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Isolation and Characterization of *Vibrio tubiashii* Outer Membrane Proteins and Determination of a *toxR* Homolog▽†

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Outer membrane proteins (OMPs) expressed by *Vibrio tubiashii* under different environmental growth conditions were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and PCR analyses. Results showed the presence of a 38- to 40-kDa OmpU-like protein and *ompU* gene, a maltoporin-like protein, several novel OMPs, and a regulatory *toxR* homolog.

Although *Vibrio tubiashii* was originally found to cause bacillary necrosis in larval and juvenile mollusks (3, 10, 27, 28), recent studies have shown that it can also cause diarrhea in suckling mice (8). Additionally, its ability to cause death in fish (1) has led to the conclusion that *V. tubiashii* may also be a finfish pathogen. In the present study, we report that *V. tubiashii* expresses a number of known *Vibrio* outer membrane proteins (OMPs) and regulatory elements which have been shown to be involved in disease processes, including the porin-like OmpU protein and a *toxR* homolog. These results may have significant implications not only in food safety and in understanding bacterial diversity but also in illuminating the survival strategies used by marine vibrio gastrointestinal pathogens.

Isolation of OMPs, identification of an OmpU-like protein in *V. tubiashii*, and effect of environmental conditions on the expression of OMPs. For routine cultivation, frozen (−80°C) cultures of *V. tubiashii* strains ATCC 19105 and ATCC 19109, *Vibrio cholerae* strain 395, *Vibrio vulnificus* strain 4965-T1, and *Escherichia coli* strain HB101 stored in Trypticase soy broth medium (TSB; Becton Dickinson Microbiology Systems, BBL, Cockeysville, MD) supplemented with 1% NaCl (TSB-S) and 25% glycerol, pH 7.3, were rapidly thawed and each streaked onto a plate containing Trypticase soy agar medium (TSA; BBL) supplemented with 1% NaCl, pH 7.3 (TSA-S). The plates were incubated at 30°C for 18 h. For OMP extraction, each inoculum was prepared by suspending cells from a TSA-S plate into TSB-S to make a 10^8-CFU/ml cell suspension. This was then applied aseptically to the surface of 1.5 liters of TSA-S or TSA-S-supplemented agar (as described below) contained in a sterile stainless steel serving pan (53 cm [length] by 32.5 cm [width] by 6.5 cm [height]). Each culture was incubated overnight at 30°C or under various growth conditions achieved by including NaCl (0 to 8%), bile (0.1 to 1%), or maltose (2%); by growing the cells on TSA-S adjusted to different pH values (6.0, 7.0, and 8.5); and/or by incubating the cultures at different temperatures (30°C, 35°C, and 43°C). Growth from each pan's agar surface was scraped off using two 3- by 2-in. sterile microscope slides. The bacterial cells were weighed, 5 ml of sterile 0.1 M lithium acetate–0.2 M LiCl buffer (pH 8.0; lithium acetate and LiCl were obtained from Sigma Aldrich Chemical Co., St. Louis, MO) per gram of bacterial cell pellet (wet weight) was added, and the OM complexes and associated OMPs were isolated according to the procedure described by Johnston et al. (13). To verify the purity of the OM complexes, the samples were negatively stained with 1% sodium phosphotungstate (Electron Microscopy Sciences, Fort Washington, PA), pH 6.8, and evaluated by transmission electron microscopy. If flagella were present in the sample, they were removed using an acid dissociation wash step involving suspension of the pellet of crude OM complexes in 40 ml of 0.1 M sodium acetate, pH 3.0. The mixture was stirred for 2 h at 4°C and then concentrated by centrifugation as described above. The final pellets were resuspended in 1 ml of 0.1 M Tris–HCl buffer (pH 8.0) and stored at −20°C. The protein concentration of each sample was estimated by the method of Bradford (5).

A prominent structure associated with the gram-negative bacterial cell surface is the OM. In addition to containing lipopolysaccharide, the OM contains a number of proteins known as OMPs, some of which play significant roles in the pathogenicity of marine vibrios (2, 4, 11). Optimal growth conditions (TSA-S, pH 7.3, as a growth medium and 30°C as a growth temperature) established by Kothary et al. (14) were used to grow the organisms for the isolation of OMPs. However, several investigators have shown that growth conditions directly affect OMP expression in marine vibrios (25, 30). The effect of environmental growth conditions on OMP expression was studied by carrying out sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analyses of the OMP preparations from cells grown under the previously described conditions using 8 to 25% gradient or homogenous gels in a PhastSystem (GE Healthcare, Piscataway, NJ) and the Laemmli procedure (16). The molecular weights of the denatured and reduced OMPs were estimated by the relative-mobility method of Weber et al. (29). Figure 1 is an SDS-PAGE gel showing the OMPs isolated from *V. tubiashii* strains ATCC 19105 and ATCC 19109 grown on TSA-S (pH 7.3) at 30°C in comparison to tho OMPs obtained for *V. cholerae*, *V. vulnifi-*

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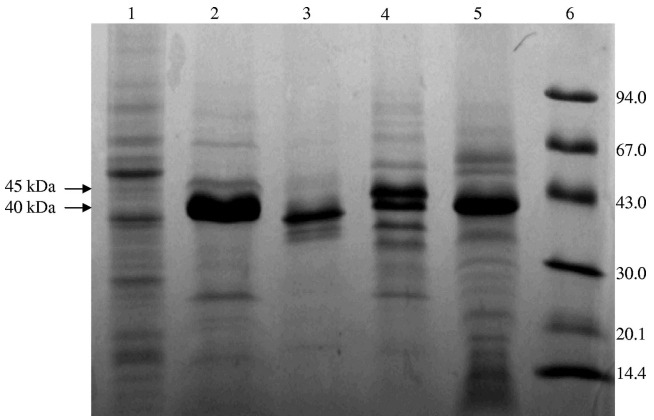

FIG. 1. SDS-PAGE analysis of the outer membrane proteins expressed by *E. coli* strain HB101, *V. cholerae* strain 395, *V. vulnificus* strain 4964-TI, *V. tubiashii* ATCC 19105, and *V. tubiashii* ATCC 19109. Lane 1, *E. coli* strain HB101; lane 2, *V. cholerae* strain 395; lane 3, *V. vulnificus* strain 4965-TI; lane 4, *V. tubiashii* ATCC 19109; lane 5, *V. tubiashii* ATCC 19105. Strains were grown at 30°C on TSA-S. Lane 6 contains molecular markers, and sizes are reported in kDa. Note that the 40- and 45-kDa OMPs are identified with an arrow.

cus, and *E. coli*. Each of the OMP preparations from the *V. tubiashii* strains included approximately 13 proteins, and a major 40-kDa OMP was observed in both preparations. However, uniquely expressed minor OMPs, varying in molecular weight and in expression level, made each strain's preparation visually different from the other and also distinctly different from OMP preparations of *V. cholerae*, *V. vulnificus*, and *E. coli*. The molecular size ranges were approximately 20 kDa to above 94 kDa for strain ATCC 19109 and 14 to approximately 80 kDa for strain ATCC 19105. The 40-kDa OMPs were also observed in OMP preparations from *V. vulnificus* and *V. cholerae* but not in those from *E. coli.* Furthermore, both *V. tubiashii* strain ATCC 19109 and *V. cholerae* strain 395 contained a 45-kDa protein that was not observed in OMP preparations from *V. tubiashii* strain ATCC 19105, *V. vulnificus* 4965-T1, or *E. coli* HB101. OMPs subjected to SDS-PAGE analysis were electrophoretically transferred onto ProBlott membranes (Applied Biosystems, Foster City, CA) for N-terminal amino acid (NTAA) sequencing (three or more repeats for each protein) using a Procise model 491 protein sequencer (Applied Biosystems). Homologies of the sequences of the proteins to known or related proteins were determined by using BLAST analysis, and sequence alignment was carried out using Clustal X analysis. NTAA and BLAST analyses of the 40-kDa protein from both *V. tubiashii* strains suggest that these OMPs were like OmpU, a known commonly expressed *Vibrio* porin, and each NTAA sequence possessed high homology (71 to 92%) to the OmpU proteins (Table 1) expressed by *V. vulnificus*, *V. cholerae*, *Vibrio parahaemolyticus*, and *Listonella anguillarum* (6, 25, 26). OmpU in *V. cholerae* is a 38-kDa protein, its expression is positively regulated by ToxR, and the 1- to 2-nm porin channel of OmpU serves as a site of entry and exit of hydrophilic, low-molecular-weight molecules (25). OmpU has also been found to act as an adherence factor involved in the colonization of epithelial cells by *V. cholerae* and plays an important role in the osmoregulation of the cell (19). It is interesting to note that our previously reported serological studies (12a) showed that antisera raised against *V. tubiashii* strains ATCC 19105 and ATCC 19109 could agglutinate each of the strains as well as *V. cholerae* and *V. vulnificus* cells. Speculatively, these results suggest that OmpU may serve as the major agglutinin responsible for the serological cross-reactivity observed in these experiments. This hypothesis also agrees with the thought expressed by Provenzano et al. (25) that porins constitute a major portion of a cell's OMP content, and it has been

TABLE 1. N-terminal amino acid sequences of OMPs expressed by *V. tubiashii* strains ATCC 19105 and ATCC 19109[a]

V. tubiashii strain	Growth condition	Mass of OMP (kDa)	N-terminal amino acid sequence of OMP	% ID	% +	Related protein[b]
ATCC 19105	TSA-S	40	AEIYNQDGNTIEMGGGAEAAL	71	85	OmpU
	3% NaCl	65	GEIYSTDVDLVGLDGDKMLDDS	None	None	None
		50	GEIYSTDSSAVGLDGEVDAYLA	None	None	None
	2% Maltose	45	EDFVQQGGDNDYGPNLQNG	None	None	None
		40	AEIYNQDGNTIEMGGRAEAALL	80	90	OmpU
	0.3% Bile	40	AEIYNSDGNTIEMGGFNRAAL	60	80	OmpU
ATCC 19109	TSA-S	45	SADGIQAGGIEGTSVYSDNGNS	None	None	None
	3% NaCl	40	GELYNQDGTSLALGGRAEXLS	85	90	OmpU
		90	EDYALRDDVVGNAALSANHHDLD	None	None	None
		70	AGDQLNPDARDESLNYSPIAIN	None	None	None
		45	SADGIQAGGIEGTTVYSGNGAD	None	None	None
	1% Bile	40	IELYNQDGVTVNMTGDVEVRYN	80	90	OmpU
		45	VDFVGYFVAGMGIQGNNNEDYL	60	70	Maltoporin OmpS
		40	GELYNQDGTSLALGGRALAALS	92	92	OmpU

[a] % ID, percentage of the protein sequence that was identified by BLAST analysis; % +, percentage of the amino acids that were positively identified from a known sequence.
[b] "None" indicates that the protein is novel.

TABLE 2. PCR primers, annealing temperatures, and theoretical sizes of the amplicons used in the study

Gene	Primer sequences	Annealing temp (°C)[a]	Amplicon size (bp)	Reference
ompU	5′-GACTTAATCATATGAACAAGACTCTGATTGCT-3′ (forward) 5′-CGCCGCGGTTAGAAGTCGTAACGTAGACCGATAGC-3′ (reverse)	40–**50**	1,026	24
ompT	5′-AGTGGATTCATATGAAAAAACTCTATTAGCA-3′ (forward) 5′-TCCCCGCGGTTACCAGTAGATACGAGCGCCGATA-3′ (reverse)	40–**50**	1,035	24
ompW	5′-CACCAAGAAGGTGACTTTATTGTG-3′ (forward) 5′GAACTTATAACCACCCGCG-3′ (reverse)	40–**60**	588	20
toxR[b]	5′-CAGGGSTTYGAGGTGGAYGAY-3′ (forward) 5′-GGAGCGACCGTTGAATGAGCA-3′ (reverse)	40–**56**	350	This study

[a] Temperature readings in bold represent the annealing temperatures that corresponded to what was reported in the report cited.
[b] Primers for the detection of *toxR* are based on a nucleotide sequence found in *V. fluvialis* (NCBI accession number AF170885 [23]).

estimated that OmpU represents ca. 30 to 60% of the total OMPs of *V. cholerae*. In addition to the OmpU-like protein, a 45-kDa protein found associated with cells of strain ATCC 19109 grown in the presence of bile salts (see Fig. S2a in the supplemental material) was identified by NTAA sequence and BLAST analyses as a maltoporin-like protein, OmpS, which has an NTAA sequence with 70% homology with that of OmpS expressed by *V. cholerae* El Tor strain N16961. Yet the 45-kDa protein identified in the other OMP samples, including the sample obtained from cells grown under optimal conditions (Fig. 1), has no homology to known proteins in the *Vibrio* proteome (data not shown). Thus, the reason for its varied levels of expression and the identity of the 45-kDa protein are unclear at this time. SDS-PAGE analysis of OMPs (Fig. S1a to b and S2a to b in the supplemental material) of both strains of *V. tubiashii* grown under various environmental conditions demonstrated that the OmpU-like protein was always expressed and further provides evidence that OmpU may be a common and constitutively expressed OMP. Between the two *V. tubiashii* strains, a total of over 15 OMPs were differentially expressed in cells grown under the various growth conditions described in this report (see Fig. S1a to b and S2a to b in the supplemental material). Many of the stained OMPs were visible but had insufficient amounts of protein for NTAA sequencing. Table 1 presents those OMPs which could be characterized using SDS-PAGE analysis and identified by NTAA and BLAST analyses. These include three OMPs (65 kDa, 50 kDa, and 45 kDa) characterized as novel hypothetical proteins expressed by *V. tubiashii* strain ATCC 19105 grown in the presence of 3% NaCl and 2% maltose and three novel OMPs (90 kDa, 70 kDa, and 45 kDa) expressed by *V. tubiashii* strain ATCC 19109 grown in the presence of 3% NaCl. Lastly, the NTAA sequences of the OmpU-like protein expressed by strain ATCC 19105 grown under different growth conditions were slightly different, suggesting that varying the growth conditions of *V. tubiashii* can affect the amino acid sequence of this OMP (Table 1). Sperandio et al. (26) suggested that in *V. cholerae*, the expression of 38-kDa OmpU and 40-kDa OmpT are regulated in opposing fashions such that the expression of OmpU is positively regulated by ToxR and the expression of OmpT is negatively regulated. This information further supports the premise that different environmental conditions can influence the expression of different OMPs.

Detection of an *ompU* homolog in *V. tubiashii*. In order to confirm the presence of the OmpU-like protein expressed by the strains and to determine whether the *V. tubiashii* strains had homologous *ompU* genes, PCR analysis was performed using primer pairs (Table 2) designed by Sperandio et al. (26) and based on the sequence of *ompU* of *V. cholerae*. DNA templates were prepared according to the procedure described by Kothary et al. (15). PCR analysis was carried out using a general master mix, and each reaction mixture contained 2 μl of a 3 μM concentration of the forward, 5′-end primer and 2 μl of a 3 μM concentration of the reverse, 3′-end primer (Invitrogen Corporation, Carlsbad, CA), 5 μl of 10× Tris-borate-EDTA buffer (Qiagen, Inc., Valencia, CA), 2 μl of 25 mM $MgCl_2$ (Qiagen, Inc.), 2 μl of a 10 mM concentration of the deoxynucleoside triphosphates or 10 μl of a 2 mM concentration of the deoxynucleoside triphosphates (Invitrogen Corporation), and 0.5 μl of Hot Star *Taq* (Qiagen, Inc.). PCR amplification of each isolate was conducted using 5 μl of the DNA template and 45 μl of a master mix containing primers, for a total volume of 50 μl. As shown in Fig. 2, a PCR product of

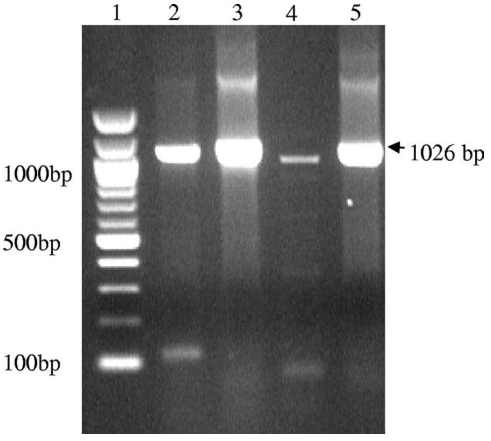

FIG. 2. PCR analysis of the *ompU* gene of *V. tubiashii* strains ATCC 19105 and ATCC 19109, *V. vulnificus* 4965-T1, and *V. cholerae* 395. Lane 1 contains molecular markers, and sizes are reported in bp. Lanes 2 to 5 contain the PCR products obtained for *V. tubiashii* strain ATCC 19105, *V. tubiashii* strain ATCC 19109, *V. vulnificus* strain 4965T1, and *V. cholerae* strain 395, respectively.

1,026 bp was obtained during a reaction using an annealing temperature of 50°C. The amplicon was sequenced, and BLAST analysis (see Fig. S3 in the supplemental material) suggests that the *ompU*-like homolog of both of the *V. tubiashii* strains has 90% homology to the *ompU* genes possessed by *V. vulnificus*, *V. cholerae*, *V. parahaemolyticus*, and *L. anguillarum*.

Lack of expression and detection of OmpT, OmpW, *ompT*, and *ompW* and suggestion of *toxR*'s presence in *V. tubiashii*. Sperandio et al. (26) found that the expression of cholera toxin (CT), toxin-coregulated pilus (TcpA), OmpT, and OmpU of *V. cholerae* was affected by changes in osmolarity and amino acid concentration, while other environmental signals, such as temperature and pH, had more-pronounced effects on the expression of CT and TcpA than on the expression of OMPs. Unfortunately, among the OMPs characterized by NTAA and BLAST analyses in our study, protein homologs of OmpT, TcpA, and other OMPs reported by Provenzano et al. (25), such as OmpA, OmpC, OmpF, and the highly immunogenic proteins OmpV, OmpW, and OmpX, were not positively identified in *V. tubiashii* by our approach (12, 17). Therefore, further investigations using molecular approaches were conducted to help identify some of the OMPs by using PCR primers based on *V. cholerae* and other *Vibrio* species. The presence of the *ompT* and *ompW* genes in *V. tubiashii* was tested by carrying out the PCRs according to conditions described in Table 2 with primers described by Provenzano et al. (25) for *ompT* and with primers described by Nandi et al. (21) for *ompW*. *V. cholerae* strain 395 was used as a control strain for these PCR analyses. *ompW* and *ompT* gene homologs were not found in *V. tubiashii*. In contrast, both *V. cholerae* and *V. vulnificus* produced amplicons of the expected sizes, which by DNA sequencing and BLAST analysis were identified as *ompW* and *ompT* homologs (data not shown). Speculatively, the absence of *ompW* and *ompT* in *V. tubiashii* could also explain why disease caused by *V. tubiashii* has not been found in humans. However, during a 3-year period (1997 to 1999), more than 40 infections reported to the CDC were caused by a number of marine vibrios that have yet to be identified at the species level (7). These data suggest that there are a number of pathogenic marine vibrios still emerging. Could some of these poorly studied, human-derived vibrios be *V. tubiashii*? Alternatively, and possibly through horizontal gene exchange, could traits currently seemingly absent in *V. tubiashii*, such as critical virulence OMPs, i.e., OmpT and OmpW, one day be acquired? These questions remain unanswered. The inability to demonstrate *ompT* and *ompW* homologs in *V. tubiashii* also suggests that if these genes are present in *V. tubiashii*, their nucleotide sequences may be much different from those of *V. cholerae* and other pathogenic vibrios that have been studied. Conversely, the identification of the OmpU-like protein and OmpS-like protein and an *ompU* homolog in *V. tubiashii* suggested the presence of the ToxR regulon in *V. tubiashii*.

Detection of a *toxR* homolog in *V. tubiashii*. Pathogenicity and the expression of virulence factors in *Vibrio* species, such as *V. cholerae*, are coordinately regulated by the *toxR* regulon (19, 20, 23). Therefore, to test the hypothesis that *V. tubiashii* may have a *toxR* homolog, PCR analyses were performed. Using degenerate *toxR* primers based on *V. parahaemolyticus* and *V. alginolyticus*, a similar *toxR* homolog was not found in *V. tubiashii* (data not shown). However, using PCR primers based on *toxR* sequences of *Vibrio fluvialis* (Table 2), a *toxR* homolog was detected in both strains of *V. tubiashii* by using a PCR protocol that incorporated an initial period of 15 min at 95°C to activate Hot Star *Taq*, followed by 45 amplification cycles that included a 2-min denaturing step at 95°C, a 45-s annealing step at 56°C, and a 45-s extension step at 72°C. This was followed by a final extension step of 5 min. *V. fluvialis* strain 807-77 was used as a control strain for the *toxR* PCR analysis. Nucleotide sequence analysis of the *V. tubiashii*/*V. fluvialis* *toxR*-like amplicon (Fig. 3) showed that it possessed 85% homology (see Fig. S4 in the supplemental material) to the *toxR* homologs of *V. fluvialis*, *V. cholerae*, *V. vulnificus*, *V. harveyi*, and *V. parahaemolyticus*. These results also indicate that the primers used to detect the *toxR*-like homolog recognized in *V. tubiashii* the transcriptional activation and membrane tether regions of *Vibrio* species *toxR* (24). ToxR was first discovered as a positive transcriptional regulator of the cholera toxin (*ctx*) gene (20). Since then, at least 17 ToxR-activated genes have been described, including genes encoding CT, Tcp, accessory colonization factor (Acf), OmpU, and OmpT (26, 30). In addition, hemolysin, protease, mucinase, neuraminidase, cytolysin, lipases, adhesins, lipopolysaccharide, fimbriae, and thermostable direct hemolysins (*tdh*) are some of the other virulence factors found among *Vibrio* species that are also regulated by ToxR (18, 22). The finding of a *toxR*-like homolog is significant, since *toxR* is a major regulator of pathogenicity in *Vibrio* species. In addition to supporting the relatedness of *V. tubiashii* to other pathogenic *Vibrio* species, the presence of *toxR* raises the possibility that *V. tubiashii* may have pathogenicity elements essential for its emergence as a pathogen. Previous reports dealing with the presence and characterization of a metalloprotease and a *vulnificus*-like cytolysin along with the genomic relatedness of *V. tubiashii* to other *Vibrio* species also support this possibility (8, 14). In conclusion, the findings reported here indicate that *V. tubiashii* expresses a number of known *Vibrio* OMPs, including OmpU- and OmpS-like proteins and novel OMPs. Furthermore, expression of these OMPs can be influenced by culture growth conditions. The findings in this report also show that *V. tubiashii* possesses a ToxR regulatory element similar to that of other pathogenic marine vibrios.

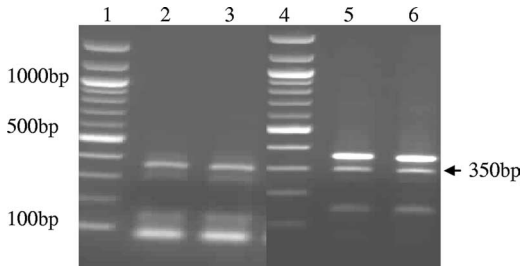

FIG. 3. PCR analysis of *toxR* gene identified by PCR based on primers designed from *V. fluvialis toxR*. Lanes 1 and 4, molecular markers (sizes are reported in bp); lanes 2 to 3, *V. tubiashii* strain ATCC 19105; lanes 5 to 6, *V. tubiashii* ATCC 19109. Note that the 350-bp PCR product is identified with an arrow.

Nucleotide sequence accession numbers. The DNA sequences for *ompU* of *V. tubiashii* strains ATCC 19105 and ATCC 19109 (accession numbers EU285490 and EU285491,

respectively) and for *toxR* of *V. tubiashii* strains ATCC 19105 and ATCC 19109 (accession numbers EU285492 and EU285493, respectively) have been deposited in the GenBank database.

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