Molecular characterization of the *Serratia marcescens* OmpF porin, and analysis of *S. marcescens* OmpF and OmpC osmoregulation

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***Serratia marcescens* is a nosocomial pathogen with a high incidence of β -lactam resistance. Reduced amounts of outer-membrane porins have been correlated with increased resistance to β -lactams but only one porin, OmpC, has been characterized at the molecular level. In this study we present the molecular characterization of a second porin, OmpF, and an analysis of the expression of *S. marcescens* porins in response to various environmental changes. Two porins were isolated from the outer membrane using urea-SDS-PAGE and the relative amounts were shown to be influenced by the osmolarity of the medium and the presence of salicylate. From a *S. marcescens* genomic DNA library an 8 kb *EcoRI* fragment was isolated that hybridized with an oligonucleotide encoding the published N-terminal amino acid sequence of the *S. marcescens* 41 kDa porin. A 41 kDa protein was detected in the outer membrane of *Escherichia coli* NM522 carrying the cloned *S. marcescens* DNA. The cloned gene was sequenced and shown to code for a protein that shared 60–70% identity with other known OmpF and OmpC sequences. The upstream DNA sequence of the *S. marcescens* gene was similar to the corresponding *E. coli* *ompF* sequence; however, a regulatory element important in repression of *E. coli* *ompF* at high osmolarity was absent. The cloned *S. marcescens* OmpF in *E. coli* increased in expression in conditions of high osmolarity. The potential involvement of *micF* in the observed osmoregulation of *S. marcescens* porins is discussed.**

Keywords: *Serratia marcescens*, OmpF, porin, outer membrane, osmoregulation

INTRODUCTION

Serratia marcescens has gained attention in the last 15 years due to its high incidence of antibiotic resistance in clinical cases. β -Lactam resistance in this organism is mainly due to β -lactamase enzymes (Sanders & Sanders, 1992); however, reductions in outer-membrane proteins have been suggested to augment the resistance levels by decreasing the outer-membrane permeability (Gutmann *et al.*, 1985; Hazishume *et al.*, 1993). It is therefore desirable to characterize the outer-membrane porins of this organism which contribute to the permeability of the outer membrane.

Abbreviations: IHF, integration host factor; OBS, OmpR-binding site(s).

The GenBank accession number for the sequence reported in this paper is U81967.

The *Escherichia coli* porins have been well characterized and data is available on their structure, function and regulation. This makes them an ideal model to use as a comparison for the study of *S. marcescens* porins. There are two major non-specific constitutive porins of *E. coli*, referred to as OmpF and OmpC. They are similar in amino acid sequence (Mizuno *et al.*, 1983), immunological reactivity (Hofstra & Dankert, 1980) and ion-selectivity (Benz *et al.*, 1985) yet different in pore size (Nikaido & Rosenberg, 1983), phage selectivity (Datta *et al.*, 1977) and regulation (Pratt *et al.*, 1996). The pore sizes, as determined by single-channel conductance, are 1.1 and 1.2 nm for OmpC and OmpF, respectively (Nikaido & Rosenberg, 1983). This seemingly slight difference in pore size translates into a twofold rate reduction in glucose permeability.

The regulation of the *E. coli* OmpF and OmpC porins is complex. They are both under the control of the *ompB*

operon such that the outer-membrane levels of each porin are inversely related (Hall & Silhavy, 1981a). Cells in high-osmolarity media have high levels of OmpC and low levels of OmpF while the opposite is true in low-osmolarity media. The *ompB* operon encodes an inner-membrane component, EnvZ, and a cytoplasmic component, OmpR (Hall & Silhavy, 1981b). EnvZ and OmpR constitute a two-component histidine kinase regulatory system (Stock *et al.*, 1989). EnvZ is responsible for sensing the external osmolarity and OmpR acts as a transcriptional activator. As EnvZ senses higher levels of osmolarity it phosphorylates OmpR (Aiba *et al.*, 1989a; Forst *et al.*, 1989) and it is the phosphorylated form of OmpR (OmpR-P) which binds the *ompF* and *ompC* regulatory regions and activates transcription (Aiba *et al.*, 1989b). The difference in transcriptional activation is a result of the different organization of the OmpR-binding sites (OBS) of the two genes. Each has three tandem 20 bp OBS but an additional binding site is present 260 bp further upstream in *ompF* (Harlocker *et al.*, 1995). In addition, the OBS have different affinities (Harlocker *et al.*, 1995; Rampersaud *et al.*, 1994). At low levels of OmpR-P the higher-affinity sites of *ompF* are preferentially bound by OmpR and transcription activated. At higher levels of OmpR-P the lower-affinity sites of *ompC* are increasingly bound, resulting in increased transcription. In addition, when the low-affinity upstream site of *ompF* is bound by OmpR-P a DNA loop is formed with the aid of integration host factor (Ramani *et al.*, 1992), which functions to decrease the transcription of *ompF*. Consequently, as external osmolarity increases the levels of OmpC increase and those of OmpF decrease. Specific residues within the OmpR recognition sites have been identified which appear to be essential for OmpR binding (Pratt & Silhavy, 1995).

OmpF levels also decrease with increased growth temperature (37 °C) (Andersen *et al.*, 1989; Lundrigan & Earhart, 1984), the presence of salicylate (Rosner *et al.*, 1991), tetracycline or chloramphenicol (Cohen *et al.*, 1989), and oxidative stress (Chou *et al.*, 1993). Under these conditions MicF (mRNA inhibitory complementing factor) has been implicated in the differential transcription. The *micF* gene is found upstream from the *ompC* gene and is transcribed in the opposite direction (Mizuno *et al.*, 1984). The 4.5S RNA product of the *micF* gene is complementary to the 5' end of the *ompF* transcript and by hybridizing to it decreases the stability of the *ompF* RNA molecule (Schmidt *et al.*, 1995). Transcription of *micF* is activated by OmpR at the same regulatory sites as are involved in *ompC* transcription (Coyer *et al.*, 1990). In addition to OmpR, the transcription of *micF* is influenced by other less clearly defined mechanisms (Andersen *et al.*, 1989; Gambino *et al.*, 1993; Gidrol & Farr, 1993). *micF* transcription has been shown to increase in conditions of high osmolarity (Takayanagi *et al.*, 1991). The role of MicF in osmoregulation, however, has been under much debate (Aiba *et al.*, 1987; Matsuyama & Mizushima, 1985). Ramani *et al.* (1994) suggested that MicF plays a

major role in OmpF osmoregulation under conditions of low to intermediate osmolarity. On the other hand, Silhavy and colleagues criticized this claim, saying that conditions used for these experiments favoured MicF production because the experiments were performed at a high temperature, which increases MicF production tenfold (Pratt *et al.*, 1996). The advantage of such regulation is that a porin with a larger pore will predominate when the cell is in an environment of poor nutrient availability, whereas a porin with a smaller pore will predominate when nutrients are abundant, such as in the human body.

Previous examinations of the *S. marcescens* porins have provided reports of anywhere from one to three porins (Gutmann *et al.*, 1985; Hazishume *et al.*, 1993; Malouin *et al.*, 1990). Two studies have revealed the presence of three porins with molecular masses close to 40 kDa in the outer membrane when using urea-SDS-PAGE (Hazishume *et al.*, 1993; Puig *et al.*, 1993). These investigators classified the protein bands observed on their gels as porins, based on heat-modifiability, or association with peptidoglycan in differential salt extractions. In a third study, only two porins were identified by visualization on conventional SDS-PAGE (Sawai *et al.*, 1987). Malouin *et al.* (1990), however, reported the presence of only one porin in the outer membrane of *S. marcescens* UOC-69. This group did not use the urea-SDS-PAGE system to check for the presence of more than one porin species. However, functional studies and N-terminal amino acid sequencing supported their report of a single porin. We have previously demonstrated the presence of two putative porins in a clinical isolate of *S. marcescens* and the ATCC type strain, based on Southern hybridization studies (Hutsul *et al.*, 1993) and more recently we described the cloning and sequence characterization of an OmpC porin in *S. marcescens* (Hutsul & Worobec, 1994).

It is not clear whether the putative *S. marcescens* porins respond to osmoregulation. Sawai *et al.* (1987) reported the absence of osmoregulation of *S. marcescens* porins, whereas Puig *et al.* (1993) suggested that osmoregulation of two of the three Omps is similar to that observed with *E. coli* porins. In response to salicylate, the synthesis of the 41 kDa porin described by Sawai *et al.* (1987) was repressed while the 40 kDa porin remained unchanged.

Here we report the molecular characterization of a porin in *S. marcescens* that is similar to the *E. coli* OmpF. We also present preliminary analyses of the regulation of both OmpF and OmpC using clinical isolate *S. marcescens* UOC-51 and the *S. marcescens* type strain ATCC 13880.

METHODS

Bacterial strains and growth conditions. *S. marcescens* UOC-51 is a clinical blood isolate whereas ATCC 13880 is the type strain of *S. marcescens*. *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^a lacZAM15]* (Promega) was used for plasmid manipulations. All strains were grown in LB at 37 °C for DNA manipulations. For osmoregulated expression studies strains were grown in LB broth modified for low salt (no NaCl added) or high salt (0.3 M NaCl). For study of salicylate-mediated porin regulation sodium salicylate was added to a final concentration of 5 mM in LB broth. Where required, media were supplemented with 100 µg ampicillin ml⁻¹.

Cloning and sequencing. Preparation of the *S. marcescens* genomic library has been previously described (Hutsul & Worobec, 1994). Plaque lifting and alkaline Southern blotting were performed as described by Maniatis *et al.* (1982) and Reed & Mann (1985). Hybridizations were carried out at 42 °C using an oligonucleotide derived from the N-terminal amino acid sequence of the 41 kDa porin of *S. marcescens* UOC-69 (Malouin *et al.*, 1990). DNA fragments were cloned into pBluescript phagemids (Stratagene). Sequencing was performed with single-stranded and double-stranded templates by the Sanger dideoxy method (Sanger *et al.*, 1977) using the Sequenase kit from USBiochemical and [³⁵S]dATPαS (DuPont). Oligonucleotides for sequencing were synthesized on an Applied Biosystems 391 DNA synthesizer.

Isolation of outer membranes. Outer membranes were isolated on a discontinuous sucrose gradient (Malouin *et al.*, 1990). Cultures (50 ml) were grown overnight and harvested by centrifugation. Cells were resuspended in 50 mM Tris/HCl, pH 8.5, and 2 mM EDTA and passed through a French pressure cell twice at 18000 p.s.i. Unlysed cells and debris were removed by centrifuging at 1200 g for 10 min. The sample was loaded on a two-step sucrose gradient (54% and 70%, w/v, sucrose in 50 mM Tris/HCl, pH 7.9) and centrifuged at 100000 g overnight, 5 °C. The lowest band, containing outer membranes, was collected, diluted with water and centrifuged at 100000 g for 1 h. Outer membranes were resuspended in 50 mM Tris/HCl, pH 8.0.

Isolation of porins. Cell envelopes were prepared following the procedure of Lugtenberg *et al.* (1975). After French-pressure lysis of the cells as described above, cell envelopes were collected by centrifuging the supernatant at 100000 g for 1 h. The cell envelopes were resuspended in 50 mM Tris/HCl, pH 8.0, or 2 mM Tris/HCl, pH 7.8. Cytoplasmic proteins and some outer-membrane proteins were then solubilized from the membranes with 2% (w/v) SDS at 37 °C for 30 min. The sample was centrifuged at 100000 g for 30 min and the pellet, which contained the insoluble porins, was resuspended in 50 mM Tris/HCl, pH 8.0. The SDS-insoluble pellet was washed with water and recentrifuged as above. To further remove OmpA the pellet was resuspended in 50 mM Tris/HCl, pH 7.2, 1% (w/v) SDS, and 5 mM EDTA. After solubilization at 37 °C, the sample was centrifuged at 100000 g for 1 h. The remaining porins were solubilized from the pellet with 50 mM Tris/HCl, pH 7.7, 1% (w/v) SDS, 5 mM EDTA, and 0.4 M NaCl for 1 h at 37 °C (Malouin *et al.*, 1990).

SDS-PAGE. Outer-membrane samples were subjected to SDS-PAGE by the Lugtenberg (1975) system at a running concentration of 11% polyacrylamide. To be able to distinguish between OmpF and OmpC 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%, w/w, glycerol; 5%, v/v, 2-mercaptoethanol; 0.025%, w/v, bromophenol blue) before loading.

RESULTS

The outer membrane of *S. marcescens* UOC-51

Prior to studying *S. marcescens* porins, the outer membrane of strain UOC-51 was examined. The outer membrane was isolated on a discontinuous sucrose gradient and subjected to SDS-PAGE (Fig. 1, lane 1). Two major bands are seen in the outer membrane. The approximately 38 kDa protein is the OmpA protein, which demonstrates heat modifiability (data not shown) with a slower mobility after heating, as described for *E. coli* OmpA protein (Heller, 1978). The *S. marcescens* *ompA* gene has been previously cloned and sequenced (Braun & Cole, 1984). The other major band at approximately 41 kDa represents what we suspected to be a porin. The protein in this SDS-PAGE band exhibits heat modifiability common to many porins, in that it migrates as multiple high-molecular-mass bands when the sample is not heated. Also characteristic of porins, this protein was extracted by differential salt extraction (Fig. 1, lane 2). Amino acid sequencing of the protein from the 41 kDa band (Hutsul *et al.*, 1993) revealed an amino-terminal sequence that closely resembled other porins sequenced, including the *S. marcescens* UOC-69 porin (Malouin *et al.*, 1990).

From the separation by SDS-PAGE (Fig. 1, lane 2) it

Fig. 1. Electrophoretic separation of the *S. marcescens* UOC-51 outer-membrane proteins. Samples (approximately 3 µg per lane) were heated at 100 °C for 10 min before loading. Lane 1, *S. marcescens* outer-membrane proteins separated by SDS-PAGE; lane 2, *S. marcescens* porins separated by SDS-PAGE; lane 3, *S. marcescens* porins separated by 4 M urea-SDS-PAGE. a, *S. marcescens* OmpF porin; b, *S. marcescens* OmpC porin. The *S. marcescens* OmpA is indicated in lane 1. The asterisk indicates the separation of the stronger band on conventional SDS-PAGE into two bands on urea-SDS-PAGE. Molecular mass standards are indicated in kDa; the markers do not apply to the urea-SDS-PAGE sample as they do not migrate as expected.

Fig. 2. Regulation of *S. marcescens* UOC-51 and ATCC 13880 porins by osmolarity (a) and salicylate (b). (a) Urea-SDS-PAGE of *S. marcescens* UOC-51 and ATCC 13880 porins from the SDS-insoluble fractions after growth in low (no NaCl added) and high (0.3 M NaCl) osmolarity media. Samples (approximately 5 µg per lane) were heated at 100 °C before loading. Lane 1, *S. marcescens* UOC-51, no NaCl; lane 2, *S. marcescens* UOC-51, 0.3 M NaCl; lane 3, *S. marcescens* ATCC 13880, no NaCl; lane 4, *S. marcescens* ATCC 13880, 0.3 M NaCl. (b) Urea-SDS-PAGE of *S. marcescens* UOC-51 and ATCC 13880 porins after growth in the presence and absence of salicylate. Samples (approximately 5 µg per lane) were heated at 100 °C before loading. Lane 1, *S. marcescens* UOC-51, no salicylate; lane 2, *S. marcescens* UOC-51, 5 mM salicylate; lane 3, *S. marcescens* ATCC 13880, no salicylate; lane 4, *S. marcescens* ATCC 13880, 5 mM salicylate.

would appear that only one porin is produced. However, it is known that the *E. coli* OmpF and OmpC porins are best separated when urea is added to the gel system (Uemura & Mizushima, 1975). Indeed the *S. marcescens* porin separated into two bands when run in a 4 M urea-SDS-PAGE system (Fig. 1, lane 3). The 40 kDa protein band (labelled b) was previously characterized as OmpC (Hutsul & Worobec, 1994). The higher molecular mass band seen in lanes 2 and 3 was not investigated but may be equivalent to the LamB porin which runs at 47 kDa in *E. coli* (Werts *et al.*, 1993). The difference in number of porins observed between the various studies may be due to different SDS-PAGE systems, growth conditions and/or strains used. In our case, it appears that strain UOC-51 possesses at least two constitutive general diffusion porins as visualized by urea-SDS-PAGE based on differential salt extraction, heat-modified SDS-PAGE mobility and gene sequencing.

Regulation of porins in response to external osmolarity and salicylate

One of the distinguishing features of the *E. coli* OmpF and OmpC porins is that they are osmoregulated (Csonka, 1989). In conditions of low osmolarity OmpF is the major porin seen in the outer membrane while OmpC is the predominant species at high osmolarity. To compare the *S. marcescens* porins with *E. coli* porins, osmoregulation was examined.

S. marcescens UOC-51 and the type strain, ATCC 13880, were grown in conditions of high (0.3 M NaCl) and low (no NaCl added) osmolarity and the porins examined on urea-SDS-PAGE (Fig. 2a). For both strains

tested the concentration of the 41 kDa protein decreased with increased osmolarity (Fig. 2a, lanes 2 and 4) while the concentration of the 40 kDa protein increased; thus both strains appear to exhibit porin osmoregulation. The pattern of osmoregulation, however, does not match that reported for *E. coli*, where characteristically the quantity of OmpC is greater than that of OmpF at high osmolarity. In *S. marcescens* the 41 kDa porin remained more abundant than the 40 kDa porin despite the changing ratio of the protein. Puig *et al.* (1993) observed osmoregulation in the *S. marcescens* strains examined and the pattern was similar to that seen in *E. coli*. Sawai *et al.* (1987) did not observe osmoregulation of *S. marcescens* porins in their study. We also previously reported the absence of osmoregulation in this strain prior to the use of urea in the gel system (Hutsul *et al.*, 1993).

Salicylate is a natural substance that bacteria may encounter in some plants or in the human host after oral intake of foods or aspirin (Budavari, 1989; Rosner, 1985). The concentration of *E. coli* OmpF porin is known to decrease in response to salicylate, and a reduction in 41 kDa and 40 kDa porins has also been observed in *S. marcescens* and *Klebsiella pneumoniae*, respectively (Sawai *et al.*, 1987). Therefore, regulation of the *S. marcescens* UOC-51 porins in response to salicylate was examined.

Following growth in the presence and absence of 5 mM salicylate, the outer membranes were isolated from both *S. marcescens* UOC-51 and ATCC 13880 and loaded on a 4 M urea-SDS-PAGE gel (Fig. 2b). For both *S. marcescens* UOC-51 (Fig. 2b, lane 2) and ATCC 13880 (Fig. 2b, lane 4) the amount of higher molecular mass protein, approximately 41 kDa, decreased when the cells were grown in 5 mM salicylate. This result was also obtained by Sawai *et al.* (1987). In addition, for both strains tested the 40 kDa protein increased when cells were grown in the presence of salicylate. Previous studies observed little or no change in the 40 kDa porin of *S. marcescens* or OmpC of *E. coli* (Rosner *et al.*, 1991; Sawai *et al.*, 1987). The reason for the difference in OmpC regulation is unclear; the OmpF result is as expected.

Cloning of the *S. marcescens* ompF gene

We previously described the cloning of an *ompC* gene from a sub-genomic phage library prepared from *S. marcescens* UOC-51 (Hutsul & Worobec, 1994). Using the same oligonucleotide probe which was designed from the N-terminal amino acid sequence of the *S. marcescens* UOC-69 porin (Malouin *et al.*, 1990) a second distinct phage isolate, λgtS1, was obtained from the library. The *EcoRI* fragment contained in λgtS1 was determined to be 8 kb in size. This fragment was mapped with restriction endonucleases and shown to differ from the 11 kb fragment carrying the *ompC* gene. The map of this isolate is shown in Fig. 3. The sizes of the two *EcoRI* fragments in the phage isolates are consistent with the sizes of the two *EcoRI* fragments that

Fig. 3. Restriction endonuclease map of the 8 kb *S. marcescens* genomic DNA isolate, λ gtS1, and its subclones. The pS1E(-) plasmid contains the entire 8 kb *Eco*RI genomic DNA fragment of λ gtS1. The location of the porin gene is boxed with the direction of transcription indicated. E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Sp, *Sph*I.

Fig. 4. Expression of the cloned *S. marcescens* OmpF porin in *E. coli*. Approximately 3–5 μ g samples were heated at 100 °C for 10 min prior to loading on SDS-PAGE. Lane 1, *S. marcescens* UOC-51 porins; lane 2, *E. coli* NM522/pS1E SDS-insoluble fraction; lane 3, *E. coli* NM522 outer membrane. a, *S. marcescens* OmpF porin; b, *E. coli* OmpF/C porins; c, *E. coli* OmpA protein. Molecular mass standards are indicated in kDa.

were detected in Southern hybridizations with the *S. marcescens* UOC-69 N-terminal oligonucleotide (data not shown).

The entire 8 kb *Eco*RI fragment was transferred into pKS(-), creating the plasmid pS1E(-), to allow expression and examination of the cloned protein. An approximately 41 kDa product was detected by SDS-PAGE of outer membranes prepared from *E. coli* NM522/pS1E(-) (Fig. 4 lane 2) which was not seen in the outer membranes prepared from *E. coli* NM522 without the plasmid (Fig. 4, lane 3). Polyclonal antibodies specific for the UOC-51 porins reacted with this protein in immunoblot experiments (data not shown), confirming that a porin gene was cloned. The protein produced

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TTTCGGCTGGGCTTTGAACGTTTAAATCGCTATGTCACCGCGTGCAAAACGTGCGCA 60
TGTGATTCCATCCCGCGACGCCGCGTAACGCAAGCTTCTAATTATACGATCACAGT 120
TTTTTAAACGATTTCGTTACAAAACAAAGGCCAGCATCGTGGCCTTTGCGATTTTTTAA 180
TATTGACGCAGTCACAAAGTTCGCCAAAAATTACATTGGTTACACATACTTCTCTTT 240
GCAACCTGATTGGACATTGGTATCATTTCGTCCTAGATTAAACCGCCTGTGAATGGAA 300
CACTGCGTTTCAGACACAGGACGACCAATCTATCAACAATAGTTCCTCAAGGATTATTG 360
-10 M M K R N
GCGGCAGTGGCAAAGGTGTCCGAATAACACCAATGAGGGTAATAATGATGAAGCGCAACA 420
I L A V V I P A L L A A G A A N A A G E I
TTCTGTCAGTGGTAATCCCGCTCTGTTGGCTGCTGGTGCAGCAACGAGCTGAAATCT 480
Y N K D G N K L D L Y G K V D G L H Y F
ACAACAAGACGCGCAACAAGCTGGATCTGTACGGCAAGTTGACGGTCTGCACACTTCT 540
S K D K G N D G D Q T Y V R F G F K G E
CCAAAGACAAAGTAATGACGGCGATCAGACCTATGTTCTGTTTCGGCTTCAAAGGTGAAA 600
T Q I T D Q L T G Y G Q W E Y N V Q S N
CCCAGATTACTGACCAACTGACCGGTTACGGCCAGTGGGAATACAACGTTCACTCAACC 660
H A E S Q G T E G T K T R L G F A G L K
ACGCCGAATCTCAGGGCACCGAAGGCACAAAACCGCTCTGGGCTTCGCGGCTGTAAT 720
F A D Y G S F D Y G R N Y G V L Y D V E
TCGCTGACTACGGCTCTTCGACTACGGCCGTAACACTGCGGTACTGTACGAGCTGGAAG 780
G W T D M L P E F G D T Y T Y S D N F
GCTGGACCGATATGCTGCCAGAGTTCGGTGGCGATACTTACACTACTCCGACAACCTCA 840
M T G R T N G V A T Y R N N N F F G L V
TGACCGGCGTACCAACGGCGTTCGACCTATCGTAACAACACTTCTTCGGTCTGGTTG 900
D G L N F A L Q Y Q G K N Q N D G R D V
ACGGTCTGAACCTCGCTCTGCAATACCGGGCAAAAACGAAACGACGCGCGTGACGTCA 960
K K Q N G D G W G I S S T Y D I G E G V
AAAAACAAAACGGCGACGGCTGGGCGATTCTCTACTTATGACATCGCGCAAGCGGTAA 1020
S F G A A Y A S S N R T D D Q K L R S N
GCTTCGGCGCTGCATACGCTTCTTCAACCGTACCGACGACGAAACTGCGTTCAAACG 1080
E R G D K A D A W T V G A K Y D A N N V
AGCGTGGCGACAAGCTGACGATGGACCGTGGGCGCAAAATATGACGCCAACACGCTCT 1140
Y L A A M Y A E T R N M T P F G G N F
ACCTGGCGCAATGTATGAGAAACCGTAACATGACTCCGTTCCGTTGGCGGTAACCTCA 1200
T N T C A A T E N C G G F A S K T Q N F
CCAACACTTCGCGAGCTGAAAACCTCGCGCGCTTCGTAAGCAAACTCAGAATCTCG 1260
E V T A Q Y Q F D F G L R P E V S Y L Q
AAGTACTGCTCAGTATCAGTTGACTTCGGTCTGCGCCAGAAAGTGTCTTACCTGCACT 1320
S K G K N L N V P G V G S D Q D L V K Y
CTAAGGGTAAAAACCTGAACGTCACGCGTGGGTTCTGACCAAGATCTGGTTAAATATG 1380
V S V G T T Y Y F N K N M S T Y V D Y K
TTTCTGTTGGTACCCTTACTTCAACAAAACATGTCCACCTATGTTGATTACAAAA 1440
I N L L D D N D F T K A T G I A T D D I
TCAACCTGCTGGATGACACGACTTCACCAAGCAACCGGATCGCAACTGACGATATCG 1500
V G V G L V Y Q F -
TAGGCGTTGGTTTGGTATACAGTCTAAGTTGTCTCGCTTATCGGCGTTCCACGTCGA 1560
CTAAGTTAAACAAACAGGCTTCGGCTGTTTGTGTATCAGCGGTAACCTTTTAT 1620
CCTGCCGATTCCTCCCTCCCATCTTTTCTGTCGAATCTTCACTGTCATAAGATCG 1680
CGGTGTTTATTCGCAACCGG 1701

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Fig. 5. Nucleotide sequence of the *S. marcescens* OmpF 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 OBS are double underlined with arrow heads bordering each one. A putative transcription 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. The cysteines present in the sequence are circled.

appeared to be the same size as the major band in the *S. marcescens* outer membrane (Fig. 4, lane 1). In addition, this protein migrated more slowly than the previously cloned *S. marcescens* OmpC, appearing to be about 1 kDa larger. These results suggest that this may be the major porin seen in the *S. marcescens* outer membrane. OmpA is not seen in lane 2 because the SDS-insoluble

Table 1. Amino acid sequence comparison between the *S. marcescens* 41 kDa porin and other OmpF and OmpC porins

Values are percentage identity between porins as determined by PALIGN. Abbreviations: SM41KDA, *S. marcescens* UOC-51 41 kDa porin; ECOOMPF, *E. coli* OmpF (Inokuchi *et al.*, 1982); ECOOMPC, *E. coli* OmpC (Mizuno *et al.*, 1983); KPNOMPC, *Klebsiella pneumoniae* OmpC (Alberti *et al.*, 1995); STYOMPC, *Salmonella typhi* OmpC (Puente *et al.*, 1989).

	SM41KDA	ECOOMPF	ECOOMPC	KPNOMPC	STYOMPC
SM41KDA	.	67.7	68.4	71.5	70.3
ECOOMPF	.	.	64.9	64.5	65.9
ECOOMPC	.	.	.	81.8	84.2
KPNOMPC	.	.	.	.	82.7
STYOMPC	.	.	.	.	.

fraction of the outer membrane was used, whereas because the sample in lane 3 was prepared from unextracted outer membranes OmpA was visible.

S. marcescens ompF sequence

The cloned gene was sequenced in both directions using various synthesized primers. The nucleic acid sequence and the deduced amino acid sequence are illustrated in Fig. 5. The gene has an ORF of 1122 bp, with a typical Shine–Dalgarno sequence centred nine nucleotides upstream of the start codon, and a potential terminator hairpin downstream. The amino acid sequence from 22 to 41 was identical to that determined by N-terminal amino acid sequencing of the extracted 41 kDa outer-membrane protein (Hutsul *et al.*, 1993) and to the OmpC porin (Hutsul & Worobec, 1994). The calculated molecular mass of the mature protein is 39.0 kDa, which is 0.5 kDa smaller than the mature *S. marcescens* OmpC porin despite a slower SDS-PAGE mobility. The primary sequence exhibited characteristics of other porins including an N-terminal phenylalanine (Struyvé *et al.*, 1991) and the conserved PEFGGD motif (Cowan *et al.*, 1992). An interesting feature seen in this porin sequence is the presence of two cysteines at positions 269 and 275. A comparison with the *E. coli* OmpF amino acid sequence showed that the cysteines would be located in the sixth external loop of the *E. coli* porin structure (Cowan *et al.*, 1992). Cysteine residues have not been reported in the sequence of any classical trimeric diffusion porins. However, cysteines have been identified in the *Pseudomonas aeruginosa* porins OprF (Duchêne *et al.*, 1988) and OprB (Wylie & Worobec, 1994), and the LamB porin of *E. coli* (Clément & Hofnung, 1981). In the *P. aeruginosa* non-selective monomeric porin, OprF, the four cysteines present are proposed to function in switching between the two functional pore sizes observed for this porin through alternate disulphide bonds (Hancock, 1987). In the case of *E. coli* LamB, replacement of the two cysteines with serine had no effect on maltodextrin binding (Ferenci & Stretton, 1989) although a slight reduction in trimer stability was noted. The function, if any, of the two cysteines in *P. aeruginosa* OprB is not known.

Amino acid sequence comparisons of the cloned *S. marcescens* porin with other enteric porins demonstrated approximately 60–70% identity but did not place it within one distinct group (Table 1). The upstream nucleotide sequence of the cloned gene most closely resembles the *E. coli* ompF upstream sequence. With the *E. coli* sequence as a guide, the OBS and promoter sequences were located. However, it is interesting to note that the similarity ends just before the three consecutive OBS. In *E. coli*, the ompF gene is located after *asnS*, a gene encoding asparaginyl tRNA synthetase (Aoki *et al.*, 1992). The intergenic region is 600 bp in size and contains regulatory components including the negative OBS at –380 to –360 and integration host factor (IHF) binding sites (Huang *et al.*, 1994). An *asnS* gene was also identified upstream to the cloned *S. marcescens* ompF gene; however, the intergenic region is approximately 300 bp shorter. Further examination showed that a region of DNA after the *asnS* terminator to just upstream of the OBS is absent (Fig. 6). Included in the segment would be the OBS and IHF recognition sequences crucial for repression of ompF transcription. Therefore, osmoregulation via the OmpR/EnvZ system as it is described in *E. coli* cannot take place in *S. marcescens* and probably instead involves *micF*. A similar result was recently observed in *Xenorhabdus nematophilus* (Forst *et al.*, 1995).

The hypothetical secondary structure of the *E. coli* 4.5S MicF RNA and its hybridization with ompF has been determined (Schmidt *et al.*, 1995). A *micF* gene from *S. marcescens* UOC-51 was previously described (Hutsul & Worobec, 1994) and the secondary structure of the *S. marcescens* 4.5S MicF has been predicted (Schmidt *et al.*, 1995). Based on this structure and the *E. coli* ompF/MicF hybridization, the hybridization of the *S. marcescens* MicF with the *S. marcescens* ompF sequence was predicted and is shown in Fig. 7. Sites in the *S. marcescens* *micF* sequence that are not conserved with the *E. coli* *micF* are specifically complementary to the *S. marcescens* ompF, thus enabling MicF/ompF hybridization. Therefore, it is predicted that *micF* could function in regulating expression of the OmpF porin in *S.*

Fig. 6. Alignment of the partial DNA sequence of *E. coli ompF* (Inokuchi *et al.*, 1982) and *asnS* (Aoki *et al.*, 1992) genes and the *S. marcescens ompF* upstream DNA sequence. The sequences shown are from within the *asnS* genes to approximately 70 bp after the translation start of the *ompF* genes. The termination codon, TAA, of each of the *asnS* genes is in bold followed shortly by the terminator hairpin designated with horizontal arrows. OBS are boxed. The IHF-binding region is double underlined. Vertical lines show sites that are conserved between the two genes. Deletions are represented by a dashed line. EC, *E. coli*; SM, *S. marcescens*.

Fig. 7. Predicted hybridization of the *S. marcescens micF* transcript with the 5' end of the *S. marcescens ompF* mRNA.

marcescens. In addition, the observed complementarity between the regions of *ompF* and MicF that are proposed to function in hybridization supports the

Fig. 8. Osmoregulation of the *S. marcescens* OmpF porin. SDS-insoluble cell envelope fractions of cells grown in the absence or presence of NaCl were run on SDS-PAGE. Samples (approximately 3 µg per lane) were loaded after heating at 100 °C for 10 min. Lane 1, *E. coli* NM522/pS1E(-), no NaCl; lane 2, *E. coli* NM522/pS1E(-), 0.3 M NaCl. The arrow indicates the cloned *S. marcescens* porin in the *E. coli* outer membrane while the band below it consists of the *E. coli* OmpF and OmpC porins.

secondary structure model proposed by Schmidt *et al.* (1995).

Regulation of the *S. marcescens* OmpF in response to osmolarity

The response of the cloned *S. marcescens* OmpF porin to the osmolarity of the medium was examined in *E. coli*. *E. coli* NM522/pS1E(-) cells were grown in LB containing 0.3 M NaCl or no added NaCl. The SDS-insoluble fraction was isolated and subjected to SDS-PAGE. From Fig. 8 it can be seen that the level of cloned *S. marcescens* OmpF increased when the cells were grown in the high-osmolarity condition (Fig. 8, lane 2). The porin samples isolated from cells grown in different osmolarity were run on non-urea SDS-PAGE and therefore the OmpF and OmpC of *E. coli* were not distinguished. However, in general there is less native *E. coli* porin present in the high-salt condition.

DISCUSSION

There has been some controversy in the literature regarding the number of porins in the *S. marcescens* outer membrane and whether any are osmoregulated. Many more outer-membrane proteins are probably present but were not detected because they are produced in lower levels than the *S. marcescens* porins and OmpA. In this study two porins were observed in *S. marcescens* UOC-51 and ATCC 13880 which were inversely regulated in response to the osmolarity of the medium.

This was only evident when a urea-SDS-PAGE system was employed. A previous report suggesting the absence of osmoregulation of *S. marcescens* porins did not use the urea-SDS-PAGE method and it is therefore possible that porins in those strains are also osmoregulated (Sawai *et al.*, 1987). In addition, the porins in our study responded to salicylate. The mechanism of decreased OmpF expression due to salicylate in *E. coli* involves *micF*, which is specific for the *ompF* transcript. Since the expression of the *S. marcescens* 41 kDa outer-membrane protein was reduced in salicylate-grown cells, our studies suggest that it is equivalent to *E. coli* OmpF. The pattern of expression of the two porins in high- and low-osmolarity conditions also indicates that the 41 kDa protein may be equivalent to OmpF while the 40 kDa protein is probably OmpC.

The amount of cloned *S. marcescens* OmpF porin increased in the *E. coli* outer membrane when cells were grown under conditions of high osmolarity. The OBS present, therefore, appear to function in *E. coli*. An OmpF porin would be expected to decrease in *E. coli* but our result is not surprising as there are no negative regulatory sites in the *S. marcescens* *ompF* gene. The MicF produced by the two organisms are homologous. Thus we would expect the *E. coli* MicF to reduce the amount of *S. marcescens* OmpF produced. This, however, may not be observed due to the high copy number of the cloned gene expected to be present in the cell. MicF may actually reduce the number of OmpF molecules; however, with the increased OmpR-P more *ompF* RNA is likely produced and thus the reduction by MicF would be masked. Aiba *et al.* (1987) found that the ratio of molecules of MicF to molecules of *ompF* mRNA was important in determining the extent of OmpF repression. It also remains possible that the sequence of *E. coli* MicF is not sufficiently similar to enable repression of *S. marcescens* OmpF production or that the OmpR/IHF mechanism is required for complete repression in conditions of high osmolarity.

The observation of osmoregulation of OmpF in *S. marcescens*, which lacks a repressor OBS, is consistent with *micF* playing a major role in osmoregulation (Ramani *et al.*, 1994). The possibility remains, however, that the observed changes in expression of the OmpF porin may have occurred through an alternative negative-OBS. OmpR is required for efficient transcription of either the *ompF* or *ompC* porin gene regardless of medium osmolarity, but if MicF can replace OmpR in osmoregulation function, what is the need for OmpR/EnvZ for this purpose in *E. coli*? MicF decreases the amount of OmpF produced but has no effect on OmpC production. Increased *ompC* transcription in conditions of high osmolarity requires OmpR. *micF* itself is under the control of the OmpR/EnvZ system and its transcription increases in high-osmolarity conditions in concert with *ompC*. The presence of both mechanisms of OmpF repression, the OmpR/IHF-mediated loop mechanism and MicF may therefore enhance the response of decreased OmpF production (Aiba *et al.*, 1987). In the case of *S. marcescens* the more

stringent OmpR/IHF mechanism of OmpF repression may not be as critical since its natural soil habitat favours OmpF expression. If choosing only one mechanism, *micF* may be considered the more advantageous mechanism of OmpF repression for *S. marcescens* since it is capable of regulation under several conditions.

micF is involved in reducing the amount of *E. coli* OmpF in the outer membrane under a number of environmental conditions, including increased growth temperature, oxidative stress and exposure to certain antibiotics (Andersen *et al.*, 1989). The phenomenon of multiple antibiotic resistant strains of *E. coli* occurs through a complicated mechanism of global transcriptional regulation which includes elevated transcription of *micF* (Gambino *et al.*, 1993). A survey of the *mar* operon in a number of Gram-negative bacteria demonstrated the presence of the operon in a variety of members of the *Enterobacteriaceae*, although not *S. marcescens* (Cohen *et al.*, 1993). This does not mean that it is necessarily absent from *S. marcescens* since it is possible that the hybridization conditions were too stringent to detect the operon in a bacterium with some divergence from *E. coli*. Reduced levels of the 41 kDa porin have been observed in strains of *S. marcescens* with high levels of antibiotic resistance (Gutmann *et al.*, 1985). It is possible that the mechanism of reduction of this protein could occur by a mechanism that is at least similar to that observed for strains with multiple antibiotic resistance.

In summary, the general diffusion porins produced by *S. marcescens* are similar to *E. coli* OmpF and OmpC. They also exhibit regulation in response to osmolarity and salicylate although with some unique features. The *S. marcescens* OmpF has an identity of 60–70% with other enteric porins but does not resemble the *E. coli* OmpF specifically. However, the upstream nucleic acid sequence and the location of the gene suggest that it encodes an OmpF-like porin. The recombinant porin does not decrease under high-osmolarity conditions when expressed in *E. coli*, but this can be explained by the absence of the DNA region which includes the site of negative regulation found in the *E. coli* *ompF* gene. The *ompF* mRNA is predicted to be able to hybridize with the *micF* transcript and thus be negatively regulated in conditions that activate *micF* transcription.

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