Expression and Characterization of Streptococcal *rgp* Genes Required for Rhamnan Synthesis in *Escherichia coli*†

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Six genes (*rgpA* through *rgpF*) that were involved in assembling the rhamnose-glucose polysaccharide (RGP) in *Streptococcus mutans* were previously identified (Y. Yamashita, Y. Tsukioka, K. Tomihisa, Y. Nakano, and T. Koga, J. Bacteriol. 180:5803–5807, 1998). The group-specific antigens of Lancefield group A, C, and E streptococci and the polysaccharide antigen of *Streptococcus sobrinus* have the same rhamnan backbone as the RGP of *S. mutans*. *Escherichia coli* harboring plasmid pRGP1 containing all six *rgp* genes did not synthesize complete RGP. However, *E. coli* carrying a plasmid with all of the *rgp* genes except for *rgpE* synthesized the rhamnan backbone of RGP without glucose side chains, suggesting that in addition to *rgpE*, another gene is required for glucose side-chain formation. Synthesis of the rhamnan backbone in *E. coli* required the initiation of transfer of *N*-acetylglucosamine to a lipid carrier and the expression of the *rgpC* and *rgpD* genes encoding the putative ABC transporter specific for RGP. The similarities in RGP synthesis between *E. coli* and *S. mutans* suggest common pathways for rhamnan synthesis. Therefore, we evaluated the rhamnosyl polymerization process in *E. coli* by high-resolution sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the lipooligosaccharide (LOS). An *E. coli* transformant harboring *rgpA* produced the LOS modified by the addition of a single rhamnose residue. Furthermore, the *rgpA*, *rgpB*, and *rgpF* genes of pRGP1 were independently mutated by an internal deletion, and the LOS chemotypes of their transformants were examined. The transformant with an *rgpA* deletion showed the same LOS profile as *E. coli* without a plasmid. The transformant with an *rgpB* deletion showed the same LOS profile as *E. coli* harboring *rgpA* alone. The transformant with an *rgpF* deletion showed the LOS band with the most retarded migration. On the basis of these results, we speculated that RgpA, RgpB, and RgpF, in that order, function in rhamnan polymerization.

Polysaccharides are the major constituents of streptococcal cell walls and are useful for the serological classification and identification of streptococci. The group-specific polysaccharide antigens of Lancefield group A, C, and E streptococci (3, 26), the serotype-specific antigen of *Streptococcus mutans* (18, 27), and the rhamnose-glucose polysaccharide (RGP) antigen of *Streptococcus sobrinus* (19) share a common structural relationship. The backbones of these polysaccharides are polymers of α 1,2- and α 1,3-linked rhamnose units. Although the rhamnan backbone has been identified in many streptococci, little is known about the mechanism of its synthesis. Rhamnan is also present in O polysaccharides of phytopathogenic bacteria (*Xanthomonas*, *Pseudomonas*, and *Stenotrophomonas*), *Yersinia enterocolitica*, and *Pseudomonas aeruginosa*, and these O polysaccharides are regarded as pathogenic factors (1, 6, 25, 28, 32, 40). However, the only report dealing with the assembly of rhamnan is that describing the synthesis of the A band, D-rhamnan polysaccharide, of *P. aeruginosa* (28).

S. mutans strains are classified into three serotypes (serotypes c, e, and f) on the basis of the immunological properties of cell wall antigens (20). These serotype-specific antigens are RGPs, which are composed of rhamnan backbones and glu-

cose side chains (18, 27). The biological function of RGPs is receiving increasing attention. In vitro stimulation of human monocytes with the serotype f-specific RGP was reported to induce the release of inflammatory cytokines, such as tumor necrosis factor alpha and interleukin-1 β (33), and to provoke nitric oxide production in the rat aorta (21). Furthermore, it was shown that the serotype-specific RGP played an important role in resistance to phagocytosis and consequent killing by human polymorphonuclear leukocytes (36).

Four loci that are involved in RGP synthesis were previously characterized. Four *rml* genes (*rmlA* through *rmlD*) are directly related to the synthesis of dTDP-L-rhamnose (37, 38), and the *gluA* gene encodes the enzyme producing UDP-D-glucose (43). The *rgpG* gene is implicated in the initiation of RGP synthesis by transfer of *N*-acetylglucosamine-1-phosphate to a lipid carrier (41). In addition, six other genes (*rgpA* through *rgpF*) required for RGP synthesis were identified in the region downstream from *rmlD*, and some of these genes were shown likely to be involved in the transport and assembly of RGP (44).

In this study, we found that *Escherichia coli* harboring these six *rgp* genes synthesized an RGP rhamnan backbone without glucose side chains, a structure which resembled an O polysaccharide linked to the lipopolysaccharide (LPS) of *E. coli*. This finding enabled us to characterize the functions of the *rgp* genes in *E. coli* by modifying the LPS core region of *E. coli* K-12 strains via the expression in *trans* of glycosyltransferases. We discuss *rgp* gene functions on the basis of changes in the lipooligosaccharide (LOS) profiles of *E. coli* transformants carrying the *rgp* genes.

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† Dedicated to the memory of Toshihiko Koga, our mentor.

‡ Deceased.

TABLE 1. *E. coli* and *S. mutans* strains used in this study

Strain	Genotype or relevant characteristics	Reference or source
<i>E. coli</i>		
AB1133	<i>thr-1 leuB6 Δ(gpt-proA)66 hisG4 argE3 thi-1 rfbD1 lacY ara-14 galK2 xyl-5 mtl-1 mgl-51 rpsL31 kdgK51 supE44</i>	22
21548	AB1133 derivative; <i>wecA::Tn10</i>	22
CS2767	<i>his leu proA argT his thi galk Δlac trpE non mtl xyl ara rpsL sup⁺</i>	2
CS2775	<i>rfaS2007::Tnlac</i> derivative of CS2767; Kan ^r	2
CS2775-KD	CS2775 derivative; <i>wecA::Tn10</i>	This study
KD401	AB1133 carrying pRGP1	This study
KD411	AB1133 carrying pRGP11	This study
KD412	AB1133 carrying pRGP12	This study
KD413	AB1133 carrying pRGP13	This study
KD414	AB1133 carrying pRGP14	This study
KD415	AB1133 carrying pRGP15	This study
KD421	21548 carrying pRGP1	This study
KD422	21548 carrying pKU58 and pRGP1	This study
KD4311	CS2775 carrying pRGP11	This study
KD4312	CS2775 carrying pRGP12	This study
KD4313	CS2775 carrying pRGP13	This study
KD4315	CS2775 carrying pRGP15	This study
KD4116	CS2775 carrying pRGP16	This study
KD4117	CS2775 carrying pRGP17	This study
KD4118	CS2775 carrying pRGP18	This study
KD432	CS2775 carrying pRGPA	This study
KD433	CS2775 carrying pRGPB	This study
KD434	CS2775 carrying pRGPF	This study
KD441	CS2775-KD carrying pRGPA	This study
KD214	CS2775 carrying pSR4	This study
KD501	AB1133 carrying pNKB26	This study
<i>S. mutans</i>		
Xc	Serotype c wild-type strain	15
Xc47	Em ^r ; strain Xc carrying p15A replicon and Em ^r gene (pResYT10) inserted into open reading frame 7	44

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *S. mutans* and *E. coli* strains and plasmids used in this study are listed in Tables 1 and 2. *E. coli* CS2775 was kindly provided by R. A. Welch, Department of Medical Microbiology and Immunology, University of Wisconsin at Madison. Strains of *S. mutans* and *E. coli* were maintained and grown routinely as described previously (42). Antibiotics were used at the following concentrations: 200 µg of erythromycin per ml, 50 µg of ampicillin per ml, 25 µg of kanamycin per ml, or 25 µg of tetracycline per ml for *E. coli* and 10 µg of erythromycin per ml for *S. mutans*.

DNA manipulation. Standard DNA recombinant procedures, such as DNA isolation, endonuclease restriction, ligation, and agarose gel electrophoresis, were carried out as described by Sambrook and Russell (30). Transformation of *S. mutans* and *E. coli* was carried out as described previously (42). To generate *E. coli* strain CS2775-KD, the *wecA* gene with a Tn 10 insertion was transferred from *E. coli* strain 21548 to *E. coli* strain CS2775 by P1 transduction (24).

DNA amplification. To improve the fidelity of the PCR, we used KOD DNA polymerase (Toyobo Co., Ltd., Osaka, Japan). PCR was performed with 0.05 U of KOD DNA polymerase/ml in 120 mM Tris-HCl buffer (pH 8.2) containing appropriate amounts of the primers, a 0.2 mM concentration of each deoxyribonucleoside triphosphate, 6 mM ammonium sulfate, 10 mM KCl, 1 mM MgCl₂, 0.1% Triton X-100, and 0.001% bovine serum albumin. The reactions were carried out for 25 cycles under the following conditions: denaturation at 94°C for 15 s, annealing at 58°C for 2 s, and extension at 74°C for 30 s.

LPS analysis. The proteinase K digestion method of Hitchcock and Brown (8) was used for the preparation of LPS from *E. coli*. Briefly, whole cells of *E. coli* were treated with 2% (wt/vol) sodium dodecyl sulfate (SDS) and 4% (vol/vol) 2-mercaptoethanol at 100°C for 10 min and then digested with proteinase K at 60°C for 1 h. The LPS samples were analyzed by standard glycine-SDS-polyacrylamide gel electrophoresis (PAGE) with 12.5% gels (16) or by Tricine-SDS-PAGE with 18% Tricine gels (17). The separated LPS was visualized by silver staining as described previously (35). In addition, LPS separated by SDS-PAGE was transferred to a nitrocellulose sheet by the Western blotting technique (34).

After transfer, immunoblots were incubated with serotype c-specific rabbit antiserum (38), rhamnan-specific rabbit antiserum raised against whole cells of *S. mutans* Xc31 (44), or monoclonal antibody to O9a antigen (41). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Zymed Laboratories, South San Francisco, Calif.) or alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Zymed). Prestained protein standards (Bio-Rad Laboratories, Richmond, Calif.) were used as molecular weight standards.

ELISA. Cell surface localization of the polymer produced in *E. coli* was estimated by an enzyme-linked immunosorbent assay (ELISA). Whole cells of *E. coli* strains were washed with phosphate-buffered saline (PBS) (pH 7.4), and the cell suspension was adjusted with PBS to an optical density at 600 nm (OD₆₀₀) of 0.3. The cell suspension was added to each well (50 µl per well) of a high-binding microtiter plate (Immunoplates I; Nunc, Roskilde, Denmark). After incubation at 4°C for 12 h, the plate was washed three times with PBS containing 0.05% Tween 20 and blocked with PBS–0.05% Tween 20 containing 1% bovine serum albumin at room temperature for 4 h. The coated plate was incubated at 37°C for 1 h with rhamnan-specific rabbit antiserum or preimmune serum that had been diluted 1:5,000 with PBS (50 µl per well). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Zymed) followed by the addition of *p*-nitrophenyl phosphate substrate solution (1 mg/ml). After incubation at 37°C for 30 min, the OD₄₀₅ was measured with a microplate reader (Bio-Rad).

Sugar composition of the LPS core oligosaccharide. LPS was extracted from lyophilized *E. coli* cells by the hot phenol-water extraction procedure; treated with DNase, RNase, and proteinase K; washed extensively with distilled water; and subjected to ultracentrifugation (39). The core oligosaccharide was detached from lipid A by hydrolysis with 1.5% (vol/vol) acetic acid at 100°C for 2 h. Nonsolubilized lipid A was separated by centrifugation. The supernatant, which contained the core oligosaccharide, was passed through a cation-exchange column (Shodex SUGAR KS-802; Showa Denko, Tokyo, Japan) with water as the eluent. The core oligosaccharide-containing fractions were collected and lyophilized.

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pACYC184	Tc ^r Cm ^r ; cloning vector	12
pBluescript II KS(+)	Ap ^r ; phagemid cloning vector	Stratagene
pNKB26	pACYC184 containing <i>E. coli</i> O9 <i>rfb</i>	12
pYT10	Em ^r ; p15A replicon	44
pSR4	pBluescript II KS(+) containing 1.6-kb <i>Sma</i> I- <i>Hinc</i> II fragment which carries <i>S. flexneri</i> 2aYSH6200 <i>rmlD</i>	38
pKU58	pBluescript II KS(+) containing 1.9-kb <i>Pst</i> I- <i>Eco</i> RI fragment which carries <i>Xc</i> <i>rgpG</i>	41
pRGP1	Em ^r ; marker rescue plasmid of 10.0-kb <i>Bst</i> EII fragment of <i>Xc</i> 47 chromosomal DNA which includes <i>rmlD</i> through <i>rgpF</i>	This study
pRGP11	pRGP1 with a deletion of 0.2 kb in the <i>rgpA</i> gene	This study
pRGP12	pRGP1 with a deletion of 0.45 kb in the <i>rgpB</i> gene	This study
pRGP13	pRGP1 with a deletion of 0.8 kb in the <i>rgpCD</i> genes	This study
pRGP14	pRGP1 with a deletion of 0.7 kb in the <i>rgpE</i> gene	This study
pRGP15	pRGP1 with a deletion of 1.35 kb in the <i>rgpF</i> gene	This study
pRGP16	pRGP1 with a deletion of 1.6 kb in the <i>rgpAB</i> genes	This study
pRGP17	pRGP1 with deletions of 0.2 kb in the <i>rgpA</i> gene and 1.35 kb in the <i>rgpF</i> gene	This study
pRGP18	pRGP1 with deletions of 1.6 kb in the <i>rgpAB</i> genes and 1.35 kb in the <i>rgpF</i> gene	This study
pRGPA	pSR4 containing a 1.1-kb PCR fragment of the <i>rgpA</i> gene	This study
pRGPB	pSR4 containing a 1.0-kb PCR fragment of the <i>rgpB</i> gene	This study
pRGPF	pSR4 containing a 1.8-kb PCR fragment of the <i>rgpF</i> gene	This study
pRGPCD	pSR4 containing a 2.1-kb PCR fragment of the <i>rgpCD</i> genes	This study
pRGPAAB	pSR4 containing a 2.1-kb PCR fragment of the <i>rgpAB</i> genes	This study
pRGPAF	pSR4 containing a 1.1-kb PCR fragment of the <i>rgpA</i> gene and a 1.8-kb PCR fragment of the <i>rgpF</i> gene	This study
pRGPAABF	pSR4 containing a 2.1-kb PCR fragment of the <i>rgpAB</i> genes and a 1.8-kb PCR fragment of the <i>rgpF</i> gene	This study

ilized. Characterization of neutral sugars was performed by high-pressure liquid chromatography (HPLC) with TSK-gel SugarAXG (Tosoh, Tokyo, Japan) following hydrolysis in 2 M trifluoroacetic acid at 100°C for 6 h (10). The buffer used for HPLC was 0.5 M potassium tetraborate buffer (pH 8.7). The flow rate was 0.4 ml/min, and the column was operated at 70°C. Characterization of amino sugars was performed by HPLC with TSK-gel SCX (Tosoh) following hydrolysis in 4 M hydrochloric acid at 100°C for 6 h (9). The buffer used for HPLC was 0.04 M sodium tetraborate buffer (pH 7.6). The flow rate was 0.3 ml/min, and the column was operated at 60°C. Monosaccharides in the eluate were monitored fluorometrically at 320 nm (excitation) and 430 nm (emission) by postcolumn labeling with L-arginine (23).

RESULTS AND DISCUSSION

Analysis of polymer production in *E. coli* harboring pRGP1.

In this study, we examined whether the six *rgp* genes (*rgpA* to *rgpF*), which seem to be involved in RGP assembly, could produce RGP in *E. coli*. The plasmid pRGP1, which contains six *rgp* genes in addition to *rmlD*, was constructed by *Bst*EII digestion of chromosomal DNA from *S. mutans* Xc47 followed by self-ligation, because the excised target fragment includes the erythromycin resistance gene and a p15A replicon in the region immediately downstream from *rgpF* (Fig. 1). The plasmid was introduced into *E. coli* K-12 strain AB1133, and polymer production by the transformant was examined by using SDS-PAGE and silver staining. *E. coli* K-12 is naturally capable of synthesizing UDP-glucose by *galU* function, but some strains, such as AB1133, cannot form dTDP-rhamnose because of a mutation in *rmlD* that encodes the enzyme catalyzing the last step in dTDP-rhamnose synthesis (14). Polymer synthesis in AB1133 harboring pRGP1 (KD401) was recognized by SDS-PAGE (Fig. 2A, lane 1), but it was not observed in AB1133 (Fig. 2A, lane 2). Although the synthesized polymer did not react with serotype c-specific rabbit antiserum in Western blot analysis (data not shown), it reacted with a rhamnan-

specific rabbit antiserum (Fig. 2B, lane 1). Because the antigenicity of *S. mutans* RGP is determined by glucose side chains, the polymer produced in *E. coli* probably represents an α 1,2- and α 1,3-linked rhamnosyl polysaccharide that lacks glucose side chains. It was previously assumed that *rgpE* encoded a glucosyltransferase for side-chain formation (44). The present results suggest either that *rgpE* is nonfunctional in *E. coli* or that the formation of glucose side chains requires an additional gene besides *rgpE*.

Rhamnan localization. We examined the localization of rhamnan in the *E. coli* transformants by an ELISA. Cells of KD401 reacted with rhamnan-specific rabbit antiserum but not with preimmune serum, and those of AB1133 did not react with either antiserum (Table 3). These results suggested that rhamnan was localized on the cell surface of the transformant. It seemed likely that rhamnan was produced as an O-polysaccharide component of the LPS in KD401. To confirm this hypothesis, the LOS profiles of the *E. coli* transformants were examined by high-resolution SDS-PAGE (Tricine-SDS-PAGE). The LOS of AB1133 consisted of three sharp bands of approximately equal intensities (Fig. 3, lane 1), whereas AB1133 transformed with pNKB26 (KD501), which synthesized typical O polysaccharide, produced a single prominent LOS band (Fig. 3, lane 2). The LOS profile of KD401 was similar to that of KD501 (Fig. 3, lane 3). The *E. coli* LOS was modified when pRGP1 was introduced into AB1133, suggesting that rhamnan was added to the lipid A core as an O polysaccharide in *E. coli*.

Complementation of the *E. coli* *wecA* mutant with *rgpG*. The *wecA* gene is known to initiate the syntheses of enterobacterial common antigen and some O polysaccharides by catalyzing the transfer of *N*-acetylglucosamine to the lipid carrier, undecaprenol phosphate, in *E. coli*. We investigated whether the syn-

FIG. 1. Restriction map of the *rgp* locus of *S. mutans* Xc. The arrows indicate the locations of the eight open reading frames (ORFs). The pResYT10 integration site for the insertional inactivation of ORF7 is indicated by the inverted open triangle. The lines in the lower portion of the diagram represent pRGP1 and its deletion derivatives. The boundaries of the internal deletions in pRGP11, pRGP12, pRGP13, pRGP14, pRGP15, pRGP16, pRGP17, and RGP18 are indicated.

thesis of rhamnan in *E. coli* required WecA. The plasmid pRGP1 was introduced into strain 21548, which is a *wecA*-defective mutant of AB1133. Strain 21548 carrying pRGP1 (KD421) did not produce rhamnan, whereas AB1133 transformed with pRGP1 (KD401) did produce rhamnan (Fig. 4, lanes 1 and 2). The *wecA* gene was also involved in the synthesis of *S. mutans* RGP in *E. coli*. Yamashita et al. previously reported functional similarities between RgpG and WecA in

the syntheses of enterobacterial common antigen and O9a antigen (41). To further confirm these similarities, both pKU58, containing the *rgpG* gene, and pRGP1 were introduced into strain 21548. Rhamnan production was observed in the resultant transformant (KD422) (Fig. 4, lane 3). As expected, the *rgpG* gene complemented the *wecA* mutation of *E. coli* for RGP synthesis.

Characterization of the *rgp* genes. There are two pathways for O-polysaccharide export across the cytoplasmic membrane in LPS synthesis. In the Wzy-dependent pathway, each O-polysaccharide repeating unit is transported across the cytoplasmic membrane by a flippase encoded by *wzx*. In the other pathway, the polymerized O polysaccharide is exported across the cytoplasmic membrane by an ATP-binding cassette (ABC) transporter. Previous studies suggested that the gene products of *rgpC* and *rgpD* were homologous to ABC transporter components and might be involved in polysaccharide export (44). To determine which pathway is used for exporting rhamnosyl polymers across the cytoplasmic membrane in *E. coli*, the *rgpC*

FIG. 2. Analysis of the polymer isolated from *E. coli* KD401(pRGP1). Plasmid pRGP1 contains *rmlD* and *rgpABCDEF* of *S. mutans*. (A) Silver-stained Tricine-SDS-PAGE gel. (B) Western immunoblot of a duplicate of the Tricine-SDS-PAGE gel shown in panel A with rhamnan-specific rabbit antiserum. Lane 1, KD401; lane 2, strain AB1133. The sizes of standard molecular mass markers are indicated to the left of each panel.

TABLE 3. Binding of rhamnan-specific rabbit antiserum and preimmune serum to *E. coli* cells

Strain	OD ₄₀₅ ^a (as determined by ELISA) with:	
	Rhamnan-specific rabbit antiserum	Preimmune serum
AB1133	0.02 ± 0.01	0.04 ± 0.01
KD401	1.48 ± 0.03	0.03 ± 0.01

^a Mean and standard deviation of triplicate determinations.

FIG. 3. Tricine-SDS-PAGE analysis of LPS samples isolated from *E. coli* K-12 strain AB1133, KD501(pNKB26), and KD401(pRGP1). LPS samples were detected by silver staining. Plasmid pNKB26 contains the genes required for O9a antigen synthesis in *E. coli*. Lane 1, strain AB1133; lane 2, KD501; lane 3, KD401. The sizes of standard molecular mass markers are indicated to the left.

and *rgpD* genes of pRGP1 were disrupted by an internal deletion, and the resultant plasmid (pRGP13) (Fig. 1) was introduced into AB1133. Western blot analysis with the rhamnan-specific rabbit antiserum revealed that the disruption of *rgpC* and *rgpD* resulted in the loss of rhamnan O polysaccharide in *E. coli* (Fig. 5, lane 4), suggesting that the latter pathway is used for rhamnan O-polysaccharide synthesis in *E. coli*. Since the first step of *N*-acetylglucosamine transfer to the lipid carrier and an ABC transporter originating from *S. mutans* are required for the synthesis of the rhamnan backbone of RGP in *E. coli*, it appears that the mechanisms for rhamnan synthesis

FIG. 4. Analysis of the effect of the *wecA* mutation on RGP synthesis in *E. coli* K-12 and complementation of the *E. coli* *wecA* mutant with *rgpG*. LPS samples were separated by SDS-PAGE and detected by immunoblotting with rhamnan-specific rabbit antiserum. Lane 1, KD401(pRGP1); lane 2, KD421(pRGP1)(*wecA*::Tn10); lane 3, KD422(pRGP1 and pKU58)(*wecA*::Tn10); lane 4, strain AB1133. The sizes (in kilodaltons) of standard molecular mass markers are indicated to the left.

FIG. 5. Effect of *rgp* gene deletions on RGP synthesis in *E. coli* K-12. LPS samples were separated by SDS-PAGE and detected by immunoblotting with rhamnan-specific rabbit antiserum. Lane 1, KD401(pRGP1); lane 2, KD411(pRGP11); lane 3, KD412(pRGP12); lane 4, KD413(pRGP13); lane 5, KD414(pRGP14); lane 6, KD415(pRGP15). Plasmids pRGP11, pRGP12, pRGP13, pRGP14, and pRGP15 are derivatives of pRGP1 that carry defects in *rgpA*, *rgpB*, *rgpCD*, *rgpE*, and *rgpF*, respectively. The sizes of standard molecular mass markers are indicated to the left.

in *E. coli* and *S. mutans* are similar, except for the final step of translocation to the lipid A core in *E. coli*.

Similarly, the contributions of other *rgp* genes to rhamnosyl polymer synthesis were assessed in the same manner by Western blot analysis. The *rgpA*, *rgpB*, *rgpE*, and *rgpF* genes of pRGP1 were individually disrupted by an internal deletion, and the resultant constructs (pRGP11, pRGP12, pRGP14, and pRGP15, respectively) (Fig. 1) were introduced independently into AB1133. A deletion in *rgpA*, *rgpB*, or *rgpF* resulted in defective rhamnan synthesis (Fig. 5, lanes 2, 3, and 6), whereas inactivation of the *rgpE* gene did not affect rhamnan synthesis (Fig. 5, lane 5). These findings corroborate previous results obtained with *S. mutans* mutants in which the corresponding *rgp* genes were disrupted (44).

The *rgp* genes are located close to each other, suggesting polycistronic transcription of these genes. To verify that the loss of rhamnan synthesis in these deletion mutants was due to the deletion in each gene and not to polar effects on the translation of the downstream genes, complementation analyses were carried out. PCR fragments containing the *rgpA*, *rgpB*, *rgpCD*, and *rgpF* genes were amplified by using the following sets of primers: for *rgpA*, 5'-TAAATGGGGGAATAGA G-3' and 5'-ACCATTTGTAGGTGGACAT-3'; for *rgpB*, 5'-A TACTTGGGAGAAGATTG-3' and 5'-CGACTAAAAAAG TCCATT-3'; for *rgpCD*, 5'-GAATCGTGCCTTTCATAC-3' and 5'-CCGACACTATATCCTATG-3'; and for *rgpF*, 5'-GA CCATTCCTACAAAAAT-3' and 5'-TTCAATTGTTTCATG ACT-3'. The PCR fragments were inserted into pSR4. The resultant plasmids, carrying the *rmlD* gene of *Shigella flexneri* and either *rgpA*, *rgpB*, *rgpCD*, or *rgpF* (pRGPA, pRGPB, pRGPCD, or pRGPF, respectively), were introduced into AB1133 harboring pRGP1 derivatives which had a deletion in the corresponding *rgp* gene (KD411, KD412, KD413, or KD415, respectively). Rhamnan syntheses in all the transformants were

FIG. 6. Analysis of the effects of *rmlD*, *rgpA*, *rgpB*, and *rgpF* on the LPS profile of CS2775. LPS samples were separated by Tricine-SDS-PAGE and silver stained. Lane 1, CS2775 (*rfaS*::*Tnlac*); lane 2, KD214(pSR4); lane 3, KD432(pRGPA); lane 4, KD433(pRGPB); lane 5, KD434(pRGPF). Plasmid pSR4 carries *S. flexneri rmlD*. Plasmids pRGPA, pRGPB, and pRGPF carry *rgpA*, *rgpB*, and *rgpF* of *S. mutans*, respectively, in addition to *S. flexneri rmlD*. The sizes of standard molecular mass markers are indicated to the left.

confirmed by using Western blot analysis with the rhamnan-specific rabbit antiserum (data not shown). The results excluded the possibility of polar effects. We concluded that all the gene products of *rgpA*, *rgpB*, and *rgpF* are responsible for rhamnosyl transfer.

Identification of the first rhamnosyltransferase. The LOS banding patterns in Tricine-SDS-PAGE are highly reproducible and provide a very sensitive assay for identifying genes involved in generating LPS heterogeneity, since a difference in the LOS profile reflects the addition or the removal of a single glycoside moiety (4, 11, 13, 29). Furthermore, the rhamnan synthetic process in *E. coli* seems to be similar to that in *S. mutans*, as described above. Therefore, we analyzed the change in the LOS banding pattern in CS2775 transformed with each of *rgpA*, *rgpB*, and *rgpF* by using Tricine-SDS-PAGE to identify the gene encoding the first rhamnosyltransferase. The LOS of AB1133 does not contain rhamnose, because the strain cannot synthesize dTDP-rhamnose. However, when *rmlD* was introduced into AB1133, a rhamnose moiety was incorporated into the LOS. This result poses the question as to whether rhamnose was incorporated into the LOS as a result of transformation with the *rgp* genes or as a result of the intrinsic rhamnosyltransferase activity of *E. coli* when dTDP-rhamnose synthesis was introduced. To resolve this issue, we used *E. coli* strain CS2775, which is defective in *rfaS*, the gene involved in the addition of rhamnose to the LOS inner core. As expected, the LOS of CS2775 was not affected by the introduction of *rmlD* (Fig. 6, lane 2).

Therefore, plasmids pRGPA, pRGPB, and pRGPF were introduced into strain CS2775, and the transformants were designated KD432, KD433, and KD434, respectively. The LOSs from these transformants were analyzed by Tricine-SDS-PAGE. The top band of the LOS from KD432 migrated slower than the top bands of the LOSs from KD433 and KD434 (Fig. 6). Assuming that the change in the banding pattern of KD432 LOS reflects the addition of rhamnose to the LOS, *rgpA* appears to encode the first rhamnosyltransferase. In this study, we discovered that a single rhamnose transferred to the *N*-acetylglucosamine residue on the lipid carrier was translocated to the periplasm, even in the absence of the *rgpC* and *rgpD* genes. This translocation might have been mediated by the enteric Wzx. Rocchetta et al. (29) detected no differences between the LOS of a wild-type *E. coli* strain and the LOSs of

TABLE 4. Sugar compositions of core oligosaccharides from CS2775 and KD432

Strain	Approx molar ratio of indicated sugar to galactose				
	Glucose	Rhamnose	Galactose	Heptose	<i>N</i> -Acetylglucosamine
CS2775	2.66	0	1.00	0.86	1.09
KD432	2.56	0.36	1.00	0.80	0.70

E. coli strains expressing *P. aeruginosa* rhamnosyltransferases in *trans*, suggesting that the enteric Wzx might not recognize partial O units containing rhamnose. The discrepancy between our results and those of Rocchetta et al. (29) might be due to the difference between D-rhamnose and L-rhamnose.

Based on the data presented above, the sugar compositions of purified core oligosaccharides from CS2775 and KD432 were determined by HPLC (Table 4). The hydrolysate of the core oligosaccharide from CS2775 contained all of the sugars (glucose, galactose, heptose, and *N*-acetylglucosamine) that are normally present in the core oligosaccharide of *E. coli* K-12 (7). In addition to these sugars, rhamnose was detected in the core oligosaccharide from KD432. These findings confirmed that the differences in LOS profiles between CS2775 and KD432 were due to the addition of a rhamnose into the LOS. Furthermore, pRGPA was introduced into CS2775-KD, which is a *wecA*-defective mutant of CS2775. No differences were observed in any of the LOS bands of KD441 and CS2775-KD (data not shown). These results suggest that RgpA is the first rhamnosyltransferase in RGP synthesis and that *N*-acetylglucosamine is required as an acceptor for rhamnose residues from dTDP-rhamnose.

We predicted that either RgpB or RgpF might transfer the second rhamnose to the initial rhamnose that was transferred by RgpA. Plasmids pRGPA, pRGPAF, and pRGPAF, derived from pSR4 carrying PCR fragments containing *rgpAB*, *rgpAF*, and *rgpABF*, respectively, were constructed in the same manner as that described above and introduced into CS2775. Contrary to our expectations, the LOS profiles of the transformants did not change as a result of the addition of *rgpB*, *rgpF*, or *rgpBF* to *rgpA* (data not shown). One explanation is that functional RgpB or RgpF was not actually expressed in the transformants. To confirm the expression of functional RgpB and RgpF in the transformants, pRGP1 derivatives in which *rgpAB*, *rgpAF*, and *rgpABF* were disrupted by internal deletions were constructed and designated pRGP16, pRGP17, and pRGP18, respectively. CS2775 cotransformed with pRGPA and pRGP16, pRGP17, and pRGP18 synthesized rhamnosyl polymers (data not shown). These complementation analyses indicated that functional RgpB and RgpF were expressed from pRGPA, pRGPAF, and pRGPAF in the CS2775 transformants. Considering the findings that a single rhamnose can be exported across the cytoplasmic membrane, even in the absence of RgpC and RgpD, and that rhamnan cannot be transported in this way, it is possible that the enteric Wzx translocates only a single rhamnose residue to the periplasm and that an ABC transporter encoded by *rgpC* and *rgpD* is required to export the oligomer composed of two or more rhamnose residues across the cytoplasmic membrane.

FIG. 7. Tricine-SDS-PAGE analysis showing the effects of deletions of *rgpA*, *rgpB*, and *rgpF*. LPS samples were detected by silver staining. Lane 1, CS2775; lane 2, KD4311(pRGP11); lane 3, KD4312(pRGP12); lane 4, KD4315(pRGP15). Plasmids pRGP11, pRGP12, and pRGP15 are derivatives of pRGP1 that carry defects in *rgpA*, *rgpB*, and *rgpF*, respectively. The sizes of standard molecular mass markers are indicated to the left.

Characterization of RgpA, RgpB, and RgpF. To determine the order of rhamnose transfer during rhamnosyl polymer synthesis, the LPSs from CS2775 transformants containing pRGP11, pRGP12, and pRGP15 were analyzed by Tricine-SDS-PAGE. Although none of the CS2775 transformants (KD4311, KD4312, and KD4315, respectively) produced rhamnan in the form of O polysaccharide (data not shown), distinct changes were identified in the LOSs from KD4312 and KD4315 (Fig. 7, lanes 3 and 4). KD4311 showed the same LOS gel profile as CS2775 (Fig. 7, lanes 1 and 2). This result is consistent with our conclusion that RgpA mediated the transfer of the first rhamnose to *N*-acetylglucosamine. The LOS profile of KD4312 was identical to that of KD432 (Fig. 6), indicating that RgpA is active even when RgpB is inactivated. Furthermore, the top band of the LOS from KD4315 migrated more slowly than that of the LOS from KD4312. RgpB may be the second rhamnosyltransferase, which is active in the absence of RgpF function. It is possible that the change in the LOS profile reflects the addition of a rhamnose residue to the rhamnose residue already appended to *N*-acetylglucosamine. We also introduced pRGP13 into CS2775. The LOS banding pattern of the resultant transformant (KD4313) was identical to that of KD4312 (data not shown). These results reinforce our hypotheses that the enteric Wzx translocates only a single rhamnose residue to the periplasm and that an ABC transporter encoded by *rgpC* and *rgpD* is required to export the oligomer composed of two or more rhamnose residues across the cytoplasmic membrane. Although we attempted to clarify the sugar composition of the purified core oligosaccharide from KD4315, we failed to obtain a sufficient amount of LOS, because pRGP15 unfortunately was unstable in *E. coli*. However, the introduction into *E. coli* of pRGP1, which contains pRGP15 and *rgpF*, produced rhamnan as an O-polysaccharide component of the lipid A core, suggesting that RgpF is the third rhamnosyltransferase.

Rhamnan polymerization probably occurs in the *E. coli* cytosol. The first step in rhamnan synthesis toward the production of RGP is the transfer of *N*-acetylglucosamine to the lipid carrier, undecaprenol phosphate, in a process that is probably mediated by WecA in *E. coli* and RgpG in *S. mutans*. Since the mechanisms by which polysaccharides are linked to cell surface components in gram-positive bacteria are currently poorly understood, the lipid carrier for RGP synthesis in the *S. mutans* cytosol is unknown. However, the initial steps in the process in both *E. coli* and *S. mutans* seem to be very similar, except for the lipid carrier. In the second stage, RgpA catalyzes the transfer of rhamnose to *N*-acetylglucosamine on the lipid carrier from dTDP-rhamnose. Since successive polymerization of rhamnan does not require this process, RgpA acts only at the initial stage of rhamnan chain growth. In the third stage, RgpB transfers the second rhamnose residue to a rhamnose residue on *N*-acetylglucosamine linked to the lipid carrier. Finally, RgpF catalyzes the transfer of the third rhamnose residue to the second rhamnose residue of the resultant glycolipid carrier, and RgpB and RgpF probably alternate in elongating the rhamnan chain. The polymerized products are exported across the cytoplasmic membrane by an ABC transporter encoded by exogenous *rgpC* and *rgpD*, and the endogenous *waaL* gene product transfers the polysaccharide to the lipid A core of *E. coli*. The RGP linker and the genuine enzyme of WaaL function in *S. mutans* are unknown. However, an evaluation of LOSs in *E. coli* transformants can be used to understand the synthetic mechanisms of cell wall-associated polysaccharides in gram-positive bacteria.

At present, the function of RgpE in glucose side-chain formation is still unclear. It was previously suggested that two steps were necessary for the formation of an α -linkage from an α -linked sugar donor (31). Glucose side chains of serotype c-specific RGP form α 1,2-linkages that are produced from α -linked UDP-glucose donors. A gene in addition to *rgpE* may be needed for glucose side-chain formation during RGP synthesis. Further characterization of *S. mutans* mutants that lack glucose side chains in RGP is needed to elucidate the details of glucose side-chain formation.

Streptococcus pyogenes (Lancefield group A streptococcus), the causative agent of a number of suppurative diseases that are sometimes followed by rheumatic fever or acute glomerulonephritis, possesses a rhamnan backbone that is identical to that of *S. mutans*. We investigated the existence in *S. pyogenes* of genes that are homologous to the *rgp* genes of *S. mutans* by using the BLAST program of the *S. pyogenes* genome sequencing database, which is based on the results of the streptococcal genome sequencing project, on the World Wide Web site of the University of Oklahoma Advanced Center for Genome Technology (5). *S. pyogenes* gene products that had 50% or greater identity to the *rgp* gene products of *S. mutans* were identified. This finding strongly suggests that the processes of rhamnan synthesis in *S. pyogenes* and *S. mutans* may be similar. The work presented here may be useful in elucidating the mechanisms of streptococcal rhamnan synthesis.

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