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*_r 39 204) 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*_r 40 000) 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 *Hind*III–*Pst*I 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-

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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 ($< 50 \mu\text{M}$) of formamide (M_r 45) or urea (M_r 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⁺ lacI^a lacZAM15) relA1 thi Δ(lac-proAB)*] and *E. coli* NM522 [*supE hsd5 F' (proAB⁺ lacI^a lacZAM15) thi Δ(lac-proAB)*]. The plasmids used in this work were pUC18, pUC19 (*amp lacZ'*), pNW3 and pNW323 (pUC19 carrying respectively a 3.2 kbp *Pst*I restriction fragment and a 2.3 kbp *Hind*III–*Pst*I restriction fragment of *M. methylotrophus* DNA; Wyborn *et al.*, 1994, 1996), and pSW1 (pUC18 carrying an 8.4 kbp *Sph*I restriction fragment of *M. methylotrophus* DNA).

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 ($50 \mu\text{g ml}^{-1}$). 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 (Silman *et al.*, 1989; Wyborn *et al.*, 1994).

Preparation of crude membrane and high-speed supernatant fractions. A formamide-limited continuous culture of *M. methylotrophus* (D 0.1 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 $40\text{--}50 \text{ mg dry wt ml}^{-1}$. The cell suspension was disrupted by passage three times through an

Aminco French pressure cell at approximately 9.0 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 $30 \text{ mg protein ml}^{-1}$. 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 $3 \text{ mg protein ml}^{-1}$ 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 through an acrodisc filter ($0.2 \mu\text{m}$ pore size; Gelman), loaded 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_r 10000 cut-off filter (Flowgen). The protein was shown subsequently by SDS-PAGE to be $\geq 95\%$ 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 Kenacid 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 (Jeanteur *et al.*, 1991).

Chemicals and enzymes. Restriction endonucleases and T4 ligase were obtained from BRL; calf intestinal phosphatase was obtained from Pharmacia. Protein M_r 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-

appropriate, as the mean $\pm$ SEM with the number of independent determinations given in parentheses.

RESULTS AND DISCUSSION

Construction and characterization of pSW1

Nucleotide sequencing of a 2.3 kbp *Hind*III–*Pst*I 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 *Sph*I-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-GC) 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 formamide-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-), 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 of FmdC, indicating that the mature protein was produced by removal of a 26 amino acid signal peptide (MMNLKKCALHTAVAMACGLGLAQLANA). The latter exhibited the expected characteristics of a prokaryotic signal sequence (see Duffaud *et al.*, 1985; Goodwin & Anthony, 1995), viz. a basic N-terminal

Fig. 1. Restriction mapping and genetic organization of part of the 8.4 kbp *Sph*I-restriction fragment of *M. methylotrophus* DNA in pSW1. (a) Restriction map and plasmids; (b) organization of the *fmd* genes.

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1  GATISFGEDKYISAGFG...FITSYNSVEDAAANGSDRSNDFTLDSARLY 47
   |:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|
1  ....QQQNSVEIEAFGRKRYFTDSVRMMKNADLYGGSIGYFLTDD...VE 42
48  LSGSFNKYIKGMLNTEKSGGGSANSNFEVIDANVQFQLTPEVAIWAGRFL 97
   |:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|
43  LALSYPEY.HDVRGTYYTGKKVHGMLTSLDAIYHFG.TPGVGLRP...YV 88
98  SPS.....DRANM.....AGPYYSMGGGYWAN..IASRYG 125
   |:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|
89  SAGLAHQNITNINSDSQGRQQTMANIGAGLKYTTTENFFAKASLDQYQG 138
126  W....NGGVIGRDDGVAVVTSFME.....DHVAVSFGAFEGDNIFRFS 164
   |:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|
139  LEKRDNGHQGEWMAGLGVGFMFGGSKAAPAPFPVADVCSDSDNDGVCNDV 188
165  GVGAQSEANGTKDKLMYAG....RVQIDF.WDAEPGYGTGNYFGAKDI 208
   |:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|
189  DKCPDTPANVTVDANGCPAAEVVRVQLDVKFDKSKVKENSYADIKN. 237
209  LAIGIAGRMKQKGDVVSTIPGVVDYKSYVDFFLEKKDVGPGTFSAAAY 258
   |:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|
238  ...LADFMKQ..YFSTSTTVEGHTDSVGTDAYNQK.....LSERRA. 273
259  YYIDTDDVFLGQKAYSAGLGYLFDNFVGWQKIMPIVRYQKFDADGLTT 308
   |:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|
274  ..NAVRDLVME...YGVGGGRV..NAVGYGESRPVA..DNATAEGRAI 313
309  AASVNTRTSADTKRFEIGANYVIAPYNSVITATYQKTDTPAASDNDFRL 358
   |:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|:::|
314  NRRVEAEVEAEAK..... 326
359  ALQMVF..... 364

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Fig. 2. Amino acid sequence alignment of FmdC from *M. methylotrophus* and outer-membrane porin OprF from *P. aeruginosa*. Identical amino acids in the two sequences are linked by vertical lines. Upper sequence, *M. methylotrophus* mature FmdC; lower sequence, *P. aeruginosa* OprF. N-terminal signal sequences have been omitted in both cases.

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 isoelectric point of 4.53 , a net charge of -10.5 , and an unusually high content (14.6%) 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_r 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* OprF (26.2% identical, 52.1% similar including conservative replacements) (Fig. 2), *E. coli* OmpF (23.3% identical, 49.0% similar) and the *Rhodopseudomonas blastica* porin (20.6% identical, 48.8% 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 OprF), as also is the high content of aromatic amino acids and the overall

Fig. 3. Purification of mature FmdC. Samples taken from each stage of the purification procedure were subjected to SDS-PAGE and stained for protein. Tracks: 1, M_r standards; 2, washed cells; 3, crude membrane fraction; 4, outer membranes; 5, Triton-solubilized outer membranes; 6, purified mature FmdC; 7, M_r standards.

negative charge (Struyve *et al.*, 1991; Nieweg & Bremer 1997).

Secondary structure predictions (Jeanteur *et al.*, 1991; Rost & Sander, 1993, 1994) showed that mature FmdC was particularly rich in β -sheet (37% β -sheet cf. 20% α -helix) and contained at least 14 putative membrane-spanning β -strands. Other porins have also been shown to have a high β -sheet content and up to 18 β -strands, commensurate with their proposed antiparallel β -barrel structures (Cowan *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_r 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_r approximately 38000) and the other M_r 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 $\geq 95\%$ pure, and by N-terminal amino acid sequencing to contain the predicted N-terminal sequence (GATISF-). SDS-PAGE yielded a subunit M_r of approximately

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 [$\mu\text{mol min}^{-1}$ (mg cells)⁻¹] are expressed as mean $\pm$ SEM (number of independent determinations).

N-source	Limiting nutrient	[Ammonia] (mM)	Formamidase		Mature FmdC concn
			Activity	Concn	
Ammonia	Ammonia	< 0.05	0.29 $\pm$ 0.14 (6)	—	—
	Methanol	5.90	0.02 $\pm$ 0.02 (3)	+	—
Formamide	Formamide	< 0.05	2.64 $\pm$ 0.31 (7)	+++	++
	Methanol	8.90	0.23 (2)	+	—
Acetamide	Acetamide	< 0.05	1.37 $\pm$ 0.35 (4)	+++	++
	Methanol	7.00	0.19 (2)	+	—
Urea	Urea	< 0.05	3.39 (2)	+++	++
	Methanol	1.80	0.08 (2)	+	—

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_r 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_r standards. Formamidase runs anomalously on SDS-polyacrylamide gels, giving a subunit M_r of 51 000 compared with a predicted M_r of only 44 438 from the nucleotide sequence of *fmdA* (Wyborn et al., 1994, 1996). Mdh- α , α -subunit of methanol dehydrogenase.

40 000 (cf. 39 204 from the derived amino acid sequence) which increased to approximately 75 000 when the protein was pre-treated with SDS at 30 °C instead of the normal 100 °C (behaviour characteristic of many outer-membrane porins). SDS-PAGE and Western blotting using antiserum raised against the purified porin showed that it cross-reacted strongly against its own antigen, but not against the other other M_r 40 000 protein (ADYPTA-) or the second outer-membrane protein.

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 M_r 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_r 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 M_r 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, M_r 60000) decreased over the first 66 generations and then levelled off, and the concentrations of several other strongly expressed proteins (M_r 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 (M_r 45), acetamide (M_r 59) or urea (M_r 60) is the limiting nutrient, but not when methanol (M_r 32) is the limiting nutrient, suggests that it may act as a substrate-specific channel for compounds with the general formula (R.CO.NH₂), rather than as a general pore for low M_r 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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