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Two *tonB* Systems Function in Iron Transport in *Vibrio anguillarum*, but Only One Is Essential for Virulence

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We have identified two functional *tonB* systems in the marine fish pathogen *Vibrio anguillarum*, *tonB1* and *tonB2*. Each of the *tonB* genes is transcribed in an operon with the cognate *exbB* and *exbD* genes in response to iron limitation. Only *tonB2* is essential for transport of ferric anguibactin and virulence.

Outer membrane receptors for ferric siderophores require energy to internalize the complexed iron. This energy is transduced from the proton motive force in the inner membrane to the outer membrane receptor by the TonB protein in complex with the ExbB and ExbD proteins (4, 10). The presence of *tonB* has been associated with bacterial virulence in bacteria such as *Vibrio cholerae* (16), *Shigella dysenteriae* (15), and *Bordetella pertussis* (13).

To be able to cause an infection in a vertebrate fish host, the marine fish pathogen *Vibrio anguillarum* requires an active iron uptake mechanism (2, 6, 8, 17, 21) mediated by the siderophore anguibactin (1); however, it can also acquire iron via transport of heme and siderophores secreted by other microorganisms, like ferrichrome and enterobactin (5). Anguibactin is synthesized via a nonribosomal peptide synthetase mechanism and secreted to the extracellular environment (9). Once bound to iron, the ferric siderophore is transported back into the cell cytosol through the specific outer membrane receptor FatA and a transport complex consisting of the periplasmic binding lipoprotein FatB and the integral membrane proteins FatC and FatD (3, 7, 12).

Ferric-anguibactin transport via the FatA receptor requires energy, suggesting the existence of a *tonB* complex in *V. anguillarum* (7).

The *tonB*, *exbB*, and *exbD* cluster in *V. anguillarum* 775 also includes the heme transport genes (Fig. 1). This *tonB* system shows homology to the *V. cholerae tonB1* cluster: TonB1, 48% (GenBank accession number NP_233295.1); ExbB1, 74% (NP_233296.1); ExbD1, 72% (NP_233297.1). When the *tonB1* gene was replaced with a chloramphenicol resistance cassette (MS533), no phenotypic difference in iron uptake could be detected by bioassays with different iron sources (Table 1). This indicates that at least one other *tonB* system must be present in *V. anguillarum*. By Tn*10* mutagenesis (11) of MS533, the *tonB1* knockout, we identified a mutant, MS570, that was unable to transport any of the iron sources tested (Table 1). The Tn*10* insertion was cloned and sequenced, showing that it occurred at bp 935 of an open reading frame homologous (66% identity) to open reading frame 1547 (*tolR*) of the *V. cholerae* genome (accession no. AF047974.1). This *V. anguillarum tolR* homologue is located upstream of a second *tonB* cluster (Fig. 1), which shares homology with the *tonB2* system in *V. cholerae*: TonB2, 68% (NP_231186.1); ExbB2, 87% (NP_231185.1); ExbD2, 62% (NP_231184.1). We then generated a *tonB2* knockout strain by inserting the kanamycin resistance (Km^r) cassette (18) into the chromosomal locus of *tonB2*, with the suicide vector pTW-MEV (19) in the wild-type *V. anguillarum* strain, generating MS801. We repeated this mutagenesis with the *tonB1* mutant strain to obtain the *tonB1-tonB2* double mutant MS658. We complemented the mutants with a construct harboring the *tonB2* operon under the control of the Km^r gene promoter in pACYC177, pMS789. The mutants and complemented mutants were used in bioassays (Table 1). While the *tonB1* mutant does not show any changes with respect to the wild type, the *tonB1-tonB2* double mutant is impaired in transport of all of the iron sources tested. TonB2, but not TonB1, functions in the transport of anguibactin and enterobactin, while both TonB proteins can operate in the transport of ferrichrome and heme. We also transformed plasmid pMS789, harboring the *tonB2* system, into *Escherichia coli tonB* mutant KP1032, showing that *tonB2* from *V. anguillarum* cannot complement the *tonB* mutation in KP1032 (Table 1), even though the *tonB2* operon of *V. anguillarum* is expressed in *E. coli* as determined by reverse transcription (RT)-PCR (data not shown). This was somewhat surprising since the *V. cholerae tonB2* gene can complement this *E. coli* strain and the TonB2 proteins from *V. anguillarum* and *V. cholerae* share high homology, as is apparent in the alignment of these two proteins (Fig. 2). Furthermore, as is also shown in Fig. 2, comparison with *E. coli* TonB does not lead to an obvious answer for the lack of complementation.

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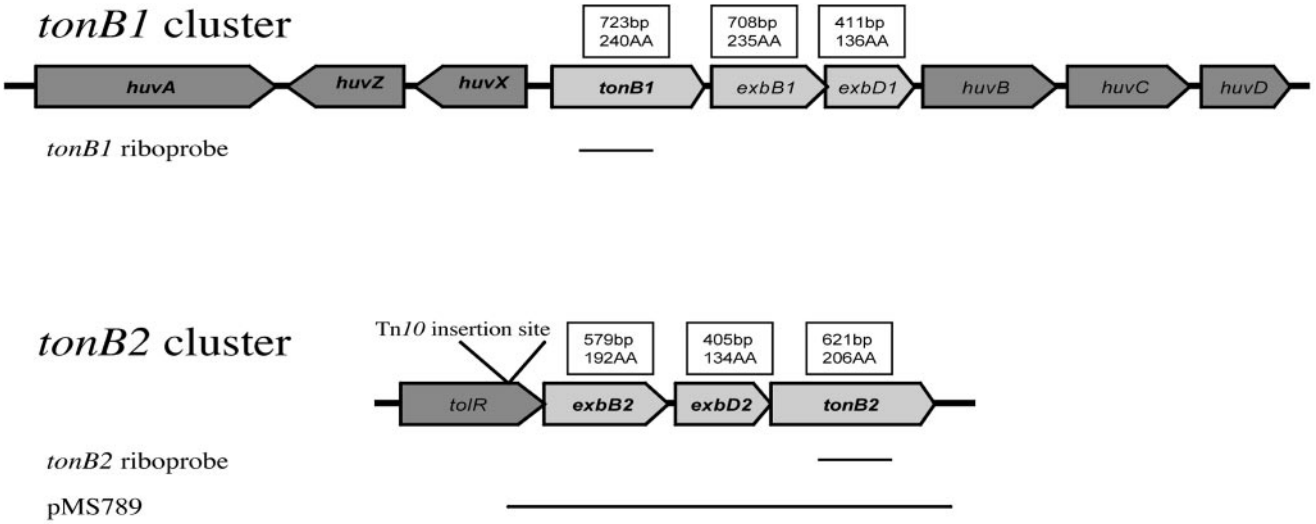

FIG. 1. Schematic representation of the *tonB1* and *tonB2* loci in *V. anguillarum*. The sizes of the genes and the corresponding proteins are shown above the *tonB* cluster genes. Also shown is the site of insertion of the Tn*10* Km^r transposon in the *tolR* homologue. The lines under the loci represent the DNA fragments cloned in the pACYC177 plasmid generating pMS789, used in complementing studies, and the riboprobes used in the RPAs. AA, amino acids.

We also analyzed the transcription of the two *V. anguillarum* *tonB* clusters by RT-PCR to determine whether the two *tonB* genes are transcribed in an operon with the respective *exbB* and *exbD* genes. As shown in Fig. 3, a band of the expected size was detected for all three genes, indicating that each one of the two *tonB* systems is indeed transcribed as an operon. Since the Tn*10* mutant has the same phenotype as *tonB2* mutant MS801, it may be that the *tolR* homologue is also part of the *tonB2* operon and that the phenotype comes from a polar effect of the transposon on downstream genes. Therefore we performed an RT-PCR with a primer in *exbB2* for the RT reaction and a primer set in *tolR* for the PCR. The results in Fig. 3C demonstrate that *tolR* is indeed also part of the *tonB2* operon.

To determine whether expression of the *tonB1* and *tonB2* systems is regulated by the iron concentration in the cell, we performed an RNase protection assay (RPA) with labeled riboprobes to detect either the *tonB1*- or the *tonB2*-specific mRNAs on total RNA obtained from cultures grown under

TABLE 1. Bioassay results

Strain	Result with following Fe source:				
	FAC[a]	Ang.[b]	Ent.[c]	Fer.[d]	Heme
V. anguillarum 775	+[e]	+	+	+	+
H775-3[h]	+	–[f]	+	+	+
MS533 (*tonB1*)	+	+	+	+	+
MS801 (*tonB2*)	+	–	–	+	+
MS658 (*tonB1 tonB2*)	+	–	–	–	–
MS801/pMS789[i]	+	+	+	+	+
MS658/pMS789	+	+	+	+	+
E. coli W3110	+	ND[g]	+	+	ND
E. coli KP1032	+	ND	–	–	ND
E. coli KP1032/pMS789	+	ND	–	–	ND

[a] FAC, ferric ammonium citrate.
[b] Ang.: anguibactin from *V. anguillarum* 775.
[c] Ent.: enterobactin from *E. coli* HB101.
[d] Fer., ferrichrome.
[e] +, positive, forming a zone of growth around the iron source.
[f] –, negative, no zone of growth around iron source.
[g] ND, not determined.
[h] Plasmidless derivative of *V. anguillarum* 775 lacking the anguibactin transport system.
[i] Plasmid containing the *V. anguillarum tonB2, exbB2,* and *exbD2* genes.

FIG. 2. Amino acid sequence alignment of the TonB2 proteins of *V. anguillarum* and *V. cholerae* (TonB2va and TonB2vc, respectively) and the TonB protein of *E. coli* (TonBec). We used the sequence analysis software package of the University of Wisconsin Genetics Computer Group. Underlined are the transmembrane domain, the proline-rich region, and the putative site of interaction with outer membrane proteins as determined for *E. coli* TonB. Identical amino acids are highlighted in gray.

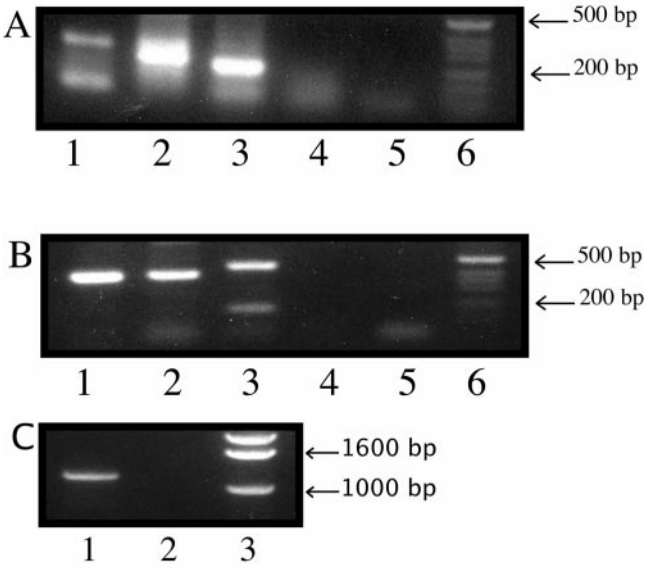

FIG. 3. The *tonB1* and *tonB2* clusters are transcribed as separate operons. RT-PCR was performed with a downstream primer for the RT reaction in *exbD1* and in *tonB2* for the *tonB1* and *tonB2* clusters, respectively. In the subsequent PCR, specific primer sets for each gene were used to identify their presence in the same cDNA sample. (A) Lanes: 1, *tonB1* RT-PCR product; 2, *exbB1* RT-PCR product; 3, *exbD1* RT-PCR product; 4, control with no reverse transcriptase; 5, H_2O control; 6, molecular weight marker. (B) Lanes: 1, *exbB2* RT-PCR product; 2, *exbD2* RT-PCR product; 3, *tonB2* RT-PCR product; 4, control with no reverse transcriptase; 5, H_2O control; 6, molecular weight marker. (C) Lanes: 1, *tolR* RT-PCR product; 2, control with no reverse transcriptase; 3, molecular weight marker.

iron-rich and iron-limiting conditions. Figure 4 shows the RPAs for *tonB1* (panel A) and *tonB2* (panel B). Comparison of lanes 1 (iron rich) and 2 (iron limiting) demonstrates that both *tonB* operons are iron regulated.

The ability of *V. anguillarum* to cause infection has been correlated with the ability to synthesize anguibactin (2, 6, 8, 20, 21). We therefore investigated if the transport of anguibactin, mediated by the TonB2 protein, is essential for virulence. We performed 50% lethal dose (LD_{50}) experiments with fish, and the results are listed in Table 2. The *tonB2* mutant is severely attenuated in virulence, more than 100-fold, while the *tonB1* mutant shows only a 10-fold decrease in virulence. The *tonB1-tonB2* double mutant is twofold more attenuated in virulence than the *tonB2* mutant, possibly because the *tonB2* mutant still has the ability to take up heme via TonB1. Complementation of the *tonB2* and *tonB1-tonB2* mutants with the wild-type *tonB2* gene results in restoration of virulence to a level close to that of the wild type. Our results demonstrate that a functional *tonB2* system is essential for ferric-anguibactin transport and virulence in *V. anguillarum*.

Nucleotide sequence accession numbers. The nucleotide sequences of the *tonB1* and *tonB2* clusters from *V. anguillarum* strain 775 have been deposited in the GenBank sequence library and assigned accession numbers AJ496544 and AY644719, respectively.

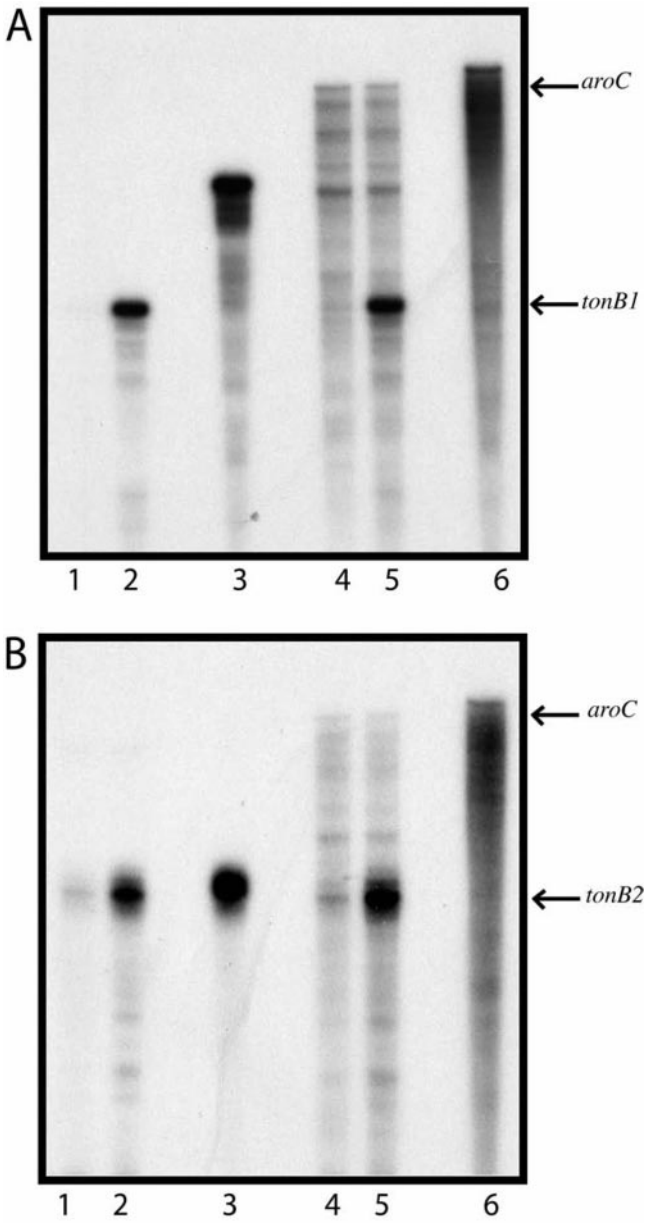

FIG. 4. Iron regulation of the two *tonB* systems. RPAs were performed with the MAXIscript kit and the RPA III RNase Protection kit, both from Ambion. RPAs for *tonB1* (A) and *tonB2* (B) were performed with RNA isolated from *V. anguillarum* 775 cultures grown under iron-rich (lanes 1 and 4) or iron-limiting (lanes 2 and 5) conditions. The *aroC* control is included in lanes 4 and 5. The riboprobes are shown in lanes 3 and 6.

TABLE 2. LD_{50} determination in rainbow trout (*Salmo gairdnerii*)

Strain	LD_{50}[a]	Fold attenuation[b]
V. anguillarum 775	1.2×10^4	1
MS533 (*tonB1*)	1.3×10^5	10
MS801 (*tonB2*)	1.3×10^6	103
MS658 (*tonB1 tonB2*)	2.9×10^6	234
MS801/pMS789[c]	7.1×10^4	6
MS658/pMS789	2.3×10^4	2

[a] LD_{50} calculated by the method of Reed and Muench (14).
[b] Fold attenuation normalized to wild-type *V. anguillarum* 775.
[c] Plasmid containing the *V. anguillarum tonB2 exbB2* and *exbD2* genes.

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