JOURNAL OF BACTERIOLOGY, Dec. 1979, p. 786-797
0021-9193/79/12-0786/12$02.00/0

Vol. 140, No. 3

Requirement for Peptidoglycan Synthesis During Sporulation of *Bacillus subtilis*

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Received for publication 21 August 1979

Cultures of *Bacillus subtilis* were treated during sporulation with antibiotics (bacitracin and vancomycin) that affect peptidoglycan synthesis. The cells were resistant to the effects of the antibiotics only when the drugs were added about 2 h after the beginning of sporulation. This was about 1 h later than the escape time of a temperature-sensitive sporulation mutant that is unable to complete prespore septation. Similar experiments were done with a mutant temperature sensitive for peptidoglycan synthesis. This showed an escape curve similar to that shown by the antibiotics. When sporulating cells were treated with antibiotics, they produced alkaline phosphatase earlier than normal. Enzyme production was unaffected by inhibition of deoxyribonucleic acid synthesis but was inhibited by chloramphenicol. Sporulation mutants that are unable to make alkaline phosphatase under normal conditions were able to make it in the presence of bacitracin. The alkaline phosphatase made under these conditions was under "sporulation-type" control since its synthesis was repressible by casein hydrolysate and unaffected by inorganic phosphate. When cells were treated with bacitracin in the growth medium as well as in the sporulation medium, alkaline phosphatase synthesis was at the same level as in an untreated control. A number of other antibiotics and surfactants were tested for the ability to cause premature production of the phosphatase of those tested, only taurodeoxycholate showed this behavior. Moreover, incubation of cells with taurodeoxycholate in the growth medium as well as in the sporulation medium prevented premature enzyme production.

The cell walls of bacilli provide rigid support, which could be an important element in the structural changes undergone during endospore formation. Peptidoglycan synthesis is certainly necessary for the production of the cortex and germ cell wall, which are incorporated into the spore after the prespore has formed (10, 40). There is also an earlier period during which the cell wall is required. Fitz-James (9) found that an intact cell wall is essential for development of the prespore in *Bacillus megaterium*. When sporulating cells were converted to protoplasts, only those that contained a free prespore were able to complete sporulation. Lawrence (21) showed that development of the prespore septum was reversibly inhibited in cells of *B. megaterium* treated with penicillin. There have been numerous reports that bacilli treated at the beginning of sporulation with other drugs that affect cell wall synthesis are unable to form prespore septa (14).

It has generally been thought that the prespore septum differs from the normal division septum in that it contains no cell wall material (15, 23). The distinction is now questionable. Evidence for the involvement of cell wall components in the early events of sporulation came from Freese et al. (11), who found that glucosamine-requiring mutants of *B. subtilis* are unable to make prespore septa in the absence of glucosamine but can make them when glucosamine is restored. Also, Holt et al. (17) showed that the prespore septum of *Bacillus sphaericus* contains material that is continuous with the cell wall. As sporulation continues, this material becomes more flexible and diffuse. If cells were treated with vancomycin to inhibit the terminal polymeraization reaction of peptidoglycan synthesis (19), then prespore septa failed to form. The question as to the involvement of cell wall components in prespore septation has been reviewed recently by Hitchins (14), who concluded that there is now increasing support for the view that small amounts of such material are included in the intermembrane space.

In this communication I report the results of experiments with *B. subtilis* which were designed to determine the period of sporulation before cortex synthesis during which peptidoglycan synthesis is required. Second, experiments were done to determine whether the biochemical sequence that parallels the morphological se-

quence of sporulation events (23) is affected by inhibition of peptidoglycan synthesis. Two approaches were used. First, cells were treated with drugs that have been shown to inhibit specific reactions in peptidoglycan synthesis. Penicillin inhibits the cross-linking between peptidoglycan strands (30), and D-cycloserine inhibits alanine racemase and D-alanyl-D-alanyl synthetase (25, 37). Bacitracin and vancomycin affect membrane-associated reactions. The former specifically prevents dephosphorylation of undecaprenol pyrophosphate (34), and the latter inhibits the terminal polymerization reaction of peptidoglycan synthesis (19). Second, a temperature-sensitive mutant defective in peptidoglycan synthesis was used. It is hoped that such a dual approach avoided some of the difficulties that may arise from using antibiotics or mutants alone. A temperature-sensitive sporulation mutant was exposed to the antibiotics to determine the stage of sporulation at which cells escaped from the effects of the drugs.

MATERIALS AND METHODS

Organisms. The strains of *B. subtilis* that were used are shown in Table 1. Strain 168 sporulates normally in the presence of tryptophan and is referred to as the parent strain. The autolysin-defective strain FJ3, which grows as long chains (8), was frequently used instead since it sporulated as well as the parent and behaved in the same way with regard to the biochemical characters used to follow sporulation (23, 38). Strain 610 is a temperature-sensitive mutant defective in *N*-acetyl-L-aminopimelate deacylase (J. B. Ward, personal communication). DNA from this strain was used at a saturating concentration to transform strain FJ3 to methionine independence by the method of Piggot (26). Strain MB77 was selected from among the transformants as a strain that was temperature sensitive for growth. It grew as long chains at 34°C and sporulated as well as strain FJ3. At 42°C growth and sporulation were inhibited, but the cells did not autolyze. Strain Ni1 was used as a thymine-requiring strain since it is defective in prophage PBSX (33) and so is less prone to lysis when its DNA synthesis is inhibited. The sporulation mutants are classified as suggested by Piggot and Coote (27).

TABLE 1. *Strains of B. subtilis used*

Strain	Genotype[a]	Source	Reference
168	*trpC2*	J. Spizizen	35
FJ3	*metC3 lyt-1*	H. Rogers	8
610	*thyA thyB trpC2 dap*	M. Sargent	33
MB77	*lyt-1 dap*	FJ3 transformed with 610 DNA	
Ni1	*thyA thyB trpC2 mit*	M. Sargent	33
34.1	*leu-8 tal-1 spo0A34*	Stock strain	
43.3	*metC3 tal-1 spo0A43*	Stock strain	
17.2	*metC3 tal-1 spo0H17*	Stock strain	
46	*trpC2 spo0H46*	Stock strain	
93	*metC3 trpC2 rif-2 spo0J93*	Stock strain	
87.1	*metC3 tal-1 spo0J87*	Stock strain	
69.1	*trpC2 rif-2 spoIIA69*	Stock strain	
1	*trpC2 spoIIA1*	Stock strain	
70	*trpC2 spoIIB70*	Stock strain	
60.1	*leu-8 rif-2 spoIIE60*	Stock strain	
20.1	*trpC2 rif-2 spoIIE20*	Stock strain	
55.1	*leu-8 tal-1 spoIIG55*	Stock strain	
49.1	*leu-8 tal-1 spoIIG49*	Stock strain	
279.1	*metC3 tal-1 spoIIG279*	Stock strain	42

[a] The genotype abbreviations are those of Young and Wilson (41) and Piggot and Coote (27). New abbreviations are: *dap*, temperature sensitive for diaminopimelic acid synthesis; *tal*, β-thienylalanine resistance; and *lyt*, autolysin deficient.

Growth and sporulation. Cells were grown and induced to sporulate by the procedure of Sterlini and Mandelstam (36). This involved growth at 35°C with vigorous aeration in a hydrolyzed casein medium. When the culture density reached 0.25 mg (dry weight) per ml, the cells were centrifuged at room temperature and transferred to a poor nutritional medium containing L-glutamate and salts; full heat resistance developed in the culture 10 h after resuspension. Sporulation was found to be more extensive and synchronous if 1 ml of growth medium per 100 ml of sporulation medium was included. Times (in hours) after resuspension are referred to as t_0, t_1, etc., and the stages of sporulation are those suggested by Ryter (31) except that stage 0 and stage I were not distinguished. Culture density was determined turbidimetrically with a Unicam SP600 spectrophotometer.

Assays. Proteases were measured as described by Dancer and Mandelstam (7). A unit of specific activity was the amount of enzyme per milligram (dry weight) of cells that solubilized 1 mg of hide power azure (Calbiochem, Bishops Stortford, England) in 30 min at 37°C. Alkaline phosphatase was assayed by the method of Grant (13); specific activity was the amount of enzyme per milligram (dry weight) of cells that hydrolyzed 1 nmol of *p*-nitrophenol phosphate per min at 30°C. Manganese-dependent DNase was assayed as described by Akrigg (1). Glucose dehydrogenase and dipicolinic acid were determined by the methods of Sadoff et al. (32) and Janssen et al. (18), respectively.

Electron microscopy. The procedure of Kay and Warren (20) was used to fix and section cells. The sections were viewed in a Phillips 200 transmission electron microscope.

Measurement of heat resistance. Heat resistance was determined by heating 1-ml culture samples at 85°C for 15 min and plating suitable dilutions on nutrient agar.

Chemicals. Bacitracin, D-cycloserine, vancomycin, phenethanol, sodium deoxycholate, Triton X-100, colistine methane sulfonate, polymixin B sulfate, and gramicidin D were obtained from Sigma, Poole, England. Benzylpenicillin (Crystapen) was from Glaxo, Greenford, England. Gramicidin S and sodium taurodeoxycholate were from Calbiochem. Tween 80 was obtained from Koch-Light, Colnbrooke, England, tyrocidine was from the U.S. Biochemical Corp. Cleveland, Ohio, and cetyl trimethylammonium bromide was from Hopkins and Williams, Essex, England. Mecillinam was a gift from Leo Pharmaceutical Products, Copenhagen, Denmark. Fosfomycin was from Merck, Sharp & Dohme Research Laboratories, Rahway, N.J.

6-Hydroxyphenylazouracil was donated by B. Langley, I.C.I. Pharmaceuticals, Macclesfield, England.

RESULTS

Inhibition of sporulation by inhibitors of peptidoglycan synthesis. For each drug the concentration required to reduce the number of phase-bright spores to less than 1% of the number in the control was determined. Cultures of the parent strain were grown, divided into several portions, and transferred to sporulation medium containing various concentrations of the drugs. For penicillin and vancomycin the minimum inhibitory concentrations were 100 and 5 μg/ml, respectively. Both drugs at the same concentrations were then used to treat cells growing exponentially in hydrolyzed casein medium. They caused growth to stop and the cells to lyse.

The lysis-resistant strain FJ3 had the same sensitivity to the drugs in sporulation medium but did not lyse in growth medium. This indicated that the lysis in the parent strain was due to autolysin action in the absence of peptidoglycan synthesis.

D-Cycloserine, even at high concentrations (500 μg/ml), only delayed sporulation. A transient respone to the drug was seen in vegetative cultures, in which growth ceased after about 40 min but was renewed after about 3 h. This was presumably due to the inducible cycloserine resistance reported by Clark and Young (4).

Bacitracin prevented sporulation of the parent strain and strain FJ3 at 50 μg/ml. At this concentration growth in hydrolyzed casein medium was scarcely affected; i.e., the mean generation time increased from 40 to 45 min. If cells were grown in sporulation medium supplemented with either 0.2% glucose or 0.28% sodium lactate, a similar, slight effect on growth rate was observed when the cells were exposed to 50 μg of bacitracin per ml. If the concentration of the drug was raised to 200 μg/ml, growth ceased in less than a generation in all three growth media.

Morphological stage of blockage produced by the drugs. Cells of the parent strain or strain FJ3 were grown in casein hydrolysate medium and transferred to sporulation medium containing the minimum inhibitory concentration of a drug. Samples were taken at t_6 and prepared for electron microscopy. Vancomycin and penicillin blocked sporulation at stage 0–I and caused the cells to become long and narrow. Cells exposed to bacitracin were shorter and broader, and about 10% contained aberrant, multiple, subterminal septa which appeared to contain cell wall material. Such cells are commonly regarded as aberrant stage II sporangia (38). Cells treated at t_0 with the concentration of bacitracin necessary to inhibit growth (200 μg/ml) resembled those inhibited by the other drugs.

Times of escape from inhibition by the drugs. The parent strain or strain FJ3 was grown in casein hydrolysate medium and suspended in sporulation medium. At intervals after transfer, portions of the culture were treated with antibiotics; the final yields of phase-bright and heat-resistant spores were determined at t_7 and t_9, respectively. With bacitracin and vancomycin the time at which 10% of the cells escaped from sensitivity was t_2 to $t_{2.5}$ (Fig. 1). With penicillin, however, the cells were affected by the drug at later times so that most cells treated as late as t_4 to t_5 produced only phase-grey spores and only a few developed full phase brightness. With all three drugs the curves for phase brightness and heat resistance were similar.

Comparison of effects of inhibiting peptidoglycan synthesis by using temperature sensitivity and antibiotics. Similar experiments were then done in which the temperature sensitivity of MB77 was used in place of antibiotic treatment. The escape curve for temperature sensitivity of sporulation was similar to that obtained with antibiotics (Fig. 2), although the time for 10% escape above the "noise" level was a little earlier (about $t_{1.8}$). However, it was difficult to compare the two types of experiments directly because of the different temperatures involved. An estimate of the temperature effect

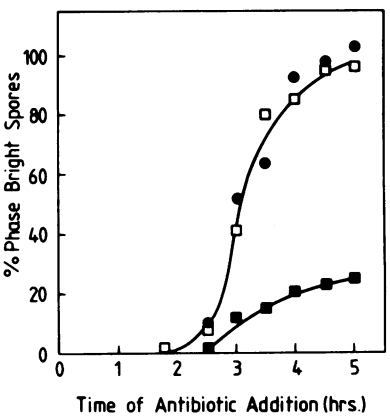

FIG. 1. *Escape from antibiotic inhibition of sporulation. A culture of strain FJ3 was transferred from hydrolyzed casein medium to sporulation medium. At intervals, 5-ml portions were exposed to either 50 μg of bacitracin per ml (●), 5 μg of vancomycin per ml (□), or 100 μg of penicillin per ml (■), and the incubation was continued. The numbers of phase-bright spores at t_7 were determined and expressed as a percentage of the number in a control flask that had not received antibiotic. The control flask typically contained about 80% spores.*

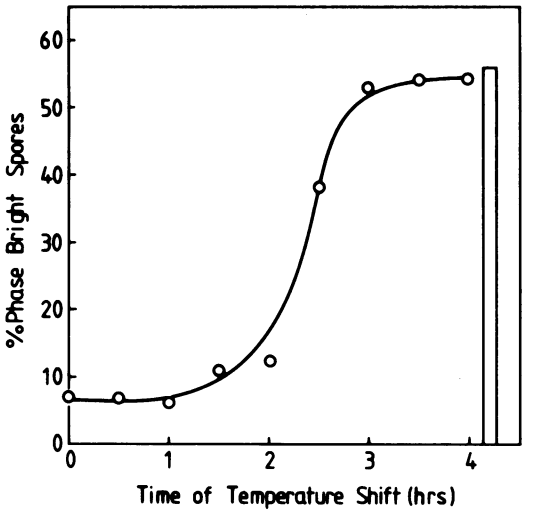

FIG. 2. *Escape of sporulation in a mutant temperature sensitive for peptidoglycan synthesis. A culture of strain MB77 was grown in casein hydrolysate medium and resuspended in sporulation medium at 34°C. At intervals, 5-ml samples were transferred to 42°C, and the incidence of phase-bright spores was determined at t_7. The bar shows the incidence in a culture maintained at 34°C throughout.*

could be obtained by comparison of the escape curve of temperature sensitivity with that of antibiotic sensitivity in strain MB77. A culture sporulating at 34°C was transferred to 42°C at $t_{2.5}$. At this time and at intervals thereafter portions of the culture were treated with bacitracin, vancomycin, or penicillin, each at its minimum inhibitory concentration. Subsequently, all of the cultures that had been incubated at 42°C contained the same percentage of phase-bright spores as did the untreated control (except those treated with penicillin, in which the spores became only phase grey, as described above). This experiment shows that at 42°C the escape points for bacitracin and vancomycin are the same or earlier than the escape point for temperature sensitivity.

Comparison of time of escape from antibiotic inhibition with time of escape from the effects of a temperature-sensitive sporulation mutation. Strain 279.1 is a temperature-sensitive mutant which at the restrictive temperature (42°C) is blocked at stage II of sporulation (prespore septation); at 34°C sporulation is normal, and growth is unaffected at either temperature (42). This strain was used in an attempt to define more closely the time at which cells escape the effects of the antibiotics.

A sporulating culture of strain 279.1 was transferred to 42°C at t_2. This culture contained about 50% phase-bright spores at t_7. If either bacitracin or vancomycin was added at the same time that the temperature shift was made, the incidence of phase-bright spores was less than 1% at t_7. Later addition of the drug allowed production of increasing numbers of spores until $t_{3.5}$, when the maximum was reached (Fig. 3). This indicates that the point of escape from antibiotic sensitivity is later than the point of escape for the sporulation mutation and so, by inference, later than prespore septation.

Escape from bacitracin inhibition of formation of phase-bright spores and production of manganese-stimulated endonuclease. If bacitracin inhibits sporulation up to the end of engulfment, then events occurring before that point would show a time of escape different from the time of escape for phase-bright spores. Production of Mn^{2+}-stimulated endonuclease is a late stage II event (2). Portions of a sporulating culture of strain FJ3 were exposed to bacitracin at t_0 and at intervals thereafter. Production of endonuclease was found to escape before production of phase-bright spores (Fig. 4). The 10% escape time for endonuclease was about $t_{1.5}$, and that for phase brightness was about $t_{2.2}$. This suggests that cell wall synthesis is required after the time of inhibition of endonuclease synthesis, i.e. toward the end of engulfment.

Biochemical events in sporulation. Cells of the parent strain or strain FJ3 were grown in hydrolyzed casein medium and transferred to sporulation medium containing the minimum inhibitory concentration of a drug. The effects

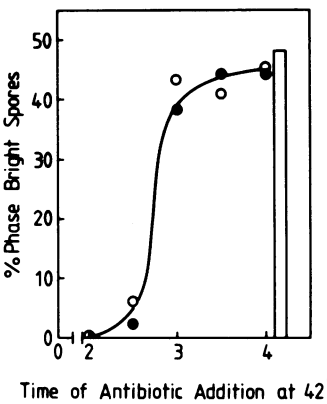

FIG. 3. *Escape time from antibiotic inhibition and from temperature sensitivity in strain 279.1. A culture (100 ml) was grown in casein hydrolysate medium and resuspended in sporulation medium at 34°C. At t_2 the culture was transferred to 42°C, and at intervals 5-ml samples were treated with either 50 μg of bacitracin per ml (○) or 5 μg of vancomycin per ml (●). The incidence of phase-bright spores was determined at t_7 and compared with that in a culture that had not received antibiotic (bar).*

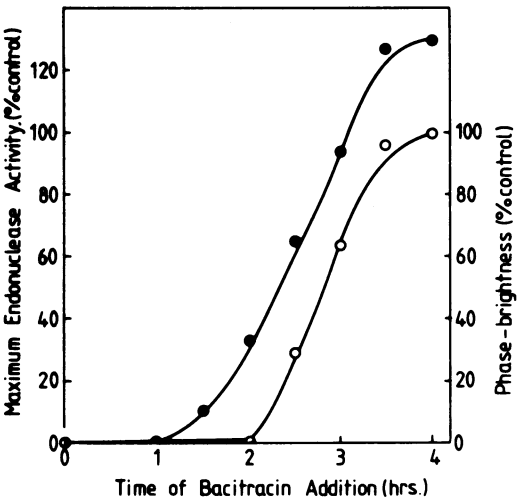

FIG. 4. *Comparison of escape time from bacitracin inhibition of production of Mn^{2+}-stimulated endonuclease and phase-bright spores. A culture of strain FJ3 was transferred to sporulation medium. At intervals 10-ml portions were treated with 200 μg of bacitracin per ml. Symbols: ●, endonuclease at t_7; ○, phase brightness at t_7.*

on synthesis of four sporulation-specific enzymes and dipicolinic acid were determined.

(i) Proteases. With bacitracin at 50 μg/ml the production of serine protease and metalloprotease was little affected (Fig. 5). However, bacitracin at 200 μg/ml, penicillin, and vancomycin depressed the rate of synthesis of protease (Fig. 5).

(ii) Alkaline phosphatase. This enzyme, associated with the transition from stage II to stage III, was not produced in the presence of penicillin. With bacitracin and vancomycin the enzyme was produced about 1 h earlier than it was in the control culture (Fig. 6). With penicillin the production of the enzyme was inhibited.

(iii) Other biochemical markers of spor-

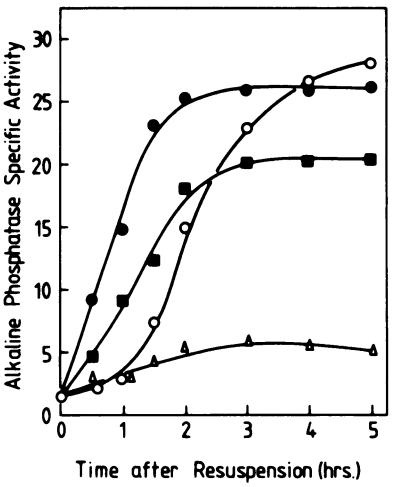

FIG. 6. *Alkaline phosphatase production in the presence of antibiotics. A culture of strain FJ3 was suspended in sporulation medium. One portion was left as a control, and other portions were exposed to the drugs; the subsequent production of alkaline phosphatase was followed. Symbols: ○, control; ●, 50 μg of bacitracin per ml; ■, 5 μg of vancomycin per ml; △, 100 μg of penicillin per ml.*

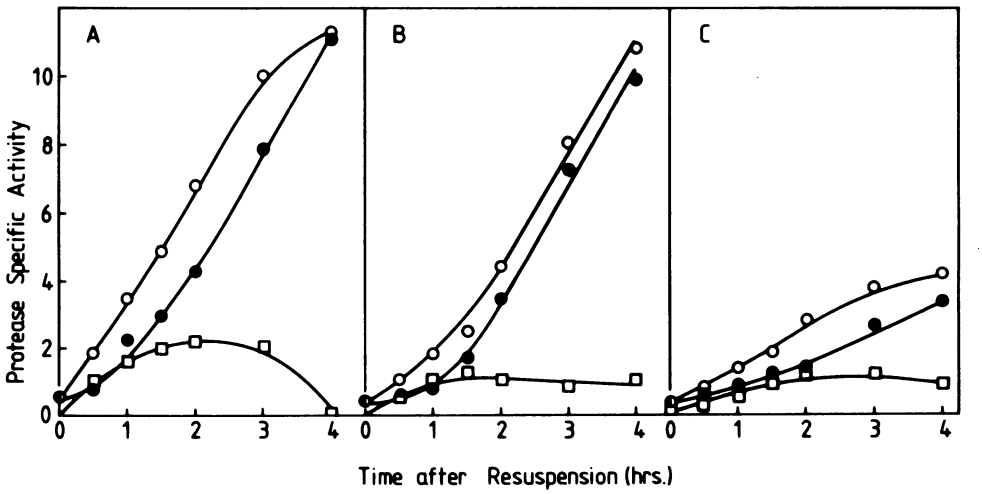

FIG. 5. *Extracellular protease production in the presence of antibiotics. A culture of strain FJ3 was suspended in sporulation medium. One portion was left as a control (A), a second received 50 μg of bacitracin per ml (B), and a third received 5 μg of vancomycin per ml (C). The subsequent production of protease was followed. Symbols: ○, total protease; ●, serine protease; □, metalloprotease.*

ulation. With bacitracin, vancomycin, or penicillin the Mn^{2+}-stimulated endonuclease (2) was not produced. Similarly, glucose dehydrogenase (stage III) and dipicolinic acid (stage IV) were not detectable.

Biochemical events in MB77. Cultures of MB77 were transferred to sporulation medium and incubated at 42°C. The pattern of biochemical events was compared with that in a control culture kept at 34°C throughout. Cells at 42°C produced protease for only 1 h, and alkaline phosphatase was produced in much smaller amounts than in the control (Fig. 7). This is similar to the effects observed with penicillin. Like the parent strain treated with any of the antibiotics, MB77 at 42°C produced no Mn^{2+}-stimulated endonuclease, glucose dehydrogenase, or dipicolinic acid.

Production of alkaline phosphatase in sporogenic cells exposed to bacitracin. When cells of the parent strain or strain FJ3 were transferred to sporulation medium containing bacitracin, alkaline phosphatase was produced immediately rather than after a 1- to 2-h delay. The effect was not the result of too low a concentration of bacitracin since even 200 μg/ml (which stops sporulation at stage 0) allowed similar production of enzyme. If bacitracin was added to cultures at intervals after resuspension, alkaline phosphatase was induced immediately after the addition (Fig. 8A). If the cells were already producing the enzyme (after $t_{1.5}$), the drug caused no further synthesis over that in the control (Fig. 8B).

Derepression of phosphatase synthesis by bacitracin occurred only in the sporulation medium. Growth in hydrolyzed casein medium or the sporulation medium supplemented with either 0.2% glucose or 0.28% sodium lactate was only slightly affected by 50 μg of bacitracin per ml, and phosphatase was produced at the same low rate (39) as in the control.

Mandelstam et al. (24) showed that sporulation-specific events were inhibited by thymidine starvation. Therefore, it was of interest to determine whether alkaline phosphatase production in cells treated with bacitracin was also inhibited in cells deprived of thymidine. Alkaline phosphatase production in the thymine-requiring strain Nil in cultures containing bacitracin began soon after resuspension and ended at about t_2 (Fig. 9A). The culture supplemented only with thymidine produced the enzyme at the usual time, whereas the culture deprived of thymidine showed no production. Similar results were obtained if DNA synthesis was inhibited by 50 μg of 6-hydroxyphenylazouracil per ml instead of by thymidine starvation (Fig. 9B). This drug is a specific inhibitor of DNA polymerase III (22). The rapid production of phosphatase in the presence of bacitracin must be assumed to be due to de novo synthesis of protein since 50 μg of chloramphenicol per ml added at the same time completely inhibited enzyme synthesis. Production of the enzyme was not inhibited, however, by resuspension in sporulation medium containing 10 mM phosphate (data not shown), indicating that the enzyme was under "sporulation-type" control and had not been derepressed by inadvertant phosphate starvation (12).

Alkaline phosphatase production in sporulation mutants. Mutants blocked at stage 0 of sporulation and those blocked early in stage II (*spoIIA*, *spoIIE*, *spoIIF*, and *spoIIG*)

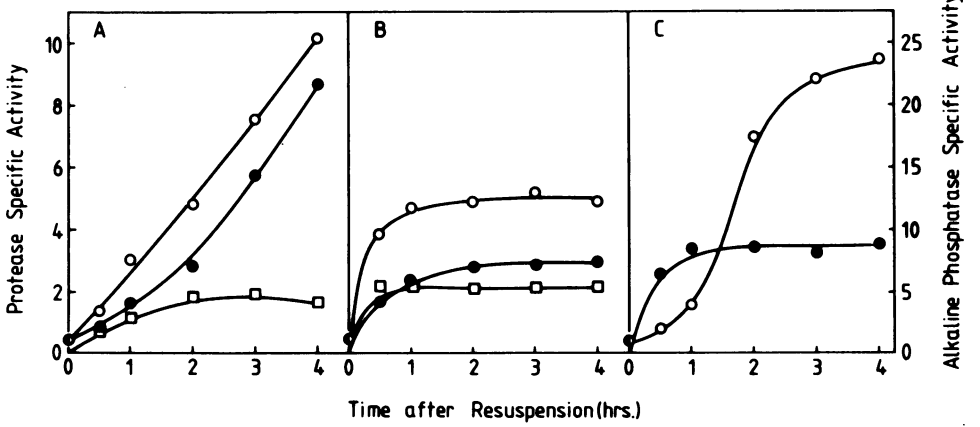

FIG. 7. *Production of protease and alkaline phosphatase in MB77 at 34 and 42°C. A culture of strain MB77 was suspended in sporulation medium. One portion was incubated at 34°C, and another was incubated at 42°C. The production of protease and the production of alkaline phosphatase were followed. (A) Protease at 34°C. (B) Protease at 42°C. Symbols: ○, total protease; ●, serine protease; □, metalloprotease. (C) Alkaline phosphatase. Symbols: ○, 34°C; ●, 42°C.*

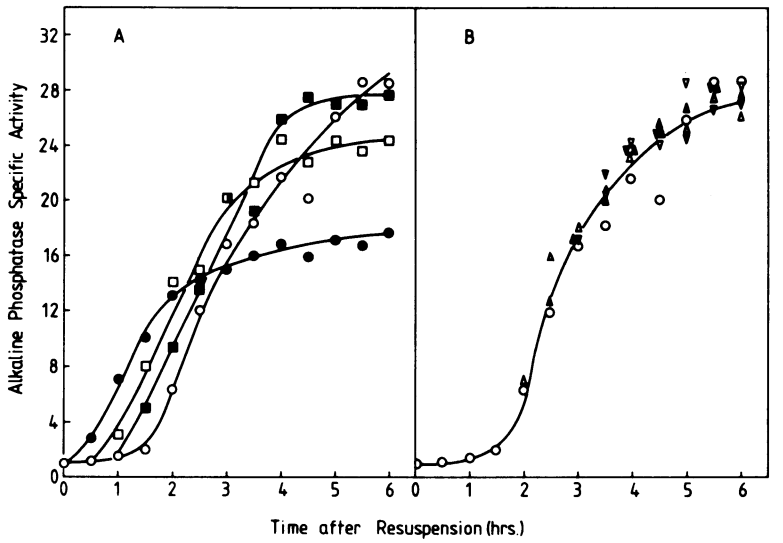

FIG. 8. *Effect of time of addition of bacitracin on production of alkaline phosphatase. A culture of the parent strain was suspended in sporulation medium. Portions were treated with 50 μg of bacitracin per ml, incubation was continued, and phosphatase production was followed. Cultures were exposed to the drug before (A) and after (B) commencement of synthesis of the enzyme in the control. Symbols: ○, control; bacitracin at* t_0 (●), $t_{0.5}$ (□), t_1 (■), $t_{1.5}$ (△), t_2 (▲), $t_{2.5}$ (▼), *and* t_3 (▽).

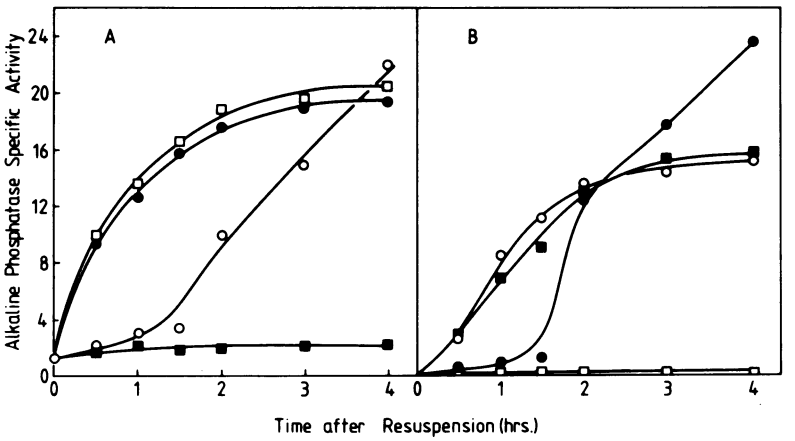

FIG. 9. *Effect of inhibition of DNA and protein syntheses on phosphatase production with bacitracin. Alkaline phosphatase production was measured in sporulating cultures of strain Ni1 treated with 200 μg of bacitracin per ml and either (A) deprived of thymidine or (B) treated with 50 μg of 6-hydroxyphenylazouracil per ml or 50 μg of chloramphenicol per ml. (A) Symbols: ○, +thymidine, −bacitracin; ●, +thymidine, +bacitracin; □, −thymidine, +bacitracin; ■, −thymidine, −bacitracin. (B) Symbols: ○, bacitracin alone; ●, control; ■, +6-hydroxyphenylazouracil, +bacitracin; □, +chloramphenicol, +bacitracin.*

are unable to produce alkaline phosphatase under sporulation conditions (28, 38). However, when such mutants were treated with bacitracin at t_0, all were able to make the enzyme (Fig. 10). Strains with mutations in *spo0A*, *spo0H*, *spo0J*, *spoIIA*, *spoIIE*, and *spoIIG* loci showed substantially the same behavior. The cells did not sporulate or produce other biochemical markers of sporulation (i.e., dipicolinic acid, Mn^{2+}-stimulated endonuclease, and glucose dehydrogenase). Those strains that did not usually produce extracellular protease still did not produce it in the presence of bacitracin.

Alkaline phosphatase was induced at any time after resuspension, although with longer preincubation times before the drug was added the response became weaker (Fig. 10).

Effect of preincubation of cells with bac-

itracin. It was possible that the effect of bacitracin on alkaline phosphatase synthesis was due to incomplete inhibition of the cells for the first hour or so of sporulation. If this were true, it would be expected that incubation with bacitracin in the growth medium would prevent enzyme synthesis in the sporulation medium. Cells of strain FJ3 were grown with and without bacitracin. After transfer to sporulation medium, each of the cultures was divided again and one-half was treated with bacitracin. Preincubation with bacitracin during growth prevented the stimulation of phosphatase production by the drug during sporulation (Fig. 11A). Similar results were obtained with the *spo0A* mutant strain 43.3 (Fig. 11B).

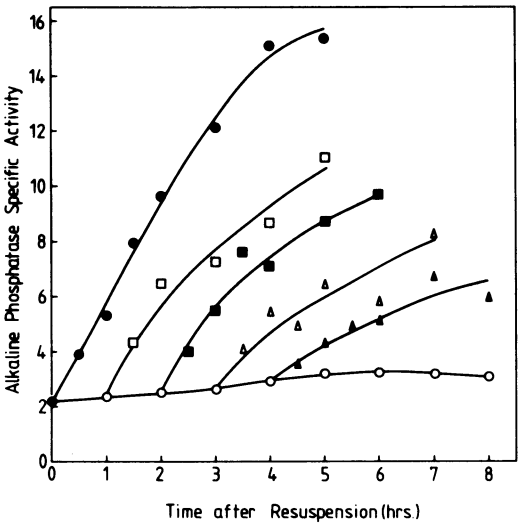

FIG. 10. *Effect of time of addition of bacitracin to a culture of spo0A mutant 43.3 in sporulation medium. A culture of strain 43.3 was suspended in sporulation medium. Portions were treated with 200 μg of bacitracin per ml, and alkaline phosphatase production was followed. Symbols: ○, control; bacitracin at* t_0 (●), t_1 (□), t_2 (■), t_3 (△), *and* t_4 (▲).

Enrichment of sporulation medium with casein hydrolysate. Alkaline phosphatase production under sporulation conditions differs from production under phosphate limitation in that it is repressible by casein hydrolysate. Thus, if alkaline phosphatase produced in the presence of bacitracin is under sporulation-type control, then it should be repressible by casein hydrolysate. The parent strain was grown in casein hydrolysate medium and resuspended in sporulation medium with and without bacitracin. At intervals thereafter casein hydrolysate was added to portions of the cultures, and the subsequent production of phosphatase was determined. In all cultures treated with hydrolyzed casein, further production of the enzyme was inhibited. Synthesis was resumed after 3 to 4 h when the casein hydrolysate became exhausted. This is exemplified by the addition of casein hydrolysate at $t_{0.75}$ (Fig. 12). Cultures exposed to bacitracin behaved in the same way as those

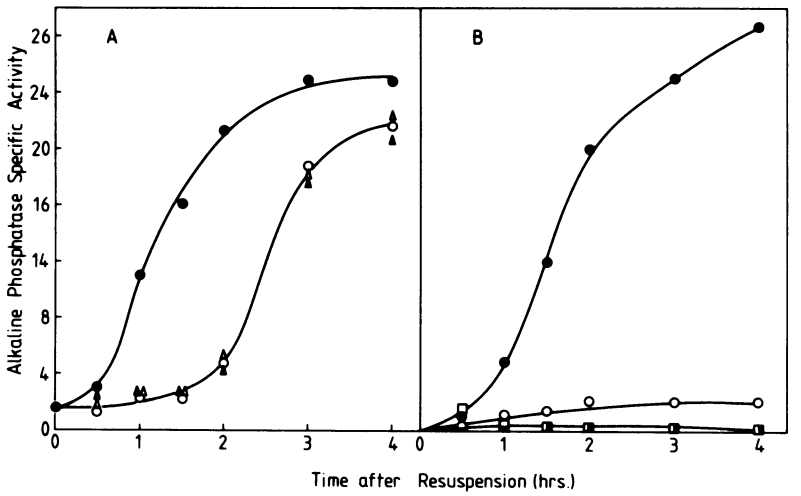

FIG. 11. *Effect of pretreatment of cells with bacitracin. Cultures (40 ml) of strains FJ3 and 43.3 (spo0A) were grown to 0.06 mg (dry weight) per ml, and then one-half of each culture was treated with 50 μg of bacitracin per ml. When the culture densities reached 0.25 mg (dry weight) per ml, the cultures were resuspended in sporulation medium with and without 50 μg of bacitracin per ml. Alkaline phosphatase production was followed after resuspension. (A) Strain FJ3. (B) Strain 43.3. Symbols: ○, control; ●, bacitracin in sporulation medium; △, bacitracin in growth medium; ▲, bacitracin in growth medium and sporulation medium.*

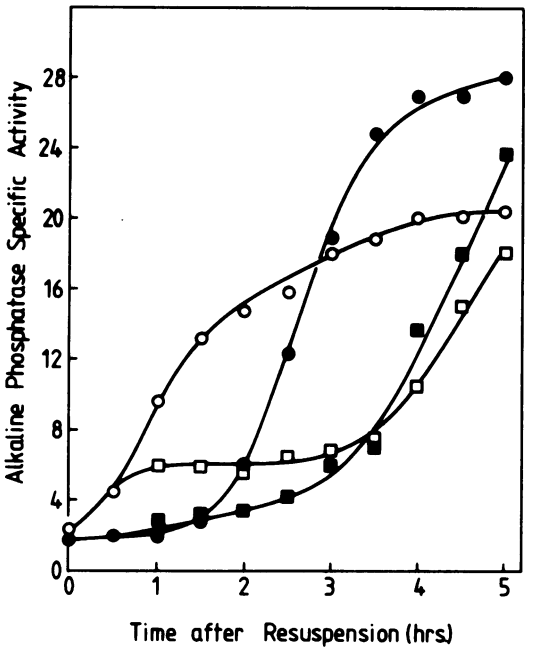

FIG. 12. *Effect of enrichment of sporulation medium with casein hydrolysate. A culture of the parent strain was grown in hydrolyzed casein medium, halved, and suspended in sporulation medium with and without 50 μg of bacitracin per ml. At t_0 and at 15-min intervals thereafter, a sample of each culture was transferred to another flask and 0.1 volume of 10% (wt/vol) casein hydrolysate was added. Incubation was continued, and alkaline phosphatase activity was followed. For simplicity only one point for hydrolyzed casein addition is shown. Symbols: ●, control; ○, +bacitracin; ■, control +hydrolyzed casein at 45 min; □, +bacitracin, +hydrolyzed casein at 45 min.*

that had not received the drug.

Effects of other antibiotics and surfactants on alkaline phosphatase production. Bacitracin is a cyclic oligopeptide with an acyl side chain that gives it detergent properties. Therefore, I tested the effects of other antibiotics and surfactants on enzyme production. Cultures of the parent strain were exposed to the test compound at t_0; the concentration used was the minimum concentration inhibitory for sporulation, except that antibiotics were used at a maximum of 100 μg/ml. The antibiotics and concentrations (in micrograms per milliliter) used were: colistin, 100; polymixin B, 20; mecillinam, 100; gramicidin S, 5; gramicidin D, 100; fosfomycin 100; and tyrocidine, 1. The surfactants used were 0.3% (vol/vol) phenethanol, 3% (vol/vol) ethanol, 0.05% (wt/vol) sodium taurodeoxycholate, 0.5% (wt/vol) sodium deoxycholate, 0.05% (vol/vol) Tween 80, 0.05% (wt/vol) sodium lauryl sarcosinate, 0.05% (wt/vol) cetyl trimethylammonium bromide, and 0.5% (vol/vol) Triton X-100. Of these, all inhibited sporulation except Tween 80, colistin, and gramicidin D. With regard to alkaline phosphatase production, only taurodeoxycholate caused early production of the enzyme; the other compounds either were without effect or inhibited production of the enzyme completely.

The premature alkaline phosphatase production with taurodeoxycholate was less than that with bacitracin and about the same as that with vancomycin (Fig. 13). Enzyme induction with taurodeoxycholate was likely to be due to a mechanism similar to that caused by the antibiotics since the detergent caused alkaline phosphatase production in strain 43.3 (*spo0A*) in sporulation medium which was abolished if the cells were preincubated with the detergent in the growth medium.

DISCUSSION

The experiments with antibiotics and with strain MB77 confirm earlier observations (14, 15, 16, 21) that there is a requirement for peptidoglycan synthesis early in sporulation. The period of sensitivity to inhibition of cell wall synthesis is from t_0 to about $t_{2.5}$ under the conditions used here. The cells escape from sensitivity to inhibition of peptidoglycan synthesis about 1 h later than the escape time of a temperature-

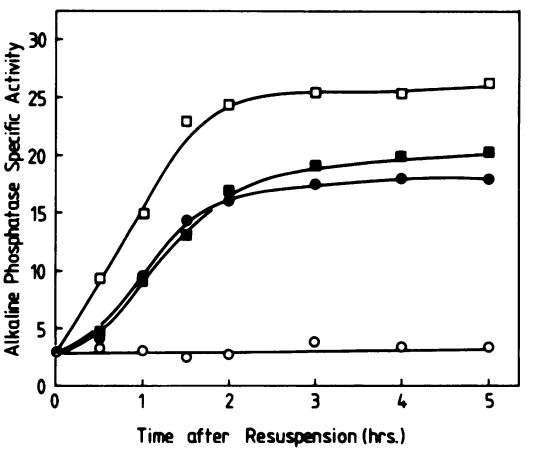

FIG. 13. *Comparison of effects of taurodeoxycholate, bacitracin, and vancomycin on alkaline phosphatase production in strain 43.3. A culture of strain 43.3 was grown in casein hydrolysate medium and suspended in sporulation medium. Portions of the culture were immediately exposed to either 0.05% taurodeoxycholate, 50 μg of bacitracin per ml, or 5 μg of vancomycin per ml. Alkaline phosphatase production was followed. Symbols: ○, control; ●, +detergent; □, +bacitracin; ■, +vancomycin.*

sensitive sporulation mutant which, at the restrictive temperature, is blocked at stage II. This later time corresponds, approximately, with the end of engulfment. The production of the Mn^{2+}-stimulated endonuclease escaped from antibiotic inhibition before the ability to form phase-bright spores. As the endonuclease is considered a later stage II event (2), this provides good evidence that cell wall synthesis is required during engulfment. These results are consistent with those of Fitz-James (9), who showed that protoplasts of sporulating *B. megaterium* were only able to complete sporulation if they had reached stage II (free prespore) before removal of the cell wall.

The required peptidoglycan synthesis could be due to surface extension, cell division, and/or incorporation into the prespore septa and the intermembrane space during engulfment. Presumably, no further sporangial extension is necessary afterwards. It is noteworthy that strain FJ3 sporulates as well as the parent strain. This shows that the vegetative autolysins have no essential role in sporulation and that either cell wall need not be turned over during sporulation or sporulation-specific enzymes are induced to carry out this function.

The escape time from inhibition of sporulation, as judged by appearance of visible spores under phase-contrast microscopy, was the same for penicillin, bacitracin, vancomycin, and strain MB77. With the exception of penicillin, the cells then developed fully phase-bright, heat-resistant spores. With penicillin, however, the spores became only weakly phase bright (phase grey) and did not develop full heat resistance. This suggests that penicillin also inhibits a later process in sporulation that is unaffected by the other treatments. This might well be cortex synthesis. Nevertheless, it is surprising that penicillin inhibits this later event when the other treatments do not. Bacitracin and vancomycin are both lipophilic compounds. It is possible that after engulfment is completed the forespore is protected from these drugs by the sporangial membrane. Penicillin, however, may cross the sporangial membrane and affect cortex synthesis in the forespore. This explanation is incomplete since the temperature sensitivity of strain MB77 would be expected to continue after engulfment; however, in fact it did not.

The combination of morphological and biochemical evidence indicates that when peptidoglycan synthesis was inhibited at t_0, the cells mostly remain at stage 0–I of sporulation. Even extracellular protease synthesis (a very early stage 0 event) was partly inhibited. However, the minimum concentration of bacitracin allowed development to stage II, and this was accompanied by full derepression of the proteases.

The production of alkaline phosphatase when cells were in sporulation medium containing bacitracin or vancomycin was anomalous in that the cells were blocked at stage 0-I (i.e., before alkaline phosphatase synthesis was normally derepressed) and synthesis was immediate rather than after a 1- to 2-h delay. Moreover, strains that would not normally produce the enzyme in sporulation conditions became able to do so in the presence of the drugs. The enzyme was under sporulation-type control since its synthesis was repressible by casein hydrolysate and not repressed by inorganic phosphate. Also, the amount of enzyme produced was similar to the amount produced under sporulation conditions, i.e. about 5% of the activity produced in phosphate-limited cultures. Sporulation-associated events are normally inhibited by prevention of DNA synthesis (24). However, bacitracin-induced alkaline phosphatase production was not inhibited by either thymidine starvation or 6-hydroxyphenylazouracil treatment.

Bacitracin and vancomycin act on membrane components (undecaprenyl phosphate derivatives). It was, therefore, of interest to investigate the effect of other membrane-active compounds. However, all of the other antibiotics used had no specific effect on synthesis of alkaline phosphatase, although some of them inhibit sporulation in general. A similar lack of effect was general with the detergents tested. Taurodeoxycholate was the exception. This compound seemed to act in a way similar to bacitracin and vancomycin with regard to its effects on alkaline phosphatase synthesis.

The role of alkaline phosphatase in sporulation has never been clear. It could be regarded as a scavenging enzyme (29) like protease, amylase, etc., providing dephosphorylated, assimilable carbon compounds. However, the other enzymes of this type are produced immediately in starvation conditions whereas alkaline phosphatase synthesis is delayed to stage II–III of sporulation in *B. subtilis* and even later in other species. As a marker event in sporulation, the enzyme is somewhat unreliable under nonstandard conditions. Thus, Coote (5) caused cells to produce alkaline phosphatase out of sequence in experiments with 5-bromodeoxyuridine. Moreover, Piggot and Taylor (28) obtained *sap* mutants in which the enzyme was produced with kinetics different from those of the wild-type strain and enzyme production was allowed in a *spo0* background. The effects shown here are similar.

Experiments with gram-negative bacteria

have suggested that alkaline phosphatase may be involved in septation. Bhatti et al. (3) obtained a temperature-sensitive mutant of *Pseudomonas aeruginosa*. A culture was kept at the restrictive temperature for a time to produce aseptate filaments. When the temperature was decreased so that septa formed, alkaline phosphatase synthesis was initiated. It is possible that a similar effect occurs during sporulation since the enzyme appears at stage II to stage III, i.e., prespore septation and engulfment. Thus, it is possible to imagine that bacitracin and vancomycin, by affecting cell wall synthesis, inhibit septation and upset the control of alkaline phosphatase production.

Previous studies on biochemical markers of sporulation have used alkaline phosphatase as a marker in epistasis experiments (6). The results presented here and elsewhere (28) indicate that the production of this enzyme in sporulating cultures should be viewed with caution as a marker.

ACKNOWLEDGMENTS

I am grateful to J. Mandelstam for his help and encouragement and to D. Torgersen for her skilled technical assistance.

This work was supported by a grant from the Guinness Trust.

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