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Morphogenesis of the bacterial division septum: A new class of septation-defective mutants

(cell division/membranes/murein/lipoprotein)

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ABSTRACT **A new class of mutants of *Salmonella typhimurium* (*lkyD* mutants) are described. The mutants are defective in morphogenesis of the division septum, and are characterized by a failure of the outer membrane to invaginate despite normal ingrowth of the cytoplasmic membrane and murein layers of the growing septum. The cell envelopes of the mutants show a significant decrease in the bound form of murein-lipoprotein and a corresponding increase in the free form of the lipoprotein. This suggests that the morphogenetic defect may result from a defect in formation of covalent bonds between the free lipoprotein of the outer membrane and the murein of the nascent septum.**

In all cells that divide by binary fission, the process of cell division requires ingrowth of a septum at the mid-point of the cell in a sequence that must be controlled both spatially and temporally. In most cases, this occurs by the circumferential invagination of the components of the cell envelope and eventually leads to complete separation of the two daughter cells. The biochemical mechanisms responsible for this complex morphogenetic event are unknown.

In Gram-negative bacteria, the cell envelope consists of three layers—cytoplasmic membrane, murein layer, and outer membrane (Fig. 1n). During normal cell division these components invaginate coordinately to form the new septum. In this paper, we describe a new class of bacterial mutants characterized by uncoupling of the normally coordinate ingrowth of these three layers during septum formation.

METHODS

Characteristics of the parental (SA722) and *lkyD* mutant strains (R71 and R53) of *Salmonella typhimurium* are described elsewhere (1). Strain R5312R was a spontaneous revertant of R53 that had regained the wild-type phenotype. Bacteria were grown at 37° in glucose-supplemented minimal medium containing 2.5% proteose peptone-beef extract (2) (Difco). Labeling experiments were performed by adding 500 μCi of [^{3}H]amino acid (1 Ci/g) or 50 μCi of [^{14}C]amino acid (0.5 Ci/g) (New England Nuclear) to 20 ml of culture. After two to three generations of exponential growth, during which steady-state labeling was achieved, 10 mM sodium azide was added, the cells were collected by centrifugation at 1000 × *g* for 10 min, and immediately frozen. For cell envelope preparations, cells were disrupted by sonication in 10 mM Tris·Cl buffer at pH 8.5, and the cell envelope fraction was collected by centrifugation at 105,000 × *g* for 2 hr at 6° after several cycles of centrifugation at 1000 × *g* (10 min) and 105,000 × *g* (2 hr) to remove unbroken cells (sedimentable at 1000 × *g*). Electron microscopy was performed on unsynchronized exponentially-growing cultures. Transmission electron microscopy was performed using a Hitachi HU11E electron microscope after fixing cells in glutaraldehyde, postfixing in OsO_4, dehydrating, embedding in Epon, and sectioning, as described previously (1). Scanning electron microscopy was performed on cells precipitated on Nucleopore filters (Nucleopore Corp., Pleasanton, Calif.), fixed with glutaraldehyde, postfixed with OsO_4, dehydrated, rinsed with Freon 113, critical point dried using Freon 13, and coated with gold-palladium. The coated cells were examined in an Etec Autoscan (Etec Corp., Haywood, Calif.).

RESULTS

Characteristics of *lkyD* mutants

The *lkyD* phenotype was identified during a study of periplasmic-leaky mutants of *Salmonella typhimurium*. All members of the periplasmic-leaky group are characterized by the spontaneous release of periplasmic proteins from growing cells without release of intracellular proteins. They are assumed to be defective in organization of the outer membrane or of other cell envelope components (1).

The *lkyD* mutants form one class within the larger group of periplasmic-leaky mutants. In addition to the defect in septum formation that is described in this paper, *lkyD* mutants are characterized by leakage of several periplasmic enzymes and binding proteins, and by increased sensitivity to several detergents and to the antibiotic rifampicin. A more complete description of the entire group of periplasmic-leaky mutants is published elsewhere (1).

Septum formation in *lkyD* mutants

The *lkyD* mutants were characterized by a defect in invagination of the outer membrane during formation of the division septum. The following sequence of events was reconstructed from electron micrographs (Fig. 1b–i).

At early stages of septation in *lkyD* cells, the outer membrane failed to follow the cytoplasmic membrane and murein layers into the developing septum (Fig. 1b). This contrasts with the situation in wild-type cells in which all three layers participate in septal morphogenesis (Fig. 1a). As septation progressed, the outer membrane began to bulge outward over the septal region, leading to formation of large blebs over the septal region of the cell (Fig. 1c and g). The membrane surrounding the blebs appeared to be continuous with the outer membrane of the body of the cell (Fig. 1m).

At later stages, the continued ingrowth of cytoplasmic membrane and murein led to segregation of cytoplasm and nuclear material into two daughter cells that were held together by a bridge of outer membrane (Fig. 1d). Complete cell separation appeared to occur by the mechanical breaking and resealing of this membranous bridge, leaving a large bleb of outer

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

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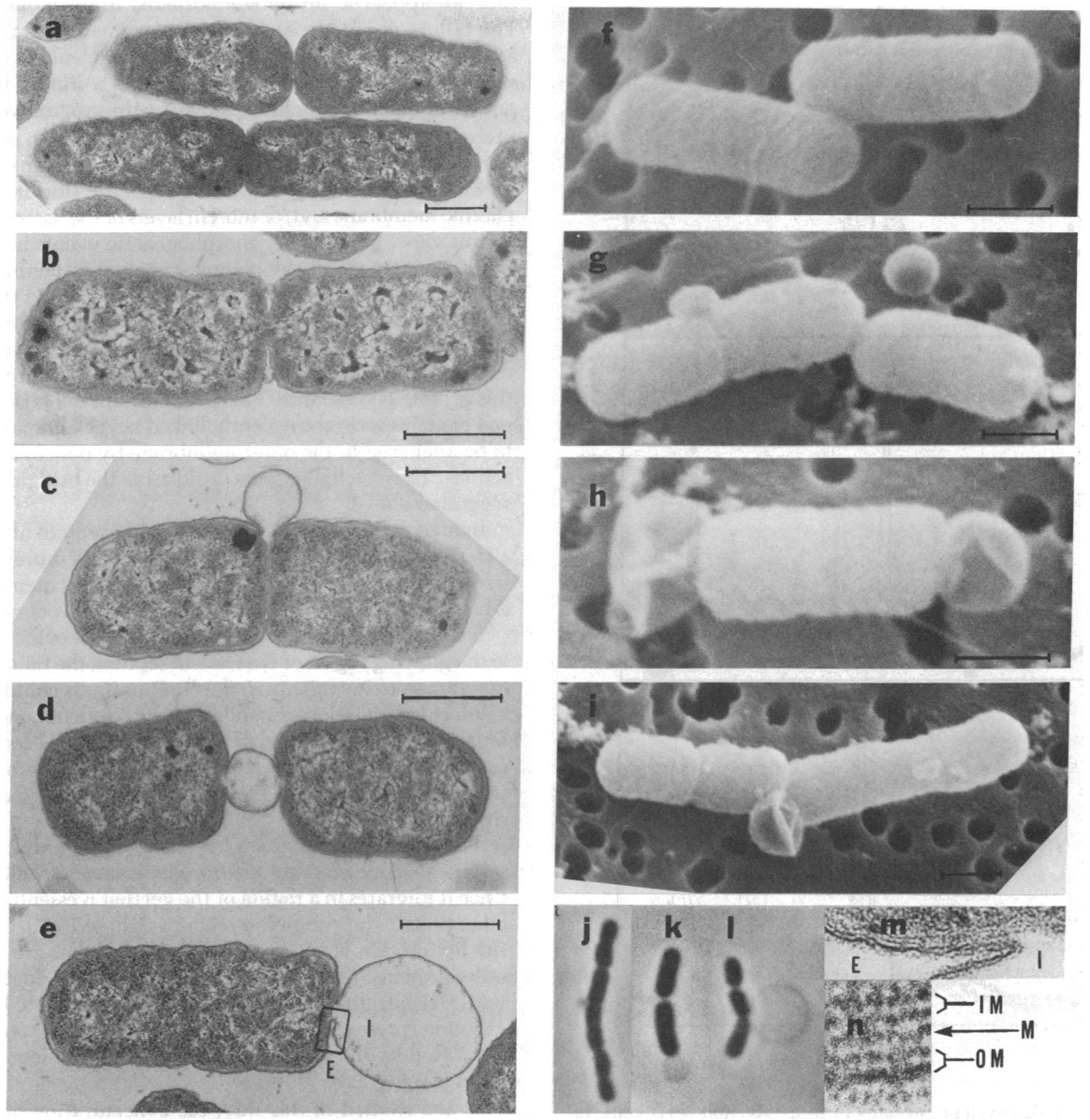

FIG. 1. Morphology of wild-type and *lkyD* cells. (a–e) Electron micrographs of thin sections of parental strain SA722 (panel a) and *lkyD* mutant R71 (panels b–e). See *text* for discussion of micrographs. The bars represent 0.5 μm. (f–i) Scanning electron micrographs of SA722 are shown in panel f and those of R71 are shown in panels g–i. A septal bleb and a free bleb are present in g. Partially collapsed blebs are present over both poles of the cell in h. A chain of cells with a collapsed bleb over one of the septal regions is present in i. The bars represent 0.5 μm. (j–l) Phase contrast micrographs of live unfixed cells of R71 at magnifications of 3320× for j and l, and 4150× for k. A chain of cells with a bleb over one of the septal regions is present in j and in l. A polar bleb is present in k. (m) Enlargement of the junction between bleb and cell body, from a region comparable to that enclosed in the rectangle in panel e, to show the continuity between outer membrane of cell body and bleb. I, internal side of bleb membrane; E, external side (see panel e). Magnification, 144,000×. (n) Cell envelope region of SA722 enlarged from an electron micrograph comparable to panel a, to show locations of inner membrane (IM), murein (M), and outer membrane (OM). Magnification, 415,000×.

membrane attached to the pole of one of the progeny cells (Fig. 1e and h). Although repeated cycles of cell separation appeared to occur in this way, many cells failed to separate completely, leading to formation of chains of incompletely separated cells that were held together only by outer membrane bridges (Fig. 1d, i, and j). Large numbers of these chains accumulated after 3 or 4 hr of culture.

Outer membrane blebs were restricted to the septal and polar regions. The larger blebs frequently broke off from their stalk-like attachment and appeared free in the medium as membrane-bounded vesicles (Fig. 1g).

Blebs over sites of septation or at the poles of daughter cells also were seen in living cells by light microscopy using phase contrast (Fig. 1j–l) or Nomarski differential-interference optics, ruling out the possibility that the apparent failure of outer membrane invagination was an artifact of preparation for electron microscopy. When a drop of distilled water was added to a suspension of *lkyD* cells, the blebs rapidly swelled and ruptured, leaving collapsed membrane sacs attached to the pole or septal region of affected cells. This sensitivity to osmotic rupture of the blebs suggests that the limiting membranes maintained their integrity and acted as barriers to the unrestricted passage of water soluble molecules.

Biochemical changes in *lkyD* mutants

The inner two layers of the septum (cytoplasmic membrane and

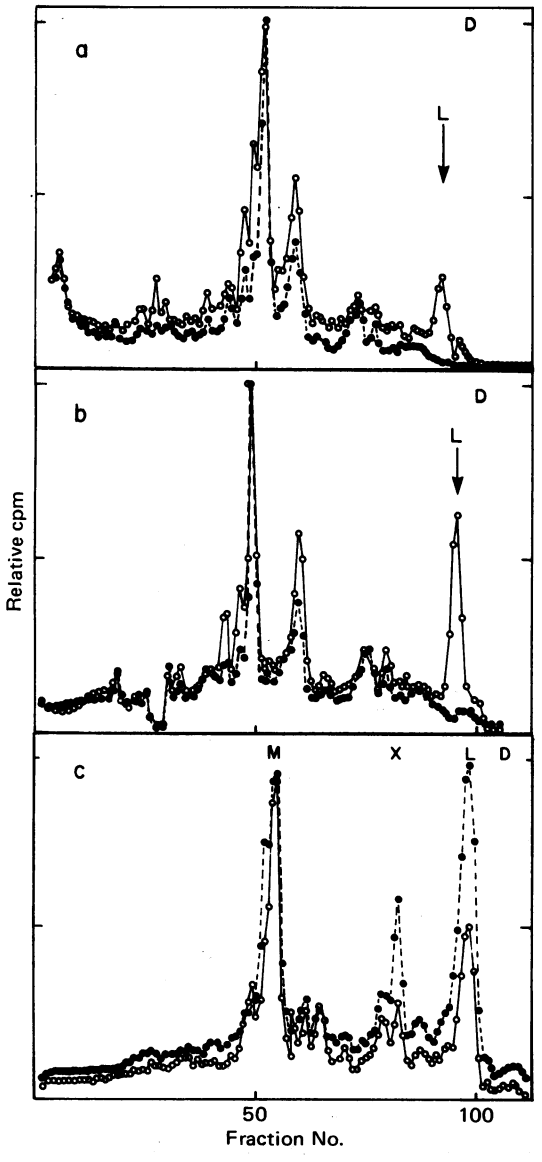

FIG. 2. Cell envelope proteins in wild-type and *lkyD* cells. (a) Histidine and leucine double labeling of wild-type cell envelope. A culture of SA722 (wild type) was labeled simultaneously with [^{3}H]-histidine and [^{14}C]leucine. The cell envelope was prepared, further purified by centrifugation on a discontinuous gradient of 30% and 55% (wt/vol) sucrose (5.5 ml each) in an SW41 rotor at 35,000 rpm for 4.5 hr at 4°, the material was collected from the 30–55% sucrose interface, and dialyzed against water to remove sucrose. A sample for electrophoresis, containing approximately 100,000 dpm of ^{3}H and 40,000 dpm of ^{14}C, was lyophilized and suspended in 0.07 ml of 0.05 M Tris·Cl (pH 6.8) containing 1% $NaDodSO_4$, 2 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol, and 0.2% bromphenol blue. The suspension was heated in a boiling water bath for 2 min, cooled to room temperature, applied to a tubular polyacrylamide gel (10 cm) prepared as described by Neville (7) (11% acrylamide lower gel, pH 9.81 lower gel buffer), and run at 1.5 mA per tube at 15° for approximately 5 hr. The gel was frozen and thawed once and fractionated into 1 mm fractions using a Gilson Aliquogel Fractionator. Each fraction was suspended in 0.5 ml of 1.25% $NaDodSO_4$, shaken at 37° for 2 hr, and suspended in a toluene-Liquiflor (New England Nuclear Corporation)–Bisolv (Beckman) mixture for determination of radioactivity. Recoveries of radioactive counts from the fractionated gels were routinely 90–100%. Scales are normalized to the cpm in peak M, the major protein peak of the outer membrane (see panel c). The origin is at the left. Symbols: D, dye front; L, location of free lipoprotein; –O–, [^{14}C]arginine; –●–, [^{3}H] histidine. (b) Histidine and leucine double labeling of *lkyD* cell envelope. A culture of R71 (*lkyD*) was treated as described above. (c) Labeling of *lkyD* and revertant strain. Cultures of R53 (*lkyD*) and the revertant strain R5312R were labeled with [^{3}H]arginine and [^{14}C]arginine respectively and the cell envelopes were prepared. The two preparations, containing approximately 150,000 dpm of ^{3}H (R53) and 60,000 dpm of ^{14}C (R5312R), were mixed, solubilized, and electrophoresed as described above. Symbols: X, location of 15,000 molecular weight peptide(s); –●–, [^{3}H]arginine (R53); –O–, [^{14}C]arginine (R5312R).

murein) appear to invaginate normally in the *lkyD* mutants. The failure of outer membrane to invaginate thus represents an uncoupling of this process from the other elements of normal septal morphogenesis. This is not due to a simple failure of growth of outer membrane as shown by the progressive increase in size of the blebs over the septal region as septation progressed. Rather, the defect may reflect the absence of a link that normally attaches outer membrane to specific sites in the cytoplasmic membrane and/or murein layers of the nascent septum. In this view, the primary morphogenetic events in septation would be ingrowth of cytoplasmic membrane and murein with outer membrane being pulled in passively behind them.

One candidate for the hypothetical link between outer membrane and nascent septum is the murein-lipoprotein described by Braun and Rehn (3). The COOH-terminal end of this protein is covalently linked to murein; the NH_2-terminal end contains several covalently linked acyl chains and appears to be anchored in the outer membrane by noncovalent interactions. Thus the lipopeptide could serve the bridging function suggested above.

Inouye *et al.* have shown that the lipoprotein also exists in a free form which is not covalently linked to murein (4). The free lipoprotein is tightly anchored in the outer membrane and is believed to be a biosynthetic precursor of the bound form (4).

If the murein-lipoprotein is required to link outer membrane to the growing region of the nascent septum, the failure of outer membrane to invaginate in the *lkyD* mutants could be due to a defect in formation or stability of the covalent bond between murein and the free form of the lipoprotein. Evidence consistent with this hypothesis was obtained from a comparison of free and bound forms of the lipoprotein in wild-type and *lkyD* strains.

The free lipoprotein can be identified in sodium dodecyl sulfate ($NaDodSO_4$) gel electropherograms of cell envelope since it migrates in a region of the gel that is essentially free of other proteins (apparent molecular weight of 7500). In addition, the lipoprotein lacks several amino acids that are present in other proteins. Therefore, when the organism is doubly labeled with [^{3}H]histidine (absent in lipoprotein) and [^{14}C]leucine or arginine (present in lipoprotein), the free lipoprotein can be identified by the absence of ^{3}H in the low-molecular-weight band (4) (Fig. 2a).

When studied in this way, the concentration of free lipoprotein was found to be severalfold higher in *lkyD* mutants than in the parental strain whether expressed relative to total cell envelope protein or to any of the other major outer membrane peptide peaks (Fig. 2a and b). Similar changes were seen in three independently isolated *lkyD* mutants whereas a spontaneous revertant from *lkyD* to wild-type phenotype showed a protein pattern that was similar to that of the original parental strain (Fig. 2c).

At least one other cell envelope peptide, of apparent molecular weight 15,000, also was increased in *lkyD* cells (Fig. 2c) although the extent of the increase varied among the different mutants. The possible relationship of this band to the lipoprotein and to an outer membrane protein of similar molecular weight

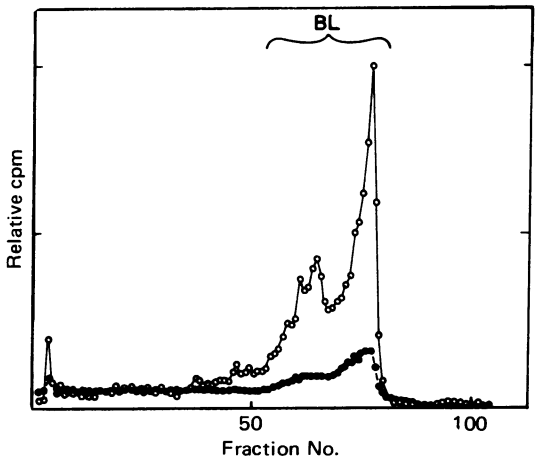

FIG. 3. Bound lipoprotein in wild-type and *lkyD* cells. Cultures of SA722 (wild type) and R71 (*lkyD*) were labeled with [^{14}C]arginine and [^{3}H]arginine, respectively, and cell envelopes were prepared. The preparations (2 ml each) were mixed and a small amount of unlabeled carrier cells was added. For murein isolation, the mixture was suspended in 4 ml of 5.6% $NaDodSO_4$ at 100°, stirred for 20 min at 100°, and then for 2 hr at room temperature. The murein pellet was collected by centrifugation at 240,000 × *g* for 90 min at room temperature, and $NaDodSO_4$ was removed by four cycles of resuspension in water and recentrifugation under the same conditions. The final murein pellet was lyophilized, treated with lysozyme (0.3 mg/ml) at 37° for 3 hr, and lyophilized again. The sample was solubilized and electrophoresed for 19 hr in 7.5% $NaDodSO_4$ gels as described by Inouye *et al.* (4). No radioactivity entered the gels when the lysozyme treatment was omitted. Complete digestion is indicated by the essentially complete recovery in the gel fractions of the radioactivity present in the sample subjected to lysozyme treatment (data not shown), and in the absence of significant radioactivity in the high molecular weight region of the gel. Scales are normalized to cpm of each isotope in the cell envelope preparations at the time of mixing. The origin is at the left. Symbols: BL, bound lipoprotein; -○-, [^{14}C]arginine (SA722); -●-, [^{3}H]arginine (R71).

that may be involved in cell elongation in *Escherichia coli* (8) remains to be determined.

The increase in free lipoprotein in the *lkyD* mutants was accompanied by a comparable decrease in bound lipoprotein. In contrast to the free form, the bound form of the lipoprotein does not enter $NaDodSO_4$ gels unless the murein layer first has been digested with lysozyme. This results in release of bound lipoprotein still covalently linked to oligosaccharide fragments of the digested murein. When purified murein is treated with lysozyme, the released lipoprotein can be identified by its characteristic migration in $NaDodSO_4$ gel electropherograms since all other cell envelope polysaccharides and proteins, including the free lipoprotein, have been removed previously during the murein isolation procedure. When examined in this way, *lkyD* cells contained substantially less bound lipoprotein than the parental strain (Fig. 3). The ratio of free lipoprotein to bound lipoprotein was 4-fold higher in *lkyD* than in parental cells whereas the total lipoprotein (free plus bound) was approximately the same in the two strains (Table 1).

Bound lipoprotein also was determined by amino acid analysis of murein sacculi from wild-type and *lkyD* cells. With the exception of those amino acids that are present in murein (alanine, glutamic acid, and diaminopimelate), the amino acid compositions of the sacculi were similar to authentic lipoprotein (5). The ratio of bound lipoprotein to murein was approximately 2-fold higher in parental than in *lkyD* cells as calculated from the relative amounts of aspartic acid and muramic acid in the murein hydrolysates, assuming 15 aspartic acid residues per mol of lipoprotein. Identical values were obtained when the indexed amino acid was threonine (2 residues), leucine (4 residues), or serine (6 residues) (Table 2).

Table 1. Relative amounts of free and bound lipoprotein in *lkyD* and wild-type cells

	Radioisotope incorporated[a]			
	SA722 (wild type)		R71 (*lkyD*)	
Fraction	cpm × 10^{-3}	percent	cpm × 10^{-3}	percent
Cells	5060	100	9380	100
Cell envelope	958	18.93	1850	19.72
Lipoprotein				
Free	86	1.70	266	2.83
Bound	48	0.95	37	0.39
Total	134	2.65	303	3.22
Free lipoprotein/ bound lipoprotein		1.8/1		7.3/1

[a] Cultures (40 ml) of SA722 (wild type) and R71 (*lkyD*) were labeled with [^{14}C]arginine and [^{3}H]arginine respectively. The labeled cultures were combined. Radioactivity in cells was determined by membrane filtration after addition of cold 5% trichloroacetic acid to an aliquot of the mixed cultures. Half of the mixture was used for cell envelope preparation and was subjected to electrophoresis as described in Fig. 2 for determination of free lipoprotein. The other half was centrifuged and immediately frozen. The frozen cells were suspended directly in 5 ml of 4% $NaDodSO_4$ at 100° and murein was isolated, treated with lysozyme, and electrophoresed as described in Fig. 3 for determination of bound lipoprotein. The amounts of free and bound lipoprotein were calculated from the total ^{3}H and ^{14}C cpm in the peaks with the mobilities of peak L, Fig. 2c (free lipoprotein) and peak BL, Fig. 3 (bound lipoprotein).

DISCUSSION

The failure of outer membrane to participate in septal morphogenesis in the *lkyD* mutants was associated with a significant decrease in bound lipoprotein and a corresponding increase in free lipoprotein in several independently isolated *lkyD* mutants. These biochemical abnormalities were restored to normal in spontaneous revertants that no longer showed the defect in septation. Although this does not prove a cause-and-effect relationship it prompts us to suggest that the morphogenetic abnormality may result from a defect in synthesis or stability of the murein-lipoprotein bond.

The observations are consistent with several models, all based on the premise that formation of covalent bonds between the lipoprotein of the outer membrane and the murein of the nascent septum is required to pull outer membrane into the growing septum. Proof that the apparent defect in murein-lipoprotein is causally related to the defect in septation and, if so, determination of the mechanism of the effect, will require identification and characterization of the enzymes responsible for formation and cleavage of the murein-lipoprotein bond.

A defect in outer membrane invagination also has been described in *envA* mutants of *E. coli* (6). Comparison of an *envA* mutant and its parental strain (kindly sent to us by Dr. S. Normark) failed to show any difference in the amount of free lipoprotein in the two strains. The *envA* mutation also differs from *lkyD* in map position (9) and it did not leak RNase or show blebs by electron microscopy. Despite these differences

Table 2. Partial composition of murein-lipoprotein

	Number of residues[a]		
	SA722 (wild type)	R71 (*lkyD*)	Braun *et al.*[b]
Asp	15.0	15.0	15
Thr	2.0	1.9	2
Ser	5.5	5.7	6
Leu	4.3	4.0	4
Pro	0	0	0
Gly	0	0	0
Muramic acid	8.2	16.2	9
Lipoprotein/ muramic acid[c]	0.13	0.06	0.11

[a] Strains SA722 and R71 were grown to mid-exponential growth (optical densities at 600 nm of 0.38 and 0.36, respectively) as described in *Methods* except that glucose was replaced by 0.5% sodium lactate in the SA722 culture to equalize the growth rates (doubling times of 70 and 67 min, respectively). Similar results were obtained when the analyses were performed on cells from cultures grown with glucose, glycerol, or lactate as carbon source and with generation times varying between 50 and 75 min. Cells were collected by centrifugation at 12,000 × *g* for 10 min and were frozen immediately. The frozen cells were suspended in cold 0.01 M Tris·HCl (pH 8.5), disrupted by sonication (temperature kept below 6°) and centrifuged at 50,000 × *g* for 100 min at 4°. The murein-lipoprotein complex was isolated by hot $NaDodSO_4$ extraction as described by Braun and Rehn (3). Duplicate samples were hydrolyzed in 6 M HCl at 110° for 20 hr under reduced pressure. Similar results were obtained when samples were hydrolyzed in 4 M HCl at 110° for 15 hr. Amino acid analyses were performed on the long column of a Beckman amino acid analyzer by a modification of the procedure of Braun and Rehn (3). The results are expressed per mol of lipoprotein, assuming 15 aspartate residues per mol. Values for valine, methionine, and isoleucine were not calculated because of interference from diaminopimelate and glucosamine, and values for alanine and glutamate are not included because these amino acids are present in both lipoprotein and murein.

[b] Values for *S. typhimurium* from Braun *et al.* (5).

[c] Molar ratio of lipoprotein to muramic acid (assuming 15 residues of aspartate per mol of lipoprotein).

it still is possible that a relationship exists between *envA* and *lkyD* mutations and the question should be considered open.

It is not known why *lkyD* mutants leak periplasmic proteins. The fact that genetic reversion of the septal defect was associated with loss of the periplasmic leaky phenotype indicates that both defects were caused by the same mutation. Release of periplasmic proteins could result from a generalized defect in outer membrane integrity (for example, due to an abnormality of murein-lipoprotein) or from spontaneous rupture and resealing of outer membrane as, for example, when large blebs break free from their stalk-like attachments. It is impossible to choose between these alternatives at this time.

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