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Role of Precursor Translocation in Coordination of Murein and Phospholipid Synthesis in *Escherichia coli*

KERSTIN EHLERT AND JOACHIM-VOLKER HÖLTJE*

Abteilung Biology, Max-Planck-Institut für Entwicklungsbiologie, 72076 Tübingen, Germany

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Inhibition of phospholipid synthesis in *Escherichia coli* by either cerulenin treatment or glycerol starvation of a glycerol-auxotrophic mutant resulted in a concomitant block of murein synthesis. The intracellular pool of cytoplasmic and lipid-linked murein precursors was not affected by an inhibition of phospholipid synthesis, nor was the activity of the penicillin-binding proteins. In addition, a decrease in the activity of the two lipoprotein murein hydrolases, the lytic transglycosylases A and B, could not be demonstrated. The indirect inhibition of murein synthesis by cerulenin resulted in a 68% decrease of trimeric muropeptide structures, proposed to represent the attachment points of newly added murein. Importantly, inhibition of phospholipid synthesis also inhibited O-antigen synthesis with a sensitivity and kinetics similar to those of murein synthesis. It is concluded that the step common for murein and O-antigen synthesis, the translocation of the respective bactoprenolphosphate-linked precursor molecules, is affected by an inhibition of phospholipid synthesis. Consistent with this assumption, it was shown that murein synthesis no longer depends on ongoing phospholipid synthesis in ether-permeabilized cells. We propose that the assembly of a murein-synthesizing machinery, a multienzyme complex consisting of murein hydrolases and synthases, at specific sites of the membrane, where integral membrane proteins such as RodA and FtsW facilitate the translocation of the lipid-linked murein precursors to the periplasm, depends on ongoing phospholipid synthesis. This would explain the well-known phenomenon that both murein synthesis and antibiotic-induced autolysis depend on phospholipid synthesis and thereby indirectly on the stringent control.

The cell wall of *Escherichia coli* is a multilayered structure in which a thin, rigid layer of murein (peptidoglycan) is sandwiched between two membranes, the cytoplasmic membrane and the outer membrane (15). Growth of the cell wall requires a coordinate increase in the surface of both membrane systems as well as the murein sacculus in order to avoid stretching or buckling of one or the other layer; hence, the rates of syntheses of phospholipids and murein have to be well adjusted to each other. In fact, it has been observed that membrane vesicles are pinched off when murein synthesis is specifically blocked but phospholipid synthesis continues normally (16).

Furthermore, both murein synthesis and phospholipid synthesis are inhibited upon induction of the stringent response, a general regulatory mechanism that affects numerous metabolic activities during stress situations such as amino acid starvation (4). The stringent response, which is mediated by an intracellular accumulation of guanosine 3′,5′-bispyrophosphate, couples protein synthesis with the synthesis of stable RNA and thereby of ribosomes and with the synthesis of phospholipids and thereby of inner and outer membrane (4, 34). Appropriately, the synthesis of the murein sacculus also appears to be regulated by the stringent control (18, 21, 22). However, recently it became clear that murein synthesis is only indirectly regulated by this control mechanism (19, 38). Murein synthesis was shown to depend on ongoing phospholipid synthesis, the latter being under stringent control.

Murein synthesis takes place in all three compartments of the cell (15, 40). In the cytoplasm, two UDP-activated precursors, the UDP-*N*-acetylmuramyl-L-Ala-D-Glu-m-A_2pm (*meso*-diaminopimelic acid)-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) and the UDP-*N*-acetylglucosamine (UDP-GlcNAc), are synthesized by a multistep biochemical pathway. These precursors have to be combined to a disaccharide-pentapeptide subunit and transported across the membrane in order to be inserted into the preexisting murein sacculus. Export of the hydrophilic UDP derivatives is facilitated by a lipid carrier molecule, undecaprenylphosphate (bactoprenol). Accordingly, first MurNAc-pentapeptide is transferred by the translocase I onto undecaprenylphosphate and then GlcNAc is added by the translocase II. In the case of gram-negative bacteria, the lipid-linked disaccharide-pentapeptide that is formed on the cytoplasmic side of the membrane is then translocated to the periplasmic side of the membrane by a still unknown mechanism. In the periplasm, the disaccharide-pentapeptide is inserted into the murein net by the concerted action of murein synthases and hydrolases, which have been speculated to be combined in a multienzyme complex (12, 14). Penicillin-sensitive enzymes, called penicillin-binding proteins (PBPs), catalyze both the attachment to the existing glycan strands by transglycosylation and the cross-linkage with peptide side chains by transpeptidation (33). Lytic transglycosylases and endopeptidases remove old murein strands to allow the insertion of new material into the stress-bearing murein sacculus (14, 42).

The indirect dependency of murein synthesis on the stringent control is of some importance for the bacteriolytic effect of murein synthesis inhibitors such as penicillins. As has been studied in detail by Ishiguro and coworkers, after induction of the stringent response, for example, after amino acid starvation, *E. coli* is tolerant toward these antibiotics (20, 28, 36, 38, 39). That is, the cells stop growing but do not lyse. In other words, bacteriolytic antibiotics become bacteriostatic ones (49). Inhibition of phospholipid synthesis apparently affects not only murein-synthesizing but also murein-hydrolyzing enzymes.

* Corresponding author. Mailing address: Max-Planck-Institut für Entwicklungsbiologie, Abteilung Biochemie, Spemannstraße 35, 72076 Tübingen, Germany. Phone: 49 7071 601 412. Fax: 49 7971 601 447.

Here we report on studies aimed to unravel the stage(s) in the murein biosynthetic pathway that is affected by an inhibition of phospholipid synthesis. It is proposed that the translocation of the lipid-linked murein precursors depends on ongoing phospholipid synthesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* W7 (*dap lys*) (11) and *E. coli* MUF16 (*sltY*) (48) were grown in M9 medium containing 0.4% glycerol as a carbon source. For strain W7, A_2pm (2 μg/ml) and L-lysine (20 μg/ml) were added. The glycerol-auxotrophic mutant *E. coli* BB2636 (1) was grown in mineral salts medium supplemented with 5 mM glycerol and 0.5% glucose and buffered with MOPS (morpholinepropanesulfonic acid)-KOH at pH 7.3 (25). To avoid clumping of the cells, sodium dodecyl sulfate (SDS) was added at a final concentration of 0.01%. The galactose-auxotrophic mutant *E. coli* CS1928 (44) transformed with plasmid pSS37, which carries all enzymes needed to synthesize the O antigen of *Shigella dysenteriae* (47), was cultured in MOPS medium containing 0.2% glycerol, 0.5% Casamino Acids, 2 mM D-fucose, 5 mM galactose, kanamycin (50 μg/ml), and chloramphenicol (20 μg/ml). Bacteria were grown with agitation at 37°C, and culture turbidity was monitored by readings of optical density at 578 nm (OD_{578}) in an Eppendorf photometer.

Inhibition of phospholipid synthesis. Phospholipid synthesis was inhibited either by the addition of cerulenin or by glycerol starvation. Cerulenin (Sigma) was dissolved in 95% ethanol to a concentration of 20 mg/ml and stored at −20°C. Exponentially growing cultures (OD_{578} of 0.1 to 0.5) were treated with 100 μg of the antibiotic per ml. The same volume of 95% ethanol was added to the control. For glycerol starvation, cells were grown in minimal medium containing 5 mM glycerol. At an OD_{578} of about 0.15, cells were filtered through a nitrocellulose membrane (pore size, 0.45 μm; Millipore) and washed several times with prewarmed glycerol-free medium, in which they were resuspended for further growth.

Determination of the rate of macromolecular syntheses. (i) Murein synthesis. Murein synthesis was measured by the incorporation of radioactively labeled A_2pm into SDS-insoluble material. At the indicated time points, samples (1 ml) of exponentially growing cultures of *E. coli* W7 or *E. coli* BB2636 were added to 5 μCi of *meso*-2,6-diamino[3,4,5-^{3}H]pimelic acid (23 Ci/mmol) in an Eppendorf cup and incubated for 3 min at 37°C. In the case of *E. coli* W7, unlabeled A_2pm (2 μg) was also added. Labeling was stopped by the addition of 1 ml of boiling SDS (8%). Samples were incubated at 100°C for 10 min, filtered through nitrocellulose membranes (pore size, 0.45 μm; Millipore), and washed six times with 5 ml of 0.1 M LiCl. Radioactivity was measured on the dried filters after the addition of 3 ml of scintillation cocktail (Ultima Gold; Packard) in a Packard Tri-Carb scintillation counter. Murein synthesis in ether-permeabilized cells (27) (15 mg of protein per ml) was determined by incubation at 30°C for 60 min in a total volume of 200 μl in the presence of 80 nmol of UDP-*N*-acetylmuramyl-pentapeptide and 0.8 nmol UDP-*N*-acetyl[^{14}C]glucosamine (304 mCi/mmol) in 50 mM Tris-HCl buffer (pH 8.3) containing 50 mM NH_4Cl, 20 mM $MgCl_2$, and 0.5 mM β-mercaptoethanol. Murein was isolated after the addition of 1 ml of boiling SDS (4%) and incubation at 100°C for 30 min by filtration as described above.

(ii) Phospholipid synthesis. Incorporation of labeled acetate and glycerol was used to determine the rate of phospholipid synthesis. Culture samples (1 ml) of exponentially growing *E. coli* W7 were incubated with 2 μCi of [2-^{14}C]acetate (55.6 mCi/mmol) and 360 μmol of sodium acetate at 37°C for 10 min; for strain *E. coli* BB2636, 4 μCi of [2-^{14}C]acetate was added. Phospholipids were extracted by the addition of 6 ml of chloroform-methanol (1:2). Samples were incubated overnight prior to the addition of 2 ml of chloroform and 2 ml of water (3). The lower phase was washed three times with 2 M potassium chloride (5 ml) containing 0.1 M sodium acetate in case of acetate labeling (50). Radioactivity was measured in 200 μl of the lower phase. Phospholipid synthesis in ether-permeabilized cells was determined by incubation of 190-μl samples containing ether-treated cells (1.4 mg of protein per ml), sodium acetate (0.037 mg/ml), 0.05 M Tris-HCl (pH 8.3), 0.05 M NH_4Cl, 20 mM $MgCl_2$, and 0.5 mM β-mercaptoethanol in the presence of 2 μCi of [2-^{14}C]acetate (200 μCi/ml; 55.6 mCi/mmol) for 5 min at 30°C. The reaction was stopped, and the phospholipids were extracted by the addition of 6 ml of chloroform-methanol as described above.

(iii) Synthesis of O antigen. The *galE* mutant *E. coli* CS1928 transformed with plasmid pSS37 was cultured overnight in the presence of D-fucose and D-galactose (44). Cells were centrifuged and resuspended in fresh medium without D-fucose and D-galactose. At various times, 0.5-ml samples were removed and pulse-labeled with 1 μCi of [^{3}H]galactose (3.6 Ci/mmol) in the presence of 15 μM unlabeled galactose for 3 min at 37°C. Incorporation was stopped by adding an equal volume of cold 10% trichloroacetic acid (TCA). Samples were kept on ice for 30 min and filtered as described above for murein synthesis. Sample cups were rinsed twice with 1 ml of 5% TCA. Filters were washed with 5 ml of 5% TCA–5 ml of 1% acetic acid and finally twice with 5 ml of 0.1 M LiCl.

PBP assay. For detection of PBPs in whole cells, samples (1 ml) of exponentially growing cultures were centrifuged, washed, and resuspended in 10 μl of 10 mM Tris-maleate-NaOH (pH 6.7) containing 10 mM $MgCl_2$. The labeling reaction was performed with a ^{125}I-labeled derivative of ampicillin prepared as described by Schwarz et al. (45). Cells were incubated in the presence of 5 μl of freshly prepared ^{125}I-labeled ampicillin derivative (about 45 kBq) for 30 min at 37°C. The reaction was stopped by the addition of 2 μl of penicillin G (60 mg/ml). Samples were subjected to SDS–12% polyacrylamide gel electrophoresis (PAGE) (32), and labeled PBPs were detected by autoradiography using Hyperfilm MP (Amersham Buchler, Braunschweig, Germany).

Murein hydrolase assay. Murein hydrolase activity was measured in solubilized membrane fractions with radioactively labeled sacculi as a substrate (29). Briefly, bacteria were harvested in the exponential phase and broken by passage through a French pressure cell (American Instrument Company) at a pressure of 18,000 lb/in^2. Intact cells were removed by low-speed centrifugation, and membranes were collected after centrifugation (350,000 × *g*, 4°C) for 30 min. The membranes were washed twice with 10 mM Tris-maleate-NaOH buffer (pH 5.2) containing 10 mM $MgCl_2$. The pellet was resuspended in wash buffer containing 0.3 M NaCl and 1% Triton X-100 and incubated overnight at 4°C with stirring. Membranes were separated by centrifugation. Aliquots of the supernatant (1.7 mg/ml) were tested for murein hydrolase activity by incubation in the presence of 0.2% Triton X-100 with 5 μg of [^{3}H]A_2pm-labeled murein sacculi (0.5 μg/μl; 2,000 cpm/μg) for 1 h at 37°C. One unit of enzyme activity was defined as the amount of enzyme that solubilizes 1 μg of murein at 37°C in 30 min.

Treatment with colicin M. Colicin M (a generous gift of Klaus Hantke, University of Tübingen) was added to the culture in the presence of 0.1% Triton X-100 and 0.1 mM $CaCl_2$ (43) to a final titer of 10,000 U. Activity of colicin was determined as described by Schaller et al. (43).

Ether permeabilization of cells. The membranes of *E. coli* cells were permeabilized for the diffusion of UDP-activated murein precursors by treatment with ether as described by Kraus et al. (27).

Analysis of murein precursors. UDP-MurNAc-pentapeptide and the lipid-linked precursors were determined by reversed-phase high-pressure liquid chromatography (HPLC) as described by Kohlrausch et al. (26) except that for UDP-MurNAc-pentapeptide, the column was eluted at room temperature at a flow rate of 1 ml/min with a linear gradient from 0.05% trifluoroacetic acid to 0.05% trifluoroacetic acid containing 60% acetonitrile that was built up within 23.5 min.

Muropeptide analysis. Isolation of murein sacculi, digestion with muramidase, and analysis of the muropeptide composition were performed as described by Glauner et al. (7).

Radiochemicals. All radiochemicals were purchased from Amersham Buchler with the exception of [^{3}H]A_2pm, which was obtained from CEA, Gif-sur-Yvette, France.

RESULTS

Dependency of murein synthesis on ongoing phospholipid synthesis. It has been shown that inhibition by cerulenin of relaxed phospholipid synthesis, that is, phospholipid synthesis in amino acid-starved cells treated with chloramphenicol, also inhibits murein synthesis in *E. coli* (20). To investigate this relationship in a more direct way, we inhibited phospholipid synthesis in exponentially growing cells either by the specific antibiotic cerulenin (35) or by glycerol starvation of a glycerol-auxotrophic mutant strain (1).

The incorporation of ^{3}H-labeled A_2pm into SDS-insoluble murein and of ^{14}C-labeled acetate into phospholipids during growth of *E. coli* W7 in A_2pm-substituted M9 minimal medium was determined at different time points after the addition of cerulenin to inhibit phospholipid synthesis. As shown in Fig. 1a, phospholipid synthesis was completely inhibited about 15 min after the addition of cerulenin. Likewise, murein synthesis was also totally blocked at 15 min (Fig. 1b). These results are consistent with those of similar experiments reported by Ishiguro and coworkers (20, 38).

When phospholipid synthesis was blocked by glycerol starvation of the auxotrophic mutant BB2636 (1), again the decrease of the rate of [^{14}C]acetate incorporation paralleled the decrease in the rate of A_2pm incorporation (Fig. 2). Compared to cerulenin treatment, glycerol starvation caused a less dramatic inhibition of phospholipid synthesis and thus of murein synthesis. However, irrespective of the method used to block phospholipid synthesis, both syntheses were inhibited with similar kinetics, indicating some kind of coupling between the two biosynthetic pathways.

Changes in the murein structure after inhibition of phospholipid synthesis. In an attempt to obtain information about

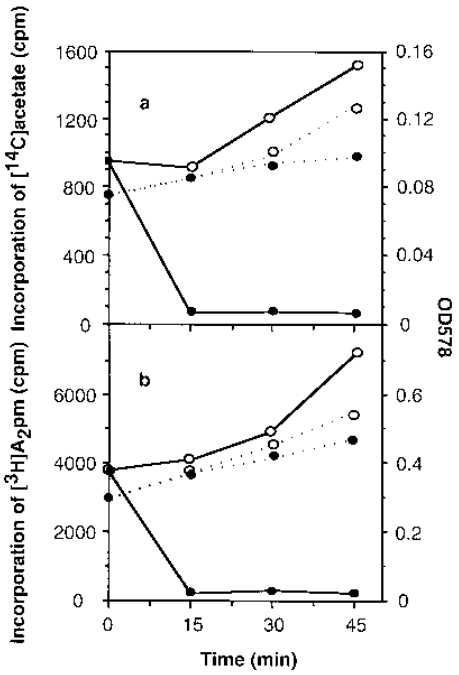

FIG. 1. Effects of cerulenin on macromolecular syntheses. *E. coli* W7 was grown in M9–0.4% glycerol medium and divided into two cultures. One subculture received cerulenin (100 μg/ml) at time zero (filled circles); the other one served as a control (open circles). The rate of phospholipid synthesis (a) was determined by incubation of 1-ml culture samples in the presence of 2 μCi of [^{14}C]acetate for 10 min; the rate of murein synthesis was determined (b) by incubation in the presence of 5 μCi of [^{3}H]A_2pm for 3 min. Samples were processed as described in Materials and Methods. Growth was monitored by readings of OD_{578} (dotted lines).

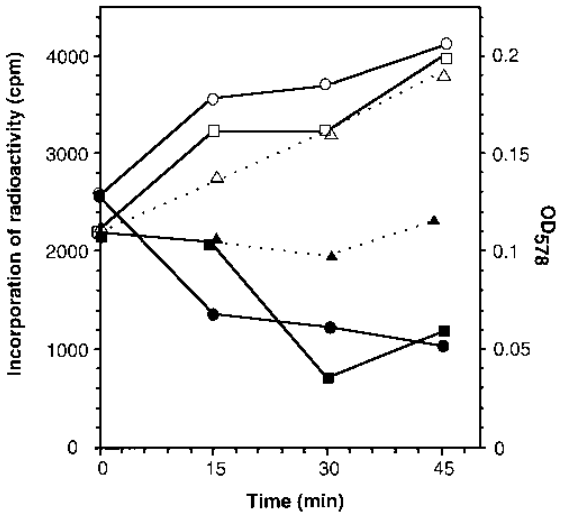

FIG. 2. Phospholipid and murein syntheses after glycerol starvation. *E. coli* BB2636 was grown in M9 medium supplemented with 5 mM glycerol and 0.5% glucose and divided into two cultures. One subculture was starved for glycerol (filled symbols) as described in Materials and Methods; the other one served as a control (open symbols). The rates of phospholipid synthesis (squares) and of murein synthesis (circles) were determined as described in the legend to Fig. 1. Growth was monitored by readings of OD_{578} (dotted lines).

the specific enzymatic step of murein synthesis that is affected, the muropeptide composition of the murein sacculus was determined after inhibition of phospholipid synthesis by cerulenin. The results are summarized in Table 1. In contrast to earlier reports, which demonstrated an increase in oligomeric muropeptides after amino acid starvation (8), cross-linked muropeptides were slightly decreased (about 12 to 17%) after cerulenin treatment. A major change compared to untreated control cells was a severe decrease in the relative amount of trimeric muropeptides. Trimers were reduced 53% 15 min after the addition of cerulenin and 68% 60 min later. A second significant change was a reduction in the amount of covalently attached lipoprotein, as manifested by a more than 40% reduction of the relative amounts of all LysArg-substituted muropeptides. The decrease in the relative amount of trimeric muropeptides is consistent with a recent growth model, called three-for-one, that claims that new material is first attached underneath the murein sacculus by the formation of trimeric cross bridges (12, 14). Analysis of the murein structure, however, did not reveal which murein biosynthetic step is inhibited when phospholipid synthesis is blocked.

Quantification of the murein intermediates. To pinpoint the site of coupling of murein with phospholipid synthesis, the intracellular concentrations of murein precursors were determined after inhibition of phospholipid synthesis by cerulenin. Specific labeling of the murein precursors was done by growth of the A_2pm-auxotrophic mutant W7 in the presence of [^{3}H]A_2pm in M9 minimal medium. The water-soluble UDP-activated MurNAc-pentapeptide and the two lipid precursors undecaprenylpyrophosphate-linked MurNAc-pentapeptide (lipid I) and undecaprenylpyrophosphate-linked GlcNAc-MurNAc-pentapeptide (lipid II) were extracted with butanol-acetic acid as described previously (26). Quantification of the compounds after separation by HPLC revealed no significant change in the concentration of any of these precursors after blockage of phospholipid synthesis by cerulenin. The radioactivity in the UDP-MurNAc-pentapeptide fraction 15 min after the addition of cerulenin was 28.0×10^3 cpm, compared to 26.0×10^3 cpm in the control cells. After prolonged growth in the presence of the drug, the radioactivity levels increased to 34.5×10^3 and 36.0×10^3 cpm at 30 and 45 min, respectively. At the same time points the radioactivity levels in the UDP-MurNAc-pen-

TABLE 1. Murein structure of cerulenin-treated *E. coli* W7[a]

Muropeptide[b]	Relative amt				
	Control	Cerulenin (15 min)	%	Cerulenin (60 min)	%
Monomers, total	51.83	57.12	+10	60.20	+16
Tri	7.72	6.39	−17	9.29	+20
Tetra	38.59	46.91	+21	46.12	+20
Dimers, total	42.68	39.73	−7	38.09	−11
Ala-A_2pm	36.77	35.58	−3	30.52	−17
A_2pm-A_2pm	5.90	4.14	−30	7.56	+28
Trimers, total	3.77	1.77	−53	1.21	−68
Anhydros	1.92	1.39	−28	2.12	+10
Cross-linkage, total	23.85	21.04	−12	19.85	−17
Ala-A_2pm	20.90	18.97	−9	16.07	−23
A_2pm-A_2pm	2.95	2.07	−30	3.78	+28
LysArg, total	4.63	2.55	−45	2.76	−41

[a] *E. coli* W7 was grown in M9–0.4% glycerol medium to an OD_{578} of 0.5. An aliquot was treated for 15 and 60 min with cerulenin (100 μg/ml), and murein sacculi were prepared and digested with Cellosyl as described previously (8).

[b] Abbreviations: Tri, disaccharide-tripeptide; Tetra, disaccharide-tetrapeptide; Ala-A_2pm, D,D-peptide bond in oligomeric muropeptides between D-Ala and m-A_2pm; A_2pm-A_2pm, L,D peptide bond in oligomeric muropeptides between A_2pm and A_2pm; Anhydros, muropeptides with a 1,6-anhydro-muramic acid; LysArg, muropeptides substituted at the A_2pm residue with a Lys-Arg dipeptide (from the murein lipoprotein).

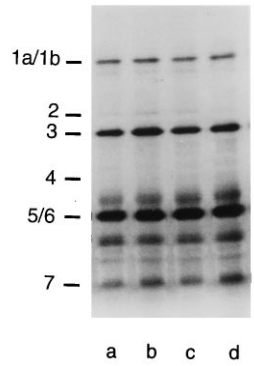

FIG. 3. Penicillin-binding activity in cerulenin-treated cells. *E. coli* W7 was grown in M9 medium. Cerulenin was added at a final concentration of 100 μg/ml, and samples were withdrawn at different time points. Whole cells were incubated with a ^{125}I-labeled ampicillin derivative. Proteins were separated by SDS–PAGE. The radiolabeled complexes were visualized by autoradiography of the dried gel. Lanes: a, untreated control; b to d, 15, 30, and 45 min, respectively, after cerulenin addition.

tapeptide pool of the control cells were 28.0×10^3 and 37.5×10^3 cpm, respectively.

The results for the lipid-linked precursors were less clear-cut due to additional peaks in the HPLC profile; however, there was no difference between the samples of the cerulenin-treated culture and the control. We conclude that none of the cytoplasmic reaction steps including the translocases I and II depends on phospholipid synthesis.

Determination of the PBPs. To determine whether the penicillin-sensitive enzymes that are involved in the final steps of murein synthesis are inhibited by an inhibition of phospholipid synthesis, we determined the enzyme-catalyzed binding of penicillin to these proteins (46). This was done with whole cells, using iodine-labeled ampicillin as described by Schwarz et al. (45). In accordance with earlier results by Rodionov et al. (38), the pattern of PBPs separated by SDS-PAGE was not altered when the cells had been treated with cerulenin (Fig. 3). When phospholipid synthesis was blocked by glycerol starvation of *E. coli* BB2636, again the PBP pattern was unchanged (data not shown).

Activity of murein hydrolases during inhibition of phospholipid synthesis. It has been claimed that murein hydrolases are indirectly regulated by the stringent control and directly by ongoing phospholipid synthesis (19, 38). In particular, it is tempting to speculate that the activity of the recently characterized lipoprotein murein hydrolases MltA and/or MltB (6) might depend on phospholipid synthesis. However, the enzymatic activity of murein hydrolases has never been determined after inhibition of phospholipid synthesis in *E. coli*, except for autolysis studies of harvested cells (30). In *Bacillus subtilis*, an altered autolytic activity by cerulenin treatment has been observed (41).

To obtain a clearer answer with respect to the lipoprotein murein hydrolases, we purposely used a mutant lacking the soluble lytic transglycosylase Slt70 (48) that grows perfectly well and induces the stringent control normally. No decrease in murein hydrolytic activity could be found after inhibition of phospholipid synthesis by cerulenin. In a range of 4.25 to 34 μg of protein per ml, the specific enzyme activities were 0.70 U/mg in control cells and 0.79 U/mg in cerulenin-treated cells.

Synthesis of the O-specific side chain of LPS. Thus far, we have obtained evidence for an inhibition of neither a cytoplasmic nor a periplasmic enzymatic step of the murein biosynthesis pathway after inhibition of phospholipid synthesis. There remains one important step that has not been looked at, because a simple method to study this step is missing, namely, the transport of the lipid-linked precursor from the inner to the outer side of the cytoplasmic membrane. Synthesis of the O-antigen side chain of the lipopolysaccharide (LPS), as well as murein synthesis, depends on the transport of undecaprenylphosphate-linked sugar subunits across the cytoplasmic membrane (37). If inhibition of phospholipid synthesis affects the translocation of the lipid-linked precursors, it may also inhibit the synthesis of the O-antigen. That we tested.

Since the *E. coli* strain used in this study is unable to synthesize the O-specific side chain, we transformed the *galE* mutant *E. coli* CS1928 with a plasmid (pSS37) that encodes the enzymes necessary to synthesize the O antigen of *S. dysenteriae* and to join it to the *E. coli* LPS (47). Synthesis of the O antigen was followed by determination of the incorporation of radioactively labeled galactose (44). Because there is only 1 molecule of galactose in the outer core, compared to 10 molecules in the O antigen, synthesis of the latter part of the LPS is predominantly measured by this method. The results shown in Fig. 4a clearly demonstrate that inhibition of phospholipid synthesis by cerulenin inhibits the synthesis of the O antigen within the first 15 min of treatment. Thus, both murein synthesis and O-antigen synthesis are affected in the same way. Further support for the speculation that it may be the translocation step that is inhibited when phospholipid synthesis is blocked comes from experiments analyzing the effect of colicin M on O-antigen synthesis (Fig. 4b). Colicin M has been shown to block the dephosphorylation of undecaprenylpyrophosphate (9, 10), a step that is needed to recycle the bactoprenol carrier molecule (15, 40). Consequently, colicin M inhibits murein synthesis and LPS–O-antigen synthesis. Interestingly, the inhibition of O-antigen synthesis by colicin M was quite similar in

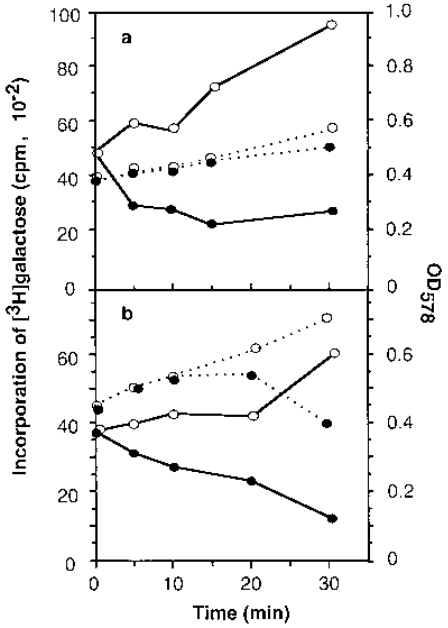

FIG. 4. O-antigen synthesis after inhibition of phospholipid synthesis. *E. coli* CS1928/pSS37 was grown in M9 medium and treated during exponential growth either with cerulenin (100 μg/ml) (a) or colicin M (final titer, 10,000 U) (b). To measure O-antigen synthesis, samples (0.5 ml) were withdrawn at the indicated time points, incubated with 1 μCi of [^{3}H]galactose for 3 min, and further processed as described in Materials and Methods. Open circles, the control culture; filled circles, treated culture. Growth was monitored by readings of OD_{578} (dotted lines).

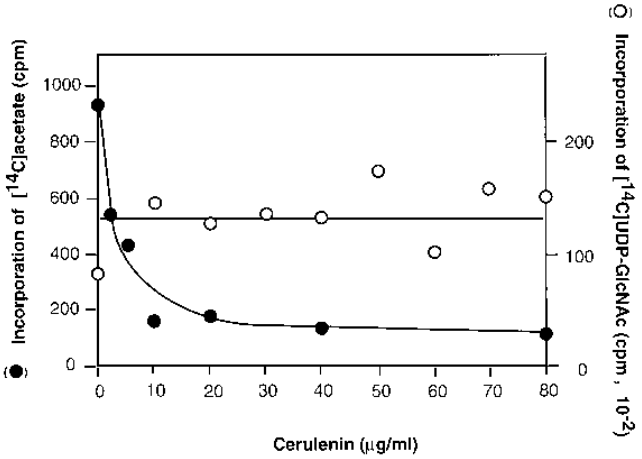

FIG. 5. Phospholipid and murein syntheses in ether-permeabilized cells. *E. coli* W7 was permeabilized by ether treatment as described previously (27), and the effects of increasing concentrations of cerulenin on the rates of phospholipid and murein synthesis were determined. Ether-treated cells were preincubated for 5 min in the presence of the indicated amounts of cerulenin. Pulse-labeling (6 min) with [^{14}C]acetate and [^{14}C]UDP-GlcNAc was done as described in Materials and Methods.

extent and kinetics to the inhibition caused by cerulenin (Fig. 4), as one would expect if colicin M and cerulenin interfere with the same part of the metabolic pathway.

Murein synthesis in ether-permeabilized cells. We were not able to find an enzymatic step of the murein biosynthesis pathway that is inhibited upon inhibition of phospholipid synthesis. However, synthesis of the O-antigen side chain of the LPS, as well as that of murein, was inhibited by cerulenin. The synthesis of both compounds depends on the transport of water-soluble sugar subunits by the lipid carrier undecaprenylphosphate. We therefore speculated that it is the transport process of the lipid-linked precursors that may be affected by inhibition of phospholipid synthesis. To test for this possibility, we decided to analyze murein and phospholipid synthesis in ether-treated cells in which the cytoplasmic membrane has been permeabilized (27). The results of these experiments are shown in Fig. 5. In ether-treated cells, murein synthesis seems greatly uncoupled from phospholipid synthesis. At cerulenin concentrations that completely blocked acetate incorporation into phospholipids, murein synthesis was only marginally affected. In whole cells, on the other hand, murein synthesis and phospholipid synthesis responded with similar sensitivities to the addition of cerulenin, and both were 100% inhibited 15 min after the addition of the drug (Fig. 1).

DISCUSSION

It seems well established that murein synthesis depends on ongoing phospholipid synthesis and thereby indirectly responds also to the stringent control (18–22). However, the site in the murein biosynthetic pathway which is affected when phospholipid synthesis is blocked could not be determined up to now. Here we report that another biosynthetic reaction of the periplasmic space, i.e., synthesis of the O-specific side chain of the LPS, was concomitantly inhibited with murein synthesis when phospholipid synthesis was blocked. It was inhibited as early as 15 min, which corresponds to the inhibition of murein synthesis. The finding that both syntheses are affected with similar sensitivities and kinetics is highly indicative for the inhibition of a step common to both biosynthetic pathways. Growth of the murein sacculus as well as LPS side chain synthesis depend on the translocation of a undecaprenylphosphate-linked precursor molecule from the cytoplasm to the periplasm. Thus, a block in this step will affect both syntheses. We therefore speculate that it is the translocation of the lipid-linked precursors that depends on ongoing phospholipid synthesis.

Consistent with this speculation, we were able to demonstrate that permeabilization of the cytoplasmic membrane by ether treatment uncouples murein synthesis from phospholipid synthesis. That is, unlike in vivo, in ether-permeabilized cells, murein synthesis was not inhibited when phospholipid synthesis was already completely abolished. Hence, it seems that destruction of the permeability barrier crucially alters the mechanism of precursor translocation across the cytoplasmic membrane. Under these conditions, it is no longer dependent on phospholipid synthesis.

The details of the mechanism of the translocation of the lipid-linked precursors from the cytoplasmic side to the periplasmic side are not known (33). However, because of the high rate of murein synthesis, in particular during septum formation, it is unlikely that this translocation is only by a flip-flop mechanism. Auxiliary proteins are likely to participate in this process. The integral membrane proteins RodA and FtsW (17) have been proposed to channel the lipid precursors by an unknown mechanism to PBP2 and PBP3, respectively (23, 24, 33). Likewise, the membrane protein RfbX has recently been shown to function as a flippase for O-unit translocation across the membrane (31, 37). Inhibition of murein synthesis and O-antigen synthesis upon inhibition of phospholipid synthesis, which seems to be at the site of precursor translocation, may thus be a consequence of inefficient or improper insertion of these transmembrane proteins into the cytoplasmic membrane. A dependency of the insertion into membranes and proper folding of proteins on phospholipids is well established (5). For example, the assembly of the outer membrane protein OmpF of *E. coli* has been shown to be prevented by the addition of cerulenin to the growing culture (2).

Importantly, inhibition of phospholipid synthesis interferes not only with murein synthesis but also with penicillin-induced bacteriolysis, causing penicillin tolerance (8, 20, 36, 38, 39). This has been explained by assuming that the enzymatic activity not only of murein synthases but also of murein hydrolases depends on phospholipid synthesis (20, 38). However, we failed to obtain evidence that inhibition of phospholipid synthesis reduces murein hydrolase activity. A recent model proposes that murein hydrolases are kept under control by forming a functional complex together with murein synthases, including PBP2 and PBP3 (12–14, 42, 51). Interestingly, evidence has been presented that these two PBPs interact with RodA and FtsW. Therefore, improper membrane insertion and/or functioning of RodA and FtsW due to an inhibition of phospholipid synthesis may affect the assembly of the multienzyme complex. As a result, not only may murein synthesis be inhibited but also the murein hydrolases may gain no access to the murein sacculus, thus establishing the phenomenon of penicillin tolerance.

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