

Biosynthesis of Inner Core Lipopolysaccharide in Enteric Bacteria Identification and Characterization of a Conserved Phosphoheptose Isomerase*

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The *lpcA* locus has been identified in *Escherichia coli* K12 novobiocin-supersensitive mutants that produce a short lipopolysaccharide (LPS) core which lacks glyceromannoheptose and terminal hexoses. We have characterized *lpcA* as a single gene mapping around 5.3 min (246 kilobases) on the *E. coli* K12 chromosome and encoding a 22.6-kDa cytosolic protein. Recombinant plasmids containing only *lpcA* restored a complete core LPS in the *E. coli* strain χ 711. We show that this strain has an IS5-mediated chromosomal deletion of 35 kilobases that eliminates *lpcA*. The LpcA protein showed discrete similarities with a family of aldose/ketose isomerases and other proteins of unknown function. The isomerization of sedoheptulose 7-phosphate, into a phosphosugar presumed to be D-glycero-D-mannoheptose 7-phosphate, was detected in enzyme reactions with cell extracts of *E. coli lpcA*⁺ and of *lpcA* mutants containing the recombinant *lpcA* gene. We concluded that LpcA is the phosphoheptose isomerase used in the first step of glyceromannoheptose synthesis. We also demonstrated that *lpcA* is conserved among enteric bacteria, all of which contain glyceromannoheptose in the inner core LPS, indicating that LpcA is an essential component in a conserved biosynthetic pathway of inner core LPS.

LPS,¹ an integral component of the outer membrane of Gram-negative bacteria, consists of lipid A attached to a core oligosaccharide, and in some microorganisms, contains an O-specific surface polysaccharide which is subsequently attached to the terminal residues of the core (1–2). The core oligosaccharide has an inner domain made of 3-deoxy-D-manno-octulosonic acid and L-glycero-D-mannoheptose, and an outer domain composed of hexoses and N-acetylglucosamine. The structure of the

inner core is relatively highly conserved among enteric (3) and non-enteric bacteria (4). Most of the genes involved in the biosynthesis and assembly of the core oligosaccharide are located within the *rfa* cluster, at about 81 min on the chromosome in *Escherichia coli* K12 and 79 min in *Salmonella enterica* LT2 (1). However, the genes involved in the early steps of the synthesis of L-glycero-D-mannoheptose are not located in the *rfa* region and they have not been characterized as yet.

LPS plays an important role in maintaining the structural integrity of the outer membrane by interacting with other components of the outer membrane and providing a physical barrier against the entry of deleterious compounds and some bacteriophages (3). *E. coli* LPS mutants with defects in the inner core display a dramatic reduction in porin proteins (5) and are unable to grow in media containing detergents, bile salts, or hydrophobic antibiotics, all of which normally have a reduced permeability across the outer membrane and are toxic only in high concentrations (6). Since these mutants lack an attachment site for the rest of the core oligosaccharide, they are resistant to LPS core-specific bacteriophages (6) and survive poorly within the host environment (7).

Early work by Tamaki *et al.* (6) resulted in the isolation of mutations conferring supersensitivity to novobiocin which mapped to two different regions on the *E. coli* K12 chromosome: between *ara* and *lac* (1–10 min) next to the *proAB* genes, and between 55 and 60 min; they were designated as *lpcA* (LPS-core synthesis) and *lpcB*, respectively (6). Similar mutations were also identified by Havekes *et al.* (8) as F plasmid conjugation-deficient mutants. The LPS of both *lpcA* and *lpcB* mutants lacks heptose (6), suggesting these loci are involved in synthesis of the inner core domain, but since their original discovery, their precise function has not been established.

This study reports the molecular analysis of the *lpcA* locus, and the biochemical characterization of its gene product. We conclude that *lpcA* encodes a phosphoheptose isomerase used in the first step of the biosynthesis of the inner core LPS precursor, ADP-L-glycero-D-mannoheptose. We also demonstrate that *lpcA* is widely conserved among enteric bacteria, suggesting that its function is part of a conserved pathway for LPS biosynthesis.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—Bacteria used in this study include: *E. coli* K12 strains χ 705 (F[−], *leu*-4, ϕ ^R, Str^R, *arg*-35, T6^R, λ [−]), χ 711 (F[−], *leu*-4, ϕ ^R, *proAB*118, Str^R, T3^R, *arg*-35, T6^R), Y10 (F[−], *thr*-1, *leu*B6, *thi*-1, *rfa*D1, *supE*44), JM109(DE3) (*endA*1, *recA*1, *syrA*96, *thi*, *hsdR*17 (*r*_k[−], *m*_k⁺), *relA*1, *supE*44, Δ (*lac*-*proAB*), [F⁺, *tra*D36, *proAB*, *lacI*^qZ Δ M15], λ (DE3)), D21e7 (*rfa*-1), CS2051 (has a deletion eliminating *rfaG*, *rfaP*, *rfaM*, *rfaN*, and *rfaB*), D31m4 (*rfa*-229, *rfa*-230); *E. coli* strains O4, UWO101; *Pseudomonas aeruginosa* strains AK44, O16; *S. enterica* strains 10749 serovar Newbrunswick group E2, 10756 serovar Thomasville group E3, G30 serovar Typhimurium; *Proteus mirabilis* PMVHL 46, *Proteus vulgaris* VHL 453; *Enterobacter cloacae* cloDF13R,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U32590.

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¹ The abbreviations used are: LPS, lipopolysaccharide; kb, kilobase(s); bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; DIG, digoxigenin; HPLC, high pressure liquid chromatography; ABEE, p-amino-benzoate ethyl ester; NS, novobiocin-supersensitivity; ORF, open reading frame; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine.

Enterobacter aerogenes 62-1, *Enterobacter agglomerans* UW0100; *Klebsiella pneumoniae* VHL/8, *Klebsiella* spp. Raph 3a, VHL/16, VHL/17; *Shigella flexneri* FH10(SF6), and *Shigella boydii* MV300 type 12. Bacteria were grown in Luria broth. The following compounds were added as appropriate: novobiocin (50 µg/ml), chloramphenicol (30 µg/ml), ampicillin (100 µg/ml), streptomycin (100 µg/ml), spectinomycin (80 µg/ml), sodium dodecyl sulfate (6 mg/ml), and deoxycholate (10 mg/ml). Cosmid pE4021 was obtained from A. Higashitani and contains *EcoRI* fragments from the chromosome of *E. coli* strain W3310, inserted into the *EcoRI* site of the plasmid vector pHC79. pJB1 is a deleted derivative of pE4021 containing a single 14-kb *EcoRI* fragment. pJB2 and pJB8 were constructed by cloning a 3-kb *Bam*HI fragment from pJB1 into the *Bam*HI site of pMAV3 (9) and pSF6 (10), respectively. pJB2-9 through to pJB2-34 are unidirectional deletion clones from pJB2 (see below). pJB15 was constructed by deletion of a *Hinc*II fragment from pJB2-25. pJB18 contains an in-frame translational fusion of *lpcA* with a histidine tag cloned into the expression vector, pQE32 (Qiagen, Chatsworth, CA). pREP4 contains the *lacI* gene encoding the *lac* repressor (Qiagen). ¹⁴C-Labeled SDS-PAGE molecular weight markers were purchased from Amersham Canada, Oakville, Ontario, Canada. Calf alkaline phosphatase was purchased from Boehringer Mannheim, Dorval, Quebec, Canada. HPLC grade acetonitrile was purchased from BDH, Toronto, Ontario, Canada. HPLC grade methanol was purchased from Fisher Scientific, Nepean, Ontario, Canada. All other chemicals and antibiotics were purchased from Sigma.

Recombinant DNA Methodologies—Small and large scale plasmid DNA extractions and electrophoresis of plasmid DNA were performed as described previously (11). Large scale preparation of RNA from *E. coli* strain χ 711(pJB2) was performed using a combination of the methods described by Deuschle *et al.* (12) and Glisin *et al.* (13). Colony hybridizations were carried out with the DIG-dUTP-labeled RNA probe (see below) using Zeta Probe membranes (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) according to the manufacturer's instructions (Boehringer Mannheim, Dorval, Quebec, Canada). Hybridizations were performed at 37 °C for 21 h, followed by two 15-min washes with 0.1 × SSC (20 × SSC: 300 mM citric acid, 3 M NaCl, pH 7.0) containing 0.1% SDS at 37 °C and development using a chemiluminescent detection system (Boehringer Mannheim). Blots were exposed to Kodak X-Omat film for 18 h at room temperature. Transformations were done by electroporation with a Gene Pulser apparatus (Bio-Rad), using 0.1-cm cuvettes following the method of Dower *et al.* (14). Restriction enzyme analysis and cloning were performed using standard protocols (15). A set of nested deletions was produced with the method of Henikoff (16) using appropriate restriction enzyme sites on either side of the 3-kb *Bam*HI fragment in pJB2. Restriction endonucleases, exonuclease III, S1 nuclease, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were obtained from Boehringer Mannheim and Pharmacia Canada Inc., Baie d'Urfe, Quebec, Canada, and used as recommended by the suppliers.

Sequence Analysis—DNA sequencing was performed using the dideoxy chain termination method (17) modified for use with the T7 Sequencing Kit (Pharmacia), with double stranded DNA as the template. Suitable deletion clones were sequenced using the T7 or SP6 promoters' primers. Gel reading in areas of high G + C content was improved as described by Beck *et al.* (18). DNA and protein sequence analysis was carried out with the University of Wisconsin GCG package version 7 (19) and compared to protein and DNA sequence data bases (GenBank, EMBL, and Swissprot) using BLAST (20). The *LpcA* amino acid sequence was also analyzed with the PROFILEGRAPH program version 1.3 (21).

Preparation of RNA and DNA Probes—Digoxigenin (DIG)-dUTP-labeled riboprobes (Boehringer Mannheim, Dorval, Quebec, Canada) were obtained by standard procedures (22, 23) using pJB15 as a template. 2.5 µg of cellular RNA from *E. coli* strain χ 711 (pJB2), 0.6 µg of pJB2 DNA, and 5 and 10 µg of cellular RNA from *E. coli* strain χ 711 were spotted onto a Zeta Probe membrane (Bio-Rad). Membranes were hybridized with the RNA DIG-dUTP probes at 42 °C for 23 h and developed as described above.

The 14-kb *EcoRI* DNA fragment of pJB1 was labeled with DIG-11-dUTP using a DIG DNA Labeling and Detection kit from Boehringer Mannheim. Chromosomal DNAs from *E. coli* strains χ 711 and χ 705 were cleaved with *EcoRI* and electrophoresed in a 0.6% agarose gel for 18–20 h at 15 mA. Southern blots were hybridized at 42 °C for 18–20 h and bands developed using a colorimetric detection system as recommended by the manufacturer (Boehringer Mannheim).

Analysis of Polypeptides—*In vivo* labeling of proteins with [³⁵S]methionine (ICN Biomedicals, Irvine, CA) was performed using the T7 promoter-polymerase directed overexpression system induced by the

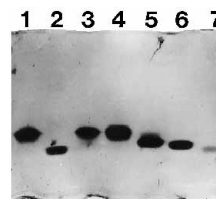


FIG. 1. Silver-stained 16.4% (w/v) T (total acrylamide), 1.9% (w/v) C (bisacrylamide) Tricine SDS-polyacrylamide gel showing the LPS profiles of *E. coli* K12 strains. 1, χ 705; 2, χ 711; 3, χ 711(pJB2); 4, Y10; 5, D21e7 (*rfa-1*); 6, CS2051 (has a deletion eliminating *rfaG*, *rfaP*, *rfaM*, *rfaN*, and *rfaB*); 7, D31m4 (*rfa-229*, *rfa-230*).

addition of 0.5 mM isopropyl-1-thio- β -D-galactopyranoside (24). *In vitro* transcription-translation was performed using the Prokaryotic DNA-directed Translation kit from Amersham with [³⁵S]methionine, as recommended by the manufacturer. Polypeptides were separated by SDS-PAGE (25) followed by treatment with EN³HANCE (DuPont NEN). Dried gels were exposed to Kodak X-Omat film at -110 °C for 16–48 h.

LPS Analysis—LPS was extracted as described by Marolda *et al.* (11) and analyzed by Tricine SDS-PAGE as described by Schagger and von Jagow (26). LPS was detected using the silver-staining procedure of Dubray and Bezaud (27).

Enzyme Reactions—Cell extracts were prepared from 800 ml of culture grown 3 h at 37 °C. Cells were harvested and resuspended in 2 ml of TDE (50 mM Tris-HCl (pH 7.8), 5 mM dithiothreitol, 1 mM EDTA) (28) and sonicated 2–3 times for 30 s in a Branson Sonic Power sonifier cell disruptor 350 with 1 min cooling between sonications. Cell debris and unbroken cells were sedimented by centrifugation at 27,000 × g at 4 °C for 20 min, and the supernatant was passed through Sephadex G-25 columns using TDE/glycerol (80:20) (v/v) as eluent to remove low molecular weight material. Protein fractions were collected and glycerol was added to a final concentration of 50% (v/v) for storage at -20 °C until use. Enzyme reaction mixtures consisted of either 0.05 or 1.0 µmol of sedoheptulose 7-phosphate, 5.0 µmol of Tris-HCl (pH 7.8), 0.5 µmol of MgCl₂, 500 µg of protein cell extract, in a final volume of 100 µl. Reactions were carried out at 37 °C and terminated by boiling for 2 min. For some experiments, samples were treated with 4 units of calf alkaline phosphatase for 1 h at 37 °C prior to termination of the reactions. To examine the reaction products, samples were dried by vacuum overnight at room temperature, and then derivatized using ABEE and sodium cyanoborohydride as described (29, 30).

HPLC Analysis—ABEE-labeled carbohydrates were separated on a C18 reverse-phase column (Brownlee RP-18 Spheri-5, 5-µm resin, 250 × 4.6 mm) run isocratically for 0 to 6 min with acetonitrile/water/diaminobutane (50:49.05:0.05), followed by a linear gradient of acetonitrile/water/diaminobutane (50:49.05:0.05) to acetonitrile/water/diaminobutane (10:89.05:0.05) over 20 min at a flow rate of 0.5 ml/min at 35 °C.

RESULTS

Characterization of *E. coli* Strain χ 711—Curtiss *et al.* (31) isolated *E. coli* strain χ 711 which has been thought to have a chromosomal deletion around the region containing the proline synthesis genes *proAB*, and is resistant to bacteriophages P1 and T3. Since the *lpcA* locus has been mapped near the *proAB* genes (6), we examined strain χ 711 for characteristics of inner core LPS defects. In contrast to the parental strain χ 705, *E. coli* χ 711 did not grow in Luria broth (LB) with SDS, MacConkey agar, LB with novobiocin, and LB with deoxycholate, suggesting a defect in inner core LPS.

Direct evidence of an altered LPS structure was obtained by a comparative analysis of the LPS profiles of strains χ 711 and χ 705 (Fig. 1). Strain χ 705 produces a core oligosaccharide identical to that of the prototypic *E. coli* K12 strain Y10 (Fig. 1, lanes 1 and 4) whereas strain χ 711 produces a much shorter core (Fig. 1, lane 2). LPS of *E. coli* strains D21e7, CS2051, and D31m4, containing different mutations in *rfa* genes, were examined and compared with the χ 711 core. The LPS core of strains D21e7 and CS2051 was shorter than the wild-type core but still longer than the χ 711 core (Fig. 1, lanes 5 and 6), whereas the LPS core of D31m4 migrated the same distance in

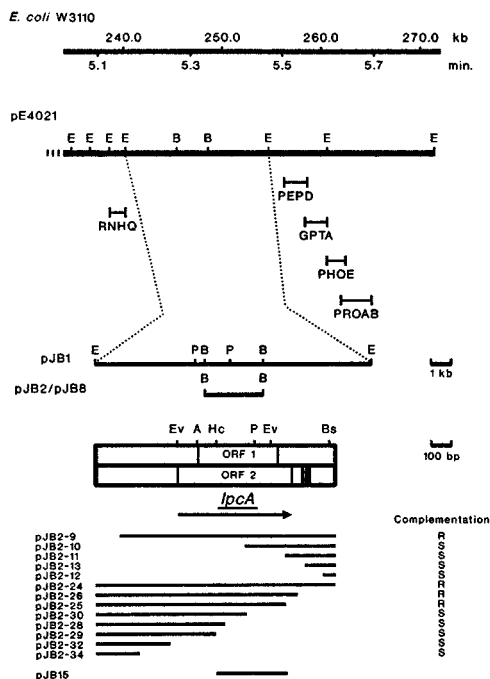


FIG. 2. Physical map of the *lpcA* region. Vector sequences are not shown. pE4021 is a cosmid clone containing chromosomal DNA from *E. coli* W3110, including the region from 4.9 to 5.8 min. *RNHQ*, *PEPD*, *GPTA*, *PHOE*, and *PROAB* indicate the location of sequenced genes. pJB1 contains a 14-kb *EcoRI* fragment cloned from pE4021. pJB2 and pJB8 contain a 3-kb *BamHI* fragment cloned from pJB1 into different vectors. pJB2-9 to pJB2-34 indicate the various deletions of pJB2 spanning the *lpcA* region. pJB15 indicates the DNA insert used for construction of DIG-labeled riboprobes. ORF1 and ORF2 are two open reading frames found on opposite strands of the DNA. The direction of transcription of *lpcA* is indicated by the arrow beneath ORF2. The complementation of the novobiocin supersensitivity phenotype by the deletion clones is indicated: R, successful complementation; S, unsuccessful complementation. Restriction enzymes indicated are: A, *AvaII*; B, *BamHI*; Bs, *BstEII*; E, *EcoRI*; Ev, *EcoRV*; Hc, *HincII*; P, *PvuI*.

the gel as the LPS core of $\chi 711$ (Fig. 1, lanes 7 and 2). Since strain D31m4 produces a heptoseless LPS made of only 3-deoxy-D-manno-octulosonic acid and lipid A (32) we conclude that $\chi 711$ also lacks heptose in its core LPS.

Identification of the *lpcA* Locus—To investigate if sequences near the *proAB* genes include the gene(s) responsible for the LPS defect in $\chi 711$, $\chi 711$ cells were transformed with the cosmid pE4021 which contains a DNA segment spanning the *proAB* region (33) (Fig. 2). Transformants appeared on plates containing novobiocin, indicating that pE4021 could carry the *lpcA* gene determinant(s). To position the *lpcA* locus, partial *EcoRI* digestion and self-ligation of pE4021 was performed, followed by transformation into strain $\chi 711$ and selection on plates with novobiocin. The surviving colonies contained plasmids carrying a single 14-kb *EcoRI* fragment; one of these plasmids was designated pJB1 (Fig. 2) and used to subclone smaller DNA fragments into pMAV3. One of the subclones, pJB2, contained a approximately 3-kb *BamHI* fragment (Fig. 2) and enabled *E. coli* $\chi 711$ cells to grow in medium containing either novobiocin or SDS. This indicated that the gene(s) of the *lpcA* locus reside(s) within the 3-kb *BamHI* fragment of pJB2 (Fig. 2). A comparison of the core LPS profiles of strains $\chi 705$ and $\chi 711$ (pJB2) revealed that this plasmid restored the core LPS defect of *E. coli* $\chi 711$ (Fig. 1, lanes 1 and 3). pJB2, however, failed to complement the core LPS defect in strain D31m4 (data not shown), indicating that the function of the *lpcA* gene, although associated with a heptoseless core, is different from the functions defined by the mutations *rfa-229* and *rfa-230* in D31m4. Complementation of the *lpcA* mutation in $\chi 711$ was

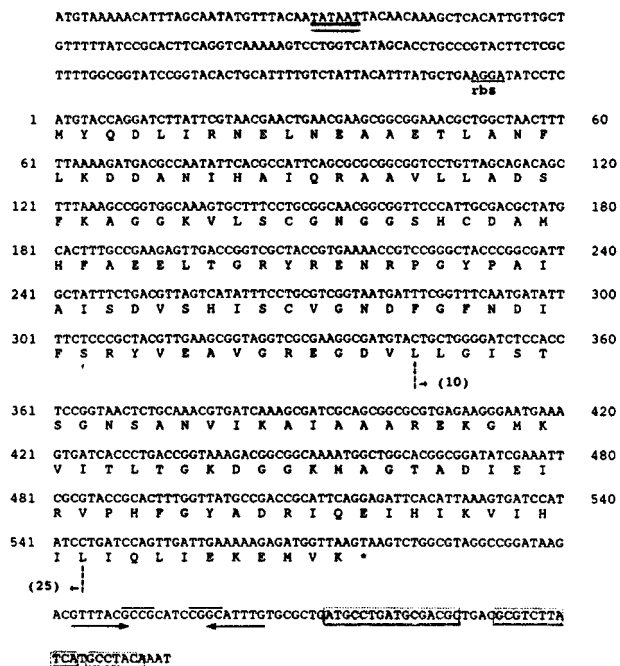


FIG. 3. Nucleotide sequence and deduced amino acid sequence of *lpcA*. The underlined sequence AGGA denotes the possible ribosomal binding site (*rbs*). The putative -10 consensus sequence is indicated by double underlining. The deletion end points of pJB2-10 and pJB2-25 (Fig. 2) are indicated by arrows followed by the numbers 10 and 25 in parentheses; the direction of the arrows indicates the sequence contained within pJB2-10 and pJB2-25, respectively. A putative transcription termination signal is indicated by arrows beneath the sequence downstream of the termination codon TAA. Lines above the sequences GCGG and CGGC indicate the complementary sequences forming the stem of the hairpin loop structure. Boxed sequences denote the location of the repetitive extragenic palindromic sequence.

also achieved with the low copy number construct pJB8 (Fig. 2 and data not shown).

Nucleotide Sequence of the *lpcA* Locus—Unidirectional deletion derivatives of pJB2 were made as described under "Experimental Procedures." Transformation of these plasmids into *E. coli* $\chi 711$ followed by selection of transformants on medium containing novobiocin, demonstrated that the *lpcA* locus lies within a 0.826-kb DNA segment located between the deletion end points of plasmids pJB2-9 and pJB2-25 (Fig. 2). DNA sequence of this region revealed two open reading frames, one on each strand, designated as ORF1 (412 bp) and ORF2 (577 bp) (Fig. 2). To determine which of the ORFs is expressed, we examined the direction of transcription of *lpcA* using *in vitro* synthesized DIG-dUTP-labeled riboprobes. Two probes of labeled mRNA independently transcribed from each of the DNA strands spanning the region where the two ORFs overlapped were prepared using either SP6 or T7 RNA polymerase-directed transcription. pJB15 was constructed for this purpose, as a *HincII* deletion of pJB2-25 (Fig. 2). pJB15 DNA was cut with *EcoRV* or *HincII* separately to linearize the DNA prior to *in vitro* transcription in the presence of DIG-dUTP. The RNA probes were then used for hybridization with total RNA obtained from *E. coli* $\chi 711$ containing pJB2. Only the probe synthesized with SP6 RNA polymerase hybridized with cellular RNA, whereas the probe corresponding to the transcript of the opposite strand did not. Therefore, we concluded that ORF2 corresponds to the *lpcA* gene. We also observed that the deletion plasmid pJB2-25 lacks a small portion of the carboxyl terminus of LpcA from Ile-183 to Lys-192 (Figs. 2 and 3). Since pJB2-25 complements the NS phenotype of *E. coli* $\chi 711$ (Fig. 2), we conclude that these 10 carboxyl-terminal amino acids are probably not essential for the function of the protein.

TABLE I
Comparison of conserved region of *LpcA* with other proteins

Protein ^a	Sequence ^b	Identity ^c	Score ^d	Accession
<i>lpcA_eco</i>	DVlllgIStSG nsAnvikAi- aaarEkGmkV -tltlTgkdGg kMagtAD	NA	NA	NA
<i>orfb_clope</i>	DViVaiSnSG tTkeViktV- kqaKEngtXl -tltlTedsdn pLrkLAD	40	110	M81878
<i>lmbn_sl</i>	-sYhggsrGg EvsanLpAva RLakERGAAV -VAVTgfdGg aLgdLAD	36	80	X79146
<i>kpsf_eco</i>	DlIlLlIsaSG ETdEilklv- pslKnfGnrI -tAlTnngns tLaknAD	29	103	L19929
<i>glms_mycol</i> [*]	ElvVaiSQSG ETADTLAav- RhakEgkAKV -tAlCNTnGS qipRECD	27	141	U00020
<i>gfal_rat</i> [*]	DVCfFISQSG ETADTLmgI- RYcKERGALT -VgINTVGS SisREtd	27	119	U00932
<i>nodm_rhll</i> [*]	saalFISQSG ETADTLasl- RYcKahGlrI -gAVvNTres tMaREAD	24	95	Y00548
<i>gfal_ys</i> [*]	DVCVFVSQSG ETADTLAl- nYclERGALT -VgIvNsVGS SisRuth	22	100	X71133
<i>gfal_ysc</i> [*]	DVCVFVShcG ETADTLAl- nYclERGALT -VgIvNsVGS SisRvth	20	90	J04719
Consensus	DVCVFISQSG ETADTL-A-- RY-KERGALT -VAINTVGS SL-READ			
Secondary Structure ^e	bbbbbbTTTT hhhhhh-h-- hh-hhhbbb- -bbbbbbb*o *o--oTTT			

^a Protein sequences are abbreviated as indicated in the DNA sequences entries. *eco* (*E. coli*), *clope* (*Clostridium perfringens*), (*Streptomyces lincolnensis*), *mycol* (*Mycobacterium leprae*), *rat* (*Mus musculus*), *rhil* (*Rhizobium leguminosarum*), *ys* (yeast), *ysc* (*Saccharomyces cerevisiae*). Asterisks indicate members of the aldose-ketone family of proteins.

^b The sequences were aligned using the computer program PILEUP and the alignments displayed with the computer program PRETTY. Gaps were represented by dashes. Shading indicates conserved amino acids present in *LpcA* that are conserved in the other proteins.

^c Sequence identities were established by pairwise comparisons using the computer program BESTFIT. The quality of all the alignments was significant as indicated by using randomly shuffled sequences (higher than average by 5 times the standard deviation). NA, not available.

^d The scores are those obtained for a given protein when the consensus was used to search the GenPept database using the computer program BLAST.

^e Secondary structure was calculated using the consensus sequence and the computer program PEPTIDESTRUCTURE (b, β -sheet; h, α -helix; T, turn; o, random coil).

The % G + C content of *lpcA* was 51%, similar to the reported values for % G + C content of the *E. coli* genome, and the codon usage was typical for *E. coli* genes. The sequence AGGA found 8 bp upstream of the AUG codon may correspond to the ribosomal binding site (Fig. 3). The sequence TATAAT located 146 bp upstream of the AUG codon has similarities with a -10 consensus sequence (Fig. 3). A repetitive extragenic palindromic sequence was noted 54 bp downstream of the termination codon of *lpcA* (Fig. 3). Inverted repeats with a potential for a hairpin secondary structure consistent with a transcriptional termination signal were also noticed between the repetitive extragenic palindromic sequence and the termination codon (Fig. 3).

DNA and deduced amino acid sequences of *lpcA* were compared to sequences from EMBL/GenBank. No homologies with known genes involved in LPS synthesis were noted. A region of the *LpcA* polypeptide spanning 45 amino acids showed significant similarities with a family of isomerases described as glutamine:fructose-6-phosphate amidotransferases, the lincomycin biosynthesis gene product LmbN from *Streptomyces lincolnensis*, and two other proteins, KpsF (*E. coli*) and OrfB (*Clostridium perfringens*) with no assigned functions (Table I). The potential significance of these similarities is discussed below.

The expression of the *lpcA* gene product *in vivo* and *in vitro* identified a 22.6-kDa polypeptide as the *LpcA* protein (data not shown), which is in agreement with the predicted molecular mass of 20.6 kDa. A hydropathy profile of the *LpcA* protein (34) deduced from the nucleotide sequence of *lpcA*, did not reveal any significant regions of hydrophobicity compatible with membrane domains suggesting that *LpcA* is a cytosolic protein.

Mapping of *lpcA* on the *E. coli* Chromosome—DNA sequencing of the *lpcA* region and restriction endonuclease mapping of cosmid pE4021 revealed a very close similarity to the established restriction map at about 5.9 min on the *E. coli* K12 chromosome corresponding to phages 7D5, 4A11, and 8F9 of the Kohara library (35). We have thus positioned *lpcA* at about 246 kb, (5.3 min) on the *E. coli* K12 chromosome map, between *rnhQ* and *pepD* (Figs. 2 and 5A).

Detection of *lpcA* in Other Bacteria—A DIG-dUTP-labeled RNA probe derived from the *lpcA* internal sequence was used to determine the conservation of *lpcA* among different bacteria. Hybridization of the probe with chromosomal DNA in colony

blots demonstrated that *lpcA* was conserved among the principal genera of enteric bacteria: *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Salmonella*, and *Shigella*. *Pseudomonas aeruginosa* strains AK44 and O16 did not hybridize with the probe. *E. coli* strain χ 711 also did not hybridize with the probe, indicating that the *lpcA* locus was deleted from the chromosome of this strain.

Characterization of the Chromosomal Deletion of *E. coli* Strain χ 711—To identify whether the chromosomal deletion of *E. coli* strain χ 711 included *lpcA* and neighboring genes, Southern blots of *EcoRI*-cleaved chromosomal DNA from χ 711 and from the parental strain χ 705 were probed with the DIG-11-dUTP-labeled 14-kb *EcoRI* DNA fragment from pJB1. A 14-kb *EcoRI* fragment was detected using χ 705 DNA as a control, whereas a 2.8-kb *EcoRI* fragment hybridized with χ 711 DNA (Fig. 4B, lanes 1 and 2); this suggested that part of the 14-kb *EcoRI* fragment is still present in the chromosome of χ 711. The 2.8-kb fragment was cloned and its DNA sequence was established. A 1.6-kb region was identical to a 1.6-kb region at the end of the 14-kb *EcoRI* fragment whereas the rest of the sequence contained an IS5 element not present in the 14-kb fragment (Fig. 4C). Therefore, an IS5 element is located at the deletion end point in strain χ 711. Since χ 711 lacks *proAB*, we conclude that the IS5 must have originated from the region rich in IS elements located clockwise from the *proAB* genes (Fig. 4, A and D). Thus we predict that the deletion removes approximately 35 kb DNA including both the *lpcA* locus and the *proAB* genes.

Role of *lpcA* in LPS Biosynthesis—A region from Asp-113 to Asp-157 in the amino acid sequence of *LpcA* showed significant similarities to the family of bacterial and eukaryotic glutamine:fructose-6-phosphate amidotransferases Gfat, GlmS, and NodM (Table I). Conservation ranged from 20% identity and 47% similarity with the Gfat of *Saccharomyces cerevisiae*, up to 27% identity and 53–60% similarity with both rat Gfat and mycobacterial GlmS (Table I, and data not shown).

Glutamine:fructose-6-phosphate amidotransferase belongs to both ketose/aldose isomerase and amidotransferase groups (36, 37). The amidotransferase reaction requires residues located in the NH₂-terminal sequence of these enzymes, notably an NH₂-terminal Cys which is the active residue for the glutamine amide transfer function (38). This region is conserved among the various glutamine:fructose-6-phosphate amido-

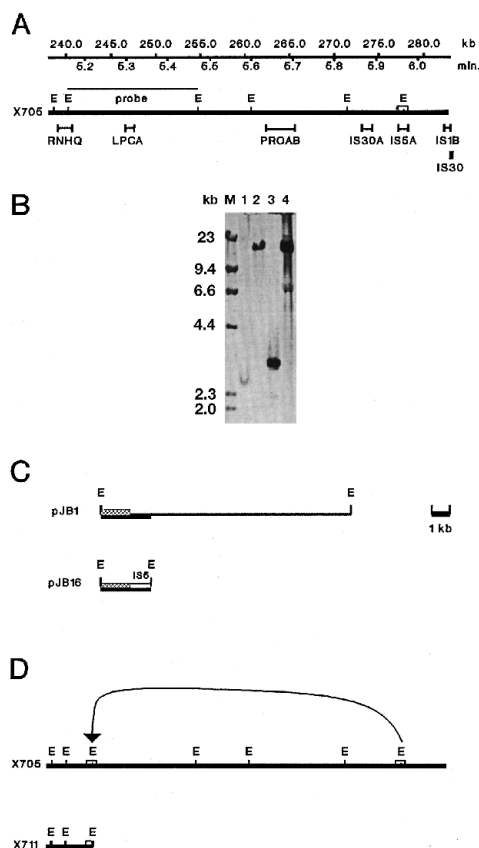


FIG. 4. A schematic representation of the proposed events leading to the chromosomal deletion of the *lpcA* locus in *E. coli* strain χ 711. Panel A, chromosomal map of *E. coli* K12 strain χ 705. *RNHQ*, *LPCA*, *PROAB*, *IS30A*, *IS5A*, *IS1B*, and *IS30* indicate the location of sequenced genes. Panel B, Southern blot showing chromosomal DNA profiles of *E. coli* strains χ 711 and χ 705 probed with a 14-kb *EcoRI* DIG-11-dUTP-labeled DNA probe. *M*, λ *HindIII* molecular weight markers; 1, χ 711 DNA digested with *EcoRI*; 2, χ 705 DNA digested with *EcoRI*; 3, pJB2 digested with *Bam*HI; 4, pJB1 digested with *EcoRI*. Panel C, restriction maps of pJB1 and pJB16 showing identical nucleotide sequence (hatched box) and the IS5 element (open box). Panel D, transposition of the IS5A insertion element from approximately 5.9 min to 5.2 min followed by replication of the element, and chromosomal map of *E. coli* strain χ 711 showing the resulting deletion of the *lpcA* locus. Restriction endonucleases indicated are: *E*, *EcoRI*.

transferases investigated to date (38); the absence of this region in *LpcA* suggests that this protein is not an amidotransferase.

To test whether *lpcA* catalyzes an isomerization reaction, cell extracts of *E. coli* strain χ 711, *E. coli* strain χ 711(pJB2), and *E. coli* strain χ 711 (pJB18, pREP4) were used in an assay containing sedoheptulose 7-phosphate. The reaction products were examined by high performance liquid chromatography (HPLC) after derivatization with aminobenzoic ethyl ester (ABEE) to facilitate their detection with UV light. Chromatograms from reactions with cell extracts of χ 711(pJB2) and χ 711-(pJB18,pREP4) revealed the appearance of a new peak with a retention time of 8.2 min (Fig. 5B and data not shown) after 2 min of incubation with the enzyme. After 60 min incubation, the peak corresponding to sedoheptulose 7-phosphate decreased considerably, 39 and 93% of initial amount for χ 711(pJB2) and χ 711(pJB18,pREP4), respectively (data not shown), suggesting that the substrate was consumed, whereas the level of sedoheptulose 7-phosphate remained constant for 60 min when incubated with boiled extract (Fig. 5D). Upon incubation of χ 711 cell extract with sedoheptulose 7-phosphate (Fig. 5A), a peak with 8.2 min retention as found with χ 711(pJB2) and χ 711(pJB18,pREP4) was not apparent, al-

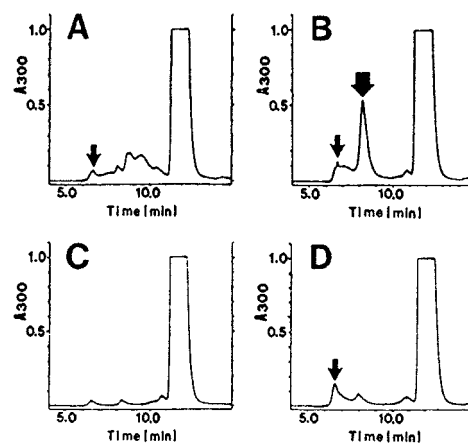


FIG. 5. Reversed-phase high performance liquid chromatography analyses of carbohydrates synthesized by *E. coli* strains χ 711 and χ 711(pJB2) cell extracts following incubation with 1.0 μ mol of sedoheptulose 7-phosphate. Panel A, χ 711 incubated 60 min. Panel B, χ 711(pJB2) incubated 2 min. Panel C, χ 711(pJB2) incubated 60 min without sedoheptulose 7-phosphate. Panel D, χ 711(pJB2) boiled extract incubated 60 min. Large arrow indicates the retention peak of the phosphorylated product. Small arrow indicates the retention peak of sedoheptulose 7-phosphate.

though other peaks with retention times ranging from 8.5 to 10.5 min were observed. These extra peaks could be due to the conversion of sedoheptulose 7-phosphate into fructose 6-phosphate and erythrose 4-phosphate by a transaldolase activity (39) in the extracts, or into D-ribose 5-phosphate and D-xylulose 5-phosphate by a transketolase activity (39), or the conversions of triose phosphates and glucose 6-phosphate which are present as contaminants in the sedoheptulose 7-phosphate preparation.

From this experiment we concluded that the 8.2-min peak corresponded to the reaction product, presumably D-glycero-D-mannoheptose 7-phosphate, however, this could not be verified directly since this phosphosugar is not commercially available. To prove that the product was a phosphorylated form of D-glycero-D-mannoheptose, reaction components were dephosphorylated by treatment with alkaline phosphatase prior to HPLC analysis. A peak with a retention time of 10.5 min corresponding to the retention time of authentic glyceromannoheptose was detected (Fig. 6). A peak with a retention time of 8.2 min corresponding to the reaction product in the absence of alkaline phosphatase was not detected in this experiment (Fig. 6, arrow). Therefore, we concluded that the product of the reaction in the presence of sedoheptulose 7-phosphate is a phosphorylated form of glyceromannoheptose. The two peaks in the glyceromannoheptose standard (Fig. 6) probably indicate the two anomeric forms of the sugar.

The HPLC analysis, however, did not allow us to determine if the product seen in the HPLC in the absence of alkaline phosphatase treatment is D-glycero-D-mannoheptose 7-phosphate or D-glycero-D-mannoheptose 1-phosphate. The latter is the product of the second reaction in the biosynthetic pathway of ADP-L-glycero-D-mannoheptose which is catalyzed by a phosphomutase (Fig. 7). However, since the phosphomutase reaction takes place after the formation of D-glycero-D-mannoheptose 7-phosphate and a peak corresponding to a phosphorylated D-glycero-D-mannoheptose is not present in the χ 711 extract with sedoheptulose 7-phosphate (Fig. 5A) we conclude that it must correspond to D-glycero-D-mannoheptose 7-phosphate and the *LpcA* protein must be the phosphoheptose isomerase.

DISCUSSION

Sequencing and mapping of the cloned *lpcA* locus revealed a single gene, *lpcA*, that is located at 5.3 min (246 kb) on the

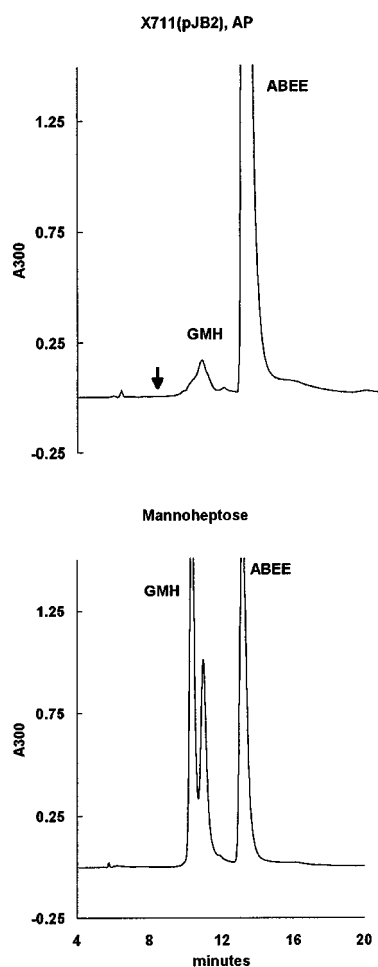


FIG. 6. Effect of alkaline phosphatase treatment in the reaction products analyzed by reversed-phase high performance liquid chromatography. Upper panel, HPLC profile of χ 711(pJB2) extract incubated with 1.0 μ mol of sedoheptulose 7-phosphate (SED-7-P) and treated with alkaline phosphatase (4 units) prior to derivatization with ABEE. Arrow indicates the location of the reaction peak of the reaction product in the absence of alkaline phosphatase treatment. Lower panel, HPLC profile of authentic glyceromannoheptose derivatized with ABEE. ABEE, p-aminobenzoic ethyl ester; AP, alkaline phosphatase; GMH, glyceromannoheptose.

chromosomal map of *E. coli*. This gene is physically unlinked to the majority of genes used in core biosynthesis that are found within the *rfa* cluster at 81 min on the chromosomal map. LpcA was expressed *in vitro* and *in vivo* as a protein of 22.6 kDa molecular mass. Results of preliminary fractionation experiments and the absence of characteristic features of membrane proteins suggested that LpcA is a soluble protein present in the cytoplasmic fraction.

The chromosomal deletion of the *lpcA* locus in *E. coli* strain χ 711 appeared to be the result of an IS5-mediated DNA rearrangement. Since an IS5 element is not present in the 14-kb *Eco*RI fragment of the parent strain χ 705 and χ 711 lacks *proAB*, we propose a transposition of an IS5 insertion element originally located in the vicinity of *proAB* within a region particularly rich in IS sequences (40). Recombination of the original IS5 insertion element and the newly replicated IS5 element may have resulted in the removal of the looped out DNA (Fig. 4D). IS5-mediated rearrangements have also been implicated in chromosomal DNA inversions in other *E. coli* K12 derivatives (41). It is also interesting that some mutations in other LPS genes have resulted from IS5 movements within the bacterial chromosome (42, 43).

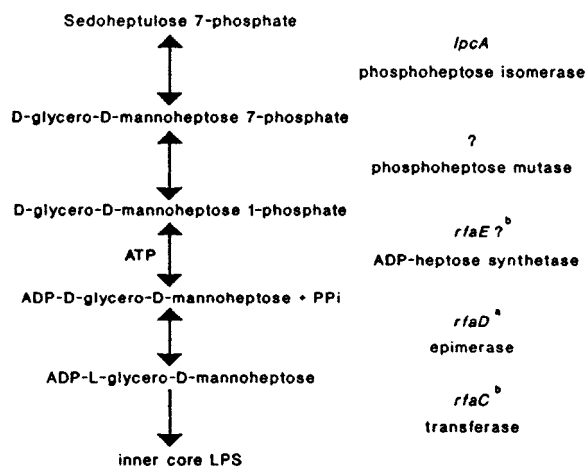


FIG. 7. A schematic diagram of the biosynthetic pathway of the nucleotide precursor ADP-L-glycero-D-mannoheptose, following Eidels and Osborn (28), Coleman (5)^a, and Sirisena *et al.* (47)^b.

In this study, we provide genetic and biochemical evidence that *lpcA* is necessary for heptose biosynthesis of inner core lipopolysaccharide in *E. coli*. Eidels and Osborn (28, 44), proposed a biosynthetic scheme for L-glycero-D-mannoheptose that uses the conversion of sedoheptulose 7-phosphate into ADP-L-glycero-D-mannoheptose. The four reactions needed for the synthesis of ADP-L-glycero-D-mannoheptose shown in Fig. 7, include: 1) conversion of sedoheptulose 7-phosphate to D-glycero-D-mannoheptose 7-phosphate by a phosphoheptose isomerase, 2) conversion of D-glycero-D-mannoheptose 7-phosphate to D-glycero-D-mannoheptose 1-phosphate by a phosphoheptose mutase, 3) conversion of D-glycero-D-mannoheptose 1-phosphate with ATP to ADP-D-glycero-D-mannoheptose and PP_i by an ADP-heptose synthetase, and 4) racemization by an epimerase of ADP-D-glycero-D-mannoheptose to the L-isomer. The completed ADP-L-glycero-D-mannoheptose is then used for the transfer of its sugar moiety onto the inner core LPS by a specific transferase. The only genes of this pathway fully characterized to date are *rfaD* and *rfaC*, encoding the epimerase (45) and the transferase (46, 47), respectively. Work by Sirisena *et al.* (47) in *Salmonella typhimurium* suggests that *rfaE* encodes the ADP-heptose synthetase since addition of ADP-glyceromannoheptose to cell extracts of *rfaE* mutants restores the synthesis of a complete core LPS, but a similar gene in *E. coli* has not been identified.

Our data demonstrate that *lpcA* restores the expression of a complete core LPS by the heptoseless mutant, *E. coli* strain χ 711, and encodes the phosphoheptose isomerase used in the biosynthesis of ADP-L-glycero-D-mannoheptose. Biochemical evidence for a phosphoheptose isomerase includes: 1) appearance of a new product upon incubation with sedoheptulose 7-phosphate with a concomitant reduction of substrate concentration, 2) the new product is a phosphorylated sugar, 3) upon dephosphorylation, the product has the same HPLC retention time as authentic glyceromannoheptose, 4) the new product does not appear in reactions with cell extracts of the *lpcA* mutant. In addition, a region of the LpcA protein has amino acid sequence homology with a family of aldo/keto isomerases.

Sedoheptulose 7-phosphate has been isolated from plants such as *Sedum spectabile* (48), and from animal tissues such as rat liver (49) and chicken muscle (50), as an intermediate in the nonoxidative portion of the pentose phosphate pathway (39). To date there are no reports of an isomerase in plants or animals that uses sedoheptulose 7-phosphate as a substrate. Thus, its conversion into D-glycero-D-mannoheptose 7-phosphate could be interpreted as a specialized branch of the pentose phosphate

pathway for LPS synthesis. This branch is likely to be present in many if not all Gram-negative bacteria.

The degree of similarity of LpcA with the lincomycin biosynthetic gene product LmbN of *S. lincolnensis*, suggests that *lmbN* may encode an isomerase needed for the synthesis of this antibiotic. Chemical synthesis of 8-carbon sugar derivatives as potential intermediates leading to the production of methyl 6-amino-6,8-dideoxy-1-thi-D-erythro- α -D-galacto-octopyranoside, the carbohydrate moiety of lincomycin, appears to require an isomerization step (51).

The fact that glyceromannoheptose is a very common component of the inner core LPS of many enteric (52) and non-enteric bacteria (4) and the conservation of *lpcA* among enteric bacteria suggests that this gene has an essential function in a conserved pathway for ADP-L-glycero-D-mannoheptose synthesis. Although the *lpcA* homolog was not identified in *Pseudomonas*, we believe that this function does exist in this genus but lack of hybridization with the probe reflects the high hybridization stringency used in this experiment.

In general, genes necessary for synthesis of the conserved lipid A and inner core components are found scattered in the *E. coli* K12 chromosome: lipid A synthesis genes (*lpxA*, *lpxB*, and *lpxD*) are found at 4 min, 3-deoxy-D-manno-octulosonic acid pathway genes (*kdsA* and *kdsB*) are found at 27 and 85 min, respectively. From the heptose pathway, only *rfaD* and *rfaC* are located at one end of the *rfa* cluster at 81 min (4) next to the other genes for synthesis of the structurally more variable outer core components whereas the *lpcA* gene is located outside of the *rfa* cluster. The G + C content of *lpcA* is similar to the G + C content of the lipid A and 3-deoxy-D-manno-octulosonic acid pathway genes, *rfaC* and *rfaD* genes, and is close to the average G + C content for *E. coli* and other enteric bacteria. This conservation of G + C supports the suggestion that biosynthesis genes required for lipid A and inner core may have been part of a common enterobacterial genome, and that outer core biosynthesis genes, which have a lower G + C content, evolved later (4).

This study reports the first molecular characterization of a novel phosphoheptose isomerase in prokaryotes that uses sedoheptulose 7-phosphate as a substrate, and it is needed for the first reaction committed to the biosynthesis and assembly of inner core lipopolysaccharide in enteric bacteria. Our findings have relevance to the area of infection since bacterial strains with defects in core LPS are more susceptible to the killing effect of serum complement and phagocytosis (7). The molecular details of the enzyme-substrate activity, currently being assessed in our laboratory, will provide further information about the use of sedoheptulose 7-phosphate as an intermediary component of the pentose phosphate pathway, and will lead to the design of enzyme inhibitors which may serve as novel antimicrobial agents.

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REFERENCES

- Schnaitman, C. A., and Klena, J. D. (1993) *Microbiol. Rev.* **57**, 655–682.
- Whitfield, C., and Valvano, M. A. (1993) *Adv. Microb. Physiol.* **35**, 135–246.
- Hancock, R. E. W., Karunaratne, D. N., and Bernegger-Egli, C. (1994) in *Bacterial Cell Wall* (Ghuysen, J.-M., and Hakenbeck, R., eds) pp. 263–279, Elsevier, New York.
- Reeves, P. (1994) in *Bacterial Cell Wall* (Ghuysen, J.-M., and Hakenbeck, R., eds) pp. 281–317, Elsevier, New York.
- Coleman, W. G., Jr., and Leive, L. (1979) *J. Bacteriol.* **139**, 899–910.
- Tamaki, S., Sato, T., and Matsuhashi, M. (1971) *J. Bacteriol.* **105**, 968–975.
- Taylor, P. W. (1983) *Microbiol. Rev.* **47**, 46–83.
- Havekes, L. M., Lugtenberg, B. J. J., and Hoekstra, W. P. M. (1976) *Mol. & Gen. Genet.* **146**, 43–50.
- Yao, Z., Liu, H., and Valvano, M. A. (1992) *J. Bacteriol.* **174**, 7500–7508.
- Selvaraj, G., Fong, Y. C., and Iyer, V. N. (1984) *Gene (Amst.)* **32**, 235–241.
- Marolda, C. L., Welsh, J., Dafee, L., and Valvano, M. A. (1990) *J. Bacteriol.* **172**, 3590–3599.
- Deuschle, U., Kammerer, W., Gentz, R., and Bujard, H. (1986) *EMBO J.* **5**, 2987–2994.
- Glisin, V., Crkvenjakov, R., and Byus, C. (1974) *Biochemistry* **13**, 2633–2637.
- Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988) *Nucleic Acids Res.* **16**, 6127–6145.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Henikoff, S. (1984) *Gene (Amst.)* **28**, 351–359.
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467.
- Beck, K.-F., Stathopoulos, I., Berninger, M. G., and Schweizer, M. (1993) *BioTechniques* **14**, 375.
- Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403–441.
- Hofmann, K., and Stoffel, W. (1992) *Comput. Appl. Biosci.* **8**, 331–337.
- Dunn, J. J., and Studier, F. W. (1983) *J. Mol. Biol.* **166**, 477–535.
- Kassavetis, G. A., Butler, E. T., Roulland, D., and Chamberlin, M. J. (1982) *J. Biol. Chem.* **257**, 5779–5788.
- Poole, K., Schiebel, E., and Braun, V. (1988) *J. Bacteriol.* **170**, 3177–3188.
- Laemmli, U. K. (1970) *Nature* **227**, 680–685.
- Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379.
- Dubray, G., and Bezard, G. (1982) *Anal. Biochem.* **119**, 325–329.
- Eidels, L., and Osborn, M. J. (1974) *J. Biol. Chem.* **249**, 5642–5648.
- Wang, W. T., LeDonne, N. C., Jr., Ackerman, B., and Sweeley, C. C. (1984) *Anal. Biochem.* **141**, 366–381.
- Kwon, H., and Kim, J. (1993) *Anal. Biochem.* **215**, 243–252.
- Curtiss, R., Charamella, J., Stallions, D. R., and Mays, J. A. (1968) *Bacteriol. Rev.* **32**, 320–348.
- Qureshi, N., Takayama, K., Mscagni, P., Honovich, J., Wong, R., and Cotter, R. J. (1988) *J. Biol. Chem.* **263**, 11971–11976.
- Tabata, S., Higashitani, A., Takanami, M., Akiyama, K., Kohara, Y., Nishimura, Y., Nishimura, A., Yasuda, S., and Hirota, Y. (1989) *J. Bacteriol.* **171**, 1214–1218.
- Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
- Kohara, Y., Akiyama, K., and Isono, K. (1987) *Cell* **50**, 495–508.
- Golinelli-Pimponeau, B., Le Goffic, F., and Badet, B. (1989) *J. Am. Chem. Soc.* **111**, 3029–3034.
- Rose, I. A. (1975) *Adv. Enzymol.* **43**, 491–517.
- Zalkin, H. (1993) *Adv. Enzymol.* **66**, 203–405.
- Wood, T. (1985) *The Pentose Phosphate Pathway*, Academic Press, New York.
- Birkenbihl, R. P., and Vielmetter, W. (1989) *Mol. & Gen. Genet.* **220**, 147–153.
- Umeda, M., and Ohtsubo, E. (1990) *J. Mol. Biol.* **213**, 229–237.
- Yao, Z., and Valvano, M. A. (1994) *J. Bacteriol.* **176**, 4133–4143.
- Liu, D., and Reeves, P. R. (1994) *Microbiology* **140**, 49–57.
- Eidels, L., and Osborn, M. J. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68**, 1673–1677.
- Coleman, W. G., Jr. (1983) *J. Biol. Chem.* **258**, 1985–1990.
- Chen, L., and Coleman, W. G., Jr. (1993) *J. Bacteriol.* **175**, 2534–2540.
- Sirisena, D. M., Brozek, K. A., MacLachlan, P. R., Sanderson, K. E., and Raetz, C. R. H. (1992) *J. Biol. Chem.* **267**, 18874–18884.
- Ujejski, L., and Waygood, E. R. (1955) *Can. J. Chem.* **33**, 687–691.
- Wood, T., and Poon, W. M. (1970) *Arch. Biochem. Biophys.* **141**, 440–446.
- Yardley, H. J., and Godfrey, G. (1963) *Biochem. J.* **86**, 101–103.
- Howarth, G. B., Lance, D. G., Szarek, W. A., and Jones, J. K. N. (1969) *Can. J. Chem.* **47**, 75–79.
- Rick, P. D. (1987) in *Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology* (Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umberger, H. E., eds) pp. 647–662, American Society for Microbiology, Washington, D. C.