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# Disruption of the *Escherichia coli cls* Gene Responsible for Cardiolipin Synthesis

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**The *cls* gene of *Escherichia coli* is responsible for the synthesis of a major membrane phospholipid, cardiolipin, and has been proposed to encode cardiolipin synthase. This gene cloned on a pBR322 derivative was disrupted by either insertion of or replacement with a kanamycin-resistant gene followed by exchange with the homologous chromosomal region. The proper genomic disruptions were confirmed by Southern blot hybridization and a transductional linkage analysis. Both types of disruptants had essentially the same properties; cardiolipin synthase activity was not detectable, but the strains grew well, although their growth rates and final culture densities were lower than those of the corresponding wild-type strains and strains with the classical *cls-1* mutation. A disruptant harboring a plasmid that carried the intact *cls* gene grew normally. The results indicate that the *cls* gene and probably the cardiolipin synthase are dispensable for *E. coli* but may confer growth or survival advantages. Low but definite levels of cardiolipin were synthesized by all the disruptants. Cardiolipin content of the *cls* mutants depended on the dosage of the *pss* gene, and attempts to transfer a null allele of the *cls* gene into a *pss-1* mutant were unsuccessful. We point out the possibilities of minor cardiolipin formation by phosphatidylserine synthase and of the essential nature of cardiolipin for the survival of *E. coli* cells.**

Cardiolipin, one of the major phospholipids of *Escherichia coli*, is unique in its structure among membrane lipids (i.e., tetraacyl structure with two phosphate groups) and has been postulated to play specific roles in membrane functions. It is synthesized from two molecules of phosphatidylglycerol by cardiolipin synthase (4, 20), which differs from its eucaryotic counterpart that utilizes CDP-diacylglycerol and phosphatidylglycerol as substrates (21). The gene *cls* is responsible for cardiolipin formation, and several lines of evidence indicate, though not definitely, that it is the structural gene for cardiolipin synthase (10).

An *E. coli* mutation (*cls-1*) that results in a defective cardiolipin synthase has been isolated (14), and the cells with this mutation have been shown to display only minor growth phenotypes, despite decreased levels of cardiolipin (14, 18). Therefore, it has been uncertain whether the *cls* gene and cardiolipin are essential in *E. coli* cells or whether the residual low levels of cardiolipin observed maintain membrane functions that are dependent on this particular phospholipid.

To determine whether the *cls* gene is essential and to analyze the physiological roles, if any, of this gene, an attempt to obtain a null mutation of this gene seemed most useful. This paper describes the construction, by genetic manipulations, of such mutants and their characteristic properties. The results show that the *cls* gene is not essential. Cardiolipin was formed at low levels even in the null *cls* mutants. We also present results that favor a hypothesis of secondary cardiolipin formation by phosphatidylserine synthase (EC 2.7.8.8) and suggest that cardiolipin is essential in *E. coli* cells.

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## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Major strains used and their genotypes are listed in Table 1. To show the genetic backgrounds of newly constructed strains, the letters CLI (for insertion into the *cls* gene), CLR (for replacement of the *cls* gene), and CLM (for a classical mutation in the *cls* gene) are added to the names of the corresponding parent strains. In addition to these derivatives listed, similar derivatives of strains D110, MM383, and OS2124 were constructed. Plasmid pCL11 is a 6.7-kilobase (kb) derivative of pBR322 which contains a 2.3-kb *E. coli* genomic fragment including the *cls* gene (10). pPS3155 (*pss*; 12), pUC4K (*kan*; 22), and pML31 (mini-F, *kan*; 6, 10) were previously described.

**Growth of bacteria and genetic manipulations.** Broth media LB (10) and NBY (18) and synthetic media M56LP fortified with amino acids (18) and minimal A (8) were used with supplementation of ampicillin or kanamycin (7). For growth of CLR strains in kanamycin-containing media, 200 μM isopropyl-β-D-thiogalactoside (IPTG) was added to induce the expression of the *kan* gene. To introduce the *cls-1* allele into strains other than CB64 (*trp-75*), strain NK5151 (*trpB83*::Tn*10*) was used to transduce them to $Trp^-$.

**DNA manipulations.** Methods for DNA isolation, sizing and separating DNA by using agarose gel electrophoresis, and bacterial transformation were described previously (7, 10). Transfer of DNA fragments to nitrocellulose for Southern hybridization analysis, preparation of DNA probes by using nick translation in the presence of [α-$^{32}$P]dCTP, hybridization to DNA bound to nitrocellulose, and detection by autoradiography were carried out as described previously (7). Enzymes for DNA manipulations were obtained from Takara Shuzo Co., Kyoto, Japan, and were used according to the recommendation of the manufacturer.

**Other assays.** Phospholipid content was determined by radioactivity measurements of the spots on two-dimensional thin-layer chromatograms for cells labeled uniformly with

TABLE 1. *E. coli* K-12 derivatives used in this work

| Strain | Genotype[a] | Source or reference |
|---|---|---|
| CB64 | *trp-75 cysB93 tfr-8* | CGSC[b] |
| CB64-CLI | CB64 *cls*::*kan*[c] *trp*$^+$ | This work |
| CB64-CLR | CB64 Δ*cls*::*kan*[d] *trp*$^+$ | This work |
| CB64-CLM | CB64 *cls-1*[e] *trp*$^+$ | This work |
| D110 | *polA1 thy dra endA* | K. Yamaguchi |
| D110-CLR | D110 Δ*cls*::*kan* | This work |
| MM383 | *polA12 thy rha lac str* | K. Yamaguchi |
| MM383-CLI | MM383 *cls*::*kan* | This work |
| NK5151 | *trpB83*::Tn*10* λ$^-$ θ(*rrnD-rrnE*)*1* | CGSC |
| OS2124 | *pyrD34 thyA33 galK35 xyl-7 mtl-2 thi-1 rpsL118 malA* λ$^r$ λ$^-$ | 11 |
| OS2101 | OS2124 *pss-1* | 11 |
| SD12 | *pyrD34 his-68 galK35 phoA8 glpD3 glpR2 glpK glpKp rpsL118* | 18 |
| SD12-CLI | SD12 *cls*::*kan* | This work |
| SD12-CLR | SD12 Δ*cls*::*kan* | This work |
| SD11 | SD12 *cls-1* | 18 |
| SD10 | SD12 *pss-1* | 18 |
| SD9 | SD12 *cls-1 pss-1* | 18 |
| SD90 | SD12 *recA1 cls-1 pss-1* | 10 |
| T16GP | *cls-1*[e] *thi-1 relA1* λ$^-$ | P. Overath |

[a] Allele numbers are those of the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn., except for *cls-1* (see footnote *e*). θ, Deletion by eduction.

[b] CGSC, *E. coli* Genetic Stock Center.

[c] *kan*, The structural gene for aminoglycoside-3′-phosphotransferase derived from pUC4K (22).

[d] Δ*cls*::*kan*, An allele of the *cls* gene with both a deletion and the insertion of the *kan* gene.

[e] This allele was originally described as *cls* (14) but is referred to as *cls-1* in this paper to avoid confusion with other mutations in the same gene.

$^{32}$P as described (10, 18). To identify the $^{32}$P-labeled spots appearing in the region of cardiolipin, they were scraped off the two-dimensional chromatographic plates, extracted, and mixed with authentic *E. coli* cardiolipin. A part of each sample was rechromatographed two dimensionally, and the rest of the sample was deacylated (19), followed by one-dimensional chromatography on cellulose plates (13, 19) in three different solvent systems, phenol-water-acetic acid-ethanol (80:20:10:12 by volume), ammonium acetate (0.5 M, pH 7.5)–ethanol (3:7, vol/vol), and methanol-formic acid-water (80:13:7, vol/vol/vol). Spots on the autoradiograms and those on the plates visualized by phosphate spray (Hanes-Isherwood reagent) were compared for their locations and intensities. Cardiolipin synthase activity in the crude membrane and soluble fractions was determined as described (10, 18). Protein was assayed by the method of Bradford (2) with crystalline bovine serum albumin as a standard.

## RESULTS

**Construction of null alleles of the *cls* gene.** The strategy for the construction consisted of the following three steps: identification of the region essential to the functional *cls* gene, disruption of the *cls* gene cloned on a pBR322-derived plasmid with the *kan* gene, and replacement of the chromosomal *cls* locus with the plasmid-borne, disrupted copy of the gene by homologous recombination in a *polA12* mutant in which plasmids of the ColE1 family cannot replicate at higher temperatures and the cells with chromosomes integrated with such plasmids show poor survival at lower temperatures (17, 23).

Figure 1 shows the starting DNA fragment contained in pCL11 and the ability to form cardiolipin by its various deletion derivatives. The analysis indicated that the functional expression of the *cls* gene to form cardiolipin required a 1.2-kb fragment (from 0.6 to 1.8 kb in the physical map).

Figure 2 illustrates the structures of two *cls*-disrupted plasmids, pCL-I1 and pCL-R1. They were constructed by inserting the 1.5-kb *kan* gene into the *Bgl*II site located inside the *cls* gene and by replacing a 1.4-kb *Hpa*I fragment with the *lac* promoter-operator region connected with the *kan* gene that was excised from pUC4K, respectively.

Strain MM383 (*polA12*) was transformed with these plasmids at 30°C on LB agar plates containing kanamycin. For pCL-R1, IPTG was included in all media. Colonies appeared at a frequency of about $1 \times 10^3$ per μg of DNA with pCL-I1 and $5 \times 10^2$ per μg of DNA with pCL-R1, whereas kanamycin-resistant (Kan$^r$) transformants appeared at far lower frequencies when plasmids were introduced at 42°C. One colony each among the transformants of both groups was cultured in LB medium containing kanamycin at 42°C overnight and plated onto LB agar containing kanamycin. All colonies appeared were Kan$^r$ and ampicillin-resistant (Amp$^r$), and all eight colonies randomly selected from the transformants of each group did not contain free plasmids as assessed by gel electrophoresis (7, 10). MM383 with pCL-I1 was cultured at 30°C in LB supplemented with kanamycin for 13 generations with repeated dilutions with a fresh medium and then plated onto LB agar plates containing

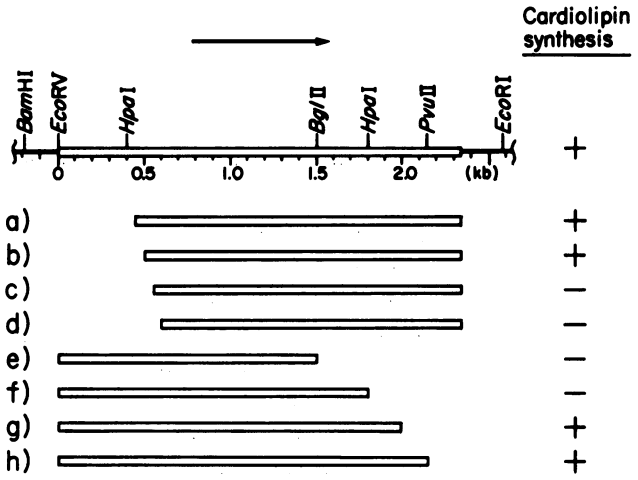


FIG. 1. Determination of the region essential to the expression of the *cls* gene. The cloned insert in pCL11 (▭), with flanking regions (▬), is shown at the top. →, Direction of transcription. Relevant restriction sites are labeled (see also the entire structure of the plasmid in Fig. 2). Bars below show deletion derivatives in which the indicated portions were cloned instead of the entire 2.3-kb insert of pCL11. Plasmids harboring *a* through *d* were constructed by digesting pCL11 with *Bam*HI and *Bal* 31, followed by filling in with DNA polymerase I Klenow fragment and ligating with a *Bam*HI linker. *e* and *f* were obtained by digesting pCL11 with *Bgl*II and *Hin*cII, respectively, followed by digestion with *Eco*RV, whereas *g* and *f* were produced by successively digesting pCL11 with *Eco*RI, *Bal* 31, and *Eco*RV. All four fragments were inserted into a *Sma*I-digested expression vector, pMC1403 (3). These deletion derivatives, together with the starting pCL11, were introduced into strain SD9, and phospholipid compositions were determined. Ability to produce cardiolipin is shown on the right of each bar. +, Cardiolipin content of more than 5% (molar phosphorus basis) of the total phospholipids in the stationary phase; −, cardiolipin content of less than 0.1%.

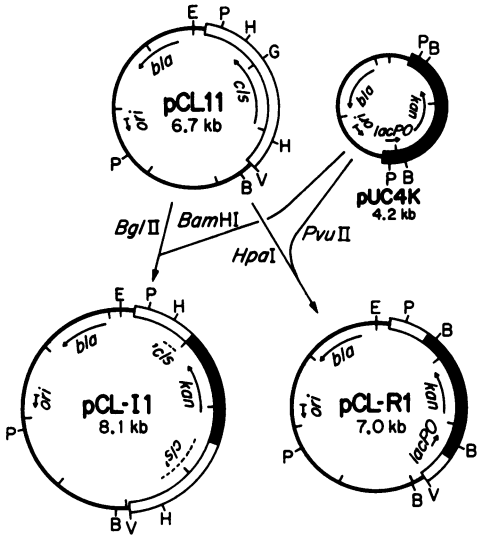


FIG. 2. Construction of pCL-I1 and pCL-R1 used for disruption of the chromosomal *cls* gene. The 1.5-kb promoterless *kan* gene excised from pUC4K by *Bam*HI digestion was inserted into the unique *Bgl*II site of pCL11 to yield pCL-I1. pCL-R1 was constructed by removing a 1.4-kb *Hpa*I fragment from pCL11 and ligating with a 1.7-kb *Pvu*II fragment from pUC4K which contained the promoter-operator region of the *lac* operon and the promoterless *kan* gene. The *lac* regulatory region was included to allow expression of the downstream foreign gene (*kan*) only when necessary. The line portions of the circles have been derived from pBR322, except for the 0.16-kb fragment between the *Eco*RI site and the *cls* fragment which originated from a mini-F plasmid (10). Abbreviations: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hpa*I; P, *Pvu*II; V, *Eco*RV; *lacPO*, promoter-operator region of the *lac* operon; *bla*, β-lactamase gene; *ori*, replication origin.

kanamycin. Among 700 Kan$^r$ colonies, 3 were Amp$^s$. Strain MM383-CLI was one of these Kan$^r$ Amp$^s$ derivatives. In the case of MM383 with pCL-R1, Kan$^r$ Amp$^s$ colonies did not emerge after a prolonged culture (16 generations) at 27 or 30°C, probably because of much shorter regions in pCL-R1 homologous to the chromosome (ca. 0.4 kb each at both ends of the *lacPO-kan* insert). Strain D110 (*polA1*) was transduced with a P1 bacteriophage lysate of strain MM383 carrying pCL-R1, and Kan$^r$ colonies were obtained. Among 800 transductants, 1 was Kan$^r$ and Amp$^s$ and did not contain a plasmid. This strain was named D110-CLR. By using P1 lysates of strains MM383-CLI and D110-CLR, two types of *cls* disruptants were constructed from strains D110, MM383, OS2124, and SD12 by transducing to Kan$^r$. The *cls-1* allele of strain T16GP was introduced by P1 transduction into the above strains to construct CLM derivatives.

**Confirmation of the disruption of the *cls* gene.** Kan$^r$ colonies obtained by P1 transduction, with strain CB64 (*trp cysB*) as a recipient and strain MM383-CLI or strain D110-CLR as a donor, were examined for their tryptophan and cysteine auxotrophies. With strain MM383-CLI, 160 of 500 and 43 of 500 were Trp$^+$ and Cys$^+$, respectively, and with D110-CLR, 46 of 200 and 15 of 200 were Trp$^+$ and Cys$^+$, respectively. Since the *cls* gene is linked to the *trp* operon and the *cysB* gene in the order *cls-trp-cysB* in the *E. coli* chromosome (1), the results indicated that the *kan* gene had been integrated into the *cls* locus on the chromosomes in both disruptants.

The disruption of the genomic *cls* gene by insertion (strain SD12-CLI) or by replacement (strain SD12-CLR) with the *kan* gene was examined by Southern blot hybridization. Chromosomal DNA of strains SD12, SD12-CLI, and SD12-CLR were prepared and digested with *Eco*RV and *Pvu*II. pCL11 was labeled with $^{32}$P by using nick translation and was used to probe the presence of the *cls* gene. Since the *kan* gene has no *Pvu*II or *Eco*RV site, the digestion should produce fragments of ca. 2.1 kb, containing the intact *cls* gene from wild-type strain SD12; 3.6 kb, containing the *cls* gene inserted with the *kan* gene from strain SD12-CLI; and 2.5 kb, containing the *cls* region, most of which was replaced by the *kan* gene from strain SD12-CLR. The results (Fig. 3) were consistent with the insertion of the *kan* gene into the chromosomal *cls* locus in strain SD12-CLI and the replacement of 1.4 kb in the *cls* locus with the *kan* gene in strain SD12-CLR.

**Characteristics of the strains with *cls*-null mutations.** All *cls* disruptants with various genetic backgrounds were able to grow well in broth and synthetic media at 37 and 42°C, and no significant difference was found between the growth characteristics of the two types of the disruptants. Figure 4 shows the growth curves of a typical set of strains, i.e., CLI and CLR strains with the corresponding wild-type and CLM strains. Similar growth characteristics were observed for other sets of strains (strains derived from D110, MM383, and OS2124; data not shown). All disruptants (both CLI and CLR types) had generation times definitely longer and final cell densities lower than those of their parent strains, although their values were somewhat variable. Strains with the *cls-1* mutation (CLM-type mutants) had growth rates only slightly lower than those of the wild-type strains. Strains CB64 and CB64-CLR, both harboring pCL11, grew at exactly the same rate (generation time, 51 min), and strain CB64-CLR harboring pCL-R1 (Fig. 2) grew more slowly (generation time, 70 min) in LB medium supplemented with tryptophan, cysteine, and ampicillin but without IPTG. The growth rates of strains SD12-CLI and SD12-CLR were also lower than those of strains SD12 and SD11, but the differences were much smaller than with those with other genetic backgrounds (generation times of strains SD12-CLI and SD12-CLR were only 1.2 times longer than that of strain SD12 in LB, NBY, and M56LP media and were sometimes almost the same as that of strain SD11), and there was no

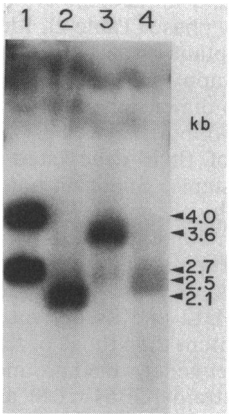


FIG. 3. Southern blot hybridization of pCL11 probe with chromosomal DNA from strains SD12 (lane 2), SD12-CLI (lane 3), and SD12-CLR (lane 4). Each genomic DNA was digested with *Eco*RV and *Pvu*II, separated on a 1% agarose gel together with bacteriophage lambda DNA molecular size markers, and transferred to nitrocellulose for hybridization. Lane 1 is pCL11 digested with *Pvu*II for reference. For restriction sites of intact and disrupted *cls* loci, see Fig. 1 and 2.

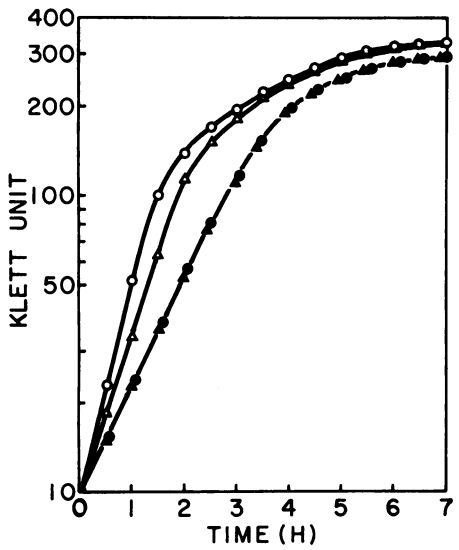


FIG. 4. Reduced growth rates and final cell densities of *cls*-disrupted mutants. Cells were cultured in LB medium supplemented with tryptophan and cysteine at 37°C with constant shaking, and Klett units were measured every 30 min. Generation times for strains CB64 (○), CB64-CLM (△), CB64-CLI (●), and CB64-CLR (▲) were 27, 33, 50, and 50 min, respectively.

significant difference in final cell densities among these strains (see Discussion). Nevertheless, these reduced growth abilities must be typical phenotypes of *cls*-disrupted strains.

Cardiolipin synthase activity was not detectable in membrane and soluble fractions from any of the *cls* mutants; cardiolipin formation was less than the detection limit of 50 nmol/g of protein during a 20-min incubation at 37°C, whereas ca. 1,000 nmol of cardiolipin was formed under identical conditions with the membrane fractions of wild-type strains. Cardiolipin synthase activities of less than the detection limit in *cls-1* mutants have been previously described (10, 18). However, cardiolipin was formed at very low but significant levels in all of the *cls*-disrupted cells in the exponential phase, and its accumulation was more clearly seen in the stationary phase (Table 2). Identity of cardiolipin formed in the disruptants was ascertained by two-dimensional cochromatography of the $^{32}$P-labeled lipid samples recovered from two-dimensional chromatograms with authentic *E. coli* cardiolipin, as well as by one-dimensional cochromatography of their deacylated products in three different solvent systems. Spots on autoradiographic films and spots visualized by phosphate spray coincided exactly in all cases. Cardiolipin contents in *cls-1* mutants were higher than those of the disruptants but was much lower than those of the wild-type cells, as previously observed (14, 18). The reason why strain SD12 and its derivatives had somewhat lower cardiolipin content than those of the CB64-derivatives (and strains with other genetic backgrounds; data not shown) is unknown. Since strains CB64-CLM and SD11 had more cardiolipin than the disruptants with the same genetic backgrounds (Table 2), the mutation *cls-1* must be leaky.

**Possible involvement of phosphatidylserine synthase in the formation of residual cardiolipin.** We have previously described a 10-fold decrease in cardiolipin content in a *cls-1* mutant upon introduction of a defective mutation (*pss-1*) in the structural gene for phosphatidylserine synthase (18). To see the effect of the *pss-1* mutation on the *cls* disruptants, we tried to transfer, by P1 transduction, one of the null alleles of the *cls* gene from strains MM383-CLI and D110-CLR to strain OS2101 (*pss-1 pyrD34*) at 30°C, but no Kan$^r$ colonies were obtained in either combination of the transduction; transduction frequency was less than $3 \times 10^{-9}$ per adsorbed phage particle with MM383-CLI and less than $1 \times 10^{-8}$ with D110-CLR. Strains CB64, MM383, D110, SD12, and OS2124 (OS2101 *pss*$^+$) were transduced to Kan$^r$ with frequencies of between $10^{-6}$ and $10^{-7}$ under identical conditions. For a control, strain OS2101 harboring either pUC4K (*kan*) or pML31 (mini-F, *kan*) was transduced with a P1 lysate of strain MM383-CLI or of strain D110-CLR to Ura$^+$ at 30°C in the presence of kanamycin on selective plates. Frequencies of these transductions were all normal (around $8 \times 10^{-7}$). The results indicated that the failure to obtain Kan$^r$ transductants from strain OS2101 was not due to its genetic background other than the *pss-1* allele or to its possible hypersensitivity to kanamycin (16). We conclude that the cells harboring both the *pss-1* and one of the null *cls* alleles are difficult to grow.

A correlation of the cellular cardiolipin content with the *pss* gene was further observed by analyzing the phospholipid compositions of various strains with different functional levels of the *pss* gene product (Table 3). An introduction of the *pss-1* allele into a *cls-1* mutant SD11 (strain SD9) caused a further decrease in cardiolipin, whereas an increase in the *pss* gene dosage by introducing a high-copy-number plasmid bearing the *pss* gene (pPS3155) increased cardiolipin significantly in both the CB64 and SD12 backgrounds. Since strains harboring the *pss-1* allele have a lowered ability to form phosphatidylserine even at lower temperatures (18), and since the activity of phosphatidylserine synthase is roughly proportional to the *pss* gene dosage (12), the results presented in Table 3 indicate a dependence of cardiolipin content on the intracellular level of phosphatidylserine synthase activity in *cls* mutants.

## DISCUSSION

We have used two methods to disrupt the *cls* gene on a plasmid, since either method alone should not give a clear-cut result; a simple insertion of the *kan* gene does not

TABLE 2. Phospholipid composition of the strains harboring various alleles of the *cls* gene[a]

| Strain | *cls* gene | % of total[b] during phase: | | | | | |
|---|---|---|---|---|---|---|---|
| | | Exponential | | | Stationary | | |
| | | PE | PG | CL | PE | PG | CL |
| CB64 | Wild type | 71.1 | 17.8 | 9.1 | 80.1 | 4.8 | 13.1 |
| CB64-CLM | *cls-1* | 74.2 | 22.7 | 1.1 | 74.4 | 18.8 | 4.9 |
| CB64-CLI | *cls*::*kan* | 72.1 | 25.5 | 0.4 | 74.6 | 21.0 | 2.4 |
| CB64-CLR | Δ*cls*::*kan* | 72.6 | 25.1 | 0.4 | 76.6 | 19.2 | 2.3 |
| SD12 | Wild type | 75.5 | 17.2 | 5.2 | 74.6 | 16.6 | 7.9 |
| SD11 | *cls-1* | 74.6 | 23.3 | 0.2 | 73.6 | 23.0 | 1.5 |
| SD12-CLI | *cls*::*kan* | 74.4 | 23.5 | 0.1 | 72.7 | 24.7 | 0.6 |
| SD12-CLR | Δ*cls*::*kan* | 75.3 | 22.6 | 0.1 | 71.9 | 25.7 | 0.6 |

[a] Cells were labeled uniformly with [$^{32}$P]phosphate (2 μCi/0.3 μmol per ml) in the synthetic medium M56LP supplemented with uracil and amino acids to exponential phase ($5 \times 10^8$ cells per ml) or to stationary phase (5 h after the cessation of turbidity increase), and lipids were extracted and quantitated as described in Materials and Methods.

[b] Molar percent of lipid phosphorus calculated from the radioactivity of each spot. Up to six minor spots, including that of phosphatidic acid, are not included in the table. They did not exceed 2% of the total extracts. The detection limit was ca. 0.05% under these conditions. Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

TABLE 3. Cardiolipin contents of strains with different dosages of the *pss* gene[a]

| Strain | Plasmid | % of total[b] during phase: | | | | | |
|---|---|---|---|---|---|---|---|
| | | Exponential | | | Stationary | | |
| | | PE | PG | CL | PE | PG | CL |
| CB64-CLR[c] | pBR322 | 76.1 | 21.6 | 0.3 | ND[d] | ND | ND |
| CB64-CLR | pPS3155 | 71.4 | 25.2 | 1.3 | 71.3 | 22.5 | 4.2 |
| SD11 | None | 80.3 | 18.3 | 0.1 | 77.8 | 20.4 | 0.6 |
| SD9 | None | 68.4 | 30.0 | <0.1 | 64.3 | 34.2 | 0.2 |
| SD90 | pBR322 | 62.5 | 35.5 | <0.1 | 64.4 | 33.9 | 0.3 |
| SD90 | pPS3155 | 80.6 | 16.4 | 0.3 | 80.1 | 16.4 | 1.7 |

[a] Cells were labeled uniformly with [$^{32}$P]phosphate in M56LP medium (CB64 derivatives; 2 μCi/ml) or in NBY medium (SD12 derivatives; 7.5 μCi/ml) with supplementation of ampicillin for plasmid-harboring strains. Lipids were quantitated in the exponential and stationary phases, as explained in Table 2.

[b] For definitions and abbreviations, see Table 2, footnote *b*.

[c] For strain CB64-CLR without plasmid, see Table 2.

[d] ND, Not determined, because of an earlier cessation of the growth for an unknown reason.

eliminate the possibility of formation of a partially enzymatically active gene product, whereas a replacement of a large part around the *cls* gene with the *kan* gene leaves open the possibility of influencing the neighboring gene(s). Very similar, if not identical, properties of the two types of disruptants and the normal growth of a disruptant harboring pCL11 indicated that the growth phenotypes observed were specifically caused by the null mutation in the *cls* gene and the polar effects of the disruptions, if any, on the downstream gene(s) were negligible as far as the growth capability was concerned. Reduced but still active growth of the disruptants indicated that the cells harboring the intact *cls* gene have a selective advantage over those without the *cls* gene but that this gene is not essential to the wild-type *E. coli* cells. Of course, there remains a possibility that the loss of this gene is lethal in some more severe natural environment.

The genetic background of strain SD12 seemed somewhat unusual, since it allowed better growth of *cls* disruptants and resulted in a lower cardiolipin content than those of all the other strains examined. We have observed that the *pgsA3* allele, isolated in this particular genetic background and exhibiting an extremely low content of acidic phospholipids (9), was not transferable by P1 transduction to strains with other genetic backgrounds, unless a mutation at around 37 min emerged in the recipient (Y. Katayose, C. Hikita, A. Ohta, and I. Shibuya, unpublished data). It remains to be elucidated whether the suppressing property of strain SD12 for the *pgsA3* mutation has any correlation with the unusual properties of *cls* disruptants of SD12 described in the present study.

Although cardiolipin synthase activity was not detectable in the *cls* disruptants, this does not necessarily mean the complete absence of the enzyme in these cells. Therefore, the formation of cardiolipin even in the disruptants indicates either a nonstructural nature of the *cls* gene or a secondary route for cardiolipin formation other than cardiolipin synthase. At present, we are not able to identify this gene rigorously as the structural gene for the enzyme because of several technical reasons; e.g., a lethal effect of gene amplification that makes gene product purification difficult, the labile nature of the enzyme that does not permit the detection of enzymatic activity after electrophoretic separation, etc. However, all available data point to a structural nature, rather than a regulatory nature, of the *cls* gene. As discussed previously (10), *cls* is the only gene found that is related to cardiolipin synthesis, and amplification of the *cls* gene results in an increase in cardiolipin synthase activity. In addition, the amino acid sequence deduced from the nucleotide sequence of the *cls* gene is consistent with a membrane-bound cardiolipin synthase, and the increase in the amount of *cls* gene product, upon introduction of the *tac* promoter as detected by denaturing gel electrophoresis, was roughly proportional to the increase in cardiolipin synthase activity (Y. Asami, N. Uetake, A. Ohta, and I. Shibuya, unpublished data). If *cls* is the structural gene, then the present results imply that cardiolipin synthase is completely absent from the disruptants and not essential to *E. coli* cells and that the residual cardiolipin found in the disruptants must have been formed by an enzyme other than cardiolipin synthase. It is interesting that a greater accumulation of cardiolipin in the stationary phase was also observed in the *cls*-disrupted cells.

The dependence of cardiolipin content on the intracellular activity of phosphatidylserine synthase (Table 3) indicated a possibility of minor cardiolipin synthesis by phosphatidylserine synthase. Since this enzyme is capable of donating a phosphatidyl residue not only to the natural substrate L-serine but also to glycerol and *sn*-glycerol-3-phosphate (5), phosphatidylglycerol might serve as a nonefficient phosphatidyl receptor. If this is the case, the mechanism for this minor route would be similar to the eucaryotic cardiolipin synthases which condense CDP-diacylglycerol and phosphatidylglycerol (21). The data that demonstrated the major cardiolipin synthesis in *E. coli* from two molecules of phosphatidylglycerol (4, 20) do not necessarily conflict with this possibility. A test with a highly purified preparation of phosphatidylserine synthase is necessary to prove this possibility.

Two alternative explanations may be possible for the failure in our attempts to introduce a null allele of the *cls* gene into a *pss-1* strain. One is based on the putative membrane fluidity; the *pss-1* mutation causes a decrease in phosphatidylethanolamine and, by an unknown mechanism, an apparently compensatory increase in cardiolipin, thus resulting in a membrane probably equivalent to that of wild-type cells as far as the membrane fluidity is concerned (11, 18). Pluschke and Overath observed similar transition temperatures for cardiolipin and phosphatidylethanolamine (15). The introduction of a null *cls* allele would have a lethal effect by inhibiting such a compensatory cardiolipin accumulation. However, this explanation, based on the bulk fluidity, is unlikely, since *E. coli* cells are viable with membranes of widely variable phospholipid compositions, e.g., 50% phosphatidylglycerol (18). Another explanation assumes the involvement of phosphatidylserine synthase in cardiolipin synthesis. If the residual levels of cardiolipin in *cls* disruptants are formed by phosphatidylserine synthase, as discussed above, then a mutationally lowered activity of this enzyme would not be sufficient to form cardiolipin that is essential for the survival of *E. coli* cells. In addition to this possibility, the observation that the wild-type cells grew more rapidly than the cells with low levels of cardiolipin implies that cardiolipin is more suited than any other lipids to some specific interactions or biochemical processes. Identification of cellular processes that depend on or prefer cardiolipin is under way in our laboratory.

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## LITERATURE CITED

1. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12, edition 7. Microbiol. Rev. **47**:180–230.
2. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **27**:248–254.
3. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143**:971–980.
4. **Hirschberg, C. B., and E. P. Kennedy.** 1972. Mechanism of the enzymatic synthesis of cardiolipin in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **69**:648–651.
5. **Larson, T. J., and W. Dowhan.** 1976. Ribosomal-associated phosphatidylserine synthase from *Escherichia coli*: purification by substrate-specific elution from phosphocellulose using cytidine 5′-diphospho-1,2-diacyl-*sn*-glycerol. Biochemistry **15**:5212–5218.
6. **Lovett, M. A., and D. R. Helinski.** 1976. Method for the isolation of the replication region of a bacterial replicon: construction of a mini-F′*km* plasmid. J. Bacteriol. **127**:982–987.
7. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Miyazaki, C., M. Kuroda, A. Ohta, and I. Shibuya.** 1985. Genetic manipulation of membrane phospholipid composition in *Escherichia coli: pgsA* mutants defective in phosphatidylglycerol synthesis. Proc. Natl. Acad. Sci. USA **82**:7530–7534.
10. **Ohta, A., T. Obara, Y. Asami, and I. Shibuya.** 1985. Molecular cloning of the *cls* gene responsible for cardiolipin synthesis in *Escherichia coli* and phenotypic consequences of its amplification. J. Bacteriol. **163**:506–514.
11. **Ohta, A., and I. Shibuya.** 1977. Membrane phospholipid synthesis and phenotypic correlation of an *Escherichia coli pss* mutant. J. Bacteriol. **132**:434–443.
12. **Ohta, A., K. Waggoner, K. Louie, and W. Dowhan.** 1981. Cloning of genes involved in membrane lipid synthesis: effects of amplification of phosphatidylserine synthase in *Escherichia coli*. J. Biol. Chem. **256**:2219–2225.
13. **Ono, Y., and D. C. White.** 1970. Cardiolipin-specific phospholipase D activity in *Haemophilus parainfluenzae*. J. Bacteriol. **103**:111–115.
14. **Pluschke, G., Y. Hirota, and P. Overath.** 1978. Function of phospholipids in *Escherichia coli*. Characterization of a mutant deficient in cardiolipin synthesis. J. Biol. Chem. **253**:5048–5055.
15. **Pluschke, G., and P. Overath.** 1981. Function of phospholipids in *Escherichia coli*. Influence of changes in polar head group composition on the lipid phase transition and characterization of a mutant containing only saturated phospholipid acyl chains. J. Biol. Chem. **256**:3207–3212.
16. **Raetz, C. R. H., and J. Foulds.** 1977. Envelope composition and antibiotic hypersensitivity of *Escherichia coli* mutants defective in phosphatidylserine synthetase. J. Biol. Chem. **252**:5911–5915.
17. **Russel, M., and P. Model.** 1984. Replacement of the *fip* gene of *Escherichia coli* by an inactive gene cloned on a plasmid. J. Bacteriol. **159**:1034–1039.
18. **Shibuya, I., C. Miyazaki, and A. Ohta.** 1985. Alteration of phospholipid composition by combined defects in phosphatidylserine and cardiolipin synthases and physiological consequences in *Escherichia coli*. J. Bacteriol. **161**:1086–1092.
19. **Shibuya, I., S. Yamagoe, C. Miyazaki, H. Matsuzaki, and A. Ohta.** 1985. Biosynthesis of novel acidic phospholipid analogs in *Escherichia coli*. J. Bacteriol. **161**:473–477.
20. **Tunaitis, E., and J. E. Cronan, Jr.** 1973. Characterization of the cardiolipin synthetase activity of *Escherichia coli* envelopes. Arch. Biochem. Biophys. **155**:420–427.
21. **van den Bosch, H.** 1974. Phosphoglyceride metabolism. Annu. Rev. Biochem. **43**:243–277.
22. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
23. **Yamaguchi, K., and J. Tomizawa.** 1980. Establishment of *Escherichia coli* cells with an integrated high copy number plasmid. Mol. Gen. Genet. **178**:525–533.