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S-Formylglutathione Hydrolase of *Paracoccus denitrificans* Is Homologous to Human Esterase D: a Universal Pathway for Formaldehyde Detoxification?

NELLIE HARMS,* JOYCE RAS, WILLEM N. M. REIJNDERS, ROB J. M. VAN SPANNING, AND ADRIAAN H. STOUTHAMER

Department of Microbial Physiology, Vrije Universiteit, 1081HV Amsterdam, The Netherlands

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Downstream of *flhA*, the *Paracoccus denitrificans* gene encoding glutathione-dependent formaldehyde dehydrogenase, an open reading frame was identified and called *fghA*. The gene product of *fghA* showed appreciable similarity with human esterase D and with the deduced amino acid sequences of open reading frames found in *Escherichia coli*, *Haemophilus influenzae*, and *Saccharomyces cerevisiae*. Mutating *fghA* strongly reduced *S*-formylglutathione hydrolase activity. The mutant was unable to grow on methanol and methylamine, indicating that the enzyme is essential for methylotrophic growth. *S*-Formylglutathione hydrolase appears to be part of a formaldehyde detoxification pathway that is universal in nature.

In *Paracoccus denitrificans*, the oxidation of methanol, methylamine, and choline leads to the transient synthesis of formaldehyde. This poses a special regulation problem, as the concentration of this toxic compound must be kept within the limits imposed by its toxicity on the one hand and the concentration required for rapid further oxidation on the other hand. The oxidation of methanol and methylamine is catalyzed by the periplasmic quinoproteins methanol dehydrogenase and methylamine dehydrogenase, respectively (11). Formaldehyde formed during these reactions is transported to the cytoplasm by a protein-mediated mechanism (14). Choline is oxidized in several steps to glycine. In this process, formaldehyde is released in the cytoplasm. In the cytoplasm, formaldehyde is coupled to reduced glutathione (GSH) to yield *S*-hydroxymethyl-glutathione. The latter compound is oxidized by NAD-dependent formaldehyde dehydrogenase (GD-FALDH) to *S*-formylglutathione (27). The *P. denitrificans* gene (*flhA*) encoding this enzyme has been isolated and sequenced (21). An *flhA* mutant was unable to grow on methanol, methylamine, or choline, indicating that GD-FALDH is essential for methylotrophic growth of *P. denitrificans*. *S*-formylglutathione is hydrolyzed to formate and GSH by *S*-formylglutathione hydrolase (FGH). This enzyme has not yet been isolated from *P. denitrificans*, but FGH activity has been demonstrated in human tissues (26) and in the methylotrophic yeasts *Candida boidinii* (20) and *Kloeckera* sp. strain No2201 (13). FGH isolated from human liver and *Kloeckera* sp. strain No2201 is a homodimer with a molecular mass of 58 kDa. The *C. boidinii* FGH is a heterodimer with subunits of 35 and 25 kDa. In these organisms, genes encoding FGH have not been identified. Studies on the polymorphism of FGH in human erythrocytes revealed that the enzyme is identical to human esterase D (7). Human esterase D is a member of a group of nonspecific esterases. The native enzyme has a molecular weight of 70,000 and consists of two identical subunits (16). 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 (15).

The capacity to detoxify formaldehyde is an important feature for every organism. Both GD-FALDH and FGH are part of a GSH-dependent formaldehyde oxidation pathway. Since little was known about FGH, we decided to study this enzyme and its gene in a methylotrophic bacterium. We here report on the isolation and characterization of a procaryotic gene encoding FGH and show that in *P. denitrificans* FGH is essential for growth on methanol and methylamine. Part of these data have been presented at the 8th International Symposium on Microbial Growth on C_1 Compounds (10).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used are listed in Table 1. *P. denitrificans* and *Escherichia coli* were routinely grown aerobically either on brain heart infusion broth (GIBCO, Life Technologies LTD, Paisley, United Kingdom) or on mineral salt medium at 35°C (4). Carbon sources and their concentrations were alternatively 25 mM methanol, 50 mM methylamine, 25 mM succinate, and 15 mM choline chloride. In minimal medium plates, final concentrations of 50 mM methylamine, 50 mM methanol, 15 mM choline chloride, and 25 mM succinate were used. Antibiotics, when added, were used at final concentrations of 40 μg of rifampin per ml, 25 μg of kanamycin per ml, 25 μg of streptomycin per ml, and 100 μg of ampicillin per ml.

DNA manipulations and analyses. DNA was manipulated essentially as described previously (2). Southern hybridizations employed positively charged nylon membranes as specified by the manufacturer (Boehringer GmbH, Mannheim, Germany). The nucleotide sequence was determined by using the dideoxy chain termination method of Sanger et al. (23) combined with the M13 cloning system, with an Automatic Sequenator (Applied Biosystems, Foster City, Calif.). Analysis of the sequences used DNA-Strider software. For homology studies on amino acid sequences, the international protein and DNA data banks were screened on-line by using GenBank (1, 9). The wild-type genes on the chromosome were replaced by the mutated ones by homologous recombination as described previously (28).

Enzyme assay. The FGH activity was measured by monitoring the rate of hydrolysis of 4-methylumbelliferyl acetate on a Titertek Fluoroskan fluorometer with an excitation filter of 355 nm and an emission filter of 480 nm. One milliliter of culture of a mid-exponential-phase-grown culture was spun down and resuspended in the assay buffer (60 mM K_2HPO4, 40 mM KH_2PO4, 100 mM NaCl). In a microtiter plate, a twofold dilution series of this suspension was made in the assay buffer. The reaction was started by the addition of 12 mM 4-methylumbelliferyl acetate, and the reaction was monitored for 35 min. A twofold dilution series of 4-methylumbelliferon was used as a calibration curve. All measurements were corrected for nonenzymatic hydrolysis of the substrate. The enzyme activities were expressed as the amount of 4-methylumbelliferon formed per minute per milligram of protein. Protein determinations were performed by a modified Lowry method with bovine serum albumin as a standard (17).

* Corresponding author. Mailing address: Department of Microbial Physiology, Vrije Universiteit, De Boelelaan 1087, 1081HV Amsterdam, The Netherlands. Phone: 31-204447179. Fax: 31-204447229. Electronic mail address: harms@bio.vu.nl.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Bacterial strains		
P. denitrificans		
Pd1222	Rif^r Spec^r; enhanced conjugation frequencies	5
Pd6422	Pd1222; *clpP*::pRTd6422	This study
Pd6521	Pd1222; *orf3*::Km^r	This study
Pd6621	Pd1222; *fghA*::Km^r	This study
E. coli S17-1	Sm^r *pro* r$^-$ m$^+$ RP4-2 integrated (Tc::Mu) (Km::Tn*7*)	24
Plasmids		
pUC4K	Km^r (Tn*903*)	29
pGRPd1	*oriV* (ColE1) Amp^r *oriT* Sm^r	28
pRTd6422	pGRPd1 derivative; *clpP′*	This study

Nucleotide sequence accession number. The nucleotide sequences have been deposited with GenBank under accession number U34346.

RESULTS

Isolation and analysis of the *fghA* gene. Recently, the gene *flhA*, encoding GD-FALDH, of *P. denitrificans* has been isolated and sequenced (21). Sequence analysis of 2 kbp of DNA downstream of *flhA* revealed three open reading frames (to be designated *clpP*, *orf3*, and *fghA*) (Fig. 1). These open reading frames have a codon usage that is typical for *P. denitrificans* genes, i.e., a G+C content of around 60% and a preference for a G or a C at position 3 in the codons of around 80%. The deduced amino acid sequence of the open reading frame immediately downstream of *flhA* showed significant identity (37 to 41%) with the caseinolytic protease proteolytic subunit (ClpP) found in many organisms (8, 18, 19). This open reading frame, designated *clpP*, encodes a protein of 212 amino acids corresponding to a molecular weight of 23,080. The deduced amino acid sequence of *orf3* has 174 amino acids, leading to a protein with a molecular weight of 18,700. This protein has an overall hydrophilic character as judged by the Hopp-Woods hydropathy plot. No homology with protein sequences in databases could be found. The third open reading frame, designated *fghA*, encodes a protein with a molecular weight of 30,986. The protein shows an identity of 49% with human esterase D (15), of 52% with the *yeiG* gene product of *E. coli* (accession number P33018), and of 49 and 39% with the deduced amino acid sequence of open reading frames of *Haemophilus influenzae* (accession number U32703) and *Saccharomyces cerevisiae* (accession number X88851), respectively (Fig. 2). The similarity between human esterase D, which was found to be identical to human FGH (7), and the *fghA* gene product of *P. denitrificans* suggests that *fghA* encodes FGH. To study this further, a mutation was generated in *fghA* and the mutant was characterized. In addition, the possible roles of ClpP and the *orf3* gene product were investigated by mutational analysis.

Isolation of mutants in *clpP*, *orf3*, and *fghA*. Two constructs that contained a kanamycin resistance gene of pUC4K in the *Bal*I site present either in *orf3* or in *fghA* were made (Fig. 1). These constructs were used to introduce the kanamycin resistance gene into the *P. denitrificans* genome by homologous recombination. The exchange of the wild-type sequences with the mutated ones was verified by Southern analysis of genomic

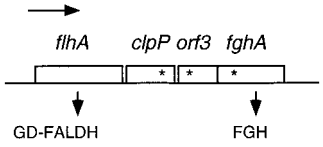

FIG. 1. Physical map of the 3.2-kb chromosomal fragment of *P. denitrificans* containing *flhA*, *clpP*, *orf3*, and *fghA*. Open bars indicate open reading frames. The arrows indicate the direction of transcription. The asterisks indicate the location of the mutations.

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P.den      MTLAYE-TVSENRSFGGIQGVYRHQSQATGTPMTFAIYLPPD-ARHGKVP   48
E.coli     MEM-----LEEHRCFEGWQQRWRHDSSTLNCPMTFSIFLPPP-RDHTPPP   44
H.inf      MKL-----IEQHQIFGGSQQVWAHNAQTLQCEMKFAVYLPNN-PENRPLG   44
Human      MAL---KQISSNKCFGGLQKVFEHDSVELNCKMKFAVYLPPK-AETGKCP   46
S.cer      MKVVKEFSVCGGRLIKLSHNSNSTKTSMNVNIYLPKHYYAQDFPRNKRIP   50

Consensus  M L----.....R.FGG.Q.V..H.S..L.C.M.FA.YLPP.-......P   50

P.den      VLWYLSGLTCTHENAMTKAGAQEWAAEYGIAVIFPDTSPRGEGVAND--E   96
E.coli     VLYWLSGLTCNDENFTTKAGAQRVAAELGIVLVMPDTSPRGEKVAND-D-   92
H.inf      VIYWLSGLTCTEQNFITKSGFQRYAAEHQVIVVAPDTSPRGEQVPND-A-   92
Human      ALYWLSGLTCTEQNFISKSGYHQSASEHGLVVIAPDTSPRGCNIKGE-DE   95
S.cer      TVFYLSGLTCTPDNASEKAFWQFQADKYGFAIVFPDTSPRGDEVANDPEG   100

Consensus  VLYWLSGLTCT..NF.TKAG.Q..AAE.G..VV.PDTSPRGE.VAND-..   100

P.den      TYDLGQGAGFYVDATEAPWAPHFRMWHYVTHELPELVFNNFPLDREAQ--   144
E.coli     GYDLGQGAGFYLNATQPPWATHYRMYDYLRDELPALVQSQFNVS-DRC--   139
H.inf      AYDLGQGAGFYLNATEQPWATNYQMYDYILNELPDLIEANFPTN-GKR--   139
Human      SWDFGTGAGFYVDATEDPWKTNYRMYSYVTEELPQLINANFPVDPQRM--   143
S.cer      SWDFGQGAGFYLNATQEPYAQHYQMYDYIHKELPQTLDSHFNKNGDVKLD   150

Consensus  .YDLGQGAGFYLNATE.PWATHYRMYDY...ELP.L...NFP......--   150

P.den      -----GITGHSMGGHGAL-TIAMTFP-ERYRSVSAFAPIAHPSESDWGRK   187
E.coli     -----AISGHSMGGHGAL-IMALKNP-GKYTSVSAFAPIVNPCSVPWGIK   182
H.inf      -----SIMGHSMGGHGAL-VLALRNR-ERYQSVSAFSPILSPSLVPWGEK   182
Human      -----SIFGHSMGGHGAL-ICALKNP-GKYKSVSAFAPICNPVLCPWGKK   186
S.cer      FLDNVAITGHSMGGYGAICGYLKGYSGKRYKSCSAFAPIVNPSNVPWGQK   200

Consensus  -----.I.GHSMGGHGAL-..AL.NP-.RY.SVSAFAPI.NPS.VPWG.K   200

P.den      QFAAYLGDDKAAWKRHDSTILMREKGYPGE--VLIDQGASDQFLD--LLK   233
E.coli     AFSSYLGEDKNAWLEWDSCALMYASNAQDAIPTLIDQGDNDQFLAD-QLQ   231
H.inf      AFSAYLGEDREKWQQYDASSLIQQGYKVQGMR--IDQGLEDEFLPT-QLR   229
Human      AFSGYLGTDQSKWKAYDATHLV-KSYPGSQLDILIDQGKDDQFLLDGQLL   235
S.cer      AFKGYLGEEKAQWEAYDPCLLIKNIRHVGDDRILIHVGDSDPFLEE-HLK   249

Consensus  AFS.YLGEDK..W..YD...L.............LIDQG..DQFL..-QL.   250

P.den      PEALAHAM-AERRQP-GTFRMQQGYDHSYFFVQSFMADHIPL-ARG-ALG   279
E.coli     PAVLAEAARQKAWP--MTLRIQPGYDHSYYFIASFIEDHLRFHAQYL-LK   278
H.inf      TEDFIETCRVANQP--VDVRFHKGYDHSYYFIASFIGEHIAYHAEFLK--   275
Human      PDNFIAACTEKKIP--VVFRLQEGYDHSYYFIATFITDHIRHHAKYL-NA   282
S.cer      PELLLEAVKATSWQDYVEIKKVHGFDHSYYFVSTFVPEHAEFHARNLGLI   299

Consensus  PE.L.EA......P--V..R.Q.GYDHSYYFIASFI.DHI..HA..L.L.   300
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FIG. 2. Comparison of the primary structures of FGH of *P. denitrificans* (P.den), *E. coli*, *H. influenzae* (H.inf), humans, and *S. cerevisiae* (S.cer). The consensus sequence is indicated.

TABLE 2. Growth characteristics of *P. denitrificans* wild-type and mutant strains[a]

Strain	Relevant genotype	Growth on carbon source					
		CH_3NH_2	CH_3OH	Succinate	Choline	Choline + CH_3NH_2	Choline + CH_3OH
1222	wt	+	+	+	+; 0.39[b]	+; 0.24[b]	+
6621	*fghA*	−	−	+	+; 0.15[b]	+; 0.10[b]	−
6521	*orf3*	+	+	+	+	ND	ND
6422	*clpP*	+	+	+	+	ND	ND

[a] wt, wild type; +, growth; −, no growth; ND, not determined.
[b] Maximal specific growth rate ($hour^{-1}$).

DNA (results not shown). The *orf3* and *fghA* mutant strains were designated Pd6521 and Pd6621, respectively. An 0.8-kb *Fsp*I-*Bgl*II fragment containing part of *clpP* was cloned in the suicide vector pGRPd1. This construct was integrated in the genome of *P. denitrificans* by homologous recombination. The resulting strain, Pd6422, contained two truncated versions of the *clpP* gene, one of which lacked the 3′ part and the other of which lacked the 5′ part including the translation start.

Characterization of the mutant strains. The physiological consequences of the mutations were studied by comparing the growth of the *P. denitrificans* parent strain with that of the mutants in batch cultures and on solid medium with various substrates as carbon and energy source. Mutants Pd6521 and Pd6422 showed no phenotype on succinate and the methylotrophic substrates methylamine, methanol, and choline: ClpP and Orf3 are not essential for methylotrophic growth (Table 2). Mutant Pd6621 was unable to grow on methanol and methylamine. Growth on choline was still possible, but the maximal specific growth rate on this substrate is reduced to 38% of that of the wild type. Pd6621 grew on a mixture of choline and methylamine but was unable to grow on a mixture of choline and methanol.

Table 3 shows FGH activities that were found in the wild-type and mutant Pd6621 grown on succinate, choline, or methylamine. In the *fghA* mutant Pd6621, only a very low amount of 4-methylumbelliferyl acetate hydrolysis was detected and this was considered not to reflect FGH activity. The data suggest that FGH activity is absent in Pd6621. FGH synthesis in the wild type was activated during growth on both methylamine and choline. A lower activity of FGH was found in wild-type cells grown on succinate. These levels are above the background level and indicate therefore that a basic amount of FGH is always present in *P. denitrificans.*

DISCUSSION

The data presented here indicate that the *P. denitrificans* gene encoding FGH has been isolated. The gene is located downstream of the gene encoding GD-FALDH but is separated from that gene by two other genes, i.e., *clpP* and *orf3*. GD-FALDH and FGH are both involved in the oxidation of formaldehyde and consequently in the detoxification of this toxic compound. This may be reflected by the basic levels of both FGH and GD-FALDH (27) that were found in succinate-grown cells. These low but significant levels may ensure that the cells can respond quickly to small amounts of formaldehyde that may be formed adventitiously or upon first challenges with methylotrophic substrates. Higher levels of both enzymes were found during growth on methanol, methylamine, and choline. For the former two C_1 substrates, the complete oxidation of formaldehyde to formate was found to be essential for survival. With these C_1 substrates, *P. denitrificans* mutants lacking either FGH or GD-FALDH were unable to grow.

TABLE 3. FGH activities in *P. denitrificans* wild-type and mutant strains grown on different carbon sources[a]

Strain	Relevant genotype	Activity on carbon source		
		CH_3NH_2	Choline	Succinate
1222	wt	150	117	26
6621	*fghA*	NG	13	12

[a] wt, wild type; NG, no growth, hence not tested. Activities are given in nanomoles of 4-methylumbelliferon formed per minute per milligram of protein.

The finding that Pd6621 was still able to grow on choline, be it at a markedly reduced growth rate, was surprising, since a GD-FALDH mutant was unable to do so. Possibly, in choline-grown cells, an enzyme other than FGH that can carry out the hydrolysis of *S*-formylglutathione to formate is induced. Indeed, the mere presence of choline in methylamine medium led to growth of Pd6621, and apparently, toxic levels of formaldehyde are not formed. This supports the hypothesis that the hydrolyzation of *S*-formylglutathione in these cells is carried out by an enzyme that is induced by the presence of choline. Important, however, is that this putative enzyme is less efficient than FGH and therefore that the growth rate is reduced. Growth on a mixture of choline and methanol did not occur. The explanation for this may be that the oxidation pathway of formaldehyde is blocked, while the formation of formaldehyde is increased. For formaldehyde is an effector in the activation of methanol dehydrogenase synthesis (6). Consequently, an accumulation of formaldehyde should lead to an increase of methanol dehydrogenase synthesis, an increase in methanol oxidation, and conceivably an increase of formaldehyde to toxic levels.

The capacity to detoxify formaldehyde is an important feature for every organism. Detoxification of this compound can be carried out by enzymes like formaldehyde dismutase (3, 30), GSH-independent formaldehyde dehydrogenase (12, 25), and methylformate synthase (22). However, these enzymes have been found in only a limited number of organisms. FGH has been found in yeasts, bacteria, and humans. It has been reported previously that FGH is identical to human esterase D (7). Lee et al. (16) showed that the latter enzyme is also present in other mammals. The genes encoding GD-FALDH and FGH are also found in *H. influenzae*, *E. coli*, *S. cerevisiae*, and *Homo sapiens*. FGH and GD-FALDH appear to be widespread in nature. It is obvious that for the oxidation of formaldehyde via the GSH-dependent route both GD-FALDH and FGH are required. Our data suggest that FGH and hence esterase D are employed widely by organisms of wide phylogeny to detoxify formaldehyde.

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