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Salmonella typhimurium LPS Confers Its Resistance to Antibacterial Agents of Baicalin of *Scutellaria baicalensis* George and Novobiocin: Complementation of the *rfaE* Gene Required for ADP-L-*glycero*-D-*manno*-heptose Biosynthesis of Lipopolysaccharide

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Abstract The antibacterial mechanism of enterobacter *Salmonella typhimurium* was studied. The *rfa* (*Waa*) gene cluster of *S. typhimurium* encodes the core oligosaccharide biosynthesis of lipopolysaccharide (LPS). Among the *rfa* gene cluster, we recently cloned the *rfaE* gene, which is involved in ADP-L-*glycero*-D-*manno*-heptose biosynthesis. The *rfaE* mutant synthesizes heptose-deficient LPS, which consists of only lipid A and 3-deoxy-D-*manno*-octulosonic acid (KDO), thus making an incomplete LPS and a rough phenotype mutant. *S. typhimurium* deep-rough mutants with the heptose region of the inner core show a reduced growth rate, sensitivity to high temperature, and hypersensitivity to hydrophobic antibiotics such as baicalin isolated from the medicinal herb of *Scutellaria baicalensis* Georgi. Thus, in this study, the cloned *rfaE* gene was added to the *S. typhimurium rfaE* mutant strain SL1102 (*rfaE543*), which makes heptose-deficient LPS and has a deep-rough phenotype. The complementation created a smooth phenotype in the SL1102 strain. The sensitivity of SL1102 to bacteriophages was also recovered to that of wild-type strain, indicating that LPS is used as the receptor for bacteriophage infection. The permeability barrier of SL1102 to hydrophobic antibiotics such as novobiocin and baicalin was restored to that of the wild-type, suggesting that antibiotic resistance of the wild-type strain is highly correlated with their LPS. Through an agar diffusion assay, the growth-inhibition activity of baicalin was fully observed in the mutant SL1102 strain. However, only a half of the inhibitory activity was detected in the *rfaE*-complemented SL1102 strain. Furthermore, the LPS produced by the *rfaE*-complemented SL1102 strain was indistinguishable from LPS biosynthesis of smooth strains.

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Key words: Bacterial resistance, baicalin, *rfaE* gene, *Salmonella typhimurium*, core oligosaccharide, lipopolysaccharide (LPS), ADP-L-*glycero*-D-*manno*-heptose, complementation

The lipopolysaccharide (LPS) of *Salmonella typhimurium* functions as an important virulence determinant in salmonella infections [18]. *Salmonella* LPS is involved in immune evasion, attachment to epithelial cells, and bactericidal antibodies. LPS consists of lipid A and an oligosaccharide core domain as an outer membrane component in enteric and nonenteric Gram-negative bacteria [20]. Lipid A of *S. typhimurium* consists of five to seven saturated fatty acids attached to a β-1,6-linked glucosamine disaccharide (Fig. 1). This is attached to the inner core with two 3-deoxy-D-*manno*-octulosonic acid (ketodeoxyoctonate; KDO) units followed by two units of heptose; the outer core region and the O-antigen are attached to one of the heptose units. LPS activates the complement, and some forms are potent toxins, leading to LPS often being

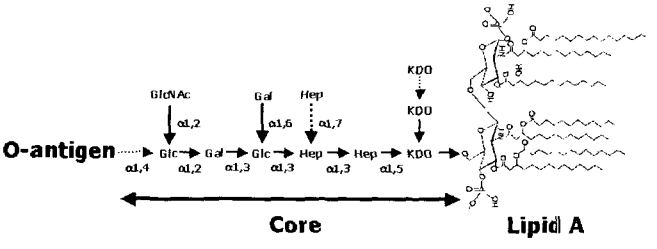

Fig. 1. Schematic illustration of the structure of *S. typhimurium* LPS.
Abbreviations: Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-octulosonic acid. Possible partial substitutions are indicated by dashed arrows.

called an endotoxin [15]. Mutants, which are lacking the O-antigen and the outer core components, are nonvirulent. However, deep-rough mutants affected in the heptose region of the inner core often show a reduced growth rate, sensitivity to elevated temperature, and hypersensitivity to detergents [3].

The *rfa* (*Waa*) genes, which encode the LPS core biosynthesis enzyme, are present as a cluster on *E. coli* chromosomes, and several *rfa* genes have been cloned and characterized. Mutants have been used to identify at least five genes, *rfaC*, *rfaD*, *rfaE*, *rfaF*, and *rfaP* of *S. typhimurium*, which are involved in the synthesis of the inner core [23]. Until now, although the *rfaE* gene of *S. typhimurium* has not been cloned, the putative *rfaE* mutant synthesized heptose-deficient LPS, having only lipid A and KDO [14, 23].

Medicinal plants have been used as traditional remedies for hundreds of years, and they are also the important medicinal herbs widely used for the treatment of various inflammatory diseases and diarrhea in China, Taiwan, Japan, and Korea [4]. These plants have been reported to contain a large number of flavonoid and other constituents, including phenethyl alcohols, sterols, essential oils, and amino acids. *Scutellaria baicalensis* Georgi is an important medicinal herb widely used for the treatment of various inflammatory diseases, hepatitis, tumors, and diarrhea in East Asian countries such as China, Korea, Taiwan, and Japan [4]. *S. baicalensis* Georgi produces a potent antibacterial agent, baicalin, which is a flavonoid and has been reported to have an antiviral effect by the inhibition of reverse transcriptase [2]. Baicalin is a glucuronic compound of baicalein which is also a component of Sho-saiko-to. Both baicalin and baicalein have been reported to induce bacterial cell death or necrosis [6], however, the precise mechanism of the flavonoid-induced cell death has not been elucidated.

In a previous paper, we reported the first cloning of the *rfaE* gene, which is involved in ADP-L-*glycero*-D-*manno*-heptose biosynthesis from *S. typhimurium* [11]. The cloned *rfaE* gene encoded a polypeptide of 477 amino acid residues with a molecular weight of 53 kDa. In the present study, the author reports a functional analysis of LPS, and examination of the bacterial resistance of two well-known agents of baicalin and novobiocin and bacteriophage infection into the strain using complementation with the *S. typhimurium* *rfaE* gene.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions

The bacterial strains, phages, and plasmids used in this study are described in Table 1. *Salmonella* strains and their phages were generously supplied by Dr. Ken Sanderson, *Salmonella* Stock Center (SSC), University of Calgary, Calgary, Alberta, Canada. *E. coli* strains were grown in LB medium, and *S. typhimurium* (wild-type) strains were grown in tryptic soy broth (TSB, Difco) or brilliant green agar (BGA, Difco) containing 25 μg/ml novobiocin (Sigma) and nalidixic acid (Sigma) at 37°C. *S. typhimurium* LT2 mutants were grown in brain heart infusion medium (BHI, Difco) without antibiotics [22]. The test for sensitivity to LPS-specific phages was performed by applying 0.1 μl of each phage stock suspension to lawns of *S. typhimurium* strains.

Table 1. Bacterial strains, plasmids, and bacteriophages.

Strain or plasmid	Genotype or characteristic[a]	Source or reference
S. typhimurium	Wild-type	MHWKG[b]
S. typhimurium LT2		
SL3770	*rfa*$^+$	SGSC[c]
SL1102	*rfaE543 metA22 trpC2 H1-b H2-e, n, x fla-66 rpsL120 xyl-404 metE551*	SGSC
SL3019	*rfaE827 rfaL446 SD14 (E1) azi gal rha his*	SGSC
SA1377	*rfaC630(P22)*$^+$	SGSC
SL3600	*rfaD657 metA22 trpC2 H1-b H2-e, n, x fla-66 rpsL120 xyl-404 metE551*	SGSC
Phage		
P22.c2	Smooth-specific phage of *S. typhimurium*	SGSC
Felix-O	Smooth-specific phage of *S. typhimurium*	SGSC
Ffm	Rough-specific phage of *S. typhimurium*	SGSC
Br60	Rough-specific phage of *S. typhimurium*	SGSC
Plasmid		
pHEPs	2.6-kb DNA from *S. typhimurium* in pUC19, Amp^r	This study
pHEPs-1	2.2-kb *Dra*I-*Hin*dIII fragment of pHEPs cloned into pUC19	This study

[a]Abbreviations for antibiotics: Amp, ampicillin; Kan, kanamycin.

[b]MHWKG, Ministry of Health and Welfare of Korean Government.

[c]SGSC, *Salmonella* Genetic Stock Center, University of Calgary, Alberta, Canada.

Isolation of Baicalin from *S. baicalensis* Roots

Baicalin was isolated from the herbal plants of *S. baicalensis* Georgi roots (5 kg) that were cut into small pieces, immersed in, and extracted with acetone (50 l×2) at room temperature for two weeks. After filtration, the residues were then reflux-extracted with 50% aqueous ethanol (20 l×2). Acetone and 50% aqueous ethanol extracts were concentrated under reduced pressure to 1 and 5 l, respectively. Ethanol was added to the concentrated 50% aqueous ethanol extracts, and a large amount of yellow precipitate were produced. A portion of the precipitate (5.0 g) was recrystallized from aqueous ethanol to obtain baicalin (3.6 g). The acetone extracts were subjected to column chromatography on silica gel (10 cm i.d.×30 cm) eluted with $CHCl_3$ and $CHCl_3$-MeOH (10:1 to 1:1 gradient) to obtained ten fractions. $CHCl_3$ eluate was coated with Celite 545 (Merck) and rechromatographed on silica gel (2 cm i.d.×30 cm) by eluting with hexane-acetone (10:1 to 2:1 gradient) to yield some baicalin (1.5 g). A portion of the $CHCl_3$-MeOH (10:1) eluate (4 g) was subjected to gel permeation chromatography on Sephadex LH-20 (2 cm i.d.×45 cm) by eluting with MeOH to yield baicalein (2.5 g). Each compound was identified by direct comparison of its spectroscopic data with authentic samples.

Purity tests of baicalin and baicalein were performed by HPLC equipped with a 280-nm detector and LiChrospher 100 RP-18e column (4 mm i.d.×125 mm). The mobile phase was composed of CH_3CN-0.1 M H_3PO_4 (28:72), and the flow rate was 1.0 ml/min. The purity of all compounds was more than 99.5%.

DNA Manipulations

Restriction enzymes, alkaline phosphatase (calf intestinal), and T4 DNA ligase were purchased from Promega, and standard DNA recombinant procedures were performed as described previously [19]. The plasmid DNAs were resolved on a 1.0% agarose gel and purified by using Geneclean II Kit (BIO 101, Inc.) [21]. The plasmids were transformed in *E. coli* and purified by the alkaline lysis method [19]. Transformation of *E. coli* strains with plasmid DNA was routinely done by the $CaCl_2$ method [8]. *Salmonella* strains were transformed by electroporation [1].

Complementation of *S. typhimurium rfa* Mutants

Overnight cultures of the *S. typhimurium rfa* mutants were inoculated into 50 ml of fresh BHI medium and grown at 37°C with vigorous shaking to an optical density of 0.5 at 600 nm. The cells were chilled on ice and centrifuged. The pellets were washed twice with ice-cold glycerol-water and resuspended with an equal volume of 15% glycerol-water (vol/vol) to that of the pellet.

Fifty microliters of the cells were electroporated with 10 ng of plasmid library DNA by using a Gene Pulser II Electroporation system (Bio-Rad), incubated in 1 ml of BHI medium at 37°C for 4 h with shaking, and then spread on BHI plates containing 50 μg/ml of ampicillin and 25 μg/ml of novobiocin. Plasmid DNA was purified from each transformant and retransformed to the mutant strain to confirm the complementation. The transformants were also tested for phage sensitivities and LPS phenotype.

Agar Diffusion Assay for Antibacterial Susceptibility Test

The bacteriostatic activity of baicalin was assayed as described [7]. Approximately 500 μl of the diluted cell suspension (1×10^6 cells/ml) was evenly spread on TSB agar plates and dried at 37°C in an incubator. Agar plates, in which holes with a diameter of 5 mm were made, were used for the diffusion assay using various concentrations of baicalin. The plates were then placed on an incubator. After incubation at 37°C for 16 h, the zone of inhibition around the hole was measured.

LPS Gel Analysis

S. typhimurium LPS was prepared from proteinase K-treated whole-cell lysates [10], separated on a 14% polyacrylamide gel containing sodium dodecyl sulfate (SDS) [13], and visualized by silver staining as described previously [24].

RESULTS

Functional Analysis of the Mutant Strain of *S. typhimurium* by Complementation with *rfaE* Gene

Previously, a plasmid carrying the *rfaE* gene was isolated from *S. typhimurium* genomic DNA using the *rfaE* mutant of *S. typhimurium* SL1102 [11], which is defective in ADP-heptose synthesis [23, 25], resulting in an incomplete LPS core. Two open reading frames (ORFs) were found. One of these ORFs encoded a polypeptide of 477 amino acid residues. The molecular weight predicted from the nucleotide sequence is 53-kDa. The expression of the genes on pHEPs with a Lac or T7 promoter, capable of transcribing this ORF in the direction inferred from the DNA sequence, yielded a 53-kDa protein. It is inferred that the other ORF terminates at base 786 and is partially homologous with the *E. coli* hypothetical 60.7-kDa protein in the GLGS-WAAE intergenic region.

Transformants carrying the *rfaE* gene showed wild-type LPS that are less permeable and thus more resistant to hydrophobic antibiotics than mutant strains with a defective LPS core structure. When the cells were plated on an BHI plate containing ampicillin (50 μg/ml), a hydrophobic antibiotic novobiocin (50 μg/ml), and baicalin (100 μg/ml), all of the transformants grew in the presence of novobiocin or baicalin, indicating that the plasmid carried a gene conferring resistance to novobiocin or baicalin (Table 2).

Table 2. Phage sensitivity and novobiocin resistance of *S. typhimurium* LT2 *rfa* mutants complemented with various subcloned plasmids.

Strain	Partial genotype	Plasmid	Phage sensitivity[a]			Novobiocin[b] (μg/ml)		Baicalin (μg/ml)	
			P22.c2	Felix-O	Br60	25	75	50	100
SL3770	*rfa*$^+$		+	+	-	r	r	r	r
SL1102	*rfaE543*		-	-	+	s	s	s	s
SL1102	*rfaE543*	pHEPs	+	+	-	r	r	r	r
SL1102	*rfaE543*	pHEPs-1	-	+	-	r	r	r	r
SL3019	*rfaE827rfaL446*		-	-	+	s	s	s	s
SL3019	*rfaE827rfaL446*	pHEPs	-	+	+	r	s	r	s
SL3019	*rfaE827rfaL446*	pHEPs-1	-	+	+	r	s	r	s
SA1377	*rfaC630*		-	-	+	s	s	s	s
SA1377	*rfaC630*	pHEPs	-	-	+	s	s	s	s
SA1377	*rfaC630*	pHEPs-1	-	-	+	s	s	s	s
SL3600	*rfaD657*		-	-	+	s	s	s	s
SL3600	*rfaD657*	pHEPs	-	-	+	s	s	s	s
SL3600	*rfaD657*	pHEPs-1	-	-	+	s	s	s	s

[a]+, sensitive; -, resistant.

[b]s, sensitive; r, resistant. Phage P22.c2 requires O-antigen, Felix-O requires a complete core, and Br60 recognizes inner core structure.

Antibacterial Activity of Baicalin Examined by Using an Agar Diffusion Assay

The chemical structure of baicalin is shown in Fig. 2. This compound is a flavonoid. The extraction and isolation of the compound from the herbal *S. baicalensis* was described in Materials and Methods, and the purity of each compound was more than 99.5%, by HPLC analysis (data not shown).

As a fast and reliable method to determine the effectiveness of antibacterial activity, an agar diffusion analysis was used to test the growth-inhibition activity of baicalin. As shown in Fig. 3, a clear zone around the disk was distinct, where baicalin had been dropped, but a high concentration (at least 2 times the amount) of baicalin to the *rfaE*-complemented SL1102 strain was required in order to obtain a zone comparable in size to that of the *rfaE*-mutant SL1102 strain.

Recovery of Sensitivity to the LPS-Specific Phages

Colonies were also tested for sensitivity to the LPS-specific phages. They were all resistant to the rough-specific phage Br60 and sensitive to the smooth-specific phages, Felix-O and P22.c2 (Table 2), indicating that they synthesized a complete LPS core structure and O-antigen repeating units. When another *rfaE* mutant strain, SL3019, was also used, SL3019 carrying pHEPs became sensitive to phage P22.c2 and Felix-O, but also retained its sensitivity to Br60. Two other *Salmonella* LPS mutant strains, SA1377 and SL 3600, have the same LPS phenotype as SL1102 (i.e., they make heptoseless LPS), but cause mutations in other genes, such as *rfaC* and *rfaD*, which encode ADP-heptose:lipopolysaccharide heptosyltransferase I and ADP-L-*glycero*-D-manno-heptose-6-epimerase, respectively [6, 23]. When these strains were transformed with pHEPs, neither

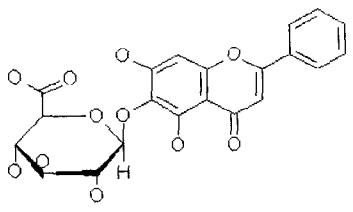

Fig. 2. The chemical structure of baicalin flavonoid.

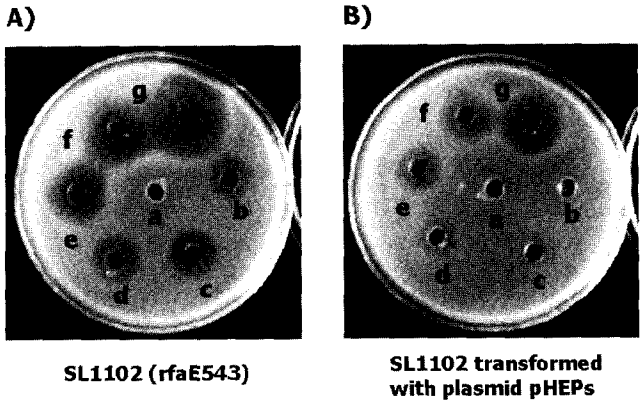

Fig. 3. Agar diffusion assay showing antibacterial activity of baicalin.

A) *S. typhimurium* SL1102 (*rfaE543* mutant) treated with various concentrations of baicalin. B) *S. typhimurium* SL1102 (*rfaE543* mutant) complemented with pHEPs carrying the *rfaE* gene was treated with various concentrations of baicalin. a, control without baicalin; b, 5 μg/ml of baicalin; c, 10 μg/ml of baicalin; d, 20 μg/ml of baicalin; e, 30 μg/ml of baicalin; f, 50 μg/ml of baicalin; g, 100 μg/ml of baicalin.

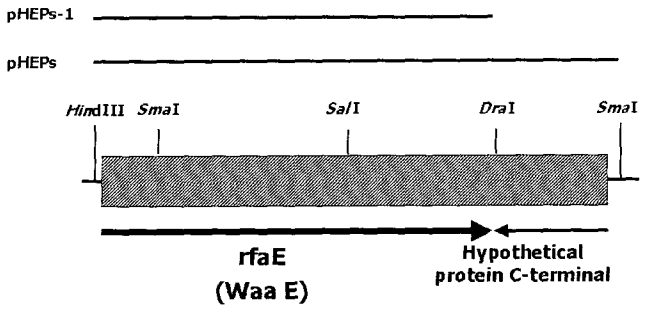

Fig. 4. Schematic structure of the *rfaE* gene of *S. typhimurium*. Plasmids of pHEPs-1 and pHEPs carrying the *rfaE* gene were illustrated. The coding regions are marked by bold arrows. Thin arrows indicate the hypothetical protein C-terminal region.

of them was complemented, as determined by phage sensitivity (Table 2).

LPS Phenotypes of the *rfaE* Mutant Complemented with pHEPs Carrying the *S. typhimurium rfaE* Gene

Using plasmids, designated pHEPs and pHEPs-1 (Fig. 4), LPS gel analysis confirmed that the LPS of SL1102 complemented with plasmid pHEPs was converted to the wild-type phenotype (Fig. 5). When analyzed by SDS-PAGE followed by silver staining (Fig. 5), LPS from SL3770, which is *rfa*$^+$, formed the ladder-like pattern indicative of the presence of the O-antigen repeat units (Fig. 5, lane 1); LPS from SL1102 contained very-fast-migrating bands representing a heptose-deficient incomplete core structure (lane 2); SL1102 complemented with pHEPs showed the LPS which migrated in a pattern similar to that obtained with the LPS of the wild-type strain (lane 3). The LPS pattern of SL1102 complemented with pHEPs-1 was the same as that of SL1102 complemented with pHEPs (data not shown).

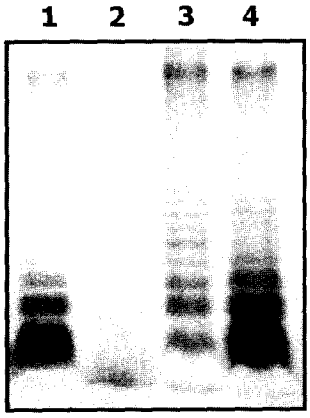

Fig. 5. Silver-stained LPS gel showing complementation of SL1102 (*rfaE543*) by plasmid carrying the *S. typhimurium rfaE* gene. Lane 1, SL3770 (*rfa*$^+$); Lane 2, SL1102 (*rfaE543*); Lane 3, SL1102 transformed with plasmid pHEPs-1; Lane 4, SL1102 transformed with plasmid pHEPs.

DISCUSSION

Previously, we cloned the *rfaE* gene which is involved in the synthesis of ADP-heptose [11] of *S. typhimurium*. In the present study, I examined the functional activity of the *rfaE* gene in antibiotic resistance and bacteriophage infectivity of the strain.

In the LPS of Enterobacteriaceae, the functions of the genes of the core oligosaccharide biosynthesis have been deduced from genetic studies of LPS [12], and 5 genes, *rfaC*, *rfaD*, *rfaE*, *rfaF*, and *rfaP*, have been suggested to function in the synthesis of the inner core region. Two genes, *rfaD* and *rfaP*, have been identified for heptose-epimerase and heptose-phosphorylase, respectively. Two others, *rfaC* and *rfaF*, were identified for heptosyltransferase which transfers heptose from ADP-heptose to the inner core. The core oligosaccharide region is not generally considered to be a virulence factor per se. But due to its virulence, it has been suggested to play an indirect role in providing the attachment site for O-antigen (polysaccharide) and a crucial role in establishing the essential barrier function of the outer membrane [9, 14].

Although bacterial LPS genetics is now well established, nothing is known on the specific synthesis steps controlled by *rfaE* [12]. However, recent reports have implicated the core oligosaccharide in the adhesion of bacteria to host cells, the high degree of structural conservation, and the adaptation away from high temperatures and hydrophobic antibiotics [9]. The *rfaE* gene produces ADP-heptose, the substrate of heptosyltransferase. The heptose region of the core oligosaccharide is known to be important for outer membrane stability in *Salmonella*. The phosphorylation of HepI and HepII may be involved in both the cross-linking of adjacent LPS, and the interaction between positively charged groups on proteins [9]. The significant compositional and structural changes in the outer membrane and the pleiotropic phenotype result from inability to synthesize or incorporate Hep or from the loss of phosphoryl derivatives [17, 20]. This increase results in the sensitivity to compounds such as baicalin and novobiocin by the changes on the outer membrane and release of periplasmic enzymes into the medium [16, 26].

In conclusion, SL1102 (*rfaE543*), which makes heptose-deficient LPS, has a deep-rough phenotype, but pHEPs and pHEPs-1 complemented the *rfaE543* mutation to give the smooth phenotype, suggesting that the complete biosynthesis of LPS was closely linked to the following biological activities of the *Salmonella* strain. Thus, the present results can be summarized as follows: (1) the sensitivity of SL1102 to bacteriophages (P22.c2, Felix-O, Br60), which use LPS as their receptor for absorption, was changed to that of the wild-type strain; (2) the permeability barrier of SL1102 to antibiotics novobiocin and baicalin was restored to that of the wild-type. These results suggested

that the heptose regions of the LPS molecule may be important therapeutic targets in bacteria, and that the biosynthetic processes that modify the heptose region of the core oligosaccharide are definitely worth for further investigation.

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