An outer-membrane porin inducible by short-chain amides and urea in the methylotrophic bacterium *Methylophilus methylotrophus*

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The *fmdA* and *fmdB* genes encoding formamidase and a putative regulatory protein, respectively, from the methylotrophic bacterium *Methylophilus methylotrophus* were recloned with additional flanking DNA (pSW1). *fmdC*, encoding a weakly hydrophilic protein containing an N-terminal signal sequence, was identified upstream of *fmdAB*. The derived amino acid sequence of mature FmdC (*M*, 39204) showed that it was rich in β-sheet and aromatic amino acids, and exhibited significant similarities to several outer-membrane porins from other bacteria. Cell fractionation studies showed that the protein was located in the outer membrane. Mature FmdC was purified and shown to consist of a single type of subunit (*M*, 40000) with the predicted N-terminal amino acid sequence (GATISF-). SDS-PAGE and Western blotting of cells grown in continuous culture under various conditions showed that mature FmdC was induced by formamide, acetamide and urea, repressed by excess ammonia, and over-expressed during prolonged growth under formamide limitation. It is concluded that mature FmdC is a porin involved in the transport of short-chain amides and urea through the outer membrane of *M. methylotrophus* under conditions where these nitrogen sources are present at very low concentration.

Keywords: *Methylophilus methylotrophus*, outer-membrane porin, amide-urea porin, *fmdC* gene

INTRODUCTION

The methylotrophic bacterium *Methylophilus methylotrophus* readily uses formamide as a source of nitrogen for growth by virtue of its ability to hydrolyse the amide to ammonia and formate using a cytoplasmic formamidase (formamide amidohydrolase; EC 3.5.1.49). The purified enzyme exhibits a high activity with formamide, very low activity with short-chain aliphatic amides and no activity with urea (Wyborn et al., 1994).

Nucleotide sequencing of a 2.3 kbp **HindIII–PstI** restriction fragment of *M. methylotrophus* DNA (pNW323) has shown the presence of two genes, *fmdA* and *fmdB*, which encode formamidase and a putative positive regulator, respectively; these genes probably comprise an operon which is transcribed in the direction *fmdAB* (Wyborn et al., 1996). *M. methylotrophus* formamidase (FmdA) is closely related on the basis of its derived amino acid sequence, secondary structure and immunological properties to the formamidase (acetamidase) from *Mycobacterium smegmatis* (Mahenthiralingam et al., 1993; Wyborn et al., 1996).

The location of the *M. methylotrophus* formamidase in the cytoplasm of the cell implies that formamide must traverse both the outer and inner membranes prior to hydrolysis. In principle, passage through the outer membrane could occur either unmediated (simple diffusion) or via the mediation of a membrane protein (facilitated diffusion). Porin-mediated facilitated dif-
fusion of molecules such as sugars, sugar acids, phosphate and various cations through bacterial outer membranes is well documented (Nikaido, 1993, 1994; Schulz, 1993). In contrast, very small uncharged molecules such as formamide can pass freely through the outer membrane by simple diffusion when present at high concentrations, but little is known about the movement of such molecules under conditions when they are limiting for growth. No porins have as yet been identified in methylotrophic bacteria, but the ability of M. methylotrophus to use a very low steady-state concentration (< 50 μM) of formamide (M, 45) or urea (M, 60) as a source of nitrogen during growth in continuous culture under formamide or urea limitation (J. Mills & C. W. Jones, unpublished) suggests that a porin(s) may be present for these substrates.

In this paper we report the characterization of the fmdC gene encoding an outer-membrane porin (FmdC) in M. methylotrophus, the purification and partial characterization of the porin, and the physiological regulation of the porin expression by short-chain amides and urea.

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains used in this work were Methylophilus methylotrophus (NCIMB 10515), Escherichia coli JM109 (recA1 supE44 endA1 hsdR17 gyrA96 F’ [traD36 proAB lacIq lacZAM15] relA1 thi [lac–pro–AB]) and E. coli NM522 [supE hsdS F’[proAB lacIq lacZAM15] thi [lac–pro–AB]). The plasmids used in this work were pUC18, pUC19 (amp’ lacZ’), pNW3 and pNW323 (pUC19 carrying respectively a 3.2 kbp PstI restriction fragment of M. methylotrophus DNA). pUC18 carrying an 8.4 kbp SphI restriction fragment of M. methylotrophus DNA was stored at -20 °C until required.

**Growth of bacteria.** M. methylotrophus was grown in continuous culture (dilution rate, D, 0.1 h-1) in methanol-mineral salts medium at 37 °C with ammonia, formamide, acetamide or urea as the source of nitrogen (Wyborn et al., 1994). E. coli was grown in batch culture in M9 glucose-mineral salts liquid medium at 37 °C with 20 mM ammonia, formamide or acetamide as the source of nitrogen (Sambrook et al., 1989; Wyborn et al., 1994); during the growth of clones carrying pUC18/19 or derivatives of pUC18/19, all media were supplemented with ampicillin (50 μg ml-1). Culture supernatants were prepared by centrifuging samples for 1 min at maximum speed in an MSE MicroCentaur bench-top centrifuge; the concentration of ammonia in the supernatants was measured colorimetrically (Silman et al., 1989).

**Preparation of washed cells and measurement of formamidase activity.** Washed cells were prepared and assayed for formamidase activity as described previously (Wyborn et al., 1994).

**Preparation of membrane and high-speed supernatant fractions.** A formamide-limited continuous culture of M. methylotrophus (D 0.4 h-1) was harvested by centrifugation at 12200 g for 15 min, then washed in 50 mM Tris/HCl buffer pH 7.5, re-centrifuged and resuspended finally in the same buffer to a cell density of 40–50 mg dry wt ml-1. The cell suspension was disrupted by passage three times through an Amino French pressure cell at approximately 90 MPa, then unbroken cells and cell debris were removed by centrifugation at 40000 g for 20 min to produce a cell-free extract. The latter was centrifuged at 175000 g for 75 min to produce a high-speed supernatant fraction, which was removed carefully with a Pasteur pipette, and a crude membrane fraction which was washed by re-centrifugation in 50 mM Tris/HCl buffer pH 7.5 and resuspended finally in the same buffer to approximately 30 mg protein ml-1. The crude membrane and high-speed supernatant fractions were stored at -20 °C until required. Protein was assayed by the method of Bradford (1976) using the Bio-Rad protein assay reagent.

**Purification of mature FmdC.** The crude membrane fraction, diluted to 3 mg protein ml-1 in 50 mM Tris/HCl buffer pH 7.5 plus 5 mM EDTA and 1% (w/v) N-lauroyl sarcosine, was stirred on ice for 30 min and then centrifuged at 120000 g for 1 h. The resultant outer-membrane pellet was resuspended in 50 mM Tris/HCl buffer pH 7.5 plus 4% (w/v) Triton X-100, then centrifuged at 120000 g for 1 h. The supernatant containing solubilized outer membrane was dialysed overnight at 4 °C in 50 mM Tris/HCl buffer pH 7.5 plus 0.1% (w/v) Triton X-100 to decrease the concentration of the detergent, then filtered on to an FPLC Mono-Q 5/5 anion-exchange column (Pharmacia) and eluted via a linear gradient of 0–1 M NaCl in the same buffer at a flow rate of 2 ml min-1 over 10 column volumes. Mature FmdC eluted at approximately 120 mM NaCl. Peak fractions were bulked, then concentrated and de-salted using an Omegacell unit containing an M, 10000 cut-off filter (Flowgen). The protein was shown subsequently by SDS-PAGE to be ≥ 95% pure.

**Preparation of antiserum to mature FmdC.** Antiserum to purified, mature FmdC was prepared essentially as described previously for antiserum to formamidase (Wyborn et al., 1996), but using two rats rather than a rabbit. The antiserum was stored at -20 °C until required.

**PAGE.** Discontinuous SDS-PAGE was carried out as described previously (Silman et al., 1989) and gels were stained for protein with Renacidin blue R. Western blotting was carried out as described previously (Greenwood et al., 1990), but using antiserum to mature FmdC.

**N-terminal amino acid sequencing.** N-terminal amino acid sequencing of proteins excised from polyacrylamide gels was carried out using an Applied Biosystems model 470 gas-phase sequencer as described previously (Wyborn et al., 1996).

**DNA sequencing and analysis.** Double-stranded plasmid DNA was isolated and sequenced as described previously (Wyborn et al., 1996). Nucleotide and derived amino acid sequence analyses were carried out using DNA Strider version 1.2, and the University of Wisconsin Genetics Computer Group sequence analysis package version 8.0 (BLAST, CODON-PREFERENCE, GAP, ISOELECTRIC and MOTIFS programs). Secondary structure predictions were obtained using the PHD program (Rost & Sander, 1993, 1994) and the turn-prediction rules for β-sheet proteins (Jeanjean et al., 1991).

**Chemicals and enzymes.** Restriction endonucleases and T4 ligase were obtained from BRL; calf intestinal phosphatase was obtained from Pharmacia. Protein M, standards were obtained from Sigma. All other chemicals used were obtained from Fisons or Sigma, and were of the highest grade obtainable.

**Presentation of results.** Results are expressed, where ap-
RESULTS AND DISCUSSION

Construction and characterization of pSW1

Nucleotide sequencing of a 2.3 kbp HindIII–PstI fragment of *M. methylotrophus* DNA in pNW323 (a subclone of pNW3) had previously shown the presence of *fmdA* and *fmdB* encoding formamidase and a putative positive regulator, respectively (Wyborn et al., 1996). To investigate flanking genes, an 8.4 kbp *SphI*-fragment of *M. methylotrophus* chromosomal DNA likely to contain these genes was identified by Southern blotting using the pNW3 insert as a probe, then cloned in pUC18 to generate pSW1 essentially as described previously for pNW3 (Wyborn et al., 1996). Restriction analysis of pSW1 showed that it contained insert DNA identical to that in pNW3 plus 5.2 kbp of additional flanking DNA (2.5 kbp upstream and 2.7 kbp downstream) (Fig. 1a).

*E. coli* NM522(pSW1) exhibited a similar phenotype to that of *E. coli* JM109(pNW3) and JM109(pNW323) (Wyborn et al., 1994, 1996) viz. (i) growth on glucose-mineral salts medium supplemented with formamide or acetamide as nitrogen source, and (ii) synthesis of formamidase as determined by measurement of formamidase activity and concentration (SDS-PAGE and Western blotting using antiserum to formamidase). SDS-PAGE also showed the presence in NM522(pSW1) of a protein(s) of *M. r* 40000 which was absent from JM109(pNW3) and JM109(pNW323). Neither of the control strains, *E. coli* JM109(pUC19) and NM522 (pUC18), grew on glucose-mineral salts medium supplemented with either formamide or acetamide as nitrogen source, or synthesized either formamidase or the *M. r* 40000 protein(s). It was concluded that the 8.4 kbp insert in pSW1 contains *fmdAB* plus an additional gene(s) encoding a protein(s) of *M. r* approximately 40000.

**Nucleotide sequence of *fmdC***

The 8.4 kbp insert in pSW1 was sequenced in both directions for approximately 1.4 kbp upstream of *fmdA*. Codon preference analysis showed the presence of an ORF (orfC, later identified as *fmdC*; Fig. 1b) which was preceded by a putative ribosome-binding site (RBS). Several potential ORFs were identified on the opposite strand of the DNA, but none of these encoded proteins of *M. r* > 5000 or were preceded by a candidate RBS.

*fmdC* contained 1170 bp and was preceded by a potential RBS (GGGAGT) centred eight nucleotides upstream of the ATG translational start codon (taken to be the first of two successive ATG codons). The presumptive RBS was similar to the consensus sequences found in *E. coli* (AGGAGG) and various methylotrophic bacteria, including *Methylophilus* spp. (AGGAGA) (see Wyborn et al., 1996), and was preceded by putative ‘−24’ (CTGGCT)/−12 (TTGCTT) promoter sequences centred 240 and 228 nucleotides, respectively, upstream of the translational start site. These sequences conformed to the consensus nitrogen-regulated ‘−24’/‘−12’ promoter sequence (GG-N10-CC) found upstream of various genes in *Klebsiella pneumoniae* and *Salmonella typhimurium* (Dixon, 1984; Merrick, 1988), of amiC in *Pseudomonas aeruginosa* (Wilson & Drew, 1991) and of *fmdA* in *M. methylotrophus* (Wyborn et al., 1996). No potential promoter sequences similar to the canonical ‘−35’/‘−10’ sequences found in *E. coli* (TTGACA and TATAAT) and various methylotrophic bacteria, including *Methylophilus* spp. (TTGACC and TATAAT) (see Wyborn et al., 1996) were identified upstream of *fmdC*. *fmdC* was terminated by a TAA translational stop codon centred 43 nucleotides upstream of the ATG translational start codon of *fmdA*. The derived amino acid sequence showed that *fmdC* encodes a protein of 390 amino acids with a predicted *M. r* of 41817.

**Analysis of predicted amino acid sequence of FmdC**

In an attempt to identify FmdC, washed cells were prepared from a formamidine-limited continuous culture (D 0.1 h⁻¹) of *M. methylotrophus* and cell proteins were separated by SDS-PAGE. A strongly expressed protein(s) of *M. r* approximately 40000 was then excised and subjected to N-terminal amino acid sequencing. This revealed two sequences (GATISF- and ADYPTA-) separated by SDS-PAGE. A strongly expressed protein(s) of *M. r* approximately 40000 was then excised and subjected to N-terminal amino acid sequencing. This revealed two sequences (GATISF- and ADYPTA-), suggesting the presence of two proteins in approximately equal concentrations. The sequence GATISF- was identical to residues 27−32 in the derived amino acid sequence (MMNLKKCALHTAVAMACGLGAQLANA). The latter exhibited the expected characteristics of a prokaryotic signal sequence (see Duffaud et al., 1985; Goodwin & Anthony, 1995), viz. a basic N-terminal...
region (two adjacent lysine residues, no acidic residues), a central region of high hydrophobicity (as evidenced by a hydropathy profile of FmdC based on Kyte-Doolittle assignments; Kyte & Doolittle, 1982; not shown), a C-terminal domain containing a helix-disrupting glycine residue, and an AxA sequence immediately upstream of the cleavage site.

Analysis of the derived amino acid sequence of mature FmdC revealed an overall hydropathy index of 0-2, an isolectric point of 4-53, a net charge of -10.5, and an unusually high content (14.6 Yo) of aromatic amino acids (Phe, Trp, and Tyr). It was concluded that mature FmdC is a weakly hydrophilic, acidic protein composed of 364 amino acids with a predicted M, of 39204.

Comparison of the derived amino acid sequence of mature FmdC with other proteins in the databases revealed significant similarities to several outer-membrane porins, viz. P. aeruginosa OmpF (26-2 Yo identical, 52.1 Yo similar including conservative replacements) (Fig. 2), E. coli OmpF (23.3 Yo identical, 49.0 Yo similar) and the Rhodopseudomonas blastica porin (20.6 Yo identical, 48.8 Yo similar) (Duchene et al., 1988; Cowan et al., 1992; Kreusch & Schulz, 1994). Weaker similarities were observed with the Rhodobacter capsulatus porin, E. coli OmpA and PhoE, and P. aeruginosa OprD.

Furthermore, the presence of phenylalanine as the C-terminal amino acid in FmdC is characteristic of many outer-membrane porins (but not OmpF), as also is the high content of aromatic amino acids and the overall negative charge (Struyve et al., 1991; Nieweg & Bremer, 1997).

Secondary structure predictions (Jeanneur et al., 1991; Rost & Sander, 1993, 1994) showed that mature FmdC was particularly rich in beta-sheet (37.0 Yo beta-sheet cf. 20.0 Yo alpha-helix) and contained at least 14 putative membrane-spanning beta-strands. Other porins have also been shown to have a high beta-sheet content and up to 18 beta-strands, commensurate with their proposed antiparallel beta-barrel structures (Cowen et al., 1992; Nikaido, 1993, 1994; Schulz, 1993; Kreusch & Schulz, 1994; Rawling et al., 1995; Schirmer et al., 1995; Nieweg & Bremer, 1997).

Analysis of subcellular fractions of M. methylotrophus showed that mature FmdC (identified using SDS-PAGE followed by N-terminal amino acid sequencing of the M, 40000 protein(s) or Western blotting using antiserum prepared against the purified protein; see below) was one of two major proteins located in the outer membrane. The identities of the second outer-membrane protein (M, approximately 38000) and the other M., 40000 protein (ADYPTA; not located in the outer membrane) are currently under investigation.

In view of its cellular location, derived amino acid composition and sequence, and predicted secondary structure, mature FmdC was tentatively identified as an outer-membrane porin.

**Purification of mature FmdC**

Mature FmdC was purified from a formamide-limited continuous culture of M. methylotrophus (Fig. 3). The purified protein was shown by SDS-PAGE to be >95 Yo pure, and by N-terminal amino acid sequencing to contain the predicted N-terminal sequence (GATISF-). SDS-PAGE yielded a subunit M, of approximately 2376.
Table 1. Physiological regulation of formamidase and mature FmdC expression in *M. methylotrophus*

*M. methylotrophus* was grown in continuous culture ($D$ 0.1 h$^{-1}$) under various nutrient limitations with methanol as the carbon source and ammonia, formamide, acetamide or urea as the nitrogen source. Formamidase activities were measured colorimetrically; formamidase and mature FmdC concentrations were determined semi-quantitatively by SDS-PAGE and Western blotting. Formamidase activities [µmol min$^{-1}$ (mg cells)$^{-1}$] are expressed as mean ± SEM (number of independent determinations).

<table>
<thead>
<tr>
<th>N-source</th>
<th>Limiting nutrient</th>
<th>[Ammonia] (mM)</th>
<th>Formamidase Activity</th>
<th>Mature FmdC concn</th>
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</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>Ammonia</td>
<td>&lt;0.05</td>
<td>0.29±0.14 (6)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>5.90</td>
<td>0.02±0.02 (3)</td>
<td>+</td>
</tr>
<tr>
<td>Formamide</td>
<td>Formamide</td>
<td>&lt;0.05</td>
<td>2.64±0.31 (7)</td>
<td>++ +</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>8.90</td>
<td>0.23 (2)</td>
<td>–</td>
</tr>
<tr>
<td>Acetamide</td>
<td>Acetamide</td>
<td>&lt;0.05</td>
<td>1.37±0.35 (4)</td>
<td>++ +</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>7.00</td>
<td>0.19 (2)</td>
<td>–</td>
</tr>
<tr>
<td>Urea</td>
<td>Urea</td>
<td>&lt;0.05</td>
<td>3.39 (2)</td>
<td>++ +</td>
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<tr>
<td></td>
<td>Methanol</td>
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</table>

Fig. 4. Physiological regulation of formamidase and mature FmdC expression in *M. methylotrophus*. *M. methylotrophus* was grown in continuous culture ($D$ 0.1 h$^{-1}$) under various nutrient limitations. Cells were harvested and cell proteins separated by SDS-PAGE (a), then transferred to nitrocellulose and probed with antiserum to formamidase (b) or mature FmdC (c). Tracks: 1, M, standards; 2, ammonia-limited; 3, methanol-limited (ammonia excess); 4, acetamide-limited; 5, methanol-limited (acetamide excess); 6, formamide-limited; 7, methanol-limited (formamide excess); 8, urea-limited; 9, methanol-limited (urea excess); 10, M, standards. Formamidase runs anomalously on SDS-polyacrylamide gels, giving a subunit $M_\text{r}$ of 51000 compared with a predicted $M_\text{r}$ of only 44438 from the nucleotide sequence of *fmdA* (Wyborn et al., 1994, 1996). Mdh-$\alpha$, $\alpha$-subunit of methanol dehydrogenase.

### Physiological regulation of formamidase and porin expression

*M. methylotrophus* was grown in continuous culture ($D$ 0.10 h$^{-1}$) under various nutrient limitations with methanol as the carbon source and either ammonia, formamide, acetamide or urea as the nitrogen source. Formamidase activities were highest during growth under amide or urea limitation, and were greatly diminished...
during growth under methanol limitation (i.e. in the presence of a high concentration of ammonia resulting either from its direct addition or from the hydrolysis of excess amides or urea) (Table 1). These variations in formamidase activity were accompanied by similar variations in formamidase concentration as determined by SDS-PAGE and Western blotting using antiserum to formamidase (Fig. 4a, b). The combined concentrations of the M₄ 40000 proteins (mature FmdC and ADYPTA) followed a similar pattern to that of formamidase, and this was confirmed for mature FmdC by Western blotting (Fig. 4c); the antiserum also cross-reacted with an unidentified protein (M, 22000), the concentration of which was unaffected by the growth conditions (not shown). Both formamidase and mature FmdC were therefore induced strongly by amides and urea, and repressed by a high concentration of ammonia.

Over-expression of the porin during prolonged growth under formamide limitation

*M. methylotrophus* was grown for 308 generations (89 d) in formamide-limited continuous culture (D 0.1 h⁻¹). SDS-PAGE of washed cells showed that the concentration of formamidase remained essentially constant over the course of the experiment, whereas the combined concentrations of the M₄ 40000 proteins (mature FmdC and ADYPTA) remained constant for at least 66 generations and then approximately doubled. This pattern was confirmed for the porin by Western blotting using antiserum to mature FmdC. In contrast, the concentration of methanol dehydrogenase (α-subunit, M₄ 60000) decreased over the first 66 generations and then levelled off, and the concentrations of several other strongly expressed proteins (M₄ 67000, 47000 and 22000) remained essentially constant over the entire experiment.

Role of porin A during the growth of *M. methylotrophus*

The probable role of mature FmdC during the growth of *M. methylotrophus* is to facilitate the passage of short-chain amides (e.g. formamide, acetamide) and urea into the periplasm when the concentrations of these compounds in the surrounding medium are extremely low. Simple considerations of solute diffusion rates at very low external concentrations highlight the requirement of micro-organisms to synthesize appropriate porins under these conditions (Nikaido & Vaara, 1985), even for small uncharged substrates such as formamide, acetamide and urea which exhibit relatively high permeability coefficients. This is supported by the much higher concentration of mature FmdC in cells grown in continuous culture under amide or urea limitation than under methanol limitation (or in batch culture), since under these latter two conditions the amides and urea are present at high concentrations (up to approximately 20 mM) and can therefore enter the cell by simple diffusion alone at a rate fast enough to support the observed growth rate. It is also supported by the over-expression of mature FmdC during prolonged growth under formamide limitation, where any improvement in the permeability of the outer membrane to formamide would lead to increased ‘biological fitness’ (Dykhuizen & Hartl, 1983) and hence be a major selective advantage.

The high concentration of the porin in cells grown under conditions where formamide (M₄ 45), acetamide (M₄ 59) or urea (M₄ 60) is the limiting nutrient, but not when methanol (M₄ 32) is the limiting nutrient, suggests that it may act as a substrate-specific channel for compounds with the general formula (R, CO–NH₃), rather than as a general pore for low M₄ substrates, i.e. more like LamB, Tsx, ScrY and various *P. aeruginosa* porins, than like OmpF, OmpA or PhoE (Nikaido, 1993, 1994; Schulz, 1993; Schirmer et al., 1995; Nieweg & Bremer, 1997).

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