

## Thin Pilus PilV Adhesins of Plasmid R64 Recognize Specific Structures of the Lipopolysaccharide Molecules of Recipient Cells

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**Inc11 plasmid R64 encodes a type IV pilus called a thin pilus, which includes PilV adhesins. Seven different sequences for the C-terminal segments of PilV adhesins can be produced by shufflon DNA rearrangement. The expression of the seven PilV adhesins determines the recipient specificity in liquid matings of plasmid R64. *Salmonella enterica* serovar Typhimurium LT2 was recognized by the PilVA' and PilVB' adhesins, while *Escherichia coli* K-12 was recognized by the PilVA', PilVC, and PilVC' adhesins. Lipopolysaccharide (LPS) on the surfaces of recipient cells was previously shown to be the specific receptor for the seven PilV adhesins. To identify the specific receptor structures of LPS for various PilV adhesins, R64 liquid matings were carried out with recipient cells consisting of various *S. enterica* serovar Typhimurium LT2 and *E. coli* K-12 *waa* mutants and their derivatives carrying various *waa* genes of different origins. From the mating experiments, including inhibition experiments, we propose that the GlcNAc( $\alpha$ 1-2)Glc and Glc( $\alpha$ 1-2)Gal structures of the LPS core of *S. enterica* serovar Typhimurium LT2 function as receptors for the PilVB' and PilVC' adhesins, respectively, while the PilVC' receptor in the wild-type LT2 LPS core may be masked. We further propose that the GlcNAc( $\beta$ 1-7)Hep and Glc( $\alpha$ 1-2)Glc structures of the LPS core of *E. coli* K-12 function as receptors for the PilVC and PilVC' adhesins, respectively.**

Bacterial adhesins are usually located at the tip of pili in gram-negative bacteria (7). Adhesins play key roles in the attachment of bacterial pathogens to host eukaryotic cells. They recognize and bind to specific receptors present on the host cells. For example, the PapG adhesin of P pili specifically binds to the Gal( $\alpha$ 1-4)Gal moiety of glycolipids on the host cells.

Plasmid R64 encodes type IV pili, called thin flexible pili (9, 30). The PilV adhesins are thought to be located at the tips of the thin pili. They are unusual among bacterial adhesins, since the C-terminal segments of the PilV adhesins are exchanged by multiple DNA inversions of the shufflon (3, 11, 15) (Fig. 1A). The R64 shufflon consists of four DNA segments, which are separated and flanked by seven *sfx* sequences. Site-specific recombination is mediated by the *rci* product and occurs between any two inverted *sfx* sequences, resulting in the inversion of the four DNA segments independently or in groups. The sequence of the *pilV* gene encoding the seven C-terminal segments is joined in frame to the constant region of the *pilV* gene. Thus, the C-terminal segments of the PilV adhesins interconvert among seven types as a result of the shufflon DNA inversions. The constant region of a PilV adhesin consists of 361 amino acids and bears a structure similar to that of a type IV prepilin at the N terminus. The variable regions range between 69 and 113 amino acids, and no remarkable homology is found among them. To identify the biological significance of the shufflon, R64 derivatives in which the *pilV* gene encoding the seven C-terminal segments was fixed were constructed by in-

activation of the *rci* gene (12). The seven PilV adhesins in donor cells were shown to determine the recipient specificity in R64 liquid matings (13, 14). The recipient *Escherichia coli* K-12 strain was recognized by the PilVA', -C, and -C' adhesins in liquid matings, while *E. coli* B was recognized only by the PilVA' adhesin. *Salmonella enterica* serovar Typhimurium LT2, *E. coli* C, and *Shigella flexneri* were recognized by the PilVA' and -B', PilVA and -A', and PilVA' and -D' adhesins, respectively.

To identify the specific PilV receptors in recipient bacterial cells, we previously performed R64 liquid matings with various *E. coli* K-12 *waa* mutants and *E. coli* B derivatives carrying the *waa* genes of *E. coli* K-12 as recipient cells (8). The *E. coli* K-12 *waa* mutants had lost the receptors for specific PilV adhesins. *E. coli* B cells carrying the *waaR* gene or *waaRUL* genes of *E. coli* K-12 were recognized by the PilVC' adhesin or the PilVC and PilVC' adhesins, respectively, in addition to the PilVA' adhesin. Addition of *E. coli* K-12 or B lipopolysaccharide (LPS) to R64 mating mixtures specifically inhibited liquid matings with *E. coli* K-12 or B recipients, respectively. Thus, it is suggested that the thin pilus PilV adhesins of the R64 donor cells recognize LPS moieties located on the surfaces of various recipient bacterial cells.

The LPS structures of *S. enterica* serovar Typhimurium LT2 and *E. coli* K-12 are illustrated in Fig. 1B and C, respectively (2, 5, 22). LPSs consist of three parts: the hydrophobic lipid A region, the core oligosaccharide region, and the O-antigen polysaccharide region. The LPS of bacterial strains forming rough colonies lacks the O-antigen region. The LPS core oligosaccharides are synthesized through the successive addition of component sugars to lipid A by the products of the *waa* genes (5, 23). Then O-antigen is attached to the LPS core by the *waaL* product. Hence, *waa* mutants produce incomplete

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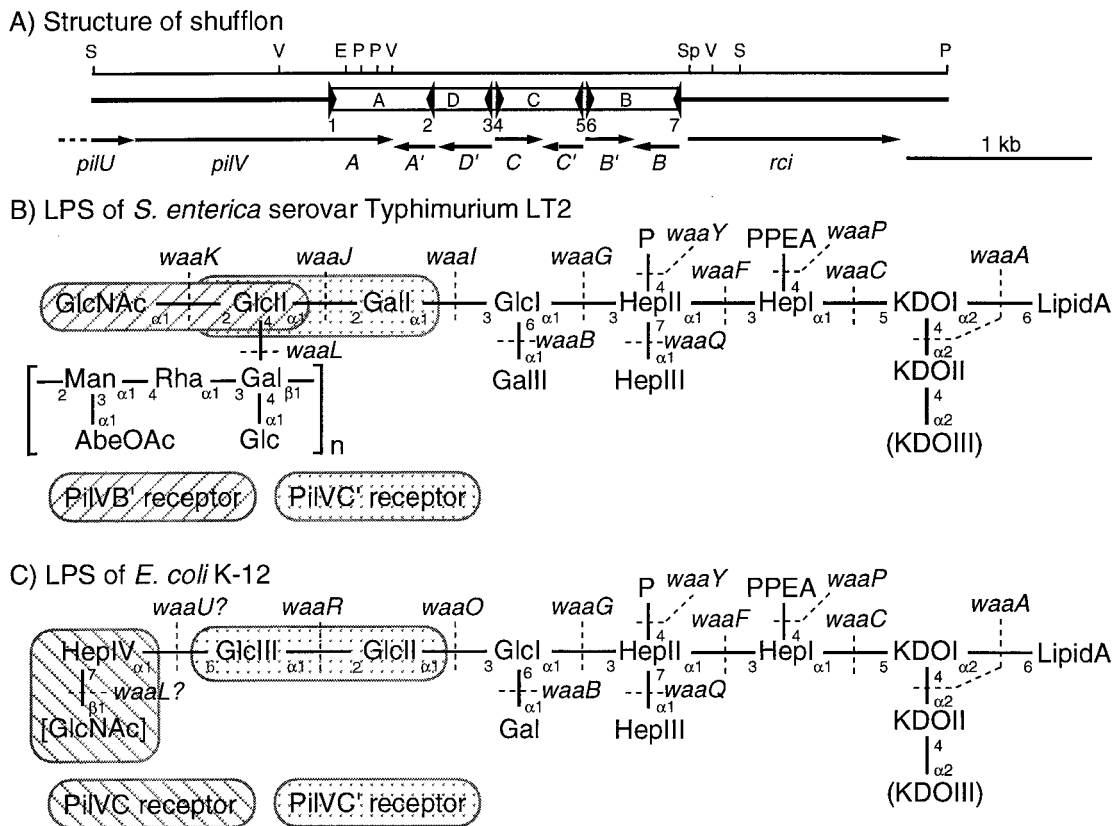


FIG. 1. (A) Structure of the shuffleon. The gene organization of *pilU*, *pilV*, the shuffleon, and the *rci* region of plasmid R64 is shown below a restriction map. E, *EcoRI*; P, *PstI*; S, *SspI*; Sp, *SphI*; V, *EcoRV*. The panel represents an arrangement (corresponding to pKK010-85) of many isomers of the shuffleon subjected to multiple DNA inversions. Open bars A, B, C, and D, shuffleon segments; filled triangles, seven *sfx* sequences; arrows, coding sequences for *pilU*, *pilV* and the variable C-terminal segments encoded by it, and *rci*. (B and C) LPS molecules of *S. enterica* serovar Typhimurium LT2 (B) and *E. coli* K-12 (C) (2, 5, 22). Shown are the proposed LPS structures together with the genes encoding the enzymes thought to be responsible for the generation of each linkage. Brackets, O-antigen repeating units. In *E. coli* K-12 LPS, the terminal GlcNAc residue in brackets was shown to be an incomplete O-antigen moiety (2). The structure of the LPS core shows heterogeneity, as indicated by parentheses. Abbreviations: AbeOAc, O-acetylchabecose; Gal, galactose; Glc, glucose; Hep, heptose; KDO, 3-deoxy-D-manno-2-octulosonic acid; Man, mannose; P, phosphate; PPEA, pyrophosphoethanolamine; Rha, rhamnose. The specific receptor structures for the PilVB', PilVC, and PilVC' adhesins in the LPS molecules are indicated.

LPS molecules. The steps in the formation of LPS involving actions by these *waa* genes are indicated in Fig. 1B and C (5). Recently, the LPS structure of *E. coli* K-12 was finally established (2) (Fig. 1C). The terminal sugar moiety of the *E. coli* K-12 LPS is GlcNAc, which is considered to be an incomplete O-antigen subunit (2). *E. coli* K-12 carries the *rml-wbb* genes, which are involved in the synthesis of the O-antigen O16, but the *wbbL* gene, encoding rhamnose transferase, has been inactivated by IS5 insertion (17). It has been demonstrated that an incomplete O-antigen subunit (even a single sugar) can be attached to the LPS core by O-antigen ligase encoded by the *waaL* gene (2).

In this study, genetic analyses were performed to determine the specific receptor structures in LPSs for each PilV adhesin encoded by plasmid R64. Various *S. enterica* serovar Typhimurium LT2 *waa* mutants, producing defined chemotypes of mutant LPS, were used as recipient strains in liquid matings. Various serovar Typhimurium LT2 and *E. coli* K-12 derivatives which produced chimeric LPS molecules were constructed by

introducing the *waa*<sup>+</sup> genes from different origins and were used as the recipient cells in liquid matings.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. Nalidixic acid- or rifampin-resistant derivatives of the listed *S. enterica* serovar Typhimurium LT2 strains were used as the recipients of conjugation.

**Media.** Luria-Bertani (LB) medium was prepared as previously described (24). Solid medium contained 1.5% agar. Antibiotics were added to the liquid and solid media at the following concentrations when necessary: ampicillin, 100 µg/ml; chloramphenicol, 25 µg/ml; kanamycin, 50 µg/ml; nalidixic acid, 20 µg/ml; rifampin, 50 µg/ml; spectinomycin, 50 µg/ml; tetracycline, 15 µg/ml.

**Recombinant DNA techniques.** Recombinant DNA techniques were performed as previously described (24). Plasmids containing the serovar Typhimurium LT2 *waaL* or *waaK* genes were constructed by modifying pKZ38 *waaL*<sup>+</sup> *waaK*<sup>+</sup> (18). To remove the 0.8-kb *MluI* segment within *waaL*, pKZ38 DNA was digested with *MluI* and self-ligated, giving pRF101 (LT2 *waaK*<sup>+</sup>). To remove the 1.75-kb *SnaBI-EcoRV* segment within *waaK*, pKZ38 DNA was digested with *SnaBI* and *EcoRV* and self-ligated, giving pRF102 (LT2 *waaL*<sup>+</sup>). To clone the *waaJ* gene of serovar Typhimurium LT2, a 1.8-kb DNA segment was amplified by

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
<b>Bacterial strains</b>		
<i>S. enterica</i> serovar Typhimurium LT2		
SL4213	<i>galE496 metA22 metE551 xyl-404 rpsL 120 hsdL6 hsdS29</i>	B. A. D. Stocker
SL3770	<i>waa</i> <sup>+</sup>	SGSC <sup>a</sup>
SA1355	<i>waa</i> <sup>+</sup>	SGSC
SL428	<i>wzy458</i>	SGSC
SA1627	$\Delta$ <i>rml-his (wba)</i>	SGSC
SL3749	<i>waaL446</i>	SGSC
SL733	<i>waaK953</i>	SGSC
RF733	<i>waaK::Tc</i>	This study
SL3750	<i>waaJ417</i>	SGSC
SL3748	<i>waaI432</i>	SGSC
SL3769	<i>waaG471</i>	SGSC
<i>E. coli</i> K-12		
JM83	<i>ara</i> $\Delta$ ( <i>lac-proAB</i> ) <i>rpsL</i> $\phi$ 80d <i>lacZ</i> $\Delta$ M15	29
W3110	Prototoroph	Laboratory stock
TN102	W3110 <i>Nal</i> <sup>r</sup>	12
CS2198	<i>waaR19::Tnlac</i> $\Delta$ <i>lac</i>	20
CS2334	<i>waaL1::TnphoA</i>	10
CS2529	<i>waaU2::Km</i>	10
<b>Plasmids</b>		
pUC119	<i>Ap</i> <sup>r</sup> ; <i>lacZ</i> '	27
pCL1921	<i>Sp</i> <sup>r</sup> ; <i>lacZ</i> '	16
pHSG415	<i>Ap</i> <sup>r</sup> <i>Km</i> <sup>r</sup> <i>Cm</i> <sup>r</sup> ; pSC101 <i>rep</i> (Ts)	4
pKK661	pHSG576 derivative carrying 35.6-kb R64 <i>tra</i> segment	12
pKK641A	pKK641 derivative carrying a fixed <i>pilVA</i> gene	12
pKK641A'	pKK641 derivative carrying a fixed <i>pilVA</i> ' gene	12
pKK641B	pKK641 derivative carrying a fixed <i>pilVB</i> gene	12
pKK641B'	pKK641 derivative carrying a fixed <i>pilVB</i> ' gene	12
pKK641C	pKK641 derivative carrying a fixed <i>pilVC</i> gene	12
pKK641C'	pKK641 derivative carrying a fixed <i>pilVC</i> ' gene	12
pKK641D'	pKK641 derivative carrying a fixed <i>pilVD</i> ' gene	12
pRF101	3.0-kb LT2 <i>waaK</i> <sup>+</sup> fragment in pBR322	This study
pRF102	2.1-kb LT2 <i>waaL</i> <sup>+</sup> fragment in pBR322	This study
pRF103	1.8-kb LT2 <i>waaJ</i> <sup>+</sup> fragment in pUC119	This study
pRF005	2.3-kb K-12 <i>waaU</i> <sup>+</sup> fragment in pCL1921	8
pRF006	2.7-kb K-12 <i>waaL</i> <sup>+</sup> fragment in pCL1921	8
pRF009	4.6-kb K-12 <i>waaU</i> <sup>+</sup> <i>waaL</i> <sup>+</sup> fragment in pCL1921	8

<sup>a</sup> SGSC, *Salmonella* Genetic Stock Center.

PCR using appropriate primers and chromosomal DNA as the template and inserted into pUC119, giving pRF103 (LT2 *waaJ*<sup>+</sup>).

To construct a *waaK*-null mutant, the LT2 *waaK* gene was first cloned into temperature-sensitive suicide vector pHSG415 (4). A tetracycline resistance gene was then inserted into the *Sna*BI site within *waaK*, and the resultant *waaK::Tc* allele was introduced into the SL3770 chromosome by a gene replacement method, giving RF733.

**Conjugal transfer.** Liquid matings were performed as previously described (12). Two sets of donor cells were used: *S. enterica* serovar Typhimurium LT2 SL4213 cells harboring pKK661 and one of the seven pKK641 series plasmids were used in the experiments using serovar Typhimurium LT2 recipients; *E. coli* K-12 W3110 cells harboring pKK661 and one of the seven pKK641 series plasmids were used in the experiments using *E. coli* K-12 recipients. A log-phase culture (200  $\mu$ l) of donor cells was mixed with an overnight culture (50  $\mu$ l) of recipient cells. The mating mixture was incubated for 90 min at 37°C and plated at various dilutions on selective media. The transfer frequencies were calculated as the ratio of the number of transconjugants to the number of donor cells.

As specific inhibitors for liquid matings, Glc( $\alpha$ 1-2)Glc (kojibiose) and Glc( $\alpha$ 1-2)Gal were purchased from Wako (Osaka, Japan) and Toronto Research Chemicals (Toronto, Canada), respectively. LPS molecules were prepared from *E. coli* K-12 TN102 cells by the phenol-chloroform-petroleum ether method as described previously (8). To examine their effects on liquid matings, both the log-phase donor culture and overnight recipient culture were diluted 100-fold with LB broth before mixing and then inhibitors were added (8). At time intervals, aliquots of mating mixture were plated onto selective media.

**Phage sensitivity analysis.** Phage sensitivity was analyzed by spotting phage stocks (P22, Felix O [FO], and Ffm) on lawns of various bacterial strains on LB agar plates.

**Preparation and gel analysis of LPS.** Outer membrane fractions containing LPS molecules from various bacterial strains were prepared by the method of Schnaitman and McDonald (25). After treatment of the outer membranes with proteinase K, the residual fractions were used as LPS samples (21). The LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (6).

## RESULTS AND DISCUSSION

**Dependency of the frequency of transfer to various *S. enterica* serovar Typhimurium LT2 *waa* recipient strains on the seven PilV adhesins.** Since various *E. coli* K-12 *waa* mutants have been shown to lack receptors for one or two PilV adhesins (8), the effects of various mutations of *S. enterica* serovar Typhimurium LT2 *waa* on PilV recognition were examined. LT2 strains with *waa*, *wzy*, and *wba* mutations were used as the recipient strains for liquid matings of R64 derivatives expressing each of the seven PilV adhesins (Table 2).

*S. enterica* serovar Typhimurium LT2 wild-type strains

TABLE 2. Dependency of the frequency of transfer to various recipient *S. enterica* serovar Typhimurium LT2 *waa* strains on the seven PilV adhesins in the donor cells

Recipient strain	Transfer frequency ratio <sup>a</sup> for donor strain expressing:						
	PilVA	PilVA'	PilVB	PilVB'	PilVC	PilVC'	PilVD'
SL3770 <i>waa</i> <sup>+</sup>	0.003	<b>0.35</b>	0.003	<b>1</b>	0.004	0.009	0.004
SA1355 <i>waa</i> <sup>+</sup>	0.002	<b>0.42</b>	0.002	<b>1</b>	0.002	0.004	>0.001
SL428 <i>wzy</i>	>0.001	<b>1</b>	0.008	<b>0.86</b>	0.002	0.001	0.001
SA1627 <i>wba</i>	0.009	<b>1</b>	0.068	<b>0.43</b>	0.005	0.009	0.007
SL3749 <i>waaL</i>	0.010	<b>0.78</b>	0.009	<b>1</b>	0.010	0.022	0.009
SL733 <i>waaK</i>	0.003	<b>0.85</b>	0.001	0.064	0.002	<b>1</b>	0.002
RF733 <i>waaK</i>	0.001	<b>0.95</b>	0.001	0.001	0.001	<b>1</b>	0.001
SL3750 <i>waaJ</i>	0.005	<b>1</b>	0.004	0.015	0.004	0.005	0.010
SL3748 <i>waaI</i>	0.001	<b>1</b>	0.002	0.025	0.001	0.001	0.002
SL3769 <i>waaG</i>	0.009	<b>1</b>	0.013	0.005	0.008	0.010	0.008
SL3749/pRF102	0.002	<b>0.80</b>	0.030	<b>1</b>	0.002	0.010	0.001
SL733/pRF101	0.001	<b>0.67</b>	0.010	<b>1</b>	0.001	<b>0.16</b>	0.002
RF733/pRF101	>0.001	<b>1</b>	0.001	<b>0.88</b>	0.001	<b>0.20</b>	0.001
SL3750/pRF103	0.001	<b>1</b>	0.001	<b>0.30</b>	0.001	0.007	0.001

<sup>a</sup> *S. enterica* serovar Typhimurium LT2 SL4213 cells harboring pKK661 and one of seven pKK641 series plasmids were used as donors. *S. enterica* serovar Typhimurium LT2 strains with various *waa* mutations were used as recipient strains. Liquid matings were carried out at 37°C for 1.5 h. For each recipient strain, the ratio of the frequency of transfer of pKK661 from *S. enterica* serovar Typhimurium LT2 donor cells expressing various PilV adhesins to the highest transfer frequency (1.0) is estimated. High ratios are indicated by boldface type.

SL3770 and SA1355 were recognized by the PilVA' and PilVB' adhesins, as was wild-type SL4213 (13, 14). The wild-type *S. enterica* serovar Typhimurium LT2 strain has LPS with O-antigen (Fig. 1B). Mutants SL428 *wzy*, SA1627 *wba*, and SL3749 *waaL*, lacking O-antigen, were recognized by the PilVA' and PilVB' adhesins, as were the wild-type strains (Table 2). These results indicate that the O-antigen of *S. enterica* serovar Typhimurium LT2 is not required for recognition by the PilVA' and PilVB' adhesins in R64 liquid matings and does not block recognition.

On the other hand, a series of *waa* mutants producing incomplete LPS cores exhibited different mating patterns (Table 2). The SL733 *waaK* mutant showed very low recognition by the PilVB' adhesin, suggesting that the PilVB' adhesin may recognize the GlcNAc(α1-2)Glc moiety, the terminal structure of the *S. enterica* serovar Typhimurium LPS core. Residual recognition by the PilVB' adhesin was observed in SL733, suggesting that SL733 is a leaky mutant and that a small number of LPS molecules carrying the PilVB' receptor structure are still produced in this strain. This postulation was confirmed by using the RF733 *waaK*-null mutant, which was not recognized by the PilVB' adhesin. To our surprise, SL733 and RF733 *waaK* mutants were recognized by the PilVC' adhesin, which did not recognize any wild-type serovar Typhimurium LT2 strains (Table 2). The SL3750 *waaJ*, SL3748 *waaI*, and SL3769 *waaG* mutants were recognized only by the PilVA' adhesin. These results suggest that the PilVC' adhesin recognizes the Glc(α1-2)Gal moiety in the LPS of serovar Typhimurium LT2, the formation of which requires the *waaJ*, *waaI*, and *waaG* genes. In the previous study, the PilVC' adhesin was shown to recognize the Glc(α1-2)Glc structure in the LPS of *E. coli* K-12 (8).

The internal Glc(α1-2)Gal structure in the wild-type serovar Typhimurium LT2 cells was not recognized by the PilVC' adhesin, while the internal Glc(α1-2)Glc structure in wild-type *E. coli* K-12 cells was recognized. This apparent discrepancy may be explained as follows. In the wild-type LT2 LPS, the PilVC' receptor is masked by the addition of GlcNAc, while, in

the wild-type K-12 LPS, the neighboring sugars attached to the PilVC' receptor do not affect PilVC' recognition. The difference in the positions of the modification may be responsible for this, since the C-2 position of GlcII in the LT2 LPS is modified by GlcNAc, while the C-6 position of GlcIII in the K-12 LPS is modified by heptose (Fig. 1B and C). Alternatively, it is possible that not all of the *E. coli* K-12 LPS core has the terminal disaccharide and that PilVC' may recognize the incomplete LPS core of *E. coli* K-12, in which the PilVC' receptor is exposed.

Complementation experiments were performed by introducing plasmids carrying the corresponding wild-type *waa*<sup>+</sup> genes into LT2 *waa* mutants (Table 2). The *waaK* and *waaJ* mutants were complemented by pRF101 and pRF103, respectively,

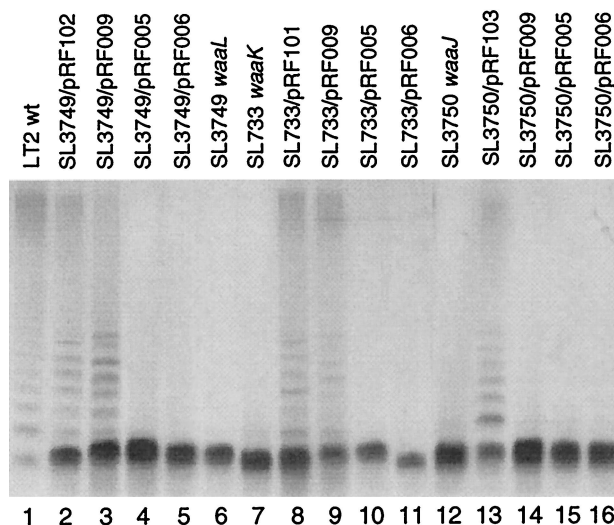


FIG. 2. Gel electrophoretic analysis of chimeric LPSs. The LPSs from *S. enterica* serovar Typhimurium LT2 strains harboring the indicated plasmids were analyzed by SDS-PAGE and visualized by silver staining. wt, wild type.

TABLE 3. PilV dependency of the frequency of transfer to various recipient *S. enterica* serovar Typhimurium LT2 and *E. coli* K-12 *waa* strains carrying various *waa*<sup>+</sup> genes from different origins

Recipient strain	Transfer frequency ratio <sup>a</sup> for donor strain expressing:							Sensitivity <sup>b</sup> to phage:		
	PilVA	PilVA'	PilVB	PilVB'	PilVC	PilVC'	PilVD'	P22	FO	Ffm
SL3770 <i>waa</i> <sup>+</sup>	0.003	<b>0.35</b>	0.003	<b>1</b>	0.004	0.009	0.004	+	+	-
SL3770/pRF009	0.014	<b>0.89</b>	0.009	<b>1</b>	<b>0.67</b>	<b>0.20</b>	0.014	+	±	-
SL3770/pRF005	0.006	<b>0.57</b>	0.003	<b>1</b>	0.007	<b>0.30</b>	0.006	+	±	±
SL3770/pRF006	>0.001	<b>0.18</b>	>0.001	<b>1</b>	>0.001	0.004	>0.001	+	+	-
SL3749 <i>waaL</i>	0.010	<b>0.78</b>	0.009	<b>1</b>	0.010	0.022	0.009	-	+	+
SL3749/pRF009	0.003	<b>1</b>	0.004	0.002	<b>0.27</b>	<b>0.03</b>	0.002	+	-	+
SL3749/pRF005	>0.001	<b>1</b>	0.001	0.007	0.001	<b>0.05</b>	0.001	-	±	+
SL3749/pRF006	0.002	<b>1</b>	0.002	<b>0.30</b>	0.003	0.009	0.002	-	+	+
SL733 <i>waaK</i>	0.003	<b>0.85</b>	0.001	0.064	0.002	<b>1</b>	0.002	-	-	+
SL733/pRF009	0.001	<b>1</b>	0.003	0.001	<b>0.30</b>	<b>0.06</b>	0.001	+	-	±
SL733/pRF005	0.003	<b>1</b>	0.002	0.002	0.003	<b>0.04</b>	0.002	-	-	+
SL733/pRF006	0.002	<b>1</b>	0.001	0.027	0.001	<b>0.42</b>	0.002	-	-	+
SL3750 <i>waaJ</i>	0.005	<b>1</b>	0.004	0.015	0.004	0.005	0.010	-	-	+
SL3750/pRF009	0.001	<b>1</b>	0.001	0.001	0.001	0.001	0.002	-	-	+
SL3750/pRF005	0.002	<b>1</b>	0.001	0.003	0.002	0.004	0.001	-	-	+
SL3750/pRF006	0.002	<b>1</b>	0.002	0.006	0.005	0.007	0.004	-	-	+
JM83	0.007	<b>0.29</b>	0.007	0.006	<b>0.62</b>	<b>1</b>	0.006	-	-	+
JM83/pRF101	0.020	<b>0.33</b>	0.020	<b>1</b>	<b>0.15</b>	<b>0.38</b>	0.013	-	+	+
JM83/pRF102	0.013	<b>1</b>	0.017	0.006	<b>0.09</b>	<b>0.32</b>	0.007	-	-	+
CS2334 <i>waaL</i>	0.016	<b>0.68</b>	0.021	0.019	0.015	<b>1</b>	0.016	-	-	+
CS2334/pRF006	0.003	<b>0.63</b>	0.003	>0.001	<b>0.94</b>	<b>1</b>	0.003	-	-	+
CS2334/pRF102	0.001	<b>1</b>	0.002	0.001	0.002	<b>0.52</b>	0.001	-	-	+
CS2529 <i>waaU</i>	0.004	<b>0.6</b>	0.006	0.005	0.005	<b>1</b>	0.005	-	-	+
CS2529/pRF005	0.039	<b>0.48</b>	0.030	0.017	<b>0.38</b>	<b>1</b>	0.017	-	-	+
CS2529/pRF101	0.004	<b>1</b>	0.005	<b>0.045</b>	0.001	<b>0.049</b>	>0.001	-	+	+
CS2198 <i>waaR</i>	0.007	<b>1</b>	0.007	0.007	0.007	0.007	0.007	-	-	+
CS2198/pRF101	0.007	<b>1</b>	0.007	0.007	0.007	0.005	0.007	-	-	+

<sup>a</sup> *S. enterica* serovar Typhimurium LT2 SL4213 and *E. coli* K-12 W3110 harboring pKK661 and one of seven pKK641 series plasmids were used as donor strains. *S. enterica* serovar Typhimurium LT2 and *E. coli* K-12 with and without various *waa*<sup>+</sup> plasmids were used as recipient strains. Liquid matings were carried out at 37°C for 1.5 h. For each recipient strain, the ratio of the frequency of transfer of pKK661 from *E. coli* K-12 or serovar Typhimurium LT2 donor cells expressing various PilV adhesins to the highest transfer frequency (1.0) is estimated. High ratios are indicated by boldface type.

<sup>b</sup> +, sensitive; ±, partially sensitive; -, resistant.

while the complementation of the *waaK* mutant by pRF101 was incomplete (recognition by PilVC'). The complementation of *waaL* by pRF102 was confirmed by SDS-PAGE analysis of the LPS (Fig. 2, lane 2) and by the sensitivity of the mutants to phage P22 (data not shown).

These results strongly suggest that the PilVB' and PilVC' adhesins recognize the GlcNAc(α1-2)Glc and Glc(α1-2)Gal structures, respectively, of the LPS core of *S. enterica* serovar Typhimurium cells in R64 liquid matings, while the PilVC' receptor in the wild-type LT2 LPS may be masked by GlcNAc.

**Various chimeric LPSs as PilV receptors.** The results described above, together with previous results (8), suggest that specific moieties of LPSs function as receptors for various PilV adhesins. To confirm this, we tried to construct the PilVC and PilVB' receptors on the LPSs of serovar Typhimurium LT2 and *E. coli* K-12, respectively. Various chimeric LPSs were constructed by introducing *waa*<sup>+</sup> genes from different bacterial strains on multicopy plasmids into wild-type or *waa* strains of serovar Typhimurium LT2 or *E. coli* K-12, which were used as recipient strains (Table 3).

*S. enterica* serovar Typhimurium LT2 SL3770 cells harboring pRF009 (carrying *waaU*<sup>+</sup> and *waaL*<sup>+</sup> of *E. coli* K-12) were recognized by the PilVC and PilVC' adhesins in addition to the PilVA' and PilVB' adhesins (Table 3). SL3770 cells harboring pRF005 (K-12 *waaU*<sup>+</sup>) were recognized by the PilVC' adhesin, while SL3770 cells harboring pRF006 (K-12 *waaL*<sup>+</sup>) were not.

These results can be explained as follows. In SL3770 cells harboring pRF005, heptose may be attached to the C-6 position of GlcII of the LT2 LPS by WaaU of *E. coli* K-12, resulting in recognition of the cells by the PilVC' adhesin. In SL3770 cells harboring pRF009, heptose and GlcNAc may be successively attached to the C-6 position of GlcII of the LT2 LPS by WaaU and WaaL of *E. coli* K-12, respectively, resulting in recognition of the cells by the PilVC' and PilVC adhesins. In SL3770 cells harboring pRF006, modification of the LT2 LPS did not occur. Thus, the GlcNAc(β1-7)Hep structure (PilVC receptor) can be constructed on the LT2 LPS by K-12 WaaU and WaaL. However, in SL3770 cells harboring pRF009 or pRF005, the attachment of heptose to GlcII of the LT2 LPS might be partial. *N*-Acetylglucosamine and O-antigen were also bound to some portion of GlcII in these LPSs, since the cells were also recognized by the PilVB' adhesin. Thus, these LPSs may be heterogeneous.

Introduction of pRF005, pRF006, or pRF009 had similar effects on PilV dependency in SL3749 *waaL* cells and SL3770 cells (Table 3). In the case of SL3749, however, modification by heptose of GlcII in the LT2 LPS appears to be nearly complete, since the cells were not recognized by the PilVB' adhesin. The reason for the differences in the levels of heptose modification between SL3770 harboring pRF009 or pRF005 and SL3749 harboring pRF009 or pRF005 is not known at present. Introduction of pRF009, pRF005, and pRF006 into

SL733 *waaK* cells affected the PiiV dependency similarly to their introduction into SL3749. In contrast, introduction of the same plasmids into SL3750 *waaJ* exhibited no effect on PiiV dependency. These results indicate that GlcII of the LT2 LPS is essential for the construction of the PiiVC receptor [GlcNAc( $\beta$ 1-7)Hep] on it.

*E. coli* K-12 JM83 cells harboring pRF101(*waaK*<sup>+</sup> of serovar Typhimurium LT2) were recognized by the PiiVB' adhesin in addition to the PiiVA', PiiVC, and PiiVC' adhesins (Table 3). Introduction of pRF102 (LT2 *waaL*<sup>+</sup>) into JM83 cells slightly affected the PiiV recognition pattern of JM83 cells. It is likely that GlcNAc was added to the C-2 position of GlcIII in the LPS of *E. coli* K-12 cells harboring pRF101 by LT2 WaaK, forming the GlcNAc( $\alpha$ 1-2)Glc moiety as the PiiVB' receptor. However, the addition of GlcNAc to GlcIII might be partial, since *E. coli* K-12 cells harboring pRF101 were still recognized by the PiiVC and PiiVC' adhesins, suggesting heterogeneity of their LPSs.

*E. coli* K-12 CS2334 *waaL* could not be complemented by pRF102 (LT2 *waaL*<sup>+</sup>), while it was complemented by pRF006 (K-12 *waaL*<sup>+</sup>) (Table 3). Although the WaaL proteins of the two strains carry O-antigen ligase activity, their substrate specificities may be different since *E. coli* K-12 and serovar Typhimurium LT2 carry distinct O-antigen and LPS core structures. In fact, only limited amino acid sequence similarity between the two WaaL proteins is found.

*E. coli* K-12 CS2529 *waaU* cells harboring pRF101 (LT2 *waaK*<sup>+</sup>) were recognized weakly by PiiVB' but not by PiiVC, while those harboring pRF005 (K-12 *waaU*<sup>+</sup>) were recognized by PiiVC (Table 3). These results confirm that both the WaaU and WaaL proteins of *E. coli* K-12 are essential for the formation of the PiiVC receptor and that WaaK of serovar Typhimurium LT2 is responsible for the formation of the PiiVB' receptor. *E. coli* K-12 CS2198 *waaR* cells harboring pRF101 (LT2 *waaK*<sup>+</sup>) were not recognized by PiiVB', suggesting that WaaK of serovar Typhimurium LT2 requires GlcIII of the K-12 LPS for the attachment of GlcNAc.

From the construction of the chimeric LPSs together with the results of the experiments using *waa* mutant versions of various bacteria, we conclude that the products of various *waa* genes are involved in the formation of the specific PiiV receptor structures within the LPS.

**SDS-PAGE analysis of chimeric LPSs.** The formation of the postulated chimeric LPSs in the strains carrying the *waa*<sup>+</sup> genes from different bacterial strains was confirmed by SDS-PAGE analysis of the chimeric LPSs. Typical results are shown in Fig. 2. The LPS samples were prepared from the serovar Typhimurium LT2 *waaL*, *waaK*, and *waaJ* mutants harboring various plasmids and analyzed by SDS-PAGE followed by silver staining. The LT2 LPSs to which heptose and GlcNAc were attached by *E. coli* K-12 WaaU and WaaL, respectively, exhibited slower mobilities than unmodified LPSs (Fig. 2, compare lanes 3 and 4 to 6 and lanes 9 and 10 to 7). Attachment of O-antigen to the LPSs in SL3749 and SL733 cells harboring pRF009 was unexpected (Fig. 2, lanes 3 and 9). In these cells, the O-antigen of LT2 is likely to be attached to the HepIV-modified LT2 LPSs by WaaL of *E. coli* K-12. Attachment of O-antigen to LPSs in these cells was confirmed by determining the sensitivity to phage P22 (Table 3; see below).

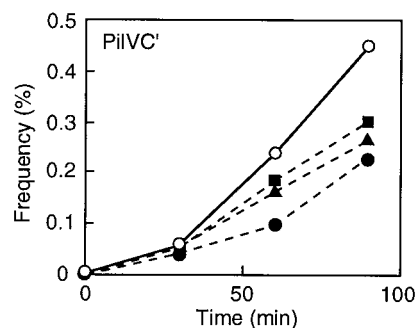


FIG. 3. Effects of Glc( $\alpha$ 1-2)Glc, Glc( $\alpha$ 1-2)Gal, and LPS on PiiVC'-mediated liquid matings. *E. coli* K-12 W3110 harboring pKK661 together with pKK641C' and TN102 were used as donor and recipient strains, respectively. Donor and recipient cultures were diluted 100-fold with LB broth before mixing. As inhibitor molecules, 1 mM Glc( $\alpha$ 1-2)Glc (filled triangles), 1 mM Glc( $\alpha$ 1-2)Gal (filled squares), and 0.1 mM LPS from *E. coli* K-12 TN102 (filled circles) were added to the mating mixture. The control mating mixture (open circles) did not contain any inhibitor molecule. The transfer frequency is expressed as a percentage of the number of donor cells and was determined at time intervals.

**Phage sensitivity analysis.** The formation of the postulated chimeric LPS was further confirmed by phage sensitivity (Table 3). Phage P22 recognizes the O-antigen unit structure of the LPS molecule of *S. enterica* serovar Typhimurium LT2 (1, 26). Phage FO recognizes the terminal structure of the *E. coli* R2-type LPS core, including that of *S. enterica* serovar Typhimurium LT2 (28). Phage Ffm recognizes the rough-type LPS (28). *S. enterica* serovar Typhimurium LT2 wild-type SL3770 strains harboring pRF009, pRF005, or pRF006 were sensitive to phage P22 (Table 3), indicating that all three strains produce LPS molecules carrying O-antigen. SL3770 cells harboring pRF009 and pRF005 exhibited weak sensitivity to FO, and SL3770 cells harboring pRF005 were partially sensitive to Ffm. All of SL3749 *waaL*, SL733 *waaK*, and SL3750 *waaJ* derivatives were sensitive to Ffm, indicating a rough-type character, while SL3749 and SL733 cells harboring pRF009 were sensitive to P22. All of the *E. coli* K-12 derivatives were sensitive to Ffm. *E. coli* JM83 harboring pRF101 and CS2529 harboring pRF101, which carried the PiiVB' receptor, were sensitive to FO. These results suggest that the PiiVB' receptor is similar to the FO receptor.

**Specific inhibition of PiiVC'-mediated liquid matings by Glc( $\alpha$ 1-2)Glc and Glc( $\alpha$ 1-2)Gal.** We have previously shown that addition of *E. coli* K-12 or B LPS specifically inhibits liquid matings (8). The effects of the addition of Glc( $\alpha$ 1-2)Glc and Glc( $\alpha$ 1-2)Gal as well as LPS from *E. coli* K-12 on the transfer frequency were examined in liquid matings with *E. coli* K-12 W3110 cells harboring pKK661 together with pKK641C' as the donor strain (Fig. 3). Addition of LPS decreased the transfer frequency, as was found previously (8). Glc( $\alpha$ 1-2)Glc and Glc( $\alpha$ 1-2)Gal also decreased the transfer frequencies, although the effects were not so marked as that of LPS, even in the presence of higher concentrations. The inhibition was dependent on the concentrations of Glc( $\alpha$ 1-2)Glc and Glc( $\alpha$ 1-2)Gal (data not shown). In contrast, addition of maltose [Glc( $\alpha$ 1-4)Glc] did not affect liquid matings (data not shown). These results further support the notion that the PiiVC' pro-

tein recognizes Glc( $\alpha$ 1-2)Glc and Glc( $\alpha$ 1-2)Gal moieties of LPS molecules in recipient cells.

**Receptor structures within LPSs for PilVB', PilVC, and PilVC' adhesins.** It is proposed that the PilVB' receptor is GlcNAc( $\alpha$ 1-2)Glc, since (i) the *S. enterica* serovar Typhimurium LT2 *waaK* mutant was not recognized by PilVB' and (ii) the *E. coli* K-12 strain carrying the LT2 *waaK* gene was recognized by PilVB'. In addition, the PilVB' adhesin also recognized *E. coli* R2 F632, in which the LPS contained the GlcNAc( $\alpha$ 1-2)Glc structure (data not shown).

It is proposed that the PilVC receptor is GlcNAc( $\beta$ 1-7)Hep, since (i) the *E. coli* K-12 *waaU* or *waaL* mutants were not recognized by PilVC and (ii) the *S. enterica* serovar Typhimurium LT2 strain carrying the K-12 *waaU* and *waaL* genes was recognized by PilVC. In addition, it was previously shown that the *E. coli* B strain carrying the K-12 *waaRUL* genes was recognized by PilVC and PilVC' (8).

The PilVC' receptor of the LT2 LPS was shown to be Glc( $\alpha$ 1-2)Gal, since the *S. enterica* serovar Typhimurium LT2 *waaJ* mutant was not recognized by PilVC'. The PilVC' receptor of the K-12 LPS was previously shown to be Glc( $\alpha$ 1-2)Glc, since the *E. coli* K-12 *waaR* mutant was not recognized by PilVC' (8). An *E. coli* B strain carrying the K-12 *waaR* gene was recognized by PilVC'. In addition, Glc( $\alpha$ 1-2)Glc and Glc( $\alpha$ 1-2)Gal were shown to inhibit PilVC'-mediated liquid matings (Fig. 3).

From these results, we propose that the GlcNAc( $\alpha$ 1-2)Glc and Glc( $\alpha$ 1-2)Gal structures of the LPS core of *S. enterica* serovar Typhimurium LT2 function as receptors for the PilVB' and PilVC' adhesins, respectively, while the PilVC' receptor in the wild-type LT2 LPS core is masked. We further propose that the GlcNAc( $\beta$ 1-7)Hep and Glc( $\alpha$ 1-2)Glc structures of the LPS core of *E. coli* K-12 function as receptors for the PilVC and PilVC' adhesins, respectively. These receptor structures are indicated in Fig. 1B and C. It is important that the disaccharide structures of the LPSs presented in Fig. 1B and C as specific receptors for the three PilV adhesins represent only the minimal essential structures. The results indicating that addition of LPS to the mating mixtures decreased the transfer frequency more efficiently than addition of disaccharides (Fig. 3) suggest the involvement of other structures of the respective LPSs in binding to the PilV adhesins.

Many bacteriophages use the LPS molecules of the host bacterial cells as receptors (19). Since gram-negative bacteria produce specific LPSs, the recognition of LPS by bacteriophages results in their host specificity. The LPS binding of the bacteriophage P22 tailspike proteins has been extensively studied (1, 26). To directly demonstrate LPS-PilV adhesin interaction, *in vitro* binding experiments may be helpful. Such work is in progress in our laboratory.

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