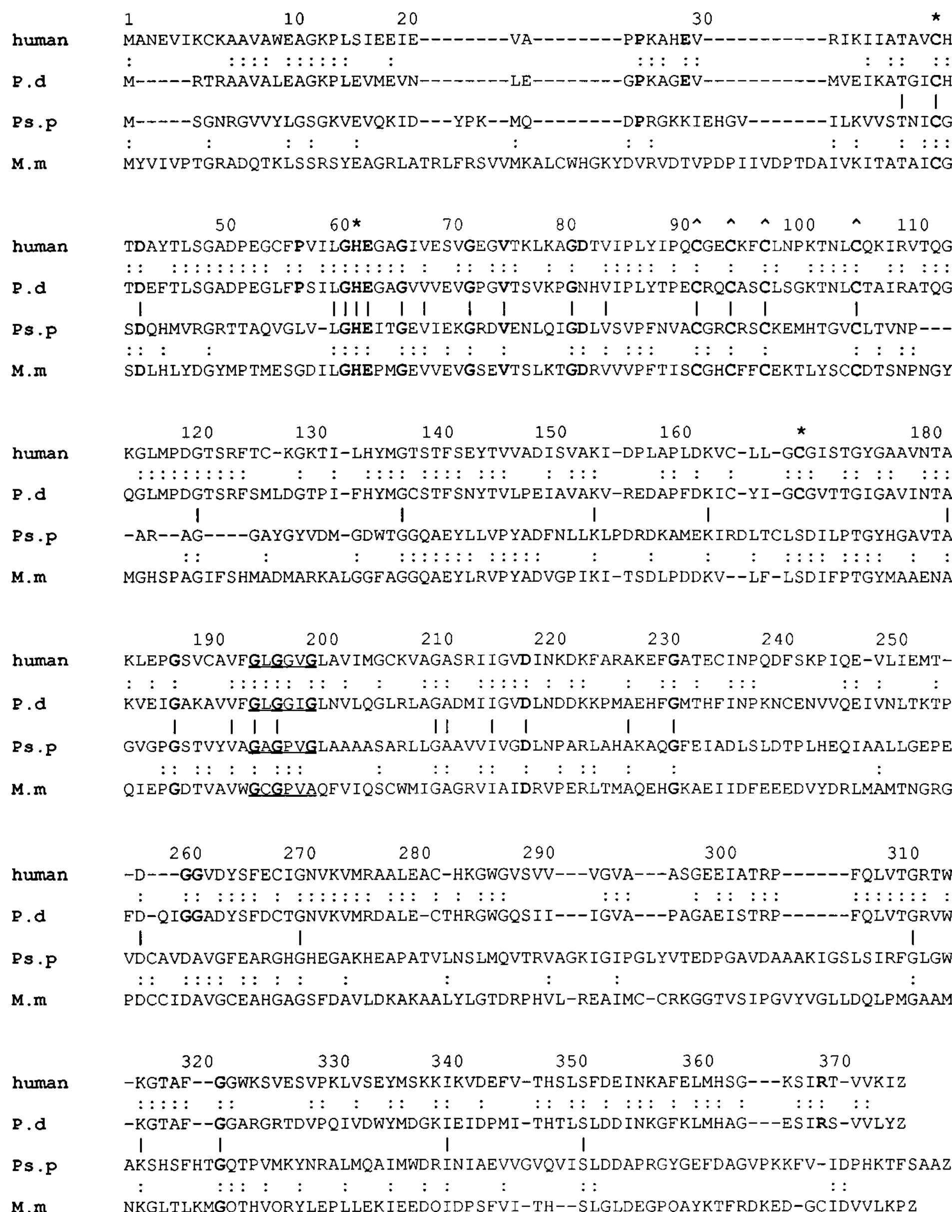

FIG. 2. Comparison of the primary structures of GD-FALDH of *P. denitrificans* (P.d) and human liver (human) (25) with the deduced amino acid sequences of FALDH genes of *Pseudomonas putida* (Ps.p) (9) and *M. marinus* A45 (M.m) (26). The conserved residues in the GD-FALDHs and FALDHs are indicated (:). The conserved residues present in all four sequences are indicted with |. The amino acids indicated in boldface are the residues conserved in group I ADHs (10). The ligands to the zinc atoms are indicated. Distinguished are the catalytic ligands (*) and the ligands to the second zinc atom (^). The conserved domain involved in NAD binding is underlined.

the same spacing as has been found in the primary structure of group I ADHs of different organisms (10). These amino acids were proven to be involved in binding the zinc atoms and the coenzyme. Cys-40, His-62, and Cys-170 are the ligands to the active zinc atom, and Cys-92, Cys-95, Cys-98, and Cys-106 are the ligands to the second zinc atom. For the purified *E. coli* enzyme, a zinc content of four atoms per dimer was determined (8). 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 involved in NAD binding (33). Besides the ligands to NAD and the zinc atoms, mammalian and plant GD-FALDHs have another 18 residues in common with group I ADHs. In (GD-)FALDHs of *P. denitrificans*, *Pseudomonas putida*, and *M. marinus* A45, 17, 12, and 10, respectively, of these conserved residues are present. The function of these amino acids is not known. It is clear from Fig. 2 that the homology of the *P. denitrificans* enzyme with the two FALDHs is low and found mainly around residues involved in zinc binding and around the NAD-binding site. This finding suggests that GD-FALDH and glutathione-independent FALDHs belong to the zinc-containing long-chain ADHs of group I, but probably to different subclasses within this group.

Construction of an *flhA* mutant. A 1.6-kb *Sal*I fragment harboring almost the complete *flhA* gene was inserted in the *Sal*I site of pUC19E, resulting in pJR67.1. In the *Eco*RI site of

this plasmid, a 1.4-kb *Eco*RI fragment containing a kanamycin resistance cassette of pUC4KISS (32) was inserted, resulting in pJR67.2. The mutated gene was subsequently inserted in the suicide vector pGRPd1 (31) and transferred from *E. coli* to *P. denitrificans* by homologous recombination as described by Van Spanning et al. (30). The mutant strain was designated Pd6721. The correctness of gene replacement was confirmed by Southern analysis of genomic DNA, using positively charged nylon membranes as specified by the manufacturer (Boehringer GmbH, Mannheim, Germany) (results not shown).

Physiological analysis of the *flhA* mutant. For determination of growth characteristics, cells were grown in 2-liter flasks filled with 500 ml of mineral salts medium described by Chang and Morris (3) supplemented with a trace element solution described by Lawford et al. (17) at 30°C; 25 mM succinate, 50 mM ethanol, 100 mM methylamine, 50 mM methanol, and 10 mM choline chloride were used as carbon sources. Growth of the mutant strain Pd6721 on a complex medium or on minimal media with either succinate or ethanol as a carbon and energy source was unimpaired. However, Pd6721 was not able to growth on methanol, methylamine, and choline. The most obvious conclusion from these results is that because of the mutated *flhA* gene, GD-FALDH is absent. The mutant is then unable to oxidize formaldehyde, and consequently no reduction equivalents and ATP are available for carbon dioxide fixation.

GD-FALDH activity in *P. denitrificans* is low in heterotrophically grown cells but increases during growth on methanol, methylamine, and choline, indicating its specific role during methylotrophic growth (18, 29). Besides GD-FALDH, also dye-linked FALDH activity has been demonstrated in *P. denitrificans* (18, 29). The in vitro measured activities were low, and no specific increase of enzyme activity was noted during methylotrophic growth, indicating that this enzyme is not directly involved in C_1 metabolism. It has also been reported that methanol dehydrogenase is able to oxidize formaldehyde in vitro (5). The results obtained here with the GD-FALDH mutant strain indicate, however, that neither dye-linked FALDH nor methanol dehydrogenase is able to restore methylotrophic growth in the mutant strain.

GD-FALDH has been isolated from several mammal liver tissues, *E. coli*, and *Pseudomonas putida*, and it has been suggested that GD-FALDH has a possible role in normal alcohol metabolism and in detoxification of exogenous formaldehyde (8, 9, 28). In the formaldehyde-resistant organism *Pseudomonas putida* F61, GD-FALDH was formed only in response to the addition of formaldehyde (13). In *P. denitrificans*, GD-FALDH activity is induced during C_1 metabolism, and from mutant studies described here, it is clear that GD-FALDH is essential for the oxidation of formaldehyde produced during methylotrophic growth.

GenBank accession number. The nucleotide sequence of the *flhA* gene of *P. denitrificans* has been deposited with GenBank under accession number L36327.

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