A 17 kDa outer-membrane protein (Omp4) from *Serratia marcescens* confers partial resistance to bacteriocin 28b when expressed in *Escherichia coli*

Joan F. Guasch, Santiago Ferrer, Josefina Enfedaque, M. Beatriz Viejo and Miguel Regué

Author for correspondence: Miguel Regué. Tel: +34 3 4024496. Fax: +34 3 4021886.
e-mail: regue@farmacia.far.ub.es

Department of Microbiology and Parasitology, Health Sciences Division, Faculty of Pharmacy, University of Barcelona, 08028 Barcelona, Spain

A cosmid-based genomic library of *Serratia marcescens* N28b was introduced into *Escherichia coli* and clones were screened for a bacteriocin 28b insensitive phenotype. One clone was found that showed partial resistance to bacteriocin 28b. By using Tn5tac1 insertions it was shown that this phenotype was due to the expression in *E. coli* of an outer-membrane protein of 17 kDa (Omp4). The DNA region defined by insertion mutagenesis was sequenced and found to contain an ORF of 515 bp. The deduced amino acid sequence has 172 residues with a theoretical molecular mass of 18.4 kDa. The protein contains an N-terminal signal sequence of 24 amino acid residues and, when compared to other enterobacterial outer-membrane proteins, most closely resembles a family of small outer-membrane proteins of *Enterobacteriaceae* whose known functions appear to be related with virulence. Immunoblotting experiments showed that Omp4 is present in 15 biotypes of *S. marcescens*. The bacteriocin 28b resistance phenotype conferred on *E. coli* by Omp4 appears to be pleiotropic since overexpression of the Omp4-encoding gene leads to a decrease in the amount of OmpA, OmpF and/or OmpC; OmpA and OmpF are the receptors for bacteriocin 28b in *E. coli*.

Keywords: *Serratia marcescens*, outer-membrane protein, bacteriocin 28b

INTRODUCTION

The outer membrane of Gram-negative bacteria, irrespective of its function, has been exploited by both bacteriophages and bacteriocins, which use outer-membrane components as receptors. In a previous work we reported on a colicin-like bacteriocin produced by *Serratia marcescens* N28b (Viejo *et al.*, 1992). This bacteriocin, termed bacteriocin 28b, is a polypeptide of 47.5 kDa which is active against *Escherichia coli*. The gene (*bss*) encoding bacteriocin 28b was cloned into *E. coli*, expressed, and sequenced. The predicted product showed amino acid sequence homology with pore-forming colicins in the C-terminal part of the protein, which is known to be responsible for the lethal activity. This result suggested that bacteriocin 28b could have a pore-forming mechanism of killing action.

Determinants for colicin production studied so far are carried by plasmids (colicinogenic or Col plasmids) classified as small high-copy-number or large low-copy-number plasmids (Hardy *et al.*, 1973; Pugsley, 1984a). An important feature of these plasmids is that they confer on their host the property of being specifically insensitive (immune) to the colicin encoded by the plasmid (Pugsley, 1984b). The gene responsible for colicin immunity is always located downstream of and adjacent to the colicin structural gene (de Graaf & Oudega, 1986; Mankovich *et al.*, 1986; Schramm *et al.*, 1987). In cells producing pore-forming colicins the homologous immunity proteins are located in the cytoplasmic membrane, where they block the lethal activity of the pore-forming colicins (Jakes & Lazdunski, 1992). The bacteriocin 28b structural gene appears to be located on the chromosome (Viejo *et al.*, 1992), and a unique feature of this system is the absence of a gene similar to those of the colicin immunity genes located downstream from the *bss* gene (Viejo *et al.*, 1995).

The EMBL accession number for the sequence reported in this paper is Z37157.

In order to identify *S. marcescens* N28b genes involved in protection against its own bacteriocin a genomic library of *S. marcescens* N28b was introduced into *E. coli* and screened for *E. coli* clones that were insensitive to bacteriocin 28b. This screening should detect clones in which the bacteriocin could not bind to its receptors (resistant phenotype) and clones in which, upon bacteriocin-receptor interaction, bacteriocin translocation through the envelope was arrested (tolerant phenotype). The term insensitive was used to denote both cases. In this work we report the characterization of an outer-membrane protein (Omp4) of *S. marcescens* that, when expressed in *E. coli*, confers partial resistance to bacteriocin 28b.

METHODS

Bacterial strains and growth conditions. *S. marcescens* N28b has been described previously (Gargallo-Viola, 1989). *E. coli* 5K F⁻ r_k⁻ m_k⁻ rpsL thr thi leu lacZ (Juárez *et al.*, 1984) was used as recipient in subcloning experiments. *E. coli* NM554 recA13 araD139 Δ(ara leu) 7696 Δ(lac)17 A galU galK hsdR rpsL mcrA mcrB (obtained from Stratagene) was used as recipient for the *S. marcescens* N28b cosmid-based genomic library. Bacteria were grown in Trypticase Soy Broth (TSB) and on Trypticase Soy Agar (TSA). When required, media were supplemented with ampicillin (50 µg ml⁻¹), chloramphenicol (50 µg ml⁻¹) or kanamycin (30 µg ml⁻¹).

Bacteriocin 28b sensitivity assays. The overlay test was used for qualitative bacteriocin sensitivity assays (Pugsley & Oudega, 1987). The procedure for the quantitative bacteriocin sensitivity assays was adapted from that described by Cavard & Lazdunski (1981). Cells were grown in TSB and adjusted to an OD₆₀₀ of 0.8. Samples (10 µl) containing different amounts of bacteriocin were added to 10 µl cells in a microtitre plate. The microtitre plates were incubated for 20 min at 37 °C with shaking. As a control, 10 mM sodium phosphate buffer (pH 7.0) was used instead of the bacteriocin dilution. TSB medium (200 µl) was then added and the cultures were incubated at 37 °C with shaking until the OD₆₀₀ reached 1.0 in the control. A unit (U) of bacteriocin activity was defined as the amount of bacteriocin reducing the growth of *E. coli* 5K tenfold.

Cloning and subcloning. Genomic DNA from *S. marcescens* N28b was isolated by the hexadecyltrimethylammonium bromide procedure (Ausubel *et al.*, 1987), and was partially digested with *Sau3A*. Cosmid SuperCos1 (Stratagene) was first digested with *Xba*I, dephosphorylated, digested with *Bam*HI and then ligated to *Sau3A* genomic DNA fragments. Ligated DNA was packaged using the Gigapack II Gold (Stratagene) according to the manufacturer's instructions. The library was introduced into *E. coli* NM554 and recombinant cosmids were selected in TSA containing ampicillin and kanamycin. Clones were screened for bacteriocin 28b insensitive phenotype. Standard procedures were used for restriction mapping of recombinant cosmid DNA (Maniatis *et al.*, 1982). To obtain subclones, partially *Sau3A*-digested recombinant cosmid DNA was ligated to *Bam*HI-cut pBR328 DNA. Ligated DNA was transformed into *E. coli* 5K and plated on TSA containing ampicillin and chloramphenicol.

Isolation of plasmids carrying Tn5tac1 insertions. *E. coli* 5K carrying recombinant plasmid pCR001 coding for Omp4 was mutagenized using λ: Tn5tac1 (Chow & Berg, 1988). After 1 h of phenotype expression, kanamycin (30 µg ml⁻¹) was added to the cultures. After overnight growth, plasmid DNA was

isolated and retransformed into *E. coli* 5K. Kanamycin-resistant transformants were assayed for bacteriocin-insensitive phenotype, and the points of Tn5tac1 insertions were determined by restriction mapping.

Purification of Omp4. Omp4 was partially purified from *E. coli* 5K harbouring pCR001 using a procedure described for partial purification of the Ail protein (Miller *et al.*, 1990). Cells from a 100 ml overnight culture grown at 37 °C in TSB were collected by centrifugation at 4000 g for 15 min (4 °C). The cell pellet was suspended in 5 ml 1% (w/v) octylglucoside (Aldrich Chemical Co.) in 20 mM Tris (pH 8.0) and incubated at 25 °C for 30 min. Cell debris was removed by centrifugation at 14000 g for 15 min (4 °C), and 340 µl 3 M KCl was added to the supernatant fluid. The mixture was incubated at 25 °C for 30 min and Omp4 was sedimented by centrifugation at 14000 g for 15 min (4 °C). Omp4 was separated by SDS-PAGE and transferred to Hybond N (Applied Biosystems) membranes.

Rabbit anti-Omp4 serum preparation. Antibodies against Omp4 were raised in a rabbit by subcutaneous injection of a solution containing 100 µg purified Omp4 combined with either Freund's complete or Freund's incomplete adjuvant. Injections were performed over an 8 week period. About 20 ml blood was collected, allowed to clot at 4 °C overnight, and serum was obtained by centrifugation.

SDS-PAGE and immunoblot analysis. Overnight cultures (10 ml) were adjusted to 1 × 10⁹ c.f.u. ml⁻¹. Outer-membrane fractions were isolated as described by Matsuyama *et al.* (1984). The outer-membrane proteins were analysed on 14% (w/v) polyacrylamide gels containing 0.2% SDS. Gels were stained with Coomassie Blue or immunoblots were prepared by electrophoretic transfer to Hybond N (Applied Biosystems) membranes (Tsang *et al.*, 1983). The membranes were incubated sequentially with a rabbit polyclonal anti-Omp4 serum and anti-rabbit IgG antibody conjugated to alkaline phosphatase (Blake *et al.*, 1984).

DNA sequencing. Sequencing of the Omp4-encoding gene was performed with double-stranded plasmid templates by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the T7 DNA sequencing kit from Pharmacia LKB Biotechnology and [³⁵S]dATP-αS (NEN-Du Pont). Oligonucleotides I (5'-CTCCCCGAGATCTGATCAA-3') and O (5'-CCCCTACTTGTGTATAAG-3') were used to sequence from the ends of Tn5tac1 insertions. Other custom-designed primers were used to complete the gene sequence.

DNA and protein sequence analysis. The DNA sequence was transformed into amino acids, using all six frames, and all ORFs greater than 100 bp were inspected. Deduced amino acid sequences were compared with those of DNA translated in all six frames from the non-redundant GenBank V.76 and EMBL V.34 database by using the BLAST network service at the NCBI (Altschul *et al.*, 1990). A codon preference table for *S. marcescens* genes was built to identify the Omp4-encoding gene. Alignments and protein similarity calculations were performed using the GCG package (Genetics Computer Group, Madison, Wisconsin). Hydrophathy profiles were calculated according to Kyte & Doolittle (1982) with a window of seven amino acid residues. Phylogenetic studies were performed by using both the neighbour joining method (Saitou & Nei, 1987) from the CLUSTAL V package (Higgins *et al.*, 1992) and a parsimony method from the PHYLIP package (Felsenstein, 1989).

Southern blot hybridization. A 351 bp *Kpn*I-*Pst*I DNA fragment containing an internal part of the Omp4-encoding gene was labelled with digoxigenin as described by the manufacturer (Boehringer Mannheim). Isolated genomic DNA

was electrophoresed, denatured and transferred to a Hybond B membrane. After baking, the membrane was prehybridized and hybridized in $5 \times \text{SSC}$, 0.5% blocking reagent (Boehringer Mannheim), 0.1% Sarkosyl and 0.02% SDS. Washing, antibody incubation and signal detection with *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim) were as described by the manufacturer.

N-terminal amino acid analysis. The sequence of the N-terminal 10 amino acids of the purified Omp4 was determined by automated Edman degradation using an Applied Biosystems 470A gas-phase sequencer equipped with a model 120A on-line phenylthiohydantoin analyser (Applied Biosystems).

Complement resistance assay. Pooled normal human serum was used for the complement resistance assay using the method described by Bliska & Falkow (1992).

RESULTS AND DISCUSSION

Cloning of an *S. marcescens* gene determining bacteriocin 28b insensitivity in *E. coli*

A cosmid-based genomic library of *S. marcescens* N28b was introduced by transduction into *E. coli* NM554. Transductants were screened for bacteriocin 28b insensitivity by the agar-overlay technique. From about 1000 colonies screened, several clones were found which were apparently bacteriocin 28b insensitive. Quantitative assays showed that one of these recombinant cosmids conferred a bacteriocin 28b insensitive phenotype upon *E. coli* NM554. Bacteriocin binding experiments showed that this clone was able to bind ten times less bacteriocin than *E. coli* NM554 harbouring vector SuperCos1 (results not shown), suggesting that the clone was bacteriocin 28b resistant. This resistance phenotype was partial since the cells become bacteriocin sensitive when higher bacteriocin 28b concentrations were used. The recombinant cosmid was isolated and mapped with restriction endonucleases. Several subclones were constructed using pBR328 as vector. The restriction map of the smallest subclone,

pCR001, still able to confer bacteriocin 28b resistance (Fig. 1) is shown in Fig. 2.

Bacteriocin 28b resistance is due to an outer-membrane protein

To identify the gene product responsible for the bacteriocin 28b resistance phenotype, cytoplasmic, periplasmic, inner and outer membrane fractions were prepared from *E. coli* 5K, carrying pCR001 or the vector pBR328. Analysis of these preparations by SDS-PAGE showed the presence of an outer-membrane protein with an apparent molecular mass of 17 kDa in the bacteriocin-insensitive clone (Fig. 3). Furthermore, this outer-membrane protein was absent from *E. coli* 5K carrying vector plasmid pBR328, and a protein band with a very similar molecular mass was present in outer membrane preparations from *S. marcescens* N28b. The small difference in mobility between the 17 kDa protein in the *E. coli* and *S. marcescens* backgrounds is probably due to the presence of O antigen containing lipopolysaccharide in the preparations of the *S. marcescens* strain, while outer-membrane preparations from the *E. coli* strains used in this study do not contain O antigen. To prove that the bacteriocin 28b resistance phenotype was indeed due to the 17 kDa outer-membrane protein, Tn5*tac*1 insertion mutants of pCR001 were obtained. Each was phenotypically characterized by determining the bacteriocin resistance phenotype, the pattern of outer-membrane proteins, and the points of Tn5*tac*1 insertion (Figs 2 and 3). All the Tn5*tac*1 insertions in pCR001 that abolished the bacteriocin-resistance phenotype appeared to cause loss of the 17 kDa outer membrane protein (Fig. 3). These results strongly suggest that the presence in *E. coli* 5K of the 17 kDa outer-membrane protein is responsible for the bacteriocin resistance phenotype. This protein was named Omp4, in keeping with the nomenclature used for *S. marcescens* outer-membrane proteins (Puig *et al.*, 1993).

Fig. 1. Bacteriocin 28b resistance conferred by pCR001. Bacteriocin 28b sensitivity assay was carried out as described in Methods. Each assay was done independently four times; the results are means $\pm$ SEM. ○, *E. coli* 5K harbouring pBR328 (Omp4⁻); ●, *E. coli* 5K harbouring the recombinant plasmid pCR001 (Omp4⁺).

Nucleotide sequence of the *S. marcescens* gene encoding Omp4

A nucleotide sequence of 667 bp was determined in both directions by using oligonucleotides I and O complementary to the ends of Tn5*tac*1 and the collection of pCR001 Tn5*tac*1 insertions (Fig. 4). Other specially designed oligonucleotides were used to complete the nucleotide sequence (Fig. 4). An ORF of 515 bp, designated *omp4*, was identified as the gene encoding Omp4. It appeared to code for a protein of 172 amino acids with a calculated molecular mass of 18.4 kDa. The nucleotide sequence preceding the ATG start codon of *omp4* contains a putative Shine-Dalgarno sequence GAGG (nucleotides 83–87). Two sequences, TATATT (nucleotides 32–37) and AATAAT (nucleotides 66–71) similar to the -10 region of the consensus *E. coli* promoters were found, but no sequences similar to the -35 region. No stable hairpin structure characteristic of rho-independent terminators of transcription in prokaryotes was found following the proposed stop codon TAA (nucleotides 608–610).

Fig. 2. Restriction map of pCR001, including adjacent regions in pBR328 (striped box). The positions and orientation of Tn5 insertions in pCR001 used for DNA sequencing, and the corresponding bacteriocin 28b sensitive phenotype, are also shown (R, resistant; S, sensitive).

Fig. 3. SDS-PAGE of outer-membrane preparations. Overnight cultures (10 ml) were adjusted to 1×10^9 c.f.u. ml⁻¹, and outer-membrane fractions were isolated and loaded on the gels. Lanes: 1, *S. marcescens* N28b; 2, *E. coli* 5K(pCR001); 3, *E. coli* 5K(pCR001::Tn5S2); 4, *E. coli* 5K(pCR001::Tn5S4); 5, *E. coli* 5K(pCR001::Tn5S1); 6, *E. coli* 5K(pCR001::Tn5R6); 7, *E. coli* 5K(pBR328). The bacteriocin 28b resistant (R) or sensitive (S) phenotype of the strains is shown below the lane number.

Analysis of the deduced Omp4 amino acid sequence

The predicted Omp4 has a higher molecular mass than the protein obtained from the outer-membrane SDS-PAGE analysis, suggesting that the protein is synthesized as a precursor with an N-terminal signal peptide, characteristic of exported proteins (Perlman & Halvorson, 1983; von Heijne, 1983). Inspection of the N-terminal part of the Omp4 reveals the presence of two basic lysine residues followed by a stretch of hydrophobic residues, characteristic of a signal sequence. According to the proposed consensus cleavage site for signal peptidase I (von Heijne, 1983) the most probable processing site is located between A₂₄ and G₂₅ (Fig. 4). This was confirmed by determining the 10 N-terminal amino acid residues of Omp4. This sequence, G-Q-S-T-V-S-A-G-Y-A, was identical to that predicted from the *omp4* DNA sequence after A₂₄.

Fig. 4. Nucleotide sequence of the ORF *omp4*, identified as the gene encoding Omp4. The predicted Omp4 amino acid sequence is shown below the DNA sequence. The stop codon is indicated by an asterisk. Sites of Tn5 insertions, restriction sites and the putative ribosome-binding site (S-D) are labelled above the DNA sequence. Oligonucleotides used to complete the nucleotide sequences are shown by arrows.

Homology between Omp4 and outer-membrane proteins PagC, Ail, Rck, OmpX_{E. cloacae}, OmpX_{E. coli} and Lom

Computer searches for similar amino acid sequences in the GenBank, EMBL, and SwissProt databases revealed homology between Omp4 and six other outer-membrane

Fig. 5. Alignments of the amino acid sequence of Omp4 (172 amino acid residues) and the outer-membrane proteins OmpX_{E. cloacae}, OmpX_{E. coli}, Ail, PagC, Rck and Lom. According to the topology model for OmpX_{E. cloacae} (Stoorvogel *et al.*, 1991a), black dots and lines correspond to amino acid residues located in cytoplasmic loops and membrane spanning regions respectively. The arrow shows the suggested site of signal peptide cleavage for Omp4.

proteins: PagC, (18 kDa), encoded by a chromosomal gene of *Salmonella typhimurium* (Pulkkinen & Miller, 1991); Ail (19.5 kDa), encoded by a chromosomal gene of *Yersinia enterocolitica* (Miller *et al.*, 1990); Rck (17 kDa), encoded by the virulence plasmid of *S. typhimurium* (Heffernan *et al.*, 1992b); OmpX_{E. cloacae} (18 kDa), encoded by a chromosomal gene of *Enterobacter cloacae* (Stoorvogel *et al.*, 1991a); Lom (21.8 kDa), encoded by lysogenic bacteriophage λ (Barondess & Beckwith, 1990); and OmpX_{E. coli} (16.3 kDa), encoded by a chromosomal gene from *Escherichia coli* (Mecbas *et al.*, 1995).

An amino acid sequence alignment of the predicted Omp4 protein with the six members of this protein family is shown in Fig. 5. The amino acid similarity among all of them is very high and 72% of the amino acid residues of Omp4 are identical to one or another of these small outer-membrane proteins. Inspection of the amino acid sequence alignment reveals the presence in Omp4 of the sequence GFNLKYRYE (residues 47–55) that fits the consensus signature sequence G[VIHLF]N[LVIH]KYRYE proposed by Heffernan *et al.* (1992b) for this family of small outer-membrane proteins. Furthermore,

Fig. 6. Immunoblot of outer-membrane preparations from *S. marcescens* strains used a rabbit polyclonal antibody raised against Omp4. Lanes: 1, *S. marcescens* G 0005 (biotype A1a); 2, *S. marcescens* G 0328 (biotype A1b); 3, *S. marcescens* SLM 5556 (biotype A2a); 4, *S. marcescens* SLM 2134 (biotype A2b); 5, *S. marcescens* G 0324 (biotype A6); 6, *S. marcescens* SLM 5797 (biotype A3a); 7, *S. marcescens* SLM 5782 (biotype A3b); 8, *S. marcescens* G 4516 (biotype A3c); 9, *S. marcescens* G 5738 (biotype A3d); 10, *S. marcescens* G 0060 (biotype A4a); 11, *S. marcescens* G 0246 (biotype A5); 12, *S. marcescens* G 0266 (biotype A8a); 13, *S. marcescens* SLM 5818 (biotype A8b); 14, *S. marcescens* G 0481 (biotype A8c); 15, *S. marcescens* G 0081 (biotype TCT).

the sequence TWNVGVGYRF, corresponding to the last 10 C-terminal amino acid residues of Omp4, fits the consensus sequence XZXZXZYXF (where X is any amino acid residue and Z is any hydrophobic residue). This consensus sequence was shown by Stoorvogel *et al.* (1991a) to be present in all the members of this family of small outer-membrane proteins, and it is also seen in many outer-membrane proteins, including the major OmpA of *E. coli* and *S. marcescens* (Beck & Bremer, 1980; Braun & Cole, 1984), the OmpC-like porin from *S. marcescens* (Hutsul & Worobec, 1994), and *E. coli* porin proteins OmpF and PhoE (Inokuchi *et al.*, 1982; Overbeeke *et al.*, 1983).

A high level of similarity, 70.4% amino acid identity, was found between the Omp4 and the OmpX_{*E. cloacae*} protein. According to the model for the topology of the OmpX protein (Stoorvogel *et al.*, 1991a), the most similar regions correspond to the three cytoplasmic loops (100%, 80% and 100% amino acid identity), the eight membrane-spanning regions (60%, 90%, 80%, 70%, 80%, 80%, 70% and 80% amino acid identity), and the fourth cell-surface-exposed region (87.5% amino acid identity). The most dissimilar regions correspond to the three first cell-surface-exposed loops (66.6%, 57.1% and 40% amino acid identity) (Fig. 5), suggesting that there could be functional differences between the Omp4 and OmpX proteins.

The hydrophobicity profile of the Omp4 protein was very similar to those of the other six members of this outer-membrane protein family (results not shown). The phylogeny of this family was found to be very similar using either the neighbour joining or the parsimony method. Five proteins are grouped in two clusters, one formed by OmpX_{*E. cloacae*}, OmpX_{*E. coli*} and Omp4, encoded by chromosomal loci from *Ent. cloacae*, *E. coli* and *S. marcescens* respectively, and another with the pair Rck and PagC encoded by loci found in *S. typhimurium*.

Ail, encoded by a chromosomal locus from *Y. enterocolitica*, appears to be evolutionarily more closely related to the OmpX_{*E. cloacae*}-OmpX_{*E. coli*}-Omp4 cluster, and Lom, encoded by lysogenic bacteriophage λ , appears to be evolutionarily less closely related to the other six proteins.

Biological characterization of Omp4

Similarly to the Ail protein (Miller *et al.*, 1990) Omp4 was successfully purified from *E. coli* overproducing this protein, by extracting whole cells with octylglucoside and selective precipitation with KCl. The presence of Omp4 in *E. coli* caused a significant decrease in the amount of major outer-membrane proteins OmpA, OmpF and/or OmpC (Fig. 3). Similarly, overexpression in *E. coli* of OmpX led to a decrease in the amount of OmpF and OmpC porins, causing resistance to β -lactam antibiotics (Stoorvogel *et al.*, 1987, 1991b). High-level Rck expression in *S. typhimurium* was also associated with a decrease in certain outer-membrane proteins (Heffernan *et al.*, 1992b). Overproduction of Omp4 in *E. coli* resulted in a five- to tenfold increase in the MICs of several β -lactam antibiotics (results not shown). Since previously the OmpA and OmpF proteins have been identified as members of the bacteriocin 28b receptor in *E. coli* (J. Enfedaque and others, unpublished results) the decrease in OmpF and OmpA probably accounts for the observed phenotype of partial resistance to bacteriocin 28b. As expected, *E. coli* 5K carrying pCR001 was able to bind significantly less bacteriocin 28b than *E. coli* 5K carrying the vector pBR328 (data not shown).

The role of Omp4 in *S. marcescens* N28b remains to be established. Four proteins that are homologous to Omp4 have been associated with virulence. PagC has been associated with *S. typhimurium* survival in macrophages and with virulence in mice (Miller *et al.*, 1992); Ail from *Y. enterocolitica* has been associated with epithelial cell adherence, invasion and resistance to complement killing

(Miller & Falkow, 1988; Bliska & Falkow, 1992); Rck encoded by an *S. typhimurium* virulence plasmid has been associated with complement resistance (Heffernan *et al.*, 1992a); and OmpX_{E. cloacae} from *E. cloacae* has been shown to be involved in invasion of rabbit ileal tissue (de Kort *et al.*, 1994). When some of these proteins are expressed in *E. coli* K12 they provide significant resistance to complement-mediated killing (Heffernan *et al.*, 1992a; Bliska & Falkow, 1992). To test whether this was the case for Omp4, *E. coli* 5K and *E. coli* HB101 either without or overexpressing Omp4 were assayed for resistance to human serum complement. No significant differences were observed in the behaviour of the two pairs of strains in these assays. Thus, despite the structural similarities among the six members of this protein family, the function of Omp4, as well as that of the OmpX_{E. coli} from *E. coli* and Lom protein from λ (Barondess & Beckwith, 1990) are presently unknown.

Omp4 is produced by different *S. marcescens* biotypes

To test whether the presence of Omp4 was a particular characteristic of *S. marcescens* N28b or was a general characteristic of this species, 25 *S. marcescens* strains belonging to 15 different biotypes were assayed. A 351 bp *KpnI*-*PstI* DNA probe of the Omp4-encoding gene hybridized with genomic DNA from all 25 strains. Analysis of the outer-membrane protein pattern by SDS-PAGE showed that in all of the strains an outer-membrane protein was present with a molecular mass similar to that of the Omp4. These bands reacted with anti-Omp4 serum in immunoblotting experiments. In Fig. 6 an immunoblot is shown with one strain of each *S. marcescens* biotype. The unspecific reactions seen in some of these strains could be due to aggregation of Omp4, higher amounts of lipopolysaccharide in these preparations, or the presence of other outer-membrane proteins able to cross-react with the anti-Omp4 serum in the *S. marcescens* strains showing this behaviour. Furthermore, the same bands reacted with a polyclonal serum raised to a synthetic peptide representing the first N-terminal peripheral loop of OmpX from *E. cloacae*. These results strongly suggest that presence of Omp4 is a general characteristic of *S. marcescens*, although they do not rule out possible minor differences among Omp4 within the species.

ACKNOWLEDGEMENTS

We thank J. A. M. van de Klundert for providing the polyclonal serum raised to a synthetic peptide corresponding to the OmpX protein, and P. A. D. Grimont for providing the 25 *S. marcescens* strains belonging to 15 different biotypes. This work was supported by grant PB 90-0468 from the Comisión Interministerial de Ciencia y Tecnología (CICYT), Spain. M. B. V. and J. F. G. were supported by FI fellowships from the Generalitat de Catalunya. J. E. and S. F. were supported by fellowships from the Spanish MEC.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (editors) (1987). *Current Protocols in Molecular Biology*. New York: John Wiley.

Barondess, J. J. & Beckwith, J. (1990). A bacterial virulence determinant encoded by lysogenic coliphage lambda. *Nature* **346**, 871–872.

Beck, E. & Bremer, E. (1980). Nucleotide sequence of the gene *ompA* coding the outer membrane protein II of *Escherichia coli* K12. *Nucleic Acids Res* **8**, 3011–3027.

Blake, M. S., Johnson, K. H., Russell-Jones, G. S. & Gotschlich, E. C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody. *Anal Biochem* **136**, 175–179.

Bliska, J. B. & Falkow, S. (1992). Bacterial resistance to complement killing mediated by the Ail protein of *Yersinia enterocolitica*. *Proc Natl Acad Sci USA* **89**, 3561–3565.

Braun, G. & Cole, S. T. (1984). DNA sequence analysis of the *Serratia marcescens ompA* gene: implications for the organization of an enterobacterial outer membrane protein. *Mol & Gen Genet* **195**, 321–328.

Cavard, D. & Lazdunski, C. (1981). Involvement of BtuB and OmpF proteins in binding and uptake of colicin A. *FEMS Microbiol Lett* **12**, 311–316.

Chow, W. Y. & Berg, D. E. (1988). Tn5tac1, a derivative of Tn5 that generates conditional mutations. *Proc Natl Acad Sci USA* **85**, 6468–6472.

Felsenstein, J. (1989). PHYLIP: phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.

Gargallo-Viola, D. V. (1989). Enzyme polymorphism, prodigiosin production and plasmid fingerprints in clinical and naturally occurring isolates of *Serratia marcescens*. *J Clin Microbiol* **27**, 860–868.

de Graaf, F. K. & Oudega, B. (1986). Production and release of cloacin DF13 and related colicins. *Curr Top Microbiol Immunol* **125**, 183–205.

Hardy, K. G., Meynell, G. G., Dowman, J. E. & Spratt, B. G. (1973). Two major groups of colicin factors: their evolutionary significance. *Mol & Gen Genet* **125**, 217–230.

Heffernan, E. J., Reed, S., Hackett, J., Fierer, J., Roudier, C. & Guiney, D. (1992a). Mechanism of resistance to complement-mediated killing of bacteria encoded by the *Salmonella typhimurium* virulence plasmid gene *rck*. *J Clin Invest* **90**, 953–964.

Heffernan, E. J., Harwood, J., Fierer, J. & Guiney, D. (1992b). The *Salmonella typhimurium* virulence plasmid complement resistance gene *rck* is homologous to a family of virulence-related outer membrane protein genes, including *pagC* and *ail*. *J Bacteriol* **174**, 84–91.

von Heijne, G. (1983). Patterns of amino acids near signal-sequence cleavage sites. *Eur J Biochem* **133**, 17–21.

Higgins, D. G., Bleasby, A. J. & Fuchs, R. (1992). CLUSTAL V: improved software for multiple sequence alignment. *Comput Appl Biosci* **8**, 189–191.

Hutsul, J. A. & Worobec, E. (1994). Molecular characterization of a 40 kDa OmpC-like porin from *Serratia marcescens*. *Microbiology* **140**, 379–387.

Inokuchi, K., Mutoh, N., Matsuyama, S. & Mizushima, S. (1982). Primary structure of the *ompF* gene that codes for a major outer membrane protein of *Escherichia coli* K-12. *Nucleic Acids Res* **10**, 6957–6968.

Jakes, K. S. & Lazdunski, C. (1992). Immunity to colicins. In *Bacteriocins, Microcins and Lantibiotics* (NATO ASI Series H: Cell

Biology, vol. 65), pp. 163–170. Edited by R. James, C. Lazdunski & F. Pattus. Berlin: Springer-Verlag.

Juárez, A., Hartlein, M. & Goebel, W. (1984). Study of regulation and transport of hemolysin by using fusion of β -galactosidase gene (*lacZ*) to hemolysin genes. *J Bacteriol* **160**, 161–168.

de Kort, G., Bolton, A., Martin, G., Stephen, J. & van de Klundert, J. A. M. (1994). Invasion of rabbit ileal tissue by *Enterobacter cloacae* varies with the concentration of OmpX in the outer membrane. *Infect Immun* **62**, 4722–4726.

Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**, 105–132.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Mankovich, J. A., Hsu, C. & Konisky, J. (1986). DNA and amino acid sequence analysis of structural and immunity genes of colicins Ia and Ib. *J Bacteriol* **168**, 228–236.

Matsuyama, S.-I. & Mizushima, S. (1985). Construction and characterization of a deletion mutant lacking *micF*, a proposed regulatory gene for OmpF synthesis in *Escherichia coli*. *J Bacteriol* **162**, 1196–1202.

Mecas, J., Welch, R., Erickson, J. W. & Gross, C. A. (1995). Identification and characterization of an outer membrane protein, OmpX, in *Escherichia coli* that is homologous to a family of outer membrane proteins including Ail of *Yersinia enterocolitica*. *J Bacteriol* **177**, 799–804.

Miller, V. L. & Falkow, S. (1988). Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. *Infect Immun* **56**, 1242–1248.

Miller, V. L., Bliska, J. B. & Falkow, S. (1990). Nucleotide sequence of the *Yersinia enterocolitica* *ail* gene and characterization of the Ail protein product. *J Bacteriol* **172**, 1062–1069.

Miller, V. L., Beer, K. B., Loomis, W. P., Olson, J. A. & Miller, S. I. (1992). An unusual *pagC*::*TnphoA* mutation leads to an invasion- and virulence-defective phenotype in salmonellae. *Infect Immun* **60**, 3763–3770.

Overbeeke, N., Bergmans, H., Van Mansfield, F. & Lugtenberg, B. (1983). Complete nucleotide sequence of *phoE*, the structural gene for the phosphate limitation inducible outer membrane pore protein of *Escherichia coli* K-12. *J Mol Biol* **163**, 513–532.

Perlman, D. & Halvorson, H. O. (1983). A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. *J Mol Biol* **167**, 391–409.

Pugsley, A. P. (1984a). Genetic analysis of ColN plasmid determinants for colicin production, release and immunity. *J Bacteriol* **158**, 523–529.

Pugsley, A. P. (1984b). The ins and outs of colicins. Part II. Lethal action, immunity and ecological implications. *Microbiol Sci* **1**, 203–205.

Pugsley, A. P. & Oudega, B. (1987). Methods for studying colicins and their plasmids. In *Plasmids, a Practical Approach*, pp. 105–161. Edited by K. G. Hardy. Oxford: IRL Press.

Puig, M., Fusté, C. & Viñas, M. (1993). Outer membrane proteins from *Serratia marcescens*. *Can J Microbiol* **39**, 108–111.

Pulkkinen, W. S. & Miller, S. I. (1991). A *Salmonella typhimurium* virulence protein is similar to a *Yersinia enterocolitica* invasion protein and a bacteriophage lambda outer membrane protein. *J Bacteriol* **173**, 108–114.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for constructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

Schramm, E., Mende, J., Braun, V. & Kamp, R. (1987). Nucleotide sequence of the colicin B activity gene *cba*: consensus pentapeptide among TonB dependent colicins and receptors. *J Bacteriol* **169**, 3350–3357.

Stoorvogel, J., van Bussel, M. J. A. W. M. & van de Klundert, J. A. M. (1987). Cloning of a beta-lactam resistance determinant of *Enterobacter cloacae* affecting outer membrane proteins of Enterobacteriaceae. *FEMS Microbiol Lett* **48**, 277–281.

Stoorvogel, J., van Bussel, M. J. A. W. M., Tommassen, J. & van de Klundert, J. A. M. (1991a). Molecular characterization of an *Enterobacter cloacae* outer membrane protein (OmpX). *J Bacteriol* **173**, 156–160.

Stoorvogel, J., van Bussel, M. J. A. W. M. & van de Klundert, J. A. M. (1991b). Biological characterization of an *Enterobacter cloacae* outer membrane protein (OmpX). *J Bacteriol* **173**, 161–167.

Tsang, V. C. W., Peralta, J. M. & Simons, A. R. (1983). Enzyme-linked immunoelectrotransfer blot techniques (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. *Methods Enzymol* **92**, 377–391.

Viejo, M. B., Ferrer, S., Enfedaque, J. & Regué, M. (1992). Cloning and DNA sequence analysis of a bacteriocin gene from *Serratia marcescens*. *J Gen Microbiol* **138**, 1737–1743.

Viejo, M. B., Enfedaque, J., Guasch, J. F., Ferrer, S. & Regué, M. (1995). Protection against bacteriocin 28b in *Serratia marcescens* is apparently not related to the expression of an immunity gene. *Can J Microbiol* **41**, 217–226.

Received 1 March 1995; revised 9 May 1995; accepted 25 May 1995.