Role for the major outer-membrane protein from *Vibrio anguillarum* in bile resistance and biofilm formation

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*Vibrio anguillarum*, a fish pathogen, produces a 38 kDa major outer-membrane porin, which may be involved in environmental adaptation. The gene encoding the 38 kDa porin was cloned and deleted. The deduced protein sequence was 75% identical to that of the major outer-membrane protein (OMP), OmpU, from *Vibrio cholerae*. LacZ expression from an ompU::lacZ transcriptional gene fusion was increased 1.5-fold in the presence of bile salts and was decreased 50- to 100-fold in a toxR mutant compared to that in the wild-type, showing that ompU expression is positively regulated by ToxR and induced by bile salts. Similar to a toxR mutant, an ompU mutant showed a slight decrease in motility, an increased sensitivity to bile salts and a thicker biofilm with better surface area coverage compared to that of the wild-type. When ompU was expressed under a ToxR-independent promoter in the toxR mutant, the phenotypes for bile resistance and biofilm formation, but not motility were complemented to that of the wild-type. In rainbow trout, the ompU mutant showed wild-type virulence via immersion into infected seawater and intraperitoneal injection. The ompU mutant produced two colony morphologies: opaque, which did not grow at 0-2 % bile, and translucent, which grew at 2 % bile. The translucent ompU mutant strain produced a second major OMP that was induced by bile. All ompU mutants showed variations in the amount and length of smooth LPS. In *V. anguillarum*, OmpU is not required for virulence, possibly due to a second OMP also critical for resistance to bile; however, outside of the fish host, OmpU limits the progression of biofilm formation.

**INTRODUCTION**

*Vibrio anguillarum* causes a terminal haemorrhagic septicemia in marine fish and is associated with high rates of mortality, resulting in great economic losses within aquaculture (for reviews see Actis et al., 1999; Austin & Austin, 1999). *V. anguillarum* is suggested to constitute part of the microflora of marine fish as well as part of the normal microflora of the aquatic environment (Muroga et al., 1986; Oppenheimer, 1962; West et al., 1983). In each of these environments, the bacterium likely utilizes its external membrane to protect its intracellular contents from damaging agents or conditions.

The outer membrane consists predominately of phospholipids, LPS and proteins, either integral membrane proteins or lipoproteins, which make up almost 50% of the outer-membrane mass (Koebnik et al., 2000). The outer-membrane protein (OMP) profiles of 10 serotypes of *V. anguillarum*, although varying in the minor proteins, contain one major OMP in the molecular size range of 35–42 kDa (Simón et al., 1996). The major OMPs of serotype O1 and O2 are classified as general diffusion porins with similar functions to that of OmpF and OmpC from *Escherichia coli* (Davey et al., 1998; Simón et al., 1996). Furthermore, the major OMP from serotype O1 exhibits weak cation selectivity and possesses a moderate surface charge (Simón et al., 1996). The N-terminal amino acid sequence suggests that these two major OMPs are possible homologues of OmpU from *V. cholerae* (Okuda et al., 2001; Wang et al., 2002).

In *Vibrio cholerae* and *Vibrio fischeri*, OmpU homologues play a role in protecting the bacterium from bile salts (Aeckersberg et al., 2001; Provenzano & Klose, 2000). In *V. cholerae*, bile resistance is suggested to be an early step in the evolution of this bacterium as an intestinal pathogen. OmpU has previously been suggested to play a role in adherence of *V. cholerae* during intestinal colonization (Sperandino et al., 1995), but conflicting results have also
been reported (Nakasone & Iwanaga, 1998). However, neither of these studies were done using an ompU null mutant. A recent study utilizing a null ompU mutant suggests that OmpU is not essential for colonization or virulence factor production (Provenzano et al., 2001). Furthermore, bile was shown not to affect membrane permeability via the OmpU pore. Thus, the role of OmpU in the virulence of V. cholerae is suggested to provide a means for nutrient uptake in the presence of bile without compromising membrane integrity (Wibbenmeyer et al., 2002).

Interestingly, in V. fischeri, a symbiont of the light-emitting organ of the squid, OmpU is suggested to play a role in the initiation of colonization of the light organ. In addition, a null mutation in the V. fischeri ompU gene also showed an increased sensitivity to membrane-disrupting agents such as bile. However, this observation may indicate the importance of OmpU in maintaining membrane integrity as opposed to survival within the intestinal environment (Aeckersberg et al., 2001).

In this study, the gene encoding the major OMP from V. anguillarum serotype O1 was cloned and designated ompU. A strain carrying a null mutation showed a significant decrease in bile resistance, but no decrease in virulence. However, a second OMP was found that was induced by low concentrations of bile and could be involved in bile resistance. In addition, OmpU was shown to have a new role in limiting biofilm formation.

### METHODS

**Strains, plasmids, phage and growth conditions.** Bacterial strains and plasmids are described in Table 1. E. coli SY327 (Δpir) was used for transformation after subcloning fragments into the pDM4 or pNQ705-1 suicide vector. All plasmids to be conjugated into V. anguillarum were transformed into E. coli S17-1 (Δpir), which was used as the donor strain. Plasmid transfers from E. coli to V. anguillarum were done as described previously (Milton et al., 1996). E. coli XL-1 Blue was used for bacteriophage λ infections and for most transformations.

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and/or relevant markers</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY327</td>
<td>Δ(lac pro) argE(am) rif malA recA56 Δpir</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td>S17-1</td>
<td>thi pro hsdR hsdM4+ recA RP4-2-Tc::Mu-Km::Tn7 Δpir</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lac-pro) [F′proAB lacI9 lacZΔM15 Tn10(TetR)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>V. anguillarum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NB10</td>
<td>Wild-type, serotype O1, clinical isolate from the Gulf of Bothnia</td>
<td>Norqvist et al. (1989)</td>
</tr>
<tr>
<td>SY10</td>
<td>NB10 derivative containing a toxR deletion</td>
<td>Wang et al. (2002)</td>
</tr>
<tr>
<td>SY12</td>
<td>NB10 derivative containing an ompU deletion of bp 966–1778</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript</td>
<td>Ap1; ColE1 origin</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBSOmpU-AC</td>
<td>Ap1; pBluescript containing a 560 bp PCR fragment from ompU (bp 1008–1567)</td>
<td>This study</td>
</tr>
<tr>
<td>pBSOmpU-8</td>
<td>Ap1; pBluescript containing a cloned fragment with the ompU gene</td>
<td>This study</td>
</tr>
<tr>
<td>pDM4</td>
<td>Ap1; pBluescript containing a promoterless lacZ gene used for transcriptional gene fusion studies</td>
<td>Milton et al. (1996)</td>
</tr>
<tr>
<td>pDM4-OmpU2</td>
<td>Cm1; pDM4 derivative containing an ompU deletion of bp 725–965 fused to bp 1779–2011</td>
<td>This study</td>
</tr>
<tr>
<td>pDM8</td>
<td>Cm1; Tc1; (ColE1 origin, RP4 mob+) , pSup202 derivative (Simon et al., 1983) containing a promoterless lacZ gene used for transcriptional gene fusion studies</td>
<td>Croxatto et al. (2002)</td>
</tr>
<tr>
<td>pDM8-OmpU1</td>
<td>Cm1; Tc1; pDM8 derivative containing an ompU::lacZ transcriptional gene fusion (bp 400–919)</td>
<td>This study</td>
</tr>
<tr>
<td>pNQ705-1</td>
<td>Cm1; suicide vector that contains an R6K origin (pir-requiring)</td>
<td>McGee et al. (1996)</td>
</tr>
<tr>
<td>pDM35-OmpU1</td>
<td>Cm1; pNQ705-1 derivative containing an ompU::lacZ transcriptional gene fusion (bp 400–919)</td>
<td>This study</td>
</tr>
<tr>
<td>pSup202P</td>
<td>Cm1; Tc1; pSup202 derivative (Simon et al., 1983) (ColE1 origin, RP4 mob+) with a polylinker cloned into PstI within the Ap1 gene</td>
<td>Milton et al. (1992)</td>
</tr>
<tr>
<td>pSup-OmpU</td>
<td>Cm1; Tc1; pSup202P derivative containing the ompU gene and its promoter (bp 442–2011)</td>
<td>This study</td>
</tr>
<tr>
<td>pMMB208</td>
<td>Cm1; IncQ lacI9 P_tac polylinker from M13mp19</td>
<td>Morales et al. (1991)</td>
</tr>
<tr>
<td>pMMB-OmpU1</td>
<td>Cm1; pMMB208 derivative containing the ompU gene lacking its promoter (bp 921–2011)</td>
<td>This study</td>
</tr>
<tr>
<td>pToxR</td>
<td>Cm1; Tc1; pSup202P derivative containing the toxR gene and its promoter</td>
<td>Wang et al. (2002)</td>
</tr>
</tbody>
</table>
E. coli was routinely grown in Luria broth, which contains Bacto Tryptone (10 g L⁻¹), Bacto yeast extract (5 g L⁻¹) and sodium chloride (10 g L⁻¹). For V. anguillarum, Trypticase soy medium (TSB) from BBL was used for routine growth and the cultures were grown with aeration at 24°C. Biofilm growth medium was minimal M63 salts (Silhavy et al., 1984) supplemented with 1% (w/v) NaCl, 1.5% (w/v) Casamino acids, 1% (w/v) glucose, 1 mM MgSO₄ and 10 μg thiamine ml⁻¹. Antibiotic concentrations for V. anguillarum were tetracycline at 5 μg ml⁻¹ and chloramphenicol at 10 μg ml⁻¹.

**PCR conditions, DNA manipulation and sequencing.** PCR was performed as described previously (Wang et al., 2002). Unless otherwise stated, all conditions for the various DNA techniques were as described by Sambrook et al. (1989). Reaction conditions for the DNA-modifying enzymes and DNA restriction enzymes were as suggested by the manufacturers. Double-strand DNA sequencing was performed using a Seq4 × 4 Automated Sequencer and a Thermo Sequenase Cy 5.5 Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech).

**Cloning of the ompU gene.** Previously, the amino-terminal sequence of the major OMP from V. anguillarum serotype O1 was sequenced and shown to be 75% identical to that of OmpU from V. cholerae (Wang et al., 2002). Using the amino-terminal sequence of V. anguillarum OmpU and an internal amino acid sequence from V. cholerae OmpU, two degenerate inosine-containing oligonucleotides were designed and used to PCR amplify a 560 bp DNA fragment from the V. anguillarum ompU gene. Primer OmpU-A [5'-GGTGAGCTCCTAAIACAAGA(TC)GG(ACTG)AC-3'] is complementary to deduced codons for the amino-terminal sequence YNQDGT of OmpU from V. anguillarum (Wang et al., 2002) and OmpU-C [5'-GGCAGTTGGTTCITTITGITC(TG)TC(TG)TC(TG)TC-3'] is complementary to V. cholerae ompU codons for amino acids DQDDQNE. This fragment was cloned into pBluescript (Stratagene), creating pBSompU-A, and sequenced. The deduced protein sequence of this fragment was 70% identical to OmpU of V. cholerae (Sperandino et al., 1996).

The 560 bp fragment was used as a probe to screen a λ Zap (Stratagene) bacteriophage-based V. anguillarum genomic library (Milton et al., 1992) using previously described methods (Milton et al., 1996). The pBluescript plasmids containing a chromosomal insert were excised from positive plaques as described previously (Milton et al., 1992). One plasmid, pBSompU-8, was chosen for sequencing ompU.

**Construction of an ompU mutant.** An ompU in-frame deletion mutation was made in the wild-type strain by allelic exchange as described previously (Milton et al., 1996). After allelic exchange using pDMOMP2, codons for the first 10 amino acids were fused to the codons encoding the last 49 amino acids. The strain containing this in-frame deletion was called SY12. PCR amplification of the deleted region from the chromosome and subsequent DNA sequencing confirmed the ompU in-frame deletion. For complementation analyses, pSup-OmpU, which carries the wild-type ompU and its promoter, was mobilized into the ompU mutant via conjugation.

**Rescue of phenotypes in a toxR mutant by OmpU.** To determine if OmpU can rescue wild-type phenotypes in a toxR mutant, a PCR fragment (residues 921–1101) containing the toxR gene plus 15 bp upstream of the start codon was amplified and fused to the inducible P_tac promoter on pMMB208 (Morales et al., 1991), creating pMMB-OmpU1. The PMMB-OmpU1 plasmid was mobilized into the toxR mutant SY10 via conjugation. To induce ompU expression in the toxR mutant carrying pMMB-OmpU1, a final concentration of 1 mM IPTG was added to the medium. After overnight growth under inducing conditions in the toxR mutant, the amount of OmpU in a whole-cell extract or in an outer-membrane preparation was approximately 80% of the OmpU levels in the wild-type strain under similar conditions. For functional analyses of the rescue of wild-type phenotypes in the toxR mutant, similar inducing conditions were used.

**Construction of ompU transcriptional gene fusion.** A transcriptional gene fusion was constructed between the promoter region of ompU and the reporter gene lacZ from E. coli. This fusion was created using previously described methods (Wang et al., 2002). pDM8-OmpU1 is described in Table 1. However, this plasmid was very unstable after conjugation into V. anguillarum strains, possibly due to the high expression of the lacZ gene using the ompU promoter. To obtain a lower copy number, theompU::lacZ gene fusion was subcloned from pDM8-OmpU1 into the suicide vector pNQ705-1, which requires the pir gene for replication. To create pDM35-OmpU1, pDM8-OmpU1 was partially digested with BamHI. The fragment containing the ompU promoter and the lacZ gene (3400 bp) was purified from an agarose gel and ligated to BglII site in the polynuclein region of pNQ705-1. pDM35-OmpU1 was then recombined into the chromosome of V. anguillarum strains. The site of insertion was verified by PCR and the insertion was stable for 30 generations of growth in the absence of chloramphenicol.

**β-Galactosidase assays.** Conditions for bacterial growth for assaying β-galactosidase activity was done as described previously (Wang et al., 2002). β-Galactosidase assays were performed according to Miller (1972).

**LPS analysis.** LPS were prepared from a Protease K digestion of whole-cell lysates as described by Preston & Penner (1987). LPS were fractionated by 18% SDS-PAGE (Laemmli, 1970) and visualized with Silver Stain Plus (Bio-Rad) following the manufacturer’s instructions.

**OMP analyses.** OMP preparations were prepared as described previously (Wang et al., 2002) and separated by 5% SDS-PAGE (Laemmli, 1970). The gels were fixed and stained with 0.1% Coomassie brilliant blue in 40% methanol/10% acetic acid and then destained in 40% methanol/10% acetic acid.

**Western analysis.** Western analysis was done as described previously (Wang et al., 2002) using a rabbit polyclonal antiserum raised against OmpU from V. cholerae (a gift from James Kaper, University of Maryland, School of Medicine, MD, USA).

**Flow cell analysis of biofilms.** Biofilm formation and analysis using a continuous flow of fresh medium was done using a modification of the previously described protocol of Christensen et al. (1999). A pre-sterilized three-channel flow cell (Stovall Life Sciences), which has an acrylic base with a #1 cover slip attached and a channel size of 4 × 40 × 1 mm was used. The flow cell was filled with biofilm growth medium and the flow rate was stabilized to 250 μl min⁻¹ throughout the experiment. The flow cell was kept at room temperature. Overnight cultures, grown in biofilm growth medium, were diluted to an OD₆₀₀ of 1.0 in fresh biofilm growth medium. The medium flow was stopped and 0.4 ml was used to inoculate a channel. The flow cell was inverted with the cover slip side down for 1 h after which the flow cell was turned right side up and the flow of medium was initiated again. Biofilms were allowed to progress for 72 h before staining with acridine orange for visualization.

To stain the biofilm, 200 μl 0.1% acridine orange in potassium phosphate buffer (pH 7.4) was injected into the flow cell and allowed to stain for 20 min after which the biofilm was washed for 60 min before viewing for images. When using the pMMB208 plasmid derivatives, 1 mM IPTG was added to the biofilm growth medium to induce expression of OmpU under the tac promoter.
Imaging of biofilms in flow cells was done using scanning confocal laser microscopy (SCLM) (Multiprobe 2001; Molecular Dynamics). The 488 nm laser line of an argon-krypton laser was used to excite the acridine orange. ImageSpace software (Molecular Dynamics) running on an O2 workstation (Silicone Graphics) was used to generate vertical cross sections through the biofilms. Images were further processed for display using Photoshop software (Adobe). Two sets of images were obtained from the centre of each flow cell channel for every experiment and all strains were done in triplicate.

**Percentage area coverage of a biofilm.** The percentage area coverage due to biofilm production was determined as described previously (Wang et al., 2002).

**Sequence analysis.** Sequence assembly was done using the Genetics Computer Group Sequence Analysis software (Devereux et al., 1984) of the Genetics Computer Group, University of Wisconsin. Database searches were done through the NCBI Web page using BLAST 2.0.

**Fish infections.** Rainbow trout (Oncorhynchus mykiss) with an approximate weight of 10–15 g were infected with *V. anguillarum* either by intraperitoneal injections or by immersion of the fish in seawater containing *V. anguillarum* as described previously (Milton et al., 1996).

**RESULTS**

**Identification and mutagenesis of the ompU gene**

To identify the *ompU* homologue from *V. anguillarum* serotype O1, degenerate PCR primers were designed using the previously sequenced amino terminus of the major OMP of *V. anguillarum* (Wang et al., 2002) and from an internal sequence within the *V. cholerae ompU* DNA sequence (Sperandino et al., 1996). Using these primers, a 560 bp PCR fragment was generated that encoded an amino acid sequence with 70% identity to *OmpU* of *V. cholerae* and was used as a probe to screen a genomic library for a chromosomal fragment carrying the *ompU* gene and flanking DNA regions were sequenced and the genetic organization is shown in Fig. 1. One complete ORF was identified and named *ompU*. The deduced OmpU protein sequence is 330 aa long and is 75% identical to OmpU from *V. cholerae* (Sperandino et al., 1996), 59% identical to OmpU of *V. fischeri* (Ackersberg et al., 2001) and 47% identical to OmpL of *Photobacterium profundum* (Welch & Bartlett, 1996).

In addition, two partial ORFs were found (Fig. 1). The deduced 127 aa sequence of ORF1 is 85% identical to the *V. cholerae* VC0634 gene product from chromosome I, which encodes the transcription elongation factor GruA (Heidelberg et al., 2000). The partial deduced protein sequence of ORF2 is 62% identical to the *V. cholerae* VC0632 gene from chromosome I, which encodes a D-alanyl-D-alanine carboxypeptidase (DacB) (Heidelberg et al., 2000). Since the *ompU* gene corresponds to the *V. cholerae* VC0633 gene from chromosome I, the genetic organization of these three *V. anguillarum* ORFs is similar to that from the sequenced *V. cholerae* strain El Tor N16961 (Heidelberg et al., 2000).

To determine the function of OmpU, an in-frame deletion mutation was made that fused codons for the first 10 aa to codons encoding the last 49 aa. Growth of the *ompU* mutant (SY12) was similar to that of the wild-type in TSB (data not shown). For complementation analyses of the *ompU* deletion, pSup-OmpU, which carries the wild-type *ompU* gene and its promoter, was mobilized into the *ompU* mutant.

**Major OMP is encoded by ompU**

To show that the *ompU* gene encodes the major OMP in *V. anguillarum* serotype O1, OMPs were isolated from overnight broth cultures of the wild-type, the *ompU* mutant and the complemented *ompU* mutant, and separated by 12.5% SDS-PAGE. Fig. 2 shows that the major OMP is absent in the *ompU* mutant and is present again when the mutation is complemented with the wild-type *ompU* gene. In the *ompU* mutant, an additional 37 kDa protein can be seen that migrates with OmpU in the wild-type preparation. To ensure that this protein is not OmpU, a Western blot analysis was done using a rabbit polyclonal antiserum against *V. cholerae* OmpU. Fig. 2(b) shows that the OmpU antiserum did not bind to any OMPs from the *ompU* mutant, indicating that the 37 kDa OMP is different to OmpU and that the *ompU* mutant is lacking the major OMP.

**ToxR positively regulates ompU**

To determine if ToxR regulates the expression from *ompU*, a transcriptional gene fusion was made to the *E. coli lacZ* gene and carried on the suicide plasmid pDM35-OmpU1. This plasmid was recombined into the wild-type, the *toxR* mutant and the complemented *toxR* mutant at the *ompU* promoter region. Since the promoter region was duplicated on the chromosome downstream of the plasmid insertion,
an ompU mutation was not made and the expression level of OmpU was still that of the wild-type (data not shown). Fig. 3 shows that the expression of ompU::lacZ in the toxR mutant is 50- to 100-fold less than in the wild-type throughout growth, indicating that ToxR positively regulates expression from the ompU promoter. When the toxR defect was complemented with the wild-type toxR gene, LacZ activity returned almost to that of the wild-type.

**Fig. 2.** OMP analyses. OMPs were isolated from the wild-type, the ompU mutant, the ompU mutant carrying a plasmid-encoded wild-type ompU gene (pSup-OmpU), the opaque (Op) variant of the ompU mutant and the translucent (Tr) variant of the ompU mutant. In some cases, the strains were grown in the presence of 0-04 % bile. OMPs were applied to a 12.5 % SDS-PAGE gel, separated and stained with (a) Coomassie brilliant blue or (b) transferred to nitrocellulose followed by Western blotting using an antiserum raised against OmpU from *V. cholerae*. Lanes 1–7 are labelled in (a) and are the same for both (a) and (b). Molecular mass markers are included in the left-most lane and sizes are indicated.

**Table 2.** Effect of bile salts on the survival of *V. anguillarum* and motility analysis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Motility (%)</th>
<th>c.f.u. with indicated bile salts concentration (%) in TSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.04</td>
</tr>
<tr>
<td>Wild-type</td>
<td>100</td>
<td>115</td>
</tr>
<tr>
<td>ΔompU</td>
<td>71 ± 1.1</td>
<td>107</td>
</tr>
<tr>
<td>ΔompU::Sup-OmpU</td>
<td>92 ± 2.3</td>
<td>81</td>
</tr>
<tr>
<td>ΔtoxR</td>
<td>84 ± 2.7</td>
<td>100</td>
</tr>
<tr>
<td>ΔtoxR::pMMB-OmpU1</td>
<td>81 ± 2.1</td>
<td>264</td>
</tr>
</tbody>
</table>

*Colony size was greatly reduced compared to wild-type.
†IPTG (1 mM) was added to the medium to induce ompU expression.

**OmpU enhances growth in the presence of bile**

To test if OmpU from *V. anguillarum* plays a role in resistance to bile, equal amounts of bacterial cells were spread onto TSA plates containing various concentrations of bile salts (Difco). After 3 days, the number of c.f.u. was counted. Results are presented in Table 2. The wild-type was resistant up to 2 % bile salts, after which the c.f.u. began to decrease. However, the ompU mutant did not grow in the presence of 0.2 % bile and even at the lowest concentration of bile salts (0.04 %), the c.f.u. was decreased. For all concentrations of bile, the colony size of the ompU mutant was much smaller than the wild-type on a similar medium. When the ompU mutation was complemented with the wild-type gene and its promoter, growth in the presence of all concentrations of bile was regained.
phenotype is routinely checked when mutants are made to ensure that a spontaneous mutation that affects virulence did not occur. Equal numbers of cells were spotted onto a TSB/0.25% agar plate. Migration of cells from the centre spot was measured and compared to the wild-type (Table 2). The ompU mutant was only 71% as motile as the wild-type and when the ompU mutation was complemented with the wild-type gene, motility returned to that of the wild-type.

Biofilm formation

Since OmpU does not appear to have an essential role in virulence, maybe it is important for survival of *V. anguillarum* outside of the fish host. OmpU is located in the outer membrane and could affect surface structures or surface charges that may be important for biofilm formation. The percentage area coverage of a biofilm on a glass surface was determined for 2–14 h of growth for the wild-type, ompU mutant and the complemented ompU strains (Fig. 5). All strains showed the same percentage area coverage through 8 h of growth. After 8 h, the percentage area coverage for the ompU mutant increased above that of the wild-type and reached a 10-fold greater percentage area coverage by 14 h. The surface area coverage for the wild-type, on the other hand, did not change much after 8 h. In some cases, the level of biofilm coverage by the wild-type often dropped after 12 h. When the ompU mutation was complemented, near wild-type levels were obtained during the later hours of growth.

In addition to the percentage area coverage determination, flow cell analysis was done to analyse the thickness of the biofilm for the wild-type, the ompU mutant and the complemented ompU mutant. Using SCLM, images of a vertical cross-section through a 3-day-old biofilm for each strain were taken. As shown in Fig. 6, the ompU mutant produced a biofilm that was thicker than that of the wild-type and complementation of the mutation with the wild-type gene resulted in a near wild-type biofilm. These data may suggest that the ompU mutant either was more proficient than the wild-type at forming a biofilm or was unable to detach from the glass surface as well as the wild-type, thus enhancing surface biofilm coverage and biofilm thickness.

**ompU expression complements some phenotypes of a toxR mutation**

For the ompU mutant, alterations in biofilm formation, bile resistance and motility were similar to the same phenotypes for the toxR mutant (Wang et al., 2002). To test if these three toxR mutant phenotypes were due to the loss of OmpU or to other ToxR-regulated proteins, ompU was fused to the tac promoter carried on pMMB208, creating pMMB-OmpU1 and this plasmid was mobilized into the toxR deletion mutant SY10. This fusion places ompU under the control of a ToxR-independent promoter. To determine if ompU was expressed when 1 mM IPTG was added to the growth medium, OMP preparations from induced and uninduced cultures of toxR carrying pMMB-OmpU1 were compared to

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**Fig. 4.** The effect of bile on the transcription of ompU. β-Galactosidase activity of an ompU::lacZ transcriptional gene fusion (pDM35-OmpU1), which was inserted into the chromosome of the wild-type (NB10), was measured in the presence (0-04 circles and 0-4% triangles) or absence (squares) of bile. Overnight cultures were diluted into fresh TSB medium to an OD600 of 0.05 and then incubated with shaking at 24°C. Samples were taken at various times and analysed for growth (OD600; dotted lines) and β-galactosidase expression (Miller units; solid lines).

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In addition, LacZ activity was measured from the ompU::lacZ transcriptional fusion in the wild-type strain in the presence (0-04 and 0-4%) or absence of bile. In Fig. 4, a 1.5-fold increase in LacZ expression occurred at the onset of stationary phase in the presence of 0.4% bile, but not in the presence of 0.04% bile. Taken together, these data suggest that OmpU is required for optimal growth of *V. anguillarum* in the presence of bile and that induction of ompU by bile occurs at the onset of stationary phase.

**Virulence analyses**

*V. anguillarum* has been shown to utilize the intestines as a site of attachment, colonization and proliferation (Horne & Baxendale, 1983; Olsson et al., 1996, 1998). Since OmpU enhances growth in the presence of bile, the ompU mutant may be defective in virulence. Virulence of the ompU mutant was assayed via both the immersion and intraperitoneal infection routes. For the intraperitoneal route, the LD50 values for the ompU mutant and the wild-type were 69 ± 14 and 107 ± 80 bacteria, respectively. For the immersion route, the LD50 values for the ompU mutant and the wild-type were 1 x 10^4 ± 9 x 10^3 and 7 x 10^4 ± 3 x 10^3 bacteria (ml seawater)⁻¹, respectively. These results indicate that ompU is not essential for *V. anguillarum* to cause disease in fish.

**Motility analysis**

Since motility aids the entry of *V. anguillarum* into the fish host (Milton et al., 1996; O’Toole et al., 1996), this phenotype is routinely checked when mutants are made to
those of the wild-type and toxR mutant (data not shown). In the toxR/pMMB-OmpU1 strain, ompU is expressed to approximately 80% of the wild-type levels in the presence of IPTG. Western analysis confirmed that this induced protein was OmpU.

Bile resistance, motility and biofilm formation were assayed under inducing conditions (1 mM IPTG) for the toxR mutant carrying pMMB-OmpU1. Figs 5 and 6 and Table 2 show that the thickness and percentage area coverage of biofilm formation and bile resistance returned to approximate wild-type levels in the toxR mutant expressing OmpU; however, motility (Table 2) was unaffected. Thus, OmpU is a major player from the ToxR regulon for biofilm formation and bile resistance, but not for motility.

A second OMP involved in bile resistance

When doing c.f.u. counts using the ompU mutant, about 2% of the colonies had an increased translucent colony morphology compared to other colonies. PCR analysis indicated that both colony types carried the ompU deletion. Moreover, the ompU mutant was made a second time and a similar phenotypic alteration occurred. Thus, the alteration did not appear to be a spontaneous mutation unrelated to ompU. Interestingly, bacteria from the translucent colony could grow in the presence of 2% bile like the wild-type, whereas the opaque colony maintained the ompU mutant phenotype and was unable to grow in the presence of 0-2% bile (data not shown). OMP preparations were made from the opaque and translucent colonies in the presence and absence of bile. Fig. 2 shows that the translucent colony contains an abundant 37 kDa OMP that is induced by a low concentration of bile and that does not bind OmpU antibodies, suggesting that there may be more than one OMP that plays a role in bile resistance. In the OMP preparations from the opaque colony, the amount of a 50 kDa protein was increased, but was not induced by bile.

LPS profiles

LPS, which constitute the majority of the outer surface of the outer membrane, have been shown to play a role in bile
resistance (Gunn, 2000). We wondered if the loss or gain of these major OMPs had any effect on the LPS in the outer membrane. LPS were isolated from the various strains and separated in an 18% SDS-PAGE gel. The wild-type LPS profile in Fig. 7 shows that the O1 serotype produced a smooth LPS with a tight O-antigen modal length distribution as shown by Boesen et al. (1999). Compared to the wild-type, the toxR, ompU and ompU (opaque) mutants have an increased amount of the medium molecular mass O-antigen, and show an increase in the modal length of the repeating O-antigen units (lanes 2, 4 and 6, respectively), which when complemented decreased to the wild-type levels (lanes 3 and 5). Interestingly, the translucent ompU mutant (lane 7) contains predominantly the medium molecular mass O-antigen and very little of the lipid A core. This LPS profile is similar to a translucent variant of a wild-type V. cholerae strain (Finkelstein et al., 1997). These data suggest that changes in the OMP content can alter the amount and length of the smooth LPS in the outer leaflet of the cell.

**DISCUSSION**

Bacterial outer membranes form an adaptive barrier to the external environment, protecting the bacterial cell contents from damaging substances while allowing the selective uptake of nutrients (for reviews see Achouak et al., 2001; Koebnik et al., 2000; Nikaido, 1996). At the basis of this selective uptake are proteins called porins, which form hydrophilic channels in the outer membrane that permit selective transport of molecules into the bacterial cell. Porins may be either specific or non-specific in their transport properties. Regulation of the synthesis of porins is often dependent on signals found in the environment, such as osmolarity, nutrient limitation, temperature and phosphate. Thus, turning the expression of various porins on and off alters outer-membrane permeability.

The *V. anguillarum* major OMP from serotypes O1 and O2 has been shown to be a general diffusion porin for which a trimeric form can be isolated similar to that of OmpF and OmpC from *E. coli* and OmpU from *V. cholerae* (Chakrabarti et al., 1996; Davey et al., 1998; Simón et al., 1996). The predominant major OMPs from 10 *V. anguillarum* serotypes are antigenically similar to each other and for serotypes O1 and O2, they are also antigenically similar to the major OMP in other *Vibrio* species, including OmpU from *V. cholerae* (Davey et al., 1998; Simón et al., 1996; Suzuki et al., 1996; Wang et al., 2002). In this study, the *ompU* gene, encoding the major OMP from *V. anguillarum* serotype O1 was cloned. OmpU is 75% identical to OmpU from *V. cholerae* (Sperandino et al., 1996) and the N-terminal sequence of this protein is 100% identical to the Omp35La from at least four different serotypes of *V. anguillarum* (Suzuki et al., 1996). This

**Fig. 6.** SCLM images of biofilms formed in a flow cell. The images represent vertical cross-sections through 72 h biofilms formed on a glass surface after continuous flow of fresh medium. The biofilms were stained with acridine orange and SCLM was used to take images of the biofilm. Bars, 20 μm.

**Fig. 7.** Silver-stained LPS profiles of *V. anguillarum* mutant strains. LPS were isolated from equal numbers of bacterial cells as described in Methods and separated on an 18% SDS-PAGE gel.
suggests that the major OMP of *V. anguillarum* serotypes is likely to be a homologue to the *V. cholerae* OmpU porin.

Porins are the most abundant OMPs in the outer membrane and have been suggested to play an important role in bile resistance. Porins exist as trimers in the outer membrane that form pores of variable size and charge, resulting in variable permeability to bile salts (for reviews see Gunn, 2000; Lin et al., 2002). By regulating specific porins, bacteria can alter their membrane permeability and thus modulate bile resistance. In *E. coli*, OmpC is more important for bile resistance than OmpF as it has a smaller pore size than OmpF (Thanassi et al., 1997). In *V. cholerae*, the positive and negative regulation of OmpU and OmpT, respectively, is ToxR-dependent and is critical for bile resistance. OmpU, like OmpC in *E. coli*, makes a smaller pore that is more cation-selective than OmpT and is less permeable to bile salts (Provenzano & Klose, 2000). A similar mechanism of bile resistance is likely to be used by *V. anguillarum* since OmpU exhibits weak cationic selectivity and a moderate surface charge (Simón et al., 1996) and is critical for bile resistance. Moreover, the translucent and opaque variants of the *ompU* mutant clearly showed an induction of several additional OMPs in the absence of OmpU. The opaque variant was still bile-sensitive; however, for the translucent variant, the induction of one major OMP and several minor OMPs resulted in a gain of bile resistance. The translucent variant represents a small population of cells that have managed to adapt to high levels of bile. Bile resistance has previously been described as an adaptive response that involves a global alteration in protein production (Gunn, 2000).

In addition to OMPs, the outer membrane of Gram-negative bacteria also consists of LPS and phospholipids (for a review see Nikaido, 1996). LPS aid in the formation of an outer membrane that has low permeability and low fluidity and LPS are required for trimeric porin formation. Loss of the O-antigen creates a rough LPS, which results in the loss of porins and an increase in phospholipids in the outer membrane. An increase in phospholipid patches increases permeability of the outer membrane and hence increases sensitivity to bile (Gunn, 2000). Loss of the major OMP in *V. anguillarum* did lead to alterations in the LPS profile and thus may result in an increase in phospholipids in the outer membrane. In addition to the possible selective permeability of porins, the LPS and phospholipid composition of the outer membrane may also play a role in bile resistance in *V. anguillarum*.

Resistance to bile should be critical for *V. anguillarum* to colonize the intestines of the fish host (Horne & Baxendale, 1983; Olsson et al., 1996, 1998). Therefore, it is puzzling why the *ompU* mutant was not decreased in virulence. An alternate OMP that also functions in bile resistance, such as the 37 kDa OMP, could be one explanation. The 37 kDa OMP may be preferentially expressed to OmpU in the intestinal environment. The expression of the 37 kDa OMP was increased at least threefold in 0·04 % bile, whereas OmpU expression was not induced in 0·04 % bile and was only mildly induced using 0·4 % bile. Hence, *V. anguillarum* may have two general porins critical for bile resistance expressed differentially as OmpC and OmpF in *E. coli* (for a review see Nikaido, 1996). OmpC expression is induced with high osmolarity (0·15 M NaCl) and high temperature (37 °C), conditions found in human body fluids. On the other hand, OmpF expression is induced under environmental conditions found outside of the human body, such as low osmolarity and low temperature. However, preliminary studies have shown that a higher proportion of translucent colonies was not observed when the *ompU* mutant was isolated from infected fish kidney tissue, indicating a more complex regulation than that initially predicted.

Under the conditions assayed, *ompU* is likely to be the most critical of the genes regulated by ToxR that play a role in biofilm formation and bile resistance. However, OmpU did not rescue the wild-type motility in a toxR mutant. In other vibrios, the OMP profile is altered in a toxR mutant, suggesting that ToxR modulates the expression of more than one OMP (Provenzano et al., 2000). Consequently, the expression of *ompU* from a ToxR-independent promoter in a toxR mutant may not create a wild-type outer membrane. The loss of other OMPs may alter the amount of LPS or phospholipid. The polar flagella of vibrios are covered in a sheath, a possible extension of the cell membrane (for a review see McCarter, 2001). The sheath may be involved in flagellar assembly, which may have implications for flagellar morphogenesis and gene expression. Variations in the content of the outer membrane may affect sheath assembly and thus flagellar assembly. On the other hand, LPS mutants of *E. coli* are reduced in motility at varying levels, suggesting that the levels of LPS may affect flagellar functions (Genevaux et al., 1999).

Survival for an aquatic bacterium in seawater outside the fish host may depend on biofilm formation. Bacteria that are part of the normal microflora of seawater, such as *V. anguillarum* (Muroga et al., 1986; West et al., 1983), are normally found attached to surfaces, as opposed to a free-swimming form, which is likely to provide an adaptive or survival advantage for bacteria in the aquatic environment (Costerton et al., 1987, 1995). The cellular location of OmpU is ideal for playing a role in bacterial cell–cell or cell–surface interactions needed for biofilm formation. In this study, a new role is proposed for the OmpU porin in biofilm formation. OmpU may limit or prohibit biofilm formation, or it may have a role in detachment from the biofilm possibly in environmental niches that are less advantageous for the bacterium. On the other hand, an *ompU* mutant may just be better at forming a biofilm than the wild-type.

The modulation of expression and the variation of the cell-surface-exposed domains of outer-membrane porins in Gram-negative bacteria is a remarkable means by which bacteria adapt and respond to environmental changes. Porins affect various different physiological phenotypes,
allowing the bacterium to adapt quickly to aid survival of the cell. The \textit{V. anguillarum} porin OmpU was shown to affect two important physiological functions, bile resistance and biofilm formation. These physiological functions are likely to be critical for the adaptation and survival of this bacterium to particular environmental niches, such as the fish host and seawater.

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