Role of the viaB locus in synthesis, transport and expression of Salmonella typhi Vi antigen

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The Vi antigen is a capsular polysaccharide expressed by Salmonella typhi, the agent of human typhoid fever. Expression of this antigen is controlled by the viaA and viaB chromosomal loci. The viaB locus is composed of 11 genes designated tviA–tviE (typhi Vi), vexA–vexE (Vi antigen export) and ORF11. We constructed S. typhi Ty2 strains carrying non-polar mutations in ten genes located at the viaB locus and examined the individual contribution of each gene to Vi phenotype. Phenotypes of the mutants and complementation experiments suggested that synthesis of Vi antigen monomer was catalysed by the TviB and TviC polypeptides. Subsequent polymerization of the polysaccharide might be catalysed by the TviE protein, but required functional TviD product. Proteins encoded by vexA, vexB and vexC directed transport of the polymer to the bacterial cell surface. Anchoring of the Vi antigen at the bacterial cell surface was dependent of the VexE protein. The TviA protein was not essential for Vi polymer synthesis. However, disruption of the tviA gene on S. typhi Ty2 chromosome strongly decreased expression of Vi antigen. This defect was fully complemented by providing tviA in trans on a recombinant plasmid. By using lacZ transcriptional fusions, it was shown that the TviA product positively regulated co-transcription of the tviA and tviB genes from a promoter located upstream of tviA. Moreover, we showed that a tviAB–lacZ fusion was not expressed in a viaA (rcsB) mutant of S. typhi. However, expression of the tviAB–lacZ fusion was restored in this viaA mutant either by the rcsB gene of Escherichia coli, or by the tviA gene of S. typhi when present in high copy number. This suggested that the tviA and viaA products could be involved in the same regulatory pathway modulating Vi antigen expression in S. typhi. Together these results demonstrated that proteins encoded by the viaB locus are not only involved in Vi polymer synthesis and translocation of the polysaccharide to the bacterial cell surface, but also in regulation of Vi antigen expression in S. typhi.

Keywords: Salmonella typhi Vi antigen, synthesis, transport, regulation, expression

INTRODUCTION

The Vi antigen of Salmonella typhi, the agent of human typhoid fever, is a capsular polysaccharide discovered by Felix & Pitt (1934). This polysaccharide is a linear homopolymer of α-1,4 2-deoxy-2-N-acetylgalactosamine uronic acid variably O-acetylated at the C3 position (Heyns & Kiesling, 1967; Daniels et al., 1989). All strains of S. typhi and Salmonella paratyphi C, as well as a few strains of Salmonella dublin and Citrobacter freundii, are capable of expressing Vi antigen (Felix & Pitt, 1936; Baker et al., 1939; Le Minor & Nicolle, 1964). Expression of Vi antigen is controlled by two widely separated loci, viaA and viaB, located at 43 and 92 min on the chromosome of S. typhi, respectively (Johnson et al., 1965; Johnson & Baron, 1969; Snellings et al., 1977). Functional viaA genes are present not only in Vi-expressing strains of Salmonella and Citrobacter, but also in Escherichia coli (Johnson & Baron, 1969). In contrast, the viaB locus is specific to Vi-expressing strains (Snellings et al., 1977). Acquisition of the viaB region by E. coli results in a Vi-positive phenotype (Johnson & Baron, 1969; Kolyva et al., 1992). In S. typhi, the viaA locus which is allelic to rcsB, a positive regulator of capsule synthesis in E. coli
(Gottesman & Stout, 1991; Houg et al., 1992), and the \textit{ompR-envZ} two-component regulatory system (Pickard et al., 1994) play an important role in the regulation of Vi antigen synthesis.

The \textit{viaB} locus of \textit{S. typhi} appears to contain at least two regions: one involved in biosynthesis of Vi antigen, and the other required for translocation of the polysaccharide to the cell surface (Hashimoto et al., 1991, 1993; Kolyva et al., 1992). The complete nucleotide sequence of the \textit{viaB} locus of \textit{S. typhi} strain GIFU 10007 has been determined and 11 ORFs were identified, all being transcribed in the same orientation from ORF1 to ORF11 (Hashimoto et al., 1993). ORFs 1, 4 and 11 were not named by these authors. ORFs 2, 3 and 5 were designated \textit{vio} (Vi polysaccharide synthesis) \textit{A}, \textit{B} and \textit{C}, respectively. ORFs 6, 7, 8, 9 and 10 were named \textit{vec} (Vi antigen export) \textit{A}, \textit{B}, \textit{C}, \textit{D} and \textit{E}, respectively. Simultaneously, we reported the nucleotide sequence of the first six ORFs of the \textit{S. typhi} strain TY2 \textit{viaB} locus which were designated \textit{tti} (tiophy V\textit{i}) \textit{A}, \textit{B}, \textit{C}, \textit{D}, \textit{E} and \textit{F} (Waxin et al., 1993). The nucleotide sequence of the first six ORFs of \textit{S. typhi} strains GIFU 10007 and TY2 was found to be strictly identical. For reasons of clarity, the 11 ORFs will be henceforth termed as follows: \textit{ttiA}, \textit{ttiB}, \textit{ttiC}, \textit{ttiD}, \textit{ttiE}, \textit{vecA}, \textit{vecB}, \textit{vecC}, \textit{vecD}, \textit{vecE} and \textit{ORF11}, respectively (Hashimoto et al., 1993; Waxin et al., 1993; see Fig. 1). It was shown that TviB shared significant homology to the GDP-mannose dehydrogenase AlgD of \textit{Pseudomonas aeruginosa} and TviC to glucose epimerases of prokaryotic organisms (Hashimoto et al., 1993; Waxin et al., 1993). VexA, B, C and D proteins had similarities to components of capsule transporters (Hashimoto et al., 1993; Waxin et al., 1993). In contrast, TviA, TviD, TviE and VexE proteins showed no significant similarity to previously reported protein sequences. The functions of these products remain unknown (Hashimoto et al., 1991, 1993; Kolyva et al., 1992).

The present work was designed to examine in detail the individual contribution of each gene located in the \textit{viaB} locus to Vi phenotype. For this purpose, \textit{S. typhi} strains carrying non-polar mutations in the genes located in the \textit{viaB} locus were constructed. We present evidence that TviB–TviE proteins are required for Vi polymer synthesis, and Vex–VexE proteins for the translocation of the polysaccharide to the bacterial cell surface. In addition, phenotypes of non-polar mutants, complementation experiments and studies of lacZ transcriptional fusions strongly suggest that the TviA protein, whilst not essential to Vi polysaccharide synthesis, is required for the expression of Vi antigen in \textit{S. typhi}.

**METHODS**

**Strains, growth conditions and plasmids.** \textit{Salmonella} and \textit{Citrobacter} strains were from the collection of the WHO collaborating centre for Reference and Research on \textit{Salmonella} (Institut Pasteur, Paris). \textit{S. typhi} strain TY2 expressed the Vi antigen and possessed the O:9,12 somatic factors. \textit{S. typhi} strain T643WSR was a \textit{viaA} mutant which did not express the Vi antigen (Johnson et al., 1966). Presence of either Vi antigen or O:9 factor on \textit{S. typhi} strains and derivatives was assayed by slide-agglutination with specific antisera (Diagnostics Pasteur). \textit{E. coli} MCI061 [araD139 D[ara-len]-7697 rpsL2 galU galK D[lacPOZY]X74] (Casadan & Cohen 1980) and \textit{E. coli} H1B101 [hisB6 RksM recA1 supE44 lacZ4 lenB6 proA2 rhi-1 rpsL1] (Boyer & Roulland-Dussoix, 1969) were used as recipients for cloning or complementation experiments. \textit{E. coli} K12-Ni was a spontaneous nalidixic acid-resistant mutant of \textit{E. coli} C600 (Appleyard, 1954).

Strains were routinely grown at 37°C in tryptic soy broth (TSB) or on tryptic soy agar (TSA; Diagnostics Pasteur). When required, antibiotics were added at the following concentrations: ampicillin (Ap), 100 μg ml⁻¹; chloramphenicol (Cm), 30 μg ml⁻¹; kanamycin (Km), 50 μg ml⁻¹; nalidixic acid (Ni), 100 μg ml⁻¹; spectinomycin (Sp), 100 μg ml⁻¹; tetracycline (Te), 20 μg ml⁻¹.

Plasmids pUC18/19 (Ap⁺; Vieira & Messing, 1982), and pG2B (Sp⁺; Churchward et al., 1984) were used as cloning vehicles. Plasmid pRK2073 (Sp⁺; Ditta et al., 1980) was used as a helper plasmid in triparental plate matings to mobilize plasmid pvT1. Plasmid pvT1 was a recombinant mobilizable-plasmid harbouring the \textit{viaB} locus of \textit{S. typhi} Ty2 (Te⁺; Kolyva et al., 1992). This plasmid conferred a Vi-positive phenotype upon \textit{E. coli} strains. Plasmid pvT2 was used as a source of DNA for construction of recombinant plasmids presented on Fig. 1. Construction of plasmids pvT22 and pvT28 was reported previously (Kolyva et al., 1992).

Plasmid pUC4K (Pharacmania) which carried the aminoglycoside 3’-phosphotransferase (aph) gene, was used as a source of the Km-cassette. From this plasmid, a second Km-cassette carrying the aminoglycoside 3’-phosphotransferase gene, but devoid of its transcription terminator (aphT), was constructed as described by Gaian et al. (1992) and cloned into pUC18. The aphbT cassette was used to introduce non-polar mutations into the \textit{viaB} locus on plasmid pvT1 and on the chromosome of \textit{S. typhi}. Plasmid pQF50Cm (Cm⁺; F. Norel, unpublished) was a derivative of pQF50 (Farinha & Kropinski, 1990) in which the ampicillin-resistance gene had been replaced by the chloramphenicol acetyltransferase (cat) gene of pACYC184 (Chang & Cohen, 1978). A lacZ-cat cassette could be retrieved from pQF50Cm by using \textit{SalI} restriction endonuclease. This cassette was used to transcribe the gene in which it had been inserted by monitoring β-galactosidase activity.

**DNA manipulation and molecular cloning.** Methods used for constructing and manipulating recombinant DNA were essentially those of Sambrook et al. (1989). Restriction endonucleases and nucleic acid-modifying enzymes were purchased from Amersham. Small-scale preparation of plasmid DNA was carried out as described by Birnboim & Doly (1979) for \textit{E. coli} strains and as described by Kado & Liu (1981) for \textit{Salmonella} strains. Plasmid transformation was performed in \textit{E. coli} by the method of Humphreys et al. (1979) and in \textit{Salmonella} by electroporation with a Bio-Rad apparatus according to the manufacturer’s instructions. To study the variation of \textit{tviA} sequences among strains of \textit{Salmonella} and \textit{Citrobacter} by Southern blot analysis, an internal fragment of the \textit{tviA} gene from \textit{S. typhi} Ty2 was amplified by the polymerase chain reaction (PCR) with a commercial kit (Perkin-Elmer Cetus) using primers 5’-CCT CGG AAT GAT GAT TAT TAT TCC CGG G-3’ and 5’-ATC CGG CAA TAA CAA CAG ATA GCG CGG G-3’. The probe was labelled with [α³²P]dATP by using a random primer labelling kit (Amersham). The \textit{tviA} gene from \textit{S. typhi} T643WSR was cloned into vector pG2B after PCR amplification using primers 5’-GCT TCG CCG GCA TGC ATA AGG TAT
TCA TTT-3' and 5'-TTC AAG AAT AAG CTT TTT ATT AAC G-3' (Waxin et al., 1993).

A PCR clone encoding the ResB protein of E. coli MC1061 was constructed by using primers 5'-ATG GCA AGG CGG ATC CGG TGG CAT TCT GGC -3' and 5'-ATC CTG CTA AAG CTT TGC CTG CAA CGG ACA -3' (Stout & Gottesman, 1990). The PCR fragment was cloned into vector pGB2 and the resulting plasmid was designated pResB.

**Construction of S. typhi viaB mutants.** To study the individual contribution of each gene of the *viaB* locus to the Vi phenotype, the *aphAT* cassette was cloned into unique sites of recombinant plasmids (from pVT22 through pVT28) presented on Fig. 1. The correct position of *aphAT* insertion was verified by restriction mapping. The construction was transformed into *E. coli* MC1061 harbouring pVT1. The resultant strain was mated with *E. coli* C600 containing pRK2073 and *E. coli* K12-NI as the recipient strain. Transconjugants were selected on Ni, Km and Tc/TTA plates. Because the helper plasmid pRK2073 could mobilize only pVT1, homologous recombination between the wild-type gene to be mutated on pVT1 and the corresponding *aphAT* allele on the recombinant plasmid was required to allow growth of *E. coli* K12-NI transconjugants on Km- and Tc-containing plates. Transconjugants that had undergone correct recombination of the *aphAT* cassette were identified by restriction analysis. Using this strategy, the *aphAT* cassette was introduced into 10 ORFs of the *viaB* locus on pVT1. Each of the resulting plasmids was transformed into *S. typhi* Ty2.

Homologous recombination between the inactivated gene on pVT1 and the corresponding wild-type gene on *S. typhi* Ty2 chromosome was obtained as follows. *S. typhi* strains carrying pVT1 with a mutation in the given gene were inoculated into 10 ml fresh TSB without antibiotics and transferred daily for 3 d in the same conditions. Cultures were isolated on Km-plates and Tc/TSA plates. Because the helper plasmid pRK2073 could mobilize only pVT1, homologous recombination between the wild-type gene to be mutated on pVT1 and the corresponding *aphAT* allele on the recombinant plasmid was required to allow growth of *E. coli* K12-NI transconjugants on Km- and Tc-containing plates. Transconjugants that had undergone correct recombination of the *aphAT* cassette were identified by restriction analysis. Using this strategy, the *aphAT* cassette was introduced into 10 ORFs of the *viaB* locus on pVT1. Each of the resulting plasmids was transformed into *S. typhi* Ty2.

**Vi antigen assays.** Presence of Vi antigen associated with cell surface was determined by slide-agglutination using Vi-specific antiserum (Diagnostics Pasteur) and further confirmed by lysis with the Vi-specific phage IV (Craigie & Felix, 1947). Accumulation of intracellular Vi antigen or release of the polysaccharide into culture supernatant was demonstrated by immunoprecipitation as described previously (Kolyva et al., 1992). Using a highly purified preparation of Vi antigen (Typhim Vi; Institut Merieux), it was possible to detect as little as 0.1 μg ml⁻¹ soluble Vi antigen. It was verified that the commercial Vi-antiserum did not react with monomer or short oligomers of Vi antigen. For this purpose, a Vi antigen-degrading enzyme was partially purified from the culture supernatant of *Bacillus sphaericus* ATCC 17932 following the procedure described by Baker & Whiteside (1965). Incubation of 0.1 ml enzyme solution with 5 μg purified Vi antigen for 2 h at 35 °C resulted in complete loss of serological reactivity of the polysaccharide in the immunoprecipitation assay.

**Measurements of β-galactosidase activity.** The assays for β-galactosidase activity were performed using overnight cultures as described by Miller (1972). Average values (± 1 sd) of activity units were calculated based on at least three independent assays in each case.

**RESULTS**

**Vi polymer synthesis requires functional TviB, TviC, TviD and TviE polypeptides**

*S. typhi* Ty2 mutants in which the *aphAT* cassette had been inserted in the *tviB*, *tviC*, *tviD* or *tviE* genes were defective in Vi antigen synthesis. These mutants did not release Vi polymer in culture supernatant, were devoid of capsular polysaccharide at the cell surface, and did not accumulate polymer in their intracellular compartment (Table 1). Introduction of plasmid carrying only the homologous wild-type gene (Fig. 1 and Table 1) restored a Vi-positive phenotype to each mutant, which were slide-agglutinated by Vi antiserum and lysed by phage IV. These results demonstrated that TviB, TviC, TviD and TviE polypeptides were involved in Vi antigen synthesis. As Hashimoto et al. (1993) reported that TviA was not essential for Vi antigen production, we wondered whether TviB, TviC, TviD and TviE polypeptides were sufficient for Vi polymer synthesis. *E. coli* HB101 was transformed with different combinations of recombinant plasmids harbouring one or more of the *tvi* genes (Fig. 1), and the presence of intracellular Vi polymer was assayed by immunoprecipitation. Providing *tviB* and *tviC in trans* on plasmid pVT37 did not lead to Vi polymer synthesis in the intracellular compartment of *E. coli* HB101. Additional presence of *tviD* on plasmid pVT31 or of *tviE* on plasmid pVT32 did not affect the phenotype of *E. coli* HB101 carrying pVT37. In contrast, Vi polymer was detected in the intracellular compartment of *E. coli* HB101 harbouring *tviB* and *tviC* on plasmid pVT37 and *tviD* and *tviE* on plasmid pVT39. The results demonstrated that Vi polymer synthesis in the intracellular compartment of *E. coli* HB101 required functional TviB, TviC, TviD and TviE proteins.

**Vex proteins direct cell surface translocation and localization of Vi polysaccharide**

Insertion of the *aphAT* cassette in *vexA*, *vexB* or *vexC* on *S. typhi* Ty2 chromosome caused intracellular accumulation of Vi polysaccharide (Table 1). The mutants failed to elaborate cell surface-associated Vi antigen or to release it in the culture supernatant. These defects were complemented by recombinant plasmids bearing the homologous wild-type gene (Fig. 1 and Table 1). The results strongly suggested that VexA, VexB and VexC products were involved in translocation of Vi polymer to the bacterial surface. Interestingly, since the commercial Vi antiserum used did not react with monomers and oligomers of Vi antigen (see Methods), these results indicated that in vivo synthesis of full-length Vi polysaccharide chains could occur independently of chain translocation.

Immunoreactive Vi polysaccharide was detected in the intracellular compartment of cells and in the culture supernatant of the *S. typhi* Ty2(*vexE::aphAT*) mutant (Table 1). Polymeric material was apparently not present at the cell surface in this mutant. Introduction of plasmid pVT36 led to the detection of Vi antigen at the cell surface by slide-agglutination (Fig. 1 and Table 1). Thus, the
VexE protein appeared to be required for anchoring Vi antigen at the bacterial surface. The S. typhi Ty2(ORFl1::aphΔT) mutant retained a Vi-positive phenotype (Table 1). It was slide-agglutinated by Vi antiserum and lysed by phage IV. This result suggested that ORFl1 was not required for Vi antigen synthesis, nor for the translocation of the polymer to the cell surface.

**Table 1. Phenotypes of non-polar S. typhi Ty2 viaB mutants**

<table>
<thead>
<tr>
<th>S. typhi Ty2 mutant</th>
<th>Detection of Vi polysaccharide*</th>
<th>Agglutination by O:9 antiserum</th>
<th>Plasmid§ used for complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>tviA::aphΔT</td>
<td>(+)</td>
<td>+</td>
<td>pVT21</td>
</tr>
<tr>
<td>tviB::aphΔT</td>
<td>−</td>
<td>−</td>
<td>pVT29</td>
</tr>
<tr>
<td>tviC::aphΔT</td>
<td>−</td>
<td>−</td>
<td>pVT30</td>
</tr>
<tr>
<td>tviD::aphΔT</td>
<td>−</td>
<td>−</td>
<td>pVT31</td>
</tr>
<tr>
<td>tviE::aphΔT</td>
<td>−</td>
<td>−</td>
<td>pVT32</td>
</tr>
<tr>
<td>vexA::aphΔT</td>
<td>+</td>
<td>−</td>
<td>pVT33</td>
</tr>
<tr>
<td>vexB::aphΔT</td>
<td>+</td>
<td>−</td>
<td>pVT34</td>
</tr>
<tr>
<td>vexC::aphΔT</td>
<td>+</td>
<td>−</td>
<td>pVT35</td>
</tr>
<tr>
<td>vexE::aphΔT</td>
<td>+</td>
<td>+</td>
<td>pVT36</td>
</tr>
<tr>
<td>ORFl1::aphΔT</td>
<td>−</td>
<td>+</td>
<td>None</td>
</tr>
</tbody>
</table>

*Detection of Vi polysaccharide at the cell surface (cell) by slide-agglutination, and in intracellular compartment (intra) or in culture supernatant (super) by immunoprecipitation. + , Positive; − , negative; (+), weak reaction.

§See Fig. 1.

Expression of the tviB::lacZ-cat fusion was dramatically decreased when the tviA gene was disrupted by the non-polar aphΔT cassette. Providing tviA in trans on plasmid pVT21 in S. typhi Ty2(tviA::aphΔT,tviB::lacZ-cat) fully restored expression of the fusion. This result showed that TviA polypeptide was required for tviB gene transcription. In an attempt to unravel the role of TviA on tviB transcription, the tviA gene was disrupted by the aph cassette. This polar mutation was further recombined in tviA on the chromosome of S. typhi Ty2(tviB::lacZ-cat), yielding the double mutant S. typhi Ty2(tviA::aph,tviB::lacZ-cat). As expected, disruption of the tviA gene by the aph cassette resulted in little expression of the tviB::lacZ-cat fusion (Table 2). In contrast to results obtained for the S. typhi Ty2(tviA::aphΔT,tviB::lacZ-cat) mutant which carried a non-polar mutation in tviA, introduction of plasmid pVT21 did not restore expression of the tviB::lacZ-cat fusion in S. typhi Ty2(tviA::aph,tviB::lacZ-cat) which carried a polar mutation in tviA (Table 2). Together, these results suggested that the tviAB genes were co-transcribed from a TviA-inducible promoter. As a test for tviAB co-transcription, plasmid pVT40 which carried the tviB coding sequence oriented in a head-to-tail manner with the lac promoter of the cloning vector (Fig. 1), was transformed into S. typhi Ty2( tviB::aphΔT). Contrary to plasmid pVT29 (Fig. 1 and Table 1), plasmid pVT40 failed to restore a Vi-positive phenotype to this mutant, thereby confirming that tviA and tviB genes were likely to be co-transcribed in S. typhi Ty2.

**TviA protein is an activator of its own synthesis**

In an attempt to study the role of the TviA protein on its own promoter, experiments were conducted with the lacZ-cat cassette integrated in the BglII site of tviA on
the *S. typhi* Ty2 chromosome. β-Galactosidase activities were measured in overnight TSB cultures. *S. typhi* Ty2 (*tviA::lacZ-cat*) expressed about 60 Miller units. This β-galactosidase level increased tenfold in the presence of plasmid pVT21 (Table 2). In contrast, introduction of plasmid pVT42, a derivative of pVT21 in which the *tviA* coding sequence was disrupted by filling-in the *BglII* site (Fig. 1), did not restore β-galactosidase activity to *S. typhi* Ty2 (*tviA::lacZ-cat*) (Table 2). This observation showed that extra copies of *tviA* DNA did not lead to activation of chromosomal *tviA::lacZ* fusion by binding a negative regulator. Together, these results indicated that *tviA* was transcribed at a basal level in the absence of TviA and that TviA protein was an activator of its own synthesis in *S. typhi* Ty2.

**Fig. 1.** Physical map of plasmid pVT1 and pVT1 subclones. Position and designation of ORFs, and molecular mass (kDa) of the corresponding products are indicated below the restriction map of pVT1. Recombinant subclones derived from pVT1 were constructed using pUC18/19 as a vehicle, except pVT21 and pVT37, which were constructed using pGB2. In pUC18/19-derivatives, the inserted fragments were oriented so that they might be transcribed from the plasmid lac promoter, except for plasmid pVT40 in which the inserted fragment was oriented in a head-to-tail manner with the lac promoter. The *BglII* restriction site located within the *tviA* coding sequence was filled-in (box) in plasmid pVT37 and pVT42. Vertical arrows indicate restriction sites in which an *aphA* cassette was inserted so as to construct non-polar mutants. Only relevant restriction sites are indicated: A, Accl; B, BamHI; *BglII*; C, ClaI; E, EcoRI; H, HindIII; *Hp*, *HpaI*; N, *Ncol*; *Ns*, *NsiI*; P, *PstI*; S, *SphI*; V, *EcoRV*.

**RcsB protein acts as a positive regulator for co-transcription of *tviA* and *tviB* genes**

It was shown that the Vi-negative strain T643WSR was a *viaA* mutant of *S. typhi* (Johnson et al., 1966) and that *viaA* from *S. typhi* was allelic to *rcsB* from *E. coli* (Houng et al., 1992). As expected from these data, providing *E. coli* *rcsB* gene in trans on plasmid pRcsB restored a Vi-positive phenotype to *S. gphi* T643WSR. As RcsB protein acted as a positive trans-acting regulator of capsule synthesis in *E. coli* (Gottesman & Stout, 1991), we further examined the potential role of RcsB on the expression of the *tviAB* genes in *S. typhi*. Since TviA was required for transcription of *tviAB* genes, the *lacZ-cat* cassette was inserted in *tviB* on the chromosome of strain T643WSR. Expression of
Transcriptional fusions in

**Table 2. β-Galactosidase activity expressed by lacZ transcriptional fusions in S. typhi Ty2 mutants harbouring the indicated plasmids**

<table>
<thead>
<tr>
<th>S. typhi Ty2 mutant</th>
<th>Plasmid*</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>tviB::lacZ−cat</td>
<td>None</td>
<td>228 ± 21</td>
</tr>
<tr>
<td>tviB::lacZ−cat</td>
<td>pGB2</td>
<td>217 ± 17</td>
</tr>
<tr>
<td>tviB::lacZ−cat</td>
<td>pVT29 (tviB*)</td>
<td>201 ± 33</td>
</tr>
<tr>
<td>tviA::aphΔT</td>
<td>None</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>tviB::lacZ−cat</td>
<td>pGB2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>tviA::aphΔT</td>
<td>pVT21 (tviA*)</td>
<td>198 ± 10</td>
</tr>
<tr>
<td>tviB::lacZ−cat</td>
<td>None</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>tviB::lacZ−cat</td>
<td>pGB2</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>tviA::aph</td>
<td>pVT21 (tviA*)</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>tviB::lacZ−cat</td>
<td>None</td>
<td>56 ± 15</td>
</tr>
<tr>
<td>tviA::aph</td>
<td>pGB2</td>
<td>61 ± 9</td>
</tr>
<tr>
<td>tviA::lacZ−cat</td>
<td>pVT21 (tviA*)</td>
<td>556 ± 34</td>
</tr>
<tr>
<td>tviA::lacZ−cat</td>
<td>pVT42 (tviA)</td>
<td>54 ± 7</td>
</tr>
</tbody>
</table>

*See Fig. 1. The allele present on the recombinant plasmids is indicated in parentheses.

**Fig. 2. Hybridization of the tviA DNA probe to total-cell DNA from Salmonella and Citrobacter strains.** Lanes contain DNA digested with EcoRI and CiaI from: 1, S. typhi Ty2 (Vi+); 2, C. freundii CNS (Vi−); 3, plasmid pVT1 (control); 4, S. typhimurium C5 (Vi−); 5, S. typhi T643 (Vi−); 6, S. paratyphi C 1535K (Vi+); 7, S. paratyphi C 32K (Vi+); 8, S. dublin 228K (Vi−); 9, S. dublin 1662K (Vi+); 10, C. freundii 7851 (Vi+). Arrowhead to the left denotes the position of the 2.1 kb fragment containing the tviA locus.

Conservation of tviA sequence among Vi-expressing strains of Salmonella and Citrobacter

To test for the presence of tviA or tviA-like sequences in Vi-expressing strains of Salmonella and Citrobacter, Southern blot hybridization analysis was performed using total DNA samples digested with EcoRI and CiaI restriction endonucleases. The probe, generated by PCR amplification, corresponded to an internal fragment of the tviA gene from S. typhi Ty2. Result of this analysis is shown in Fig. 2. The 2.1 kb EcoRI–CiaI fragment containing tviA-homologous sequence (Fig. 1) was present in all Vi-positive strains of Salmonella and Citrobacter, and in the Vi-negative S. typhi T643WSR mutant. This sequence was not detected in the Vi-negative strains of S. typhimurium C5, S. dublin 228K or C. freundii CNS. These results indicated that regions of high sequence similarity to S. typhi tviA gene were conserved among Vi-expressing strains of Salmonella and Citrobacter.

**DISCUSSION**

**Synthesis of the Vi polysaccharide**

The bacterial capsular polysaccharides are classified into two groups (designated I and II) by chemical, physical and genetic criteria (Jann & Jann, 1990). In general, group I polysaccharides have high molecular masses, contain uronic acid as the acidic component, are expressed below 20 °C, and are co-expressed with specific O polysaccharides. The Vi antigen of S. typhi shares all these

from a multicopy plasmid. For this purpose, plasmid pRcsB was introduced into S. typhi Ty2(tviA::aphΔT). The resultant strain synthesized low amounts of Vi antigen and remained agglutinated by O:9 antiserum. Moreover, presence of pRcsB in S. typhi Ty2(tviA::aphΔT,tviB::lacZ−cat) did not increase expression of the lacZ fusion (4 ± 1 Miller units). These results demonstrated that TviA was required for full expression of Vi antigen even when RcsB was expressed from a multicopy plasmid.

**Clinical microbiology and food safety**

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The viAB locus of S. typhi is composed of 11 genes designated tviA–E, vexA–E and ORF11. Results presented in this communication confirm that TviB and TviC polypeptides are required for the synthesis of Vi polysaccharide chains. On the basis of primary sequence similarity and identification of a potential dinucleotided-binding site, Hashimoto et al. (1993) proposed that tviB and tviC might encode the NAD- or NADP-dependent enzymes required to synthesize the nucleotide sugar for the Vi polysaccharide synthesis. Their view is supported by our data based on the phenotypes of tviB and tviC mutants and complementation experiments (Table 1). In addition, the TviE and TviD polypeptides are required for synthesis of Vi antigen (Table 1). Recent comparison of the amino acid sequence of TviE with the Swiss-Prot database (release 29) showed that the C-terminal domain of TviE possesses a significant level of homology to UDP-galactosyltransferases of prokaryotic and eukaryotic organisms. Based on this similarity and on results presented in Table 1, we suggest that TviE may be the transferase required for Vi polymerization. The function of the TviD product remains unclear. A non-polar tviD mutant of S. typhi Ty2 failed to synthesize detectable Vi antigen (Table 1). In contrast, Vi polysaccharide was detected in the cytoplasmic compartment of E. coli HB101 carrying multicopies of the viAB locus in which the tviD coding sequence had been disrupted by a transposon insertion and in which the transport system was functional (Kolyva et al., 1992; Hashimoto et al., 1993). These latter observations suggest that TviE expressed from a multicopy plasmid could initiate polymerization of the Vi polysaccharide in the absence of TviD, but that these Vi chains are not recognized by the components of the translocation system. From these data, it is tempting to speculate that the TviD protein is the endogenous lipid carrier essential for the initiation of Vi polymer synthesis by the TviE protein and for the polysaccharide translocation to the bacterial cell surface. This mechanism is still speculative and direct biochemical assays to confirm this hypothesis are presently underway in our laboratory.

Translocation of the Vi polysaccharide to the bacterial cell surface

The transport systems of group II capsular polysaccharides in Gram-negative bacteria fit the characteristics of the ABC (ATP-binding cassette) active transporters (Frosch et al., 1991; Higgins, 1992; Kroll et al., 1990). Proteins VexA, VexB, VexC and VexD encoded by the viaB locus of S. typhi shared similarities with components of ABC transporters (Hashimoto et al., 1993; Waxin et al., 1993). Indeed, non-polar mutation in vexA, vexB or vexC gene of S. typhi Ty2 caused intracellular accumulation of Vi polymer. Thus, it appears that the VexA, B and C polypeptides are components of the Vi polysaccharide export apparatus in S. typhi, and probably belong to the ABC transporter family.

In E. coli, the periplasmic KpsD protein is necessary for the cell surface expression of the K1 capsule, a group II capsular polysaccharide. A chromosomal mutation in kpsD resulted in loss of surface expression of the K1 capsule (Silver et al., 1987). Like KpsD, the VexE protein is a hydrophilic protein (Hashimoto et al., 1993) with positively charged residues throughout the sequence (unpublished data), which may be relevant to interaction with the negatively charged Vi polysaccharide. In the vexE mutant of S. typhi Ty2, the Vi polymer was detected in the intracellular compartment and in the culture supernatant, but was undetectable at the bacterial cell surface (Table 1). However, no homology was detected between KpsD and VexE proteins and the amino-terminal region of VexE did not contain a characteristic signal sequence required for secretion to the periplasmic space.

Together, these data suggest that a conserved mechanism may exist for the energy-dependent translocation of group I and group II capsular polysaccharides in Gram-negative bacteria.

Regulation of Vi antigen expression

The tviA gene of the viaB locus encodes a 21 kDa protein which shares no significant homology with available sequences in databases (Hashimoto et al., 1993; Waxin et al., 1993). Though not essential for Vi polymer synthesis (Table 1; Hashimoto et al., 1993), the TviA protein was required for full expression of Vi antigen. Indeed, disrupting the tviA coding sequence on the chromosome of S. typhi by a non-polar cassette resulted in little expression of the polysaccharide. This defect was trans-complemented when tviA was provided on multicopy plasmid (Table 1). By using lacZ transcriptional fusions, we provided evidence that the transcription of the tviA and tviB genes proceeded from a promoter located upstream of tviA coding sequence (Table 2). In addition, it was shown that tviA was expressed at a low basal level in the absence of TviA protein. Expression of tviA increased tenfold upon introduction of TviA-expressing plasmid pVT21 (Table 2). Together, these results strongly suggest that tviA and tviB are co-transcribed from a TviA-regulated promoter.

The viaA product is also required for Vi antigen expression in S. typhi (Houng et al., 1992). Since viaA from S. typhi is allelic to resB from E. coli, it is likely that Vi antigen expression is regulated at least partially by a system similar to the res regulatory system involved in colanic acid synthesis in E. coli (Gottesman & Stout, 1991; Houng et al., 1992). The 60-fold increase of β-
galactosidase activity expressed by the S. typhi T643WSR(tviB::lacZ-cat) mutant, when the E. coli rcsB gene was provided in trans on a multicopy plasmid, confirmed that RcsB or a RcsB-like protein was required for the tviAB gene transcription in S. typhi. Since the chromosomal tviA gene was shown to be functional in S. typhi T643WSR, the fact that over-expression of TviA from a multicopy plasmid restored a Vi-positive phenotype to this strain, however, suggested that a high copy number of the tviA gene could bypass the regulatory pathway involving the viaA product. However, we observed that TviA did not contain a domain with any similarity to the C-terminal DNA-binding domain of RcsB. On the basis of results presented here, it is tempting to speculate that both TviA and ViaA (or RcsB) products act as positive regulators for Vi antigen synthesis by transcriptionally activating the tviAB genes, either directly or indirectly. The precise mechanism of tviA and viaA activity in modulating Vi antigen expression remains to be elucidated. Interestingly, regions of high sequence similarity to the tviA gene of S. typhi were detected in Citrobacter strains and other Salmonella serovars expressing the Vi antigen.

Given the complexity of capsule synthesis regulation and the diversity of microenvironment encountered by S. typhi within the host, it is not surprising that various regulatory circuits up- or down-regulate expression of the virulence-associated Vi antigen. Analysis of the possible interaction between the rcs system, the ompR-envZ regulon and the TviA protein should provide new insights into the transcriptional events modulating Vi antigen expression in S. typhi.

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REFERENCES


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