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Synthesis of Cell Envelope Components by Anucleate Cells (Minicells) of *Bacillus subtilis*

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Minicells produced by *Bacillus subtilis* CU403 (*divIVB1*) are capable of mucopeptide biosynthesis as shown by the incorporation of L-alanine, D-alanine, and *N*-acetylglucosamine into trichloroacetic acid-precipitable material, which can be degraded to trichloroacetic acid-soluble material by lysozyme digestion. Incorporation of the precursors is sensitive to vancomycin and D-cycloserine and insensitive to chloramphenicol. Penicillin inhibits the incorporation of D- and L-alanine *N*-acetylglucosamine at concentrations in excess of 10 μg of penicillin per ml; however, minicells are insensitive to penicillin-induced lysis. The material synthesized in minicells from *N*-acetylglucosamine is not subject to turnover during a subsequent 6-h incubation period. [2-^{3}H]glycerol is converted to a cold trichloroacetic acid-precipitable form by minicells. This synthesis is not inhibited by vancomycin, penicillin, D-cycloserine, or chloramphenicol. Fractionation of the material synthesized from glycerol into hot trichloroacetic acid-soluble material and chloroform/methanol-extractable material indicates that minicells convert glycerol into teichoic acid and lipid.

Minicells are small anucleate cells produced by misplaced cell division in rod-shaped bacteria (1, 31, 35, 43). A very important and useful property of minicells is that if they are supplied with a deoxyribonucleic acid template, then they are capable of transcribing and translating this template into ribonucleic acid and protein products. (See reference 9 for an extensive review of minicell uses.) This property has been extensively used in *Escherichia coli* to characterize plasmid-coded products, since small plasmid molecules segregate into minicells and, after separation of the plasmid-containing minicells from the nucleated cells, it is possible to analyze the plasmid-directed synthesis occurring in the minicells in the absence of chromosomally directed syntheses (8, 9, 13, 17-19, 21, 34, 42). An alternative method of introducing a deoxyribonucleic acid molecule into minicells has been to infect minicells of *E. coli* (34, 45) or *Bacillus subtilis* (29) with bacteriophage. The introduction of a phage genome results in the syntheses of ribonucleic acid and protein and permits an analysis of phage-coded products in the absence of host cell products. It has been, however, consistently reported that anucleate minicells in the absence of a plasmid or an infecting phage genome have the capacity to produce small amounts of trichloroacetic acid-precipitable material from amino acid precursors (9, 17, 19, 20, 30, 34). This anomaly was apparently explained for *E. coli* minicells by the observation that stable messenger ribonucleic acid is present in minicells and is translated into protein found in the outer cell membrane (20). We have analyzed minicells produced by *B. subtilis* in an attempt to determine what synthetic abilities these deoxyribonucleic acid-less cells have in view of the fact that gram-positive organisms do not have an outer cell membrane (32). We report here that anucleate minicells actively synthesize teichoic acid(s), mucopeptide, and lipid and that the anomalous incorporation of amino acids found in anucleate *B. subtilis* minicells can be almost totally accounted for by these syntheses.

(Part of this work was taken from the doctoral thesis of G. M., which is to be submitted to the Freie Universität, Berlin.)

MATERIALS AND METHODS

Bacteria and bacteriophage. *B. subtilis* CU403 (*divIVB1 thyA thyB metB*) (31) was used throughout this study as a source of minicells. SP82G was obtained from D. M. Green. Phage stocks were produced as previously described for bacteriophage SPO1 (29).

Minicell production. *B. subtilis* CU403 (*divIVB1 thyA thyB metB*) was grown in minimal medium consisting of, per liter: KH_2PO_4, 6 g; K_2HPO_4, 14 g; $(NH_4)_2SO_4$, 2 g; sodium citrate, 1 g; glucose, 0.4 g; thymine, 20 mg; methionine, 20 mg; plus 10^{-3} M $MgSO_4$, 5×10^{-4} M $CaCl_2$, 10^{-4} M $MnSO_4$, and 5×10^{-6} M $FeCl_3$. Ten-liter cultures were grown at 30°C with aeration in a New Brunswick Microferm fer-

mentor (New Brunswick, N.J.) to an absorbancy at 600 nm (A_{600}) of approximately 0.6. The culture was rapidly cooled to 0°C, and cells were harvested by centrifugation at 18,000 rpm (36,000 × g) and 0°C in an SZ-14 flow-through rotor in a Sorvall RC2B centrifuge. The pellet was vigorously resuspended in 40 ml of ice-cold minimal medium lacking glucose, thymine, and methionine. The cell suspension was layered on top of 4× 200-ml, 5 to 30% sucrose gradients held in clear, polycarbonate centrifuge bottles. The bottles were centrifuged at 0°C at 4,000 rpm (3,000 × g) in an HS4 swinging-bucket rotor in the RC2B centrifuge. The supernatants were collected and recentrifuged at 13,000 rpm (27,000 × g) in a GSA rotor at 0°C for 10 min. The resulting pellets, containing mostly minicells, were resuspended in a small volume of minimal medium (ca. 20 ml) and layered on top of four 5 to 30% 30-ml sucrose gradients. The gradients were centrifuged at 4,000 rpm (2,500 × g) at 0°C in an HB4 swinging-bucket rotor in the RC2B centrifuge. A band of minicells resulted at approximately one-third of the distance from the top of the gradient. This band was removed from the gradient, and the minicells were pelleted by centrifugation at 18,000 × g for 10 min at 0°C. The minicells were resuspended in minimal medium lacking nutrients, and the 30-ml sucrose gradient centrifugation was repeated. The minicell band was again removed, and the minicells were pelleted, washed twice with ice-cold minimal medium without the nutrients, and resuspended at an A_{600} of 2.5 in minimal medium lacking nutrients but containing 10% glycerol. The minicell suspension was very rapidly frozen in 1-ml aliquots by immersion in liquid nitrogen. Frozen stocks of minicells were held at −70°C and were used within 1 month of preparation. Preliminary experiments have shown that minicells may be frozen and thawed without apparent loss of activity, as measured by protein synthesis after bacteriophage infection (Mertens, unpublished data). Frozen stocks of minicells were thawed and resuspended in 10 volumes of minimal medium, pelleted by centrifugation at 10,000 × g for 10 min, washed once with minimal medium, and resuspended at the required density for the subsequent experiment. A minicell suspension of A_{600} = 0.5 contained approximately 4 × 10^9 minicells per ml, as measured by either phase-contrast microscopy using a Petroff-Hausser counting chamber (C. A. Hausser, Philadelphia, Pa.) or electronically by using a Coulter counter combined with a Nuclear Data multichannel analyzer. The parental cells remaining as contaminants within a suspension of minicells were assayed as colony-forming units. Minicell suspensions were diluted in ice-cold minimal medium, and appropriate dilutions were plated on nutrient agar plates (Difco) containing 20 μg of thymine per ml, which were incubated overnight at 37°C. A minicell suspension of A_{600} = 0.5 contained 10^5 to 2 × 10^5 colony-forming units/ml before freezing at −70°C.

Phage infection. Minicells were infected at 0°C in minimal medium at an input multiplicity of infection of approximately 5. After 10 min at 0°C, the suspension was diluted to the appropriate A_{600}, the temperature was raised to 37°C, and appropriate radioactive precursors and/or inhibitors were added.

Labeling procedures. Minicells, suspended in minimal medium at 37°C, were added to various radioactive precursors as designated below. The mixture of uniformly labeled ^{14}C-amino acids used consisted, per millicurie of mixture, of: L-alanine, 80 μCi (129 mCi/mmol); L-aspartic acid, 70 μCi (172 mCi/mmol); L-glutamic acid, 125 μCi (215 mCi/mmol); glycine, 40 μCi (93 mCi/mmol); L-histidine, 15 μCi (258 mCi/mmol); L-isoleucine, 50 μCi (258 mCi/mmol); L-leucine, 140 μCi (280 mCi/mmol); lysine, 60 μCi (258 mCi/mmol); L-phenylalanine, 80 μCi (414 mCi/mmol); L-proline, 50 μCi (210 mCi/mmol); L-serine, 40 μCi (129 mCi/mmol); L-threonine, 50 μCi (172 mCi/mmol); L-tyrosine, 40 μCi (400 mCi/mmol); L-valine, 80 μCi (215 mCi/mmol). The mixture contained 1 mCi/ml.

Fractionation procedures. Samples (volumes as in figure legends and tables) were removed from the minicell suspension and added to 20 volumes of ice-cold 5% trichloroacetic acid. "Cold" trichloroacetic acid-precipitable material within the suspension was measured within 1 h of addition of trichloroacetic acid by filtration using 0.45-μm Sartorius filters (Membranfilter GmbH, Göttingen, Germany). The material retained on the filter was washed three times with 5 ml of cold 5% trichloroacetic acid and twice with 5 ml of cold water. The filters were dried, and the radioactivity bound to each filter was counted in a liquid scintillation counting system.

"Hot" trichloroacetic acid-precipitable material was measured as for cold trichloroacetic acid-precipitable material; however, the samples in 5% trichloroacetic acid were heated to 90°C for 30 min before filtration.

Lysozyme digestion of material was done at 37°C after samples had been precipitated in cold 5% trichloroacetic acid, pelleted at 10,000 rpm for 10 min in an SS34 rotor at 0°C in a Sorvall RC2B centrifuge, and resuspended in 1/10 concentration of minimal medium. Lysozyme was added to a final concentration of 500 μg/ml. After lysozyme digestion, 20 volumes of cold 5% trichloroacetic acid was added to the digest and the samples were placed at 90°C for 30 min. Hot trichloroacetic acid-precipitable material in each sample was measured by filtration as described above.

Samples for chloroform/methanol extraction (7, 25) were precipitated in 20 volumes of cold 5% trichloroacetic acid. The precipitate was collected by centrifugation at 10,000 × g for 10 min and resuspended in 0.5 ml of methanol at 65°C for 5 min. One milliliter of chloroform was added, and the suspension was held at 45°C for a further 20 min. The suspension was filtered through a GF/C glass-fiber filter (Whatman, England) and the filtrate was collected. The filtrates were placed in scintillation vials and evaporated to dryness. A dry Sartorius filter was added to each scintillation vial before the addition of scintillation fluid. Samples for fatty acid extraction (25) were pelleted by centrifugation at 10,000 × g for 10 min and resuspended in 0.5 ml of 50% saturated KOH solution. A 0.5-ml portion of absolute ethanol was added, and the samples were held at 90°C for 30 min in sealed tubes, followed by

10 min at 90°C with the tubes open. The samples were cooled, and 0.22 ml of concentrated HCl was added plus 0.5 ml of water and 1.5 ml of pentane. The samples were vigorously shaken for two 10-s periods, and the upper pentane phase was removed. A 1.25-ml amount of water was added to the pentane phase, and the mixture was again vigorously shaken for two additional 10-s periods. The upper pentane phase was removed and transferred to a scintillation vial, where it was evaporated to dryness. A dry Sartorius filter was added to each scintillation vial before the addition of scintillation fluid.

Source of materials. L-[U-^{14}C]alanine, D-[U-^{14}C]alanine, L-[U-^{14}C]proline, L-[4,5-^{3}H]leucine, *N*-acetyl-D-[1-^{14}C]glucosamine, [2-^{3}H]glycerol, and L-[U-^{14}C]leucine were obtained from the Radiochemical Centre, Amersham, England. L-[U-^{3}H]histidine and DL-[U-^{3}H]lysine were from Schwartz Bioresearch Inc., Orangeburg, N.Y. The uniformly labeled ^{14}C-amino acid mixture was obtained from New England Nuclear Corp., Boston, Mass. Lysozyme was from Worthington Biochemicals Corp., Freehold, N.J.; chloramphenicol (CAM) and D-cycloserine from Sigma Chemical Co., St. Louis, Mo.; vancomycin (VAN) from Eli Lilly GmbH, Giessen, Germany; and penicillin G (sodium salt) (PEN) (5 U/3 μg) from Hoechst AG, Frankfurt, Germany. All antibiotics were dissolved in minimal medium identical to that used to resuspend the minicells.

RESULTS

Incorporation of amino acids into mucopeptide by minicells. When minicells, produced by *B. subtilis* CU403 *divIVB1 thyA thyB metB*, are incubated in minimal medium plus a radioactive amino acid, they actively transport the amino acid, and in some cases a small percentage of the incorporated amino acid becomes trichloroacetic acid precipitable (30). L-Alanine was found to be converted to a trichloroacetic acid-precipitable form most readily, and this incorporation was only slightly inhibited by CAM (Fig. 1). However, the synthesis is strongly inhibited by D-cycloserine and VAN, both of which would be expected to inhibit the synthesis of mucopeptide (15, 26).

The possibility that uninfected minicells are capable of synthesizing cell envelope components led to a more detailed analysis of the fate of the incorporated amino acids. In Table 1 a comparison of the incorporation of L-alanine, D-alanine, and L-leucine into various minicell fractions is presented. L- and D-alanine are known to be incorporated into mucopeptide, and it is clear that their incorporation is significantly higher than the incorporation of L-leucine, which is not a component of mucopeptide. The difference between hot and cold trichloroacetic acid-precipitable material can be taken as a measure of the synthesis of nucleic acids and teichoic acids (33, 39). As minicells do not contain deoxyribonucleic acid (31), the synthesis of nucleic acids is very unlikely. Incorporation of D-alanine in teichoic acid is known to occur in *B. subtilis* (32), and the present results indicate that this also occurs in minicells of *B. subtilis*. However, minicells are, in general, very resistant to lysis, and it is probable that some free amino acids are not extracted from minicells by cold trichloroacetic acid extraction, making a quantitative assessment of teichoic acid biosynthesis very unreliable. The type(s) and amounts of teichoic acid synthesized by minicells are currently under further investigation. Exposure of hot trichloroacetic acid-precipitable material synthesized from L- and D-alanine to lysozyme digestion results in over 70% conversion to trichloroacetic acid-soluble material, whereas less than 20% of the small amount of material synthesized from L-leucine is digested by lysozyme.

All of the D-alanine and most of the L-alanine and L-leucine incorporated into hot trichloroa-

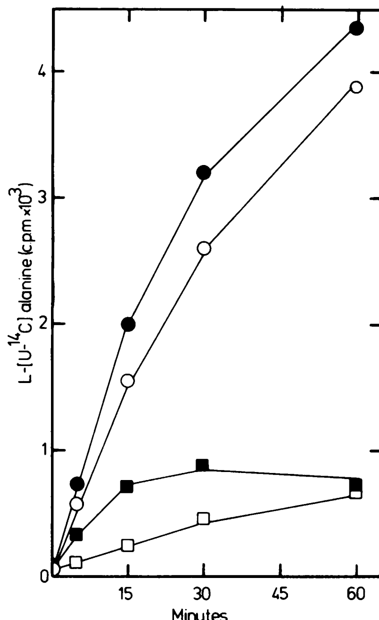

FIG. 1. *Incorporation of L-[U-^{14}C]alanine by minicells. Minicells (A_{600} = 0.5) were incubated at 37°C in minimal medium containing 20 μl of L-[U-^{14}C]alanine (164 mCi/mmol; 50 μCi/980 μl) per ml plus 4 μg of unlabeled L-alanine per ml. Samples (100 μl) were removed at intervals, and the radioactivity in the hot trichloroacetic acid-precipitable material in each sample was measured (●). At 0 min CAM (final concentration, 150 μg/ml) (○), D-cycloserine (final concentration, 10 μg/ml) (■), or VAN (final concentration, 50 μg/ml) (□) was added to portions of the minicell suspension.*

TABLE 1. *Incorporation of amino acids by minicells*

Label incorporated[a]	Cold trichloroacetic acid precipitate[b] (ng)	Hot trichloroacetic acid precipitate[b]			Chloroform/methanol extract[b]		Pentane[b] extract (ng)
		Direct (ng)	After lysozyme digestion[c] (ng)	% Digested by lysozyme	Amt extracted (ng)	% of lysozyme-insoluble material in extract	
L-[U-^{14}C]alanine	34.8	27.4	7.6	72	5.0	66	0.18
D-[U-^{14}C]alanine	24.8	18.3	5.1	72	5.3	104	0.12
L-[U-^{14}C]leucine	4.3	3.6	2.9	19	2.1	72	0.026

[a] Minicells (1 ml; A_{600} = 0.6) were incubated in minimal medium for 30 min at 37°C in the presence of either 1 μCi of L-[U-^{14}C]alanine (198 mCi/mmol) or 1 μCi of D-[U-^{14}C]alanine (37 mCi/mmol) or 1 μCi of L-[U-^{14}C]leucine (311 mCi/mmol). Synthesis of 1 ng of material from L-alanine, D-alanine, and L-leucine was equivalent to the incorporation of 413, 775, and 4,480 cpm, respectively.

[b] Fractionation of labeled minicells as in Materials and Methods.

[c] Digestion at 37°C for 30 min; 500 μg of lysozyme per ml was the final concentration.

cetic acid-precipitable material and not converted to trichloroacetic acid-soluble material by lysozyme digestion are found to be extracted by exposure to chloroform/methanol. This would indicate incorporation into lipid. It is known that the precursors of mucopeptide biosynthesis are bound to a lipid carrier during their assembly (2), and it appears likely that the material extracted by chloroform/methanol represents partially synthesized mucopeptide precursors.

It has been reported previously that valine and leucine can be converted to fatty acids by *B. subtilis* (16); however, we were unable to show an increased incorporation of L-leucine into fatty acids in comparison with L- and D-alanine (Table 1).

There remains a very small amount of incorporated L-alanine and L-leucine that cannot be accounted for by teichoic acid, mucopeptide, mucopeptide precursors, lipid, or fatty acid. It is most likely that this material is protein, although we have so far been unable to demonstrate protease sensitivity or to locate distinct bands of protein by polyacrylamide gel electrophoresis (results not shown).

Protein synthesis induced in minicells is sensitive to CAM. It has been shown previously that protein synthesis occurs after phage infection of *B. subtilis* minicells (29). As a control for the effect of CAM and VAN on uninfected minicells, we checked the effect of CAM and VAN on phage-infected minicells. It can be seen in Fig. 2 that after infection of minicells with SP82G phage, a dramatic increase in incorporation of amino acids occurs and that this increase is completely inhibited by CAM but is not affected by VAN, which inhibits infected and uninfected minicells to the same extent. In Table 2 a similar experiment is presented in which the stimulation of L-[^{14}C]alanine incor-

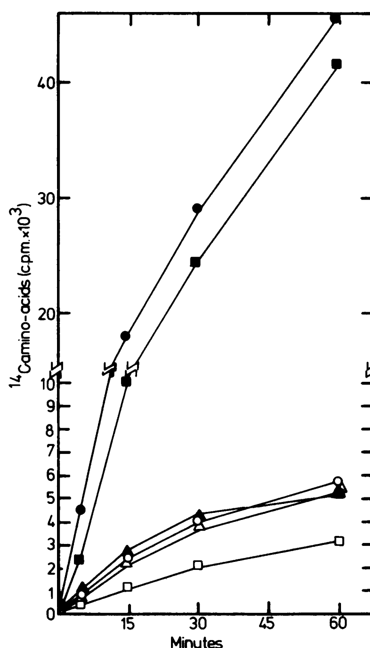

FIG. 2. *Incorporation of ^{14}C-labeled amino acids by SP82G-infected and uninfected minicells. Infected (●) or uninfected (○) minicells (A_{600} = 0.5) were incubated at 37°C in minimal medium containing 10 μCi of ^{14}C-labeled amino acid mixture per ml (see Materials and Methods). Samples (20 μl) were removed at intervals, and radioactivity contained in the hot trichloroacetic acid-precipitable material was measured. At 0 min the suspensions were placed at 37°C, and portions of the infected (solid symbols) and uninfected (open symbols) minicell suspensions were added to CAM (▲, △) (final concentration, 150 μg/ml) or VAN (■, □) (final concentration, 50 μg/ml).*

poration after SP82G infection of minicells is shown to be sensitive to CAM and dependent on the presence of methionine (the parental *B.*

subtilis strain is *metB*$^-$), whereas synthesis by uninfected minicells is insensitive to CAM and does not depend on the presence of methionine. The incorporation of L-alanine by infected minicells is 62% inhibited by VAN, whereas incorporation by uninfected minicells is 85% inhibited by VAN.

Effect of PEN on the incorporation of amino acids. The addition of PEN to *E. coli* minicell suspensions has been reported either to reduce incorporation of amino acids (34) or to have no effect on amino acid incorporation (19). We have therefore investigated the effect of PEN on the incorporation of amino acids by minicells of *B. subtilis* (Table 3). It was found that L- or D-alanine was incorporated into hot trichloroacetic acid-precipitable material to a much larger extent than the other amino acids tested (L-histidine, D,L-lysine, L-leucine, L-proline) and, indeed, as previously reported (29, 30) we were unable to detect incorporation of L-proline into trichloroacetic acid-precipitable material. At a concentration of 50 μg of PEN per ml the incorporation of L-alanine is inhibited by 84%, whereas the incorporation of D-alanine is inhibited by 76% (Table 3). The inhibition of L-alanine incorporation was consistently found to be greater than the inhibition of D-alanine incorporation over a range (10 to 100 μg/ml) of PEN concentrations. The amino acids L-leucine and D,L-lysine were incorporated into hot trichloroacetic acid-precipitable material in low, but detectable, amounts. This incorporation was only slightly (23%) inhibited by the addition of 50 μg of PEN per ml.

Although PEN does effect the biosynthesis of mucopeptide as demonstrated by the inhibition of D- and L-alanine incorporation, it does not cause lysis of the minicells (Fig. 3). PEN does, however, cause a reduction in the viable count of the few remaining contaminating parental cells in the minicell population, showing that they are sensitive to PEN (Fig. 3).

***N*-acetylglucosamine incorporation by minicells.** Incorporation of amino acids into lysozyme-soluble material indicated that the pentapeptide unit of the mucopeptide precursor molecule was synthesized in minicells. To conclude that the whole precursor unit was being synthesized, it was necessary to show incorporation of the sugar moiety, *N*-acetylglucosamine, into lysozyme-soluble material (5, 27, 32, 33). In Fig. 4 is shown the incorporation of *N*-acetyl-D-[1-^{14}C]glucosamine into hot trichloroacetic acid-precipitable material. This incorporation is resistant to CAM but sensitive to VAN. Figure 5 shows that the incorporation of *N*-acetylglucosamine is also sensitive to PEN and D-cycloserine. The inhibition of *N*-acetylglucosamine incorporation by penicillin shows a concentration dependence similar to that found for the inhibition of D- and L-alanine incorporation by PEN (unpublished data; Table 3). Inhibition of *N*-

TABLE 2. *Incorporation of L-alanine by infected and uninfected minicells*

Minicells[a]	Complete medium (cpm)	Medium lacking methionine (cpm)	CAM (cpm)	Complete medium plus[b]: VAN (cpm)	Complete medium plus[b]: CAM + VAN (cpm)
Uninfected	6,641	6,893	6,509	1,034	879
SP82 infected	12,818	8,107	5,708	4,837	1,140

[a] SP82-infected (multiplicity of infection ~ 5) or uninfected minicells (0.5 ml; A_{600} = 0.5) were incubated in minimal medium with or without methionine at 37°C for 60 min. Samples (100 μl) were placed in 2 ml of 5% trichloroacetic acid and held at 90°C for 30 min. The samples were filtered and washed, and the radioactivity contained in the hot trichloroacetic acid-precipitable material in each sample was counted as described in Materials and Methods.

[b] Final concentration of antibiotics: CAM, 150 μg/ml; VAN, 50 μg/ml.

TABLE 3. *Effect of PEN on the incorporation of amino acids by minicells*[a]

Amino acid	Concn (×10^{-5} M)	Sp act (mCi/μmol)	Amt incorporated (ng): −PEN	Amt incorporated (ng): +PEN	% Inhibition by PEN
L-[U-^{3}H]histidine	1.50	0.67	0.051[b]	0.067[b]	0
D,L-[U-^{3}H]lysine	1.14	1.75	0.26	0.20	23
L-[4,5-^{3}H]leucine	1.56	0.64	0.81	0.62	23
L-[U-^{14}C]alanine	2.55	0.021	6.71	1.07	84
D-[U-^{14}C]alanine	2.70	0.037	10.39	2.46	76
L-[U-^{14}C]proline	1.91	0.026	—[c]	—[c]	0

[a] Minicells (0.1 ml; A_{600} = 0.4) were incubated in minimal medium for 60 min at 37°C in the presence or absence of 25 μg of PEN per ml. Incorporation of the labeled amino acids into hot trichloroacetic acid-precipitable material was measured as described in Materials and Methods.

[b] Minimal detectable level, 0.03 ng/sample.

[c] —, Below detectable level of 0.05 ng/sample.

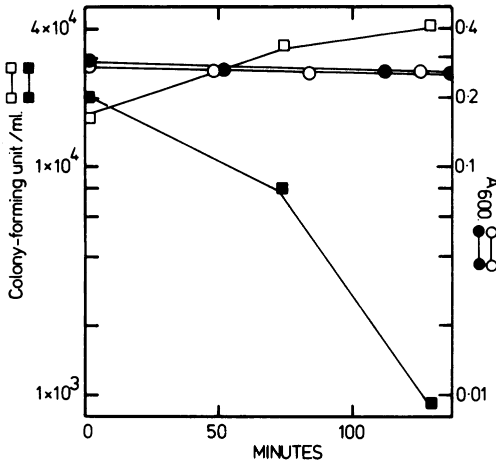

FIG. 3. *Effect of PEN on the viable count and absorbance of a suspension of minicells. Minicells were incubated in minimal medium with (solid symbols) or without (open symbols) 1 mg of PEN per ml of suspension. The absorbance (●, ○) and colony-forming units resulting from contaminating parental cells (■, □) in each suspension were measured.*

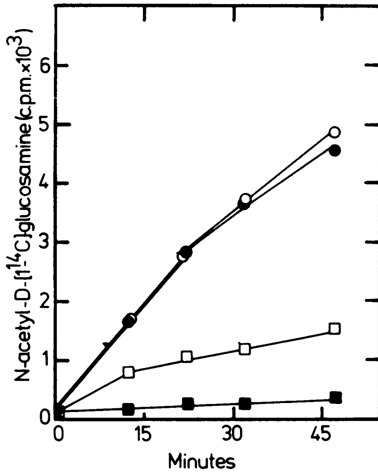

FIG. 4. *Incorporation of N-acetyl-D-[1-^{14}C]glucosamine by minicells. Minicells (A_{600} = 0.25) were incubated at 37°C in minimal medium containing 5 µl of N-acetyl-D-[1-^{14}C]glucosamine (52 mCi/mmol; 50 µCi/250 µl) per ml. Samples (100 µl) were removed, and the radioactivity in hot trichloroacetic acid-precipitable material was measured in each sample (○). At 0 min, portions of the minicell suspension were added to CAM (●) (final concentration, 150 µg/ml), VAN (■) (final concentration, 50 µg/ml), and PEN (□) (final concentration, 25 µg/ml).*

acetylglucosamine incorporation by 25 µg of PEN per ml is included in Fig. 4 for comparison with the effect of VAN and CAM.

The material synthesized by minicells from N-acetylglucosamine in the presence or absence of CAM was found to be over 90% digested to trichloroacetic acid-soluble material by lysozyme (Table 4). In the presence of VAN no lysozyme-digestible material was synthesized, whereas PEN (25 µg/ml, final concentration) only partially inhibited the incorporation of N-acetylglucosamine and the material resulting from the residual synthesis in the presence of PEN was 88% solubilized by lysozyme digestion.

Absence of turnover of mucopeptide in minicells. Analyses of cell wall biosynthesis have focused on the question of whether cell wall, once synthesized, is stable or whether turnover occurs (4, 12, 22, 27, 28, 36). In growing, nucleated *B. subtilis* cells, it has been shown that newly synthesized material is stable for up to 1.5 generations (22, 27). Turnover then occurs with a loss of 30% of the synthesized material

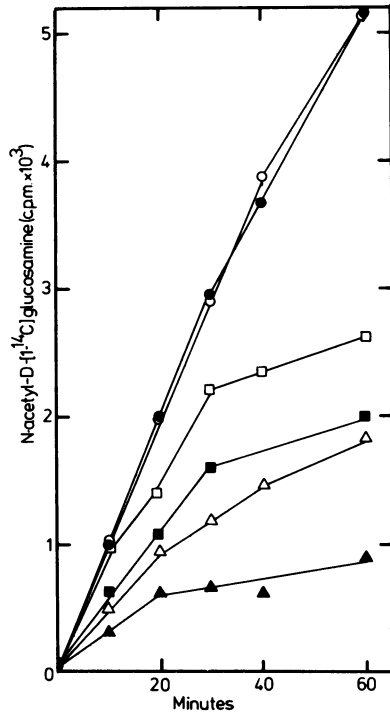

FIG. 5. *Inhibition of N-acetyl-D-[1-^{14}C]glucosamine incorporation by PEN and cycloserine. Minicells (A_{600} = 0.25) were incubated at 37°C in minimal medium containing 5 µl of N-acetyl-D-[1-^{14}C]glucosamine (52 mCi/mmol; 50 µCi/250 µl) per 0.8 ml. Samples (100 µl) were removed, and the radioactivity in hot trichloroacetic acid-precipitable material was measured (●). At 0 min, portions of the minicell suspension were added to PEN at the following concentrations, per milliliter: 1 µg (○); 10 µg (□); 25 µg (■); and 50 µg (△). A portion of the suspension was added to D-cycloserine (▲) at a final concentration of 10 µg/ml.*

TABLE 4. *Incorporation of N-acetyl-D-glucosamine by minicells*

Antibiotic added[b]	*N*-acetyl-D-[1-^{14}C]glucosamine incorporated (ng)[a]		% Converted to trichloroacetic acid-soluble material by lysozyme digestion
	Before lysozyme digestion	After lysozyme digestion	
None	15.9	1.1	92
VAN	0.7	1.1	0
PEN	4.3	0.54	88
CAM	18.1	0.57	97

[a] Minicells (1 ml; A_{600} = 0.25) were incubated at 37°C for 60 min in minimal medium containing 1 μCi of *N*-acetyl-D-[1-^{14}C]glucosamine (53 mCi/mmol; 233 μCi/mg). A total of 200 μl was removed, and the minicells were washed twice by centrifugation using minimal medium minus *N*-acetylglucosamine and resuspended in 200 μl of minimal medium plus 500 μg of lysozyme. A 100-μl amount was immediately placed in 2 ml of 5% trichloroacetic acid, and the remainder was incubated for 30 min at 37°C before addition of 2 ml of 5% trichloroacetic acid. The radioactivity contained in the trichloroacetic acid-precipitable material in each sample was counted. Incorporation of 1 ng was equivalent to the incorporation of 449 cpm.

[b] Final antibiotic concentrations: VAN, 50 μg/ml; PEN, 25 μg/ml; CAM, 150 μg/ml.

within the next 1.5 generations (27). The hot trichloroacetic acid-precipitable material synthesized in minicells produced by *B. subtilis* CU403 *divIVB1* from *N*-acetylglucosamine is apparently stable (Fig. 6). After incorporation of *N*-acetyl-D-[1-^{14}C]glucosamine for 30 min, a 500-fold excess of unlabeled *N*-acetylglucosamine was added. Incorporation continued at a substantial rate for approximately 30 min and subsequently for a 5 h period at a much slower rate. No decrease in incorporated material was found. Essentially identical results have been obtained in experiments replacing *N*-acetylglucosamine with D-alanine (results not shown.)

Incorporation of [2-^{3}H]glycerol by minicells. It has been shown that *B. subtilis* 168 strains incorporate [2-^{3}H]glycerol into teichoic acid and into lipid (25), both of which are precipitated by the addition of cold trichloroacetic acid. However, teichoic acid is rapidly degraded into soluble material by incubation at 90°C in trichloroacetic acid, whereas lipid remains hot trichloroacetic acid precipitable (25, 32, 39). Lipid material can be removed from teichoic acid by extraction with chloroform/methanol (7, 25). In Table 5 it can be seen that minicells of *B. subtilis* also incorporate glycerol into cold trichloroacetic acid-precipitable material and that this incorporation is essentially unaffected by the presence of VAN, PEN, D-cycloserine, or CAM. Fractionation of the cold trichloroacetic acid-precipitable material labeled with [2-^{3}H]glycerol into hot trichloroacetic acid-soluble material (teichoic acid) and chloroform/methanol-extractable material (lipid) indicates that approximately twice as much [2-^{3}H]glycerol is incorporated into lipid as compared with tei-

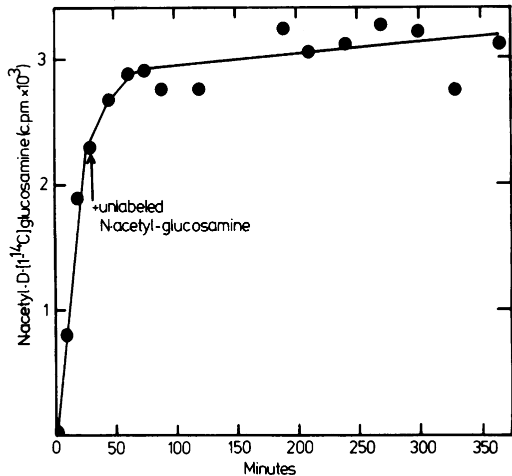

FIG. 6. *Incorporation of N-acetyl-D-[1-^{14}C]glucosamine by minicells. Minicells (A_{600} = 0.25) were incubated at 37°C in minimal medium containing 2.5 μl of N-acetyl-D-[1-^{14}C]glucosamine (52 mCi/mmol; 50 μCi/250 μl) per ml. Samples (100 μl) were removed, and the radioactivity in hot trichloroacetic acid-precipitable material was measured. After 30 min of incorporation, a 500-fold excess of unlabeled N-acetylglucosamine was added.*

TABLE 5. *Incorporation of [2-^{3}H]glycerol by minicells*

Inhibitor added[b]	[2-^{3}H]glycerol incorporation into cold trichloroacetic acid precipitate[a]		% Soluble hot trichloroacetic acid[a]	% in chloroform/methanol extract[a]
	cpm	ng		
None	36,330	183	32	79
VAN	39,549	199	37	76
PEN	34,206	172	36	65
CYC	37,246	187	43	72
CAM	31,633	160	35	72

[a] Minicells (2 ml; A_{600} = 0.1) were incubated at 37°C for 30 min in minimal medium containing 10 μl of [2-^{3}H]glycerol (1 mCi/ml; 143 mCi/mmol). A 2-ml amount of ice-cold 10% trichloroacetic acid was added, and the precipitated material was washed twice with unlabeled minimal medium and fractionated as described in Materials and Methods.

[b] Final concentration of inhibitors: VAN, 50 μg/ml; PEN, 25 μg/ml; D-cycloserine (CYC), 10 μg/ml; CAM, 150 μg/ml.

choic acid. Exposure of the radioactively labeled hot trichloroacetic acid precipitate to lysozyme digestion does not result in conversion of radioactive material to a trichloroacetic acid-soluble form (results not shown).

DISCUSSION

We have demonstrated that minicells produced by *B. subtilis* CU403 *divIVB1* are capable of synthesizing mucopeptide by the following criteria: (i) incorporation of L- and D-alanine and *N*-acetylglucosamine into trichloroacetic acid-precipitable material, which is solubilized by lysozyme digestion; (ii) inhibition of this incorporation by the known inhibitors of cell wall biosynthesis, D-cycloserine and VAN (15, 26; Fig. 1, Tables 2 and 4). The biosynthesis is insensitive to CAM (Fig. 1, Table 2) and is sensitive to PEN at concentrations of the antibiotic in excess of 10 μg/ml (Fig. 5). It has been shown previously that cell wall biosynthesis continues in *B. subtilis* (14, 24), *Staphylococcus aureus* (12), and *Streptococcus faecalis* (36) nucleated cells in the absence of protein synthesis; however, the demonstration of mucopeptide synthesis in anucleate cells is a novel observation. Cell wall synthesis in the absence of protein synthesis results in a thickening of the cell wall (10, 12, 14, 24, 36) and has led to the proposition that at least two types of cell wall growth normally occur in rod-shaped bacteria, i.e., cell wall growth involved in cell elongation and cell wall growth involved in cell wall thickening (4, 36). In minicells the situation would appear to be one in which cell wall elongation has been eliminated and only cell wall thickening can occur. Electron microscopy of minicells incubated for extended periods (23) showed that both cell wall synthesis and minicell autolysis occur. As reported previously, it is very difficult to quantitate the results obtained by electron microscopy; however, within the population of minicells that have been incubated for extended periods, there are many minicells with thickened and striated cell walls very similar to those described by Hughes et al. (14). The evidence from electron microscopy suggests that the incorporation of precursors into cell wall material probably occurs within a subpopulation of minicells. Heterogeneity within a minicell population is known to occur (19) and is to be expected, as minicells represent a variety of cell ages and have been exposed to concentrated sucrose solutions and frozen and thawed before use in experiments. For this reason no attempt has been made to calculate incorporation of precursors in terms of minicell number or per unit of protein.

It has been suggested that the cell poles of *B. subtilis* are less active in cell wall metabolism than the rod-shaped part of the cell (6). The present results suggest that the poles are active in at least some type(s) of cell wall metabolism. The observation that turnover of cell wall synthesized in minicells produced by *B. subtilis* CU403 *divIVB1* (Fig. 6) does not occur agrees with the suggestion of a cell wall thickening process, since only cell wall synthesized by the cell wall elongation process is thought to be subject to turnover whereas cell wall synthesized in the cell wall thickening process has been found to be stable and not to turn over (4).

It was recently found that pulse-labeled (3 min) cell walls of growing *B. subtilis* do not exhibit turnover of newly synthesized material for 1.5 generations, and then turnover takes place (27). In the minicell system, newly synthesized material did not turn over during a subsequent 6-h incubation. The normal generation time of the parental *B. subtilis* strain in the medium used at 37°C is approximately 90 min; however, it is, of course, impossible to define a generation time for a minicell population.

It will be of interest to determine the location of addition of mucopeptide in minicells and the degree and type of linkage of new mucopeptide to the existing mucopeptide. Minicells are essentially an "in vitro" system in that they cannot respond to metabolites, inhibitors, etc., by synthesizing new enzymes and therefore should be very valuable in dissecting the biochemistry of mucopeptide assembly. The addition of PEN has the predictable effect of increasing the relative content of D-alanine as compared to L-alanine (Table 3) in the cell wall by inhibiting cross-bridge formation (40, 44) and shows increasing inhibition of synthesis (Fig. 5) with increasing concentration, as would be predicted from the knowledge that many enzymes are sensitive to PEN over a range of inhibitor concentrations (37, 38). The observation that PEN does not cause lysis in minicells (Fig. 3), although it inhibits incorporation of *N*-acetylglucosamine and D- and L-alanine, indicates that the type of cell wall synthesis occurring in minicells is not the type that, when inhibited in nucleated growing cells, leads to cell lysis. This would tend to support the idea of a cell wall thickening type of synthesis.

In addition to mucopeptide, the cell envelope of *B. subtilis* 168 contains teichoic acid (a glycerol-phosphate polymer) and cell membrane (lipid and protein). Teichoic acid is initially precipitated by cold trichloroacetic acid but is eventually degraded over a period of days at

low temperatures by trichloroacetic acid. Teichoic acid is rapidly solubilized at 90°C in trichloroacetic acid (39). Minicells do synthesize material exhibiting cold trichloroacetic acid precipitability and hot trichloroacetic acid solubility from amino acids (teichoic acid also contains alanine) and glycerol; however, as yet a firm confirmation of teichoic acid synthesis has not been obtained. Isolation and characterization of this material is currently under investigation. The synthesis of lipid is demonstrated by the conversion of [2-^{3}H]glycerol into chloroform/methanol-extractable, trichloroacetic acid-precipitable material (25). Minicells are capable of such synthesis, and this synthesis is insensitive to antibiotics that inhibit protein or mucopeptide synthesis (Table 5). It has been reported that *B. subtilis* can convert amino acids into fatty acids (16). However, after isolation of the fatty acid fraction of minicells labeled with amino acids, essentially no label was found in the fatty acid fraction (Table 1). The accumulation of amino acids in the chloroform/methanol-extractable fraction is most likely a reflection of amino acids that are bound to the lipid carrier molecules located in the cell membrane, which are responsible for the assembly of the mucopeptide and teichoic acid precursors (2, 32). This is supported by the finding of a consistent increase in amino acids in chloroform/methanol-extractable material when VAN is present (results not shown), as VAN is known to inhibit the transfer of mucopeptide precursors from the lipid carrier to the mucopeptide acceptor molecule (2, 15). *B. subtilis* lipids also contain a small amount of lipoamino acids (3); however, no attempt has been made as yet to determine whether they are synthesized in minicells.

The question of protein synthesis in anucleate minicells of *B. subtilis* remains open. We consistently find that a small percentage (<20%) of the incorporation of amino acids is inhibited by CAM, although there is almost no inhibition of incorporation by rifampin (unpublished data). The difference between CAM sensitivity and rifampin resistance has been taken as a measure of stable messenger ribonucleic acid in *E. coli* minicells (20), and this may also be the case in *B. subtilis*. We are, however, currently unable to demonstrate the synthesis of a distinct protein(s) by minicells of *B. subtilis* using gel electrophoresis technology. The amount of CAM-sensitive synthesis in *B. subtilis* minicells is very variable (0 to 20%). This has also been found to be the case for minicells produced by *E. coli* and probably results from the heterogeneity of minicell populations (19, 20).

It has been reported that *S. aureus* is capable of synthesizing a membrane protein in the presence of CAM but that this synthesis is puromycin sensitive (41). The incorporation of the ^{14}C-labeled acid mix and L- and D-alanine by minicells is, however, resistant to both puromycin (up to 100 μg/ml) and chlortetracycline (up to 5 μg/ml). Addition of chlortetracycline at higher concentrations (50 μg/ml) inhibits both protein and cell wall biosynthesis (11), and this has also been found to be the case for biosynthesis in minicells of *B. subtilis* (Mertens, unpublished data).

It has been demonstrated previously (23) that the stability of minicells produced by *B. subtilis* against autolysis is dependent on both the growth conditions and the genotype of the minicell-producing strain. We are currently pursuing both genetic and physiological analyses of cell envelope biosynthesis in minicells and have recently found that it is possible to construct strains of *B. subtilis* that produce minicells that exhibit very high rates of autolysis (Reeve, unpublished data). In addition, we have begun an investigation of the synthetic abilities of minicells produced by *E. coli*. It is apparent from initial observations that *E. coli* minicells do synthesize mucopeptide, albeit in much lower amounts than *B. subtilis* minicells. Levy (personal communication) has also been able to demonstrate lipid biosynthesis by *E. coli* minicells.

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