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Defect in Biosynthesis of the Linkage Unit Between Peptidoglycan and Teichoic Acid in a Bacteriophage-Resistant Mutant of *Staphylococcus aureus*

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The biosynthesis of the linkage region between peptidoglycan and the ribitol teichoic acid was investigated in the bacteriophage-resistant, teichoic acid-less mutant *Staphylococcus aureus* 52A5 (Chatterjee et al., J. Bacteriol. **100**:846-853, 1969). Membrane preparations of this strain were found to be incapable of forming the first intermediate of the biosynthetic pathway, namely, the transfer of *N*-acetyl-D-glucosamine (GlcNAc) from UDP-GlcNAc to the acceptor molecule, which presumably is undecaprenol phosphate (R. Bracha and L. Glaser, Biochem. Biophys. Res. Commun. **72**:1091-1098, 1976). The addition of heat-inactivated membrane preparations of *S. aureus* 52A2 (which normally has ribitol teichoic acid) that had been preincubated with UDP-GlcNAc to membranes of strain 52A5 enabled the synthesis of teichoic acid. These data suggest that the mutational defect in the teichoic acid-less organism is in the synthesis of the first compound of the linkage unit, and this is apparently the reason for its absence in the cell walls.

For a long time it was believed that ribitol-containing teichoic acids of gram-positive organisms were covalently linked to the cell wall peptidoglycan by a phosphate-diester bond that bound the first ribitol moiety to *N*-acetylmuramic acid (MurNAc) (1, 9). During the last few years, in vitro studies of the biosynthesis of ribitol teichoic acid in *Staphylococcus aureus* and *Bacillus subtilis* have conclusively shown that a linkage region exists between the peptidoglycan and the ribitol phosphate polymer. This region consists of an oligomer containing glycerol phosphate and *N*-acetyl-D-glucosamine (GlcNAc) (3, 4, 7, 8, 11). Recent evidence obtained by Wyke and Ward (18, 19) suggests that the linkage between teichoic acid and peptidoglycan is via a phosphodiester linkage between GlcNAc and MurNAc and that the phosphate is derived from the UDP-GlcNAc moiety. The biosynthetic pathway of this linkage unit was investigated and found to be composed of three reactions (3, 4; R. Bracha, M. Chang, F. Fiedler, and L. Glaser, Methods Enzymol., in press), which are given below. Acc is an acceptor molecule which is presumably undecaprenol phosphate (2; Bracha et al., in press).

$$\text{Acc} + \text{UDP-GlcNAc} \rightarrow \text{Acc-P-(GlcNAc)}_{1\text{-}2} \text{ (compound I)} + \text{UMP} + \text{UDP} \quad \text{(I)}$$

$$\text{Acc-P-(GlcNAc)}_{1\text{-}2} + 3\ \text{CDP-glycerol} \rightarrow \text{Acc-P-(GlcNAc)}_{1\text{-}2}\text{-(glycerol-P)}_3 \text{ (compound II)} + 3\ \text{CMP} \quad \text{(II)}$$

$$\text{Acc-P-(GlcNAc)}_{1\text{-}2}\text{-(glycerol-P)}_3 + 30\ \text{CDP-ribitol} \rightarrow \text{Acc-P-(GlcNAc)}_{1\text{-}2}\text{-(glycerol-P)}_3\text{-(ribitol-P)}_{30} \text{ (compound III)} + 30\ \text{CMP} \quad \text{(III)}$$

Reaction I has been found to be sensitive to tunicamycin, and in its presence the synthesis of ribitol teichoic acid is inhibited (3, 10).

In a previous investigation with the teichoic acid-less mutant *S. aureus* 52A5 (6, 17), we suggested that the mutational defect was not due to the absence of polyribitol phosphate polymerase activity but to the inability of the organism to bind the teichoic acid to its peptidoglycan acceptor. We report here evidence that *S. aureus* 52A5 is incapable of forming compound I of the linkage region, namely, the GlcNAc-containing acceptor molecule.

METHODS AND MATERIALS

Cells of the bacteriophage-resistant mutants *S. aureus* 52A2, which contains ribitol teichoic acid but lacks the GlcNAc residues on the ribitol moieties (5), and 52A5, which lacks teichoic acid, were used in these studies (6). Cells were grown in Difco antibiotic medium 3 to an early logarithmic phase (2×10^8 cells per ml) and harvested by sedimentation in a Sharples centrifuge. Cells were stored at −20°C.

Particulate membranes were prepared as described by Bracha and Glaser (3). Assays for the different stages of teichoic acid biosynthesis using the particulate membranes were as follows. The reaction mixture in a final volume of 50 μl contained 50 mM

tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.8), 20 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10 mM spermidine, 45 mM NH_4Cl, 2.8 mM ATP, and membranes (approximately 400 μg of protein). Labeled and unlabeled substrates were added to reaction mixtures as indicated for each experiment.

In all experiments, Triton X-100 (final concentration, 0.05%) was added because this was found to stimulate the incorporation of [^{3}H]glycerol, presumably by facilitating the penetration of substrate into the membrane particles.

Incubations were done at 25°C for 60 min. The reaction was stopped by the addition of 500 μl of a solution of ethanol–1 M ammonium acetate, pH 7.0 (3:7.5, vol/vol), and the suspension was filtered through a membrane filter (Millipore Corp., 0.45-μm) and washed three times with the same ethanol-ammonium acetate solution (3 ml each time). Filters were dried at 80°C for 1 h and counted with a toluene-based scintillator in a Packard 3003 Tri-Carb liquid scintillation spectrometer.

Crude cell walls were prepared as described by Mirelman and Sharon (14). Assays for teichoic acid and peptidoglycan biosynthesis using crude cell wall preparations were done essentially as described above. The reaction mixture contained, in a final volume of 200 μl, 50 mM Tris-hydrochloride, pH 7.8, 20 mM $MgCl_2$, 1 mM mercaptoethanol, 10 mM spermidine, 45 mM NH_4Cl, 2.8 mM ATP, 4 mg (dry weight) of crude cell walls, and labeled and unlabeled substrates as indicated for each experiment. Incubations were done at 25°C for 30 min. After the reaction, the crude walls were purified from membrane components by heating in 1% sodium dodecyl sulfate (SDS; 1 ml, 20 min, 100°C), sedimentation in an Eppendorf centrifuge (10,000 × *g*, 5 min), and washing with water (four times with 1 ml each time). The purified walls were suspended in a Triton X-100-based scintillator and counted for radioactivity. All biosynthesis experiments were repeated at least four times. The results given are of one typical experiment in which all the reactions were carried out with the same membrane preparations.

Preparations of the [^{3}H]glycerol-labeled and unlabeled compound II (see above) and its extraction by 80% ethanol were done as described by Bracha and Glaser (3) and Bracha et al. (in press).

The preparation of crude compound I was done with membranes of strain 52A2 (5) that were preincubated (in an experiment scaled up 50 times) under the conditions described above (final volume, 2.5 ml). The particulate membrane preparations of 52A2 were incubated with 2.8 mM ATP and 4 mM UDP-GlcNAc. Control experiments were incubated only with ATP. After incubation, the membranes were washed free of unreacted substrate by sedimentation (140,000 × *g*, 60 min), resuspended, and washed again by centrifugation with a buffer containing 50 mM Tris-hydrochloride, pH 7.8, 20 mM $MgCl_2$, and 1 mM 2-mercaptoethanol (TMM buffer). After the second sedimentation the membranes were resuspended in TMM buffer (0.5 ml, 20 mg of protein) and heated in a bath (60°C for 10 min) to inactivate the enzymes. Membrane preparations that had been heat treated in this manner were found to be inactive in teichoic acid biosynthesis.

UDP-GlcNAc and CDP-glycerol were obtained from Sigma Chemical Co., St. Louis, Mo. [2-^{3}H]glycerol, [*U*-^{14}C]glucose, and NaB^3H_4 were obtained from New England Nuclear Corp., Boston, Mass. CDP-[^{3}H]glycerol (400 cpm/pmol) was prepared from [2-^{3}H]glycerol as previously described (16). [^{3}H]ribitol-5-phosphate was prepared from ribose-5-phosphate after reduction with NaB^3H_4. CDP-[^{3}H]ribitol (30 cpm/pmol) was synthesized from [^{3}H]ribitol-5-phosphate and cytidine monophosphoromorpholidate as described previously (16). UDP-MurNAc-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala (UDP-MurNAc-pentapeptide; 24 cpm/pmol) was prepared from *Micrococcus luteus* by accumulation in the presence of [*U*-^{14}C]glucose and vancomycin (50 μg/ml) as described previously (13).

SDS-acrylamide gel electrophoresis (20% cross-linkage) was done by the method of Laemmli (12). The radioactively labeled gels were cut into 3.5-mm-thick slices and counted after oxidation in a Packard oxidizer. Counting efficiency was 42% for ^{3}H and 70% for ^{14}C.

RESULTS

Incorporation of [^{3}H]glycerol. A comparison between the particulate membrane fractions of the teichoic acid-containing *S. aureus* 52A2 with those of the teichoic acid-less strain 52A5 in their ability to synthesize the second step in the teichoic acid biosynthesis, i.e., the synthesis of compound II of the linkage region, is shown in Table 1. In contrast to membranes of 52A2, those of strain 52A5 were incapable of

TABLE 1. *Incorporation of glycerol from CDP-[^{3}H]glycerol into compound I[a] by membrane preparations of S. aureus strains 52A2 and 52A5*

Unlabeled substrate added (200 nmol)	Addition of membranes of strain 52A2[b]	[^{3}H]glycerol incorporated (cpm/mg of protein) into:	
		Strain 52A2	Strain 52A5
None	None	665	1,137
UDP-GlcNAc	None	45,334	5,406
None	Preincubated with ATP	5,337	2,296
None	Preincubated with ATP and UDP-GlcNAc	34,544	56,175
UDP-GlcNAc	Preincubated with ATP	46,886	4,679
UDP-GlcNAc	Preincubated with ATP and UDP-GlcNAc	101,939	44,344

[a] Compound I, as recently defined by Bracha et al. (in press), is acceptor-P-$(GlcNAc)_{1-2}$, which is the first unit of the linkage area of teichoic acid to peptidoglycan (3).

[b] Membrane preparations of strain 52A2 were preincubated under standard conditions (see text) in TMM buffer, and substrates were added as indicated. After incubation, the membranes were washed free from the unreacted substrate, heat inactivated (60°C, 10 min), and added (400 μg of protein) to the new incubation mixtures in a final concentration of 0.05% Triton X-100, as described in the text.

incorporating [^{3}H]glycerol from CDP-[^{3}H]glycerol into compound II (Fig. 1). Incorporation of [^{3}H]glycerol into compound II was observed only when heat-inactivated membranes of strain 52A2, which had been preincubated with UDP-GlcNAc and ATP, were added to the reaction mixture (Table 1). These membrane preparations presumably contain the initial GlcNAc residue linked to the undecaprenol phosphate moiety (compound I). The addition of this preparation to active membrane preparations of *S. aureus* 52A2 enhanced the incorporation of [^{3}H]glycerol from CDP-[^{3}H]glycerol even in the absence of UDP-GlcNAc (R. Bracha and L. Glaser, unpublished data). In the presence of UDP-GlcNAc and preincubated membranes, there was a higher incorporation of [^{3}H]glycerol due to the endogenous synthesis of compound I by the strain (Table 1). Control experiments in which membranes of 52A2 were preincubated with ATP but without UDP-GlcNAc and added to preparations of 52A5 as above failed to stimulate the incorporation of [^{3}H]glycerol (Table 1). On the other hand, in a parallel experiment the addition of membranes of strain 52A5 to those of 52A2 did not cause any inhibition in the incorporation of [^{3}H]glycerol from CDP-[^{3}H]-glycerol by membrane preparations of the latter strain (data not shown).

The incorporation of [^{3}H]glycerol by membranes of 52A5, which were incubated together with the heat-inactivated membranes of 52A2 that had been preincubated with UDP-GlcNAc and ATP, afforded upon gel electrophoresis a labeled band that migrated similarly to that obtained by incubation of membranes of strain 52A2 with UDP-GlcNAc and CDP-[^{3}H]glycerol (Fig. 1, compound II). No incorporation of [^{3}H]glycerol into compound II by membranes of 52A5 was observed in the absence of added compound I from the 52A2 membranes.

Incorporation of [^{3}H]ribitol. Incubation of particulate membranes of strains 52A2 and 52A5 with CDP-[^{3}H]ribitol afforded, as previously observed (17), the incorporation of [^{3}H]ribitol into a macromolecular acceptor (Table 2). A strong stimulation of incorporation of [^{3}H]ribitol was observed when membranes of 52A2 were incubated in the presence of UDP-GlcNAc and CDP-glycerol, whereas with strain 52A5 some inhibitory effect was observed. An increase in the incorporation of [^{3}H]ribitol in the absence of CDP-glycerol and UDP-GlcNAc was observed with both 52A2 and 52A5 when 80% alcohol extracts of membrane preparations of strain 52A2 (that had been preincubated with UDP-GlcNAc and CDP-glycerol) were added to the incubation mixtures (Table 2).

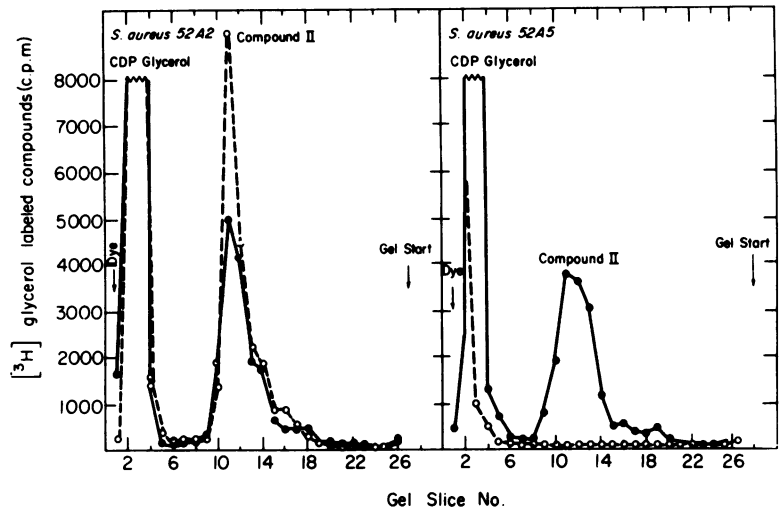

FIG. 1. *SDS-acrylamide gel electrophoresis of [^{3}H]glycerol-labeled intermediates. Standard assay conditions were used as described in the text. At the end of 60 min of incubation, the reaction was stopped by boiling in SDS, and the entire sample was subjected to acrylamide gel electrophoresis (20% cross-linked) (12). The gels were cut into 3.5-mm slices and oxidized with a Packard oxidizer, and the tritiated water was counted. Slice no. 1 is the tracking dye front. Symbols: ○, reaction mixtures with both S. aureus 52A2 and 52A5 containing UDP-GlcNAc (200 nmol) and CDP-[^{3}H]glycerol (2 × 10^5 cpm); ●, reaction mixtures containing CDP-[^{3}H]glycerol as well as heat-inactivated membranes of strain 52A2 that had been preincubated with ATP and UDP-GlcNAc (200 nmol), as described in the text. The large number of counts at the front is unreacted CDP-[^{3}H]glycerol. Compound II, as recently defined by Bracha et al. (in press), is acceptor-P-(GlcNAc)$_{1-2}$-(glycerol-P)$_3$.*

TABLE 2. *Incorporation of ribitol from CDP-[^{3}H]ribitol into compound II[a] by membrane preparations of S. aureus strains 52A2 and 52A5*

Unlabeled compounds added[b]	Incorporation (cpm/mg of protein) of [^{3}H]ribitol into[c]:	
	Strain 52A2	Strain 52A5
None	39,070	22,620
UDP-GlcNAc + CDP-glycerol	162,376	14,230
UDP-GlcNAc + CDP-glycerol + ethanol	183,616	6,948
Preincubated 52A2 membrane extracts	88,716	57,708
Preincubated 52A2 membrane extracts + CDP-glycerol + UDP-GlcNAc[c]	249,348	40,475

[a] Compound II, as recently defined by Bracha et al. (in press), is acceptor-P-(GlcNAc)$_{1-2}$-(glycerol-P)$_3$, which is the linkage unit between the teichoic acid and the peptidoglycan.

[b] CDP-glycerol, 10 nmol; 80% ethanol, 7.5 μl. Membrane preparations of strain 52A2, which were preincubated with ATP, UDP-GlcNAc, and CDP-glycerol for 60 min, were extracted with 80% ethanol (for details, see the text), and portions of this extract (7.5 μl) were added to the incubation mixtures.

[c] CDP-[^{3}H]ribitol (200,000 cpm, 30 cpm/pmol) was the labeled substrate added. Reaction mixtures contained approximately 400 μg of protein.

The transfer and polymerization of ribitol phosphate from CDP-ribitol to compound II (the linkage region) convert it into compound III, which can be easily separated from compound II by gel electrophoresis (Fig. 2). The addition of CDP-ribitol to membrane preparations of either strain 52A2 or strain 52A5 to which [^{3}H]glycerol-labeled alcohol extracts of compound II had been added converted approximately 50% of the labeled compound into compound III (Fig. 2). In contrast to 52A2, however, membrane preparations of 52A5 were capable of converting nearly 25% of the added [^{3}H]glycerol-labeled compound II into compound III even in the absence of any added CDP-ribitol. This was probably due to the fact that the teichoic acid-less 52A5 mutant cells accumulate considerable amounts of CDP-ribitol during their normal growth (6), and membrane preparations of these cells probably contained endogenous CDP-ribitol that, upon the addition of the [^{3}H]glycerol-labeled precursor (compound II), converted it into compound III.

The poly-[^{3}H]ribitol compound obtained from both 52A2 and 52A5 membranes that were incubated with CDP-[^{3}H]ribitol in the absence of added CDP-glycerol and UDP-GlcNAc or of any alcohol extracts (compound II) migrated on the gel electrophoresis with the same mobility as compound III.

Attachment of teichoic acid to the preexisting cell wall. As shown in Table 3, the incorporation and covalent attachment of ribitol to the preexisting wall of strain 52A2 has been done with a crude cell wall preparation. This incorporation was dependent upon the addition of UDP-GlcNAc and CDP-glycerol (3, 4). The incorporation and covalent attachment of glycerol from CDP-glycerol to the preexisting cell wall were dependent upon the addition of UDP-GlcNAc as previously shown (3, 4). No covalent attachment of either ribitol or glycerol to the preexisting wall was observed with crude cell wall preparations of strain 52A5, although these wall preparations were as active as those of strain 52A2 in binding newly synthesized peptidoglycan to the preexisting wall (Table 3; 14). Additions of alcohol extracts of 52A2 membranes that were preincubated with UDP-GlcNAc and with or without CDP-glycerol to crude cell wall preparations of strain 52A5 did not enhance the binding of either glycerol (from CDP-glycerol) or ribitol (from CDP-ribitol) to the preexisting wall.

DISCUSSION

In a study carried out several years ago to determine the mutational defect of the teichoic acid-less mutant *S. aureus* 52A5, it was postulated that the defect may be in the process of attachment of the teichoic acid to its peptidoglycan acceptor (15, 17). The results obtained in the present investigation clearly show that the first step (reaction I) in the synthesis of the linkage unit to peptidoglycan is defective in this mutant, and its inability to form compound I is apparently the reason for the absence of teichoic acid in this organism.

The enzyme that catalyzes the transfer of GlcNAc to the initial acceptor has not yet been isolated, and compound I from *S. aureus* has not been purified to homogeneity. An undecaprenyl [*N*-acetylglucosaminyl$_{(1-6)}$-pyrophosphate] from *B. subtilis*, which perhaps is compound I, has been isolated and characterized (2).

It has been shown previously that membrane preparations of strains 52A5 and 52A2 were capable of polymerizing CDP-ribitol even in the absence of any added UDP-GlcNAc and CDP-glycerol (17). Recently, Bracha et al. (in press) have shown that in vitro incorporations of [^{3}H]ribitol from CDP-[^{3}H]ribitol into a high-molecular-weight acceptor can occur via two parallel pathways, one which is most likely the lipoteichoic acid and the other which is compound II, the "linkage region" acceptor. The incorporation of [^{3}H]ribitol into the first type of acceptor is independent of any addition of CDP-glycerol or UDP-GlcNAc and apparently uses

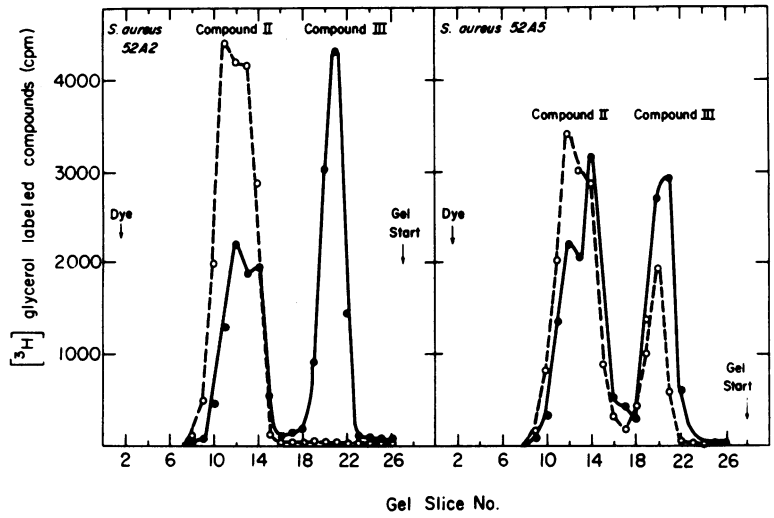

FIG. 2. *SDS-acrylamide gel electrophoresis of [^{3}H]glycerol-labeled compounds. Gels were run and analyzed as described in the legend to Fig. 1. Standard assay conditions were used as described in the text. Symbols: ○, reaction mixtures with both S. aureus 52A2 and 52A5 containing [^{3}H]glycerol-labeled extracts of compound II (200,000 cpm in 7.5 μl of 80% ethanol; compound II was extracted from membranes of strain 52A2 that had been preincubated with UDP-GlcNAc and CDP-[^{3}H]glycerol as described in the text); ●, reaction mixtures containing both [^{3}H]glycerol-labeled compound II and CDP-ribitol (100 nmol).*

TABLE 3. *Incorporation and covalent attachment of teichoic acid and peptidoglycan components to preexisting cell walls*[a]

Labeled substrate added[b]	Unlabeled substrate added[c]	Incorporation (cpm/4 mg of crude cell walls) into cell walls of:	
		S. aureus 52A2	*S. aureus* 52A5
CDP-[^{3}H]ribitol	None	483	10
CDP-[^{3}H]ribitol	UDP-GlcNAc and CDP-glycerol	2,995	41
CDP-[^{3}H]glycerol	None	937	121
CDP-[^{3}H]glycerol	UDP-GlcNAc	2,091	94
UDP-[^{14}C]MurNAc-pentapeptide	None	73	68
UDP-[^{14}C]MurNAc-pentapeptide	UDP-GlcNAc	7,104	4,851

[a] Crude cell wall preparations of *S. aureus* 52A2 and 52A5 (14) were used throughout these experiments. Reactions were carried out under conditions as described in the text. After incubation, the cell walls were isolated and purified, and their radioactivity was determined as described in the text. Results are given in counts per minute per 4 mg of crude cell walls.

[b] CDP-[^{3}H]ribitol (200,000 cpm, 30 cpm/pmol); CDP-[^{3}H]glycerol (200,000 cpm, 400 cpm/pmol); UDP-[^{14}C]-MurNAc-pentapeptide (100,000 cpm, 24 cpm/pmol).

[c] UDP-GlcNAc (1 μmol) and CDP-glycerol (10 nmol) were added to reaction mixtures where specified.

lipoteichoic acid as an analog for compound II, whereas the second type of incorporation is dependent upon the addition of these precursors. Because strain 52A5 is incapable of producing any ribitol phosphate polymer in vivo, the observed in vitro polymerization of ribitol phosphate from CDP-ribitol apparently occurs on a lipoteichoic acid acceptor, and this could be an experimental artifact. Not entirely discounted, however, is the possible pathway by which polyribitol phosphate is transferred from lipoteichoic acid to compound II (10). Because the teichoic acid-less mutant apparently fails to synthesize the linkage region, both the transfer of preassembled polyribitol phosphate units and the polymerization reaction from CDP-ribitol would be prevented in vivo.

Particulate membrane preparations of strain 52A5, when supplemented with either crude compound I or compound II (which were prepared by preincubating membranes of 52A2 with UDP-GlcNAc or with UDP-GlcNAc and CDP-glycerol [3; Bracha et al., in press]), behaved similarly to membranes of strain 52A2 in the synthesis of ribitol teichoic acid (Fig. 2 and Table 2). On the other hand, crude cell wall preparations of strain 52A5, even when supplemented with exogenously prepared compound I or II, were incapable of binding the newly synthesized teichoic acid to the cell wall peptidoglycan (Table 3). The apparent inability of the crude cell wall system to bind the newly synthesized teichoic acid to peptidoglycan is not yet understood. One of the reasons that we have not yet discounted may be that under the various experimental conditions used there is still a lack of penetration of the exogenously added precur-

sors (compound I or II) to the site within the crude cell walls where the attachment of teichoic acid to peptidoglycan occurs. This could prevent the final coupling of macromolecular precursors and the covalent attachment of teichoic acid in our in vitro system.

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