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

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**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 & Kiesling, 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
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 m\text{M} Tris base, 89 m\text{M} boric acid, 2-5 m\text{M} 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 cosmid pLA2917. The ligation mixture was packaged into bacteriophage \( \lambda \) 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. Transformants were selected on TSA plates supplemented with appropriate antibiotics and were then mated with E. coli K12-N1. 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 (Willetts, 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 \(^{32}\text{P}\) 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 \(^{35}\text{S}\) 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 \(^{14}\text{C}\)-labelled molecular mass standards (Amersham).

**Results**

**Screening of recombinant cosmid library**

Bacteriophage \( \lambda \) transducing particles carrying recombinant cosmid molecules with fragments (25–35 kb) of
The viaB locus of S. typhi

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 BamHI, BglII, Clai, EcoRI and HindIII 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 Sau3A endonuclease and ligated to the BamHI 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 in1 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; Km8) was mated with S. typhimurium HMXSr (Inl+; Sm8). Transconjugants were selected on Sm and Km-TSA plates. Of 100 Km8 transconjugants examined, 65 had lost the capacity to produce acid from inositol. This result indicated that the Km marker was linked to the in1 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 SES000(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 fragment of pVT3, encoded six polypeptides, of 80, 65, 59, 44, 39 and 35 kDa (Fig. 2). Plasmid pVT6, containing the 7.2 kb EcoRI fragment of pVT3, encoded two polypeptides, of 48 and 80 kDa. Plasmid pVT5 expressed the 48 kDa product and plasmid pVT1, the 80 kDa polypeptide (Fig. 2). From pVT1::22 and pVT1::27, the 7-2 kb EcoRI fragment containing Tn5 insertion 22 or 27 (see Fig. 1) was subcloned into pUC19. The 80 kDa
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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 ClaI–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), 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 BamHI 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 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)

<table>
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<th>Strain</th>
<th>Plasmid</th>
<th>Aggl.</th>
<th>Phage IV</th>
<th>Intra-</th>
<th>Super-</th>
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<td>-</td>
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<td>+</td>
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<td>10</td>
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<tr>
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* Presence of cell surface-associated Vi antigen determined by agglutination (Aggl.) and by lysis with Vi-phage IV (Phage IV): +, positive reaction; -, negative reaction.
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; Liu et al., 1974; Robbins et al., 1974; Leontein et al., 1983; Boulois & 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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References


