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# A Novel Protein, TtpC, Is a Required Component of the TonB2 Complex for Specific Iron Transport in the Pathogens *Vibrio anguillarum* and *Vibrio cholerae*[∇]

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Active transport across the outer membrane in gram-negative bacteria requires the energy that is generated by the proton motive force in the inner membrane. This energy is transduced to the outer membrane by the TonB protein in complex with the proteins ExbB and ExbD. In the pathogen *Vibrio anguillarum* we have identified two TonB systems, TonB1 and TonB2, the latter is used for ferric-anguibactin transport and is transcribed as part of an operon that consists of *orf2*, *exbB2*, *exbD2*, and *tonB2*. This cluster was identified by a polar transposon insertion in *orf2* that resulted in a strain deficient for ferric-anguibactin transport. Only the entire cluster (*orf2*, *exbB2*, *exbD2* and *tonB2*) could complement for ferric-anguibactin transport, while just the *exbB2*, *exbD2*, and *tonB2* genes were unable to restore transport. This suggests an essential role for this Orf2, designated TtpC, in TonB2-mediated transport in *V. anguillarum*. A similar gene cluster exists in *V. cholerae*, i.e., with the homologues of *ttpC-exbB2-exbD2-tonB2*, and we demonstrate that TtpC from *V. cholerae* also plays a role in the TonB2-mediated transport of enterobactin in this human pathogen. Furthermore, we also show that in *V. anguillarum* the TtpC protein is found as part of a complex that might also contain the TonB2, ExbB2, and ExbD2 proteins. This novel component of the TonB2 system found in *V. anguillarum* and *V. cholerae* is perhaps a general feature in bacteria harboring the *Vibrio*-like TonB2 system.

Iron is an essential element for all organisms but is not freely available because in the environment iron is found in the form of insoluble ferric-oxyhydroxides, and in vertebrates this metal is mainly bound to heme, transferrin, lactoferrin, and ferritin (29, 40). Thus, bacteria have evolved mechanisms to acquire the otherwise-unavailable iron. Bacterial iron acquisition systems can be divided in two main groups: those that utilize iron directly from the host proteins, such as transferrin and heme/hemoglobin, and those that produce intermediaries, such as hemophores and siderophores, which scavenge heme or iron, respectively, from any iron-chelating compound (13, 29, 30, 40). In both cases, uptake of the iron source requires a specific outer membrane receptor. The transport across the outer membrane is an active process in which the energy required is generated from the proton motive force in the inner membrane and transduced by the TonB protein in complex with ExbB and ExbD to the outer membrane receptor (28). The latter proteins belong to the MotB/TolR/ExbB and MotA/TolQ/ExbD family of proteins, respectively. Uptake of group B colicins and infection by phages T1 and φ80 also requires the energy transduced by the TonB system (35). The TonB system is a complex consisting of the proteins TonB, ExbB, and ExbD, and it was demonstrated in *Escherichia coli* to be required for

the TonB protein to bind the outer membrane receptor at a conserved region called the TonB box (2, 28). This complex is believed to have more than one molecule of each protein, and the ratio between TonB and the outer membrane receptor is 2:1 (18). In *E. coli*, TonB was found to be part of both the inner and outer membranes, where it is possibly associated with the outer membrane receptor (22).

We have previously identified in the fish pathogen *Vibrio anguillarum* two TonB systems (36). The first, TonB1, is able to transport heme and ferrichrome, whereas the second TonB system, TonB2, in addition to the transport of heme and ferrichrome, can also facilitate the uptake of catechol siderophores, including the endogenous catechol/hydroxamate siderophore anguibactin (36). The TonB2 system is also essential for virulence in rainbow trout, and its genes are transcribed as an iron-regulated operon (36). We describe here the previously uncharacterized gene, *ttpC*, that is located upstream of the *exbB2*, *exbD2*, and *tonB2* genes and demonstrate that this novel protein is essential for TonB2-mediated iron transport in *V. anguillarum*. Homologues of the *ttpC* gene are found as part of the *tonB2*-like system in all other *Vibrio* species examined, suggesting that it could also play a role in the *tonB2*-mediated iron transport in these bacteria. In the present study we confirmed this hypothesis in one of these microorganisms, *V. cholerae*, and we show that TtpC is essential for TonB2-mediated transport of enterobactin in this bacterium.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in the present study are described in Table 1. *V. anguillarum* and

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Genotype and relevant characteristics | Source or reference |
|---|---|---|
| **Strains** | | |
| *V. anguillarum* 775(pJM1) | Wild type | 12 |
| *V. anguillarum* MS570 | *V. anguillarum* 775 *ttpC*::Tn*10*Km$^r$ TonB1::Cm$^r$ | 36 |
| *V. anguillarum* MS801 | *V. anguillarum* 775 *tonB2*::Km$^r$ | 36 |
| *V. anguillarum* MS995 | *V. anguillarum* 775 *ttpC*::Tp$^r$ | This study |
| *V. cholerae* CA401 | Wild type | 4 |
| *V. cholerae* DOV221 | *V. cholerae* Δ*exbB2* in strain CA401 | 26 |
| *V. cholerae* DOV300 | *V. cholerae* Δ*exbB2* Δ*exbB1* in strain CA401 | 26 |
| *V. cholerae* MS1033 | *V. cholerae ttpC* mutant *in* strain CA401 | This study |
| *E. coli* HB101 | *supE44 hsd20* (r$_B^-$ m$_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* | 8 |
| *E. coli* JM109λ*pir* | *recA1 endA1 gyrA96 thi hsdR17 supE4relA1*Δ(*lac-proAB*$^+$) (F′ *traD36 proAB lacI*$^q$ *lacZ*ΔM15) λ*pir* | Lab collection |
| *E. coli* W3110 | F$^-$ IN(*rrnD-rrnE*)1 | 19 |
| *E. coli* KP1032 | *tonB* mutant of *E. coli* W3110 | 23 |
| **Plasmids** | | |
| pJM1 | Indigenous plasmid in strain 775 | 12 |
| pRK2073 | Helper plasmid for conjugation; Tp$^r$ Tra$^+$ | 16 |
| pCR2.1-TOPO | Cloning vector; Km$^r$ Ap$^r$ | Invitrogen |
| pACYC177 | Cloning vector; Km$^r$ Ap$^r$ | 10 |
| pTW-MEV | Suicide vector, R6K ori, *sacB*; Ap$^r$ | 39 |
| pDM4 | Suicide vector R6K ori, *sacB*; Cm$^r$ | 25 |
| p34E-Tp | Vector harboring the Tp$^r$ cassette | 14 |
| pUC4K | Vector harboring the Km$^r$ cassette | 37 |
| pCR8 | Cloning vector; Sp$^r$ | Invitrogen |
| pMS989 | *V. anguillarum* Δ*ttpC*::Tp$^r$ in pDM4 | This study |
| pMS1029 | *V. cholerae ttpC*::Km$^r$ in SalI in pTW-MEV | This study |
| pMS789 | pACYC177 pKm$^r$ *exbB2 exbD2 tonB2* | 36 |
| pMS800 | pACYC177 pKm$^r$ *ttpC exbB2 exbD2 tonB2* | This study |
| pMS800-Δ*exbB2* | pMS800 with internal deletion in *exbB2* | This study |
| pMS800-Δ*exbD2* | pMS800 with internal deletion in *exbD2* | This study |

*V. cholerae* were cultured at 25°C in either Trypticase soy broth or agar supplemented with 1% NaCl (TSBS or TSAS, respectively). For experiments determining iron uptake characteristics, the strains were first grown on TSAS supplemented with the appropriate antibiotics and passed to M9 minimal medium (32) supplemented with 0.2% Casamino Acids, 5% NaCl, and the appropriate antibiotics. To achieve iron-limiting conditions, 2 μM ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) (EDDA) was added to the medium, while for iron-rich conditions the medium was supplemented with 4 μg of ferric ammonium citrate/ml. The antibiotic concentrations used for *V. anguillarum* were ampicillin at 250 to 1,000 μg/ml, rifampin at 100 μg/ml, chloramphenicol (Cm) at 10 μg/ml, kanamycin (Km) at 200 μg/ml and for *V. cholerae* were Km at 200 μg/ml and trimethoprim (Tp) at 100 μg/ml.

*E. coli* strains were grown in Luria-Bertani (LB) medium in the presence of the appropriate antibiotics. Antibiotic concentrations used for *E. coli* were ampicillin at 100 μg/ml, Cm at 30 μg/ml, Km at 100 μg/ml, Tp at 100 μg/ml, and spectinomycin at 100 μg/ml.

**General DNA procedures.** Plasmid DNA preparations were performed by using the alkaline lysis method (6). Sequence quality plasmid DNA was generated by using the Qiaprep spin miniprep kit (QIAGEN) and Wizard Plus SV Minipreps (Promega). Restriction endonuclease digestion of DNA was performed under the conditions recommended by the suppliers (Invitrogen, Roche, and New England Biolabs). Transformations of plasmid DNA to *E. coli* strains and other cloning strategies were performed according to standard protocols (32). Plasmids were transferred from *E. coli* to *V. anguillarum* and *V. cholerae* by triparental conjugation as previously described (38). Primers were designed by using Oligo 6.8 primer analysis software and purchased from Invitrogen. DNA and protein sequence analysis were carried out at the National Center for Biotechnology Information using the BLAST network service (3), with the Sequencher program version 4.2 (Genecodes) and also using the Tcoffee server (27).

**Nucleotide and protein sequence accession numbers.** The nucleotide sequence of the *tonB2* cluster of *V. anguillarum* is deposited in GenBank under accession number AY644719. Accession numbers for additional sequences are referred to in the tables and figure legends.

**Construction of plasmids.** Plasmid pMS789 was generated by cloning the 1.7-kb PCR product, obtained using primers TonB2L and ExbB2U (Table 2), into vector pCR2.1-TOPO (Invitrogen). The following cycles were used to obtain the PCR product: 3 min at 95°C, followed by 30 cycles of 1 min at 95°C, 45 s at 55°C, and 2 min at 72°C. The XhoI-BamHI fragment from the PCR2.1-TOPO vector containing *exbB2*, *exbD2*, and *tonB2* was subcloned into pACYC177 digested with XhoI-BamHI so that the *exbB2*, *exbD2*, and *tonB2* genes are under

TABLE 2. Primers used in this study

| Primer | Sequence (5′–3′) |
|---|---|
| TonB2L | AATGGCTTGAGTGAGGTTCT |
| ExbB2U | TTATGCAGCAAGGGGGTATC |
| TtpCmutU1 | CAAACCATTAAAGCGCGAAA |
| TtpCmutL1 | GCTAATAATAGTGGAATCGCAA ATCTTGGCGCTGAGCTTCTA |
| TtpCmutU2 | TAGAAGCTCAGCGCCAAGATTT GCGATTCCACTATTATTAGC |
| TtpCmutL2 | AGATGCCATCAATTTAGGAT |
| TtpCvcU | CAATTGGCTCAGCAACAGATT |
| TtpCvcL | TCGTCACGGATAGGCAAGA |
| ExbB2invU | TTGGCCATGGGCTGTTCATTACG |
| ExbB2invL | TGTCCATGGTCGTCTTGCAAAAA |
| ExbD2invU | CATTCCATGGTTGGCGTCTG |
| ExbD2invL | GCGGCCATGGGATTCAAGCGGA |
| TtpCvaU | AAGCTCAGAGGTGGCTTGAT |
| ExbD2vaL | GGCTGGTCGGTTAATAACTG |
| RT-PCRvaTonB2U | AATCGCAACCATCTCTTTAG |
| RT-PCRvaTonB2L | TTATGGCGTGGCTGGTGGAT |
| RT-PCRvcTonB2U | CTGAGCAGGAGCAAGATGTA |
| RT-PCRvcTonB2L | AGCTTTCGCTGGATAGTTGG |

the control of the constitutive Km resistance (Km$^r$) gene promoter. Plasmid pMS800 was generated by cloning the PCR product that contains the *ttpC*, *exbB2*, *exbD2*, and *tonB2* genes in pCR2.1-TOPO using the TOPO cloning strategy from Invitrogen. The PCR product was obtained using total DNA from *V. anguillarum* 775 and the primers TtpCvaU and ExbD2vaL (Table 2) for 3 min at 95°C, followed by 30 cycles of 1 min at 95°C, 45 s at 55°C, and 2 min at 72°C. From the pCR2.1 construct, the 2-kb XhoI-BamHI fragment was cloned into pACYC177 digested with XhoI-BamHI, so that the four *V. anguillarum* genes are under the control of the constitutive Km$^r$ gene promoter. To generate plasmids pMS800-Δ*exbB2* and pMS800-Δ*exbD2*, we used inverse PCR with the primers exbB2invU and exbB2invL for Δ*exbB2* and exbD2invU and exbD2invL for Δ*exbD2* (Table 2) and pMS800 as template DNA. All four primers were engineered to contain an NcoI site. The same PCR cycles were used as described above with an extension time of 5 min. The PCR product was then digested with NcoI and ligated prior to electroporation into HB101 competent cells. The plasmids pMS789 and pMS800 and its mutant derivatives were all sequenced to assure that no mutation was induced during the PCR and cloning steps. Plasmid pMS989 was generated by first creating a construct containing an internal deletion of the *ttpC* open reading frame of *V. anguillarum* from two separate PCR products amplified with primers TtpCmutU1 and TtpCmutL1 for the first 500 bp and primers TtpCmutU2 and TtpCmutL2 (Table 2) for the last 500 bp. These two products were then used in a second PCR with primers TtpCmutU1 and TtpCmutL2 to obtain the ligated product of the first and last 500 bp of *ttpC*. This product was then cloned into pCR2.1-TOPO using the TOPO cloning strategy from Invitrogen. The Tp resistance (Tp$^r$) gene from p34E-TP (14) was then inserted as a blunt EcoRI product in the unique SspI site within the PCR product cloned in pCR2.1. The deleted *ttpC* gene, including the Tp$^r$ gene, was cloned as a SpeI-XbaI product into the SpeI site of pDM4, resulting in pMS989. To generate pMS1029, a PCR product made with primers TtpCvcU and TtpCvcL (Table 2), containing the complete *ttpC* gene of *V. cholerae* was cloned in pCR8 by using the TOPO cloning strategy from Invitrogen. The Km$^r$ gene from pUC4K (37) was cloned as a SalI fragment into the unique SalI site within the *ttpC* open reading frame. The *ttpC* gene with the Km$^r$ gene inserted was then removed by restriction endonuclease digestion with EcoRI, followed by treatment with the Klenow fragment of DNA polymerase I, and cloned in the EcoRV site of pTW-MEV (39), resulting in pMS1029.

**Construction of *V. anguillarum* and *V. cholerae* strains by conjugation and allelic exchange.** To generate the nonpolar mutant in *ttpC* strain MS995, the plasmid pMS989, harboring the *ttpC* deletion and insertion of the Tp cassette, was transferred to *V. anguillarum* 775 by conjugation. Exconjugants were plated on plates containing rifampin (selection for *V. anguillarum* 775), Tp (selection for mutant *ttpC* gene), and 5% sucrose. Only cells that have recombined and have the Tp cassette in *ttpC* inserted at the wild-type locus will be able to grow on 5% sucrose. The mutants were confirmed by PCR to assure that the wild-type copy was replaced by the deletion containing the Tp$^r$ cassette (data not shown). Strain MS1033 was generated by conjugating the suicide plasmid pMS1029, containing the *ttpC* gene of *V. cholerae* with the Km$^r$ gene inserted in the SalI site, to *V. cholerae* CA401. Exconjugants were selected on TCBS agar plates supplemented with Km to obtain colonies that have the whole pM1029 integrated in the chromosome of *V. cholerae*. These strains were then streaked on plates containing 10% sucrose and Km to select for colonies that lost the vector but maintained the Km$^r$ insertion. Loss of the vector was verified by PCR (data not shown).

**RNA isolation.** A 1:100 inoculum from an overnight culture was grown in minimal medium with appropriate antibiotics. Cultures were grown either with 4 μg of ferric ammonium citrate/ml (iron-rich) or with 2 μM EDDA (iron limiting). Total RNA was prepared when the culture reached an optical density at 600 nm (OD$_{600}$) of 0.3 to 0.5 using the RNAwiz (Ambion) isolation kit according to the manufacturer's recommendations.

**RT-PCR analysis.** One microgram of total RNA was reverse-transcribed to generate cDNA, using Moloney murine leukemia virus-reverse transcriptase (RT) from Ambion according to the manufacturer's recommendations. Prior to reverse transcription, total RNA was made DNA free by using TURBO DNA-free (Ambion) according to the manufacturer's recommendation. As a control, parallel samples were run in which RT was omitted from the reaction mixture. The primers used for the RT reaction were, RT-PCRvaTonB2L for *V. anguillarum tonB2* and RT-PCRvcTonB2L for *tonB2* of *V. cholerae* (Table 2). The cDNA was diluted five times, and a 10% aliquot was used in the subsequent PCR with the primers RT-PCRvaTonB2U and RT-PCRvaTonB2L for *V. anguillarum* and RT-PCRvcTonB2U and RT-PCRvcTonB2L for *V. cholerae* as listed in Table 2. For the PCR, *Taq* polymerase from New England Biolabs was used according to the manufacturer's recommendations.

**Bioassays.** A 50-μl portion of an overnight culture of each strain to be tested was seeded in a plate containing minimal medium, 0.7% agarose, and the iron

chelator EDDA. The purified compounds spotted on top of the bioassay plates to determine the functionality of the TonB cluster genes in *V. anguillarum* and *V. cholerae* were anguibactin (1 mg/ml; our laboratory), enterobactin (1 mg/ml; Biophore Research), heme (20 μM; Sigma), ferrichrome (1 mg/ml; Sigma), and ferric ammonium citrate (500 μg/ml; Sigma). From all iron sources, 5 μl was spotted onto the bioassay plate.

**Antibody generation.** One rabbit per protein was immunized with a peptide for either TtpC-CSVYNKEQNRSVEALE- or TonB2-EQEHDLQRRQRSVPEC- of *V. anguillarum*. The peptides and antibodies were generated by Lampire Biological Laboratories according to their standard protocols.

**Protein isolation.** *V. anguillarum* cells grown in minimal medium were pelleted and resuspended in phosphate buffer (pH 6.7). Cells were lysed by six 10-s sonications at 10-s intervals. The lysates were centrifuged for 1 min at 13,000 rpm to remove whole cells. These samples were used as total proteins. To obtain total membrane proteins, the total protein suspension was pelleted for 40 min at $30,000 \times g$. The pellet contains the total membrane fraction, and the supernatant was used as a soluble fraction containing the periplasmic and cytoplasmic proteins. To obtain outer and inner membrane proteins, the total membrane fraction was extracted with a 1.5% final concentration of Sarkosyl. After centrifugation of 30 min at $30,000 \times g$, the outer membranes are in the pellet while the inner membrane proteins are in the supernatant.

**Sucrose density gradients.** *V. anguillarum* cells were grown in minimal medium, and whole-cell lysates were extracted as described above, with the exception that 10 mM HEPES, 25% sucrose, and 5 mM EDTA buffer was used. A 1-ml portion of the cell lysate was pipetted on top of a sucrose gradient in Ultra-Clear centrifuge tubes from Beckman (14 by 89 mm). Sucrose gradients were made by pipetting 2 ml of sucrose solutions in 5 mM EDTA at 55, 50, 45, 40, 35, and 30%. Sucrose gradients were run in a Beckman ultracentrifuge at $37,000 \times g$ for 24 h. A total of 24 0.5-ml fractions were collected from each tube, and 20 to 80 μl was used for Western blot analysis.

**In vivo formaldehyde cross-linking.** These experiments were performed as described by Skare et al. (34). In brief, *V. anguillarum* cells were grown in minimal medium to an OD$_{600}$ of 0.8. Cells were washed twice in phosphate buffer (pH 6.7) and resuspended in the same buffer to an OD$_{600}$ of 0.5. A one-seventh aliquot was taken for the total membrane preparation, and formaldehyde was added to a 1% final concentration to the remaining cells suspension. Immediately after the addition of formaldehyde a sample was taken (0.5-min time point), and samples of equal volume were then taken at 5, 10, 20, and 30 min. The 30-min time point was done in duplicate, and one these samples was used for the boiled sample. From all samples total membrane proteins were obtained for Western blot analysis.

**Western blot analysis.** Protein samples were mixed with 2× Laemmli buffer (32), and the proteins were separated on 10% Bis-Tris criterion XT precast gels in 1× XT morpholinepropanesulfonic acid buffer (Bio-Rad). Gels were soaked for 15 min in transfer buffer (0.3% Tris, 1.44% glycine, and 20% methanol) and transferred to Protran nitrocellulose membrane (Schleicher & Schuell) in transfer buffer for 4 h at 300 mA in a Transblot cell (Bio-Rad). Membranes were blocked for 1 h in 5% skim milk in phosphate-buffered saline containing 0.5% Tween 20 (PBST). After the blocking step, membranes were washed for 10 min in PBST and then incubated with the primary antibody diluted in PBST for 16 h. The antibody concentrations used were as follows: TtpC, 1/4,000; TonB2, 1/4,000; FatA, 1/20,000; and FatB, 1/10,000. Membranes were washed three times for 10 min in PBST and incubated for 1 h in the secondary antibody goat anti-rabbit horseradish peroxidase. Membranes were washed six times for 10 min each time and then incubated for 5 min in SuperSignal West Pico chemiluminescent substrate (Pierce) according to the manufacturer's recommendations. Membranes were then exposed to Kodak X-Omat Blue XB-1 film and developed in a Kodak M35A X-Omat processor.

## RESULTS

**A novel protein is essential for iron transport mediated by the TonB2 system.** We recently reported that a transposon-generated mutant of a gene upstream of *exbB2* in *V. anguillarum* resulted in an impairment of ferric-anguibactin transport (36). Sequence analysis indicated that this mutation occurred in an open reading frame that in *V. cholerae* was reported to encode a predicted protein with homology to the *V. cholerae* TolR, a designation that we adopted for this gene in *V. anguillarum*, and it has also now been used for other *Vibrio* spp. We
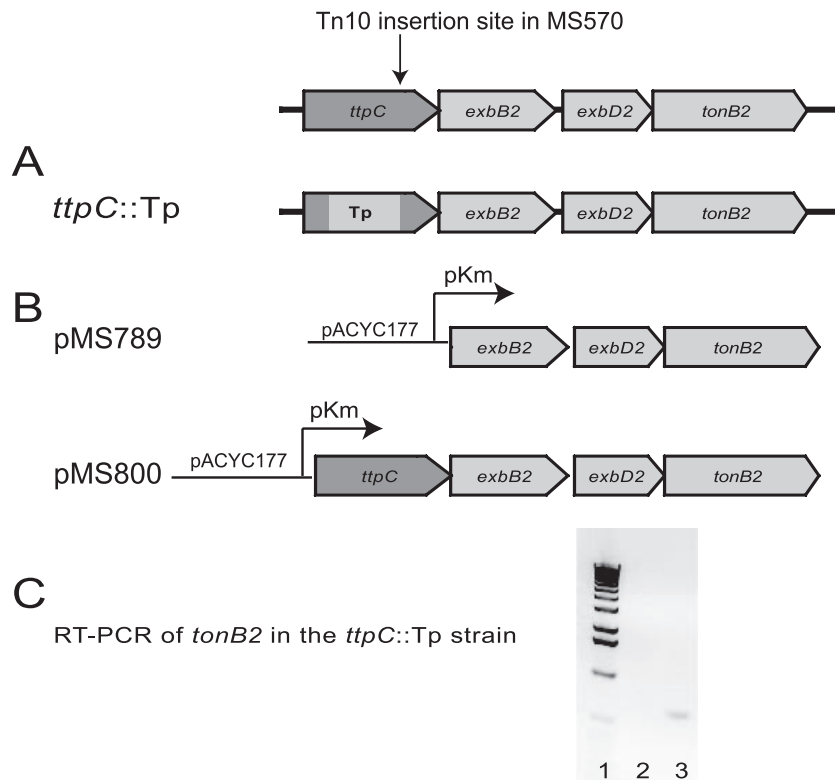
FIG. 1. Schematic representation of the TonB2 cluster genes in *V. anguillarum* and plasmids used. (A) Schematic of the mutants used. Also shown is the site of insertion of the Tn*10*Km[r] transposon. (B) Schematics of the complementing clones pMS789 and pMS800 used throughout the present study. (C) RT-PCR results of the *ttpC*::Tp mutant. Lane 1, molecular weight marker (1-kb ladder; New England Biolabs); lane 2, no RT enzyme used; lane 3, RT enzyme used in the RT reaction.

also demonstrated that this mutation is polar on the transcription of the downstream genes that include *exbB2*, *exbD2*, and *tonB2* (36). In the annotation of the *V. cholerae* genome there are two *tolR* genes. One of the *tolR* genes is part of the *tolQRAB* operon and encodes a predicted protein of 142 amino acids that shares homology with the *tolR* gene of *E. coli*. The second *tolR* gene of *V. cholerae* is located upstream of *exbB2* and encodes a predicted protein of 457 amino acids that shares no significant general homology with the sequence of the other *tolR* gene in *V. cholerae*. On the other hand, this second *tolR* gene product shares 65% identity with the 444 amino acids of the open reading frame we identified in *V. anguillarum* by transposition mutagenesis, and as a consequence we named it TolR (36). However, these assignations were incorrect, likely because of the difficulty in assessing the divergence between these genes in the vibrios and in *E. coli*. The taxonomic issue is complicated even further because the homology of this open reading frame to Exb-related proteins is confined to just the carboxy-terminal region that inserts in the membrane, with three transmembrane segments. The remaining part of the protein has no homology to this family of proteins. Thus, although this open reading frame has a domain with homology to the MotA/TolQ/ExbB family, it falls into a class of its own. Based on our results in the present study we propose that the *tolR* gene found upstream of *exbB2* in both *V. anguillarum* and *V. cholerae* be renamed *ttpC*, encoding TtpC (for TonB2 complex-associated transport protein C).

To investigate the possible function of the *ttpC* gene product in iron transport, we used the double mutant strain MS570 (Fig. 1A) that is affected in the expression of TonB1 (Cm cassette insertion in the *tonB1* gene) and harbors the polar insertion in *ttpC* that also affects the expression of *exbB2*, *exbD2*, and *tonB2* (*exbB2-exbD2-tonB2*) (36). This strain cannot transport any of the TonB-dependent ferric-iron sources tested, including ferric-anguibactin. Ferric ammonium citrate is an iron source that does not require TonB for internalization and is used as a positive control for the viability of the cells. Table 3 shows that when this strain was complemented with the cluster *exbB2*, *exbD2*, and *tonB2* harbored by plasmid pMS789 (Fig. 1B), none of the TonB-dependent iron sources tested were positive in bioassays with this strain. Transport of ferric-anguibactin and other iron sources could only be restored by using plasmid pMS800 harboring *ttpC* in addition to *exbB2-exbD2-tonB2* (Fig. 1B). This result demonstrates that TtpC is required for TonB2-mediated iron transport. To determine whether ExbB2 and ExbD2 are also required for transport by the TonB2 system, we complemented strain MS570 harboring the polar *ttpC* and the *tonB1*::Cm[r] mutation with two pMS800 plasmid derivatives with internal deletions in either *exbB2* (pMS800-Δ*exbB2*) or *exbD2* (pMS800-Δ*exbD2*). Table 3 shows the results of the bioassays performed with these strains, and it can be concluded that besides TtpC, both ExbB2 and ExbD2 are essential for TonB2-mediated iron transport. To assess whether TtpC is also necessary for TonB1-mediated iron trans-

TABLE 3. Bioassay results

| V. anguillarum strain | Growth[a] on iron source | | | | |
|---|---|---|---|---|---|
| | Ferric ammonium citrate | Anguibactin | Enterobactin | Ferrichrome | Heme |
| 775 | + | + | + | + | + |
| MS570 | + | − | − | − | − |
| MS570(pMS789)[b] | + | − | − | − | − |
| MS570(pMS800)[c] | + | + | + | + | + |
| MS570(pMS800-ΔexbB2) | + | − | − | − | − |
| MS570(pMS800-ΔexbD2) | + | − | − | − | − |
| MS995[d] | + | − | − | + | + |

[a] +, zone of growth around the iron source; −, no growth around the iron source.
[b] Plasmid expressing *exbB2*, *exbD2*, and *tonB2*.
[c] Plasmid expressing *ttpC*, *exbB2*, *exbD2*, and *tonB2*.
[d] *V. anguillarum* 775 *ttpC*::Tp[r].

port, we used a strain containing the wild-type *tonB1* gene in which the *ttpC* gene was disrupted by a Tp cassette, 775 *ttpC*::Tp, resulting in a knockout of *ttpC* (Fig. 1A). This mutation was nonpolar on the expression of *exbB2-exbD2-tonB2*, as determined by RT-PCR analysis of the *tonB2* transcripts in this strain (Fig. 1C). Table 3 clearly illustrates that this *ttpC* mutant is still able to transport heme and ferrichrome, but not anguibactin and enterobactin, which are only transported when a functional TonB2 system is present. This demonstrates that

TonB1-mediated iron transport does not require TtpC and emphasizes the need for TtpC for the TonB2 system.

**Subcellular localization and cross-linking studies of TtpC.** TtpC is necessary for TonB2-mediated iron uptake and, since in *E. coli* TonB forms a complex with ExbB and ExbD, we hypothesize that in *V. anguillarum* the TonB2 system might form a complex consisting of TonB2, ExbB2, ExbD2, and TtpC. To test our hypothesis, we used in vivo formaldehyde cross-linking (see Materials and Methods and reference 34).
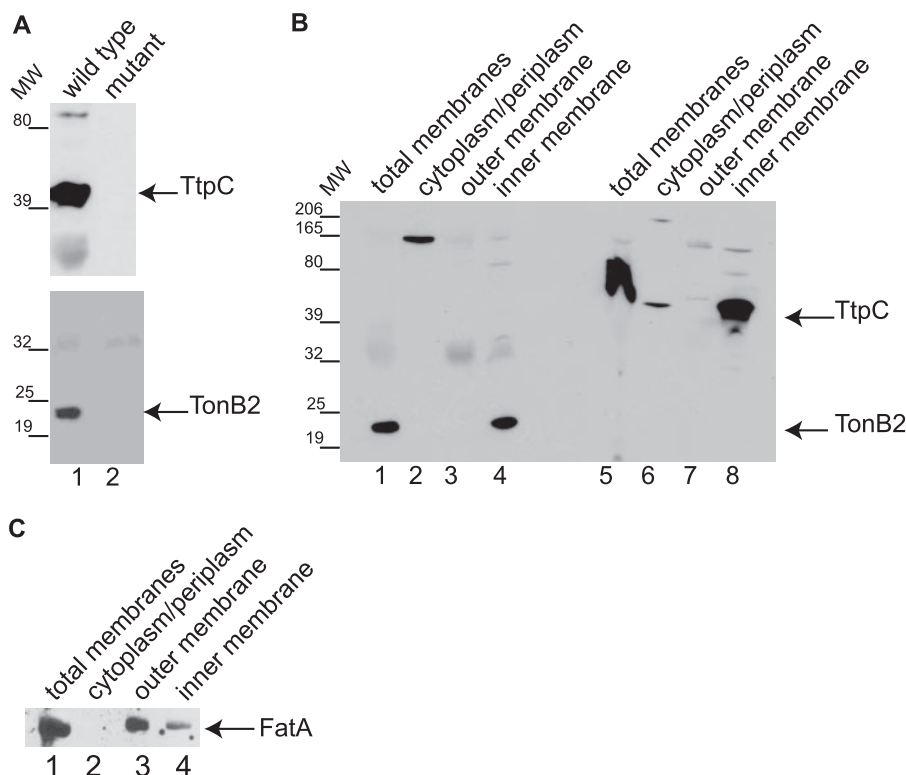


FIG. 2. Western blot analysis of the cellular localization of TonB2 and TtpC. Proteins were isolated as described in Materials and Methods. (A) Western blot with antibodies against TtpC (above) and TonB2 (below) on wild-type *V. anguillarum* and the respective mutant strains. (B) Western blot with TonB2 antibodies (lanes 1 to 4) and TtpC antibodies (lanes 5 to 8). Cellular fractions loaded are indicated on top of the figure. (C) Western blot using the same samples as in panel B but antibodies to FatA were used to indicate the purity of the fractionation. MW, molecular weight (in thousands).
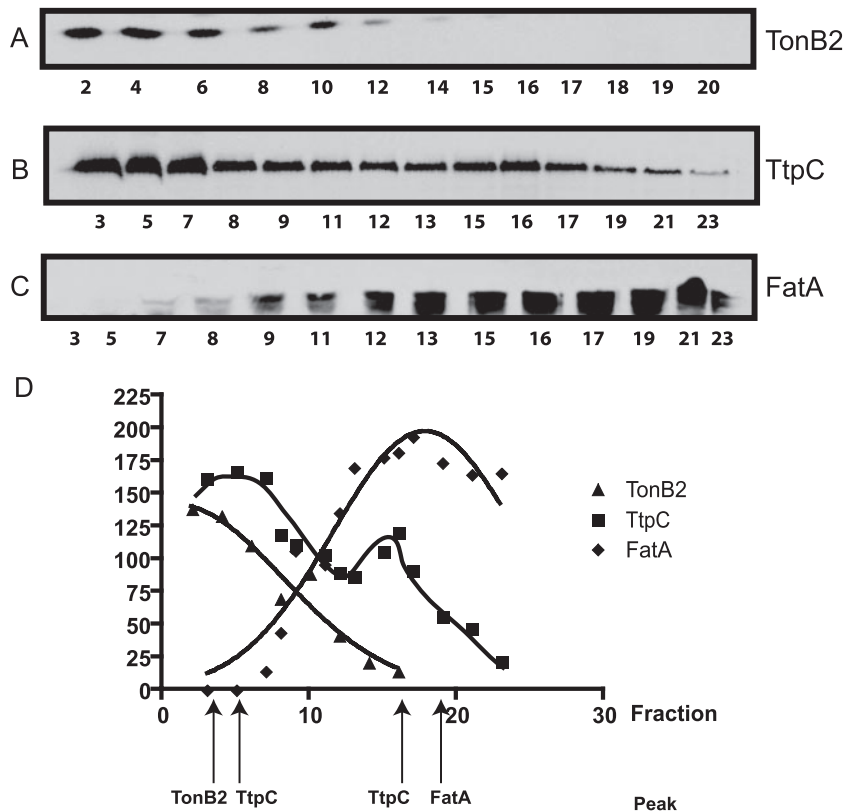
FIG. 3. Sucrose density gradients. (A to C) Western blot analysis on fractions from the sucrose gradients using antibodies to TonB2 (A), TtpC (B), and FatA (C). (D) Density of each band in panels A to C plotted against the sample number.

However, before performing the cross-linking experiments we needed to ascertain the subcellular localization of the TonB2 and TtpC proteins in *V. anguillarum* cells.

To determine the subcellular localization of these proteins, we prepared total cell lysates from *V. anguillarum* strain 775 and isolated total, outer, inner, and cytoplasmic/periplamic proteins (see Materials and Methods). Using polyclonal antibodies generated against TtpC- and TonB2-specific peptides, we performed Western blot analysis to assess the localization of these proteins and their complexes. Figure 2A shows that both the TonB2 and the TtpC antisera are specific for the respective proteins since we do not see a reaction in the mutant, although the TonB2 antiserum shows a cross-reaction with another protein of higher molecular weight. When the cells are fractionated and the total membranes are treated with Sarkosyl we found both TonB2 and TtpC in the total membrane fraction and in the inner membrane fraction (Fig. 2B, lanes 4 and 8). We do not find any TtpC or TonB2 in the cytoplasm or periplasm or in the outer membrane fraction. We did see a reaction with the TonB2 antiserum with a larger protein in the cytoplasm or periplasm; we do not know the nature of this protein. When the same fractions were probed with antiserum to the highly abundant outer membrane receptor FatA, we found that FatA is present in the total and outer membrane fraction (Fig. 2C, lanes 1 and 3) and in minor amounts in the inner membrane fraction (Fig. 2C, lane 4), indicating that the fractions have little contamination from other cellular compartments. However, from this experiment

alone we cannot conclude that both the TonB2 and the TtpC proteins are exclusively inner membrane proteins, since extraction with Sarkosyl can solubilize some outer membrane proteins (11). It should be noted that in silico prediction programs predict that both the TonB2 and the TtpC proteins are located in the inner membrane. To determine the exact cellular localization of these two proteins, we sedimented the proteins from the whole-cell lysates in sucrose density gradients (see Materials and Methods and reference 24) and used the specific antibodies for their detection in the fractions. We also used specific antibodies to FatA (1) as a control for the outer membrane fractions. The status of the sucrose gradients was determined by measuring the refractive index from a sucrose gradient in which no protein sample was added (not shown). After we collected the fractions, the proteins were transferred to nitrocellulose membranes, and the subsequent Western blots show that TonB2 is found in fractions 2 to 12 (Fig. 3A), whereas TtpC is found in fractions 3 to 23 (Fig. 3B). FatA spans fractions 8 to 23 with its peak from fraction 17 to 20 (Fig. 3C). Figure 3D shows the quantification of these results by densitometry. Both TtpC and TonB2 show a curve that is shifted to the left (top of gradient), where there is no or little FatA. Consequently, from these results together with the fractionation experiments in Fig. 2, we can conclude that TonB2 and TtpC must be inner membrane proteins, although it seems that TtpC can also be found in a small proportion in the outer membrane, as indicated by the TtpC second peak that overlaps with the region where FatA is found in large amounts.
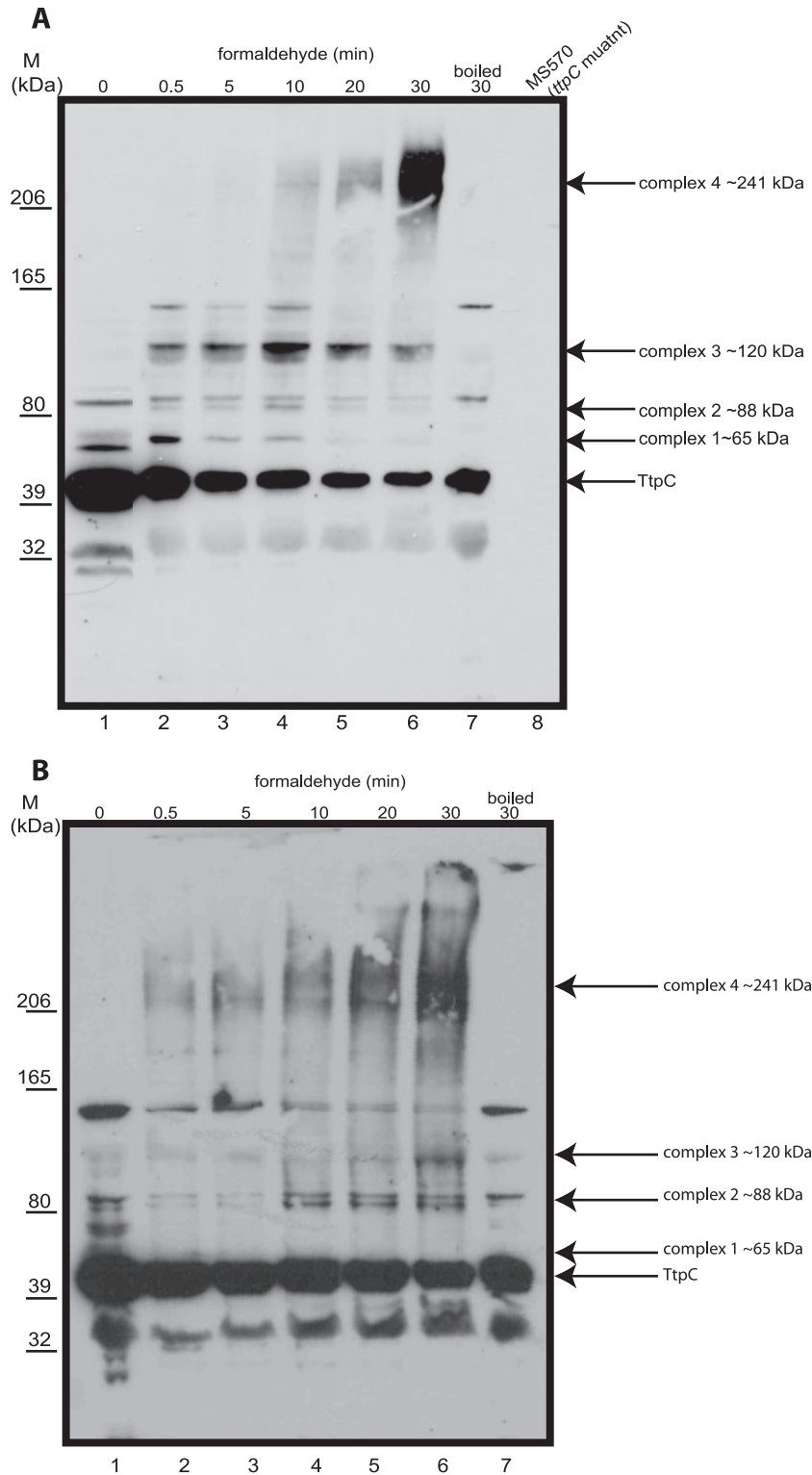
FIG. 4. Western blot on total membrane proteins from formaldehyde cross-linked cells. The TtpC antibody was used to detect TtpC containing complexes. (A) Cross-linking of wild-type *V. anguillarum* strain 775. Marker (M) is shown schematically on the left hand side (Kaleidoscope Bio-Rad). Different complexes are indicated on the right-hand side. Lane 1, no formaldehyde used (more sample was loaded than lanes 2 to 6); lanes 2 to 6, 1% formaldehyde for 0.5 to 30 min as indicated on top of each lane; lane 7, 1% formaldehyde for 30 min, followed by 10 min of incubation at 100°C; lane 8, cross-linking of the *ttpC* mutant MS570 for 30 min, followed by 10 min of incubation at 100°C. (B) Same as panel A but with strain MS570 complemented with plasmid pMS800.

**A**  *tonB2-*



formaldehyde (min)

← TtpC

**B**  *exbB2-*



formaldehyde (min)

← complex 4 ~241 kDa

← TtpC

**C**  *exbD2-*



formaldehyde (min)

← complex 4 ~241 kDa

← TtpC
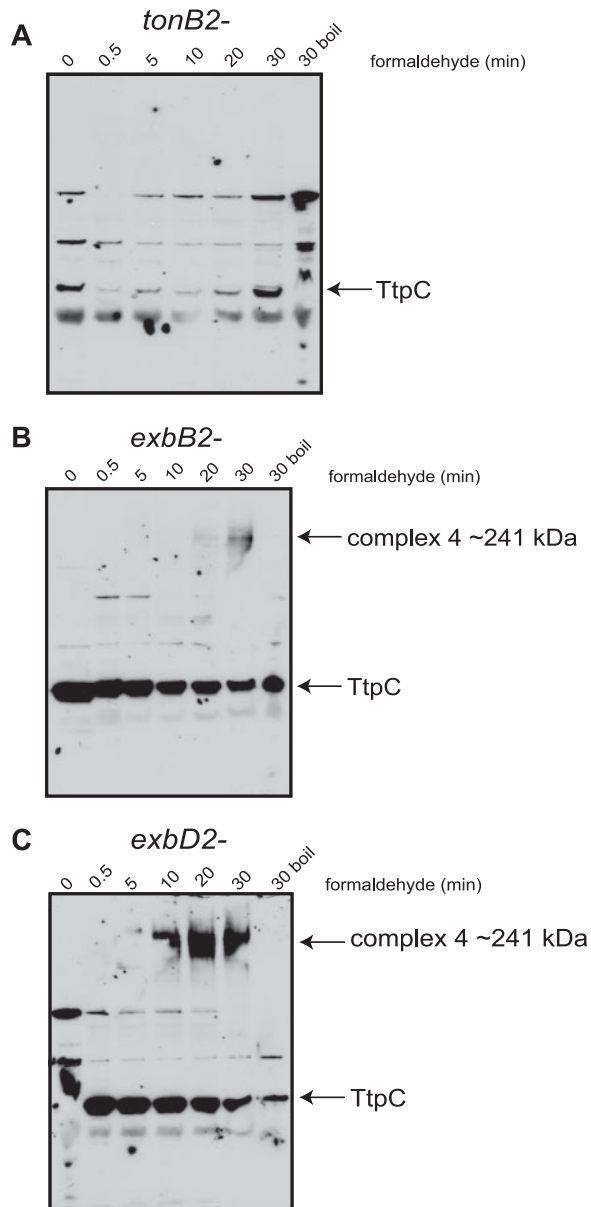
FIG. 5. Western blot of total membrane proteins from formaldehyde cross-linked samples of different mutant *V. anguillarum* strains. Cells were incubated for various times in 1% formaldehyde as indicated at the top of each lane. The complexes present are indicated on the right-hand side using the same numbering used for the wild-type strain in Fig. 4. (A) *tonB2* mutant (MS801); (B) *exbB2* mutant (MS570/pMS800-ΔexbB2); (C) *exbD2* mutant (MS570/pMS800-ΔexbD2).

In the cross-linking experiments we used the TtpC antiserum, rather than the TonB2 antiserum, because the latter has a cross-reaction with another membrane protein of higher molecular weight, as indicated by Western blot analysis (Fig. 2). Although there are several bands that can be detected with anti-TtpC in Western blots (Fig. 2), none of these bands can be detected in a *ttpC* mutant; we hypothesize that these bands result from oligomerization of TtpC, perhaps due to disulfide bonding of the sole cysteine in the inner membrane domain of this protein. To perform the cross-linking experiment, wild-

TABLE 4. Bioassay results with complemented *V. cholerae* and *E. coli* strains

| Strain | Growth[a] on iron source | | | | |
|---|---|---|---|---|---|
| | Enterobactin | Heme | Ferrichrome | Ferric ammonium citrate | φ80 |
| *E. coli* | | | | | |
| W3110 | + | ND | + | + | + |
| KP1032[b] | − | ND | − | + | − |
| KP1032(pMS789)[c] | − | ND | − | + | − |
| KP1032(pMS800)[d] | − | ND | − | + | − |
| *V. cholerae* | | | | | |
| CA401 | + | + | + | + | ND |
| DOV221[e] | − | + | + | + | ND |
| DOV221(pMS789) | − | + | + | + | ND |
| DOV221(pMS800) | + | + | + | + | ND |
| DOV300[f] | − | − | − | + | ND |
| DOV300(pMS789) | − | − | − | + | ND |
| DOV300(pMS800) | + | + | + | + | ND |

[a] +, zone of growth around the iron source; −, no growth around the iron source. ND, not determined.
[b] *E. coli tonB* mutant.
[c] Plasmid expressing *exbB2*, *exbD2*, and *tonB2*.
[d] Plasmid expressing *ttpC*, *exbB2*, *exbD2*, and *tonB2*.
[e] *V. cholerae exbB2* polar mutant.
[f] *V. cholerae exbB1* nonpolar mutant; *exbB2* polar mutant.

type *V. anguillarum* was incubated in phosphate buffer (pH 6.7) containing 1% formaldehyde prior to the isolation of total membrane proteins. The Western blot of cross-linked samples shown in Fig. 4, allowed us to identify four complexes that contain TtpC. These four complexes disappear when the cross-linked sample is boiled. Based on their size, the two bands that can still be detected in the boiled sample are probably TtpC dimers and tetramers even though 2-mercaptoethanol was added to the loading buffer since they are not present in a cross-linked sample (30 min boiled) of the TtpC mutant strain MS570 (Fig. 4A, lane 8). One of the cross-linked complexes (complex 1) forms after a few seconds but disappears with time (compare lane 2 with lane 5), possibly shifting to higher-molecular-weight complexes by the addition of other proteins, whereas complexes 2 to 4 form later, and some complexes stay for longer periods of time. These four complexes are also detected when the polar TtpC mutant (*ttpC*::Tn*10*Km) is com-



← TonB2

1    2
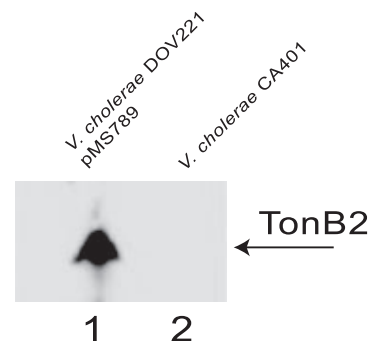
FIG. 6. Western blot analysis of TonB2 of *V. anguillarum* expressed in *V. cholerae*. Lane 1, *V. cholerae* DOV221 expressing TonB2 from *V. anguillarum* from plasmid pMS789; lane 2, wild-type *V. cholerae* CA401.

```
            1          10              20          30            40            50            60
TtpC_Va  MVFSLSSLCLTLLAFNSF.....ASTELVSNAKVENKQQQQHNIEREAGFKQTEQQLRRAKAELEAQRQD
TtpC_Vc  MTLRKTTLALALSLPFAFVSVANASNSLVQQATQEKAQQQQHNQQREAGFVQTAQELQAAKAELLAERNR

            70          80            90            100          110          120          130
TtpC_Va  LQAQADKLSTQFSNNENRLARLEEQLRLETGSLGELFGVVRQNAKALSSELESSITAVDRHQYTSRVQEI
TtpC_Vc  LQKEADQLSSQFSDNENTLARLEETLRLETGSLGEMFGVVRQNAKELQSELDQSVTGVEPRAHQQSIDDV

            140          150            160          170          180          190          200
TtpC_Va  VAAKSLPSMPQLTGLWHSYVEQIIASGELSRVQIAFINGDGVTQNVAAYRIGTFGLVTEQGYVKWNGDKQ
TtpC_Vc  VAAKTLPSMAQLRGLWQAMSEEIIASGQVKTTEIQWLNGQGETQTVPALRLGSLGLISEQGYVKWDNARQ

            210          220            230          240          250          260          270
TtpC_Va  AATAYLKQPDNGPIFSGLNT...GEIESLVVDPSRGIMLEQLALTPTLAERLQMGGAVGNIILVLLGIGL
TtpC_Vc  QALSYQQLPSDFPTFSHIRTLVDGDVVTMKVDPSRGVILEQLALTPTFSQRLQAGGVIGNVILVLLGVGL

            280          290            300          310          320          330          340
TtpC_Va  IIAFYRGFALVMTHHKIKAQLRDVQKIGNNPLGRVLSVYNKEQNRSVEALELRLLEAVVDEQAHLEKGLS
TtpC_Vc  IIALYRGAILATLRQKIKAQLKNPEQPGNNPLGRILAVYNKEQQRSVEALELRLLEAVVDEQNHLETGLS

            350          360            370          380          390          400          410
TtpC_Va  MLKLLAAIAPMLGLLGTVTGMIETFQVITQFGNGDPKVMAGGISMALVTTVLGLVSAIPLLLAHNILSSQ
TtpC_Vc  MLKLLAAIAPMLGLLGTVTGMIETFQVITQFGNGDPKVMAGGISMALVTTVEGLIAAIPLLLAHNILSAQ

            420          430            440
TtpC_Va  AENIRSILEKQGIGLVAAQAEQECQASMRNAA...............................
TtpC_Vc  AEAIRNILEKQGIGLVAQQAERDCGAAVTQSRVEQAAVAQQAEQQLPSLTPTMDKVGNAA
```

FIG. 7. Amino acid sequence alignment of TtpC from *V. anguillarum* and *V. cholerae*. Va, *Vibrio anguillarum*; Vc, *Vibrio cholerae*. Gray-shaded amino acids indicate identical residues, and light gray amino acids indicate similar amino acids. Domains identified by using the computer program PSORT are underlined.

plemented with the wild-type cluster *ttpC*, *exbB2*, *exbD2*, and *tonB2* cloned in plasmid pMS800 that restores the wild-type phenotype (Fig. 4B). The in vivo formaldehyde cross-linking experiment was also carried out with mutants of *tonB2*, *exbB2*, and *exbD2*. In cross-linking experiments with cells deficient in TonB2 (strain MS801) none of the four complexes could be found (Fig. 5A). Since we do see less free TtpC in the membrane of the *tonB2* mutant, it is possible that the complexes are not abundant enough to be detected. In both the *exbB2* and the *exbD2* mutant stains (MS570/pMS800-Δ*exbB2* and strain MS570/pMS800-Δ*exbD2*, respectively), only complex 4 could be detected (Fig. 5B and C). Complex 4 that forms after extended incubation with formaldehyde possibly consists of TtpC and TonB2. Since we do find this complex in the *exbB2* and *exbD2* mutant strains, we can conclude that the proteins encoded by these genes are not part of complex 4. However, the resolution of the area where complex 4 runs is not very good due to the large size of this complex. It could be that complex 4 consists of several complexes and that some do contain ExbD2 or ExbB2. Complex 4 could also include an outer membrane receptor such as the ferric-anguibactin receptor FatA. We tried cross-linking experiments with a strain lacking FatA, but in this strain complex 4 is still present (data not shown). This could be simply explained by the fact that the TonB2 complex can interact with several receptors, such as those for heme, ferrichrome, enterobactin, and vanchrobactin, and these receptors are all expressed in the FatA-deficient strain. From the cross-linking experiment it can be concluded that TtpC is found in at least four distinct complexes; these complexes are all absent in a TonB2-deficient strain, and complexes 1 to 3 are also not present in the *exbB2* and *exbD2* mutants.

We found TtpC in the membrane in all mutant strains used, but it seemed to be present in lower amounts in the *tonB2* mutant (Fig. 5A). Although the same amounts of cells as mea-sured by $OD_{600}$ were loaded in each lane, the Western blots cannot be compared directly. Perhaps TonB2 is in some way stabilizing TtpC in the membrane, whereas TtpC is not required to stabilize TonB2, as can be seen by the fact that we could detect wild-type levels of TonB2 in the TtpC mutant strain (data not shown).

**Complementation of *E. coli* and *V. cholerae tonB* mutants with the *tonB2* cluster genes from *V. anguillarum*.** The genes *exbB2*, *exbD2*, and *tonB2* of *V. anguillarum* cannot complement the *tonB* mutant strain KP1032 of *E. coli* (36). Because in the present study we showed that TtpC is necessary for TonB2-mediated iron uptake, we complemented *E. coli* KP1032 with a construct that expresses *ttpC*, *exbB2*, *exbD2*, and *tonB2*. The resulting *E. coli* strain is still not able to transport any of the TonB-dependent iron sources tested (Table 4) and was still resistant to phage ϕ80.

We therefore investigated whether the TonB2 cluster genes can complement the TonB2 mutant strains of the more closely

TABLE 5. Similarity of *V. anguillarum* TtpC with the TtpC encoded by other bacteria

| Species | % Identity | % Similarity | Accession no. |
|---|---|---|---|
| *Vibrio cholerae* | 66 | 80 | AAC69453.1 |
| *Vibrio parahaemolyticus* | 66 | 80 | BAC55123.1 |
| *Photobacterium profundum* | 63 | 77 | YP_132367.1 |
| *Vibrio fisheri* | 61 | 77 | YP_206737.1 |
| *Photobacterium damselae* | 59 | 74 | CAD68978.1 |
| *Vibrio vulnificus* | 57 | 73 | AAO07321.1 |
| *Shewanella oneidensis* | 38 | 58 | NP_717433.1 |
| *Idiomarina loihiensis* | 38 | 59 | YP_156239.1 |
| *Microbulbifer degradans* | 34 | 54 | ZP_00318099.1 |
| *Methylococcus capsulatus* | 37 | 55 | YP_112966.1 |
| *Desulfovibrio desulfuricans* | 30 | 48 | ZP_01129903.1 |
| *Desulfotalea psychrophila* | 30 | 45 | CAG37703.1 |

```
                    1         10        20
TonB_Ec    .........................MTLDLPRRFPWPTLLSVCIHGAVVAGLL
TonB2_Pa   MATPQPVDARTQPWRETPGGDLVALGRPVRQALHLVRHNPAQGRVLSRRETILLVLFALTLHGAVIHWLS
TonB2_So_1 ..........................MGQNV...IVNRTGIIVVSALIPLGLFVFMA
TonB2_Il_1 ..........................MMRFLAALLVAAFVTIGLFYLMQ
TonB2_Vp_1 ..........................MRYLASIALALIVSLGLFWGMD
TonB2_Vv_1 ..........................MRYLASIALALVVSLGLFWGMD
TonB2_So_2 ..........................MLRALVSIIIGAAVTLGLFWFMA
TonB2_Il2_2 .........................MVRFLVSLLLGAAVTFALFAFMA
TonB2_Vv_2 ..........................MMRLVIAFPVALALVFALFSAMA
TonB2_Vv_2 ..........................MMRLVIAFPVALALVFALFSAMA
TonB2_Pd   ..........................MLRVLMAFPLAILLAGGLFTFMA
TonB2_Pp   .......MAVVSAPTKASFSVFQTGVFQTS...IFRLVLALPISMFVALGLFTFMA
TonB2_Vf_1 ..........................MWRLFLAMPIAISMTFGIFTFMA
TonB2_Vf_2 ..........................MFRLLMALPIAVAISIALFSLMA
TonB2_Va   ..........................MGRLLLALPIAGAISLALFSFMA
TonB2_Vc   ..........................MGRLILASPIALLVTLALFSLMA
TonB2_Vp_2 ..........................MGRLLIALPASLLIAVSLFSFMA
TonB2_Ap   ..........................MKKKHSRIGLTGSILIHTAIFGGLA
TonB2_As   ..........................MKKKHSRIGLISSIVIHTFVFAGFI

           30        40        50              60        70
TonB_Ec    YTSVHQVIELPAPAQPISVTMVA.....PA...............DLEPPQAVQPPPEPVVEPEPE..PE
TonB2_Pa   QQRTPALPEVPPQVPPMTIEFTA.....PAPPVVEPPPPEPLPPVVEEPPPPVVDENAVK.PPPPKPVPK
TonB2_So_1 QLIHNPQPLSGQATDAPQINI..........LMSERTP.IPPKESRK.PEPPKP...
TonB2_Il_1 SLISGGEGAMSEPPRGSVLDF..............VRTPEPEEVQEKERK.PERP...
TonB2_Vp_1 KLVNKERHELVSNDDQRMVDF..............IRIKPDEKVQEKKREL.PKPPPP...
TonB2_Vv_1 KLVNKERHELASNDDLRMVDF..............IRVKPEEKVQEKKREL.PKPPPP...
TonB2_So_2 FLVGGGAQRAETSAETPVIEI..............TMDRQDSAQSKQRVV.PQPPPP...
TonB2_Il2_2 YLIGGGAQRNEAPAPTPVIDI.............VTSPPESDVQERRRTP.PPPPP...
TonB2_Vv_2 LLVNSGKKQAIDQTPSLSFNM..............VMVEQDTEVTRRQRSV.PPPPEI...
TonB2_Vv_2 LLVNSGKKQAIDQTPSLSFNM..............VMVEQDTEVTRRQRSV.PPPPEI...
TonB2_Pd   WMVDNGSRQEPESTPPLSFNM..............VMVEQESEVQRRQRVV.PPKPEA...
TonB2_Pp   WMVDNGHQRSPEQSQSFSFDM..............VMVEQEKEAQRRQRTV.PEKPET...
TonB2_Vf_1 WMVDNGTQEKPESSETVRFDM..............VMAEQEQDVQRRQRRV.PEKPDT...
TonB2_Vf_2 WMVDNCNQAKPEASEQLSFTM..............MMVEPESEIQRRQRSV.PEQPKP...
TonB2_Va   WMVDNGNQRAVKPTQALRFNM..............VMMEQEHDLQRRQSV.PEPLKL...
TonB2_Vc   WMVDNGGKSIPKPTAALSFTM..............VMAEQEQDVQRRQRSV.PEQPQV...
TonB2_Vp_2 WMVDNGNQRAPKPSEAVRFDM..............VMVENDADVQRRQRSV.PEQPEP...
TonB2_Ap   SMVTHSHSDDLPQEETMSMELVAALLEQPQVAVAQEEPAPPEPE.......KVEVEPEPEPEAEAIPELK
TonB2_As   SIVKSSHSDGQPEDNVMSMELVAALLEQPQVAVAEEQPTQAEPE.......KQQAEPEPEPEAEFIPEPK

           80        90        100       110       120       130       140
TonB_Ec    PIPEPPKEAPVVIEKPKPKPKPKPVKKVQEQQ.KRDVKPVESRPASPFE.N..TAPARPTSSTATAAT
TonB2_Pa   PKPKPKPQP............RPKPAP....KA.VEPAPPAPPQPAAPPAPP..A.......PAAAPAP
TonB2_So_1 ..IPTRERI...........TTPGES...TE.VVDFNPQTFTPEMPIQ............TT
TonB2_Il_1 PEPEQPPED...........LPQ.PQ...MN.NDIADT.DGGSGYDFSAN..MDSGADIG.......
TonB2_Vp_1 KRPPPPPEM...........KVTSTV...KP.VMDQVPM.DMPKLDLPVN..VTGGSVL........
TonB2_Vv_1 KRPPPPPEM...........KITSTV...KP.VMEQIPM.DMPKLDLPVN..ITGGSVL........
TonB2_So_2 ..PAQP.EK...........PQAAPP...ET.SSNIDTA....MSF..N..MGGVEAGGHSAGGFK
TonB2_Il2_2 .PEQPPET...........PQSEPD...TS.DSGMNMN....MGFDVD..VGGADTG......LSG
TonB2_Vv_2 .PEPPQPE...........TPKMTQ...TP.MQVTSTQVATPNIDMRFN..VSGLAVSIPATVSAP
TonB2_Vv_2 .PEPPQPE...........TPKMTQ...TP.MQVTSTQVATPNIDMRFN..VSGLAVSIPATVSAP
TonB2_Pd   ..PKPPPQV...........MPSQSK...PT.SVSTLPVASISALGLDTS..ISGIAVS......AP
TonB2_Pp   .PEPLPEA...........VPKSSQ...SA.TS..TPAPSMPNLGLDTA..ITGLSIN......LP
TonB2_Vf_1 .PEPPPQA...........TPMAAQ...TQ.TSMATPQSPMPLIGLDSS..ISGLAIS......AP
TonB2_Vf_2 .LEQPPEA...........KLSQSQ...SE.VSSVMPVTPMSSSGLDTA..INGLKIS......AP
TonB2_Va   .PEPPPQA...........QVTQSQ...TT.ATAATSLTSLPALGLDTA..LNGVAIS......AP
TonB2_Vc   ..PQVPTQA...........PAKAEQ...TA.ALDVSDLNPVMDLNLSTA..MEGVAVN......AP
TonB2_Vp_2 .ELSQAN...........TQ.VEPMSQVTPISALGLNTA..LDGIAIN......AP
TonB2_Ap   PQPKPKE.KPKPKEKPKEKPKPEPPKPKEKEKPKKEKVKDKPIKALEKGPEAKQGIVAKAIPNAVQGTQLR
TonB2_As   PQPKP.....KEKPKEKPKPKEQPKPKEKEKPKKEKAKDKPIKALEKGPEAKQGIVAKAIPNAVQGTQAR

                           150       160       170
TonB_Ec    .......................SKP............VTSVASGPRALSRNQPQYPARAQALRIE.GQV
TonB2_Pa   ...........................LTPPSANAGYLHNPAPEYPALAMRRGWE.GTV
TonB2_So_1 ............LFT............QSSMSAEALPLVQVSPRYPIEAAQNGKE.GYV
TonB2_Il_1 ...............SGMS...LQASDGEYLPIVKVQAAYPRRALQRGIE.GYV
TonB2_Vp_1 ...............GHYSQGGGA...RLAGSGGPVPLATFQPQMPRKAARSGLEKGIV
TonB2_Vv_1 ...............GHYSQGGGA...QVAGTGGPMPLATFQPQMPRKAAKAGLEKGLV
TonB2_So_2 ............LGN............MMSRDGDATPIVRIEPQYPDIAAARDGKE.GWV
TonB2_Il2_2 ............PGG............GLSSDGDATPIVRIEPRYPPSAARNGTE.GWV
TonB2_Vv_2 ............TQN............ASNTTKQLMPLSRVEAVYPAKAKKRRIE.GYV
TonB2_Vv_2 ............TQN............ASNTTKQLMPLSRVEAVYPAKAKKRRIE.GYV
TonB2_Pd   ............KFGKIEGAANLGAGI..NVGSNQQAMPLYRVEPRYPARALKQGAE.GYV
TonB2_Pp   ............TFS............DFGANQQAMPLYRVEPRYPARAMKQKAE.GYV
TonB2_Vf_1 ............NFA............DFTGNQQAMPLYRVEPRYPSKAMKRGAE.GFV
TonB2_Vf_2 ............TFG............SFKTNQQAMPLYRVEPKYPSRALKRRIE.GYV
TonB2_Va   ............TFG............DFGSNQQAMPLYRVEPVYPSKALKRGVE.GFV
TonB2_Vc   ............QFG............DFAVNQQVMPLHRVEPNYPAKALQRGVE.GYV
TonB2_Vp_2 ............NLK............GTMGNQQALPLYKVEPRYPSKALKRKVE.GYV
TonB2_Ap   AGIPNGKPEGNPNGLSANGSQNGAVGGSGSGASGSEIGAYKAALQRALQRRANNAYPTREKMMRKT.GVV
TonB2_As   AGIPNGKPNGNPNGTSANGSQNGAAGGSGSGSNGSEIGAYKAALQRALQRRANNAYPAREKMMRKT.GVV

           180       190       200       210       220       230
TonB_Ec    KVKFDVTPDGRVDNVQILSAKPANM.FEREVKNAMRRWRYEPGKPG......SGIVVNILFKINGTTEIQ
TonB2_Pa   LLRVHVLASGSPSEIQVQKSSGREA.LDQAAVKAVKRWSFVPAK.RGDKAEDGWVSVPIDFKLN.....
TonB2_So_1 VVGFDITADGTVSNVRVLDANPKRI.FDKEALSAVQNWKYKPKFDAGKAVPQLNQQVQLDFKLDQKI....
TonB2_Il_1 VVEFTVTKQGSVRDPVVVQAEPEGI.FDQAAMDAVLKFKYKPRVIDGEAVEVDGVRNRMTFEMN.....
TonB2_Vp_1 LVEFTVNERGSVPDVKVLKETPRKLGLGKAVASS.RLSQEIEFSTN.....
TonB2_Vv_1 VLEFTVNERGGVEDIKVLKEEPRRMDLGKEARKTVSKWTFKPKMVDGKPVSS.RMAQEIEFSVN.....
TonB2_So_2 QLRFTINELGGVEDVIQAEPKML.FDQAAMDAVLWKHKYKPKIVDGKPLKQPGMTVQLDFTLEKGGN..
TonB2_Il2_2 QLRFTIDQQGGVTDVEVIDSEPRRV.FDREARRALLRWKYKPKVVDGKPIRQEGMTVQDFTMDGN....
TonB2_Vv_2 ILKFDIDETGRTKNIEVVEAQPARF.FERNAMDAIKRWRYQPQIVDGQAQTIYGYTKIEFKMAQ.....
TonB2_Vv_2 ILKFDIDETGRTKNIEVVEAQPARF.FERNAMDAIKRWRYQPQIVDGQAQTIYGYTKIEFKMAQ.....
TonB2_Pd   VLQFTIDTQGRPSDISVLEAKPRRL.FERDAMRALRKWKYQAKVIDGQAGEQPGQTVKLEFKINQ....
TonB2_Pp   VLSFTIDEQGRPTTIDVIEAKPRRL.FEKEAVRALRKWKYQPKVVDGKSIAQVGQTVRLEFKISQ....
TonB2_Vf_1 VMSFTIDEQGRPTDVVIEAKPRRL.FEREAVKALRKWKYQPKVIDGKTIAQVGQTVRLEFPTLAK....
TonB2_Vf_2 IMSFSIDETGKPFAISVQEAKPRRL.FDRDAMRALKQWKYQPKLVEGKAIIQQNQTVRLEFKLAE....
TonB2_Va   TLSFTIDTTGKAVDINVVDANPKRL.FEREAMLALRNWKYQPKIVDGQPVSQPGQTVRLEFKLNK....
TonB2_Vc   ILRFTIDELGKTRDIEVVDANPKRY.FEREAMLALRNWKYQPKIVDGQPVSQPGQTVRLEFKLNK....
TonB2_Vp_2 IMRFTIDTTGRPKDIEVVDAEPKRM.FEKEAISALKKWKYQPKVENGVSIEQFGQTAKVEFKLGK....
TonB2_Ap   TIGFTVSSSGELINVKVLNSSGNGN.LDNAAVKAAQSTKVSPPPAGFP....SNVTVPVKFSIE.....
TonB2_As   TIGFTVSSSGDLTNVKVLNSSGNSN.LDNAAVKAAQSTKVSPPPAGFP....SSVTVPVKFSIE.....
```
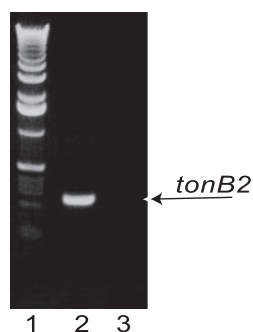
FIG. 9. RT-PCR analysis of the *ttpC*::Km mutant of *V. cholerae*. Lane 1, marker (1-kb ladder; New England Biolabs); lane 2, RT-PCR of *tonB2* in the *ttpC*::Km mutant; lane 3, no RT enzyme used.

TABLE 6. Bioassays results for the *V. cholerae ttpC* mutant

| Strain | Growth[a] on iron source | | |
| --- | --- | --- | --- |
| | Ferric ammonium citrate | Enterobactin | Heme |
| *V. cholerae* CA401 | + | + | + |
| *V. cholerae ttpC*::Km | + | − | + |

[a] +, Zone of growth around the iron source; −, no growth around the iron source.

related *V. cholerae*. As shown in Table 4, the *V. cholerae exbB2* polar mutant DOV221 (26) cannot be complemented with the *exbB2*, *exbD2*, and *tonB2* genes from *V. anguillarum* (expressed from plasmid pMS789), even though the *V. anguillarum* TonB2$_{Va}$ protein is expressed in this *V. cholerae* strain, as detected by Western blot analysis (Fig. 6, lane 1). Surprisingly, the antibody against TonB2$_{Va}$ of *V. anguillarum* does not react with the TonB2$_{Vc}$ of *V. cholerae* (Fig. 6, lane 2). When the plasmid pMS800 that includes the *V. anguillarum ttpC* was used, the *V. cholerae* strain recovered the ability to transport enterobactin, a siderophore whose transport is mediated by the TonB2 complexes of *V. cholerae* and *V. anguillarum* (33, 36). Results similar to those obtained for the single *V. cholerae exbB2* mutant were obtained with the *V. cholerae exbB2* polar and *exbB1* nonpolar mutant strain DOV300 (26). In the latter double *exbB1* and *exbB2* knockouts of *V. cholerae* we could demonstrate that both heme and ferrichrome could also be transported by the TonB2 system of *V. anguillarum* as long as TtpC of *V. anguillarum* is present (Table 4). Since the *V. cholerae* mutants used have the wild-type *ttpC*$_{Vc}$ gene of *V. cholerae*, it is clear that the TonB2 system of *V. anguillarum* needs its own TtpC$_{Va}$, even though the *ttpC* genes of *V. anguillarum* and *V. cholerae* share 65% identity. An alignment of the two TtpC proteins is shown in Fig. 7 with domain features identified by the program PSORT (31) underlined.

**TtpC-containing bacteria and correlation with the presence of TonB2.** TtpC homologues are found in all *Vibrio* species sequenced thus far and in a few other bacteria not only limited to the γ-proteobacteria (Table 5). Among the vibrios, the TtpC proteins share high homology, ranging from 57 to 66% identity and from 73 to 80% similarity. However, there is a species specificity for the TtpC protein since the *ttpC* gene from *V. cholerae* cannot replace the *V. anguillarum ttpC* in the TonB2 system of *V. anguillarum*. In general, microorganisms in which we could identify a TtpC homologue also have at least two

TonB systems, and the *ttpC* gene is part of the *tonB2* cluster. It is quite possible that *ttpC* is only found associated with the TonB2 systems, but some of the bacterial genomes are not sufficiently annotated to easily identify all of the TonB systems present. To understand some of the evolutionary possibilities, we aligned known TonB2 proteins sequences and TonB from *E. coli* (Fig. 8). We did not include the TonB2 proteins of *Microbulbifer degradans*, *Methylococcus capsulatus*, *Desulfovibrio desulfuricans*, and *Desulfotalea psychrophila* since these TonB proteins are not annotated as such.

Figure 8 shows that the TonB2 sequences from *Pseudomonas aeruginosa*, *Actinobacillus pleuropneumoniae*, and *A. suis* are generally longer than the *Vibrio* TonB2 proteins and are like the *E. coli* TonB, containing the proline-rich domain that spans amino acids 64 to 100. Interestingly, these TonB2 systems do not have a TtpC homologue, and there seems to be a correlation between the presence of TtpC and the absence of the proline-rich region in TonB2.

**The *V. cholerae* TtpC is essential for TonB2-mediated transport of enterobactin in this bacterium.** To explore whether TtpC is also essential for TonB2-mediated transport in other bacteria, we mutated the *ttpC* gene of *V. cholerae* CA401 by inserting the Km$^r$ gene cassette in the unique SalI site. This insertion is nonpolar, since we can still detect *tonB2* transcripts in this mutant by RT-PCR (Fig. 9). Seliger et al. (33) showed that in *V. cholerae* the transport of enterobactin is exclusively dependent on TonB2. We therefore used the ability to transport enterobactin to measure whether the *V. cholerae* TtpC is necessary for TonB2-mediated transport. The *ttpC* mutant and wild-type *V. cholerae* strains were then used in a bioassay with ferric ammonium citrate, heme, and enterobactin as iron sources. As shown in Table 6, the *ttpC* mutant fails to transport enterobactin, whereas ferric ammonium citrate is still transported, indicating that, as in *V. anguillarum*, TonB2-mediated iron uptake in *V. cholerae* also requires TtpC. We included heme as an iron source since heme transport depends on either the TonB1 or the TonB2 system. As expected, the bioassay is positive for heme uptake in the *ttpC* mutant, suggesting that

FIG. 8. Amino acid sequence alignment of most TonB2 proteins and TonB from *E. coli*. The suffixes _1 and _2 are randomly assigned to bacteria that harbor more than one TonB2. The bacterial species (and GenBank accession numbers) of the TonB sequences used for the alignment are as follows: Ec, *Escherichia coli* (AAB59066); Pa, *Pseudomonas aeruginosa* (AAF04082); So, *Shewanella oneidensis* (NP_720081 and AAN54880); Il, *Idiomarina loihiensis* (YP_156236 and YP_156496); Vp, *Vibrio parahaemolyticus* (NP_799665 and BAC58426); Vv, *Vibrio vulnificus* (NP_759823 and AAO09350); Pd, *Photobacterium damselae* (CAD68981); Pp, *Photobacterium profundum* (CAG23089); Vf, *Vibrio fischeri* (YP_206734 AND YP_206154); Va, *Vibrio anguillarum* (AAV48777); Vc, *Vibrio cholerae* (AAC69456); As, *Actinobacillus suis* (AAW21313); and Ap, *Actinobacillus pleuropneumoniae* (AAR95695).

transport mediated by the TonB1 system in *V. cholerae* does not require TtpC.

## DISCUSSION

The siderophore anguibactin is synthesized via a nonribosomal peptide synthetase mechanism that is entirely encoded on the virulence plasmid pJM1 of the fish pathogen *V. anguillarum* (15). The secreted siderophore is, once bound to ferric-iron, transported back into the cells cytosol via a specific transport system that starts with the outer membrane receptor FatA (1). FatA binds the ferric-siderophore and transports it to the periplasm, where it must interact with the lipoprotein FatB. Once in the periplasm, the iron complex is then further internalized by the integral membrane proteins FatC and FatD (21). It was demonstrated in *E. coli* that transport across the outer membrane requires energy from the proton motive force of the inner membrane that is transduced to the outer membrane (9). The energy-transducing complex consists of the proteins TonB, ExbB, and ExbD. The TonB protein is the actual energy transducer, whereas the ExbB and ExbD proteins are necessary for the stability of TonB (2). TonB systems have been found in almost all bacteria sequenced thus far, and some bacteria have more than one TonB system. Studies of TonB proteins in other systems have focused mainly on their importance in iron uptake and virulence, and many similarities with *E. coli* TonB have been observed (5, 17, 20, 33).

*V. anguillarum* harbors two TonB systems (36), TonB1 and TonB2, and these systems are redundant with respect to the transport of heme and ferrichrome. Only TonB2, however, can also transport anguibactin, enterobactin, and vanchrobactin (36). In the present study we have shown that just the classic TonB2 system (TonB2, ExbB2, and ExbD2) is not sufficient for the transport of the TonB2-specific iron compounds in *V. anguillarum*. A fourth protein, TtpC, was identified that is essential for iron transport mediated by the TonB2 system. The *ttpC* gene encodes a polypeptide of ~49 kDa with three predicted transmembrane domains. TtpC, however, is not essential for transport mediated by the TonB1 system. Furthermore, the cross-linking experiments show that TtpC can be cross-linked in at least four complexes, and some of these complexes are missing in mutant strains of TonB2, ExbB2, and ExbD2, suggesting possible complex formation of TtpC, TonB2, ExbB2, and ExbD2 in *V. anguillarum*. This differs from the *E. coli* TonB system, where the complex consists of only TonB, ExbB, and ExbD. Unlike the *E. coli* TonB, TonB2 from *V. anguillarum* does not seem to shuttle between the membranes and is solely found in the inner membrane.

We identified *ttpC* gene homologues in many bacteria, including all *Vibrio* species sequenced thus far, with the TtpC homologues sharing high similarity. Even though the TtpC proteins from *V. anguillarum* and *V. cholerae* share 66% identity and 80% similarity, the TtpC$_{Vc}$ protein from *V. cholerae* cannot complement a mutation in the *ttpC$_{Va}$* gene of *V. anguillarum* in *V. cholerae*. However, the *V. cholerae* TtpC$_{Vc}$ is necessary for TonB2$_{Vc}$-mediated transport in *V. cholerae*, since a *ttpC$_{Vc}$* mutant in *V. cholerae* fails to transport enterobactin. We speculate that each specific TtpC plays an essential role in the specific TonB2-mediated transport in all bacteria that harbor this protein. The dissection of the mechanism by which TtpC operates together with the TonB2 cluster in specific iron transport will contribute to our understanding of the mechanism of energy transduction in bacteria other than *E. coli*.

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