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Inhibition of Peptidoglycan Biosynthesis at a Postcytoplasmic Reaction in a Stable L-Phase Variant of *Streptococcus faecium*

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Cultures of a stable L-phase variant of *Streptococcus faecium* F24 produced and retained peptidoglycan precursors intracellularly over the entire growth cycle in a chemically defined medium. The identity of the most abundant precursor, UDP *N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine (UDP-MurNAc-pentapeptide), was confirmed by demonstrating in vitro the presence of enzymes required for the cytoplasmic stage of peptidoglycan biosynthesis. The initial membrane-bound reaction in peptidoglycan biosynthesis involving phospho-MurNAc-pentapeptide translocase and undecaprenyl-phosphate membrane carrier was catalyzed by protoplast membrane preparations but not by L-phase membrane preparations. However, both protoplast and L-phase membranes incorporated radioactivity from dTDP-L-[^{14}C]rhamnose, the presumed precursor to a non-peptidoglycan cell surface component, into high-molecular-weight material. dTDP-L-rhamnose did not accumulate in growing cultures but was synthesized from D-glucose-1-phosphate and dTTP by cell-free extracts of the streptococcus and L-phase variant. Neither rhamnose- nor muramic acid-containing compounds were detected in culture fluids. It is suggested that continued inhibition of cell wall biosynthesis in this stable L-phase variant is the result of a defect expressed at the membrane stage of peptidoglycan biosynthesis specifically involving the translocation step.

Muralytic enzymes (such as lysozyme and bacterial autolysins) and various antibiotics (including the penicillins, cycloserine, bacitracin, and phosphonomycin) act via several different mechanisms to interrupt either the structural integrity of preformed bacterial cell walls or the sequence of biochemical reactions which ultimately leads to the synthesis of complete walls (7). Bacteria treated with one of these agents in an appropriate environment may exhibit dramatic phenotypic changes as a result of cell wall damage (12). Phenotypic variants produced by such treatment can retain physiological and metabolic activity and presumably contain a complete complement of parental cell wall biosynthetic machinery. Indeed, after the wall-active agent is neutralized or eliminated, certain wall-defective variants can immediately resume the assembly of intact cell walls and then quickly regain the morphology of parent organisms (2, 5, 18). The tendency to revert to the parental phenotype is usually greatest immediately after exposure to the inducing agent and decreases with continued cultivation. Upon extended subculture in a selective environment, a population may emerge composed of "stable" variants that have apparently lost the ability to reassemble cell walls and cannot revert to parental morphology. To describe the stable L-phase phenotype in biochemical terms, marker compounds were used to assess cell wall biosynthesis in a nonreverting L-phase variant. Specifically, rhamnose and muramic acid were selected to determine the stage at which cell wall biosynthesis is inhibited in a lysozyme-produced, stable L-phase variant of *Streptococcus faecium* F24.

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MATERIALS AND METHODS

Organisms and growth conditions. *S. faecium* strain F24 (ATCC 19634) and a nonreverting L-phase variant (ATCC 19635), which was derived from a lysozyme-converted protoplast inoculum of *S. faecium* F24 (17), were propagated at 37°C in a 43-component, chemically defined medium (SM) without antibiotics (11). The variant exhibited typical L-form morphology when grown on solid media; liquid SM was used to aid the task of analyzing spent media for products of L-phase metabolism. Growth was monitored with a Klett-Summerson colorimeter (green filter, 540 nm).

Cells were harvested and washed (2.3% [wt/vol] NH_4Cl) by centrifugation (4°C) at 600 × *g* for 15 min (streptococci) or at 480 × *g* for 25 min (L-phase variant). The lysozyme method of King and Gooder (17) was used to prepare fresh protoplasts in 8% (wt/vol) polyethylene glycol. Protoplasts produced by this method are free of cell walls as determined previously (10, 17) by (i) release of the Lancefield streptococcal group D antigen located between the cell wall and plasma membrane, (ii) inability of phage P13 to attach to protoplasts, (iii) loss of more than 90% of total cellular rhamnose, and (iv) absence of cell wall fragments when examined by transmission electron microscopy.

Characterization of accumulated precursors. Total bound *N*-acetylhexosamine (HexNAc) in cultures was estimated from 40-ml samples which were removed at various times from cultures growing in SM. Washed cells were suspended in distilled water and were subjected to three cycles of flash freezing and thawing. The resulting suspension was centrifuged at 17,300 × *g* for 30 min, the pellet was extracted with 5% (wt/vol) cold trichloroacetic acid, and the two supernatants were combined. Trichloroacetic acid was removed with ether, and the aqueous portion was analyzed for total HexNAc or concentrated by lyophilization for nucleotide characterization. For preparation of UDP *N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysine (UDP-MurNAc-tripeptide), streptococci were grown in medium containing the L isomer of alanine (200 μg/ml) rather than the usual isomeric mixture (11) and then were harvested after an additional 45-min incubation in fresh medium containing 20 μg of D-cycloserine (Sigma Chemical Co.) per ml.

Nucleotides were separated by chromatography (15) on a Dowex 1-X4 formate column (1.2 by 30 cm) with an elution gradient approaching (i) 4 N formic acid, (ii) 0.2 M ammonium formate in 4 N formic acid, and (iii) 0.4 M ammonium formate in 4 N formic acid. The flow rate was approximately 35 ml/h. Fractions (5.0 ml) of peaks absorbing at 260 nm and containing bound HexNAc were pooled appropriately and adjusted to pH 4.5 with 2 M NH_4OH. Darco G-60 (20 mg/μmol of nucleotide) was added; after 30 min the charcoal was removed by centrifugation and washed with water. Nucleotides were eluted with 50% ethanol–0.03 N NH_4OH and purified by paper chromatography using either solvent system B or solvent system C (described below). Samples were eluted from chromatograms, hydrolyzed, and analyzed for amino acids and amino sugars.

Culture fluids were examined for nucleotide-bound wall precursors after concentration of harvest supernatants by lyophilization and charcoal adsorption.

Enzyme preparation. Cells used as a source of L-alanine racemase (EC 5.1.1.1) and D-alanine:D-alanine ligase (EC 6.3.2.4) were suspended in 100 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.8) containing 2.5 mM reduced glutathione (Sigma) and passed through a cold French pressure cell (American Instrument Co.) at a pressure of 20,000 pounds (ca. 9,071 kg). Enzyme preparations were clarified by centrifugation (4°C) at 34,000 × *g* for 20 min. The enzyme used in the alanine dipeptide adding system (UDP-MurNAc-tripeptide:D-alanyl-D-alanine ligase; EC 6.3.2.10) was prepared from cells which were broken in 10 mM phosphate buffer (pH 6.8) with a Branson Sonifier-Cell Disruptor (Heat Systems-Ultrasonics, Inc.) with microtip at a 50-W output. These preparations were cleared of cell debris by centrifugation and were partially purified by protamine and ammonium sulfate fractionation as described by Neuhaus and Struve (24). Crude enzyme preparations for the synthesis of dTDP-L-rhamnose were prepared in 50 mM Tris-hydrochloride (pH 8.0) containing 10 mM $MgCl_2$ and 1 mM ethylenediaminetetraacetic acid by similar sonic treatment and centrifugation.

Membrane-associated enzyme preparations for the phospho-MurNAc-pentapeptide translocase (EC 2.7.8.13) system were obtained from whole-cell suspensions of the L-phase variant and protoplasts by a combination of osmotic lysis in 50 mM Tris-hydrochloride (pH 7.8) containing 1.0 M KCl and 20-s sonic bursts. Membrane fragments were collected by centrifugation at 39,100 × *g* for 25 min (4°C) after each of the three resuspensions and sonic treatments. The enzyme system was partially purified by the method of Struve et al. (33) by centrifugation four times between 1,085 × *g* (45 min) and 39,100 × *g* (25 min). Endogenous bound precursor was removed from these preparations by the UMP-exchange reaction described by Hammes and Neuhaus (13). Membrane preparations for the rhamnosyl translocase reaction were obtained essentially as described by Panos and Cohen (26) by sonic treatment of cells suspended in 50 mM Tris-hydrochloride (pH 7.4) containing 5 mM mercaptoethanol and 40 mM $MgCl_2$. Membranes were collected by centrifugation at 34,000 × *g* for 25 min (4°C).

Reaction systems. Assays for L-alanine racemase and D-alanine:D-alanine ligase were by the method of Neuhaus and Struve (24). Reaction mixtures for the synthesis of radioactive D-alanyl-D-alanine contained 50 μl of 500 mM Tris-hydrochloride (pH 7.8), 80 μl of 1.0 M KCl, 20 μl of 100 mM ATP (Sigma), 5 μCi of D-[^{14}C]alanine, and enzyme extract in a final volume of 500 μl. L-[^{14}C]alanine replaced the D isomer for the combined racemase-ligase assay; alanine dipeptide was separated from the reaction mixture on a column (1.5 by 50 cm) of Bio-Rad AG 50W-X8 with 0.5 and 1.0 N HCl (25).

UDP-MurNAc-tripeptide:D-alanyl-D-alanine ligase was assayed and radioactive UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine (UDP-MurNAc-pentapeptide) was prepared by the procedure of Neuhaus and Struve (24) from UDP-MurNAc-tripeptide accumulated in cycloserine-treated streptococci and D-[^{14}C]alanyl-D-alanine synthesized enzymatically using extracts of the L-phase variant. Reaction mixtures contained 10 μl of 100 μM Tris-hydrochloride (pH 7.8), 10 μl of 10 μM $MgCl_2$, 20 μl of 0.1 μM UDP-MurNAc-tripeptide, 20 μl of 10 μM ATP, 20 μl of D-[^{14}C]alanyl-D-alanine (approximately 167,580 cpm), and partially purified enzyme in a total volume of 100 μl. UDP-MurNAc-^{14}C-pentapeptide for use in the translocase assay was separated from unreacted UDP-MurNAc-tripeptide by paper chromatography with solvent system C. The appropriate nucleotide areas were located by UV light, cut out, eluted with water, and concentrated by lyophilization.

Transfer of phospho-MurNAc-pentapeptide from

UDP-MurNAc-pentapeptide to membrane undecaprenyl-phosphate carrier was assayed by the method of Hammes and Neuhaus (13). The assay used was based on the amount of acid-precipitable radioactivity recovered in $HClO_4$ washes after incubation of the reaction mixture. Approximately 12,100 cpm of UDP-MurNAc-^{14}C-pentapeptide was supplied in reaction mixtures.

The general methods of Glaser et al. (9) were used for the synthesis of dTDP-L-[^{14}C]rhamnose. A typical reaction mixture contained: 25 μl of 50 mM Tris-hydrochloride (pH 8.0), 25 μl of 0.01 mM dTTP, 25 μl of 0.01 mM reduced nicotinamide adenine dinucleotide phosphate, 25 μl of 0.02 mM α-D-glucose-1-phosphate, 5 μCi of α-D-[^{14}C]glucose-1-phosphate, and enzyme preparation in a final volume of 600 μl. At various times reactions at 37°C were terminated (0°C), and 20-μl samples were paper chromatographed in solvent system C. Portions were rechromatographed in solvent system B to obtain dTDP-[^{14}C]rhamnose for membrane uptake experiments.

The rhamnosyl translocase system was similar to that described by Panos and Cohen (26). A complete reaction mixture contained 400 μl of dTDP-[^{14}C]rhamnose (14,150 cpm), 500 μl of membrane preparation in 50 mM Tris-hydrochloride (pH 7.4) containing 5 mM mercaptoethanol and 40 mM $MgCl_2$, and 100 μl of 25 mM mercaptoethanol in 50 mM Tris-hydrochloride (pH 7.4). After 60 min of incubation at 37°C, membrane fragments were collected (34,800 × *g*, 25 min, 4°C) and then washed repeatedly with cold 5% trichloroacetic acid–50 mM pyrophosphate. Rhamnose was extracted from the pelleted material with hot trichloroacetic acid and subjected to gel filtration as indicated in a subsequent section.

Rhamnose analysis. The experimental design described by Panos and Cohen (26) was followed in analyzing hot 5% trichloroacetic acid extracts of lyophilized samples of washed whole-cell preparations or membrane fragments from the rhamnosyl translocase system. After removal of trichloroacetic acid with ether, samples were subjected to chromatography on a Sephadex G-25 column (1.6 by 90 cm) using 0.01 N acetic acid at a flow rate of approximately 25 ml/h. Fractions (2.0 ml) were collected, and rhamnose was quantitated by the cysteine-hot sulfuric acid method of Dische and Shettles (3). Portions of fractions were paper chromatographed in solvent system A.

Chromatography. Paper chromatography was descending at 25°C on washed Whatman no. 1 or 3MM paper in the following solvent systems (by volume): (A) 1-butanol–pyridine–water (45:25:50), (B) isobutyric acid–1.0 M ammonium hydroxide (5:3), (C) ethanol–1.0 M ammonium acetate, pH 7.5 (7:3), (D) pyridine-water (4:1), and (E) 1-butanol–acetic acid–water (4:1:1). Nucleotides were located by absorption of UV light and amino acids and amino sugars were located after chromatograms were sprayed with a solution of 0.3% ninhydrin in ethanol (Sigma) and heated (105°C, 3 min). Carbohydrates were detected after heating (105°C, 2 min) chromatograms which had been sprayed with a 0.2% solution of acidified aniline diphenylamine (Sigma).

Thin-layer chromatography was ascending at 25°C on plates of 0.25-mm-thick Silica Gel G (Brinkman Instruments, Inc.) impregnated with 50 ml of 0.1 N boric acid per 25 g of silica. The following solvent systems were used (by volume): (A) 1-propanol–ammonium hydroxide–water (6:2:1), (B) acetone-water (9:1), and (C) methyl ethyl ketone-acetic acid-methanol (3:1:1). Detection of carbohydrates and amino acids was as on paper, and, in addition, a spray consisting of 95% ethanol–concentrated sulfuric acid–anisaldehyde (18:1:1) was used for sugar detection. Sprayed plates were heated at 90°C for 10 min to detect as little as 0.05 μg of sugar.

Column chromatographies were prepared and run at 25°C according to technical information supplied by the respective manufacturers. Gel filtration was on Sephadex G-25 (Pharmacia Fine Chemicals AB). Dowex 1-X4 (Bio-Rad; 50 to 100 mesh) was used for nucleotide separations (formate form) and Bio-Rad AG 50 W-X8 (200 to 400 mesh) was used as supplied in the hydrogen form for the alanine ligase assay or converted to the sodium form for the alanine dipeptide adding assay and optical configuration determinations. Column dimensions, buffer systems, flow rates and fraction volumes are indicated in appropriate sections along with specific applications.

Amino acids and amino sugars were quantitated with a Beckman automated amino acid analyzer after acid hydrolysis of samples. Samples of nucleotide-bound precursors were hydrolyzed under partial vacuum with 2.0 ml of 5 N HCl for 4 h for amino sugars and 18 h for amino acids at 105°C.

Other procedures. The HexNAc content of nucleotide-bound precursors was determined by the method of Reissig et al. (27) after samples were hydrolyzed for 15 min at 100°C in sealed vials containing 0.5 to 0.01 N HCl. Total protein added to enzyme assays was determined by the Lowry procedure (20).

Products separated by ion-exchange chromatography from alanine ligase reaction mixtures were subjected to paper chromatography using solvent system B, D, or E with alanine, alanine dipeptide, and alanine tripeptide standards. The optical configuration of alanine in reaction products of alanine ligase assays was determined after treatment with D-amino-acid oxidase (EC 1.4.3.3; Sigma) or L-alanine aminotransferase (EC 2.6.1.2; Sigma) by the procedure of Lynch and Neuhaus (21).

Radioactive samples were counted in either a toluene-based (for samples on paper) or toluene–Triton X-100 (for liquid samples) scintillation fluid using a Packard Tri-Carb liquid scintillation spectrometer.

Chemicals. α-D-[U-^{14}C]glucose-1-phosphate (210 mCi/mmol), L-[U-^{14}C]alanine (120 mCi/mmol), and D-[^{14}C]alanine (56.3 mCi/mmol) were purchased from the Isotope and Nuclear Division of International Chemical and Nuclear Co. Non-radioactive amino acids were from Calbiochem, except alanine peptides (Sigma). Inorganic compounds and organic solvents were reagent grade.

RESULTS

Peptidoglycan precursors in growing cultures. Peptidoglycan precursors in cultures were measured as nucleotide-bound HexNAc, because UDP-*N*-acetylglucosamine is the first

HexNAc-containing compound in the biosynthetic sequence which produces peptidoglycan precursors (7). The content of bound HexNAc extracted from growing cultures with cold trichloroacetic acid is shown in Fig. 1. The L-phase variant produced and retained bound HexNAc intracellularly over the entire growth cycle. Maximal accumulation in the variant appeared to be at 6 h of incubation, when approximately 250 nmol of bound HexNAc was accumulated by 2.4×10^{10} colony-forming units in a 40-ml culture sample. Precursor accumulated in the variant until late in the stationary period of growth, when SM-grown L-phase cultures incubated at 37°C usually begin to lose viability. For the parent, the amount of precursor was negligible until cultures had begun stationary growth. When culture turbidity had stopped increasing, 20 nmol of precursor was found per 8×10^{10} streptococcal colony-forming units. Bound HexNAc was not detected in material concentrated from L-phase or streptococcal culture supernatants; precursor was isolated by charcoal adsorption from culture fluids only after 24 h of incubation at 37°C.

Intracellular accumulation of bound HexNAc in streptococci could be obtained after treatment of growing cultures with 20 μg of D-cycloserine per ml. Rapid accumulation of precursor occurred soon after introduction of the drug; maximal accumulation, 340 nmol per 40-ml culture sample, was observed after 45 min of incubation in the presence of the drug (data not shown).

Characterization of peptidoglycan precursors. Cold trichloroacetic acid extracts of freeze-thaw cell preparations were fractionated by ion-exchange chromatography as shown in Fig. 2. Elution from the column resulted in precursor-containing peaks which absorbed at 260 nm and were positive for bound HexNAc. The elution pattern of extracts from the L-phase variant (Fig. 2A) contains a major peak, desig-

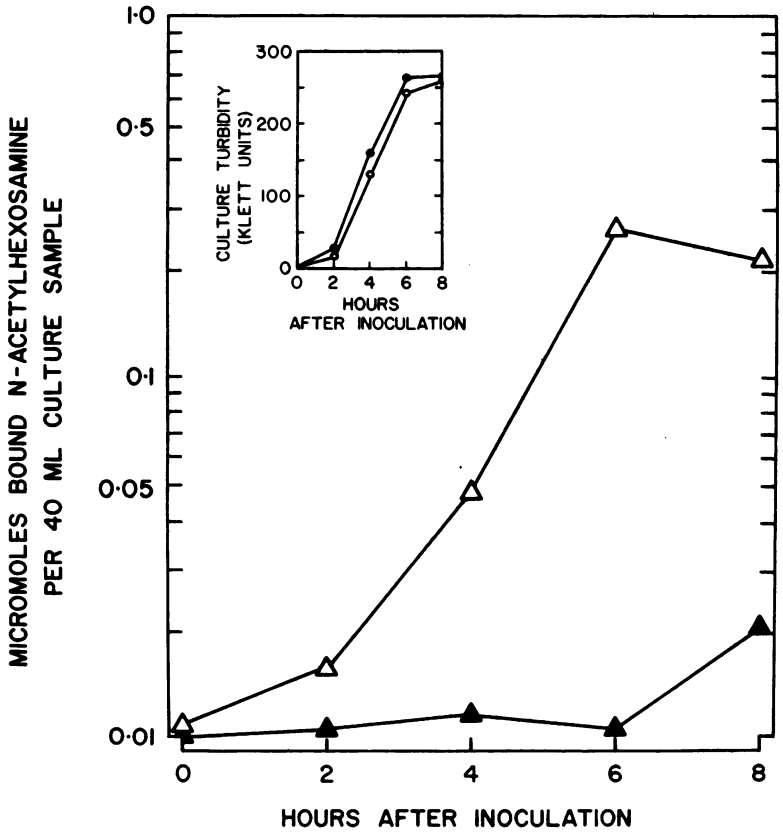

FIG. 1. *Intracellular accumulation of nucleotide-bound HexNAc in cultures of S. faecium F24 and a derived, stable L-phase variant growing in chemically defined media. At the indicated times 40-ml samples of each culture were harvested, and cells were assayed for bound HexNAc as described in the text. Symbols:* ▲, *bound HexNAc content of streptococci;* △, *bound HexNAc content of the L-phase variant; inset:* ●, *streptococcal growth;* ○, *L-phase variant growth.*

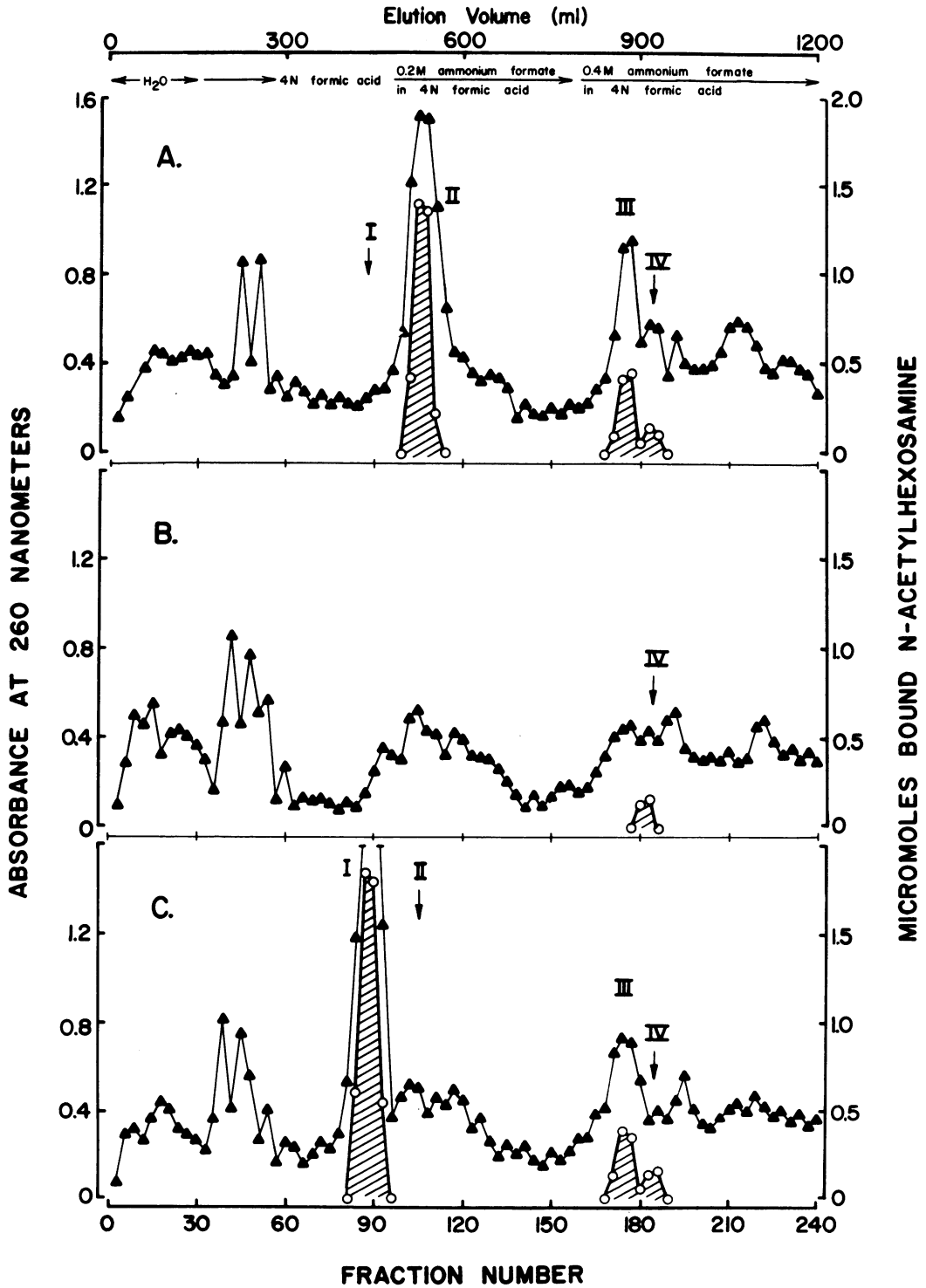

FIG. 2. *Elution profiles of cold trichloroacetic acid extracts of cultures of (A) a stable L-phase variant of S. faecium F24, (B) S. faecium F24, and (C) the streptococcus treated with* D*-cycloserine. Extracts were applied to a Dowex 1-X4 column and eluted with a formic acid-ammonium formate gradient as described in the text. Hatched areas in the figure indicate UV-absorbing fractions of effluent which also contained bound HexNAc. Data are presented for each fourth fraction collected. Symbols: ▲, absorbance at 260 nm; ○, bound HexNAc.*

nated peak II, and two minor precursor-containing peaks, referred to as peaks III and IV. Streptococcal extracts, however, contain only a single minor peak of bound precursor (Fig. 2B). This peak corresponds to peak IV in the elution profile of the variant. Extracts of cycloserine-treated streptococci (Fig. 2C) were eluted from the column in a major precursor-containing peak, peak I, just ahead of the peak II position (Fig. 2A), and two minor peaks.

Identification and quantitative analysis of the components were done on material from appropriately pooled fractions which had been desalted and concentrated by charcoal adsorption. The UV absorption spectra at pH 7.0 of all samples examined were similar to uridine nucleotides: absorption maximums and minimums were 262 and 230 nm, respectively, whereas 250/260- and 280/260-nm absorbance ratios were 0.72 to 0.74 and 0.35 to 0.39, respectively. Nucleotides were hydrolyzed and subjected to quantitative analysis (Table 1). Material from peak I, isolated from cycloserine-treated streptococci, appears to be UDP-MurNAc-tripeptide, and peak II material, from the variant, is apparently UDP-MurNAc-pentapeptide. Peaks III and IV from both organisms appear to contain UDP-*N*-acetylglucosamine and UDP-MurNAc as major components, respectively, but probably represent mixtures of these two precursors plus small amounts of UDP-MurNAc-L-alanine. The pentapeptide-containing precursor (peak II) accounts for approximately 62% of the total bound HexNAc recovered from the L-phase variant (Fig. 2), whereas 29 and 9% of the total are in peaks III and IV, respectively. UDP-MurNAc-tripeptide (peak I) accounts for 76% of the total bound HexNAc from cycloserine-treated streptococci and only 18 and 6%, respectively, are in peaks III and IV.

Cytoplasmic stage of peptidoglycan synthesis. The identity of the major peptidoglycan precursor accumulated by the L-phase variant was confirmed enzymatically, as shown in Table 2, by assaying L-alanine racemase, D-alanine:D-alanine ligase, and UDP-MurNAc-tripeptide:D-alanyl-D-alanine ligase. Functional abilities to convert L-alanine to the D isomer and to synthesize alanine dipeptide from D-alanine were apparent in extracts of the L-phase variant and streptococcus. Results of paper chromatography in solvent systems B, D, and E before and after acid hydrolysis of the reaction product were consistent with the synthesis of alanine dipeptide; results of treating dipeptide hydrolysates with D-amino acid oxidase or L-alanine aminotransferase indicated that the synthetic product of the combined racemase-ligase reaction system was composed of D isomers of alanine (data not shown). Extracts of both organisms also formed the completed cytoplasmic precursor to peptidoglycan from alanine dipeptide and UDP-MurNAc-tripeptide. The product of this reaction, UDP-MurNAc-pentapeptide, had an R_{UMP} of 0.57 (versus an R_{UMP} of 0.38 for unreacted UDP-MurNAc-tripeptide) on paper chromato-

TABLE 2. *Enzymes required for the incorporation of alanine into peptidoglycan precursors: functional presence in cell-free extracts from S. faecium F24 and a derived stable L-phase variant*

Organism	Alanine racemase[a]	D-Alanine:D-alanine ligase[b]	UDP-MurNAc-tripeptide:D-alanyl-D-alanine ligase[c]
Streptococcus	0.12	0.15	192
L-phase variant	0.10	0.11	213

[a] Nanomoles of alanine dipeptide formed in 120 min from L-[^{14}C]alanine per milligram of protein in the combined racemase-ligase assay.

[b] Nanomoles of alanine dipeptide formed in 120 min from D-[^{14}C]alanine per milligram of protein.

[c] Nanomoles of UDP-MurNAc-pentapeptide formed in 30 min from D-[^{14}C]alanyl-D-alanine and UDP-MurNAc-tripeptide per milligram of protein (partially purified enzyme preparation).

TABLE 1. *Amino acid and amino sugar analyses of UDP-HexNAc accumulated by a stable L-phase variant of S. faecium F24 and by the streptococcus treated with D-cycloserine*

Organism	Peak no.[a]	Amino acid (nmol/nmol of total hexosamine)			Amino sugar ratio[b]	
		Alanine	Glutamic acid	Lysine	Muramic acid	Glucosamine
L-phase variant	II	2.91	0.92	1.02	1.0	
	III	0.09			0.10	0.90
	IV	0.11			0.94	0.06
Streptococcus plus D-cycloserine	I	1.12	0.97	1.14	1.0	
	III	0.06			0.08	0.92
	IV	0.29			0.95	0.05

[a] Peaks as designated in Fig. 2.

[b] Total hexosamine was arbitrarily designated as unity.

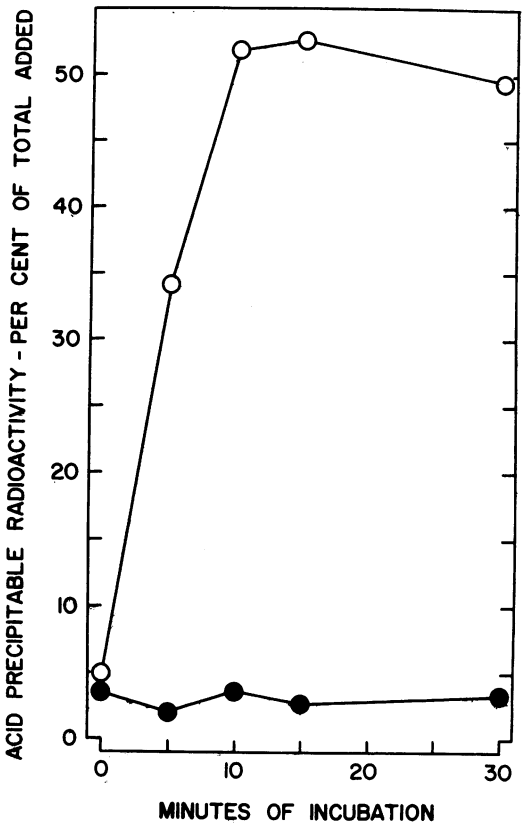

FIG. 3. *Phospho-MurNAc-pentapeptide translocase activity in protoplast and L-phase variant membrane preparations measured by incorporation of radioactivity from UDP-MurNAc-^{14}C-pentapeptide into acid-precipitable material as described in the text. Symbols: ○, protoplast system; ●, L-phase variant system.*

grams run with solvent system C. Because completed peptidoglycan precursor is accumulated intracellularly by the variant, these enzyme activities imply the functional presence in this variant of all enzymes required in the cytoplasmic stage of peptidoglycan biosynthesis.

Membrane stage of peptidoglycan biosynthesis. The initial membrane reaction in peptidoglycan synthesis, catalyzed by phospho-MurNAc-pentapeptide translocase, was measured with an appropriate substrate synthesized from UDP-MurNAc-tripeptide accumulated in cycloserine-treated streptococci and enzymatically prepared D-[^{14}C]alanyl-D-alanine. Acid-precipitable radioactivity associated with fragmented protoplast membranes increased with time (Fig. 3). After 15 min of incubation, approximately 52% of available label was taken up by protoplast membranes, whereas uptake by the L-phase variant preparation was minimal (less than 3% of input) even after 30 min of incubation. Any endogenous membrane-bound precursor had been removed from membrane preparations in a preincubation exchange reaction with UMP. These data imply that this L-phase variant is unable to catalyze the initial membrane-bound reaction in peptidoglycan biosynthesis due to a translocase defect or unavailability of membrane carrier.

Synthesis of accessory wall polymer. Lysozyme production of protoplasts from *S. faecium* F24 results in cell wall dissolution and concomitant release of secondary wall polymers, as determined by the loss of at least 90% of the total rhamnose found in parent cocci (10, 17; Table 3). Subsequent sustained growth in the L-phase results in a further reduction of total cellular rhamnose to only 2% of the rhamnose content of the parent. Crude enzyme preparations were employed to investigate the cell-free synthesis of cytoplasmic precursor to cell wall rhamnose, because nucleotide-bound rhamnose was detected neither in intracellular material nor in concentrated culture fluids, which were analyzed by thin-layer chromatography. The formation of dTDP-L-rhamnose from D-glucose-1-phosphate and dTTP by streptococcal and variant enzyme preparations was readily apparent (Table 3). The major product of this reaction had an R_f of 0.39 when paper chromatographed in solvent system B, and an acid hydrolysate had an R_f of 0.52 on thin-layer plates run with solvent system B. In addition, both protoplast and L-phase variant membrane preparations could take up radioactivity from dTDP-L-[^{14}C] rhamnose (Table 3). Although the total membrane uptake (acid-precipitable radioactivity) was greater in the protoplast system than in the L-phase variant system, about 75% of hot trichloroacetic acid-extracted radioactivity from both membrane systems was eluted in the void volume as a presumptive intermediate to a non-peptidoglycan cell surface component with a molecular weight exceeding that of monomeric rhamnose (or dTDP-rhamnose). In addition, this material did not migrate on thin-layer plates with solvent system B, but rhamnose was detected in an acid hydrolysate analyzed with the same system.

DISCUSSION

Microorganisms are capable of remarkably varied responses when they are placed in a new environment. For example, when lysozyme-induced protoplasts of *S. faecium* F24 are incubated on medium solidified with gelatin, they resynthesize cell walls and readily revert to apparently intact streptococci (18). On soft agar,

TABLE 3. *Total rhamnose in S. faecium F24 and derived cell wall-defective variants, synthesis of dTDP-L-rhamnose from D-glucose-1-phosphate and dTTP by cell-free extracts, and transfer of radioactivity from dTDP-L-[^{14}C]rhamnose to membrane preparations*

Organism	Total rhamnose content[a]	dTDP-L-rhamnose formation[b]	Incorporation of radioactivity from dTDP-L-[^{14}C]rhamnose into membrane fragments	
			Total uptake[c]	Non-peptidoglycan cell wall intermediate[d]
Streptococcus	4.7	94	ND[e]	ND
Protoplast	0.46	ND	48	62
L-phase variant	0.10	136	8.8	56

[a] Percent dry weight of whole cells.

[b] Picomoles of dTDP-L-[^{14}C]rhamnose formed per milligram of protein after 30 min of incubation of the dTDP-L-rhamnose-forming system.

[c] Acid-precipitable radioactivity in membrane fragments as percentage of the total radioactivity added to the rhamnosyl translocase system.

[d] Radioactivity which was extracted from membrane fragments (rhamnosyl translocase system) with hot trichloroacetic acid and which was eluted in the void volume of Sephadex G-25 chromatography; expressed as percent of total uptake in the respective membrane system.

[e] ND, Not determined.

however, only sporadic revertants are observed, and protoplasts are quantitatively converted to L-phase variants. Shockman and co-workers have recently reported that growing protoplasts (autoplasts) of *S. faecalis* ATCC 9790 synthesize and excrete soluble peptidoglycan fragments (29) and glycan chains (31). These freshly prepared autoplasts synthesize soluble peptidoglycan fragments in amounts equivalent to the total peptidoglycan synthesized by streptococci for insertion into intact cell walls (29). In addition, reverting protoplasts of *Bacillus licheniformis* produce peptidoglycan and teichoic acid polymers (5). Not only are soluble fragments produced by the reverting *B. licheniformis* protoplasts, but peptidoglycan fibrils are also extruded into incubation medium by protoplasts in suspension. The peptidoglycan fragments produced by *S. faecalis* protoplasts are peptide cross-linked, although the cross-linking is only 15% of that found in exponentially growing streptococci (30). These reports indicate that osmotically fragile organisms can synthesize and excrete glycosidically linked peptidoglycan polymers which can then become peptide cross-linked. The implication of these studies is that all wall biosynthetic enzymes can be present and functional in the absence of the bacterial cell wall. In stable L-phase variants, however, this biosynthetic sequence appears to be permanently interrupted.

Biochemical data have implicated lesions in the peptidoglycan biosynthetic pathway as underlying factors in the continued inability of stable L-phase variants to synthesize cell walls. Several L-phase variants derived from a single strain of *Staphylococcus aureus* synthesize various quantities of nucleotide-bound HexNAc, but do not incorporate these presumed peptidoglycan precursors into cell wall material (6). More complete studies by Panos and co-workers indicate that a broth-grown, stable L-phase variant of *Streptococcus pyogenes* with functional alanine racemase and D-alanine:D-alanine ligase (25) accumulates peptidoglycan precursors (4) due to a defect in lipid metabolism affecting the membrane carrier (28). In addition, Ward has recently reported enzymatic lesions in stable L-phase variants of *B. licheniformis* and *B. subtilis* (34). Defects in the synthesis of diaminopimelic acid, addition of L-alanine to UDP-MurNAc, or uptake of completed cytoplasmic precursor by L-phase membranes were correlated with the accumulation of appropriate peptidoglycan precursors. The two translocase defects in *B. licheniformis* variants (34) and the defect in synthesis of lipid intermediate by an *S. pyogenes* variant (28) affect peptidoglycan biosynthesis at the same reaction as does the lesion we have described for an L-phase variant of *S. faecium*. In other experiments, L-phase variants derived from other strains of *S. aureus* (1) and *B. subtilis* (8) did not appear to accumulate cell wall precursors intracellularly. Differences between membrane proteins of this latter L-phase variant and the bacillus have been reported (8), but have not been related to a specific defect in cell wall biosynthesis. These observations suggest that lesions in cell wall biosynthesis, perhaps genetic in nature, can occur at different stages in the biosynthetic sequence.

The results reported in this paper indicate that peptidoglycan precursors are accumulated intracellularly by a stable L-phase variant of *S.*

faecium F24 rather than extruded into the growth medium or not synthesized at all. The presumptive lesion is in the membrane stage of peptidoglycan biosynthesis at the reaction catalyzed by phospho-MurNAc-pentapeptide translocase. This conclusion is based on the identity of the predominant precursor accumulated, the lack of demonstrable translocase activity in L-phase membrane preparations, and the absence of muramic acid-containing compounds in culture fluids. The significance of small amounts of incomplete precursor is unknown, but it may reflect the kinetics of the UDP-MurNAc:L-alanine ligase reaction, which is the rate-limiting reaction in the cytoplasmic sequence of peptidoglycan synthesis (16). Alternatives to a defect in the translocase itself include a defective membrane carrier, perhaps brought about by structural rearrangements within the L-phase membrane. Such membrane rearrangements could inhibit carrier function or prevent regeneration (dephosphorylation) of undecaprenyl-pyrophosphate, but this is unlikely (1, 34). However, efficient regeneration of membrane carrier would not have been accomplished in the preincubation reaction containing excess UMP if peptidoglycan synthetase (transglycosylase) were defective (22). A lesion in peptidoglycan synthetase could lead to undecaprenyl diphosphate-disaccharide accumulation in the membrane, thus limiting available undecaprenyl phosphate and resulting in the observed lack of translocase activity in the variant despite preincubation with UMP. It is not known whether this variant responds to inhibition of cell wall biosynthesis by secreting lipids into the medium, which occurs in several streptococcal species when they are treated with inhibitors of peptidoglycan biosynthesis (14). Membrane carrier is available, however, in protoplasts of *S. faecium* F24.

Environmental factors may have selectively favored a change to the stable L-phase phenotype in this particular variant during passage in various environments. For example, the growth medium (1.5% ammonium chloride) may act indirectly to inhibit the transfer of D-aspartic acid to the ϵ-amino group of L-lysine on the disaccharide(-pentapeptide)-diphospho-undecaprenyl carrier intermediate in the membrane, because the D-aspartic acid-activating enzyme can be released from the membrane by high salt concentrations (32). Without the cross-linking amino acid component attached, available carrier might become quickly saturated with incomplete disaccharide-pentapeptide subunits. This, again, could limit the availability of uncharged carrier for the translocase assay despite preincubation of membrane preparations in the UMP exchange reaction (13, 22).

Lesions affecting wall biosynthesis in L-phase variants have been characterized as defects in peptidoglycan biosynthesis and not in the synthesis of accessory wall polymers (26, 34); thus, the production of peptidoglycan in vivo may dominate the biosynthetic interface of peptidoglycan and accessory polymer incorporation into cell walls. Extracts of the L-phase variant analyzed here retain the ability to synthesize dTDP-L-rhamnose, presumed cytoplasmic precursor to a non-peptidoglycan cell surface polymer, but rhamnose-containing compounds are not produced in stoichiometric amounts by growing cultures of the variant. The lack of accumulation of rhamnosyl nucleotide in either the L-phase variant or cycloserine-treated streptococci may reflect the efficient regulation of dTDP-L-rhamnose production in vivo. A previous report has shown that dTDP-D-glucose pyrophosphorylase is subject to feedback inhibition by dTDP-L-rhamnose (23). Because rhamnose can be incorporated in vitro from precursor into a high-molecular-weight membrane-associated component in the absence of a preformed cell wall, the membrane lesion affecting peptidoglycan synthesis does not prohibit membrane uptake of rhamnose. However, only low levels of rhamnose were detected in whole L-phase organisms. These observations may indicate the existence of a mechanism(s) for the regulation of the synthesis of precursors to secondary cell wall polymers that is independent of the synthesis of peptidoglycan precursors. Perhaps dTDP-L-rhamnose is another cell wall precursor which shares the undecaprenyl-phosphate carrier (35). The data presented here imply that the lipid carrier, if involved in rhamnose assimilation, was intact and operational in this stable L-phase variant.

The synthesis of peptidoglycan is a strongly conserved trait, because several wall-defective variants continue to produce precursors even on extended subculture in osmotically balanced media when intact peptidoglycan apparently ceases to be a survival prerequisite. L-phase variants with defects at the postmembrane stage of peptidoglycan biosynthesis have not been described; perhaps reactions which occur outside the osmotic barrier of cell wall-damaged cells are at a selective disadvantage in competitive microbial populations (34) or are masked by the preeminence of autolysins (2). We do not understand the selective pressures which act on these organisms, but the outgrowth of stable L-phase variants in established cultures may be the expression of genotypic changes. For example, Leder-

berg and St. Clair have described the induction of L-phase growth from a diaminopimelic acid auxotroph of *Escherichia coli* on a diaminopimelic acid-free medium (19). These variants regained parental morphology when the amino acid was again supplied in the growth medium. In addition, Wyrick et al. reported that the stable L-phase phenotype could be transferred from lysozyme-derived variants of *B. subtilis* to intact bacilli by genetic transformation (36). The L-phase transformants (about 10% of the recombinants) retained nutritional markers and did not revert to bacilli on hard agar or on medium containing 25% gelatin.

We now have a basis for understanding the molecular nature of continued cell wall inhibition in stable L-phase variants: microorganisms can permanently lose the ability to synthesize cell walls as the result of specific lesions which affect any of the several reactions unique to the cell wall biosynthetic sequence. Future genetic and biochemical analyses of stable L-phase variants should provide a direct approach to study the control of cell wall biosynthesis.

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