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*-acetylglactosamine uronic acid variably *O*-acetylated at the C3 position (Heyns & Kiessling, 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.*, 1959; 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; Hough *et al.*, 1992), and the *ompR- ϵ WZ* two-component regulatory system (Pickard *et al.*, 1994) play an important role in the regulation of Vi antigen synthesis.

The *viaB* locus of *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 *viaB* locus of *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 *vip* (Vi polysaccharide synthesis) *A*, *B* and *C*, respectively. ORFs 6, 7, 8, 9 and 10 were named *vex* (Vi antigen export) *A*, *B*, *C*, *D* and *E*, respectively. Simultaneously, we reported the nucleotide sequence of the first six ORFs of the *S. typhi* strain Ty2 *viaB* locus which were designated *tvi* (*typhi Vi*) *A*, *B*, *C*, *D*, *E* and *F* (Waxin *et al.*, 1993). The nucleotide sequence of the first six ORFs of *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: *tviA*, *tviB*, *tviC*, *tviD*, *tviE*, *vexA*, *vexB*, *vexC*, *vexD*, *vexE* and 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 *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 *viaB* locus to Vi phenotype. For this purpose, *S. typhi* strains carrying non-polar mutations in the genes located in the *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 *S. typhi*.

METHODS

Strains, growth conditions and plasmids. *Salmonella* and *Citrobacter* strains were from the collection of the WHO collaborating centre for Reference and Research on *Salmonella* (Institut Pasteur, Paris). *S. typhi* strain Ty2 expressed the Vi antigen and possessed the O:9,12 somatic factors. *S. typhi* strain T643WSR was a *viaA* mutant which did not express the Vi antigen (Johnson *et al.*, 1966). Presence of either Vi antigen or O:9 factor on *S. typhi* strains and derivatives was assayed by

slide-agglutination with specific antisera (Diagnostics Pasteur). *E. coli* MC1061 [*araD139 D(ara-leu)-7697 rpsL galU galK D(lacIPOZY)X74*] (Casadaban & Cohen 1980) and *E. coli* HB101 [*hsdR hsdM recA13 supE44 lacZ4 leuB6 proA2 thi-1 rpsL*] (Boyer & Roulland-Dussoix, 1969) were used as recipients for cloning or complementation experiments. *E. coli* K12-NI was a spontaneous nalidixic acid-resistant mutant of *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 (Tc), 20 µg ml⁻¹.

Plasmids pUC18/19 (Ap^R; Vieira & Messing, 1982), and pGB2 (Sp^R; Churchward *et al.*, 1984) were used as cloning vehicles. Plasmid pRK2073 (Sp^R; 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 *viaB* locus of *S. typhi* Ty2 (Tc^R; Kolyva *et al.*, 1992). This plasmid conferred a Vi-positive phenotype upon *E. coli* strains. Plasmid pVT1 was used as a source of DNA for construction of recombinant plasmids presented on Fig. 1. Construction of plasmids pVT22 to pVT28 was reported previously (Kolyva *et al.*, 1992).

Plasmid pUC4K (Pharmacia) 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 (*aphΔT*), was constructed as described by Galan *et al.* (1992) and cloned into pUC18. The *aphΔT* cassette was used to introduce non-polar mutations into the *viaB* locus on plasmid pVT1 and on the chromosome of *S. typhi*. Plasmid pQF50Cm (Cm^R; 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 *SalI* restriction endonuclease. This cassette was used to study the transcription of 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 *E. coli* strains and as described by Kado & Liu (1981) for *Salmonella* strains. Plasmid transformation was performed in *E. coli* by the method of Humphreys *et al.* (1979) and in *Salmonella* by electroporation with a Bio-Rad apparatus according to the manufacturer's instructions. To study the distribution of *tviA* sequences among strains of *Salmonella* and *Citrobacter* by Southern blot analysis, an internal fragment of the *tviA* gene from *S. typhi* Ty2 was amplified by the polymerase chain reaction (PCR) with a commercial kit (Perkin-Elmer Cetus) using primers 5'-CCT CCG AAT GAT ATC TAT TTC GGG G-3' and 5'-ATC CGG CAA TAA CAG ATA GCG CGG C-3'. The probe was labelled with [α-³²P]dATP by using a random primer labelling kit (Amersham). The *tviA* gene from *S. typhi* T643WSR was cloned into vector pGB2 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 RcsB protein of *E. coli* MC1061 was constructed by using primers 5'-ATG GCA AGG CGG ATC CGC TGG CAT TCT GGC -3' and 5'-ATC CTG CTA AAG CTT TGC CGT CAA CGG ACA -3' (Stout & Gottesman, 1990). The PCR fragment was cloned into vector pGB2 and the resulting plasmid was designated pRcsB.

Construction of *S. typhi* *viaB* mutants. To study the individual contribution of each gene of the *viaB* locus to the Vi phenotype, the *aphΔT* cassette was cloned into unique sites of recombinant plasmids (from pVT22 through pVT28) presented on Fig. 1. The correct position of *aphΔT* 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-Nl as the recipient strain. Transconjugants were selected on Nl, Km 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 *aphΔT* allele on the recombinant plasmid was required to allow growth of *E. coli* K12-Nl transconjugants on Km- and Tc-containing plates. Transconjugants that had undergone correct recombination of the *aphΔT* cassette were identified by restriction analysis. Using this strategy, the *aphΔT* 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 colonies were screened for their Tc-sensitivity. Double recombination was further confirmed by Southern analysis (data not shown). The mutated allele will be hereafter referred to as *gene name::aphΔT* (e.g. *viB::aphΔT*).

The strategy described above was used to insert the *aph* cassette in the *viA* gene, and the *lacZ-cat* cassette in the *viA* or *viB* gene on the chromosome of *S. typhi*.

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 *aphΔT* cassette had been inserted in the *viB*, *viC*, *viD* or *viE* 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 *vi* genes (Fig. 1), and the presence of intracellular Vi polymer was assayed by immunoprecipitation. Providing *viB* and *viC* *in trans* on plasmid pVT37 did not lead to Vi polymer synthesis in the intracellular compartment of *E. coli* HB101. Additional presence of *viD* on plasmid pVT31 or of *viE* 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 *viB* and *viC* on plasmid pVT37 and *viD* and *viE* 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 *aphΔT* 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::aphΔT*) 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

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

<i>S. typhi</i> Ty2 mutant	Detection of Vi polysaccharide*			Agglutination by O:9 antiserum	Plasmid§ used for complementation
	Intra	Cell	Super		
<i>tvfA::aphΔT</i>	(+)	(+)	(+)	+	pVT21
<i>tvfB::aphΔT</i>	—	—	—	+	pVT29
<i>tvfC::aphΔT</i>	—	—	—	+	pVT30
<i>tvfD::aphΔT</i>	—	—	—	+	pVT31
<i>tvfE::aphΔT</i>	—	—	—	+	pVT32
<i>vexA::aphΔT</i>	+	—	—	+	pVT33
<i>vexB::aphΔT</i>	+	—	—	+	pVT34
<i>vexC::aphΔT</i>	+	—	—	+	pVT35
<i>vexE::aphΔT</i>	+	—	+	+	pVT36
ORF11::aphΔT	+	+	+	—	None

* 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.

VexE protein appeared to be required for anchoring Vi antigen at the bacterial surface.

The *S. typhi* Ty2(ORF11::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 ORF11 was not required for Vi antigen synthesis, nor for the translocation of the polymer to the cell surface.

TviA protein is required for co-transcription of *tvfA* and *tvfB* genes

The *S. typhi* Ty2 mutant in which the *aphΔT* cassette had been inserted in the *tvfA* gene was weakly agglutinated with Vi antiserum (Table 1), but unlike the parent strain Ty2, this mutant was agglutinated with O:9 antiserum indicating that the Vi polysaccharide was no longer masking the lipopolysaccharide antigen. This observation suggested that Vi antigen synthesis was decreased in the *tvfA* mutant. Indeed, as measured with the immunoprecipitation assay, the amount of Vi antigen produced by the *S. typhi* Ty2(*tvfA::aphΔT*) mutant ($1.1 \mu\text{g ml}^{-1}$) was 20-fold less than that produced by *S. typhi* Ty2 ($23.5 \mu\text{g ml}^{-1}$). Introduction of plasmid pVT21 (Fig. 1) fully restored a Vi-positive phenotype to *S. typhi* Ty2(*tvfA::aphΔT*). This construct was strongly agglutinated with Vi antiserum, but no longer with O:9 antiserum, and produced $41.5 \mu\text{g Vi antigen ml}^{-1}$. These results demonstrated that the TviA polypeptide was not essential for Vi antigen synthesis, and suggested that TviA might be involved in Vi antigen expression.

To determine the effect of TviA product on Vi antigen expression, we inserted the *lacZ-cat* cassette into the *tvfB* gene which is involved in Vi polysaccharide synthesis. β -Galactosidase activities of *tvfB::lacZ* transcriptional fusions were measured in overnight TSB cultures of *S. typhi* Ty2 mutants. Results are shown in Table 2. *S. typhi* Ty2(*tvfB::lacZ-cat*) expressed about 200 Miller units.

Expression of the *tvfB::lacZ-cat* fusion was dramatically decreased when the *tvfA* gene was disrupted by the non-polar *aphΔT* cassette. Providing *tvfA* in trans on plasmid pVT21 in *S. typhi* Ty2(*tvfA::aphΔT*, *tvfB::lacZ-cat*) fully restored expression of the fusion. This result showed that TviA polypeptide was required for *tvfB* gene transcription. In an attempt to unravel the role of TviA on *tvfB* transcription, the *tvfA* gene was disrupted by the *aph* cassette. This polar mutation was further recombined in *tvfA* on the chromosome of *S. typhi* Ty2(*tvfB::lacZ-cat*), yielding the double mutant *S. typhi* Ty2(*tvfA::aph*, *tvfB::lacZ-cat*). As expected, disruption of the *tvfA* gene by the *aph* cassette resulted in little expression of the *tvfB::lacZ-cat* fusion (Table 2). In contrast to results obtained for the *S. typhi* Ty2(*tvfA::aphΔT*, *tvfB::lacZ-cat*) mutant which carried a non-polar mutation in *tvfA*, introduction of plasmid pVT21 did not restore expression of the *tvfB::lacZ-cat* fusion in *S. typhi* Ty2(*tvfA::aph*, *tvfB::lacZ-cat*) which carried a polar mutation in *tvfA* (Table 2). Together, these results suggested that the *tvfAB* genes were co-transcribed from a TviA-inducible promoter. As a test for *tvfAB* co-transcription, plasmid pVT40 which carried the *tvfB* 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(*tvfB::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 *tvfA* and *tvfB* 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 *Bgl*II site of *tvfA* on

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 *Bgl*II 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 *aph* Δ T cassette was inserted so as to construct non-polar mutants. Only relevant restriction sites are indicated: A, *AccI*; B, *Bam*HI; Bg, *Bgl*II; C, *Cl*AI; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; N, *Nco*I; Ns, *Nsi*I; P, *Pst*I; S, *Sph*I; V, *Eco*RV.

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 *Bgl*II 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.

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 *rscB* from *E. coli* (Houng *et al.*, 1992). As expected from these data, providing *E. coli* *rscB* gene *in trans* on plasmid pRcsB restored a Vi-positive phenotype to *S. typhi* 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

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

<i>S. typhi</i> Ty2 mutant	Plasmid*	β -Galactosidase activity
<i>tviB::lacZ-cat</i>	None	228 $\pm$ 21
<i>tviB::lacZ-cat</i>	pGB2	217 $\pm$ 17
<i>tviB::lacZ-cat</i>	pVT29 (<i>tviB</i> ⁺)	201 $\pm$ 33
<i>tviA::aphΔT</i> , <i>tviB::lacZ-cat</i>	None	7 $\pm$ 1
<i>tviA::aphΔT</i> , <i>tviB::lacZ-cat</i>	pGB2	8 $\pm$ 2
<i>tviA::aphΔT</i> , <i>tviB::lacZ-cat</i>	pVT21 (<i>tviA</i> ⁺)	198 $\pm$ 10
<i>tviA::aph</i> , <i>tviB::lacZ-cat</i>	None	2 $\pm$ 1
<i>tviA::aph</i> , <i>tviB::lacZ-cat</i>	pGB2	4 $\pm$ 2
<i>tviA::aph</i> , <i>tviB::lacZ-cat</i>	pVT21 (<i>tviA</i> ⁺)	26 $\pm$ 3
<i>tviA::lacZ-cat</i>	None	56 $\pm$ 15
<i>tviA::lacZ-cat</i>	pGB2	61 $\pm$ 9
<i>tviA::lacZ-cat</i>	pVT21 (<i>tviA</i> ⁺)	556 $\pm$ 34
<i>tviA::lacZ-cat</i>	pVT42 (<i>tviA</i>)	54 $\pm$ 7

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

this transcriptional fusion was measured in overnight TSB cultures of *S. typhi* T643WSR(*tviB::lacZ-cat*). In this strain, the *tviB::lacZ-cat* fusion was expressed at a very low level (3 $\pm$ 1 Miller units). Providing the *E. coli rcsB* gene *in trans* on plasmid pRcsB increased expression of this fusion to the level measured in *S. typhi* Ty2(*tviB::lacZ-cat*), i.e. 182 $\pm$ 7 versus 228 $\pm$ 21 Miller units, respectively. To preclude the possibility that *rcsB* in multicopy on plasmid pRcsB might bypass a *tviA* mutation, we cloned the *tviA* gene of strain T643WSR to examine whether it was intact in this mutant. The resulting recombinant plasmid restored a Vi-positive phenotype to Ty2(*tviA::aph Δ T*) mutant, confirming that the *tviA* gene was functional on the chromosome of *S. typhi* T643WSR. Thus, these results demonstrated that RcsB polypeptide was involved in *tviAB* transcription.

Unexpectedly, presence of *tviA* in multicopy on plasmid pVT21 increased expression of the *tviB::lacZ-cat* fusion in the *S. typhi* T643WSR mutant (115 $\pm$ 28 Miller units). This increase suggested that the requirement for RcsB for *tviAB* co-transcription could be at least partially bypassed by *tviA* multicopies. Thus, plasmid pVT21 was transformed into *S. typhi* T643WSR. In this construct, Vi antigen was detected in the intracellular compartment, at the cell surface and in the culture supernatant. This result confirmed that TviA expressed from multicopy plasmid could bypass an *rcsB* mutation in *S. typhi*. Since RcsB was not essential when TviA was overproduced, we wondered whether TviA was essential when RcsB was produced

Fig. 2. Hybridization of the *tviA* DNA probe to total-cell DNA from *Salmonella* and *Citrobacter* strains. Lanes contain DNA digested with *EcoRI* and *ClaI* 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.

from a multicopy plasmid. For this purpose, plasmid pRcsB was introduced into *S. typhi* Ty2(*tviA::aph Δ T*). 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::aph Δ T*,*tviB::lacZ-cat*) did not increase expression of the *lacZ* fusion (4 $\pm$ 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.

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 *ClaI* 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*–*ClaI* 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

structural and biochemical properties and might therefore be considered as a group I polysaccharide. Little is known about the biosynthesis of group I polysaccharides. Production of capsular polysaccharides begins with the synthesis of sugar components which are converted to nucleotide derivatives in the cytoplasmic compartment. The subsequent polymerization is catalysed by an inner-membrane-bound transferase complex. Generally, polymerization is believed to involve a lipid carrier on which monosaccharides or oligosaccharides are assembled (Boulnois & Roberts, 1990). In addition, this lipid substitution is required for translocation of the polysaccharide to the bacterial cell surface (Frosch & Müller, 1993).

The *viaB* locus of *S. typhi* is composed of 11 genes designated *twiA-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 dinucleotide-binding site, Hashimoto *et al.* (1993) proposed that *twiB* and *twiC* 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 *twiB* and *twiC* 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 *twiD* 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 *twiD* 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 trans-

porters (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 *twiA* 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 *twiA* 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 *twiA* was provided on multicopy plasmid (Table 1). By using *lacZ* transcriptional fusions, we provided evidence that the transcription of the *twiA* and *twiB* genes proceeded from a promoter located upstream of *twiA* coding sequence (Table 2). In addition, it was shown that *twiA* was expressed at a low basal level in the absence of TviA protein. Expression of *twiA* increased tenfold upon introduction of TviA-expressing plasmid pVT21 (Table 2). Together, these results strongly suggest that *twiA* and *twiB* 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 *rcsB* from *E. coli*, it is likely that Vi antigen expression is regulated at least partially by a system similar to the *rcs* 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(*twiB::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 *twiAB* gene transcription in *S. typhi*. Since the chromosomal *twiA* 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 *twiA* 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 *twiAB* genes, either directly or indirectly. The precise mechanism of *twiA* and *viaA* activity in modulating Vi antigen expression remains to be elucidated. Interestingly, regions of high sequence similarity to the *twiA* 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*.

ACKNOWLEDGEMENTS

We thank F. Norel and R. Ferrero for critical review of this manuscript.

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Received 22 May 1995; revised 7 August 1995; accepted 14 August 1995.