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Expression of Vi Antigen in *Escherichia coli* K-12: Characterization of ViaB from *Citrobacter freundii* and Identity of ViaA with RcsB

HUO-SHU H. HOUNG,[1]* KERRY F. NOON,[1] JONATHAN T. OU,[2] AND LOUIS S. BARON[1]

Department of Bacterial Immunology, Walter Reed Army Institute of Research, Washington, D.C. 20307,[1] and Department of Microbiology and Immunology, Chang Gung Medical College, Taoyuan, Taiwan, Republic of China[2]

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The Vi antigen in *Salmonella typhi* is stably expressed and may act to protect the strain against the defensive system of the host. *Citrobacter freundii*, not usually a common human pathogen, also expresses the Vi antigen but expresses it unstably, exhibiting a reversible transition between the Vi^+ and Vi^- states. Two widely separated chromosomal regions, ViaA and ViaB, are needed for Vi synthesis. *Escherichia coli* K-12 harboring a functional ViaB plasmid can also express Vi antigen, but the cloned ViaB sequence can only be stably maintained and expressed in *recA* hosts. Vi^- derivatives arise either through IS*1*-like insertional events occurring in ViaB sequences or by chromosomal mutations at the ViaA region. P1*vir* mapping indicates that the ViaA mutations are located at min 47.75 on the *E. coli* chromosome. All the spontaneous *viaA* mutants isolated from *E. coli* and *S. typhi* were identified as *rcsB* mutants by complementation tests using plasmid pJB100. Introduction of *rcsA*::Tn*10* into *E. coli* harboring functional ViaB sequences eliminates the expression of Vi antigen. These results indicate that Vi antigen synthesis is regulated by the same regulatory proteins involved in colanic acid synthesis in *E. coli*.

The virulence (Vi) antigen of *Salmonella typhi*, first described by Felix and Pitt in 1934, is a capsular surface antigen consisting of a homopolymer of galactosaminuronic acid (7, 9). All strains of *S. typhi* and *Salmonella paratyphi* C, as well as some strains of *Citrobacter* and a few strains of *Salmonella dublin*, are capable of synthesizing Vi antigen (19, 22, 23, 31). The Vi antigen in *S. typhi* is stably expressed and may act to protect the strain against the defensive system of the host by shielding bacteria from the host immune system. In certain strains of *Citrobacter*, the Vi antigen is expressed in an unstable state characterized by a reversible transition between cells exhibiting full Vi antigen expression and cells that appear not to produce the Vi antigen (2, 31). The actual mechanism that controls Vi antigen reversible expression in *Citrobacter freundii* is still unknown.

Two widely separated chromosomal regions necessary for Vi antigen expression, ViaA and ViaB, have been identified in *S. typhi* and *C. freundii* through conjugal mating experiments (18, 19, 20). Although *Escherichia coli* K-12 and *Salmonella typhimurium* do not express Vi antigen, both strains are capable of expressing this antigen after acquisition of the ViaB region from either *S. typhi* or *C. freundii* (18, 30). This result indicated that both *E. coli* K-12 and *S. typhimurium* possess a native, functional ViaA determinant. The ViaB region of *C. freundii* has been previously cloned and results in expression of the Vi antigen in HB101 (27, 28). In HB101 harboring this ViaB plasmid, loss of Vi expression occurs at much lower frequencies and is due in most instances to IS*1*-like insertional events occurring in several clustered regions of the ViaB sequences (27).

We have performed a detailed mapping of the ViaB region and characterized the locations of several of these insertion sites. Loss of Vi expression in HB101 can also be due to chromosomal *viaA* mutations. On the basis of this fact, *viaA* mutants were isolated in HB101 and were used in genetic mapping studies designed to identify and characterize the ViaA region.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this study are listed in Table 1. Plasmid pRK290 coding for kanamycin resistance is a broad-host-range vector that was employed to construct the recombinant plasmid, pWR127, which codes for the functional ViaB sequence of *C. freundii* (27, 28). Vi^+ and Vi^- bacterial colonies were readily identified visually with a dissecting microscope by using oblique lighting (22). Vi^+ colonies appear as bright, orange-hued, and dense, whereas Vi^- colonies exhibit an opaque grayish appearance. For further confirmation, slide agglutination tests were performed with rabbit Vi-specific antiserum (19).

Media. All the bacterial strains were routinely maintained on nutrient agar (Difco Laboratories, Detroit, Mich.) and were grown in Penassay broth (Difco Laboratories). Appropriate concentrations of antibiotics were added to media to maintain the presence of plasmids (24).

Plasmid isolation and transformation. Plasmid DNA was isolated by an alkaline lysis method described by Birnboim and Doly (3) and further purified on a cesium chloride density gradient (24). Mid-log-phase bacterial cultures were induced to competence through $CaCl_2$ treatment (24).

Plasmid curing. The plasmid was cured from its host by inoculation of 0.1 ml of an overnight culture into 50 ml of fresh nutrient broth without any antibiotic selection and daily transfer for 3 to 5 days. The plasmid-cured isolates could be easily identified by screening for antibiotic sensitive colonies.

P1 transduction. P1*vir* phage was harvested from soft-agar

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype(s)	Source or reference(s)
Strains		
E. coli HB101	F⁻ *hsdS20*(r_B^- m_B^+) *recA13 ara-14 proA2 leuB6 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 thi-1 supE44* λ^-	24
E. coli HB101-derived strains		
WR3011	*viaA recA13*, pWR127	This study
WR3012	*viaA recA*⁺, pWR127	This study
E. coli C600	F⁻ *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* λ^-	24
E. coli C600-derived strains		
WR3013	*srlR301*::Tn*10 recA35*	This study
WR3014	*rcsB 11*::Tn*10*	This study
WR3015	*rcsA 72*::Tn*10*	This study
E. coli DH5α	F⁻ φ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR supE44 thi-1* λ^- *gyrA96 relA1*	Bethesda Research Laboratories
E. coli JC10285	*thr-1 leuB86 proA2 his-4 argE3 thi-1 mtl-1 ara-14 lacY1 galK2 xyl-5 rpsL31 supE44 srlR*::Tn*10 recA35*	5
E. coli SA2529	*srl*::Tn*10 recA*⁺	S. Adhya
E. coli SG20797	*cpsB10*::*lac*-Mu-*imm*λ Δ*lon-510 rcsB11*::ΔTn*10*	S. Gottesman
E. coli ATC5112	*cps-11*::*lac*-Mu dl *rcsA72*::ΔTn*10* Δ*lon-100*	S. Gottesman
E. coli CAG12098	MG1655, *zeg-722*::Tn*10*	29
E. coli CAG12178	MG1655, *zei-723*::Tn*10*	29
E. coli CS1230	*ompC161 gyrA261 zeh-298*::Tn*10*	29
E. coli LS6917	*his hadR16 fadA30 zeh*::Tn*10* 80% cotransducible with *atoCAB*⁺	15
E. coli LS6919	*his fadR16 fadA30 zeh*::Tn*10* 84% cotransducible with *atoCAB*⁺	15
S. typhi WR4205	*cys trp viaA*	20
S. typhi Ty21a	*galE viaB*	10
Plasmids		
pWR127	viaB⁺ Kan^r	28
pWR150	*viaB*⁺ *bla*⁺	This study
pWR151	*viaB*⁺ *bla*⁺	This study
pWR152	*viaB bla*⁺	This study
pWR153	*viaB bla*⁺	This study
pJB100	*rcsB*⁺ *bla*⁺	4, 32

plates with *E. coli* strains containing Tn*10* transposons at the desired locations for preparation of the lysates (26, 29). The phages obtained were then used to transduce the Tn*10* marker into the recipient strains according to the procedures described by Miller (26).

RESULTS

Restriction analysis and mapping of the *viaB* determinant of *C. freundii*. Plasmid pWR127 was constructed by ligating two contiguous *Eco*RI fragments, *Eco*A (8.5 kb) and *Eco*B (9.8 kb), coding for the functional *viaB* determinants of *C. freundii* into the *Eco*RI site of pRK290 (8, 28). This plasmid can stably express the Vi antigen in *E. coli* HB101 and was subjected to detailed restriction endonuclease analysis. The results of these experiments are shown in Fig. 1. Subcloning experiments were conducted to determine the minimal nucleotides required to code for the functional *viaB* loci. An approximately 2.0-kb *Eco*RI-*Bam*HI fragment was deleted from the insert of pWR150 through partial *Bam*HI digestion followed by T4 DNA ligation (shown in Fig. 1). The resultant plasmid, pWR151, still possesses functional *viaB* determinants but contains a 2.4-kb *Bam*HI deletion of the parental pWR150 DNA, which has an additional *Bam*HI site at 375 bp from the *Eco*RI site (within the cloning vector, pBR322). No other *Bam*HI deletion plasmids smaller than pWR151 which can code for functional *viaB* in the HB101 host were identified. This indicates that the other *Bam*HI sites are located in the essential coding region of ViaB. Similar deletions were also generated by using *Mlu*I and *Pvu*I endonucleases. However, the *Mlu*I and *Pvu*I deletions shown in Fig. 1 eliminated Vi antigen expression. The cloning vector of

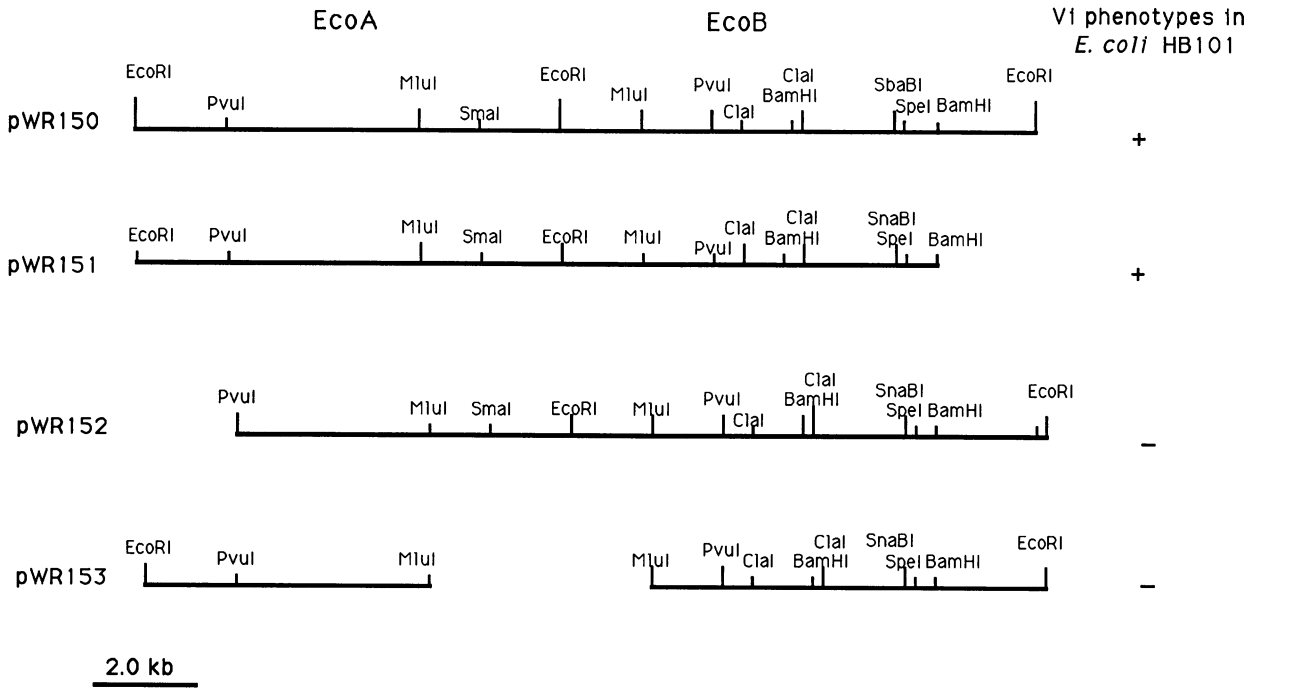

FIG. 1. Restriction map for the cloned ViaB-coding region of *C. freundii*. Two contiguous *Eco*RI inserts, 8.5-kb *Eco*A and 9.8-kb *Eco*B, of pWR127 are shown. The same *Eco*RI inserts of pWR127 were also cloned into the *Eco*RI site of pBR322. The resultant plasmid, pWR150, was subjected to partial or complete endonuclease digestions (*Bam*HI, *Pvu*I, and *Mlu*I) followed by T4 DNA ligase incubation to generate pWR151, pWR152, and pWR153 plasmids, as shown.

pWR152, pBR322, also contains a unique *Pvu*I site. The actual *Pvu*I deletion of pWR152 is shown as an *Eco*RI-*Pvu*I deletion in Fig. 1. It was determined that at least 15 kb of nucleotides is required to code for the intact *viaB* loci to express the Vi antigen in HB101.

Stability of recombinant plasmids coding for ViaB is affected by *recA*. It was found that pWR127 could stably express the Vi antigen in *E. coli* HB101, a *recA* mutant. However, Vi expression of pWR127 was extremely unstable in *recA*$^+$ strains, such as *E. coli* C600 and *S. typhi* Ty21a (10, 11), a *viaB* mutant. All Vi$^+$ transformants in a *recA*$^+$ background rapidly lose the Vi phenotype after being restreaked on antibiotic selection media. None of the Vi$^+$ colonies maintains a homogenous Vi$^+$ population. Since no pure or homogenous Vi$^+$ colony in a *recA*$^+$ background is available for use as a seed culture, it is not possible to calculate the actual percent loss of Vi expression per generation. Examination of the plasmid DNA established that a large portion of ViaB-encoding sequences was deleted from these Vi$^-$ isolates. We also examined Vi expression of pWR127 in a pair of isogenic strains of *E. coli* DH5α (obtained from Bethesda Research Laboratories, Gaithersburg, Md.) whose only difference is in their *recA* genotypes, one being *recA*$^+$ and the other being a *recA* mutant. It was found that the Vi antigen was stably expressed in the *recA* host but was unstable in the *recA*$^+$ background. In Vi$^-$ isolates from the *recA*$^+$ host, most of the ViaB-coding sequences of pWR127 were deleted. It was further demonstrated that once a *recA* mutation was introduced into the C600 host, by using P1*vir* phages derived from *E. coli* JC10285 *srlR*301::Tn*10* (5, 6), the resulting *recA* transductant, WR3013, could stably express the Vi antigen encoded by pWR127.

Isolation and mapping of IS*1*-like insertions that inactivate the cloned ViaB coding sequences for Vi antigen expression. Although HB101 harboring pWR127 stably expresses the Vi antigen, it is possible to isolate Vi$^-$ colonies of HB101 containing pWR127 by inoculating 0.1 ml of an overnight culture into 50 ml of fresh nutrient broth supplemented with 50 μg of kanamycin per ml and transferred daily for 3 to 4 days. The frequency of isolation of Vi$^-$ mutants is approximately 10^{-3} to 10^{-4}. Plasmids isolated from most of the Vi$^-$ isolates showed slower mobilities compared with those of the parental pWR127. It was found that those plasmids contained extra IS*1*-like inserts, estimated at approximately 700 to 800 bp, within the ViaB-coding sequences (27). The sites of several of these inserts were mapped through endonuclease digestion analyses and were found to be scattered over several specific ViaB-coding regions, as shown in Fig. 2.

Isolation and identification of *viaA* mutants of *E. coli* HB101. Among the Vi$^-$ transformants of HB101 harboring pWR127, 10 to 15% of these isolates were found to contain plasmids that had mobilities identical to those of the parental pWR127. These plasmids were subsequently found to be capable of conferring Vi antigen expression when they were retransformed into competent HB101 cells. Evidently, these Vi$^-$ isolates must have undergone chromosomal mutations that impair their ability to express Vi antigen. The presence of such mutations was further demonstrated by curing the plasmid content of these Vi$^-$ isolates and retransforming them with parental pWR127. The resultant plasmid-cured strains did not express the Vi antigen when they acquired pWR127 through transformation.

Mapping of the *viaA* allele of *E. coli* HB101. The *viaA* determinant(s) present in *S. typhi* was tentatively mapped

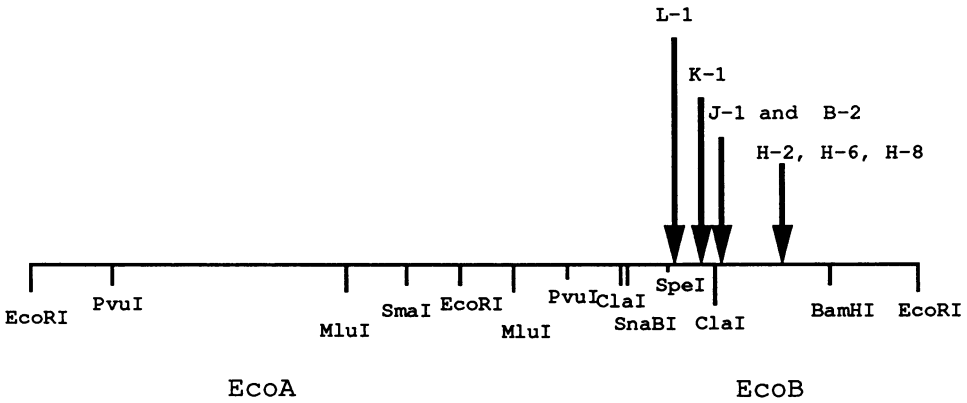

FIG. 2. IS*1*-like insertion events in the ViaB-coding region of pWR127. WR3011 can stably express the Vi antigen of *C. freundii*. Loss of Vi expression occurs at very low frequencies because of either IS*1*-like insertional events or chromosomal mutations. It was found that the IS*1*-like insertions are confined to four separate clustered regions (H-2, H-6, and H-8; J-1 and B-2; K-1; and L-1) of the cloned ViaB region, as shown by the arrows.

near the histidine biosynthesis region in Hfr conjugation studies (18, 19, 20). Twelve independent *viaA* mutants were isolated and identified among the Vi$^-$ isolates of HB101 harboring pWR127 by using the procedure described in the previous section. One of these mutants was designated WR3011 for the mapping experiments. We employed a collection of *E. coli* strains containing Tn*10* transposon elements located in the vicinity of the *his* region as donor strains for transductional mapping experiments to determine the exact location of the *viaA* mutations (15–17, 29). To conduct these experiments, WR3011 had to be converted from the *recA* mutant state back to the *recA*$^+$ state. P1*vir* phage isolated from *E. coli* SA2529 *srl*::Tn*10* *recA*$^+$ was employed to transduce the linked Tn*10* and *recA*$^+$ to the HB101 recipient (6, 25). The Tn*10* marker of the resultant transductant was eliminated by second-stage P1 transduction to replace *srl*::Tn*10* with the *srl*$^+$ marker (6, 25). The resulting *recA*$^+$ version of WR3011 was designated WR3012. Various P1*vir* lysates derived from hosts containing Tn*10* transposons at chromosomal regions from min 44 to 49 were employed to transduce the Tn*10* marker to WR3012 (1, 15–17, 29). The ratios of Vi positive transductants to total tetracycline-resistant transductants are expressed as percentages of the cotransduction rate of the Tn*10* site and the ViaA region. The results of the transduction experiments are shown in Table 2. It was found that the ViaA region is closely linked, at almost 100%, to min 47.75 on the chromosomal map of *E. coli* K-12. P1*vir* lysates derived from strains CAG12098 and CAG12178 yielded cotransduction rates which fall below 50% because these two strains have their Tn*10* insertions located at min 46.5 and 48.5, respectively (26). The linkage map of *E. coli* shows that the *ato* operon is located very close to min 47.75 (1). Strains LS6917, LS6919, and LS6920 with their Tn*10* insertions located in the vicinity of the *ato* operon (80 to 90% cotransducing frequencies with the *ato* operon) are 50 to 92% cotransducible with the ViaA region (15–17).

TABLE 2. P1*vir* transduction of WR3012[a]

Strain	Tn*10* location (min)	Cotransduction rate (%)	Reference
CAG12098	46.50	43.0	29
CS1230	47.75	99.9	29
LS6920	47.80[b]	92.0	15
LS6917	47.80[b]	93.0	15
LS6919	48.20[b]	50.0	15
CAG12178	48.50	21.0	29

[a] P1*vir* lysates derived from various *E. coli* strains containing Tn*10* insertions at known chromosomal locations were employed to transduce WR3012. Tetracycline-resistant transductants were examined for their Vi phenotypes. The ratios of Vi$^+$ colonies to the total tetracycline-resistant colonies were expressed as the cotransduction rate between the ViaA-coding region and the Tn*10* insertion sites.

[b] The Tn*10* location was estimated on the basis of the cotransduction rates with the *ato* operon, which is located near min 47.80 (1).

ViaA is identical to the positive regulator for capsule synthesis RcsB in *E. coli* HB101. The results of the P1 mapping experiments demonstrated that the *viaA* mutations are located at min 47.75. The K-12 linkage map shows that the linked *rcsB* and *rcsC* genes map near min 48 of the K-12 chromosome. It is known that capsular polysaccharide synthesis of *E. coli* K-12 is under the control of two positive regulators encoded by the *rcsA* and *rcsB* genes (12, 13, 32, 33). Thus, it seemed very likely that *viaA* was, in fact, identical to either *rcsA* or *rcsB*. To test this hypothesis, we used plasmid pJB100, which codes for the functional *rcsB* locus and ampicillin resistance (4, 32). We found that WR3011 was capable of expressing Vi antigen when it acquired the pJB100 plasmid through transformation. We also examined the other 11 individual *viaA* mutants isolated in this study and found that all of them could also be complemented by pJB100. It was further demonstrated that P1*vir* prepared with SG20797 *rcsB*::Tn*10* could transduce Tn*10* into the homologous *rcsB* sequence of C600 containing pWR127 and abolish its ability to express the Vi antigen (4, 32).

The *viaA* mutation of *S. typhi* WR4205 can be complemented by the RcsB locus of pJB100. Johnson et al. (18, 20) isolated a Vi$^-$ mutant of *S. typhi* WR4205 and classified it as a *viaA* mutant on the basis of Hfr mating experiments. This mutant of *S. typhi* WR4205 was transformed with pJB100 to determine whether it was mutated in the *rcsB* locus. All the transformants of *S. typhi* WR4205 were found to express the Vi antigen. This result indicated that the mutation of WR4205 can be complemented by pJB100, and it is therefore an *rcsB* mutant.

The RcsA locus is also essential for Vi expression in *E. coli* HB101. All the *viaA* mutants in this study were found to be

rcsB mutants through complementation tests. We also prepared a P1*vir* lysate of *E. coli* ATC5112 *rcsA*::Tn*10* to transduce the *rcsA* mutation into WR3012 (4). It was found that all the *rcsA*::Tn*10* transductants harboring pWR127 plasmid could no longer express the Vi antigen.

DISCUSSION

In this study, we characterized the ViaB-coding region of *C. freundii* using *E. coli* HB101 as the cloning host. On the basis of restriction analysis and subcloning of pWR127 coding for the functional ViaB sequences, it was determined that at least 15 kb of contiguous nucleotides is required to express the Vi antigen in *E. coli* HB101. The cloned ViaB sequences could stably express the Vi antigen in *recA* mutant hosts, such as *E. coli* HB101. The isolation of Vi^- colonies of *E. coli* HB101 containing pWR127 occurs at a frequency lower than 10^{-3}. This does not reflect the true transition frequency of the Vi antigen in *C. freundii*, which ranges from 20 to 50%. Furthermore, the transition of the Vi antigen in *C. freundii* is reversible, whereas Vi expression in *E. coli* HB101 harboring pWR127 is irreversibly shut off by IS*1*-like insertional events occurring at several coding regions of the *viaB* sequences. Vi antigen expression encoded by pWR127 is extremely unstable in $recA^+$ strains because of deletion of the ViaB-coding region or loss of the entire plasmid. On the basis of the differences in transition frequency, transition reversibility, and dependence on the *recA* mutation to stabilize Vi expression of pWR127, it is unlikely that the mechanism of IS*1*-like insertion in pWR127 is the same mechanism that causes Vi transitions in *C. freundii*.

The ViaB region of *S. typhi* was recently cloned and characterized (14, 21). It was shown that the ViaB of *S. typhi* was also encoded by at least 14- or 15-kb nucleotides. This is approximately the same size as the ViaB-coding region of *C. freundii*, as defined in this study. The restriction maps of the ViaB regions for these two organisms are similar but not identical.

Using *lac* fusions to the *cps* genes, Trisler and Gottesman (34) demonstrated that there is transcriptional regulation of a group of genes encoding enzymes necessary for capsule synthesis. At least four *trans*-acting regulatory genes play a role in setting the level of transcription of the *cps* genes. Secondary mutations which reduce capsule synthesis in *lon* mutant hosts by decreasing transcription of the *cps* genes were identified in two genes, *rcsA* and *rcsB*. It was found that *rcsB*, a positive regulator for capsule synthesis, is closely linked with *rcsC* (32). Genetic data and sequence analyses suggest that *rcsB* and *rcsC* constitute a two-component regulatory pair. The predicted amino acid sequence of *rcsC* suggests that it produces an inner membrane protein with protein kinase activity, and its target is likely to be RcsB. The phosphorylated form of RcsB would then act as a positive regulator for capsule synthesis (12). In this study, we found that the regulation of Vi expression in *E. coli* employs the same regulatory circuit as is used for capsule synthesis in *E. coli*. Two separate loci, *rcsA* and *rcsB* located at min 43 and 48, respectively, are essential for Vi antigen expression in *E. coli* (4, 13). All of the *viaA* mutants isolated in this study were found to be *rcsB* mutants. The *rcsB* chromosomal mutants in this study evolved spontaneously and were identified as Vi^- colonies. It is possible that the *rcsA* mutations are leaky, a fact which would allow identification of only *rcsB* mutations. We do not know the exact mechanism, i.e., deletion, or insertional events that give rise to the *rcsB* mutations identified in this study.

RcsA seems to act to stimulate RcsB-dependent transcription and is necessary for maximal expression of *cps* genes, but it is not absolutely essential for low levels of capsule expression (4). When the *rcsA*::Tn*10* element was transduced into *E. coli* strains harboring pWR127, none of the transductants produced a quantity of the Vi antigen large enough to be detected by the gross morphological changes used in this study.

Johnson et al. isolated a *viaA* mutant of *S. typhi* WR4205 which does not express Vi antigen even though it carries functional ViaB-coding sequences on its chromosome (18, 19). Previous conjugal mating experiments with *S. typhi* did not permit a discrete mapping study of the ViaA region. In this study, all of the mutants isolated in *E. coli* HB101 were identified as *rcsB* mutants by transformation and P1 transduction experiments. With this information, we were able to show that the original *viaA* mutant *S. typhi* WR4205 is an *rcsB* mutant because it can be complemented by plasmid pJB100 coding for the *rcsB* locus. We thus demonstrated that both *E. coli* and *S. typhi* employ the *rcs* regulatory system to express Vi antigen. This finding also allows us to differentiate Vi^- mutations by a simple complementation test using plasmids pJB100 and pWR127.

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