The Vi antigen of *Salmonella typhi*: molecular analysis of the *viaB* locus

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Strains of *Salmonella typhi* isolated from the blood of patients with typhoid fever invariably express a capsular polysaccharide, termed the Vi antigen. Vi antigen expression is controlled by two separate chromosomal loci, *viaA* and *viaB*. The *viaA* locus is commonly found in enteric bacteria. In contrast, the *viaB* locus appears to be specific to Vi-expressing strains of *Salmonella* and *Citrobacter*. Here the cloning, expression and analysis of *viaB* determinants from *S. typhi* Ty2 is described. Whole-cell DNA from strain Ty2 was size-fractionated and cloned into the pLA2917 cosmid vector. A recombinant cosmid, pVT1, conferring a Vi-positive phenotype upon *Escherichia coli* and upon the Vi-non-expressing strain Ty21a of *S. typhi*, was characterized and used for further studies. Transposon Tn5 insertion mutagenesis demonstrated that the Vi-antigen-encoding region on pVT1 consisted of a 15 kb fragment. A subclone, designated pVT3, which contained an 18 kb insert, was sufficient to confer Vi antigen expression upon *E. coli* and *S. typhi* Ty21a. Results of recombination experiments indicated that this DNA sequence was the *viaB* locus of *S. typhi* Ty2. In *E. coli* SE5000 maxicells, the *viaB* determinants encoded at least eight polypeptides, with molecular masses of 80, 65, 59, 48, 44, 39, 35 and 28 kDa. Functional characterization of *viaB* mutations in *S. typhi* Ty2 suggested that the 80 and 65 kDa proteins were required for cell-surface localization of the Vi antigen.

Introduction

The Vi antigen, discovered by Felix & Pitt (1934), is a capsular polysaccharide found mainly in *Salmonella typhi* and *S. paratyphi* C, as well as in a few strains of *S. dublin* and *Citrobacter freundii* (Felix & Pitt, 1936; Baker *et al.* 1959). Purified Vi antigen from *S. typhi* is a linear homopolymer of α -1,4 2-deoxy-2-*N*-acetylgalacturonic acid variably *O*-acetylated at the C3 position (Heyns & Kiessling, 1967; Daniels *et al.*, 1989). Determinants of Vi antigen occupy two widely separated chromosomal loci, designated *viaA* and *viaB* (Johnson *et al.*, 1965; Snellings *et al.*, 1981). Functional *viaA* genes, located at 43 min on the chromosome of *S. typhi* (Johnson *et al.* 1966), are present not only in Vi-expressing strains of *Salmonella* and *Citrobacter*, but also in *Escherichia coli* and *S. typhimurium* (Johnson & Baron, 1969; Johnson *et al.*, 1965). The *viaB* locus, specific to Vi-expressing strains, maps adjacent to the melibiose (*mel*) and inositol (*inl*) utilization genes at 92 min on the chromosome of *S. typhi* (Johnson & Baron, 1969; Johnson *et al.*, 1965, 1966; Snellings *et al.*, 1977). In *C. freundii*, Vi-expressing strains undergo a rapid and reversible transition between

forms that express the Vi antigen and forms that do not (Snellings *et al.*, 1981). The genetic switch mechanism that controls reversible expression of Vi antigen is linked to the *viaB* locus in *Citrobacter* (Snellings *et al.*, 1981). It is now known that this reversible expression of the Vi antigen of *C. freundii* in *E. coli* is caused by specific insertion and excision of an IS1-like insertion element (Ou *et al.*, 1988). In contrast, expression of Vi antigen in *S. typhi* is stable. However, Vi-non-producing variants can be isolated from Vi-producing strains of *S. typhi* after repeated subcultures on solid medium. Reversion of such non-expressing variants to Vi-expressing forms is rarely seen (Snellings *et al.*, 1977).

The present study was undertaken to better define the *viaB* locus of *S. typhi*. The cloning, analysis and expression of the DNA sequence corresponding to the *viaB* locus of *S. typhi* are presented in this paper.

Methods

Bacterial strains and plasmids. *S. typhi* strain Ty2 was from the collection of the WHO collaborating centre for reference and research on *Salmonella* (Institut Pasteur, Paris). This strain expressed the Vi antigen and possessed the O:9,12 somatic factors. *S. typhi* strain Ty21a, a UDP-glucose 4-epimerase (*galE*) mutant of strain Ty2

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(Germanier & Fürer, 1975), possessed several additional mutations and, notably, did not synthesize Vi antigen (Cryz *et al.*, 1989). *S. typhi* Hfr strain TD7 (*cys try int*), which expressed Vi antigen, and *S. typhimurium* strain HMXSr (*his metA* Sm^R), which produced acid from inositol, were previously used by Johnson *et al.* (1965) for genetic mapping of Vi antigen determinants in *Salmonella*. *E. coli* HB101 (Boyer & Roulland-Dussoix, 1969) (*hsdR hsdM recA13 supE44 lacZ4 leuB6 proA2 thi-1 rpsL*) was used as host for cloning experiments. *E. coli* S17-1 (Simon *et al.*, 1983) (*pro thi recA hsdR* chromosomal RP4-2; Tn1::ISR1 Tc::Mu Km::Tn7) carried the transfer genes of plasmid RP4 integrated in the chromosome and allowed mobilization of cloning vectors in which the Mob (*oriT*) region of plasmid RP4 was cloned (e.g. pLA2917 and pSUP203; see below). *E. coli* S17-1::Tn5 was a mutant of *E. coli* S17-1 with transposon Tn5 inserted in the chromosome (Deneffe *et al.*, 1987). *E. coli* K12-NI was a spontaneous nalidixic acid-resistant mutant of *E. coli* C600 (Appleyard, 1954). *E. coli* SE5000 (Silhavy *et al.*, 1984) (F⁻ *araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR recA56*) was used as the source of maxicells.

The cosmid pLA2917 (Allen & Hanson, 1985) was used for gene library construction. Plasmids pSUP203 (Simon *et al.*, 1983) and pUC19 (Vieira & Messing, 1982) were used as subcloning vehicles.

Media and growth conditions. Strains were routinely grown at 37 °C in tryptic soy broth (TSB) or on tryptic soy agar (TSA; Diagnostics Pasteur). When necessary, 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; streptomycin (Sm), 500 µg ml⁻¹; and tetracycline (Tc), 20 µg ml⁻¹. Production of acid from inositol was tested in peptone water containing 1% (w/v) inositol and bromophenol blue (Diagnostics Pasteur). Antigenic formulae of *Salmonella* strains were verified by slide-agglutination in antisera specific for O somatic factors and Vi antigen (Diagnostics Pasteur).

Assays for Vi antigen. The presence of Vi antigen associated with the cell surface was determined by slide-agglutination. The presence of cell-surface-associated Vi antigen was further confirmed by testing the sensitivity of the bacteria to Vi-specific phage IV (Craigie & Felix, 1947). A drop (approximately 10⁸ phage particles) was spotted onto a TSA plate that was seeded with the bacterial culture to be tested. After overnight incubation at 37 °C, cell lysis was observed only in the case of bacteria expressing surface-associated Vi antigen.

To detect accumulation of intracellular Vi antigen or release of this polymer into culture supernatant, cells were grown in 10 ml TSB at 37 °C for 18 h with vigorous shaking. The OD₆₀₀ was recorded and adjusted to OD₆₀₀ = 4 (about 2 × 10⁹ viable cells ml⁻¹) with fresh TSB. After centrifugation (7500 g for 15 min), the supernatant was carefully collected and filter-sterilized. The bacterial pellet was washed twice in 0.85% (w/v) NaCl and suspended in 0.85% NaCl at OD₆₀₀ = 4. After disruption by sonication, unbroken cells were removed by centrifugation at 20000 g for 15 min. The resulting supernatant (sonicated extract) was filter-sterilized. The protein concentration of sonicated extract was determined using a Coomassie protein assay kit (Pierce) and the extracts were standardized with 0.85% NaCl to give 150 µg protein ml⁻¹. Presence of soluble Vi antigen in these preparations was demonstrated by immunoprecipitation using a single-diffusion method (Blair *et al.*, 1970). Briefly, in a narrow-bore test tube, 100 µl of either culture supernatant or sonicated extract was overlaid onto 100 µl agar gel containing 50 µl Vi antiserum mixed with 50 µl 0.8% (w/v) low-melting-point agarose (Appligene) in 0.85% NaCl. After overnight incubation at room temperature, formation of a visible precipitate at the interface of reactants demonstrated the presence of Vi soluble antigen in culture supernatant or sonicated extract. Using a highly purified preparation of Vi antigen (Typhim Vi; Institut Merieux, Lyon, France) as a reference standard, it was possible to detect as little as 0.2 µg soluble Vi antigen ml⁻¹.

DNA manipulation and molecular cloning. Small-scale isolation 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. For large-scale isolation, the latter technique was used. DNA was further purified by centrifugation through a caesium chloride/ethidium bromide density gradient. Plasmids and their restriction products were routinely analysed on horizontal 0.7% (w/v) agarose gels in Tris/borate buffer (89 mM-Tris base, 89 mM-boric acid, 2.5 mM-EDTA; pH 8.0).

The methods used for constructing and manipulating recombinant DNA were essentially those of Maniatis *et al.* (1982). To obtain a recombinant cosmid library, chromosomal DNA from *S. typhi* Ty2 was partially digested with the restriction endonuclease *Sau3A* and DNA fragments were separated by gel electrophoresis. Fragments with an average size of 30 kb were recovered by electroelution and ligated to the *BglII* dephosphorylated site of the cosmid pLA2917. The ligation mixture was packaged into bacteriophage λ particles and used to infect *E. coli* HB101 under conditions specified by the manufacturer (*In Vitro* Packaging System for Lambda DNA; Amersham).

Restriction endonucleases and T4 DNA ligase were purchased from Amersham and calf-intestinal alkaline phosphatase was from Boehringer Mannheim.

Tn5 transposon-insertion mutagenesis. Tn5 mutagenesis was performed as previously described (Norel *et al.*, 1989). Briefly, recombinant plasmid DNA to be tagged by Tn5 was transformed into *E. coli* S17-1::Tn5. Transformants were selected on TSA plates supplemented with appropriate antibiotics and were then mated with *E. coli* K12-NI. Transconjugants were selected and purified on TSA plates containing NI, Km and required antibiotics. Tn5-tagged derivatives were subsequently analysed with restriction endonucleases.

Genetic exchange. Plasmid transformation in *E. coli* and *Salmonella* was performed by the method of Humphreys *et al.* (1979). Large recombinant plasmids, constructed with cosmid pLA2917 and plasmid pSUP203 as cloning vectors, were introduced into *Salmonella* recipient strains by conjugation using plate matings (Willets, 1984). As a first step, recombinant DNA was introduced into *E. coli* S17-1 by transformation. The recombinant plasmid was then transferred by conjugation from *E. coli* S17-1 to *Salmonella* strains. Conjugation was carried out overnight at 37 °C.

DNA-DNA hybridization and probe preparation. DNA-DNA hybridization was performed on Hybond N membranes as recommended by the supplier (Amersham). The probe was radiolabelled with [³²P]dCTP (specific activity 3000 Ci mmol⁻¹, 111 TBq mmol⁻¹; Amersham) by using the Pharmacia oligolabelling system.

Analysis of proteins expressed in maxicells. Analysis of plasmid-encoded proteins was performed in *E. coli* SE5000, a maxicell-producing strain. Preparation of maxicells, labelling of plasmid-encoded proteins with [³⁵S]methionine (Amersham), preparation of whole-cell extracts, SDS-PAGE (final acrylamide concentration 10%, w/v), and visualization of polypeptides by fluorography were carried out as described by Silhavy *et al.* (1984). Molecular masses of proteins were estimated using ¹⁴C-labelled molecular mass standards (Amersham).

Results

Screening of recombinant cosmid library

Bacteriophage λ transducing particles carrying recombinant cosmid molecules with fragments (25–35 kb) of

Fig. 1. Physical map of plasmids pVT1 and pVT3, Tn5-insertion localization, *viaB* locus subclones and estimated positions of *viaB* genes. Plain lines represent *S. typhi* Ty2 DNA. Dashed lines indicate deleted sequences in recombinant plasmids. Circles represent Tn5 insertions: open circle, Vi-positive phenotype; filled circle, Vi-negative phenotype; hatched circle, Vi-intermediate phenotype (see text). Boxes, drawn to scale, represent estimated positions of *viaB* genes. The molecular masses (kDa) of the corresponding proteins are indicated inside the boxes. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; N, *Nco*I; P, *Pst*I. Only Hp, N and P sites used for constructing recombinant plasmids are indicated.

S. typhi Ty2 total DNA were prepared and used to transduce *E. coli* HB101. About 500 Tc-resistant and Km-sensitive transductants were screened for Vi antigen expression by slide-agglutination with Vi antiserum. Two clones were strongly agglutinated by the antiserum, indicating the presence of cell surface-associated Vi antigen. Subcultures of these two clones were lysed by the Vi-specific phage IV. Soluble Vi antigen was also found in culture supernatants and in sonicated extracts.

The two Vi-expressing clones of *E. coli* HB101 harboured recombinant plasmids designated pVT1 and pVT2. The *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI and *Hind*III restriction sites of pVT1 and pVT2 were located by single and double restriction endonuclease digestions. This analysis showed that pVT1 and pVT2 carried a 22 kb and a 20 kb DNA insert, respectively. Comparison of the physical maps of pVT1 and pVT2 showed that they shared a common DNA sequence of about 19 kb. Plasmids pVT1 and pVT2 conferred a Vi-positive phenotype upon *E. coli* K12-N1, *E. coli* S17-1, *E. coli* S17-1::Tn5 and *S. typhi* Ty21a. Plasmid pVT1 was chosen as the prototype for subsequent studies (Fig. 1).

Location of the pVT1 sequence required for Vi antigen expression in *E. coli*

To define the smallest DNA fragment able to confer a Vi-positive phenotype upon *E. coli*, transposon Tn5 was randomly inserted into plasmid pVT1 using *E. coli* S17-1::Tn5 as the delivery system and *E. coli* K12-N1 as the recipient strain. The sites of Tn5 insertions were identified by restriction endonuclease analysis of the pVT1 derivatives. The location of transposons selected for further study is indicated on the map of pVT1 in Fig. 1. Tn5-tagged plasmids will be hereafter designated by pVT1:: followed by the Tn5 insertion number (see Fig. 1). The phenotype of *E. coli* K12-N1 (pVT1::Tn5) mutants was assessed by slide-agglutination and by the Vi phage IV test. It is not known why transposon Tn5 could not be inserted in the 4.2 kb region between insertions 7 and 15.

The pVT1::Tn5 derivatives could be classified into three groups as indicated in Fig. 1. The six derivatives of the first group conserved the ability to confer a Vi-positive phenotype upon *E. coli* K12-N1 (agglutination in

Vi antiserum and lysis by phage IV). The Tn5 insertions in these derivatives were localized to two regions: from insertion 55 to the left end of the pVT1 insert, and from insertion 59 to the right end of the insert. In the second group, there were ten derivatives that had lost the capacity to confer a Vi-positive phenotype upon the host strain. These *E. coli* K12-N1 (pVT1::Tn5) mutants were not agglutinated in Vi antiserum and were resistant to lysis by Vi phage IV. This result defined two DNA sequences required for expression of Vi antigen associated with the cell surface: a 6.8 kb region between insertions 40 and 44, and a 3.8 kb region between insertions 18 and 39. The five pVT1::Tn5 derivatives of the third group contained insertions clustered within a 2.3 kb sequence (from insertions 24 to 27). They conferred an intermediate phenotype upon *E. coli* K12-N1. These mutants were partially and slowly agglutinated in Vi antiserum; but they were resistant to the lytic activity of phage IV. These results suggested that a 15 kb DNA sequence of pVT1 insert was necessary for Vi antigen expression in *E. coli* K12-N1.

The 15 kb sequence of plasmid pVT1 able to confer a Vi-positive phenotype upon *E. coli* was subcloned in vector pSUP203. Plasmid pVT1 DNA was partially digested with *Sau*3A endonuclease and ligated to the *Bam*HI dephosphorylated site of pSUP203. This mixture was used to transform *E. coli* HB101. Of 50 transformants tested, one agglutinated Vi antiserum and was lysed by Vi phage IV. This Vi-expressing clone harboured a recombinant plasmid, designated pVT3, which contained an 18 kb insert. A physical restriction map of plasmid pVT3 is presented in Fig. 1.

Construction and analysis of *viaB* mutants of *S. typhi* Ty2

To test the effect of Tn5 insertions on Vi antigen expression in *S. typhi*, insertions 59, 18, 27, 22, 13, 15, 7, 40 and 55 were recombined from plasmid pVT1 derivatives into the chromosome of strain Ty2 in the following manner. Plasmid pVT1::Tn5 DNA was transformed into *E. coli* S17-1. Transformants were selected on Tc and Km-TSA plates. The correct position of Tn5 insertion was verified by restriction endonuclease mapping. The plasmid was then transferred from *E. coli* S17-1 to *S. typhi* Ty2 by conjugation. Transconjugants, selected on Km-TSA plates, were tested for Tc-resistance. One colony resistant to Km and susceptible to Tc was selected and the correct position of Tn5 insertion was verified by DNA-DNA hybridization analysis using pVT1 as radiolabelled probe (data not shown). Loss of the cosmid vector after the recombination event was verified using pLA2917 as probe. This colony was then assayed for Vi antigen production by slide-agglutination and with Vi phage IV. These mutants will be referred to

as *S. typhi* Ty2:: followed by the Tn5 insertion number.

Results of Vi antigen detection were similar for *S. typhi* Ty2::Tn5 and *E. coli*(pVT1::Tn5) mutants, except for insertions 22 and 27 (Fig. 1). These two insertions, which conferred an intermediate Vi phenotype upon *E. coli* (pVT1::22/27), completely eliminated detection of cell-surface-associated Vi antigen in *S. typhi* Ty2::22/27 mutants (no agglutination in Vi antiserum; no lysis by Vi phage IV). Vi-negative *S. typhi* Ty2::18/27/22/13/15/7/40 mutants possessed O:9 and O:12 somatic factors, as verified by slide-agglutination in specific antisera.

The *S. typhi viaB* locus occupies a position close to determinants of inositol utilization. To confirm that the Vi antigen determinants of the pVT1 insert corresponded to the *viaB* locus, it was determined whether Tn5 insertion 13 was linked to *inl* on the chromosome of *S. typhi*. For this purpose, insertion 13 from derivative pVT1::13 was recombined into the chromosome of *S. typhi* Hfr TD7 (*Inl*⁻; Vi positive). This *S. typhi* Hfr TD7::13 mutant (*Inl*⁻; Vi negative; Km^R) was mated with *S. typhimurium* HMXSr (*Inl*⁺; Sm^R). Transconjugants were selected on Sm and Km-TSA plates. Of 100 Km^R transconjugants examined, 65 had lost the capacity to produce acid from inositol. This result indicated that the Km marker was linked to the *inl* locus and strongly suggested that insertion 13 lay within the functional region of the *viaB* locus of *S. typhi* Hfr TD7. Taken together, these data confirmed that the 15 kb sequence of pVT1 required to confer a Vi-positive phenotype to *E. coli* contained the *viaB* locus of *S. typhi* Ty2.

Identification of the products encoded by the *viaB* locus

To identify the polypeptides encoded by the *viaB* locus, plasmid pVT3 was introduced into the *E. coli* maxicell-producing strain SE5000. Protein labelling of maxicells indicated eight polypeptides with molecular masses of 80, 65, 59, 48, 44, 39, 35 and 28 kDa that were produced by SE5000(pVT3) but not by SE5000(pSUP203) (Fig. 2). Expression of proteins from various subclones of plasmid pVT3 and from Tn5 derivatives was further analysed to localize the corresponding genes (Figs 1, 2 and 3). Plasmid pUC19 was used as the subcloning vehicle.

Plasmid pVT4, carrying the central 10.7 kb *Cla*I fragment of pVT3, encoded six polypeptides, of 80, 65, 59, 44, 39 and 35 kDa (Fig. 2). Plasmid pVT6, containing the 7.2 kb *Eco*RI fragment of pVT3, encoded two polypeptides, of 48 and 80 kDa. Plasmid pVT5 expressed the 48 kDa product and plasmid pVT11, the 80 kDa polypeptide (Fig. 2). From pVT1::22 and pVT1::27, the 7.2 kb *Eco*RI fragment containing Tn5 insertion 22 or 27 (see Fig. 1) was subcloned into pUC19. The 80 kDa

Fig. 2. Proteins expressed in *E. coli* SE5000 maxicells by pSUP203 (lane 1), pVT3 (lane 2), pUC19 (lane 3), pVT4 (lane 4), pVT5 (lane 5), pVT11 (lane 6), and pVT10 (lane 7). Molecular masses of proteins (kDa) are indicated.

protein was no longer expressed by these two derivatives; it was not possible to examine production of the 48 kDa protein as several Tn5-specific polypeptides migrated in this region. The 39 and 35 kDa products were expressed by pVT10 (Figs 1 and 2). Subclones expressing only one of these two products were not obtained. Products of 65, 44 and 42 kDa were expressed by pVT7 (Fig. 3). Plasmid pVT9 encoded the 65 kDa protein (Fig. 3). This product was no longer expressed by recombinant plasmids containing the 7.7 kb *Clal*-*EcoRI* fragment tagged by Tn5 insertion 7 or 40 (see Fig. 1); expression of the 44 and 42 kDa products could not be examined as they co-migrated with Tn5-encoded polypeptides. Plasmids pVT3, 4, and 7 synthesized the 44 kDa product, whereas plasmid pVT9 did not (Figs 2 and 3). This result strongly suggested that the right-hand part of pVT7 encoded the 44 kDa protein. The 42 kDa product was not encoded by plasmids pVT3, 4 and 9 (Figs 2 and 3). This product was only visualized after expression of pVT7 in maxicells (Fig. 3). Plasmids pVT4 and pVT8 expressed a 59 kDa product (Figs 2 and 3),

Fig. 3. Proteins expressed in *E. coli* SE5000 maxicells by pSUP203 (lane 1), pVT12 (lane 2), pVT9 (lane 3), pVT7 (lane 4), pVT8 (lane 5), and pUC19 (lane 6). Molecular masses of proteins (kDa) are indicated.

whereas plasmid pVT6 did not. It was concluded that the 59 kDa protein coding sequence overlapped the *EcoRI* site located in the central region of pVT3 and pVT8. The 28 kDa polypeptide was expressed by pVT12, a derivative constructed after *Bam*HI deletion of pVT3. As the *S. typhi* DNA sequence (0.3 kb) in the right-hand part of pVT12 was not long enough to encode a 28 kDa product, it was suggested that the 28 kDa protein was encoded by the left-hand end of pVT12 (see Fig. 1).

Functional characterization of *viaB* mutations in *S. typhi* Ty2 and *E. coli*(pVT1)

To perform a functional investigation of the *viaB* locus, we examined Vi antigen production in the seven Tn5-chromosomal Vi-negative mutants of *S. typhi* Ty2 and in *S. typhi* Ty2::59, a Vi-producing mutant included as a control. The characterized mutants fell into three categories as compared with the parental strain Ty2 (Table 1). No immunoreactive Vi antigen could be detected either in sonicated extracts or in culture supernatants of *S. typhi* Ty2::13/15/18 mutants. In the second category of mutants, represented by *S. typhi* Ty2::22/27, Vi antigen was evident only in sonicated extracts. It should be noted that the amount of intracellular polymer was four times higher in these mutants than in the parental strain Ty2. Vi antigen was detected in sonicated extracts and in culture supernatants of *S. typhi* Ty2::7/40 mutants. The amount of Vi antigen that either accumulated intracellularly or was released into culture supernatants by this third category of mutants was comparable to that of *S. typhi* Ty2.

S. typhi Ty21a(pVT1), *E. coli* HB101(pVT1) and *E. coli* HB101(pVT1::59) produced four times more Vi antigen than did *S. typhi* Ty2, both in the intracellular compartment and in the culture supernatant (Table 1). This Vi overproduction might be regarded as a consequence of cloning the *viaB* determinants on the vector pLA2917. *E. coli* HB101(pVT1::13/15/18) mutants did not synthesize immunoreactive Vi antigen (Table 1). Compared with *E. coli* HB101(pVT1), strains harbouring pVT1::22/27 derivatives produced similar amounts of intracellular polymer, but released 10 times less Vi antigen (Table 1). Finally, *E. coli* HB101(pVT1::7/40) mutants still contained a low level of Vi antigen in their cellular compartment, but released twice as much immunoreactive polysaccharide as did *E. coli* HB101(pVT1).

Discussion

The objectives of this work were to define the genetic organization and the functions of the *viaB* locus of *S. typhi*. The recombinant cosmid pVT1, carrying a 22 kb insert of *S. typhi* chromosomal DNA, conferred a Vi positive phenotype upon several strains of *E. coli* and upon the Vi-non-expressing strain *S. typhi* Ty21a. Further molecular cloning experiments and Tn5 mutagenesis revealed that the genetic determinants of the *viaB* locus were located within a 15 kb DNA fragment on pVT1 and on the *S. typhi* Ty2 chromosome. By mating experiments, this sequence was confirmed to be located closely adjacent to *inl* determinants on the *S. typhi*

Table 1. Amount of Vi antigen detected in the intracellular compartment of bacteria or released into culture supernatant

Strain	Plasmid	Vi phenotype*		Vi antigen ($\mu\text{g ml}^{-1}$)	
		Aggl.	Phage IV	Intra-cellular	Super-natant
<i>S. typhi</i> Ty2	—	+	+	1.25	10
<i>S. typhi</i> Ty2::59	—	+	+	1.25	10
<i>S. typhi</i> Ty2::18	—	—	—	<0.2	<0.2
<i>S. typhi</i> Ty2::13	—	—	—	<0.2	<0.2
<i>S. typhi</i> Ty2::15	—	—	—	<0.2	<0.2
<i>S. typhi</i> Ty2::22	—	—	—	5.25	<0.2
<i>S. typhi</i> Ty2::27	—	—	—	5.0	<0.2
<i>S. typhi</i> Ty2::7	—	—	—	0.75	12.25
<i>S. typhi</i> Ty2::40	—	—	—	0.75	12.25
<i>S. typhi</i> Ty21a	—	—	—	<0.2	<0.2
<i>S. typhi</i> Ty21a	pVT1	+	+	4.75	39.75
<i>E. coli</i> HB101	pVT1	+	+	4.75	39.75
<i>E. coli</i> HB101	pVT1::59	+	+	4.75	39.75
<i>E. coli</i> HB101	pVT1::18	—	—	<0.2	<0.2
<i>E. coli</i> HB101	pVT1::13	—	—	<0.2	<0.2
<i>E. coli</i> HB101	pVT1::15	—	—	<0.2	<0.2
<i>E. coli</i> HB101	pVT1::22	+	—	4.75	4.75
<i>E. coli</i> HB101	pVT1::27	+	—	4.75	2.5
<i>E. coli</i> HB101	pVT1::7	—	—	2.5	79.0
<i>E. coli</i> HB101	pVT1::40	—	—	2.5	79.0

* Presence of cell surface-associated Vi antigen determined by agglutination (Aggl.) and by lysis with Vi-phage IV (Phage IV): +, positive reaction; —, negative reaction.

chromosome. The *viaB* locus of *C. freundii* has been cloned by Rubin *et al.* (1985). They demonstrated that a 18 kb fragment was sufficient to confer a Vi-positive phenotype upon *E. coli* HB101. Although the *viaB* locus of *S. typhi* Ty2 has already been cloned, the restriction map of the recombinant cosmid was not published and its genetic organization was not analysed further (Cryz *et al.*, 1989).

Plasmid pVT3, a subclone of plasmid pVT1 with a 18 kb insert, conferred Vi antigen expression upon *E. coli* SE5000. In this maxicell-producing system, pVT3 expressed eight polypeptides, of 80, 65, 59, 48, 44, 39, 35, and 28 kDa. After subcloning experiments, it was possible to map the coding sequences for the proteins of 80, 65, 59, 48, 44 and 28 kDa within the *viaB* locus. The 39 and 35 kDa products were expressed by pVT10, which contained a 2.5 kb *HpaI*–*PstI* fragment of the *viaB* locus (Fig. 1). Subclones expressing these two products independently were not obtained, and no Tn5 insertion in this *HpaI*–*PstI* fragment could be isolated on pVT1. Consequently, the respective positions of the DNA sequences encoding these two products could not be accurately determined. The possibilities exist that: (1) the 2.5 kb *HpaI*–*PstI* fragment encodes a protein that migrated on a 10% (w/v) SDS-polyacrylamide gel as two products of apparent molecular mass 39 and 35 kDa; (2)

the 39 and 35 kDa coding genes are co-transcribed; and (3) one of these products is required for the expression or the stability of the other. The 42 kDa product was expressed by plasmid pVT7 in maxicells, but not by plasmids pVT3, 4 and 9 (Fig. 1). This polypeptide might result from translation re-initiated at a secondary start codon or from premature termination of translation within the 44 kDa protein coding sequence. The 28 kDa product did not appear to be associated with Vi antigen synthesis, as insertion 55, located by restriction mapping in this protein-coding sequence (Fig. 1), did not affect Vi expression in *S. typhi* Ty2::55 or in *E. coli*(pVT1::55). Nevertheless, it was not possible to examine expression of the 28 kDa polypeptide using a subclone with this Tn5 insertion as this protein co-migrated with the Km-resistance gene product from Tn5.

The mode of biosynthesis and biogenesis of the Vi antigen remain unknown. In order to address this problem, representative Tn5 mutants were studied further. *S. typhi* Ty2::22/27 mutants which did not express the 80 kDa polypeptide accumulated intracellular Vi antigen but were devoid of capsular polysaccharide at the cell surface and did not release antigen into culture supernatant. We propose that in *S. typhi*, the 80 kDa protein might act in the transport of the polysaccharide chains across the cytoplasmic membrane to the cell surface or in the maturation of the intracellular Vi antigen. In contrast, insertion 22/27 on pVT1 in *E. coli* did not abolish expression of the polymer at the cell surface or its release into the culture supernatant. However, these mutants released four times less Vi antigen than *E. coli*(pVT1). The fact that *E. coli*(pVT1::22/27) mutants were not lysed by the Vi-specific phage IV indicated that the Vi antigen evidenced by agglutination was incapable of acting as a Vi phage IV receptor and for this reason might not be cell-associated. It was inferred that insertion 22 or 27 on plasmid pVT1 in *E. coli* resulted in synthesis of only intracellular Vi antigen and might have caused polymer leakage by a passive and non-specific process. In *E. coli*(pVT1::7/40) and *S. typhi* Ty2::7/40 mutants, Vi antigen was detected in the cytoplasm and in the culture supernatant but not at the cell surface. Insertion 7/40 resulted in a decrease of Vi antigen accumulated within the cell and in an increase of Vi antigen released into supernatants when compared to parental strains (Table 1). These mutants did not express the 65 kDa polypeptide. We suggest that the 65 kDa product may be a protein necessary for cell-surface localization of the Vi antigen. Studies are under way in our laboratory to further define the role of the 80 and 65 kDa proteins and to identify other *viaB*-encoded functions pertinent to assembly, translocation and regulation mechanisms.

Like *S. typhi*, many invasive organisms possess a

capsular polysaccharide that interferes with the host's specific and non-specific defences. These surface antigens are usually of high molecular mass and comprise predominantly hexosaminuronic acids. Such polysaccharides have been found in *E. coli* strains expressing K antigen, *Haemophilus influenzae* types b, d and e, *Staphylococcus aureus* strain Smith and *Streptococcus pneumoniae* (Williamson & Zamenhof, 1963; Liao *et al.*, 1974; Robbins *et al.*, 1974; Leontin *et al.*, 1983; Boulnois & Jann, 1989). Capsule or capsule-like structures are considered a major virulence factor of these bacteria (Robbins & Robbins, 1984). Nevertheless the relationship of these capsular polymers to bacterial invasiveness remains an unresolved problem. Further analysis should provide new insights into the specific virulence-associated functions of capsular-polysaccharide antigens. In that light, the results presented in this study may help to focus attention for further research on the pathogenic role of *S. typhi* Vi antigen.

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