JOURNAL OF BACTERIOLOGY, Oct. 1986, p. 152–159
0021-9193/86/100152-08$02.00/0

Vol. 168, No. 1

Molecular Cloning of the Genes for Lipid A Disaccharide Synthase and UDP-*N*-Acetylglucosamine Acyltransferase in *Escherichia coli*

DRING N. CROWELL, MATT S. ANDERSON, AND CHRISTIAN R. H. RAETZ*

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 13 March 1986/Accepted 15 June 1986

Several enzymes have been discovered recently in crude extracts of *Escherichia coli* that appear to be involved in the biosynthesis of the lipid A component of lipopolysaccharide. Two of these are lipid A disaccharide synthase and UDP-*N*-acetylglucosamine acyltransferase. Lipid A disaccharide synthase activity is barely detectable in cells harboring a lesion in the *lpxB* (*pgsB*) gene. We subcloned the *lpxB* gene from plasmid pLC26-43 of the Clarke and Carbon collection (L. Clarke and J. Carbon, Cell 9:91–99, 1976) and localized it to a 1.7-kilobase-pair fragment of DNA counterclockwise of *dnaE* on the *E. coli* chromosome. Furthermore, we discovered a new gene (*lpxA*) located adjacent to and counterclockwise of *lpxB* that encodes or controls UDP-*N*-acetylglucosamine acyltransferase. Our data prove that *lpxB* and *lpxA* are transcribed in the clockwise direction and suggest that they may be cotranscribed.

The *Escherichia coli* envelope consists of two membranes separated by a rigid peptidoglycan cell wall. It is the lipopolysaccharide component of the outer membrane that gives *E. coli* many of its pathogenic properties (16, 20, 23). Lipopolysaccharide is a B-cell mitogen as well as a potent toxin to many mammalian species (23). It consists of a phosphorylated glycolipid, called lipid A, that constitutes the outer leaflet of the outer membrane, a core oligosaccharide domain that is relatively conserved among gram-negative bacteria, and an O-antigen chain that extends into the medium (23). The lipid A moiety is responsible for most of the immunostimulatory and toxic properties of lipopolysaccharide (7, 15, 23).

Lipid A biosynthesis has not been studied in detail, but several enzymes believed to be involved in lipid A biosynthesis have been discovered recently in extracts of *E. coli* (1, 4, 22). Acylation of UDP-*N*-acetylglucosamine (UDP-GlcNAc) at the 3 position of the glucosamine moiety, which is thought to be the first committed step (Fig. 1), occurs in a β-hydroxymyristoyl-acyl carrier protein (ACP)-dependent reaction that is catalyzed by UDP-GlcNAc acyltransferase (1). The product of this reaction then undergoes substitution at the 2 position of the glucosamine ring (M. S. Anderson and C. R. H. Raetz, manuscript in preparation) in the presence of *E. coli* crude extracts and β-hydroxymyristoyl-ACP to form UDP-2,3-diacyl-glucosamine (Fig. 1), which is enzymatically hydrolyzed to 2,3-diacyl-GlcN-1-P (1). Nothing has been reported about the genes involved in these reactions.

E. coli cells harboring a lesion in the *lpxB* gene, previously designated *pgsB*, massively accumulate UDP-2,3-diacyl-GlcN and 2,3-diacyl-GlcN-1-P (4, 18, 19, 26). The *lpxB* gene maps near minute 4 on the *E. coli* chromosome, in close proximity to the gene encoding the α subunit of DNA polymerase III (*dnaE*) (17, 25). Strains bearing the *lpxB1* mutation accumulate these compounds because they lack lipid A disaccharide synthase (22), which catalyzes the following reaction: UDP-2,3-diacyl-GlcN + 2,3-diacyl-GlcN-1-P → 2′,3′-diacyl-GlcN (β,1 → 6) 2,3-diacyl-GlcN-1-P + UDP (Fig. 1). In the presence of the *pgsA444* lesion (near minute 42), which causes a deficiency in phosphatidylglycerol synthesis, *lpxB1* strains accumulate monosaccharide lipid A precursors to an even greater extent and become temperature sensitive for growth (17). The interaction between the *lpxB* and *pgsA* genes is still unexplained (17, 18).

We subcloned the *lpxB* gene from plasmid pLC26-43 of the Clarke and Carbon collection (5, 17) and demonstrated overproduction of lipid A disaccharide synthase in *E. coli* cells harboring certain *lpxB*$^+$ plasmids. We found that *lpxB* is situated 500 base pairs counterclockwise of *dnaE* on a 1.7-kilobase-pair (kbp) *Pvu*II-*Nru*I fragment and that a new gene (*lpxA*), encoding or controlling UDP-GlcNAc acyltransferase, is located adjacent to and counterclockwise of *lpxB*. We present evidence that *lpxB* and *lpxA* are transcribed in the clockwise direction and might be part of an operon.

MATERIALS AND METHODS

Materials. Restriction and modification enzymes were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or from New England Biolabs (Beverly, Mass.). Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). T4 DNA ligase, $^{32}P_i$, and L-[^{35}S]methionine were obtained from New England Nuclear Corp. (Boston, Mass.). Antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.). Agarose and reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were from Bethesda Research Laboratories. Tryptone, yeast extract, and agar were obtained from Difco Laboratories (Detroit, Mich.). Kodak XAR-5 film was used for autoradiography.

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Strain DC1 was constructed by Hfr-mediated transfer of *recA56 srl-300*::Tn*10* from strain JC10240 into strain MN7 (*pgsA444 lpxB1 rpsL136*). The resulting tetracycline-resistant, streptomycin-resistant exconjugants were then scored for temperature sensitivity (*pgsA444 lpxB1*) and methyl methanesulfonate sensitivity (*recA56*). DS410 was used to radiolabel plasmid-encoded proteins (24). Except where otherwise noted, all strains were grown in LB medium (14) at 30°C. Plasmids pING1, pSR1,

* Corresponding author.

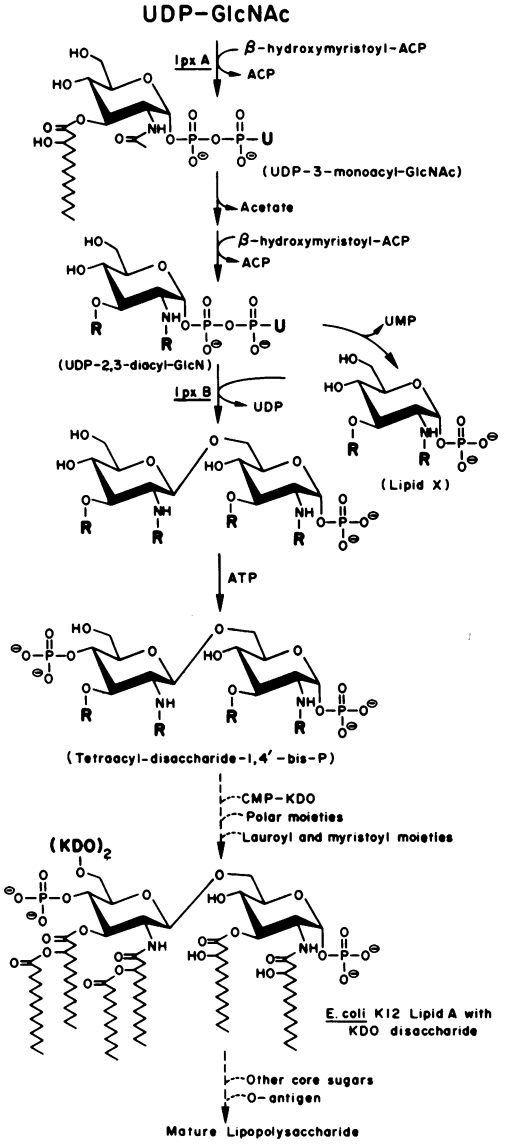


FIG. 1. Biosynthesis of lipid A disaccharides from monosaccharide precursors in extracts of *E. coli*. Evidence for this scheme has been presented previously (1, 4, 22, 26). Abbreviations: R, a β-hydroxymyristoyl moiety; U, uridine; KDO, 2-keto-3-deoxyoctulosonic acid.

and pSR8 were used in strain MC1061 (10), which was grown at 30°C in LB medium (supplemented with 0.5% fructose and 50 mM potassium phosphate at pH 7.4) to a cell density (A_{550}) of 0.7 and then induced to express the *araB* promoter by growth in the presence of 0.5% L-(+)-arabinose at 30°C.

Plasmids. Vector plasmids used were pBR322 (3) and pING1 (10). Plasmid pLC26-43 of the Clarke and Carbon collection (5) was the starting material for the cloning of *lpxB* and *lpxA*.

Recombinant DNA techniques. Plasmid preparation and cloning techniques were carried out as described by Maniatis et al. (13). Dephosphorylation of vector DNA prior to ligation was achieved by digestion with calf intestine alkaline phosphatase. DNA fragments were isolated from agarose gels by electroelution into 7.5 M ammonium acetate with an apparatus purchased from International Biotechnologies, Inc., or, alternatively, by adsorption onto DEAE-cellulose paper and elution with 1.7 M NaCl (6).

Transformation of *E. coli* cells. *E. coli* cells were made competent for transformation by $CaCl_2$ treatment as described previously (13). Transformants were spread onto LB plates at 30 μg/ml of the appropriate antibiotic and incubated at 30°C.

Phospholipid composition. The phospholipid compositions of strains DC1, DC1(pDC2), DC1(pDC4), and R477 were analyzed by growing 1-ml cultures in G56 medium (8) at 30°C to a cell density (A_{550}) of 0.7. The cultures were then labeled with 50 μCi of $^{32}P_i$ and shifted to 42°C for 3 h. The cells were sedimented and extracted (2) with chloroform–methanol–0.1 M aqueous HCl (2:2:1.8; vol/vol/vol), and the lower phases were dried under N_2 gas. The dried residues were redissolved in chloroform-methanol (4:1; vol/vol) to 4,000 cpm/μl. A 5-μl portion of each sample was then spotted onto a silica gel 60 (E. Merck AG, Darmstadt, Federal Republic of Germany) thin-layer plate, which was developed in chloroform-methanol-water-ammonium hydroxide (40:25:4:2; vol/vol/vol/vol) and autoradiographed.

Enzymatic assays. All assays were performed on crude cell extracts of stationary-phase cultures, which were prepared by passage through a French pressure cell at 18,000 lb/in^2. Unbroken cells were removed before assay by centrifugation at 2,000 × *g* for 10 min. Protein concentrations were determined by the method of Lowry et al. (12).

Lipid A disaccharide synthase assays were performed as described by Ray et al. (22), with each substrate present at 1 mM. UDP-GlcNAc acyltransferase assays were performed essentially as described by Anderson et al. (1). Briefly, β-[^{32}P]-UDP-GlcNAc (116 μM, 1.1 × 10^5 cpm/nmol), β-hydroxylmyristoyl-ACP (300 μM), octyl-β-D-glucoside (1%), and HEPES (*N*-2-hydoxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (40 mM, pH 8.0) were mixed with crude extract to a final volume of 20 μl at 30°C. Portions (5 μl) of the reaction were taken at 1 and 2 min, quenched with 20 μl of chloroform-methanol (1:2; vol/vol), and spotted on silica gel 60 (E. Merck) thin-layer plates that were then developed with chloroform-methanol-water-acetic acid (25:15:4:2; vol/vol/vol/vol). The spot corresponding to UDP-3-monoacyl-GlcNAc (1; Anderson and Raetz, manuscript in preparation) was localized by autoradiography, scraped, and counted in 10 ml of the scintillation cocktail described by Patterson and Green (21). Assay tubes contained 0.05 to 1.20 nmol of enzymatic activity per min. Octyl-β-D-glucoside was included to inhibit the formation of additional metabolites of UDP-3-monoacyl-GlcNAc (Anderson and Raetz, manuscript in preparation).

Enzyme specific activites in Figure 4 and Table 2 are normalized to 1.0 (1) for both enzymes because of twofold variability in UDP-GlcNAc acyltransferase activity from culture to culture.

TABLE 1. Strains of *E. coli* K-12 used in this study

Strain	Relevant genotype	Source or reference
R477	*pgsA*$^+$ *lpxB*$^+$ *recA*$^+$ *rpsL136*	18
MN7	*pgsA444 lpxB1 recA*$^+$ *rpsL136*	17 and 18
JC10240	Hfr *recA56 srl-300*::Tn*10*	CGSC[a]
DC1	*pgsA444 lpxB1 recA56 rpsL136 srl-300*::Tn*10*	This study
DS410	*minA minB*	24
MC1061	*araD139* Δ(*ara-leu*)*7697 hsdR hsdM*$^+$	10

[a] *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

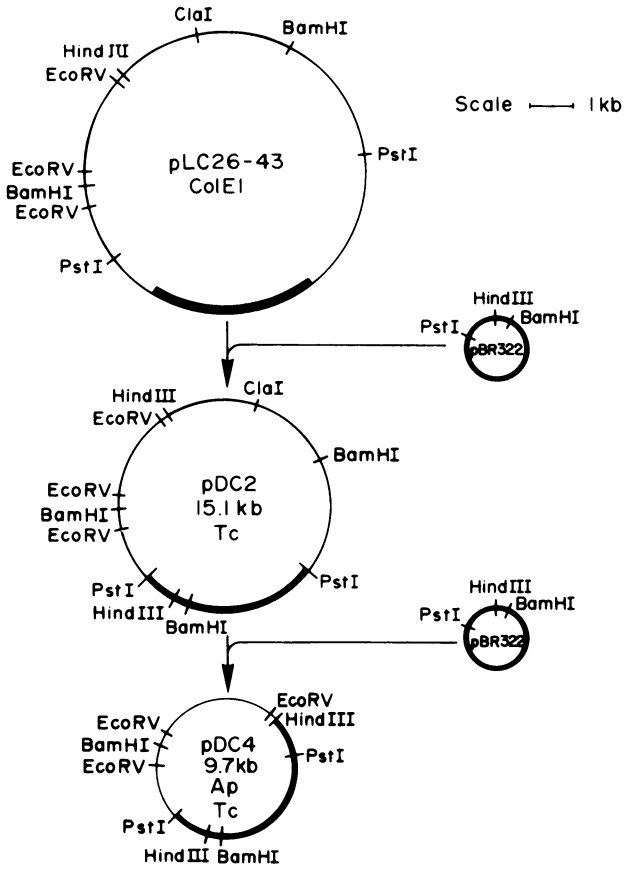


FIG. 2. Construction of plasmid pDC4. The cloning steps that led to the construction of pDC4 are shown. A description of these steps is given in the text. All hybrid plasmids shown correct the *lpxB1* phenotype. The precise size and structure of pLC26-43 is not known. pDC2 is probably identical to pMWE103, which has been described by Welch and McHenry (27). Plasmid sizes, antibiotic resistances, and relevant restriction enzyme recognition sites are shown. Fine lines represent *E. coli* chromosomal DNA and heavy lines represent vector DNA. All chromosomal inserts are shown with correct clockwise orientation. Abbreviations: Ap, ampicillin resistance; Tc, tetracycline resistance. Descriptions of relevant procedures are given in the text.

Radiolabeling of plasmid-encoded proteins. L-[^{35}S]methionine labeling of plasmid-encoded proteins was done in strain DS410, as described by Roozen et al. (24). Minicells carrying pSR1, pSR8, or pING1 were incubated at 37°C for 1 h in the presence of 0.5% L-(+)-arabinose before radiolabeling. Sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis was performed as described by Laemmli (11). Equal amounts of protein (approximately 50 μg) were loaded in each lane.

RESULTS

Subcloning of the *E. coli lpxB* gene. The *lpxB* gene of *E. coli* (referred to as *pgsB* in earlier studies [17]) was subcloned from pLC26-43 of the Clarke and Carbon collection (5), because this plasmid is capable of correcting the *lpxB1* phenotype (17). The sequence of cloning steps leading to pDC4 is shown in Fig. 2. As reported by Welch and McHenry (27), a complete *Pst*I digestion of pLC26-43 liberates a 10.7-kbp chromosomal DNA fragment. This fragment was inserted into the *Pst*I site of pBR322 to obtain the 15.1-kbp plasmid pDC2. Plasmid pDC2 was then digested completely with *Hin*dIII, and the resulting mixture was ligated into a *Hin*dIII digest of pBR322. This procedure yielded the 9.7-kbp plasmid pDC4. (Note that redundant vector DNA on pDC4 restores the tetracycline resistance gene.)

Overproduction of lipid A disaccharide synthase by plasmids pDC2 and pDC4. After successfully subcloning the *lpxB* gene from pLC26-43 (5), as judged by its ability to render strain DC1 temperature resistant, it was necessary to show that the plasmids obtained overproduce lipid A disaccharide synthase. Accordingly, crude cell extracts of DC1, DC1(pDC2), DC1(pDC4), and R477 (*pgsA*$^+$ *lpxB*$^+$) were prepared and assayed for lipid A disaccharide synthase activity. As expected, plasmids pDC2 and pDC4 directed 15-fold overproduction of lipid A disaccharide synthase activity (data not shown), demonstrating that these plasmids correct the enzyme deficiency associated with the *lpxB1* lesion and arguing that *lpxB* is the structural gene for this enzyme. Because DC1 harbors the *recA56* mutation, correction of the enzymatic lesion must be due to complementation rather than recombination.

Suppression of monosaccharide lipid A precursor accumulation in vivo by plasmids pDC2 and pDC4. To confirm the correction of the *lpxB1* lesion by plasmids pDC2 and pDC4, the ability of these plasmids to suppress the accumulation in vivo of monosaccharide lipid A precursors was also examined. The procedure for determining phospholipid composition is discussed above. Strain DC1 accumulated polar phospholipid species, after a temperature shift to 42°C, that did not appear in the *pgsA*$^+$ *lpxB*$^+$ parental strain R477 (Fig. 3). DC1 harboring either plasmid pDC2 or pDC4, however, was restored to the wild-type phenotype and did not accumulate these compounds at 42°C, indicating that the same mutation (*lpxB1*) is responsible for the temperature-sensitive growth, the disaccharide synthase deficiency, and the accumulation of precursors.

Enzyme activities of pDC4 subclones. All subclones of pDC4 are shown in Fig. 4 and were constructed as follows. (i) Fragments to be cloned were obtained by digestion of pDC4 with the appropriate enzymes (Fig. 4). These DNAs were purified by preparative agarose gel electrophoresis and harvested as described above. (ii) Fragments not blunt at both ends were treated with the Klenow fragment of DNA polymerase I in the presence of the four deoxyribonucleoside triphosphates. (iii) All fragments were ligated into pBR322, which was linearized by digestion with *Cla*I, blunt ended by treatment with the Klenow fragment of DNA polymerase I as described above, and dephosphorylated as described above. (iv) Ligation mixtures were used to transform R477 to ampicillin resistance, and plasmids were screened by restriction analysis. Plasmids were then used to transform DC1, in which lipid A disaccharide synthase and UDP-GlcNAc acyltransferase activities were determined. All of these plasmids, except pDC25 and pDC27, corrected the *lpxB1* lesion, and all existed at approximately equivalent copy numbers, as judged by plasmid yield after CsCl density gradient equilibrium centrifugation (data not shown). The largest subclones of pDC4, called pCR6 and pCR9, carried a 3.5-kbp *Xho*I-*Pst*I fragment and expressed both enzyme activities (Fig. 4). These results demonstrate that the *Xho*I-*Pst*I fragment carried not only *lpxB* but also a gene (*lpxA*) that encodes or controls UDP-GlcNAc acyltransferase. Plasmid pCR6, however, produced levels of these enzyme activities that were 5- to 10-fold lower than the levels produced by

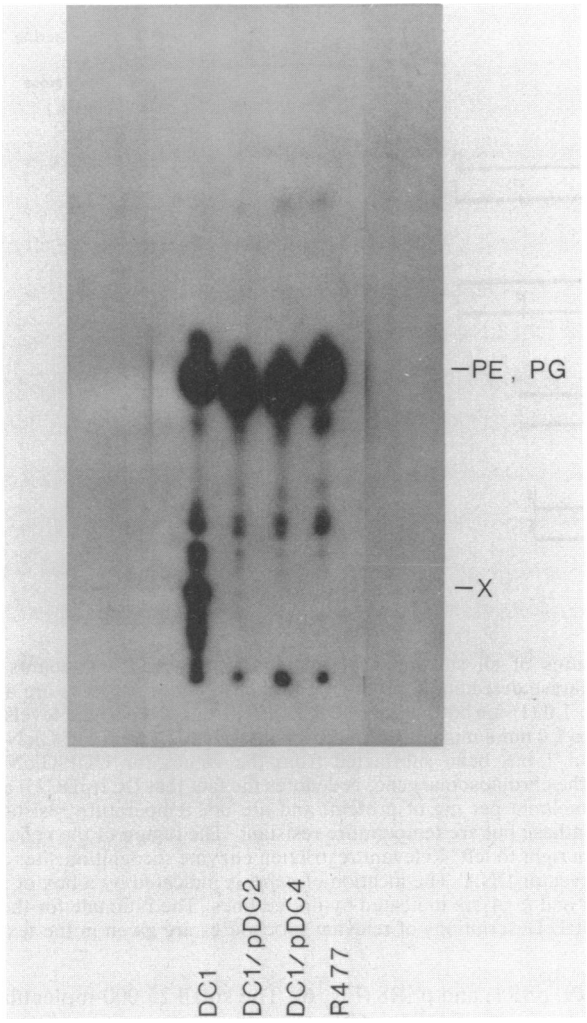


FIG. 3. Plasmids pDC2 and pDC4 suppress the accumulation in vivo of monosaccharide lipid A precursors. Phospholipid profiles of strains DC1, DC1(pDC2), DC1(pDC4), and R477 are shown. Lanes are labeled at the bottom. Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; X, 2,3-diacyl-GlcN-1-P. The procedure for demonstrating phospholipid composition is described in the text.

pCR9, which has the opposite vector orientation. This orientation effect on enzyme activity can be explained in various ways, including different vector promoter effects or different vector influences on promoter activity from the insert.

Plasmids pDC28 and pDC29 are analogous to pCR6 and pCR9, respectively, but they carry a 2.4-kbp *Pvu*II-*Pst*I chromosomal insert. Hence, they lack 1.1 kbp of genomic DNA clockwise of *lpxB* present on pCR6 and pCR9. This loss of clockwise-flanking DNA had no significant effect on *lpxB* or *lpxA* expression in either orientation (Fig. 4), arguing that this DNA is unimportant for plasmid expression of these genes. Removal of chromosomal DNA counterclockwise of *lpxB*, however, dramatically influenced expression of both *lpxB* and *lpxA*, as demonstrated by plasmids pDC24 to pDC27. Plasmids pDC24 and pDC25 harbored a 1.7-kbp *Pvu*II-*Nru*I DNA fragment with the same vector orientations as pCR6 and pCR9, respectively, and either failed to express *lpxB* altogether (pDC25) or expressed *lpxB* at very low levels (pDC24). Expression of *lpxA* from pDC24 and pDC25 above the chromosomally encoded background was undetectable. The low level of *lpxB* expression from pDC24 conferred temperature resistance to strain DC1, proving that the *lpxB* gene is present on the 1.7-kbp *Pvu*II-*Nru*I fragment carried by both pDC24 and pDC25. Plasmid pDC25, however, did not confer temperature resistance to strain DC1 and failed to produce any lipid A disaccharide synthase, suggesting that the *lpxB* gene is not expressed from this plasmid. Plasmids pDC26 and pDC27, which carry a 2.4-kbp *Hin*cII fragment with the same vector orientations as pCR6 and pCR9, respectively, confirmed the results obtained with pDC24 and pDC25. These results argue that removal of the 0.7-kbp *Nru*I-*Pst*I fragment or the 0.8-kbp *Hin*cII-*Pst*I fragment counterclockwise of *lpxB* deletes sequences necessary for *lpxB* and *lpxA* expression.

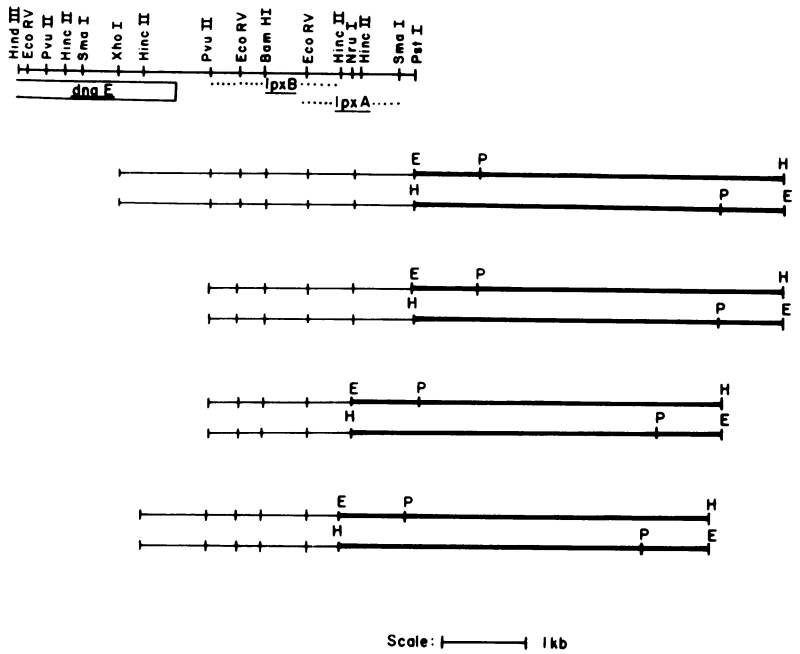
Overproduction of lipid A disaccharide synthase and UDP-GlcNAc acyltransferase by pING1-derived plasmids. To determine the gene order and direction of transcription of *lpxB* and *lpxA*, two DNA fragments were inserted into the expression vector pING1 (10), which is constructed such that cloned genes are placed under the control of the inducible *araB* promoter of the *Salmonella typhimurium* arabinose operon (Fig. 5). The hybrid plasmids pSR1 and pSR8 carry the 2.4-kpb *Pvu*II-*Pst*I fragment of pDC28 and pDC29 and the 1.7-kbp *Pvu*II-*Nru*I fragment of pDC24 and pDC25, respectively. Enzyme assays were then done on crude cell extracts of strain MC1061 carrying pING1, pSR1, or pSR8. (The cells were induced for 0, 4, and 8 h with 0.5% L-(+)-arabinose, as described above, before preparation of crude extracts.) Arabinose-inducible lipid A disaccharide synthase activity and UDP-GlcNAc acyltransferase activity were observed in cells carrying pSR1 (Table 2). Plasmid pSR8, on the other hand, was only capable of encoding arabinose-inducible lipid A disaccharide synthase activity. These results prove that *lpxB* and *lpxA* are distinct genes and are transcribed in the clockwise direction. Furthermore, it demonstrates that *lpxA* is upstream, or counterclockwise, of *lpxB*.

Radiolabeling of plasmid-encoded lipid A disaccharide synthase and UDP-GlcNAc acyltransferase in minicells. To demonstrate the molecular weights of the *lpxB* and *lpxA* gene products, and thus deduce the size of the *lpxB* and *lpxA* genes, minicell radiolabeling of plasmid-encoded proteins in strain DS410 (*minA minB*) was performed as described by Roozen et al. (24). Very little L-[^{35}S]methionine was incorporated into protein in strain DS410 after purification of the minicells by sucrose gradient centrifugation (Fig. 6, lanes marked "no DNA"). DS410(pBR322) (Fig. 6, lane pBR322)

TABLE 2. Relative enzyme specific activities encoded by pING1-derived plasmids

Time (h)	Sp act of the following strains[a]:					
	MC1061(pING1)		MC1061(pSR1)		MC1061(pSR8)	
	DSS (*lpxB*)	AT (*lpxA*)	DSS (*lpxB*)	AT (*lpxA*)	DSS (*lpxB*)	AT (*lpxA*)
0	1.0	1	1.1	1	1.1	1
4	0.7	1	13.5	74	48.0	1
8	1.1	1	10.4	140	55.2	1

[a] Enzyme assays were carried out as described in the text. For lipid A disaccharide synthase (DSS), a value of 1.0 corresponds to 2.0 nmol/min per mg of protein, the specific enzyme activity detected in MC1016(pING1) at time zero. For UDP-GlcNAc acyltransferase (AT), a value of 1 corresponds to 0.7 nmol/min per mg of protein, the specific enzyme activity detected in MC1061(pING1) at time zero.



Plasmid	Lipid A Disaccharide Synthase (lpxB)	UDP-GlcNAc Acyl-transferase (lpxA)
pCR6	1.0	1
pCR9	13.2	11
pDC28	1.0	1
pDC29	21.4	20
pDC24	0.2	0
pDC25	ts	0
pDC26	0.2	0
pDC27	ts	0

FIG. 4. Enzyme activities of pDC4 subclones. The names and structures of all subclones of pDC4 are shown. The amounts of plasmid-encoded lipid A disaccharide synthase and UDP-GlcNAc acyltransferase detected in strain DC1 harboring these plasmids are also shown. The enzyme specific activities shown in this figure are normalized to 1.0 (1) for both enzymes, which represents wild-type levels of these enzymes. A value of 1.0 for lipid A disaccharide synthase corresponds to 1.0 nmol/min per mg of protein. A value of 1 for UDP-GlcNAc acyltransferase corresponds to 2 nmol/min per mg of protein. A value of 1 has been subtracted from the values for UDP-GlcNAc acyltransferase, because DC1 produces wild-type levels of this enzyme from the chromosomal gene. ts denotes the fact that DC1(pDC25) and DC1(pDC27) produce no detectable lipid A disaccharide synthase (<0.1 nmol/min per mg of protein) and are not temperature resistant. DC1(pDC24) and DC1(pDC26) produce low levels of lipid A disaccharide synthase but are temperature resistant. The region of the genome (i.e., the chromosomal DNA of pDC4) carrying *lpxB* is shown clockwise from right to left. Relevant restriction enzyme recognition sites are marked. Fine lines depict *E. coli* chromosomal DNA and heavy lines depict vector DNA. The location of *dnaE* is indicated by a box of the appropriate size (25). The approximate locations and maximum sizes of *lpxB* and *lpxA* are indicated by dotted lines. The rationale for these assignments is given in the text. Abbreviations: E, *Eco*RI; P, *Pst*I; H, *Hin*dIII. Descriptions of relevant procedures are given in the text.

and DS410(pING1) (Fig. 6, lane pING1), however, synthesized two L-[^{35}S]methionine-labeled proteins, the molecular weights of which suggest that they were the two forms of the ampicillin resistance (*bla*) gene product (pre-β-lactamase and mature β-lactamase). Interestingly, two unique L-[^{35}S]methionine-labeled proteins with apparent molecular weights of 42,000 and 28,000 are seen in Fig. 6, lanes pCR9 and pDC29. These lanes correspond to DS410(pCR9) and DS410(pDC29), which were expected to overproduce both lipid A disaccharide synthase and UDP-GlcNAc acyltransferase. Hence, these two proteins, which are the only two proteins common to lanes pCR9 and pDC29 (Fig. 6) that were not present in the vector control lane pBR322 (Fig. 6), probably represent lipid A disaccharide synthase and UDP-GlcNAc acyltransferase. The 42,000-molecular-weight protein also appears in lanes pSR1 and pSR8 (Fig. 6), which represent radiolabelings of DS410(pSR1) and DS410(pSR8), respectively. This protein band was consistently broad and correlated with lipid A disaccharide synthase activity (Fig. 4 and Table 2). The 28,000-molecular-weight band, on the other hand, was present in lane pSR1 (Fig. 6) but not in lane pSR8 (Fig. 6) and thus correlates with UDP-GlcNAc acyltransferase activity.

These results suggest that *lpxB* is 1.1 to 1.2 kbp in length and that *lpxA* is approximately 0.8 kbp in length. Purification to homogeneity and characterization of these two proteins, as well as nucleotide sequencing of this region of the chromosome, will be required to make these assignments final. It is also worth noting that other bands appear in lanes pCR9, pSR1, and pSR8 (Fig. 6). The small 25,000-molecular-weight protein in lane pCR9 (Fig. 6) may correspond to the 5′ end of the *dnaE* gene present on pCR9 (Fig. 4). The proteins in lanes pSR1 and pSR8 (Fig. 6) with molecular weights of 33,000 to 35,000 may be fusion proteins with the *araB* gene product.

DISCUSSION

The cloning of the *lpxB* and *lpxA* genes not only facilitates the purification of the *E. coli* lipid A disaccharide synthase and UDP-GlcNAc acyltransferase but also permits the elucidation of the sequences and possible regulation of *lpxB* and *lpxA*. It seems likely that lipid A biosynthesis is essential for growth and division in *E. coli*, and the temperature-sensitive phenotype of *pgsA lpxB* strains is consistent with this belief (17). However, conditionally lethal alleles of *lpxB* with a phenotype that is independent of *pgsA444* would aid in fully demonstrating the physiological role of the *lpxB* gene. Similarly, mutations in *lpxA* would help to reveal the function of the *lpxA* gene. The inherent importance of the *lpxB* and *lpxA* genes, as well as the spatial relationship of these genes to other genes in the minute 4 region of the *E. coli* chromosome, makes the genomic organization and possible interaction of the genes in this region a subject of great interest.

The results of the study described here offer several new conclusions. The correction of the DC1 (*pgsA444 lpxB1 recA56*) phenotype by *lpxB*$^+$ plasmids confirms the finding by Nishijima et al. (17) that the *lpxB1* lesion can be comple-

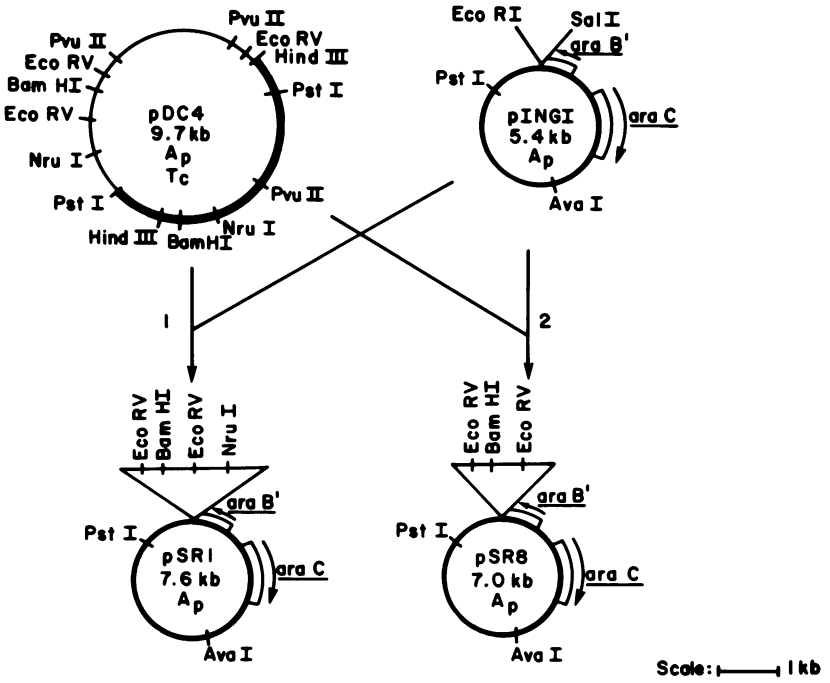


FIG. 5. Construction of pING1-derived plasmids. The cloning steps that led to the contruction of pSR1 and pSR8 are shown. pDC4 was digested with (i) *Pvu*II and *Pst*I or (ii) *Pvu*II and *Nru*I. In both cases the *lpxB*$^+$ fragment, was then isolated by gel electrophoresis and treated in the presence of the four deoxyribonucleoside triphosphates with the Klenow fragment of DNA polymerase I. pING1 (10) was digested with *Eco*RI and *Sal*I, blunt-ended by treatment with the Klenow fragment as described above, and dephosphorylated with calf intestine alkaline phosphatase (13). These DNAs were then mixed and treated with T4 DNA ligase. Plasmid sizes and antibiotic resistances are indicated. Not all restriction enzyme recognition sites are shown. Fine lines represent *E. coli* chromosomal DNA and heavy lines represent vector DNA. pDC4 is shown with the correct clockwise orientation. pSR1 and pSR8 inserts are shown clockwise from right to left. Abbreviations: Ap, ampicillin resistance; Tc, tetracycline resistance. Cloning procedures are described in the text.

mented and proves that *lpxB1* is responsible for the temperature-sensitive growth, the lipid A disaccharide synthase deficiency, and the accumulation of lipid A precursors in strain DC1. Various *lpxB*$^+$ plasmids also reveal a new gene, called *lpxA*, that encodes or controls UDP-GlcNAc acyltransferase activity. In addition, the cloning of *lpxB* and *lpxA* demonstrates that overproduction of lipid A disaccharide synthase and UDP-GlcNAc acyltransferase by gene dosage is possible. This enzyme overproduction suggests that *lpxB* and *lpxA* are the structural genes for lipid A disaccharide synthase and UDP-GlcNAc acyltransferase, respectively. Purification and characterization of these enzymes, followed

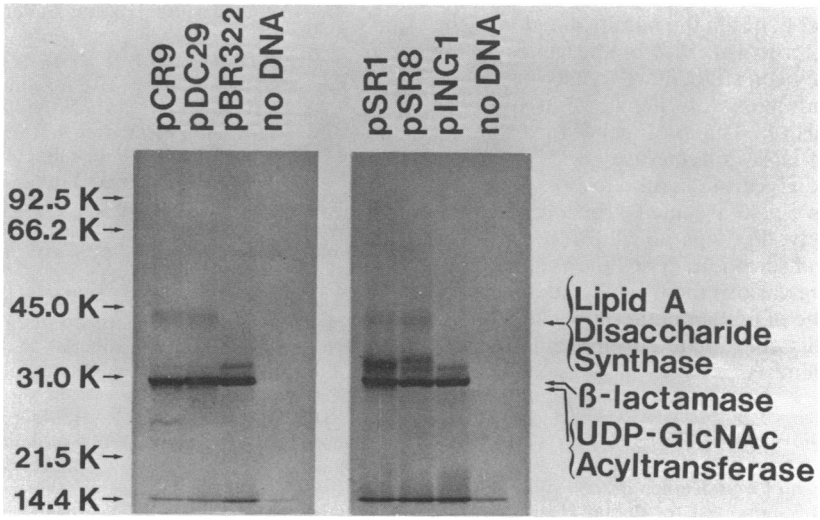


FIG. 6. Minicell radiolabeling of plasmid-encoded lipid A disaccharide synthase and UDP-GlcNAc acyltransferase. L-[^{35}S]methionine labeling of proteins encoded by pCR9, pDC29, pBR322, pSR1, pSR8, and pING1 is shown. Molecular weight standards are as follows: phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400. Descriptions of relevant procedures are given in the text.

by nucleotide sequencing of *lpxB* and *lpxA*, however, will be necessary to prove that *lpxB* and *lpxA* are indeed the structural genes for these enzymes.

The cloning of *lpxB* places the *lpxB* gene on a 1.7-kbp *Pvu*II-*Nru*I fragment counterclockwise of *dnaE*, and minicell radiolabeling of proteins encoded by *lpxB*$^+$ plasmids suggests that lipid A disaccharide synthase has a molecular weight of 42,000. We also showed, by cloning into the expression vector pING1, that the *lpxA* gene lies adjacent to and counterclockwise of *lpxB* on the *E. coli* chromosome and that both genes are transcribed in the clockwise direction. Minicell radiolabeling of proteins encoded by *lpxA*$^+$ plasmids suggests that UDP-GlcNAc acyltransferase has a molecular weight of 28,000.

We have evidence that *lpxB* and *lpxA* are cotranscribed and thus constitute part of a previously unknown operon. The levels of expression of *lpxB* and *lpxA* from the plasmids shown in Fig. 4 suggest that these two genes are coordinately expressed. The vector orientation effect on *lpxB* and *lpxA* expression from plasmids pCR6 and pCR9 and from plasmids pDC28 and pDC29, for example, argues that DNA upstream of *lpxB* and *lpxA* can influence the expression of both genes identically. Plasmids pDC28 and pDC29 also demonstrate that DNA downstream of *lpxB* does not affect plasmid expression of *lpxB* and *lpxA*. Removal of DNA upstream of *lpxB*, however, dramatically reduces expression of *lpxB* and *lpxA*, as illustrated by plasmids pDC24, pDC25, pDC26, and pDC27. We believe that these plasmids lack the 5′ end of *lpxA* because the 1.7-kbp *Pvu*II-*Nru*I fragment of pDC24 and pDC25 and the 2.4-kbp *Hin*cII fragment of pDC26 and pDC27 are too small counterclockwise of the *Pvu*II site to carry both *lpxB* and *lpxA*, and we know that both fragments carry *lpxB*. We also know that *lpxA* (0.8 kbp) must lie between *lpxB* (1.2 kbp) and the genomic *Sma*I site counterclockwise of *lpxB* because DNA inserted into the *Sma*I site does not abolish *lpxB* or *lpxA* expression (data not shown). Hence, transcription of *lpxB* is likely to begin upstream of *lpxA*, because removal of the 5′ end of *lpxA* has such a large effect on *lpxB* expression (Fig. 4). Nuclease S1 mapping of chromosomal RNA transcripts will be necessary to prove that *lpxB* and *lpxA* are cotranscribed.

The expression of *lpxB* and *lpxA* from plasmids described here provides the first clue that *lpxB* may exist as part of an operon that includes other genes in the minute 4 region of the *E. coli* chromosome. Interestingly, all of the genes in the minute 4 region that have been studied are transcribed in the clockwise direction, and many of them are involved in macromolecular synthesis (9). The *dnaE* gene, for example, encodes the α subunit of DNA polymerase III (27), and the *cds* gene encodes CDP-diglyceride synthetase (8, 9). These genes are located 0.5 kbp clockwise and 6 kbp counterclockwise of *lpxB*, respectively. Perhaps all of these genes are related at the level of transcription. If so, the regulation of such an operon, encoding various membrane and macromolecular functions, might be of fundamental significance to the mechanism by which cells coordinate chromosome replication and membrane biogenesis.

ACKNOWLEDGMENTS

We thank Susan Schlafmann for assistance in molecular cloning.

D.N.C. and M.S.A. were supported by Public Health Service training Grant for Cellular and Molecular Biology 2 T32 GM07215 from the National Institutes of Health. This work was supported in part by Public Health Service grant AM-19551 from the National Institutes of Health to C.R.H.R.

LITERATURE CITED

1. **Anderson, M. S., C. E. Bulawa, and C. R. H. Raetz.** 1985. The biosynthesis of gram-negative endotoxin: formation of lipid A precursors from UDP-GlcNAc in extracts of *Escherichia coli*. J. Biol. Chem. **260:**15536–15541.
2. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
4. **Bulawa, C. E., and C. R. H. Raetz.** 1984. The biosynthesis of gram-negative endotoxin: identification and function of UDP-2,3-diacylglucosamine in *Escherichia coli*. J. Biol. Chem. **259:**4846–4851.
5. **Clarke, L., and J. Carbon.** 1976. A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire *E. coli* genome. Cell **9:**91–99.
6. **Dretzen, G., M. Bellord, P. Sassone-Corsi, and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. **112:**295–298.
7. **Galanos, C., M. A. Freudenberg, O. Lüderitz, E. T. Rietschel, and O. Westphal.** 1979. Chemical, physiochemical and biological properties of bacterial lipopolysaccharides, p. 321–332. *In* E. Cohen (ed.), Biomedical application of the Horseshore crab (*Limulidae*). Alan R. Liss, Inc., New York.
8. **Ganong, B. R., J. M. Leonard, and C. R. H. Raetz.** 1980. Phosphatidic acid accumulation in the membranes of *Escherichia coli* mutants defective in CDP-diglyceride synthetase. J. Biol. Chem. **255:**1623–1629.
9. **Icho, T., C. P. Sparrow, and C. R. H. Raetz.** 1985. Molecular cloning and sequencing of the gene for CDP-diglyceride synthetase of *Escherichia coli*. J. Biol. Chem. **260:**12078–12083.
10. **Johnston, S., J. Lee, and D. S. Ray.** 1985. High-level expression of M13 gene II protein from an inducible polycistronic messenger RNA. Gene **34:**137–145.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
12. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431–435. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Morrison, D. C., and J. L. Ryan.** 1979. Bacterial endotoxins and host immune responses. Adv. Immunol. **28:**293–450.
16. **Nikaido, H.** 1973. Biosynthesis and assembly of lipopolysaccharide and the outer membrane layer of gram-negative cell wall, p. 131–208. *In* L. Leive (ed.), Bacterial membranes and walls. Marcel Dekker, Inc., New York.
17. **Nishijima, M., C. E. Bulawa, and C. R. H. Raetz.** 1981. Two interacting mutations causing temperature-sensitive phosphatidylglycerol synthesis in *Escherichia coli* membranes. J. Bacteriol. **145:**113–121.
18. **Nishijima, M., and C. R. H. Raetz.** 1979. Membrane lipid biogenesis in *Escherichia coli*: identification of genetic loci for phosphatidylglycerophosphate synthetase and construction of mutants lacking phosphatidylglycerol. J. Biol. Chem. **254:**7837–7844.
19. **Nishijima, M., and C. R. H. Raetz.** 1981. Characterization of two membrane-associated glycolipids from an *Escherichia coli* mutant deficient in phosphatidylglycerol. J. Biol. Chem. **256:**10690–10696.
20. **Osborn, M. J.** 1979. Biosynthesis and assembly of the lipopolysaccharide of the outer membrane, p. 15–34. *In* M. Inouye (ed.), Bacterial outer membranes. John Wiley & Sons, Inc., New York.
21. **Patterson, M. S., and R. C. Green.** 1965. Measurement of low

β-emitters in aqueous solution by liquid scintillation counting in emulsions. Anal. Chem. **37**:854–857.

22. **Ray, B. L., G. Painter, and C. R. H. Raetz.** 1984. The biosynthesis of gram-negative endotoxin: formation of lipid A disaccharides from monosaccharide precursors in extracts of *Escherichia coli*. J. Biol. Chem. **259**:4852–4859.
23. **Rietschel, E. T. (ed.).** 1984. Handbook of endotoxin, vol. 1. Elsevier Science Publishers B.V., Amsterdam.
24. **Roozen, K. J., R. G. Fenwick, Jr., and R. Curtiss. III.** 1971. Synthesis of ribonucleic acid and protein in plasmid-containing minicells of *Escherichia coli* K-12. J. Bacteriol. **107**:21–33.
25. **Shepard, D. D., R. W. Oberfelder, M. M. Welch, and C. S. McHenry.** 1984. Determination of the precise location and orientation of the *dnaE* gene of *Escherichia coli*. J. Bacteriol. **158**:455–459.
26. **Takayama, K., N. Qureshi, P. Mascagni, M. A. Nashed, L. Anderson, and C. R. H. Raetz.** 1983. Fatty acyl derivatives of glucosamine 1-phosphate in *Escherichia coli* and their relation to lipid A. J. Biol. Chem. **258**:7379–7385.
27. **Welch, M. M., and C. S. McHenry.** 1982. Cloning and identification of the product of the *dnaE* gene of *Escherichia coli*. J. Bacteriol. **152**:351–356.