Molecular characterization of a 40 kDa OmpC-like porin from *Serratia marcescens*

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An oligonucleotide that encodes the N-terminal portion of a 41 kDa porin of *Serratia marcescens* was used to probe *S. marcescens* UOC-51 genomic DNA. An 11 kb *EcoRI* fragment which hybridized with the oligonucleotide was subcloned into *Escherichia coli*, examined for expression, and sequenced. The product expressed by the cloned gene was 40 kDa. The nucleotide sequence has an ORF of 1·13 kb. When the deduced amino acid sequence was aligned and compared to other enterobacterial porins the cloned *S. marcescens* porin most closely resembled *E. coli* OmpC. Although we did not detect osmoregulation or thermoregulation of any porins in *S. marcescens* UOC-51, sequences analogous to the *E. coli* osmoregulator OmpR-binding regions are seen upstream to the cloned gene. We examined the regulation of the *S. marcescens* porin in *E. coli* and found that its expression increased in a high salt environment. A *micF* gene, whose transcriptional product functions to inhibit synthesis of OmpF by hybridizing with the *ompF* transcript, was also seen upstream of the *S. marcescens* *ompC*. An alignment with the *E. coli* *micF* gene revealed that the functional region of the *S. marcescens* *micF* gene is conserved. Based on the results obtained we have determined that *S. marcescens* UOC-51 produces a 40 kDa porin similar to the *E. coli* OmpC porin.

Keywords: *Serratia marcescens*, outer membrane, porin, OmpC, *micF* gene

INTRODUCTION

The outer membrane of Gram-negative bacteria is a semi-permeable barrier to many compounds. The diffusion of small hydrophilic molecules across the outer membrane is possible mainly due to the presence of trimeric protein structures called porins which form membrane-spanning water-filled channels. Porins may be classified into two types: non-specific porins, which allow non-specific diffusion of substrates, and specific porins, which have substrate-binding sites (Nikaido, 1992). The best characterized enterobacterial porins are OmpF, OmpC and PhoE of *Escherichia coli*. OmpF and OmpC are considered to be non-selective porins and their combined total number present in the membrane is constant while the amount of each one may vary depending on various external factors (Csonka, 1989). The PhoE porin is anion selective and its gene is induced under phosphate-limiting conditions (Benz *et al.*, 1984; Overbeeke & Lugtenberg, 1980). An example of a specific porin is LamB, the

maltodextrin-selective porin of *E. coli*. LamB has a maltose-binding site and its expression, along with other components of the maltose operon, is induced by growth on maltose (Nikaido & Saier, 1992).

In comparison to the *E. coli* porins, the porins of *Serratia marcescens* have not been well characterized. The *S. marcescens* outer membrane has at least one porin with an estimated molecular mass of 41 kDa (Malouin *et al.*, 1990). This porin has been shown to fall into the non-selective group, based on black lipid bilayer experiments, and has a calculated pore size of 1·1 nm. Other outer-membrane proteins in the 40 kDa range have been identified and classified as porins based on heat-modifiable mobility changes on SDS-PAGE and differential solubilization in NaCl/SDS/EDTA (Gutmann *et al.*, 1985; Hashizume *et al.*, 1993). No specific functional studies have been performed using these putative porins.

A reduction in the production of porins, as determined by SDS-PAGE, has been correlated with an increase in resistance of many Gram-negative bacteria to β -lactam antibiotics and a number of other antibiotics (Bellido *et al.*, 1992; Chow & Shlaes, 1991; Mitsuyama *et al.*, 1987). There have been reports of reduced protein content in the

The GenBank accession number for the sequence reported in this paper is L24960.

40 kDa range in the outer membrane of *S. marcescens* strains cross-resistant to a number of antibiotics (Gutmann *et al.*, 1985; Hashizume *et al.*, 1993). While the possession of a periplasmic β -lactamase is an effective means of resistance, an outer-membrane barrier together with the presence of a β -lactamase can provide high resistance levels (Sanders, 1992), thus any alterations in this barrier are worthy of molecular examination.

In order to characterize the *S. marcescens* outer-membrane porins and study the role they play in antibiotic resistance we have taken a molecular cloning approach. Here we report the cloning, sequencing and characterization of a 40 kDa porin from *S. marcescens*. Based on similarities in sequence and gene regulation to *E. coli* OmpC, we have chosen to call this porin *S. marcescens* OmpC.

METHODS

Bacterial strains and growth conditions. *Serratia marcescens* UOC-51 is a clinical blood isolate (Hutsul *et al.*, 1993). *E. coli* strain LE392 [*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*] (Bio/Can Scientific) was used for λ propagation while *E. coli* strain NM522 [*supE thi Δ(lac-proAB) hsd5 F' (proAB⁺ lacI^q lacZAM15)*] (Promega) was used for plasmid manipulations. All strains were grown in Luria broth at 37 °C for DNA manipulations. For osmoregulated-expression studies strains were grown in L broth modified for low salt (no NaCl added) or high salt (0.3 M NaCl) and at a temperature of 28 °C (Lundrigan & Earhardt, 1984). Where required, media were supplemented with 100 µg ampicillin ml⁻¹.

Cloning and sequencing. *S. marcescens* UOC-51 genomic DNA was isolated using the hexadecyltrimethylammonium bromide procedure (Ausubel *et al.*, 1987). The vector used for library construction was λ gtWES. λ B (Gibco-BRL). Plaque lifting and alkaline Southern blotting were performed as described previously by Maniatis (1982) and Reed & Mann (1985). Hybridizations were carried out at 42 °C. The plasmid pTZ19 (Pharmacia) was used for the expression of the cloned gene in *E. coli*. For sequencing, fragments were cloned into pBluescript phagemids (Stratagene) and deletion clones created using the Erase-a-Base kit (Promega) as outlined by the manufacturer. Sequencing was performed with single-stranded and double-stranded templates by the Sanger dideoxy method (Sanger *et al.*, 1977) using the Sequenase kit from US Biochemicals and [³⁵S]dATP α S (DuPont). Oligonucleotides for sequencing were synthesized on an Applied Biosystems 391 DNA synthesizer.

Computer analyses. Sequence analysis was performed with various programs included in the PC/GENE genetic analysis package (Intelligenetics). Sequences were retrieved from the EMBL/GenBank/DBJ nucleotide sequence data libraries using the FETCH program included in BIRCH (Biological Research Computer Hierarchy), a collection of programs set within the framework of the SUN Unix system as compiled by Fristensky (1991).

Expression of the cloned porin in *E. coli*. The *S. marcescens* porin was expressed in *E. coli* NM522/p8SX, when grown overnight in L broth. Outer membranes were isolated and purified on a discontinuous sucrose density gradient as described by Malouin *et al.* (1990). To analyse porin content in the outer membranes after growth of *E. coli* NM522/p8SX in different osmotic conditions, cell envelopes (Lugtenberg *et al.*, 1975) were incubated in 2% (w/v) SDS for 30 min at 37 °C and the

SDS-insoluble fraction recovered by ultracentrifugation at 100 000 g (Nakae, 1976). Samples were then resuspended in 50 mM Tris/HCl, pH 8.0.

SDS-PAGE, Western blotting and immunodetection. Outer-membrane samples were subjected to SDS-PAGE by the Lugtenberg (1975) system at a running concentration of 11% (w/v) polyacrylamide. To be able to distinguish between OmpF and OmpC in the osmoregulated-expression study the samples were run in a urea gel system as described by Uemura & Mizushima (1975) modified to contain 4 M urea. In both cases, all samples were heated to 100 °C for 10 min in 4 vols electrophoresis sample buffer (0.06 M Tris/HCl, pH 6.8, 2%, w/v, SDS, 10%, v/v, glycerol, 5%, v/v, 2-mercaptoethanol, 0.025% Bromophenol blue) before loading. Western blotting was performed as described by Burnette (1981). Immunodetections were carried out essentially as described by Towbin (1979) using antibodies specific for the *S. marcescens* 41 kDa porin prepared earlier (Hutsul *et al.*, 1993) and anti-rabbit IgG peroxidase conjugate (Sigma).

RESULTS

Cloning of the *S. marcescens* porin

An oligonucleotide (5'-GAAAT^ATATAATAAG-A^TGG-3') deduced from the N-terminal amino acid sequence of the *S. marcescens* 41 kDa porin (Malouin *et al.*, 1990) was used to probe genomic DNA from *S. marcescens* UOC-51 in a Southern hybridization. An 11 kb *Eco*RI fragment hybridized (data not shown). A subgenomic library was then prepared by fractionating *Eco*RI-digested *S. marcescens* genomic DNA on a continuous sucrose gradient and ligating fragments in the range 6–12 kb into the arms of the vector, λ gtWES. λ B. The library was screened using the oligonucleotide above and positive clones with an insert size of 11 kb were obtained. The cloned insert was mapped with restriction endonucleases and hybridized against the oligonucleotide to identify smaller fragments for subcloning. Initially, a 2.1 kb *Kpn*I/*Bam*HI fragment, which hybridized with the oligonucleotide, was subcloned into pTZ19 for further analy-

Fig. 1. Restriction map and sequencing strategy of the *S. marcescens* OmpC porin. The map of the 11 kb *Eco*RI fragment is shown with the scale above it. An enlarged map of the area sequenced is shown below with the sequencing strategy illustrated. The bold area is the coding region of the porin gene in 5' to 3' direction. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sst*I; X, *Xba*I.

Fig. 2. Expression of the cloned *S. marcescens* porin in *E. coli*. Immunoblot of the outer-membrane proteins probed with the 41 kDa porin specific antibodies. Antibodies were used at a 1:200 dilution. lane 1, *E. coli* NM522/p8SX; lane 2, *S. marcescens* UOC-51; lane 3, *E. coli* NM522. A 10 µg sample of protein was loaded in each lane after heating at 100 °C for 10 min. The asterisk denotes the cloned product seen in the *E. coli* outer membrane.

sis. The map of the 11 kb clone, λgtS8, is presented in Fig. 1.

Expression of the cloned *S. marcescens* porin

Outer membranes were isolated from the *E. coli* *Kpn*I/*Bam*HI clone p8BK and analysed by SDS-PAGE; however, no expressed protein was detected. This indicated that either a regulatory region and/or some other portion of the gene may be missing from the 2.1 kb *Kpn*I/*Bam*HI fragment, and therefore a larger fragment would be required for complete expression. A 6 kb *Sst*I/*Xba*I fragment from λgtS8 was then selected; when subcloned into the plasmid pTZ19 it expressed an outer-membrane protein in *E. coli* NM522 with a size of 40 kDa. This protein also reacted with *S. marcescens* 41 kDa porin specific antibodies as seen in Fig. 2. The *E. coli* porins OmpF and OmpC also react with our antibodies. However, this cross-reaction does not in any way influence the interpretation of the results from this experiment. Such a cross-reaction has been previously demonstrated and is primarily due to the strong conservation of porin structure (Hofstra *et al.*, 1980; Hutsul *et al.*, 1993).

The cloned *S. marcescens* porin gene product, when expressed in the *E. coli* outer membrane, appeared to run approximately 1 kDa faster than the 41 kDa porin, which was considered to be the major porin found in the *S. marcescens* outer membrane. This difference in mobility observed in SDS-PAGE may be due to the differences in LPS from *E. coli* and *S. marcescens* since LPS remains tightly associated with porin proteins. Alternatively, we may have cloned another porin. When the 41 kDa porin was run on a 4 M urea/SDS-PAGE system it separated into two bands, the lower of which had the same mobility as the cloned product (see Fig. 6, lane 1). The amount of the 40 kDa porin in the *S. marcescens* outer membrane is low, which may make it indistinguishable from the highly

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CTATATCGCGAGGTATTTTATTGCGTGCTATTGTGCGGCTATTGAAATTGTGAGGACA 60
GTGTGGAGGAAAAATAAAACCGGCCAGAACCGGTTAAAGGTATGCAGAAATATCTTTC 120
GGATAATGAAGTAATAATGAAATAATGATGATAGCGAGGTGATTATAGCGGCTGCGTG 180
CAGGCGCGGTTTTGGCACAATGTGCTTATTGTCCAGTACAATTGTATGGTTGGCTTTTT 240
TTCTTAAAGATTGTATACTACATGATTTTTTATCAATTAATCTATTCTTTGTCTTTAA 300
GTGCTTTATTTTGTTCGGTAATGTTTGTGAAAGTTCGCGCACATTACATTTTGAA 360
ACCTTTATTAATCAGCGTGAACACTATCGGACTTGGTATCATTTCTTTCTGGATTA 420
ATATAACCTTGTACAGAAATGGTAATGCACAGTCGTCTAATAATGCTCGAAAGGGC 480
AGTGGCCGTAGCTCGACATAATTAACGAGGATAATAACGATGAACTTCGAGTACTCTCT 540
LMVPAALLVAGTAGAAEIIYNK 600
CTGATGTACCCGCACTGCTGTTGACGACACAGCGCGCGGCGAAATCTACAACAAA 660
DGNKLDLYGKVDGLHYFSSN 660
GACGGTAATAAACTGGATTGTACGGTAAAGTCGACGCTCTTCACTATTCTCCAGCAAC 660
NGVDDGDQSYMRFGRLRGETQI 720
AACGGCTGGACGGTACCTTACATGCGTTTGGCCCTCGCGGCGAAACGCAAAATC 720
SDQLTG YVGQWEYQANLNHAE 780
AGCGATCAGTGACCGCTACGCCAGTGGGAATATCAGGCTAACCTGAAACCGCTGAA 780
NQDNKNFTTRYGGFAGLKF G D Y 840
AACCGACAAACAAGACTTACCCGTTACGGCTTCGCGGCTCTGAAATTTCGGCGATC 840
GSDFYGRNTGVLYDVAAAYTD 900
GGTTCCTTCGATACGCGGTAATACCGCGTGTGTGACGAGTCGCGCGGTATACCGAC 900
LQPEFDGMYVGA D Q F M F Q R S 960
TTGCAGCTGAGTTTCGACGTTGACCTACGGCGCGCACGAGTTCATGTTTCGGCGTTC 960
SGLATYRNNDF F G L V D G L N F 1020
AGCGGCTGGCGACCTACCGTAACAACGACTTCTTCGGTCTGGTTGACGGCTGAACCTC 1020
ALQYQ GKNGNGEETNNGRDV 1080
GCCCTGCAGTACCGGTAACAAACGGTAATGGCAAGAGACCAACACGCTCGCGATGC 1080
LGQNGEGYGMSSMSYDMGYGI 1140
CTTGGCAGAACCGGCAAGGCTACGGTATGTCATGAGCTATGACATGGGCTACGGCATC 1140
SAAAGAFFNSRRTSEQNGANG 1200
AGCGCGCGCGGCGCTTCTCAACTCTCGCGCACAGGAGCAGAACGCGGCTAACGGT 1200
HQNIMGRGDKAEGYSGLKLY 1260
CACCAGAACATCATGGCCGCGCGGACAAAGCGGAGGTTACTCGCGGCTCTGAAATAT 1260
DANDVYLA VMFTQS YN A A R F 1320
GAGCGCAACGAGTCTACCTGGCGGTGATGTTCAACCGATCTCAACAGCGCGCGGTTTC 1320
GSSDSSSVYGYANKAQSF E A Y 1380
GGCAGCTCCGACAGCAGCGTTTACGGCTACGCCAACAGGCGCAGAGCTTCAAGCCCTAC 1380
AHYQFD F G L R P F V G Y N Q T K G 1440
GGCGCACTACGAGTTCGATTTCGGTCTCGGTCTCGGTCTGATGATGATGATGATGATG 1440
KDLGRAGNGKDYGDQDLVKF 1500
AAAGACCTGGGCGCGCGGTAACGGCAAGGACTACGGCGATCAAGACCTGGTCAAAATC 1500
VDLGATYFFFNKNMSTYVDYK 1560
GTGACCTGGGTGCGACCTACTTCTTCAACAAAAACATGCTACCTATGTTGATTACAAA 1560
INLVDDNNDFTD A A G I N T D N V 1620
ATCAACCTGGTGGACAAACGACTTCACCGACGCTGCCGGGATCAACACCGACAACGTG 1620
VAVGLVYQF - 1680
GTTGCGGTGGGCGTGTATACAGTTCTAAGCACCTCAGTGTGAACGTCTATCGCGGCG 1680
TTGCGGCGCGGTTTTTTTGTGCTGTAACCGGTAAAGACGAGGATGATCGCTATCGG 1740
CACGGGAAGCACCGTGTTCCTCTCTAAGCCTACGCTGTGAGTACAAAAAGAGGGG 1800
GATGATATGAACTCAAGATCGATAAGGTGATCGAAACGGTG 1842

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Fig. 3. Nucleotide sequence of the *S. marcescens* OmpC porin gene. The complete coding sequence and its translation plus upstream and downstream elements are shown. The Shine–Dalgarno box and –10 and –35 regions are underlined. Possible OmpR-binding sites are double underlined with arrow heads bordering each one. A putative rho-independent terminator (underlined) is seen after the coding sequence. An arrow in the amino acid sequence is used to show the cleavage site for removal of the leader peptide.

expressed 41 kDa porin on conventional SDS-PAGE due to similarity in size. The 40 and 41 kDa porins may be analogous to the OmpC and OmpF porins of *E. coli* which have similar N-terminal amino acid sequences (Malouin *et al.*, 1990). It is, therefore, not surprising that the oligonucleotide derived from the 41 kDa porin may have

Fig. 4. Alignment of the *micF* genes of *E. coli* and *S. marcescens*. In *E. coli* three hairpins, (a), (b) and (c) are shown (→ ←). The -10 and -35 regions are boxed. The region of *E. coli micF* that binds to *ompF* mRNA is also shown. Corresponding putative structures are also underlined in *S. marcescens*. | denotes conserved bases.

identified the 40 kDa porin gene in the subgenomic library.

Nucleotide sequence of the cloned *S. marcescens* porin

The 6 kb *SstI/XbaI* clone, p8SX, expressing the porin protein was sequenced in both directions using various subclones, deletion clones and synthesized primers. The sequencing strategy is illustrated in Fig. 1 and the nucleotide sequence of the cloned *S. marcescens* porin and its translation are presented in Fig. 3. The oligonucleotide probe deduced from the N-terminal sequence used to clone the gene was found to have only 2–4 mismatches present within the degenerate 20 base probe sequence. Amino acid residues 22 to 41 are identical to the N-terminal amino acid sequence determined by Edman degradation of the native protein (Malouin *et al.*, 1990; Hutsul *et al.*, 1993).

The nucleotide sequence contains an ORF of 1128 nucleotides which translates into 376 amino acids, including a 21-amino-acid signal peptide. A characteristic Shine–Dalgarno sequence, GAGG, can be seen centred approximately 11 bases upstream from the initiation codon. A putative transcription terminator consisting of a hairpin with a free energy of -17 kcal (about -71 kJ), followed by a string of T residues, is present downstream from the ORF. The calculated molecular mass of the mature monomer is 39.5 kDa, which is close to the molecular mass of 40 kDa for the native protein estimated by SDS-PAGE.

The regulatory region of the cloned *S. marcescens* 40 kDa porin gene is very similar to that of the *E. coli ompF* and *ompC* genes. The location of the promoter region of the *S. marcescens* gene was determined by comparison to the *E. coli ompF* and *ompC* upstream sequences and the -10 and -35 sequences were found to be almost identical to that of the *E. coli ompC* gene. The -10 and -35 regions from these three genes do not follow the consensus sequences well. However, the efficient transcription of the *E. coli ompF* and *ompC* genes are controlled by three consecutive *cis*-acting activation sequences upstream from the -35 region, to which the transcriptional activator, OmpR, will

bind (Maeda *et al.*, 1991). Putative OmpR-binding regions are also found upstream from the -35 region of the *S. marcescens* 40 kDa porin gene.

Another regulatory element that is found upstream to the *E. coli ompC* gene is the *micF* transcript (Mizuno *et al.*, 1984). *micF* is a short RNA transcript, transcribed in the opposite direction from *ompC*, which can hybridize to the 5' end of the *E. coli ompF* transcript and subsequently prevent translation of OmpF. The osmoregulation of OmpF and OmpC is not thought to be greatly influenced by the *micF* transcript. However, *micF* definitely plays a role in the regulation of OmpF under other conditions (Chou *et al.*, 1993). A *micF*-like sequence is seen upstream of the cloned *S. marcescens ompC* gene. Fig. 4 illustrates the alignment obtained with the *S. marcescens* and *E. coli micF* genes. The secondary structure of *E. coli micF* includes three hairpin structures, with the last one functioning as a terminator. The *S. marcescens micF* is similar; however, it does not appear to have an analogous 5' end hairpin. The differences noted between the two *micF* genes are generally within the secondary structure of the transcript while the region which functions in binding to the *ompF* transcript is well conserved.

Sequence alignments and comparisons

The protein sequence of the cloned *S. marcescens* porin was compared and aligned with other enterobacterial porins. The ALIGN program was used to obtain the percentage identity between each porin sequence (Table 1). The PhoE proteins form a cluster with identities ranging from 84.3–91.1% while the two OmpC sequences, from *E. coli* and *Sal. typhi*, are 84.2% identical. The percentage identity obtained between porins outside the two groups (the PhoE and OmpC porins form separate groups) range from 57.8–66.3%. The cloned *S. marcescens* porin has the highest similarity with the OmpC sequences; 68.6% with *Sal. typhi* OmpC and 70.3% with *E. coli* OmpC, although these values are intermediate to those found within a group of similar porins and those found outside that group.

The sequences were aligned using the CLUSTAL program and then manipulated manually for the best alignments

Table 1. Similarity between enterobacterial porins

Values are percentage similarity between porins as determined by ALIGN. Acronyms are as in Fig. 5.

	SMPORIN	ECOOMPF	ECOOMPC	STYOMPC	STYPHOE	CFRPHOE	KPNPHOE	ENTPHOE	ECOPHOE
SMPORIN	•	62.2	*70.3	68.6	59.4	59.8	57.8	59.4	62.1
ECOOMPF	•	•	64.9	66.0	60.3	63.0	62.1	63.7	65.5
ECOOMPC	•	•	•	†84.2	60.9	62.7	62.4	64.0	64.7
STYOMPC	•	•	•	•	63.1	64.1	64.7	66.3	65.2
STYPHOE	•	•	•	•	•	†91.1	84.6	86.0	88.6
CFRPHOE	•	•	•	•	•	•	84.3	86.3	90.9
KPNPHOE	•	•	•	•	•	•	•	85.7	84.3
ENTPHOE	•	•	•	•	•	•	•	•	87.4
ECOPHOE	•	•	•	•	•	•	•	•	•

* The boxed region identifies the porins with which the *S. marcescens* porin has the highest similarity.

† The PhoE and OmpC porins, which each form groups of 80–90%, are enclosed by triangles.

SMPORIN	MKLRVLSL--MVPALLVAGTAGAAEYINKDGNKLDLYGKVDGLHYFSSNN	48	SMPORIN	GRDVLQNGEGYGMSSYDMG--YGISAAGAFFNSRRTEQ--NGANGHONI	231
ECOOMPC	MKVRVLSL--LVPALLVAGAAAEYINKDGNKLDLYGKVDGLHYFSSNN	48	ECOOMPC	GRDALRQNGDVGGSITYDY--EGFGIGCAITSSSKRTDAQ--NTA--A--Y	232
STYOMPC	MKVRVLSL--LVPALLVAGAAAEYINKDGNKLDLYGKVDGLHYFSSNN	48	STYOMPC	GRSLINQNGDVGGSITYAIG--EGFSVGGAITTSKRTDAQ--NNT--ANARL	230
ECOOMPF	MKKRN--TLAIVPALVAGTANAAYINKDGNKLDLYGKVDGLHYFSSNN	49	ECOOMPF	-DTARRSNGDVGGSITYEY--EGFGIVGAGAAORTNLQEAQPL--	227
ECOPHOE	M-KKS--TLALVVMGIVASASVQAAEYINKDGNKLDLYGKVKAMHYMSDNA	48	ECOPHOE	-RDVKKQNGDVGFTSLTYDFGGSDFAIISGAYTNSDRTNQNLQSR--	222
CFRPHOE	M-KKS--TLALVVMGIVASASVQAAEYINKDGNKLDLYGKVKAMHYMSDNA	48	CFRPHOE	-RDVKKQNGDVGFTSLTYDFGGSDFAVSGAYTNSDRTNQNLQSR--	222
STYPHOE	M-NKS--TLAIVV--SIIASASVHAAEYINKDGNKLDLYGKVKAMHYMSDNA	47	STYPHOE	-RDVKKQNGDVGFTSLTYDFGGSDFAVSGAYTNSDRTNQNLQSR--	221
ENTPHOE	M-KKS--TLALVVMGIVASASVHAAEYINKDGNKLDLYGKVKAMHYMSDNA	48	ENTPHOE	-RDVKKQNGDVGFTSLTYDFGGSDFAVSGAYTNSDRTNQNLQSR--	221
KPNPHOE	M-KKS--TLALVVMGIVASASVHAAEYINKDGNKLDLYGKVKAMHYMSDNA	48	KPNPHOE	-REAKKQNGDVGFTSLTYDFGGSDFAVSGAYTNSDRTNQNLQSR--	222
	• * * *			• * * *	
SMPORIN	-----GVDGDSYMRFLRGETQISDQLTYGQWEYQANLNHAENQDN-K	92	SMPORIN	MGRGDKAEGYSGLKYDANDVYLAVMFTQSYNAARFGSSDSS--VYGY	277
ECOOMPC	-----DVGDDQTYMRFLRGKGETQVTQDQLTYGQWEYQIQGNSAENENN	91	ECOOMPC	IGNGDRAETITGLKYDANNIYLAAGYQTYNATRVG-----SLGW	273
STYOMPC	-----GSDGDDQTYMRFLRGKGETQVNDQLTYGQWEYQIQGNTGEGSND	91	STYOMPC	YVNGDRAETITGLKYDANNIYLAAGYQTYNATRVG-----SLGW	280
ECOOMPF	GENSYGGNGDMTYARLGFKGETQINSDLTYGQWEYQIQGNSAENENN	99	ECOOMPF	-CNCCKAEQWATGLKYDANNIYLAAGYQTYNATRVG-----FTNS--GF	272
ECOPHOE	SK-----DGDQSYIRFGKGETQINDQLTYGQWEYQIQGNSAENENN	92	ECOPHOE	-GTGKRAEAWATGLKYDANNIYLAAGYQTYNATRVG-----FTNS--GF	261
CFRPHOE	SK-----DGDQSYIRFGKGETQINDQLTYGQWEYQIQGNSAENENN	92	CFRPHOE	-GTGKRAEAWATGLKYDANNIYLAAGYQTYNATRVG-----FTNS--GF	261
STYPHOE	SK-----DGDQSYIRFGKGETQINDQLTYGQWEYQIQGNSAENENN	91	STYPHOE	-GTGKRAEAWATGLKYDANNIYLAAGYQTYNATRVG-----FTNS--GF	260
ENTPHOE	TK-----DGDQSYIRFGKGETQINDQLTYGQWEYQIQGNSAENENN	92	ENTPHOE	-AQGKAEAWATGLKYDANNIYLAAGYQTYNATRVG-----FTNS--GF	260
KPNPHOE	SK-----DGDQSYIRFGKGETQINDQLTYGQWEYQIQGNSAENENN	92	KPNPHOE	-AQGKAEAWATGLKYDANNIYLAAGYQTYNATRVG-----FTNS--GF	261
	• * * *			• * * *	
SMPORIN	-NFTRYFAGLKFQDYGSDYGRNGLVLYDVAAYTDLPEDFMDTYG-AD	140	SMPORIN	ANKAQSFYEAHYQDFGLRPSLVYQNTKGGKDLGRAGNGKDYQDQLVKF	327
ECOOMPC	-SWTRVAFAGLKFQDYGSDYGRNGLVLYDVAAYTDLPEDFMDTYG-AD	139	ECOOMPC	ANKAQSFYEAHYQDFGLRPSLVYQNTKGGKDLGRAGNGKDYQDQLVKF	318
STYOMPC	-SWTRVAFAGLKFQDYGSDYGRNGLVLYDVAAYTDLPEDFMDTYG-AD	139	STYOMPC	ANKAQSFYEAHYQDFGLRPSLVYQNTKGGKDLGRAGNGKDYQDQLVKF	329
ECOOMPF	GKTRLAFAGLKFQDYGSDYGRNGLVLYDVAAYTDLPEDFMDTYG-AD	148	ECOOMPF	ANKAQSFYEAHYQDFGLRPSLVYQNTKGGKDLGRAGNGKDYQDQLVKF	316
ECOPHOE	-QKTRLAFAGLKFQDYGSDYGRNGLVLYDVAAYTDLPEDFMDTYG-AD	141	ECOPHOE	ANKAQSFYEAHYQDFGLRPSLVYQNTKGGKDLGRAGNGKDYQDQLVKF	305
CFRPHOE	-QKTRLAFAGLKFQDYGSDYGRNGLVLYDVAAYTDLPEDFMDTYG-AD	141	CFRPHOE	ANKAQSFYEAHYQDFGLRPSLVYQNTKGGKDLGRAGNGKDYQDQLVKF	304
STYPHOE	-QKTRLAFAGLKFQDYGSDYGRNGLVLYDVAAYTDLPEDFMDTYG-AD	140	STYPHOE	ANKAQSFYEAHYQDFGLRPSLVYQNTKGGKDLGRAGNGKDYQDQLVKF	304
ENTPHOE	-QKTRLAFAGLKFQDYGSDYGRNGLVLYDVAAYTDLPEDFMDTYG-AD	140	ENTPHOE	ANKAQSFYEAHYQDFGLRPSLVYQNTKGGKDLGRAGNGKDYQDQLVKF	304
KPNPHOE	-QKTRLAFAGLKFQDYGSDYGRNGLVLYDVAAYTDLPEDFMDTYG-AD	141	KPNPHOE	ANKAQSFYEAHYQDFGLRPSLVYQNTKGGKDLGRAGNGKDYQDQLVKF	305
	• * * *			• * * *	
SMPORIN	QFMFORSSGLATYRNNDFFGLVGLNLFALQYQGNKGN--GEE-----TNN	183	SMPORIN	VDLGATYFFNKNMSTYVDYKINLVDNNDFTDAAGINTDNVAVGLVYQF	376
ECOOMPC	NFMQQRNGFATYRNNDFFGLVGLNLFALQYQGNKGN--GEE-----TNN	189	ECOOMPC	VDVGATYFFNKNMSTYVDYKINLVDNNDFTDAAGINTDNVAVGLVYQF	367
STYOMPC	NFMQQRNGFATYRNNDFFGLVGLNLFALQYQGNKGN--GEE-----TNN	183	STYOMPC	VDVGATYFFNKNMSTYVDYKINLVDNNDFTDAAGINTDNVAVGLVYQF	378
ECOOMPF	NFMQQRNGFATYRNNDFFGLVGLNLFALQYQGNKGN--GEE-----TNN	185	ECOOMPF	VDVGATYFFNKNMSTYVDYKINLVDNNDFTDAAGINTDNVAVGLVYQF	362
ECOPHOE	NFMTRASGLATYRNNDFFGVVGLNLFALQYQGNKGN--GEE-----TNN	178	ECOPHOE	IDVGATYFFNKNMSTYVDYKINLVDNNDFTDAAGINTDNVAVGLVYQF	351
CFRPHOE	NFMTRASGLATYRNNDFFGVVGLNLFALQYQGNKGN--GEE-----TNN	178	CFRPHOE	IDVGATYFFNKNMSTYVDYKINLVDNNDFTDAAGINTDNVAVGLVYQF	351
STYPHOE	NFMTRASGLATYRNNDFFGVVGLNLFALQYQGNKGN--GEE-----TNN	177	STYPHOE	IDVGATYFFNKNMSTYVDYKINLVDNNDFTDAAGINTDNVAVGLVYQF	350
ENTPHOE	NFMTRASGLATYRNNDFFGVVGLNLFALQYQGNKGN--GEE-----TNN	177	ENTPHOE	IDVGATYFFNKNMSTYVDYKINLVDNNDFTDAAGINTDNVAVGLVYQF	350
KPNPHOE	NFMTRASGLATYRNNDFFGVVGLNLFALQYQGNKGN--GEE-----TNN	178	KPNPHOE	IDVGATYFFNKNMSTYVDYKINLVDNNDFTDAAGINTDNVAVGLVYQF	351
	• * * *			• * * *	

Fig. 5. Alignment of the *S. marcescens* OmpC porin with the porins of the *Enterobacteriaceae*. The initial alignment was made using CLUSTAL and then completed manually for the best alignment. Asterisks and dots are used to indicate perfectly and well conserved amino acids, respectively, as determined by CLUSTAL. The PEFXG motif is boxed. SMPORIN, *S. marcescens* OmpC porin; ECOOMP, *E. coli* OmpF (Inokuchi *et al.*, 1982); ECOOMP, *E. coli* OmpC (Mizuno *et al.*, 1983); STYOMPC, *Salmonella typhi* OmpC (Puente *et al.*, 1989); STYPHOE, *Salmonella typhimurium* PhoE (Spierings *et al.*, 1992a); CFRPHOE, *Citrobacter freundii* PhoE (Spierings *et al.*, 1992b); KPNPHOE, *Klebsiella pneumoniae* PhoE, and ENTPHOE, *Enterobacter cloacae* PhoE (van der Ley *et al.*, 1987); ECOPHOE, *E. coli* PhoE (Overbeek *et al.*, 1983).

(Fig. 5). An important change that can be seen is in the conserved PEFXG motif found in enterobacterial porins. In all other enterobacterial porins X = glycine and the motif is followed by an aspartic acid residue (Jeanteur *et al.*, 1991). In the cloned *S. marcescens* porin X = aspartic acid and the motif is followed by a methionine. Only in neisserial porins is X an amino acid other than glycine and the PEFXG motif is followed by a hydrophobic amino

acid residue. The cloned *S. marcescens* porin is only 37% similar to neisserial porins. The PEFXG motif forms a turn in the third external loop of the porin. This loop is important because it is responsible for the size constriction of porin pores by folding down into the pore (Cowan *et al.*, 1992). It is also the region where a lysine residue responsible for the anion specificity of PhoE porins is located (Bauer *et al.*, 1989).

Fig. 6. Osmoregulation of the *S. marcescens* OmpC porin. SDS-insoluble cell envelope fractions of cells grown in the absence or presence of NaCl were run on a 4 M urea/SDS-PAGE system. A 10 µg sample of protein was loaded in each lane after heating at 100 °C for 10 min. Lane 1, *S. marcescens* in 0.086 M NaCl; lane 2, *E. coli* NM522, no NaCl; lane 3, *E. coli* NM522, 0.3 M NaCl; lane 4, *E. coli* NM522/p8SX, no NaCl; lane 5, *E. coli* NM522/p8SX, 0.3 M NaCl. The asterisk denotes the cloned *S. marcescens* porin in the *E. coli* outer membrane.

A conserved feature of most outer-membrane proteins, including porins, is a C-terminal phenylalanine (Struyvé *et al.*, 1991). The terminal phenylalanine is important for the correct assembly of the outer-membrane proteins into the outer membrane. In keeping with this function, the *S. marcescens* OmpC porin also has a C-terminal phenylalanine.

Osmoregulation study

When outer membranes were first isolated from *E. coli* NM522/p8SX, which expresses the *S. marcescens* cloned porin, we noticed that there appeared to be a decrease in the *E. coli* OmpF and/or OmpC content in the outer membrane. We decided to examine the effect osmotic strength of the media may have on the expression of the cloned *S. marcescens* porin as well as the effect the expression of this cloned porin may have, in turn, on the expression of the two *E. coli* porins. With no added NaCl in the medium the expression of *E. coli* OmpF is increased over that of the *E. coli* OmpC (Fig. 6, lane 2), whereas, in 0.3 M NaCl OmpC is preferentially expressed (Fig. 6, lane 3), as would be expected (Csonka, 1989). When the *S. marcescens* cloned porin is expressed in *E. coli*, OmpF still exhibits regulation in response to high osmotic strength (Fig. 6, lane 5), although there is an apparent decrease in the overall content of OmpF in the membrane under both conditions. The amount of *E. coli* OmpC does not appear to increase in the high salt condition (Fig. 6, lane 5) when the *S. marcescens* cloned porin is expressed; however, a slight increase in the amount of the *S. marcescens* cloned porin is noted in the *E. coli* NM522/p8SX outer membrane. These results suggest that the OmpR-binding sites, located upstream to the *S. marcescens* cloned porin gene, are functional in *E. coli* NM522. The lower bands seen in lanes 4 and 5 are OmpA which was not removed completely during the porin isolation of these samples.

DISCUSSION

The *E. coli* outer membrane has been well characterized and major proteins normally expressed include the osmoregulated OmpF and OmpC porins and the structural protein OmpA. A large body of functional (reviewed in Nikaido, 1992), regulatory (Waukau & Forst, 1992; Mizuno *et al.*, 1984) and structural (Cowan *et al.*, 1992) information is available on the *E. coli* porins. However, the *S. marcescens* outer membrane has not been as well characterized. Early reports on the *S. marcescens* outer membrane recognize the presence of two putative porins in the 40 kDa range (Gutmann *et al.*, 1985; Sawai *et al.*, 1987). Malouin *et al.* (1990) reported the first functional characterization of a porin isolated from the *S. marcescens* outer membrane. They described the presence of a single constitutive porin with a molecular mass of 41 kDa and a single channel conductance of 1.6 nS. Recently, Puig *et al.* (1993) reported the resolution of three proteins in a urea/SDS-PAGE electrophoresis system. Two of these proteins were regulated by the osmotic strength of the media, suggesting that they may be analogous to the *E. coli* OmpF and OmpC porins. However, there were no functional data reported to confirm that any of these three proteins were porins. In addition, we do not observe the osmoregulation reported by Puig *et al.* (1993), although this may be due to differences between the strains used in each study. Here we describe the cloning, sequencing and expression of a porin from *S. marcescens* UOC-51. This is the first molecular description of a *S. marcescens* porin.

As determined by the cloning and sequencing of a porin structural gene from *S. marcescens* UOC-51 genomic DNA we have found that *S. marcescens* produces a porin with a molecular mass of 39.5 kDa for the mature peptide. The sequence of this porin has the highest similarity, ~70%, with *E. coli* and *Sal. typhi* OmpC porins; however, this value is intermediate to that within a porin group (~80–90%) and between porin groups (~60%). The upstream sequence of the *S. marcescens* porin gene also resembles the *ompC* genes, with three consecutive OmpR-binding regions and an upstream sequence resembling *micF*, which is also found upstream from *E. coli* and *Sal. typhi* *ompC* genes. The *S. marcescens* porin is very similar in size to the *E. coli* OmpC and *Sal. typhi* OmpC porins, which are 38.5 and 40 kDa, respectively. Like the *E. coli* OmpC, the expression of the cloned *S. marcescens* porin appears to be regulated in *E. coli* in response to NaCl concentration. Taking all of these factors into consideration, we chose to call the cloned *S. marcescens* porin OmpC.

OmpR-binding sites found upstream to the *S. marcescens* porin gene suggest a potential for regulation by a system similar to *E. coli* EnvZ/OmpR (Waukau & Forst, 1992). Indeed, we observed that when grown in medium containing high salt (0.3 M NaCl) the concentration of the cloned *S. marcescens* OmpC porin in *E. coli* appeared to increase. However, as mentioned earlier, we were unable to detect similar regulation of any outer-membrane proteins in the 40 kDa range in response to either osmotic stress or temperature in *S. marcescens* UOC-51. There may

be a number of reasons for the lack of osmoregulation in some *S. marcescens* strains. It is possible that there may have been a loss of OmpR/EnvZ function in this strain of *S. marcescens* since an *ompB* locus, the OmpR/EnvZ operon (Waukau & Forst, 1992), has not yet been identified in *S. marcescens*. Alternatively, there may not be another porin capable of being coordinately regulated with the *S. marcescens* OmpC porin, either through mutation to OmpR-binding sites of a second porin or due to an absence of another porin gene. Indeed, Malouin *et al.* (1990) described the presence of only one porin, with a molecular mass of 41 kDa, in the outer membrane of the *S. marcescens* strain examined. In our examination of the outer membrane of *S. marcescens* UOC-51, we have detected putative porins with molecular masses of 40 and 41 kDa; however, sequence information is available for only one of these porins. Further analysis should help explain why osmoregulation is not detected in *S. marcescens* UOC-51.

Under high osmotic stress *E. coli* OmpC levels normally rise. In our osmoregulation experiments little or no increase in *E. coli* OmpC was detected in the outer membrane when the *S. marcescens ompC* gene was present. The similarity of the regulatory regions, or OmpR-binding sites, between the *E. coli* and *S. marcescens ompC* genes and the fact that there are more copies of the cloned *S. marcescens* gene present in the clone could explain the absence of an increase in *E. coli* OmpC expression. A fraction of the *E. coli* OmpR protein could bind to *S. marcescens* DNA thus leaving a smaller than normal proportion of the OmpR pool free to bind to the binding sites preceding the *E. coli ompC* gene. Similarly, the lower content of the *E. coli* OmpF in the outer membrane under both osmotic growth conditions could be explained by the abundance of *S. marcescens* OmpR-binding sites. Probably more important than the OmpR effect is the high copy number of *micF* that would be present if it is expressed. While *micF* is thought not to play an important role in the osmoregulation of *E. coli* OmpF and OmpC it has been shown that when present in high quantities it can inhibit OmpF synthesis (Matsuyama & Mizushima, 1985). *E. coli* OmpF is repressed in the presence of salicylate and *micF* has been shown to play a role in this regulation (Rosner *et al.*, 1991). Repression of the 41 kDa porin in *S. marcescens* by salicylate has also been noted (Sawai *et al.*, 1987). The *micF* sequence seen upstream to the *S. marcescens ompC* gene may therefore play a role in the repression of the 41 kDa porin by salicylate. Alternatively, we did not observe regulation of the porins of *S. marcescens* in response to temperature, even though *micF* plays a role in the thermoregulation of the *E. coli* OmpF and OmpC porins (Andersen *et al.*, 1989).

When aligned with the amino acid sequences of other enterobacterial porins the cloned *S. marcescens* porin most closely resembles OmpC. The alignments also show that there is a strong conservation between the *S. marcescens* OmpC porin and all porins of the *Enterobacteriaceae* that have been sequenced thus far. This is not surprising since previous studies by Jeanteur *et al.* (1991), using sequence alignments, demonstrated the similarities between entero-

bacterial porins. Also, we previously demonstrated the similarity of porins between *Serratia* spp. and other *Enterobacteriaceae* through Southern and Western analyses, N-terminal amino acid sequencing and porin functional analyses (Hutsul *et al.*, 1993).

Although considerable similarity has been observed between the *S. marcescens* OmpC porin and those from the *Enterobacteriaceae*, we detected a change in a conserved region of the third external loop of the *S. marcescens* OmpC gene in our sequence alignments. This loop folds down into the pore and serves to constrict the pore size with its bulkiness. At the tip of the loop is a turn formed by the PEFXG motif. The PEFXG motif is seen in all enterobacterial and neisserial porins (Jeanteur *et al.*, 1991) with X = glycine (G) in those of enterobacterial origin and X = alanine (A) or serine (S) in neisserial porins. The G-G sequence of enterobacterial porins is thought to be conserved because of the unique torsion angles it forms which would aid in the turn formation of the PEFXG region (Cowan *et al.*, 1992). In the *S. marcescens* OmpC the X is aspartate (D) not G, as normally found with the other enterobacterial porins. Aspartic acid is also turn promoting (Paul & Rosenbusch, 1985), which therefore may not be expected to disrupt the function of the PEFXG motif. This motif is followed by an aspartic acid residue in enterobacterial porins and a hydrophobic phenylalanine residue in neisserial porins (Jeanteur *et al.*, 1991). Again the *S. marcescens* OmpC porin differs from the enterobacterial porins as the motif is followed by the hydrophobic residue methionine. We do not intend to suggest that the *S. marcescens* 40 kDa porin is more similar to neisserial porins (the average similarity is 37%) than to enterobacterial porins; however, the changes seen in this conserved motif may be a reflection of the divergence this porin has taken from other OmpC porins. In addition, these changes may indicate a possible difference in the pore characteristic of the *S. marcescens* porin.

Interest in *S. marcescens* has increased due to a high incidence of antibiotic resistance in clinical isolates. Alteration of outer-membrane permeability has been implicated as a factor in this resistance. A reduction in the level of outer-membrane proteins, possibly porins, has been observed in antibiotic-resistant strains of *S. marcescens*, as well as other members of the *Enterobacteriaceae*. The gene for the OmpC porin of *S. marcescens* has now been sequenced which will allow further analyses into its regulation, structure and function, and role in antibiotic resistance.

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