TolC Affects Virulence Gene Expression in *Vibrio cholerae*[▽]

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A *Vibrio cholerae* tolC mutant showed increased *toxT* expression in M9 medium, but not in the presence of four amino acids that induce cholera toxin production, and in LB with high osmolarity but not high pH or temperature. TolC did not affect expression of other regulatory genes in the ToxR regulon.

TolC is a major outer membrane protein involved in bacterial multidrug resistance and survival of pathogens during infection in several Gram-negative bacteria (12). In *Vibrio cholerae*, TolC is important for bile resistance, intestinal colonization (1), and RTX toxin secretion (2); however, the role of TolC in virulence gene expression has not been reported.

Expression of the main virulence factors, toxin-coregulated pilus (TCP) and cholera toxin (CT), in *V. cholerae* is tightly regulated in response to various signals, but relatively little is known about the mechanisms of sensing environmental conditions. We screened over 10,000 transposon mutants of the classical biotype of *V. cholerae* O395N1 (10) and isolated a mutant strain carrying a transposon insertion in the *tolC* gene that showed increased *toxT::lacZ* expression compared to that of the parent strain when grown in M9 medium-glycerol (data not shown). Defined *tolC* mutant strains were generated by homologous recombination essentially as described previously (7). In brief, a two-step PCR of a *cat* cassette flanked by long (1,000-nucleotide) homologous extensions of the target gene was performed. The resulting PCR product was cloned into pCR2.1 (Invitrogen) using *Escherichia coli* TOP10 as a host. The resulting plasmid was then restricted by BcuI and NotI, and the desired DNA fragment of approximately 3 kbp was purified from an agarose gel using the QIAquick gel extraction kit (Qiagen), cloned into the BcuI and NotI sites of pWM91 (18), and transformed into the *E. coli* SM10 λ pir strain (19). The resulting strain was conjugated with *V. cholerae* O395N1 or its *toxT::lacZ* derivative strain (10, 17). The *tolC* mutations in these strains were verified by PCR.

It is known that *V. cholerae* grown in minimal medium expresses TCP and CT only in the presence of certain amino acids (asparagine, arginine, serine, and glutamate; NRES) (19). The defined *V. cholerae* O395N1 *toxT::lacZ tolC* mutant strain revealed higher *toxT* expression in M9 medium supplemented with different carbon sources but was insensitive to the addition of NRES, regardless of the carbon source (Fig. 1). Consistent with the *toxT::lacZ* expression data, the defined *V. cholerae* O395N1 *tolC* mutant strain showed considerably higher CT production than the wild-type parent strain in M9

medium supplemented with different carbon sources (Table 1), whereas these strains produced comparable amounts of CT when grown in the presence of NRES (Table 1). CT levels were measured essentially as described previously (8). Together, these results showed that a loss of TolC positively affects *toxT* expression and CT production in *V. cholerae* O395N1 under specific growth conditions. It was recently suggested that TolC exports cyclic AMP (cAMP) in *E. coli* (9), and in *V. cholerae*, the cAMP receptor protein-cAMP complex (CRP-cAMP) is known to negatively affect *toxT* expression by directly repressing *tcpP* expression (21). Therefore, the observed differences in *toxT* expression in the *V. cholerae tolC* mutant could be caused by differences in intracellular cAMP levels. Because our findings showed an increase in *toxT* expression and no change in *tcpP* expression in the *V. cholerae tolC* mutant strain, we believe that changes in cAMP levels are likely not the explanation for our observations.

In *V. cholerae*, *toxT* expression is positively regulated by two membrane regulators, ToxR/S and TcpP/H, with the latter being regulated by the AphA and AphB proteins and with these regulators and proteins encoded by *toxRS*, *tcpPH*, *aphA*, and *aphB*, respectively (6, 11, 13, 14, 16, 22). We assessed the expression levels of these genes in the defined *tolC* mutant. As expected, quantitative reverse transcription-PCR (qRT-PCR) results for bacteria grown in M9 medium-glycerol showed in-

FIG. 1. Effects of growth medium on ToxT-LacZ expression in the *V. cholerae tolC* mutant (TZtolC) and parent (TZ) strains. To analyze the effects of different carbon sources, bacteria were inoculated into M9 medium supplemented with different carbon sources (final concentration, 0.4%) and incubated for 24 h. The four amino acids (asparagine, arginine, serine, and glutamate; NRES) were added to M9 medium at 25 mM each. β -Galactosidase activities were measured essentially as previously described (10). Error bars indicate standard deviations ($n \geq 3$).

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TABLE 1. Effect of the *tolC* mutation on CT production in minimal media

Medium	CT production ($\mu\text{g/ml/OD}_{600}$) by ^a :	
	WT parent ^b	<i>tolC</i> mutant
M9-glycerol	0.08 ± 0.02	0.71 ± 0.08
M9-glycerol-NRES	1.24 ± 0.04	2.06 ± 0.13
M9-glucose	0.29 ± 0.06	0.45 ± 0.04
M9-glucose-NRES	3.22 ± 0.54	4.06 ± 0.17
M9-succinate	0.31 ± 0.03	0.61 ± 0.09
M9-succinate-NRES	1.61 ± 0.27	1.33 ± 0.19

^a CT production was measured after 48 h of shaking at 30°C. OD₆₀₀, optical density at 600 nm.

^b WT, wild type.

creases in *toxT* and *ctxB* transcription levels (Fig. 2). However, expression levels of the *toxR*, *tcpP*, *aphA*, and *aphB* genes in the *V. cholerae tolC* mutant strain were similar to those in the wild-type strain (data not shown), indicating that the upregulation of *toxT* expression in the *tolC* mutant in M9 medium-glycerol is not caused by an increase in ToxR or TcpP expression levels. Thus, the loss of TolC might affect the function of TcpP and/or ToxR or might independently affect *toxT* expression.

It has been reported that the three-component signal transduction system VieSAB plays a role in *V. cholerae toxT* expression when bacteria are grown under nutrient-limiting conditions by affecting intracellular 3',5'-cyclic diguanylic acid (c-di-GMP) levels (23). Since the response regulator VieA is controlled at the transcriptional level (15), we measured the expression of the *vieSAB* genes in the *tolC* mutant. However, the expression levels of the *vieA*, *vieS*, and *vieB* genes were similar in the mutant and parent strains (data not shown), indicating that the VieSAB system is not involved in the TolC-mediated effects on *toxT* regulation.

Interestingly, the *tolC* mutant did not show increased *toxT::lacZ* expression when grown in LB at 30°C or 37°C or in LB with a high starting pH (pH 8.5) (Fig. 3). However, under high-osmolarity conditions, such as high NaCl or KCl, the *tolC* mutant showed increased *toxT* expression (Fig. 3). High osmolarity is known to negatively affect TcpP/H protein activity (10), whereas a temperature of 37°C and high pH are known to

FIG. 2. Effects of the *tolC* mutation on virulence gene expression. The *V. cholerae* O395N1 strain and the *tolC* mutant strain were grown in M9 medium-glycerol for 24 h at 30°C. Total RNA was extracted and analyzed by qRT-PCR. Gene expression levels were normalized between the samples by using 16S rRNA. All experiments were repeated three times. Error bars indicate standard deviations ($n = 3$).

FIG. 3. Effects of growth conditions on ToxT-LacZ expression in the *V. cholerae tolC* mutant (TZtolC) and parent (TZ) strains. To analyze the effects of temperature, osmolarity, and pH, the bacteria were inoculated into LB at pH 6.5, LB at pH 6.5 supplemented with 200 mM NaCl or KCl (osmolarity effect), or LB at pH 8.5, cultured at 37°C or 30°C, and incubated for 6 h. β -Galactosidase activities were measured essentially as previously described (10). Error bars indicate standard deviations ($n \geq 3$).

repress *tcpP* expression (3), suggesting that a loss of TolC might modulate TcpP/H protein activity and that this modulation might protect TcpP/H from the negative effects of high osmolarity. Since expression levels of *tcpP* were not affected by a *tolC* mutation in M9 medium-glycerol, it is tempting to speculate that the TcpP/H proteins might also be modulated by TolC in M9 medium-glycerol. Future analysis of TcpP/H protein stability is needed to better understand these observations.

Taken together, our data demonstrate that TolC affects *toxT* expression only under certain growth conditions. Recently, a lack of TolC was reported to affect cellular metabolism in *E. coli* (5), including reports that some metabolites that are exported via TolC modulate gene expression in *E. coli* (4, 20). Thus, we propose that TolC modulates *toxT* gene expression by modulating TcpP/H activity via accumulation of certain metabolites in *V. cholerae*. The nature of these metabolites and the exact mechanism by which they affect TcpP/H activity in *V. cholerae* are not yet understood, and more global approaches, such as metabolomics, should be utilized to better understand this intriguing link between TolC and virulence gene regulation.

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