TolC Affects Virulence Gene Expression in Vibrio cholerae

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Received 3 May 2011/Accepted 10 August 2011

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 Apri 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. 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-

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† Published ahead of print on 19 August 2011.

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 ≥ 3).
TABLE 1. Effect of the tolC mutation on CT production in minimal media

<table>
<thead>
<tr>
<th>Medium</th>
<th>WT parent a</th>
<th>tolC mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9-glycerol</td>
<td>0.08 ± 0.02</td>
<td>0.71 ± 0.08</td>
</tr>
<tr>
<td>M9-glycerol-NRES</td>
<td>1.24 ± 0.04</td>
<td>2.06 ± 0.13</td>
</tr>
<tr>
<td>M9-glucose</td>
<td>0.29 ± 0.06</td>
<td>0.45 ± 0.04</td>
</tr>
<tr>
<td>M9-glucose-NRES</td>
<td>3.22 ± 0.54</td>
<td>4.06 ± 0.17</td>
</tr>
<tr>
<td>M9-succinate</td>
<td>0.31 ± 0.03</td>
<td>0.61 ± 0.09</td>
</tr>
<tr>
<td>M9-succinate-NRES</td>
<td>1.61 ± 0.27</td>
<td>1.33 ± 0.19</td>
</tr>
</tbody>
</table>

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, 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 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.

We thank Barry Wanner for providing plasmids pWM91 and pKD3. We thank Sara R. Fassio and Cinthia Costa-Jones for their excellent technical assistance.

REFERENCES


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 ≥ 3).


