Analysis of two formaldehyde oxidation pathways in *Methylobacillus flagellatus* KT, a ribulose monophosphate cycle methylotroph

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**INTRODUCTION**

In aerobic methylotrophic bacteria, a key step in energy metabolism is the oxidation of formaldehyde to the formyl level. Five possible formaldehyde oxidation pathways are known in bacteria: a linear glutathione-linked pathway, found in some autotrophic methylotrophs (Barber & Donohue, 1998; Harms *et al.*, 1996); linear oxidation by specific or non-specific formaldehyde dehydrogenases, which has been suggested for various groups of methylotrophs (Anthony, 1982); two linear pterin-linked pathways, one using tetrahydrofolate (H\(_4\)F), found in serine cycle facultative methylotrophs, and another using tetrahydromethanopterin (H\(_4\)MPT), found in a variety of methylotrophs (Vorholt *et al.*, 1999); and the cyclic oxidation pathway, found in some ribulose monophosphate (RMP) cycle methylotrophs (Anthony, 1982). In all cases, the oxidative pathways mentioned have been postulated to contribute to the energy metabolism of the cell, generating reduced pyridine nucleotides or reduced cytochromes. In addition, since formaldehyde is a toxic intermediate, it is possible that one or more of these pathways plays a role in formaldehyde detoxification, a protective function.

Our recent discovery that a novel pathway similar to a part of the methanogenesis pathway in archaea is apparently involved in formaldehyde oxidation in *Methylobacterium extorquens* AM1 (Chistoserdova *et al.*, 1998) and is widespread in various groups of methylotrophs (Vorholt *et al.*, 1999) suggests that several methylotrophs have multiple formaldehyde oxidation pathways. These results raise questions concerning the functions and the significance of these pathways in methylotrophs. Multiple pathways might be redundant, or they might play different physiological roles, for instance in dissimilatory metabolism, in the generation of reduced cofactors for biosynthetic purposes, or in formaldehyde detoxification.

Keywords: methylotroph, ribulose monophosphate cycle, formaldehyde oxidation, tetrahydromethanopterin
One methylotroph with multiple formaldehyde oxidation pathways is the obligate RMP pathway methanol utilizer Methyllobacillus flagellatus KT, which possesses high activities for both cyclic oxidation of formaldehyde (Kletsova et al., 1987) and linear oxidation via H₂MPT-linked derivatives (Vorholt et al., 1999). The cyclic oxidation pathway (Fig. 1) has been postulated to be the major formaldehyde oxidation route in RMP cycle methanol utilizers, such as M. flagellatus KT, a conclusion supported by high activities of the key oxidative enzyme, 6-phosphogluconate dehydrogenase in these strains and also by ¹⁴C distribution experiments (Anthony, 1982). However, our discovery of an alternative formaldehyde oxidation pathway at high activity in M. flagellatus KT questioned this conclusion. Therefore, we have undertaken a mutation analysis of the two pathways in this methylotroph. Since no alternative to C, substrates is possible for selection of null mutants in obligate methylotrophs, we made the following predictions regarding alternative outcomes: (1) if the pathway of interest is not essential for survival of the organism, double-crossover (null) insertion mutants will be readily selected; or (2) if the pathway of interest is indispensable, null mutants will not be obtained, but single-crossover mutants can be selected that retain an intact copy of the gene of interest and consequently retain the corresponding function. Two target genes were chosen: the gene (gndA) encoding 6-phosphogluconate dehydrogenase, the key enzyme of the cyclic formaldehyde oxidation pathway, and the gene (mch) for methenyl H₂MPT cyclohydrolase (CH), participating in the direct oxidation of formaldehyde via H₂MPT derivatives. Two control genes were selected: zuf, encoding glucose-6-phosphate dehydrogenase, an enzyme participating in both assimilatory and putative dissimilatory parts of the RMP cycle, and orf-1, a gene of unknown function in the mau (methylamine utilization) cluster characterized earlier (Gak et al., 1997) and shown to be dispensable for growth. The following predictions were made for mutants in the control genes: (1) no null mutants will be obtained in zuf, as the gene encodes an essential (formaldehyde assimilation) function (Kletsova et al., 1988); (2) null mutants will be readily obtained in orf-1, as the gene encodes a dispensable function (Gak et al., 1997).

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Escherichia coli strains DH5α (Bethesda Research Laboratories) and S17-1 (Simon et al., 1983) were grown in LB medium in the presence of appropriate antibiotics as described by Maniatis et al. (1982). E. coli DF214 [F’ pgI-Mu zef α] (Vinopal et al., 1975) was grown in M9 medium (Maniatis et al., 1982) containing glycerol as a carbon source and supplemented with histidine and methionine (20 μg ml⁻¹). M. flagellatus KT was grown in the minimal medium described previously (Harder et al., 1973). Methanol (600 mM) was used as a substrate. In most experiments, pyruvate (10 mM) was added to solid media as a growth stimulator (Kletsova et al., 1988). The following antibiotic concentrations were used for M. flagellatus KT: kanamycin (Km), 100 μg ml⁻¹, rifamycin (Rif), 50 μg ml⁻¹. The following cloning vectors were used: pULB113 (Van Gijsegem & Toussaint, 1982) for R-prime formation, pUC19 (Pharmacia) and pACYC63 (Chistoserdov et al., 1994) for cloning and subcloning, pACYC61 (Chistoserdov et al., 1994) as a suicide vector, pRK310 (Ditta et al., 1985) as an expression vector, and pRK2013 (Ditta et al., 1985) as a helper plasmid for conjugation.

**DNA--DNA hybridization.** For colony hybridization, M. flagellatus KT recombinants were streaked onto nitrocellulose filters placed on top of minimal agar plates and grown for 40 h. After that the filters were treated and hybridized with appropriate probes essentially as described by Maniatis et al. (1982) except for omitting the steps involving prehybridization and preincubation with hybridization solution. To analyse chromosomal patterns, DNA--DNA hybridizations were carried out with dried agarose gels as described by Meinloth & Wahl (1984) at 55 °C. For hybridizations, 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) was used, and 0.5× SSC was used for washes.

**DNA manipulations.** Plasmid isolation, E. coli transformation, restriction enzyme digestion, ligation, blunting ends with T4 DNA polymerase, or filling in ends with Klenow enzyme, were carried out as described by Maniatis et al. (1982). The chromosomal DNA of M. flagellatus KT was isolated by the procedure of Saito & Miura (1963).

**DNA sequencing.** DNA sequencing from both strands was carried out with an Applied Biosystems automated sequencer by the Department of Biochemistry, University of Washington Sequencing Facility, and by the Department of Biology, Moscow State University.

**Computer analysis.** Translation and analyses of DNA and DNA-derived polypeptide sequences were carried out using...
Genetic Computer Group (Wisconsin) and ORF Finder (NCBI) programs.

Enzyme assays. Enzyme activities were determined in M. flagellatus KT crude extracts obtained by passing cells through a French pressure cell at $1.2 \times 10^8$ Pa, followed by centrifugation for 10 min at approximately 15,000 g. All measurements were made at room temperature in a total volume of 1 ml. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were assayed as described by Kletsova et al. (1990), methenyl H$_4$MPT CH (EC 3.4.5.27) was assayed as described by Pomper et al. (1999). Enzyme assays were done in triplicate and the values obtained agreed within 20%. Protein concentration was measured at 280 nm. Glucose-6-phosphate dehydrogenase, indicating that both 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase activities were present in the extracts.

Matings. For mutant selection, biparental matings were performed overnight at 37°C on minimal agar (containing methanol and pyruvate) to which LB medium had been added to 10% of normal concentration to allow for growth of E. coli. Cells were then washed and enriched for recombinants by incubating in a selective (methanol, Km, Rif) liquid medium overnight. After that, cultures were pelleted, resuspended in a small volume and plated onto selective plates at appropriate dilutions. Isolation of R-prime plasmids in M. flagellatus KT was performed at 42°C as previously described (Tsygankov et al., 1990).

RESULTS

Cloning of the gndA-containing region

The gndA–zwf locus was cloned by heterologous complementation of the glucose-negative mutant of E. coli DF214 (Vinopal et al., 1975). Wild-type M. flagellatus KT containing pULB113 was employed to induce the formation of R-prime plasmids and this strain was mated with E. coli DF214. E. coli transconjugants were obtained on glucose-containing medium in the presence of tetracycline to select for pULB113 containing complementing M. flagellatus KT DNA. These transconjugants contained activities of both 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase (data not shown). An R-prime plasmid from one of the transconjugants was chosen for further analysis and designated R'2D. A HindIII library of this plasmid was constructed using pUC19. Plasmid p42, containing a 10-kb HindIII fragment from R'2D, enabled DF214 to grow on glucose. Crude extracts of DF214(p42) contained activities of both 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase, indicating that gndA and zwf are tightly linked in M. flagellatus KT. After further subcloning, a 2.5-kb region containing gndA was sequenced.

The sequence revealed three ORFs (Fig. 2). One of these encoded a putative polypeptide of 303 amino acids, which showed high identity to known Gnd proteins (46% identity to Gnd from Streptomyces coelicolor, accession number L27063; 43 and 36% identity to Gnd proteins from Bacillus subtilis, accession numbers D84432 and J02584; 34% identity to Gnd from E. coli, accession number M63827). A second, incomplete, ORF was identified 63 bp downstream of the putative gnd gene. The translated polypeptide shared sequence identity with Zwf proteins (38% identity to Zwf from Pseudomonas aeruginosa, accession number AF029673; 36% identity to Zwf from B. subtilis, accession number D84432; 32% identity to Zwf from E. coli, accession number M55005). The third ORF is divergently transcribed and remains unidentified.

Cloning of the mch-containing region

A PCR fragment of 430 nucleotides containing a portion of M. flagellatus KT mch (Vorholt et al., 1999) was used as a probe to identify a 2.7-kb SacI positive fragment of M. flagellatus KT chromosomal DNA using Southern (DNA–DNA) hybridization in gels. A partial chromosomal library of M. flagellatus KT was created using a corresponding purified fraction of SacI-digested DNA cloned into pAYC63 (Chistoserdov et al., 1994), and a positive clone was identified by DNA–DNA hybridization on filters using the same probe. The insert in this clone was sequenced. Two complete and one partial ORFs were identified in the fragment (Fig. 2). The polypeptide translated from the first ORF showed 65% amino acid identity with the product of mch (formerly orfZ), encoding methenyl H$_4$MPT CH of Methylobacterium extorquens AM1 (Pomper et al., 1999), and the second and third (partial) ORFs showed identity with orf5 and orf7 of M. extorquens AM1 (46% and 52% identity, respectively, at the amino acid level; Chistoserdova et al., 1998). In M. extorquens AM1, orfY is found upstream of mch (Chistoserdova et al., 1998). However, no counterpart of orfY was found upstream of mch in M. flagellatus KT.

Mutant construction and analysis

Insertion mutations in gndA, zwf and mch were constructed in vitro using the kanamycin (Km) resistance gene cartridge as described earlier (Chistoserdov et al., 1998).
Table 1. Enzyme activities in mutant strains described in this study

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Activity* [nmol min(^{-1}) (mg protein(^{-1})]</th>
<th>Gnd (NAD)</th>
<th>Zwf (NAD)</th>
<th>H(_4)MPT CH</th>
</tr>
</thead>
<tbody>
<tr>
<td>gnd::Km (single crossover)</td>
<td>160</td>
<td>800</td>
<td>1100</td>
<td></td>
</tr>
<tr>
<td>zwf::Km (single crossover)</td>
<td>170</td>
<td>780</td>
<td>990</td>
<td></td>
</tr>
<tr>
<td>orf1::Km (double crossover)</td>
<td>150</td>
<td>720</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>mch::Km (double crossover)</td>
<td>140</td>
<td>830</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Gnd, 6-phosphogluconate dehydrogenase; Zwf, glucose-6-phosphate dehydrogenase.

1994). The mutation sites for each gene are indicated in Fig. 2. Mutants in orf-1 similar to those reported previously were constructed using the same donor strain (Gak et al., 1987). Mutants were selected in the presence of Km and screened for possible double-crossover versus single-crossover events using colony DNA–DNA hybridization analysis with the Km-resistance gene cartridge and pUC19 DNA as probes (See Methods). At least 100 recombinant clones were analysed in every case. For orf-1, a dispensable gene of unknown function in the mch cluster, double-crossover recombinants (negative for hybridization with pUC19 DNA) were indicated for about half of the Km-resistant clones. In the case of zwf and gndA, all Km-resistant clones tested showed hybridization with the vector sequence (data not shown), suggesting that only single-crossover insertions were obtained. Chromosomal DNA samples isolated from representatives of these mutants were digested by appropriate restriction enzymes and hybridized in gels with the probes for the Km-resistance gene, the vector and the gene of interest. Hybridization patterns predicted for single-crossover recombinants were revealed (data not shown). Mutants in mch that were negative for the pUC19 sequence were found with a frequency of approximately 4%. The double-crossover nature of these mutants was confirmed by diagnostic PCR and also by Southern hybridization analysis (data not shown). Mch-deficient strains grew more slowly than the wild-type on methanol-containing solid media. However, in liquid cultures the mutants grew at the wild-type rate on methanol (data not shown), indicating that the H\(_4\)MPT-linked linear pathway for formaldehyde oxidation is not essential for growth on C\(_2\) substrates. To test if the mutants were more sensitive to formaldehyde, various concentrations of free formaldehyde were added to methanol-containing solid media. Formaldehyde in the concentration range 1–1.5 mM completely inhibited growth of mch null mutants, while the wild-type and single-crossover mch mutants were not affected.

Activities of 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase and methenyl H\(_4\)MPT CH were measured in representatives of each mutant group (Table 1). As expected, wild-type activities were found in the single-crossover mutants as well as in orf-1 double-crossover mutants, but the activity of methenyl H\(_4\)MPT CH was undetectable in mch mutants.

Identity of gndA

Two isoenzymes of 6-phosphogluconate dehydrogenase have been found in M. flagellatus KT, one NAD-specific (the major isoenzyme) and one NADP-specific (Kiriuchin et al., 1988; Kletsova et al., 1990). To identify the product of gndA, the gene was expressed in E. coli. The activities of 6-phosphogluconate dehydrogenase with NAD and NADP were measured in E. coli containing gndA and also in plasmid-free E. coli. In E. coli carrying gndA, high levels of NAD-linked 6-phosphogluconate dehydrogenase [4000 nmol min\(^{-1}\) (mg protein\(^{-1}\)] were found, while no activity was detected in the plasmid-free strain. The NADP-linked activity was at the same low level [about 200 nmol min\(^{-1}\) (mg protein\(^{-1}\)] in the control and plasmid-bearing strain. This identifies gndA as the gene for the NAD-specific enzyme.

DISCUSSION

Data available so far indicate that some methylotrophs contain multiple pathways for formaldehyde oxidation. Two pterin-linked pathways, one involving tetrahydrofolate and one involving H\(_4\)MPT, are present in the facultative serine cycle methylotroph Methylobacterium extorquens AM1 (an \(\alpha\)-proteobacterium) and both seem to be required for growth (Chistoserdova et al., 1998).

Multiple potential pathways for formaldehyde oxidation are also present in the obligate RMP pathway methylotroph Methylobacillus flagellatus KT (a \(\beta\)-proteobacterium). Two possibilities, the direct oxidation by a NAD-linked formaldehyde dehydrogenase, and oxidation by a "dye-linked" periplasmic formaldehyde dehydrogenase, seem unlikely to be major dissimilatory routes, due to the low activities of these enzymes in the cells (Kletsova et al., 1987; Chistoserdova et al., 1991). However, the other two pathways, the cyclic oxidation via 6-phosphogluconate dehydrogenase and the linear oxidation via H\(_4\)MPT derivatives, involve much higher enzyme activities and each could be important for methylotrophic growth (Kletsova et al., 1987; Vorholt et al., 1999). In this study we addressed
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the necessity and the function of these two latter pathways in *M. flagellatus* KT. A random mutagenesis approach for selection of temperature-sensitive mutants in methylotrophy genes has previously been used for this organism, but no mutants in 6-phosphogluconate dehydrogenase (Gnd) were obtained (Kletsova et al., 1988). In this study we used a direct mutagenesis approach based on homologous recombination, assuming that only disruption of non-essential genes will result in mutants with null function.

No null mutants were obtained defective in 6-phosphogluconate dehydrogenase, a key enzyme of the cyclic oxidation pathway, suggesting that this pathway is essential for growth on methylotrophic substrates. However, null mutants were obtained that exhibited no detectable activity of methenyl H$_2$MPT CH$_4$, a key enzyme of the linear H$_2$MPT-linked pathway, suggesting that this alternative pathway is not required for growth. These results confirm previous suggestions that the cyclic formaldehyde oxidation pathway plays a crucial role in C$_1$ metabolism of *M. flagellatus* KT, most probably as the major energy-generating pathway.

Our results show that the H$_2$MPT-linked pathway cannot substitute for the cyclic oxidation pathway in vivo. Both pathways have high *in vitro* enzyme activities and both can potentially generate NADH and NADPH, so it is unclear why they are not functionally interchangeable. It is possible that *in vivo*, the H$_2$MPT-linked pathway preferentially produces NADPH, while the cyclic pathway produces NADH, therefore fulfilling the important energetic function. Alternatively, the *in vivo* activity of the H$_2$MPT-linked pathway may be restricted by availability of specific cofactors, such as H$_2$MPT or methanofuran.

Although the H$_2$MPT-linked pathway is not essential for growth, it does appear to play a role in methylotrophic metabolism. Mutants defective in this pathway were more sensitive to formaldehyde than wild-type for cells grown on solid media but not in shaken liquid cultures. These results suggest this pathway may serve to protect the cells from excess formaldehyde, especially during growth on solid surfaces with the attendant diffusion gradients. Alternatively, this pathway may play a role in providing reducing equivalents for a function that is physiologically more significant for cells grown on solid surfaces, for instance, polysaccharide production or the production of storage materials. If formaldehyde is not drawn away by this pathway under such physiological conditions, it is possible that the cells would become more sensitive to formaldehyde excess.

This proposed conditional role for the H$_2$MPT-linked pathway is in contrast to the role of this pathway in *Methylobacterium extorquens* AM1, where it is essential and is likely to be the main energy-generating pathway for methylotrophic growth. Since it has been suggested that this pathway may have been transferred to an ancestor of a proteobacterial methylotroph from a methanogen at some time after the two groups diverged in evolution (Vorholt et al., 1999), it is interesting that the role of this pathway is different in these two physiologically and phylogenetically distinct types of methylotrophs. An analysis of the phylogenetic relationship between the methenyl H$_2$MPT CH$_4$s of a variety of methylotrophs has shown that the major branchpoint between the representatives of α-, β- and γ-Proteobacteria reflects the 16S rRNA branchpoints, consistent with a single, early transfer event (Vorholt et al., 1999). The clearly divergent roles of this pathway in an α- and a β-proteobacterium suggest that as methylotrophic metabolism evolved, different species took advantage of the same capabilities in divergent ways. Of those methylotrophs that utilize the RMP cycle for assimilatory metabolism, only the β-proteobacterial group contains both the cyclic and the linear H$_2$MPT-linked formaldehyde oxidation pathways (Vorholt et al., 1999). The RMP cycle methanotrophs (γ-Proteobacteria) contain only the linear H$_2$MPT-linked pathway, where it is assumed to be the main energy-generating pathway, and in Gram-positive methanotrophs this pathway could not be found (Vorholt et al., 1999). Therefore, it seems likely that at some point in the evolution of methylotrophic metabolism in the β-Proteobacteria, the linear H$_2$MPT-linked oxidation pathway took on a new role, perhaps after acquisition of a gene encoding the (NAD-linked) 6-phosphogluconate dehydrogenase, which would have generated the cyclic oxidation pathway.

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