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Molecular cloning and functional expression of the *rfaE* gene required for lipopolysaccharide biosynthesis in *Salmonella typhimurium*

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The *rfaE* (*WaaE*) gene of *Salmonella typhimurium* is known to be located at 76min on the genetic map outside of the *rfa* gene cluster encoding core oligosaccharide biosynthesis of lipopolysaccharide(LPS). The *rfaE* mutant synthesizes heptose-deficient LPS; its LPS consists of only lipid A and 3-deoxy-D-*manno*-octulosonic acid (KDO), and the *rfaE* gene is believed to be involved in the formation of ADP-L-*glycero*-D-*manno*-heptose. Mutants, which make incomplete LPS, are known as rough mutants. *Salmonella typhimurium* deep-rough mutants affected in the heptose region of the inner core often show reduced growth rate, sensitivity to high temperature and hypersensitivity to hydrophobic antibiotics. We have cloned the *rfaE* gene of *S. typhimurium*. The chromosomal region carrying this gene was isolated by screening a genomic library of *S. typhimurium* using the complementation of *S. typhimurium rfaE* mutant. The 2.6-Kb insert in the plasmid pHEPs appears to carry a functional *rfaE* gene. SL1102 (*rfaE543*) makes heptose-deficient LPS and has a deep rough phenotype, but pHEPs complement the *rfaE543* mutation to give the smooth phenotype. The sensitivity of SL1102 to bacteriophages (P22.c2, Felix-O, Br60) which use LPS as their receptor for adsorption is changed to that of wild-type strain. The permeability barrier of SL1102 to hydrophobic antibiotics (novobiocin) is restored to that of wild-type. LPS produced by SL1102 (*rfaE543*) carrying pHEPs makes LPS indistinguishable from that of smooth strains. The *rfaE* gene encoded a polypeptide of 477 amino acid residues highly homologous to the *S. enterica rfaE* protein (98% identity), *E. coli* (93% identity), *Yersenia pestis* (85% identity), *Haemophilus influenzae* (70% identity) and *Helicobacter pyroli* (41% identity) with a molecular weight 53 kDa.

***Keywords:* *rfaE* gene, *Salmonella typhimurium*, core oligosaccharide, lipopolysaccharide (LPS), 3-deoxy-D-*manno*-octulosonic acid (KDO), ADP-L-*glycero*-D-*manno*-heptose, complementation**

Introduction

Lipopolysaccharide (LPS), a key component of the outer membrane which characterizes enteric and nonenteric gram-negative bacteria. LPS is a amphipathic molecule consisting of lipid A and and oligosaccharide core domain [1–4]. Lipid A of *Salmonella typhimurium* consists of five to seven saturated fatty acids attached to a β-1,6-linked glucosamine disaccharide (Figure 1). This is attached to the inner core composed of at least two 3-deoxy-D-*manno*-octulosonic acid (also called 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 is used as a receptor by bacteriophage; it also activates complement and some forms are potent toxins, leading to LPS often being called endotoxin [5]. Mutants, which are lacking the O antigen and the outer core components, are viable and not much reduced in growth rate in culture, though they are nonvirulent. However, deep-rough mutants affected in the heptose region of the inner core often show reduced growth rate, sensitivity to elevated temperature [6] and hypersensitivity to detergents and hydrophobic antibiotics (such as novobiocin)[7], as reported in case of *Escherichia coli* K-12 mutants lacking heptose in the LPS [8]. For other

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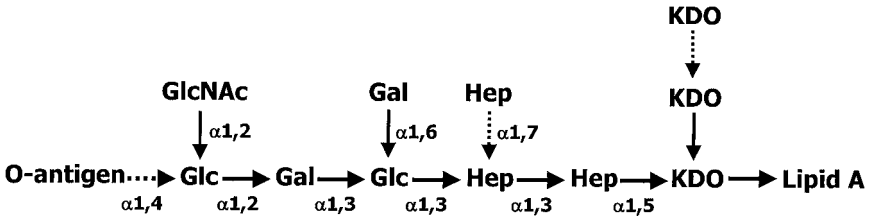

Figure 1. Schematic illustration of the structure of *S. typhimurium* LPS. Abbreviations: Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-D-*manno*-octulosonic acid. Possible partial substitutions are indicated with dashed arrows.

organisms, such *as Haemophilus influenzae*, a heptoseless mutant was found to be serum sensitive and displayed a reduced virulence in an animal model [9,10].

The *rfa* (*Waa*) gene, which encode LPS core biosynthesis enzyme, are present as a cluster on *S. typhimurium* and *Escherichi coli* K-12 chromosomes, and several *rfa* genes have been cloned and characterized. Mutants have been used to identify at least five genes of *S. typhimurium* involved in the synthesis of the inner core; these are *rfaC* (ADP-heptose:LPS heptosyltransferase I), *rfaD* (ADP-L-*glycero*-D-*manno*-heptose-6-epimerase), *rfaE* (ADP-heptose synthase), *rfaF* (ADP-heptose:LPS heptosyltransferase III), and *rfaP* (gene for heptose phosphorylation) [10]. The *rfaE* gene of *S. typhimurium* is known to be located at 76min on the genetic map outside of the *rfa* gene cluster [11]. The *rfaE* mutant synthesizes heptose-deficient LPS (i.e., its LPS consists of only lipid A and KDO), and the *rfaE* gene is believed to be involved in the formation of ADP-heptose [10,12]. Recently, it was reported that the *rfaE* gene from *E. coli* encodes a bifunctional 2 domains and Domain I involves in the synthesis of D-glycero-D-manno-heptose 1-phosphate, whereas Domain-II catalyzes the ADP transfer to form ADP-d-glycero-D-manno-heptose [13]. Up to date, *H. influenzae rfaE* gene has been cloned [12], however, cloning of the *rfaE* gene from *S. typhimurium* has not been reported yet.

In the present study, we have cloned *rfaE* gene from *S. typhimurium* by complementing *Salmonella rfaE* mutants, and thereore, we report the cloning, expression, and characterization of the *S. typhimurium rfaE* gene.

Materials and methods

Strains, plasmids, and culture conditions

The bacterial strains, phages, and plasmid used in this study are described in Table 1. Salmonella strains and their phages were gratefully supplied from 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 ug/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. The test for sensitivity to LPS-specific phages was performed by applying 0.1 ul of each phage stock suspension to lawns of *S. typhimurium* strains.

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 [14]. Transformation of *E. coli* strains with plasmid DNA was routinely done by the $CaCl_2$ method [15]; *Salmonella* strains were transformed by electroporation [16].

Construction of a genomic libaray

The chromosomal DNA from *S. typhimurium* was partially digested with *Sau*3AI and resolved on a 0.7% agarose gel. A DNA fraction ranging from 2 to 4 Kb was excised, purified by using GenecleanII Kit (BIO 101, Inc.), and ligated to *Bam*HI-digested pUC19 vector. The plasmids were transformed in *E. coli* and purified by the alkaline lysis method [14].

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 at 600 nm of 0.5. The cell were chilled on ice and centrifuged. The pellets were washed twice with ice-cold glycerol-water and resuspended with a volume of 15% glycerol-water (vol/vol) equal 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 ug/ml of ampicillin and 25 ug/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.

DNA sequencing and analysis

Construction of unidirectional deletion mutants was performed with the Erase-A-Base system (Promega) following the manufacturer's recommended procedure. DNA sequence was determined by the dideoxychain termination method [17], using

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
E. coli K-12		
JM 109	*endA*1 *recA*1 *gryA*96 *thi hsdR*17(r_k^+, m_k^+) *relA*1 *supE*44 Δ(*lac-proAB*) [F′, *traD*36 *proAB lac*^{q}Z△M15]	Progema
XL1-Blue MRF′	(mcrA)183 △(mcrCB-hsdSMR-mrr)173 *endA*1 *supE*44 *recA*1 *gryA*96 *thi*-1 *relA*1 *lac* [F′ *traD*36 *proAB lac*^{q}Z△M15 Tn*10*(Tel^r)]	Stratagene
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		
pUC19	Cloning and expression vecror, Amp^r	Gibco BRL
pET29a	Cloning and expression vecror, Kan^r	Novagen
pGEM-7Z(f$^+$)	Cloning vecror, Amp^r	Promega
pHEPs	2.6-Kb DNA from *S. typhimurium* in pUC19, Amp^r	This study
pHEPs-1	2.2-Kb *Dra*I-*Hind*III fragment of pHEPs cloned into pUC19.	This study
pHEPs-2	1.2-Kb *Sal*I-*Hind*III fragment of pHEPs cloned into pUC19.	This study
pHEPs-3	2.5-Kb *Sma*I-fragment of pHEPs cloned into pUC19.	This study
pHEPs-4	1.9-Kb *Dra*I-*Sma*I fragment of pHEPs cloned into pUC19.	This study
pHEPs-5	1.1-Kb *Sal*I-*Sma*I fragment of pHEPs cloned into pUC19.	This study
pHEPs-6	0.2-Kb *Sma*I-*Hind*III fragment of pHEPs cloned into pUC19.	This study
pUN-ctm	1.4-Kb *Sma*II-*Sal*I fragment of pHEPs cloned into pUC19.	This study
pHEPsExoI-6	ExoIII deletion mutants (400 bp ladder) of pHEPs.	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.

a Silver Sequence Kit (Promega) with pUC/M13 forward (24 mer) primer (Promega). DNA and Protein sequence information was analyzed through the National Center for Biotechnology Information, using the BLAST network service to search the Genbank database [18] and with DNAsis software (Hitachi Software Engineering Co., Ltd.).

Expression of *S. typhimurium rfaE* in *E. coli*

The *rfaE* gene was cloned in pUC19 plasmid vector. Expression of recombinant protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to cultures at an optical density at 600 nm of 0.6. After 2 h at 37°C, the cells were harvested, washed, and resuspended at 0.2 g/ml in 50 mM Tris-Hcl, freeze-thawed once, and lysed by sonication. The Insoluble proteins were obtained by centrifugation at 20,000 × g for 40 min. Most of the recombinant *rfaE* protein was precipitated in inclusion body.

Genomic Southern hybridization

S. typhimurium genomic DNA was digested with restriction enzymes, resolved on a 0.8% agarose gel, transferred to Hybond-N membrane (Amersham) by capillary blotting overnight, and coss-linked to the membrane by using a Stratalinker (Stratagene). After prehybridization, the membrane was hybridized with a digoxigenin-dUTP-labeled DNA probe and washed. The hybridized probe was detected by using a DIG Luminescent Detection Kit (Boehringer Mannheim Biochemicals).

LPS gel analysis

S. typhimurium LPS was prepared from proteinase K-treated whole-cell lysates [19], separated on a 14% polyacylamide gel containing sodium dodecyl sulfate (SDS) [20], and visualized by silver staining as described previously [21].

Nucleotide sequence accession number

The DNA sequence of the *rfaE* gene has been submitted to GeneBank, NCBI, NIH, USA and assigned accession number AF155126.

Results

Cloning of a plasmid carrying the *rfaE* gene from *S. typhimurium* genomic DNA library

A plasmid carrying the *rfaE* gene was isolated from *S. typhimurium* genomic DNA by complementing the *rfaE* mutant of *S. typhimurium* LT2. To accomplish this, the *S. typhimurium* plasmid library DNA was transformed into strain SL1102, which is *rfaE* and thus defective in ADP-heptose synthesis [10,22], resulting in an incomplete LPS core. Transformants carrying a plasmid containing the *rfaE* gene were selected on the basis of the properties of *Salmonella* strains with a wild-type LPS that are less permeable and thus more resistant to hydrophobic antibiotics than mutant strains with a defective LPS core structure [23]. Cells were plated on an BHI plate containing ampicillin (50 μg/ml) and a hydrophobic antibiotic, novobiocin (50 μg/ml). Plasmid DNA was extracted from one of the colonies and retransformed into SL1102 with selection for ampicillin and novobiocin resistance. All of the transformants tested grew in the presence of novobiocin, indicating that the plasmid carried a gene conferring resistance to novobiocin. Colonies were also tested for sensitivity to the LPS-specific phages. All of them were 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. Restriction mapping revealed that this plasmid contained an insert of 2.6-Kb, which was designated pHEPs (Figure 2). LPS gel analysis confirmed that the LPS of SL1102 complemented with plasmid pHEPs was converted to the wild-type phenotype (Figure 6).

Another *rfaE* mutant strain, SL3019, was also used to isolate the *rfaE* gene from *S. typhimurium* wild-type by the procedure described above. SL3019 transformed with pHEPs became sensitive to phage P22.c2 and Felix-O but also retained the sensitivity to Br60.

Two other *Salmonella* LPS mutant strains, SA1377 and SL 3600, have the the same LPS phenotype as SL1102, i.e., make heptoseless LPS, but have mutations in other genes, *rfaC* and *rfaD*, encoding ADP-heptose:lipopolysaccharide heptosyltransferase I and ADP-L-glycero-D-mannoheptose-6-epimerase, respectively [10,24]. When these strains were transformed with pHEPs, neither of them was complemented, as determined by phage sensitivity (Table 2).

To delimit the *rfaE* gene region, a series of subclones was made from pHEPs (Figure 2). Plasmids pUN-ctm, pHEPs-1, pHEPs-2, pHEPs-3, pHEPs-4, pHEPs-5 were generated by subcloning 1.4-Kb *Sma*I-*Sal*I, 2.2-Kb *Dra*I-*Hin*dIII, 1.2-Kb *Sal*I-*Hin*dIII, 2.5-Kb *Sal*I, 1.9-Kb *Dra*I-*Sma*I, 1.1-Kb *Sal*I-*Sma*I

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

			Phage sensitivity[a]			*Novobiocin*[b] *(μ g/ml)*	
Strain	*Partial genotype*	*Plasmid*	*P22.c2*	*Felix-O*	*Br60*	*25*	*75*
SL3770	*rfa*$^+$		+	+	−	r	r
SL1102	*rfaE543*		−	−	+	s	s
SL1102	*rfaE543*	pHEPs	+	+	−	r	r
SL1102	*rfaE543*	pHEPs-1	+	+	−	r	r
SL1102	*rfaE543*	pHEPs-2	+	+	−	r	r
SL1102	*rfaE543*	pHEPs-3	+	+	−	r	s
SL1102	*rfaE543*	pHEPs-4	+	+	−	r	s
SL1102	*rfaE543*	pHEPs-5	−	−	+	s	s
SL1102	*rfaE543*	pHEPs-6	−	−	+	s	s
SL3019	*rfaE827rfaL446*		−	−	+	s	s
SL3019	*rfaE827rfaL446*	pHEPs	+	+	+	r	s
SL3019	*rfaE827rfaL446*	pHEPs-1	+	+	+	r	s
SA1377	*rfaC630*		−	−	+	s	s
SA1377	*rfaC630*	pHEPs	−	−	+	s	s
SA1377	*rfaC630*	pHEPs-1	−	−	+	s	s
SL3600	*rfaD657*		−	−	+	s	s
SL3600	*rfaD657*	pHEPs	−	−	+	s	s
SL3600	*rfaD657*	pHEPs-1	−	−	+	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.

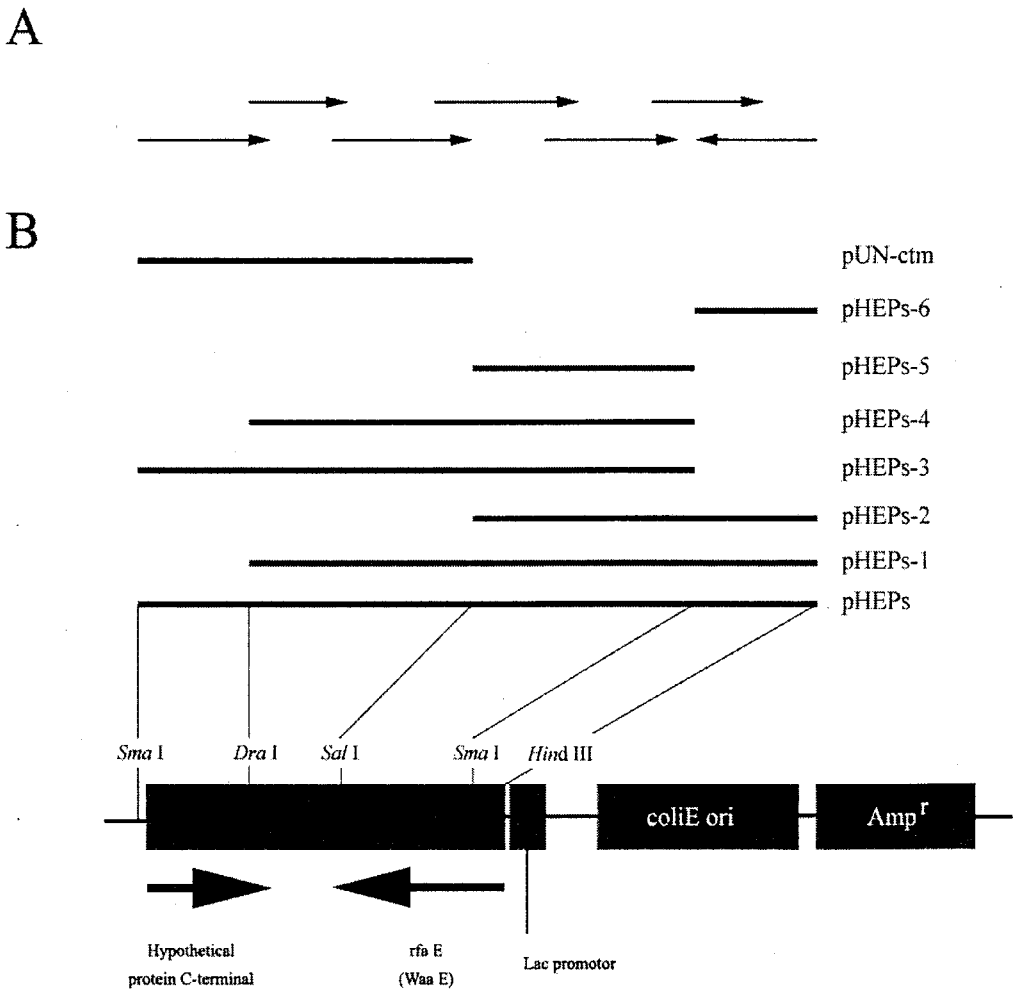

Figure 2. Restriction map of subclones (B) and sequencing strategy (A) of the *S. typhimurium rfaE* clone. The coding regions are marked by bold arrows and bold bars indicate the various subclones. Thin arrows indicate the individual sequence runs.

fragments into the vector pUC19, respectively. pHEPs-6 was made by deleting 2.5-Kb *Sma*I fragment from pHEPs. These subclones were tested for the ability to complement SL1102. SL1102 transformed with each subclone was tested for resistance to novobiocin and changes in the sensitivity to the LPS-specific phages (Table 2). pHEPs-1, pHEPs-3, and pHEPs-4 were able to complement SL1102 (data not shown).

Nucleotide sequence of the *rfaE* gene

The nucleotide sequence of the pHEPs carring the *rfaE* gene was determined by strategy, as shown in Figure 2A. A set of nested deletion mutants, pHEPsExo1-6, generated from pHEPs was used to sequence. Two open reading frames (ORFs) were found. One of these ORFs encoded a polypeptide of 477 amino acid residues (Figure 3) high homologous to the *S. enterica rfaE* protein (98% identity and 98% similarity, GenBank Accession No. AAD49846), *E. coli* (93% identity and 96% similarity, GenBank Accession No. AAC76088), *Yersenia pestis* (85% identity and 92% similarity, GenBank Accession No. CAC89508), *Haemophilus influenzae* (70% identity and 80% similarity, GenBank Accession No. AAC23172) and *Helicobacter pyroli* (41% identity and 58% similarity, GenBank Accession No. AAD06368, data not shown): the molecular weight predicted from the nucleotide sequence is 53-kDa. Amino acid sequence comparison of these genes is shown in Figure 4. Additionally, a database search for sequence homology revealed that this ORF was high homologous to the *E. coli* putative kinase protein (GenBank accession AE000387, g1789432) (93% identity and 98% similarity, data not shown) unpublished. 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 (Figure 5).

The other ORF is inferred to terminate at base 786 and partially homologous to the *E. coli* hypothetical 60.7-kDa protein in GLGS-WAAE intergenic region (Figure 2B). Because it is incomplete and lacks an initiation codon, no functional protein is expected. LPS pattern of SL1102 complemented with pHEPs-1 was the same as that of SL1102 complemented with pHEPs (data not shown). This result indicates that this ORF has no effect on *rfaE* activity.

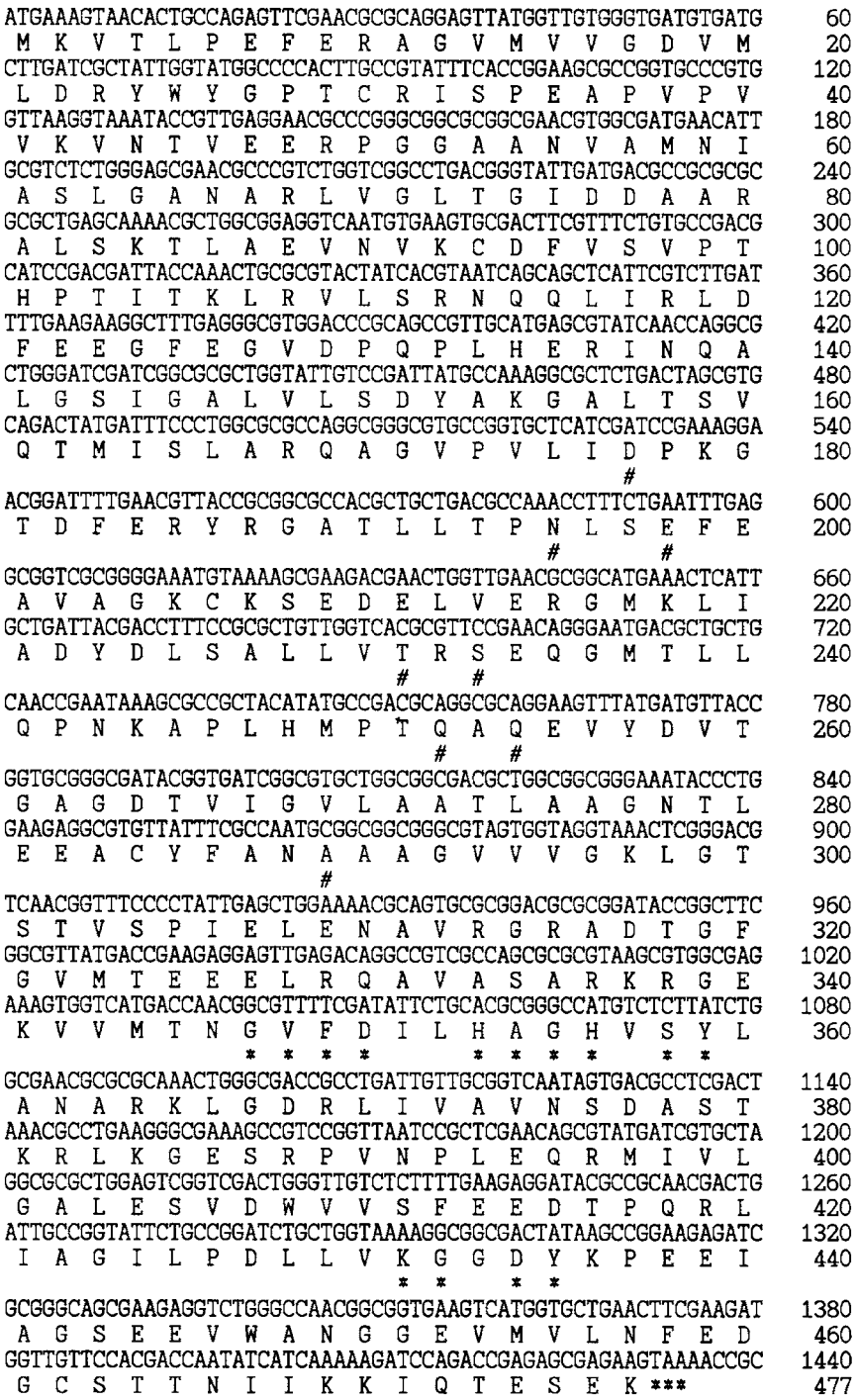

Figure 3. Base sequence and deduced amino acid sequence of the *rfaE* gene from *S. typhimurium*. #, conserved residue that bind ADP; *, conserved residue that lines the ATP binding pocket for more details on the structure of *rfa*E from E. coli see reference 13). *** indicates the stop condon of the gene.

Detection of *rfaE* gene from *S. typimurium* wild-type strain

To confirm that the *rfaE* gene clone isolated is genuine *S. typhimurium* genomic DNA, genomic Southern hybridization was carried out as described in Materials and Methods. The probe used for hybridization was a *Sal*I-*Hind*III fragment from pHEPs-2. As seen in Figure 6, the *Sal*I digest yielded a 2.1-Kb fragment, and the *Eco*RI, *Hind*III, and *Sac*I digest produced high-size bands hybridizing with the probe, which was consistent with the results expected from the restriction pattern of pHEPs, suggesting the presence of the *rfaE* gene in the wild-type *S. typhimurium* used this study.

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ST    1:MKVTLPEFERAGVMVVGDVMLDRYWYGPTCRISPEAPVPVVKVNTVEERPGGAANVAMNI 60
SE    1:MRVNLPAFERAGVMVVGDVMLDRYWYGPTCRISPEAPVPVVKVNTVEERPGGAANVAMNI 60
EC    1:MKVTLPEFERAGVMVVGDVMLDRYWYGPTSRISPEAPVPVVKVNTIEERPGGAANVAMNI 60
YP    1:MKVTLPDFRRAGVLVVGDVMLDRYWYGPTCRISPEAPVPVVKVDTIEERPGGAANVAMNI 60
HI    1:MAQYSAEFKQAKVLVLGDVMLDRYWFGATNRISPEAPVPVVRVQENEERAGGAANVAMNI 60
        *      *  * * * ********** * * *********** *   *** **********

ST   61:ASLGANARLVGLTGIDDAARALSKTLAEVNVKCDFVSVPTHPTITKLRVLSRNQQLIRLD 120
SE   61:ASLGANARLVGLTGIDDAARALSKTLAEVNVKCDFVSVPTHPTITKLRVLSRNQQLIRLD 120
EC   61:ASLGANARLVGLTGIDDAARALSKSLADVNVKCDFVSVPTHPTITKLRVLSRNQQLIRLD 120
YP   61:ASLGAVARLVGLTGIDDAARALICKLSEVRVRCDFVSVPTHPTITKLRVLSRNQQLIRLD 120
HI   61:ASLNVPVQLMGLIGQDETGSALSLLLEKQKIDCNFVALETHPTITKLRILSRHQQLLRLD 120
        ***     * ** * *    **   *      * **   ********** *** *** ***

ST  121:FEEGFEGVDPQPLHERINQALGSIGALVLSDYAKGALTSVQTMISLARQAGVPVLIDPKG 180
SE  121:FEEGFEGVDPQPLHERINQALGSIGALVLSDYAKGALTSVQTMISLARQAGVPVLIDPKG 180
EC  121:FEEGFEGVDPQPLHERINQALSSIGALVLSDYAKGALASVQQMIQLARKAGVPVLIDPKG 180
YP  121:FEEGFDGVDPTPIFERIQLALPQIGALVLSDYAKGALNSVQPMIQLARKANVPVLIDPKG 180
HI  121:FEEDFNNVDCKDLLAKLESAVKNYGALILSDYGKGTLKDVQKMIQIARKANVPVLIDPKG 180
        *** *  **          *    *** **** ** *  ** **  ** * **********

ST  181:TDFERYRGATLLTPNLSEFEAVAGKCKSEDELVERGMKLIADYDLSALLVTRSEQGMTLL 240
SE  181:TDFERYRGATLLTPNLSEFEAVAGKCKSEDELVERGMKLIADYDLSALLVTRSEQGMTLL 240
EC  181:TDFERYRGATLLTPNLSEFEAVVGKCKTEEEIVERGMKLIADYELSALLVTRSEQGMSLL 240
YP  181:SDFERYRGATLLTPNLSEFEAVVGRCKNEEELVNRGMQLVADFELSALLVTRSEQGMTLL 240
HI  181:TDFERYRGATLLTPNMSEFEAVVGKCNTEEEIIEKGLKLISDIELTALLVTRSEKGMTLL 240
         ************** ****** * *  * *    *  *  *  * ******** ** **

ST  241:QPNKAPLHMPTQAQEVYDVTGAGDTVIGVLAATLAAGNTLEEACYFANAAAGVVVGKLGT 300
SE  241:QPNKAPLHMPTQAQEVYDVTGAGDTVIGVLAATLAAGNTLEEACYFANAAAGVVVGKLGT 300
EC  241:QPGKAPLHMPTQAQEVYDVTGAGDTVIGVLAATLAAGNSLEEACFFANAAAGVVVGKLGT 300
YP  241:QLGKPPLHLPTQAKEVFDVTGAGDTVIGVLAAALAAGNSLEESCFLANAAAGVVVGKLGT 300
HI  241:RPNQEPYHLPTVAKEVFDVTGAGDTVISVLATALADGRSFEESCYLANVAAGIVVGKLGT 300
           * * ** * ** ********** ***  ** *   ** *  ** *** *******

ST  301:STVSPIELENAVRGRADTGFGVMTEEELRQAVASARKRGEKVVMTNGVFDILHAGHVSYL 360
SE  301:STVSPIELENAVRGPPDTGFGVMTEEELRQAVASARKRGEKVVMTNGVFDILHAGHVSYL 360
EC  301:STVSPIELENAVRGRADTGFGVMTEEELKLAVAAARKRGEKVVMTNGVFDILHAGHVSYL 360
YP  301:STVSPIELENAIRGRAETGFGVMDEQQLKIAVAQARQRGEKVVMTNGIFDILHAGHVSYL 360
HI  301:STVSTVELENAIHARPETGFGIMSEAELKDAVAQAKARGEKIVMTNGCFDILHPGHISYL 360
        ****  *****      **** * *  *  *** *  **** ***** ***** ** ***

ST  361:ANARKLGDRLIVAVNSDASTKRLKGESRPVNPLEQRMIVLGALESVDWVVSFEEDTPQRL 420
SE  361:ANARKLGDRLIVAVNSDASTKRLKGESRPVNPLEQRMIVLGALESVDWVVSFEEDTPQRL 420
EC  361:ANARKLGDRLIVAVNSDASTKRLKGDSRPVNPLEQRMIVLGALEAVDWVVSFEEDTPQRL 420
YP  361:ANARKLGDRLIVAVNSDASTKRLKGEKRPVNPLEQRMVVLGALEAVDWVVPFEEDTPQRL 420
HI  361:ENARKLGDRLIVAVNSDDSVKRLKGESRPINNLENRMAVLAGLASVDWLVPFTEDTPQRL 420
         ***************** * *****  ** * ** ** **  *  *** * * ********

ST  421:IAGILPDLLVKGGDYKPEEIAGSEEVWANGGEVMVLNFEDGCSTTNIIKKIQTESEK    477
SE  421:IAGILPDLLVKGGDYKPEEIAGSEEVWANGGEVMVLNFEDGCSTTNIIKKIQTESEK    477
EC  421:IAGILPDLLVKGGDYKPEEIAGSKEVWANGGEVLVLNFEDGCSTTNIIKKIQQDKKG    477
YP  421:IADILPDLLVKGGDYKPHEIAGSEEVWAAGGEVKVLNFEDGVSTTNIIQSIKNGRG-    476
HI  421:IGEILPDLLVKGGDYKPEEIAGSKEVWANGGDVKVLNFENGCSTTNVIEKIKLLKD-    476
        *  *************** ***** **** ** * ***** * **** *  *
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Figure 4. Comparison of the deduced amino acid sequences of the *rfaE* proteins from *S. typhimurium* and *Haemophilus influenzae* (Strain Rd KW20, GenBank Accession number U32828, 1574367). The asterisks indicate identical amino acid residues. ST, *S. typhimurium*; SE, *S. enterica*; EC, *E. coli*; YP, *Yersinia pestis*; HI, *Haemophilus influenzae.*

LPS phenotypes of the *rfaE* mutant complemented with pHEPs carrying *S. typhimurium rfaE* gene

LPS from the *rfaE* mutant carrying the *S. typhimurium rfaE* gene was analyzed by SDS-PAGE followed by silver staining (Figure 7). LPS from SL3770, which is *rfa*$^+$, formed the ladder-like pattern indicative of the presence of the O antigen repeat units (Figure 7, lane 1). LPS from SL1102 contained very-fast-migrating bands representing heptose-deficient incomplete core structure (lane 2). SL1102 complemented with pHEPs showed LPS which migrated in a similar pattern to that obtained with the LPS of the wild-type strain (lane 3).

Discussion

In LPS of Enterobacteriaceae, the functions of the genes of core oligosaccharide biosynthesis have been deduced from genetic

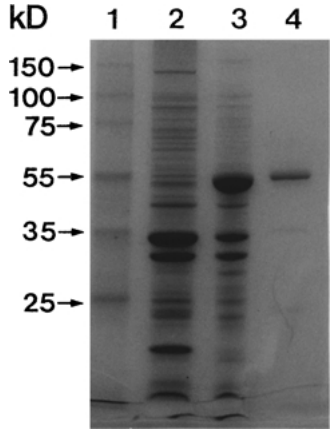

Figure 5. Expression of the *rfaE* gene, the pHEPs carrying *rfaE* gene was transformed into *E. coli* strain JM109, as described in Method. Lane 1, protein size marker (the size of protein markers are indicated on the left); lane 2, Insoluble protein of JM109 containing only pUC19 vector; Lane 3, Insoluble protein of JM109 containing pHEPs; Lane 4, purified *rfaE* protein.

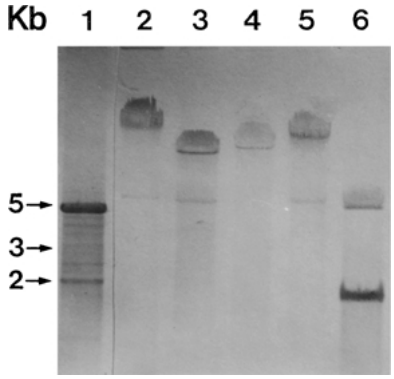

Figure 6. Genomic Southern hybridization of *S. typhimurium*. Genomic DNAs were digested with various restriction enzyme, resolved on a 0.8% agarose gel, blotted to a Hybond-N membrane, and hybridized with the *Sal*I-*Hin*dIII fragment from a pHEPs-2 (Figure 2) after labeling with digoxigenin-dUTP by random priming. The size of DNA markers are indicated on the left. Lane1, pHEPs digested with *Eco*RI; Lane 2, untreated S, typhimurium genomic DNA; Lane 3-6, genomic DNA digested with *Eco*RI (lane 3), *Hin*dIII (lane 4), *Sac*I (lane 5), and *Sal*I (lane 6).

studies, in conjunction with partial physical and chemical characterizations of LPS [25]. Five genes, *rfaC*, *rfaD*, *rfaE*, *rfaF*, and *rfaP*, for synthesis of inner core region have been identified. Two genes, *rfaD* and *rfaP*, are identified for heptose-epimerase and heptose-phosphorylase, respectively. Others, *rfaC* and *rfaF*, are identified for heptosyltransferase for transfer of heptose from ADP-heptose to the inner core, and *rfaE* gene is believed to be involved in the synthesis of ADP-heptose [5,10]. The core oligosaccharide region is not generally considered a virulence factor per se. In virulence, it has some indirect role that provides the attachment site for O-antigen (polysaccharide) and plays a

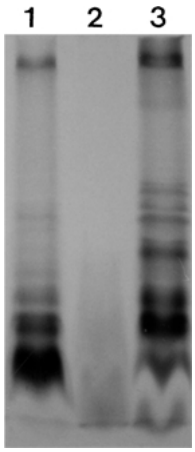

Figure 7. Silver-stained LPS gel showing complementation of SL1102 (*rfaE543*) by plasmid carrying the *S. typhimurium rfaE* gene. Lane 1, SL3770 (*rfa*$^+$); Lane 2, SL1102 (*rfaE543*); Lane3, SL1102 transformed with plasmid pHEPs.

crucial role in establishing the essential barrier function of the out membrane [12,23,26].

We have cloned the *rfaE* gene of *S. typhimurium*. The chromosomal region carrying this gene was isolated by screening a genomic library of *S. typhimurium* using the complementation of *S. typhimurium rfaE* mutant. The 2.6-Kb insert in the plasmid pHEPs (Figure 2) appears to carry a functional *rfaE* gene. SL1102 (*rfaE543*) makes heptose-deficient LPS and has a deep rough phenotype, but pHEPs or some nested deletion mutants of pHEPs complement the *rfaE543* mutation to give the smooth phenotype. This conclusion is based on the following data: (*a*) the sensitivity of SL1102 to bacteriophages (P22.c2, Felix-O, Br60) which use LPS as their receptor for adsorption is changed to that of wild-type strain (Table 2); (*b*) the permeability barrier of SL1102 to hydrophobic antibiotics (novobiocin) is restored to that of wild-type (Table 2); (*c*) LPS produced by mutant SL1102 shows only a single band of low molecular weigh on silver stained SDS-PAGE, but the same strain carrying pHEPs makes LPS indistinguishable from that of smooth strains (Figure 7).

Among the large number of LPS genes which have been sequenced from these *rfa* and *rfb* clusters, these are none which encode protein that appear to be secreted across the cytoplasmic membrane and few which encoded integral membrane proteins or proteins with extensive hydrophobic domains. Certainly, available sequence data predict that the majority of these enzymes are peripheral membrane proteins [26]. Although the bacterial LPS genetics are now well established, nothing is known of the specific step in synthesis controlled by *rfaE* [25]. However, recent reports have implicated the core oligosaccharide in the adhesion of bacteria to host cells, the high degree of structural conservation, and adaptation from the high temperature and hydrophobic antibiotics [26].

The *rfaE* gene products produce ADP-heptose, the substrate of heptosyltransferase. The heptose region of the core

oligosaccharide is known to be important for outer membrane stability in *E. coli* and *Salmonella*, The phosphorylation of HepI and HepII may be involved in both cross-linking of adjacent LPS and interaction with positively charged groups on proteins [26]. The significant compositional and structural changes in the outer membrane and the pleiotropic phenotype result from inability to synthesis or incorporate Hep, or the loss of phosphoryl derivatives [4,27]. Several evidences are known for this. This increase in phospholipid of the outer membrane is shown in deep-rough mutants [28]. This increase in results in the sensitivity to hydrophobic compounds by the changes on the outer membrane and release of periplasmic enzymes into the medium [29]. Also, lack of inner core oligosaccharide phosphorylation lead to an inactive form of secreted haemolysin in *E. coli* [30,31]. These suggest that the heptose region of the LPS molecule may be important therapeutic targets in bacteria and the biosynthetic processes for the modification of heptose region of the core oligosaccharide may provide a interesting way for further investigation.

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