JOURNAL OF BACTERIOLOGY, Apr. 1983, p. 261–268
0021-9193/83/040261-08$02.00/0

Vol. 154, No. 1

Effect of Decoyinine on Peptidoglycan Synthesis and Turnover in *Bacillus subtilis*

BRENDA URATANI, JUAN M. LOPEZ,† AND ERNST FREESE*

Laboratory of Molecular Biology, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, Maryland 20205

Received 25 October 1982/Accepted 28 January 1983

The sporulation of *Bacillus subtilis* can be induced in the presence of amino acids and glucose by partially depriving the cells of guanine nucleotides. This can be achieved, e.g., by the addition of decoyinine, a specific inhibitor of GMP synthetase. To determine the effect of this and other inhibitors on cell wall synthesis, we measured in their presence the incorporation of acetylglucosamine into acid-precipitable material. The rate of wall synthesis decreased by 50% within 5 min after decoyinine addition; this decrease was prevented by the presence of guanosine. A comparison with the effects of other inhibitors of cell wall synthesis indicated that decoyinine inhibited the final portion of the cell wall biosynthetic pathway, i.e., after the steps inhibited by bacitracin or vancomycin. Decoyinine addition also prevented cellular autolysis and cell wall turnover. It is not known whether these two effects of decoyinine on cell wall synthesis are causally related.

The sporulation of bacilli generally starts when carbon, nitrogen, or phosphate sources become growth limiting. The sporulation of *Bacillus subtilis* can also be initiated by the stringent response to amino acid deprivation or, more specifically, by the partial inhibition of GMP synthesis (4, 5, 14). All of these conditions lead to a decrease in GTP, which seems to control the initiation of sporulation (12). The first morphological event clearly needed for sporulation is the formation of an asymmetric septum, which has to be flexible enough that it can later engulf the small prespore cell compartment. Normally, this septum contains very little cell wall; mutants in which cell wall synthesis continues, producing a thick septum, cannot sporulate but instead produce multiple asymmetric septa (7, 15). Apparently, early during sporulation the rate of cell wall synthesis must be reduced, at least within the prespore septum. Pitel and Gilvary (17) have shown that the incorporation of diaminopimelate (DAP) into *B. megaterium* cell wall greatly decreases during the early stages of sporulation and resumes only later when new peptidoglycan is needed for the synthesis of the spore cortex.

The undecaprenyl disaccharide peptides, from which the cell wall is assembled, are produced in a pathway whose intermediates contain uracil nucleotides as peptidoglycan precursors and require ATP to produce the undecaprenyl lipid carrier. Guanine nucleotides are not known to be directly involved in any aspect of cell wall synthesis. We wanted to see whether the partial inhibition of guanine nucleotide synthesis, which initiates sporulation, also causes the inhibition of cell wall synthesis and how this inhibition might come about. To inhibit GMP synthesis, we used decoyinine, a specific inhibitor of GMP synthetase. Under the conditions employed here, decoyinine induced the production of 10 to 30% of the spores in *B. subtilis*. We found that decoyinine also inhibited cell wall synthesis but, in contrast to other inhibitors of cell wall synthesis, it neither prevented the synthesis nor caused the accumulation of the UDP-pentapeptide intermediate, indicating that the block is in the last stages of wall synthesis.

During this synthesis, the long peptidoglycan chains are covalently cross-linked to each other and to teichoic acid chains. It is not clear how space is provided for the insertion of new peptidoglycan chains into the wall, so that the wall can expand laterally. One possibility is that the cell contains sites or annuli at which new glycan chains are continually inserted, as long as the cell is expanding (in length); no covalent bonds would have to be broken to make space for the new chains. Another possibility is that the existing peptidoglycan network has to be loosened by hydrolytic enzymes so that new chains can be inserted. In that case, the inhibition of wall synthesis by GTP deprivation could result from the inhibition of a critical hydrolytic enzyme. *B. subtilis* has highly active peptidoglycan hydro-

† Present address: Institut für Wasser-Boden und Lufthygiene, 1000 West Berlin, West Germany.

lases, some of which cause the eventual lysis of resting cells (autolysis) and some of which enable cell wall turnover (2). Mutants lacking 95% of the autolytic enzyme activity can still undergo normal length growth, although they produce chains of cells which can no longer separate (3, 19). It remains unclear whether the residual hydrolytic activity of the mutants is required to loosen the cell wall for the insertion of new peptidoglycan chains. As this loosening is not easy to measure, we used the overall cell wall turnover as a measure for the extent to which murein hydrolysis remains active after decoyinine addition. We show here that decoyinine, at the concentration at which it causes optimal sporulation, inhibits cell wall turnover almost completely.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Two *B. subtilis* strains were used, our standard strain 60015 (*metC7 trpC2*) and a uracil-requiring mutant 61411 (*pyrA3*), which can use orotic acid in place of uracil; both are derivatives of the 168 strain. Cells were grown overnight on plates containing a tryptose-blood agar base (33 g/liter; Difco Laboratories, Detroit, Mich.) plus 100 μg of uracil per ml. They were inoculated at an initial absorbancy at 600 nm (OD_{600}) of 0.02 to 0.05 into synthetic medium (12) containing 100 mM glucose and a mixture of most amino acids. Where indicated, the medium also contained 0.2 mM *N*-acetylglucosamine (GlcNAc), which induces its own uptake system (H. L. T. Mobley, R. J. Doyle, and U. N. Streips, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K60, p. 147). For strain 61411, 0.5 mM uracil was also added. Growth was measured by the increase in the OD_{600}.

Chemicals. Decoyinine was a gift from J. Grady of The Upjohn Co., Kalamazoo, Mich. [2-^{14}C]uracil and *N*-acetyl-D-[1-^{3}H]glucosamine ([^{3}H]GlcNAc) were purchased from Amersham Corp., Arlington Heights, Ill., and Aquasol scintillation fluid was purchased from New England Nuclear Corp., Boston, Mass. UDP-GlcNAc was purchased from Sigma Chemical Co., St. Louis, Mo. UDP-*N*-acetylmuramyl-L-Ala-D-Glu-*meso*DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) was a gift from E. Ishiguro of the University of British Columbia, and UDP-MurNAc-L-Ala-D-Glu-*meso*DAP (UDP-MurNAc-tripeptide) was a gift from T. Oka of Kyowa Hakko Co.

Uptake of [^{3}H]GlcNAc. Strain 61411 was grown in a synthetic medium containing 0.2 mM GlcNAc to an OD_{600} of 0.5. [^{3}H]GlcNAc (0.25 μCi/ml final concentration) was added, and the culture was divided into two parts: 1.4 mM decoyinine was added to one part, whereas the other remained untreated. At 1-min intervals, 0.5 ml of culture was added to a membrane filter (0.45-μm pore size, 2.5-cm diameter), which was immediately washed with 5 ml of 0.1 M $LiCl_2$. The filter was dried and counted in 5 ml of Aquasol.

Assay of fructose-6-phosphate amidotransferase. Strain 60015 was grown in a synthetic medium to an OD_{600} of 0.5, the cells were washed, and lysed with lysozyme, and the extract was cleared of debris as described elsewhere (21). Fructose-6-phosphate amidotransferase was assayed as described previously (6).

Determination of UDP-GlcNAc, UDP-MurNAc-pentapeptide, and UDP-MurNAc-tripeptide concentrations. Strain 61411 was grown in a synthetic medium, containing 0.5 mM [^{14}C]uracil (36 Ci/mol) and 0.2 mM GlcNAc, from an OD_{600} of 0.02 to an OD_{600} of 0.5, to uniformly label the cells with [^{14}C]uracil. [^{3}H]GlcNAc (1 μCi/ml) was added, and the culture was divided into portions, one of which received decoyinine (1.4 mM final concentration); the second received decoyinine and guanosine (1 mM final concentration), and the third served as an untreated control. At different times, cells from 0.5-ml portions were rapidly collected on membrane filters (2.5-cm diameter, 0.45-μm pore size), which were laid upside down onto 50 μl of ice-cold 1 M formic acid to extract the cell wall intermediates, by the method of Uratani et al. (21). A 10-μl portion of the formic acid extract was applied to a corner of a thin-layer cellulose plate (20 by 20 cm, 0.1-mm thickness; MN polygram Cel 400 UV; Brinkmann Instruments Co., Westbury, N.Y.). Standards of 10 μg of UDP-GlcNAc, UDP-MurNAc-pentapeptide, and UDP-MurNAc-tripeptide were applied to the same spot. These compounds were separated from the other uracil-containing compounds by two-dimensional chromatography with a 7:3 mixture of ethanol and 1 M ammonium acetate (pH 7.5) in the first dimension and, after being dried, with a 5:3 mixture of isobutyric acid and 1 N NH_4OH in the second dimension. The dried plates were placed against X-Omat-R X-ray film (Kodak Co., Rochester, N.Y.) and incubated for 72 h. A typical chromatogram is shown in Fig. 1. Radioactive spots of interest were cut out of the cellulose plate, and the radioactive material was eluted by 2 h of incubation in 0.5 ml of distilled water. The radioactivity of both the eluate and that remaining in the cut-out portion of the plate was determined in a scintillation counter with 10 ml of Aquasol. For each spot, the sum of the two counts was used as the total amount of radioactivity. The results were expressed in picomoles per OD_{600}; 1 OD_{600} U is the amount of cells which, if contained in 1 ml, gives an OD_{600} of 1.

Measurement of cell wall synthesis. Cells were grown exponentially in a synthetic medium containing GlcNAc and uracil to an OD_{600} of 0.5. [^{3}H]GlcNAc (1 μCi/ml) was added, and the culture was divided into three parts: 1.4 mM (final concentration) decoyinine was added to 1 part; 1.4 mM decoyinine and 1 mM guanosine were added to the second; and the third remained as an untreated control. A 0.5-ml amount of culture was placed into 0.5 ml of 10% ice-cold trichloroacetic acid (TCA) containing 100 mM GlcNAc at 2-min time intervals. After 30 min, the TCA-precipitable materials were collected by filtration on 0.45-μm membrane filters and washed with 5% cold TCA containing 10 mM GlcNAc. The nonradioactive GlcNAc was added to allow the exchange of radioactive GlcNAc adsorbed to the cells. The radioactivity on the filter was determined in a liquid scintillation counter.

We calculated the total amount GlcNAc incorporated during the experiment into cells of the uracil-requiring mutant 61411 from the counts of [^{3}H]GlcNAc incorporated and the specific activity of intracellular GlcNAc. The latter was obtained by uniformly labeling the cells (61411) with [^{14}C]uracil, pulsing them with [^{3}H]GlcNAc, and then measuring

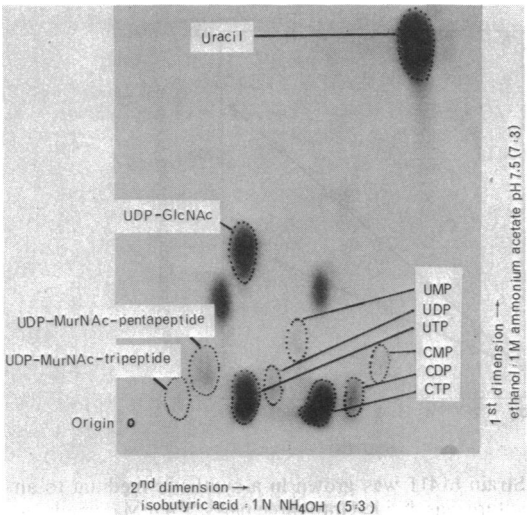

FIG. 1. Two-dimensional thin-layer autoradiogram of [^{14}C]uracil-labeled compounds in formic acid extracts of *B. subtilis*. Strain 61411 was uniformly labeled by growth for 3 h in a synthetic medium containing [2-^{14}C]uracil (36 Ci/mol, 100 μg/ml). A 10-μl amount of a formic acid extract was applied, together with 10 μg of each standard, to a cellulose thin-layer plate. After chromatography, the radioactive spots were identified by 2 days of incubation with a photographic film, and the UV-absorbing spots were marked with ink (dotted lines).

the ratio of the ^{14}C to ^{3}H counts in chromatographically separated UDP-GlcNAc. For this purpose, an exponential culture of 61411 was exposed (starting at an OD_{600} of 0.02) to [^{14}C]uracil (0.5 mM, 36 μCi/μmol). When the OD_{600} was 0.5, [^{3}H]GlcNAc (0.2 mM, 100 μCi/μmol) was added. Two minutes later, the culture was divided into three parts: one part received no addition, one part received 1.4 mM (final concentration) decoyinine, and the third part received both decoyinine and guanosine (1 mM final concentration). After 2 min of continued aeration at 37°C, cells from 0.5 ml of culture were rapidly collected on a membrane filter and extracted with 50 μl of 1 M formic acid. UDP-GlcNAc was separated from the other uracil-containing compounds in the extract by two-dimensional chromatography as described above. The specific activity of UDP-GlcNAc was equal to the ratio of the ^{3}H to ^{14}C counts in the UDP-GlcNAc multiplied by the specific activity of uracil (counts per minute of [^{14}C]uracil per mmol), which had been separately measured.

Cellular autolysis. Cell autolysis was measured by the method of Shungu et al. (20). Strain 60015 was grown exponentially in a synthetic medium until the OD_{600} was 0.5. The culture was divided into three parts: 1.4 mM (final concentration) decoyinine was added to one part; 1.4 mM decoyinine and 1 mM guanosine were added to the second; and the third part served as the untreated control. After different times of continued aeration at 37°C, cells of 10 ml of culture were rapidly collected by membrane filtration, washed with ice-cold distilled water, and suspended in 0.3 M sodium phosphate buffer (pH 7.0). The decrease in turbidity was monitored (for 6 min) at 37°C with a Gilford spectrophotometer at 600 nm. The rate of autolysis was calculated from the slope of the OD_{600} decrease plotted semilogarithmically.

Measurement of cell wall turnover. Cell wall turnover was measured by the methods of Pooley (18) and Glaser and Lindsay (8). Cultures were grown for more than six generations in the presence of [^{3}H]GlcNAc (1 μCi/ml). When the OD_{600} was 0.5, the cells were collected on membrane filters (pore size, 0.45 μm) under suction, washed with prewarmed medium, and suspended in a prewarmed synthetic medium containing 100 mM GlcNAc. At the time indicated on the figures, 5 ml of culture was transferred to flasks containing decoyinine (1.4 mM final concentration), or decoyinine plus guanosine (1 mM final concentration). The flasks were aerated at 37°C, and at intervals, samples were placed into an equal volume of ice-cold 10% TCA (containing 100 mM [final concentration] GlcNAc). The TCA-insoluble material was collected on a membrane filter (pore size, 0.45 μm) and washed with 5% TCA containing 10 mM GlcNAc, and its radioactivity was measured in a liquid scintillation counter with Aquasol.

RESULTS

Effect of limitation of guanine nucleotides on cell wall synthesis. When either one of the two strains used here was grown in a synthetic medium (plus uracil for strain 61411) to an OD_{600} of 0.5 and then exposed to decoyinine (1.4 mM), the OD_{600} increased much more slowly, and 10 h later 10 to 30% of the cells contained heat-resistant spores. Without the addition of decoyinine, less than 0.0001% of the cells contained spores. The addition of decoyinine caused a decrease in intracellular GTP, as reported earlier (12).

We measured cell wall synthesis by the incorporation of [^{3}H]GlcNAc into TCA-insoluble material. As Pooley had already observed (19), we found that more than 90% of the TCA-precipitable material was lysozyme soluble, indicating the high specificity with which GlcNAc was incorporated into the cell wall (data not shown). To determine the effect of decoyinine on cell wall synthesis, we inoculated cells of the uracil-requiring strain (61411) in a synthetic medium containing 0.2 mM GlcNAc and 0.5 mM uracil. When the OD_{600} was 0.5, [^{3}H]GlcNAc (1 μCi/ml) was added. The culture was then split into three parts, one of which received decoyinine (1.4 mM final concentration); the second received decoyinine and guanosine (1 mM final concentration), and the third served as an untreated control. At different times, 0.5 ml was placed into 0.5 ml of ice-cold TCA containing unlabeled 100 mM GlcNAc. The TCA-precipitable material was collected on a membrane filter and counted. These counts represented the incorporation of extracellular GlcNAc. Because the ratio of extracellular GlcNAc to de novo-produced GlcNAc might change during the inhi-

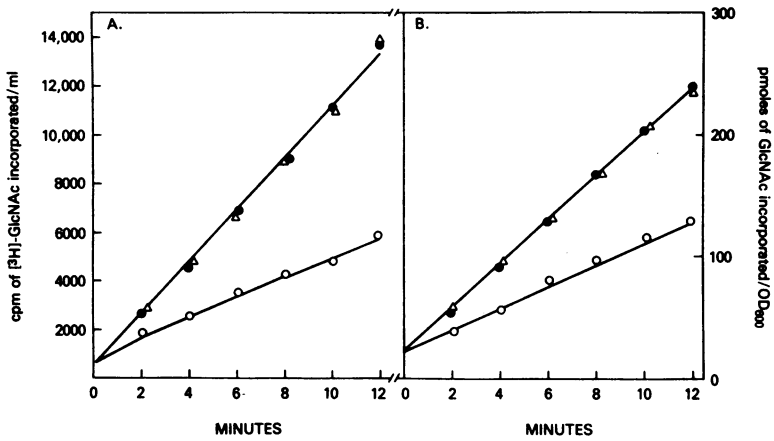

FIG. 2. Incorporation of [^{3}H]GlcNAc into cell wall. Strain 61411 was grown in a synthetic medium to an OD_{600} of 0.5. [^{3}H]GlcNAc (1 μCi/ml) was added, and the culture was divided into three parts: 1.4 mM decoyinine was added to the first, decoyinine and 1 mM guanosine were added to the second; the third remained untreated. The incorporation of [^{3}H]GlcNAc was determined in TCA-insoluble material. The results are plotted for counts per minute per ml (A) and picomoles per OD_{600} (B); the latter values were determined as described in the text. Symbols: △, untreated; ○, decoyinine treated; and ●, treated with decoyinine and guanosine.

bition of decoyinine, we determined the total amount of GlcNAc incorporated into the cell wall by dividing the counts of [^{3}H]GlcNAc incorporated by the specific activity (counts per minute per picomole) of intracellular UDP-GlcNAc determined in parallel cultures as described above. Within 2 min, this specific activity reached a constant value which was 45% of the extracellular specific activity of GlcNAc. Figure 2 shows the amount of both extracellular [^{3}H]GlcNAc and total GlcNAc incorporated into the cell wall. The immediate decrease in cell wall synthesis caused by decoyinine is apparent in both curves; the decrease in the total GlcNAc incorporation was slightly less than that of extracellular [^{3}H]GlcNAc. The decrease was prevented when decoyinine and guanosine were added together.

In control experiments, we showed that the uptake rate of GlcNAc was unaffected by decoyinine (Fig. 3). We also measured the specific activity of fructose-6-phosphate amidotransferase, the first enzyme specifically needed for de novo peptidoglycan synthesis. This activity, measured 9 min after the exposure of the cells to decoyinine, was 12 nmol/min per mg of protein, which was slightly higher than that of the control culture (5 nmol/min per mg of protein). For this reason and also because decoyinine does not inhibit this enzyme, there is no reason to believe that the de novo synthesis of glucosamine-6-phosphate was decreased by decoyinine treatment.

Comparison with the effect of other inhibitors of cell wall synthesis. The decrease in cell wall synthesis observed after the addition of decoyin-

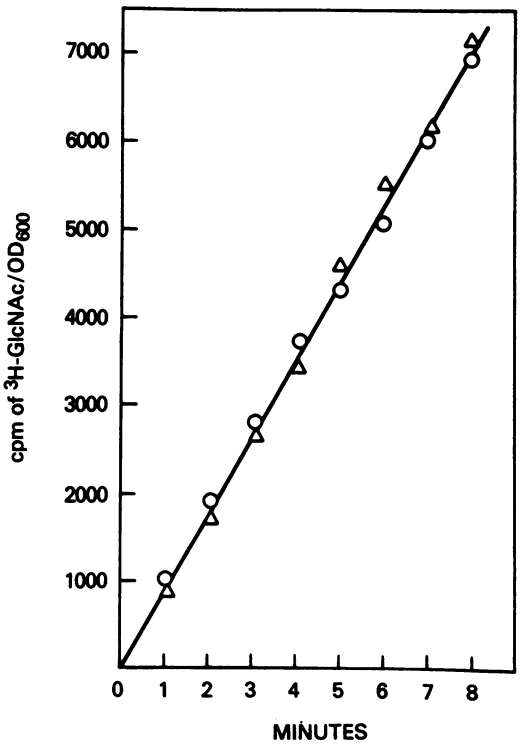

FIG. 3. Uptake of [^{3}H]GlcNAc by whole cells. Strain 61411 was grown as described in the legend to Fig. 1, and [^{3}H]GlcNAc (0.25 μCi/ml) was added when the OD_{600} was 0.5. The culture was divided into two parts, one of which received 1.4 mM decoyinine. At 1-min intervals, 1-ml samples were applied to membrane filters and washed, and the radioactivity retained on the filter was determined. Symbols: ○, decoyinine-treated culture; and △, untreated culture.

TABLE 1. Effect of different inhibitors on growth and cell wall synthesis

Compound	Concn (μg/ml)	Relative OD_{600} increase in 10 min[a]	Incorporation of [^{3}H]GlcNAc[b]	
			(pmol/ml)	(pmol/OD_{600})
None		0.35	105	200
Decoyinine	400	0.2	57	118
Decoyinine plus guanosine	400 283	0.35	105	200
Cerulenin	10	0.35	105	210
Chloramphenicol	50	0.14	162	330
HPUra	50	0.35	105	200
Rifampin	0.1	0.12	100	190

[a] The relative OD_{600} increase in 10 min indicated the degree of growth inhibition caused by the drug during the time of the experiment.

[b] Strain 61411 was grown in a synthetic medium to an OD_{600} of 0.5. [^{3}H]GlcNAc was added, and 5 ml of the culture was pipetted into flasks containing the drug(s). The incorporation of [^{3}H]GlcNAc was determined by measuring the radioactivity of material insoluble in 5% TCA. The specific activity of GlcNAc was determined as described in the text. The results are expressed in picomoles per milliliter and picomoles per OD_{600}.

ine might not result directly from a decrease in guanine nucleotides but indirectly from the inhibition of DNA, RNA, protein, or lipid synthesis caused by the deprivation of GTP. Therefore, we measured how rapidly cell wall synthesis was affected in cultures treated with 6-(*p*-hydroxyphenylazo)-uracil (HPUra), which specifically inhibits DNA synthesis (1); cerulenin, which inhibits membrane synthesis (9, 16); rifampin; and chloramphenicol. At the concentrations used (Table 1), rifampin and chloramphenicol inhibited growth almost immediately, whereas cerulenin and HPUra inhibited growth only after a lag (of 60 min). The inhibition of cell wall synthesis in the first 12 min after the addition of an inhibitor was measured as described above for decoyinine. The rate of GlcNAc incorporation into the cell wall was constant throughout this time regardless of the inhibitor used. The rate of GlcNAc incorporation per milliliter (or per OD_{600}) did not decrease in any of the cultures and increased in the culture treated with chloramphenicol (Table 1).

Accumulation of cell wall intermediates in the presence of decoyinine or cell wall inhibitors. In the attempt to locate the biochemical step in cell wall synthesis which depends on the concentration of guanine nucleotides, we measured the amounts of the cell wall intermediates UDP-MurNAc-tripeptide and UDP-MurNAc-pentapeptide in cultures treated either with decoyinine or, for comparison, with known inhibitors of cell wall synthesis. Strain 61411 was labeled uniformly with [^{14}C]uracil (0.53 mM, 37.7 Ci/mol) during growth from an OD_{600} of 0.02 to an OD_{600} of 0.5 in a synthetic medium. Culture portions (2 ml) were added to flasks containing nothing, decoyinine, or a known cell wall inhibitor. Samples (0.5 ml) were taken before the treatment and at different times thereafter; the cells were rapidly collected on a membrane filter and extracted with 1 M formic acid, and the uracil-containing peptidoglycan precursors were separated by two-dimensional chromatography (Fig. 1). We observed no accumulation of any intermediate in cultures treated with decoyinine (1.4 mM) or penicillin (1 mg/ml), but much accumulation of UDP-MurNAc-pentapeptide in cultures treated with vancomycin (10 μg/ml) or bacitracin (100 μg/ml) (Fig. 4A); the latter compounds inhibit the membrane cycle of cell wall biosynthesis.

These results showed that decoyinine inhibited either the cytoplasmic portion of peptidoglycan biosynthesis, before the UDP-MurNAc-pentapeptide, or a reaction needed for the production of a completed (cross-linked) wall. In the former case, decoyinine should reduce the pentapeptide accumulation that was observed after the addition of vancomycin, a compound inhibiting the incorporation of the pentapeptide into peptidoglycan. Therefore, we grew a culture of strain 61411 as above, treated it with decoyinine plus vancomycin, and measured the accumulation of UDP-MurNAc-pentapeptide. Exposure to decoyinine did not reduce this accumulation (Fig. 4B). Similar results were obtained when decoyinine was added to the culture 5 min before the addition of vancomycin, eliminating the possibility of competition or preferential uptake of vancomycin.

Cellular autolysis of decoyinine-treated cells. It was now apparent that the limitation of guanine nucleotides, caused by decoyinine, somehow interfered with the incorporation of the isoprenoid-linked MurNAc-pentapeptide into the cross-linked cell wall. Because this process in wall synthesis might depend on the activity of peptidoglycan hydrolytic enzymes (opening up existing wall to provide empty or acceptor sites for

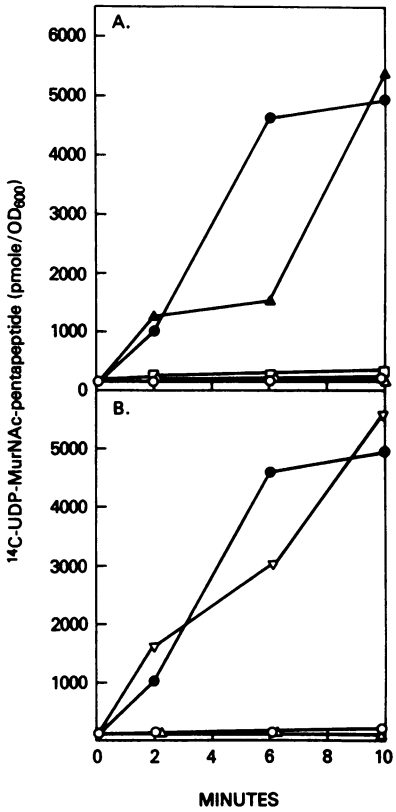

FIG. 4. Effect of decoyinine and cell wall inhibitors on the concentration of UDP-MurNAc-pentapeptide. A culture of strain 61411 (uniformly labeled with [^{14}C]uracil) was grown to an OD_{600} of 0.5 and then exposed to decoyinine or to cell wall inhibitors. At different times after drug addition, samples were extracted with formic acid. The UDP-MurNAc-pentapeptide was separated by two-dimensional chromatography as described in the legend to Fig. 1, and the radioactivity in this spot was measured. Symbols: (A) △, untreated; ○, decoyinine treated; □, penicillin G treated; ▲, vancomycin treated; and ●, bacitracin treated. (B) △, untreated; ○, decoyinine treated; ▽, vancomycin treated; and ●, treated with both decoyinine and vancomycin.

polymerization), we measured the rate of cellular autolysis. We grew strain 60015 in a synthetic medium to an OD_{600} of 0.5 and divided the culture into three parts: to one part we added decoyinine (1.4 mM final concentration), to the second part we added both decoyinine and guanosine (1 mM final concentration); and the third part served as the control. At different times, cells were collected, washed, and suspended in phosphate buffer, and the rate of decrease in the OD_{600} was followed at 37°C. Cells of a culture grown in the presence of decoyinine subsequently showed a significantly lower rate of autolysis; guanosine present in addition to decoyinine counteracted this effect of decoyinine (Fig. 5).

Cell wall turnover of decoyinine-treated cells. During growth, the cell wall of *B. subtilis* turns over continuously (13). Wall material released into the medium comes from wall synthesized more than one generation earlier, presumably because peptidoglycans slowly move during cell growth from the inside to the outside of the wall (18). If new wall synthesis should be required for the turnover, decoyinine should prevent the release of label from prelabeled cell wall, and guanosine should restore the release. To measure this effect, we grew cells of our standard strain 60015 in a synthetic medium, labeling them uniformly with [^{3}H]GlcNAc. At an OD_{600} of 0.5, the cells were rapidly washed and suspended in the same volume of warm synthetic medium containing unlabeled GlcNAc. The effect of decoyinine on cell wall turnover was determined as shown in Fig. 6A. At different times, a portion of the culture was transferred

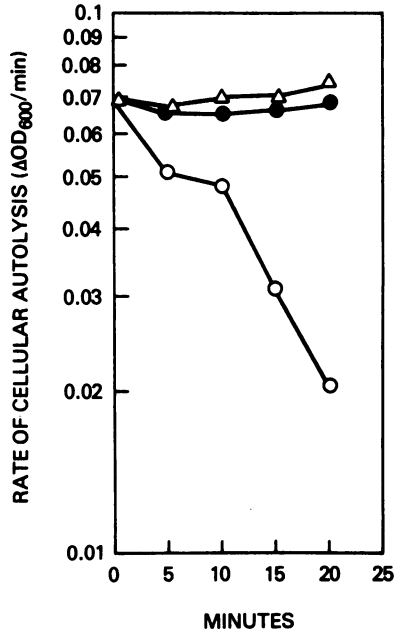

FIG. 5. Effect of decoyinine on the rate of cellular autolysis. Strain 60015 was grown to an OD_{600} of 0.5, and the culture was divided into three parts: 1.4 mM decoyinine was added to one part; 1.4 mM decoyinine and 1 mM guanosine were added to the second; the third part served as a control. At indicated times, the cells of 10 ml of culture were rapidly collected on a membrane filter, washed with, and suspended in, phosphate buffer. The rate of cellular autolysis was measured by the rate of the decrease in the OD_{600} at 37°C. Symbols: ●, decoyinine treated; ○, decoyinine plus guanosine treated; and △, untreated.

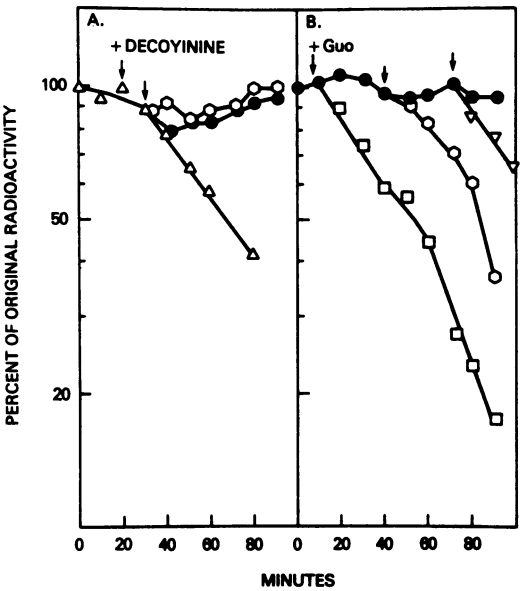

FIG. 6. Measurement of cell wall turnover. A culture of strain 60015, uniformly labeled with [^{3}H]GlcNAc, was grown to an OD_{600} of 0.5. The cells were collected on a membrane filter, washed, and suspended in a prewarmed (37°C) synthetic medium containing unlabeled 100 mM GlcNAc. (A) At different times (indicated by the arrows), 5 ml of the culture was transferred to a flask containing decoyinine (to give 1.4 mM). Every 10 min, samples (0.5 ml) were added to 10% ice-cold TCA (0.5 ml). After 30 min or more, the precipitate was collected on a membrane filter and washed, and the radioactivity was measured (see the text). The values were plotted as a percentage of the average of two zero time values. Symbols: △, untreated culture; ○, decoyinine added after 20 min; and ●, decoyinine added after 30 min. (B) At zero time, decoyinine (1.4 mM final concentration) was added to the culture. At the times indicated by the arrows, 5 ml of culture was transferred to a flask containing guanosine (1 mM final concentration). Cell wall turnover was measured as described for (A). Symbols: ●, no guanosine added; □, guanosine added at 5 min; ○, guanosine added at 40 min; and ▽, guanosine added at 60 min.

into a flask containing decoyinine (to give 1.44 mM). At this and at later times, samples were mixed with equal amounts of 10% TCA, and the radioactivity in the precipitate was counted. The release of radioactivity increased for the first 20 to 30 min and continued thereafter at a constant rate (Fig. 6A). Decoyinine arrested this release soon after its addition. On the other hand, the addition of guanosine counteracted the decoyinine effect almost immediately (Fig. 6B). It even shortened the lag, after which the release reached a constant rate (with or without decoyinine).

DISCUSSION

Our results have shown that the addition of decoyinine, which causes a partial deprivation of guanine nucleotides and induces sporulation, inhibited the net synthesis of cell wall almost immediately. In contrast, inhibitors of the synthesis of other macromolecules (rifampin, cerulenine, or HPUra) did not inhibit wall synthesis within 12 min after their addition. The addition of chloramphenicol, which inhibited growth (OD_{600} increase) immediately, increased the rate of cell wall synthesis. This observation agrees with the frequent finding that chloramphenicol causes a thickening of the cell wall because peptidoglycan synthesis continues (19). It is apparent that cell wall synthesis is inhibited earlier by the deprivation of guanine nucleotides than it is by the inhibitors that primarily inhibit the synthesis of other macromolecules but eventually also cause a decrease in wall synthesis. *Escherichia coli* cell wall synthesis shows a "stringent response" to amino acid deprivation, which also produces an increase in ppGpp and a decrease in GTP (10, 11). If one assumes that the stringent response prevented the cell wall synthesis of *E. coli* not because ppGpp increased but because GTP decreased, our findings are consistent with those for *E. coli*.

To determine the cause of this inhibition of cell wall synthesis, we compared the effect of decoyinine with that of various known inhibitors of cell wall synthesis. Whereas the addition of bacitracin or vancomycin caused the accumulation of the cell wall intermediate UDP-MurNAc-pentapeptide, the addition of decoyinine did not, nor did it prevent the accumulation caused by vancomycin. Therefore, decoyinine seems to inhibit the incorporation of peptidoglycan into acid-precipitable cell wall. Its effect was somewhat similar to that of penicillin, which also did not cause the accumulation of UDP-MurNAc-pentapeptide. Penicillin did, however, prevent the accumulation caused by vancomycin. Moreover, in contrast to penicillin, decoyinine did not cause overall cell lysis, probably because it also reduced the rate of cell expansion as is seen by the slow increase in the OD_{600}.

The presence of decoyinine during growth also inhibited cellular autolysis and the turnover of prelabeled cell wall. The addition of guanosine counteracted this effect. Guanosine even shortened the lag observed before the release occurred at a constant rate, as if it activated peptidoglycan hydrolysis. If cell wall synthesis should require a loosening of existing wall by means of a hydrolytic enzyme, and if a guanine nucleotide should be directly involved in this hydrolysis, then the inhibition of wall synthesis by decoyinine would be explained. However, it is also possible that decoyinine caused the inhi-

bition of autolysis and wall turnover indirectly because the decrease in GTP reduces the rate of cell growth. Mauck et al. (13) have reported that the addition of chloramphenicol or amino acid deprivation inhibits cell wall turnover by an unknown mechanism. The peptidoglycan hydrolytic enzymes were still present in these cells, as was shown by their extraction from the cell wall and their activity on isolated wall; chloramphenicol did not rapidly destroy these enzymes but prevented their activation. Conceivably, the peptidoglycan hydrolytic enzymes are activated only if the cell expands so that the latent enzyme can separate from its known inhibitor, lipoteichoic acid. It is, therefore, not clear whether the inhibitions of cell wall synthesis and of turnover, both caused by decoyinine and reversed by guanosine, are causally related or result independently from the deprivation of guanine nucleotides.

LITERATURE CITED

1. **Brown, W. C., and F. E. Young.** 1970. Dynamic interaction between cell wall polymers, extracellular proteases and autolytic enzymes. Biochem. Biophys. Res. Commun. **38**:564–568.
2. **Fan, D. P., and M. M. Beckman.** 1971. Mutant of *Bacillus subtilis* demonstrating the requirement of lysis for growth. J. Bacteriol. **105**:629–636.
3. **Fan, D. P., M. M. Beckman, and W. P. Cunningham.** 1972. Ultrastructural studies on a mutant of *Bacillus subtilis* whose growth is inhibited due to insufficient autolysin production. J. Bacteriol. **109**:1247–1257.
4. **Freese, E., J. Heinze, and E. M. Galliers.** 1979. Partial purine deprivation causes sporulation of *Bacillus subtilis* in the presence of excess ammonium, glucose and phosphate. J. Gen. Microbiol. **115**:193–205.
5. **Freese, E., J. Heinze, T. Mitani, and E. B. Freese.** 1978. Limitation of nucleotides induces sporulation, p. 277–285. *In* G. Chambliss and J. C. Vary (ed.), Spores VII. American Society for Microbiology, Washington, D.C.
6. **Freese, E. B., R. M. Cole, W. Klofat, and E. Freese.** 1970. Growth, sporulation, and enzyme defects of glucosamine mutants of *Bacillus subtilis*. J. Bacteriol. **101**:1046–1062.
7. **Frehel, C., A. M. Benfils, and A. Ryter.** 1971. Etude au microscope électronique de la croissance du paroi chez *B. subtilis* et *B. megaterium*. Ann. Inst. Pasteur Paris **121**:139–148.
8. **Glaser, L., and B. Lindsay.** 1977. Relation between cell wall turnover and cell growth in *Bacillus subtilis*. J. Bacteriol. **130**:610–619.
9. **Goldberg, I., J. R. Walker, and K. Bloch.** 1973. Inhibition of lipid synthesis in *Escherichia coli* cells by the antibiotic cerulenin. Antimicrob. Agents Chemother. **3**:549–554.
10. **Ishiguro, E. E., and W. D. Ramey.** 1976. Stringent control of peptidoglycan biosynthesis in *Escherichia coli* K-12. J. Bacteriol. **127**:1119–1126.
11. **Ishiguro, E. E., and W. D. Ramey.** 1978. Site of inhibition of peptidoglycan biosynthesis during the stringent response in *Escherichia coli*. J. Bacteriol. **135**:71–77.
12. **Lopez, J. M., C. L. Marks, and E. Freese.** 1979. The decrease of guanine nucleotides initiates sporulation in *Bacillus subtilis*. Biochim. Biophys. Acta **587**:238–252.
13. **Mauck, J., L. Chan, and L. Glaser.** 1971. Turnover of cell wall of gram-positive bacteria. J. Biol. Chem. **246**:820–827.
14. **Ochi, K., J. C. Kandala, and E. Freese.** 1981. Initiation of *Bacillus subtilis* sporulation by the stringent response to partial amino acid deprivation. J. Biol. Chem. **256**:6866–6875.
15. **Oh, Y. K., E. B. Freese, and E. Freese.** 1973. Abnormal septation and inhibition of sporulation by accumulation of L-alpha-glycerophosphate in *Bacillus subtilis* mutants. J. Bacteriol. **113**:1034–1045.
16. **Omura, S.** 1976. The antibiotic cerelenin, a novel tool for the biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev. **40**:681–697.
17. **Pitel, W. D., and C. Gilvary.** 1970. Mucopeptide metabolism during growth and sporulation in *Bacillus megaterium*. J. Biol. Chem. **245**:6711–6717.
18. **Pooley, H. M.** 1976. Turnover and spreading of the old wall during cell growth of *Bacillus subtilis*. J. Bacteriol. **125**:1127–1138.
19. **Rogers, H. J.** 1979. Biogenesis of the wall in bacterial morphogenesis. Adv. Microb. Physiol. **19**:1–63.
20. **Shungu, D. L., J. B. Cornett, and G. D. Shockman.** 1979. Morphological and physiological study of autolytic-defective *Streptococcus faecium* strains. J. Bacteriol. **138**:598–608.
21. **Uratani-Wong, B., J. M. Lopez, and E. Freese.** 1981. Induction of citric acid cycle enzymes during initiation of sporulation by guanine nucleotide deprivation. J. Bacteriol. **146**:337–344.