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Binding of Radioactive Benzylpenicillin to Asporogenous Mutants of *Bacillus subtilis* During Postexponential Growth

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The specific penicillin binding capacity of a postexponential culture of *Staphylococcus aureus* remains constant, but that of a sporulating *Bacillus subtilis* culture fluctuates dramatically. An initial decrease in binding capacity during presporulation events is followed by two distinct intervals of enhanced specific binding capacity during the postlogarithmic growth of a sporulating *B. subtilis* culture. The first peak of enhanced binding occurs during septation, when enzymes for germ cell wall formation are present; and the second peak coincides with cortical biosynthesis. The specific postlogarithmic binding capacities of a number of Spo$^-$ mutants of *B. subtilis* were examined to ascertain if specific asporogenous mutations altered the binding pattern observed with the wild-type organism. Four distinct postexponential binding patterns were recognized: (i) a low, constant binding capacity resembling the binding pattern of *S. aureus*, (ii) a decrease in binding capacity with no subsequent significant peaks, (iii) a decrease in binding capacity followed by a single peak corresponding to the first peak seen with the wild type, (iv) a pattern similar to the wild type. The fourth pattern was observed in a mutant blocked during stage III of sporogenesis which produced forespores that never became refractile. Mutations blocking either one or both periods of enhanced postlogarithmic binding were interspersed throughout a linkage group of spore genes next to *lys-2* on the *B. subtilis* chomosome.

The covalent binding of radioactive benzylpenicillin to intact bacterial cells (6, 7, 10, 21, 22, 25) and to bacterial membrane preparations (19, 20) has been extensively studied. These studies were confined primarily to either exponentially growing bacteria or to their membrane fragments since penicillins exert their bacteriocidal effects on rapidly growing cultures. The binding studies and investigations which elucidated the detailed chemical structure of bacterial cell wall peptidoglycan (PG) and its biosynthesis established that penicillins exert their bacteriocidal effect by inhibiting the terminal steps in the biosynthesis of functional cell wall PG. Specifically, bound penicillins have been shown to inhibit the glycopeptide transpeptidase activity of intact cells (30, 36; Fed. Proc. **25**:344, 1966) or of membrane particles (1, 2, 17, 18). Inactivation of this enzyme is dependent upon the irreversible binding of the antibiotic (J. T. Park and E. M. Wise, Int. Congr. Biochem., 7th, Abstr. D-44, 1969). The nature of this inhibition supports the hypothesis that penicillin binds directly to the glycopeptide transpeptidase. Bound penicillin also irreversibly inhibits a D-alanine carboxypeptidase in intact *Bacillus subtilis* cells (3) and in membrane preparations from this organism (20). The particulate D-alanine carboxypeptidase activity seen in *B. subtilis* Porton can function as a modified transpeptidase (Jay Umbreit, personal communication). The irreversible inhibition of the transpeptidase from *Escherichia coli* and the D-alanine carboxypeptidase in *B. subtilis* by bound penicillin supports the suggestions that penicillin is a substrate analogue of a D-alanyl-D-alanine residue at the carboxyl end of a pentapeptide chain in a PG polymer before transpeptidation or the removal of an alanine residue (29). To date, these represent the only bacterial enzymes known to be inhibited by

bound penicillins. Membranes from *B. subtilis* appear to contain more than one penicillin-binding protein, but at least 70% of the penicillin bound to the membranes is covalently attached to the D-alanine carboxypeptidase (3; P. M. Blumberg, Fed. Proc. 31:841, 1972). The quantity of antibiotic bound to the transpeptidase has not yet been established. Nonetheless, the transpeptidase and the D-alanine carboxypeptidase are acylated by penicillin, and bound radioactive antibiotic provides an estimate of the quantity of enzymes which function in the terminal stages of PG synthesis.

Bacterial sporulation has been described as a modified or atypical cell division (14). The sporulation process involves an orderly sequence of seven clearly defined morphological stages (14). Stage II in this sequence is characterized by the formation of the forespore septum which together with a portion of the terminal cell membrane forms the inner forespore membrane (IFSM) (32). During septation (stage II) and presumably during envelopment (stage III), it is believed that the IFSM contains the germ cell wall-synthesizing enzymes which function in PG polymerization (32). The germ cell wall is a structural layer made during sporulation which develops into the vegetative cell wall of the newly germinated cell (32). The fourth stage in the sporulation process includes the biosynthesis of a mucopeptide polymer called the spore cortex. This polymer is believed to be synthesized by the outer forespore membrane (OFSM) (32). Penicillin and other antibiotics which inhibit cell division by interfering with functional PG synthesis can prevent spore septum formation (13) and cortex biosynthesis (11, 23) if added to a sporulating culture during the appropriate time intervals. Because of the obvious importance of the penicillin target proteins in the bacterial sporulation process, the binding of radioactive benzylpenicillin to sporulating cultures of *B. subtilis*, *B. megaterium*, and *B. cereus* was examined and compared to a vegetative culture of *Staphylococcus aureus* during the stationary phase of growth (21). This study indicated that the binding of the radioactive antibiotic to the sporulating cultures is chemically similar to that of both vegetative cells of *S. aureus* and membrane fragments derived from a vegetative culture of *B. subtilis*. Nonetheless, distinct differences were noted in the specific binding patterns of the sporulating organisms as compared to that of *S. aureus* in the stationary phase of growth over a comparable time period. The specific binding activity of the *S. aureus* cells in the stationary phase of growth is essentially the same as that of vegetative cells, and the binding activity remains constant over a 9-h stationary period. Alternatively, sporulating cells of *B. subtilis*, *B. megaterium*, and *B. cereus* exhibit two intervals of enhanced specific binding capacity during their postlogarithmic phase of growth (21). The reproducible fluctuations in a specific binding activity of sporulating cultures as contrasted with the constant binding activity of a nonsporulating organism during postlogarithmic growth agrees with other observations which conclude that functional PG biosynthesis is required during two distinct phases of sporulation (5, 11, 13, 23, 31-34).

Numerous genes associated with sporulation have been mapped on the chromosome of *B. subtilis* (15, 16, 27, 37). The control mechanisms which regulate spore gene expression are poorly understood, and most of the products of these genes remain unidentified. The importance of the penicillin target proteins in the overall sporulation process (11, 13, 23) and the observed fluctuations in specific binding capacity of sporulating bacteria (21) suggested that some genes may function by regulating the synthesis of enzymes required for PG synthesis in either the germ cell wall or in the cortex and that specific mutations might prevent functional regulation of these genes. Therefore, asporogenous mutants genetically blocked prior to germ cell wall synthesis would not be expected to produce enhanced postlogarithmic binding, whereas mutants blocked after germ cell wall synthesis would be expected to produce either one or both enhanced postlogarithmic binding increments. The specific binding capacities of a number of Spo^- mutants of *B. subtilis* during their postlogarithmic phase of growth were examined to ascertain if specific asporogenous mutations altered the binding pattern previously seen with the wild-type organism.

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MATERIALS AND METHODS

Organisms. The sporulation mutants SpoIII 7Z and SpoIII 94U were isolated by Ionesco et al. from the *B. subtilis* strains GSY 184 and 168, respectively (16). The other sporulation mutants used in these studies were isolated from *B. subtilis* Marburg according to procedures described elsewhere (27). The asporogenous mutants have been classified according to the scheme of Young and Wilson (37).

Growth and media. The mutants and wild type were grown in 14 liters of sporulation medium at 37 C

with forced aeration in a 20-liter carboy. The medium used was that described by Donnellan et al. (9) modified as described previously (21) and adjusted to a final pH of 7.2. Under these conditions the wild-type organism showed optimal sporulation. The inoculum consisted of two 300-ml samples of an 8-h culture of the test organism grown in Spizizen minimal medium (28). Each sample was grown in a separate 2,800-ml Fernbach flask in a New Brunswick Scientific gyrotory shaker at 175 rpm prior to inoculation of the carboy. Samples (500–1,000 ml) were taken from the carboy at appropriate intervals, harvested, washed, resuspended, and frozen according to the procedures used previously (21). Nonrefractile forespores, sporangia, and spores were distinguished by dark contrast phase microscopy.

Binding assay. The binding of ^{14}C-benzylpenicillin (Amersham Searle, specific activity: 25 Ci/mole) to the wild-type organism and to the asporogenous mutants was determined by the procedure used previously (21). Specific binding capacity is expressed as nanomoles of ^{14}C-benzylpenicillin bound per gram dry weight of cells or picomoles bound per milligram of protein. Protein was determined by the method of Lowry et al. (24).

Genetic analysis. The map distance of SpoIII 7Z to *lys-2* was determined by PBS-1 mediated transduction. Details of the transduction procedure have been described elsewhere (26).

RESULTS

Chemistry of ^{14}C-benzylpenicillin binding to sporulating B. subtilis Marburg cells and asporogenous mutants. Vegetative and sporulating cultures of *B. subtilis* Marburg and the asporogenous mutants tested exhibit penicillin-binding properties similar to that of previously studied vegetative cultures of *S. aureus* (7, 10, 21, 22), membrane fragments of *B. subtilis* (19), and sporulating cultures of *B. subtilis* Porton, *B. megaterium*, and *B. cereus* (21). The antibiotic concentration required for half maximum binding (Fig. 1) to vegetative (2-h culture) *B. subtilis* Marburg cells (0.20 μg/ml) is similar to that required for intact sporulating *B. subtilis* Porton cells (21) and membrane fragments obtained from vegetative cells of the same organism (19). The concentration of ^{14}C-benzylpenicillin required for half maximal binding to sporulating cultures (16 h) of this organism is approximately 0.6 μg/ml (Fig. 1). The reasons for this difference are not clear. In the following experiments, cells were exposed to the antibiotic at a final concentration of 1.3 μg/ml, a concentration which produces maximal binding in both cell populations. As was seen with sporulating cultures of *B. subtilis* Porton, *B. megaterium* and vegetative *S. aureus* cells (21), unlabeled semisynthetic penicillins compete with radioactive benzylpenicillin for the available binding sites in sporulating *B. subtilis* Marburg cells and in Spo$^-$ mutants during the stationary phase of growth. An intact β-lactam ring is required for binding, since unlabeled benzylpenicilloic acid and benzylpenilloic acid do not prevent binding of radioactive penicillin G to any of the organisms tested (P. Lawrence, unpublished results).

Binding of ^{14}C-benzylpenicillin to sporulating B. subtilis Marburg cells. The specific binding pattern of wild-type *B. subtilis* Marburg cells is similar to that previously described for *B. subtilis* Porton, *B. megaterium*, and *B. cereus* (21). The specific binding capacities of Spo$^+$ *B. subtilis* cultures prior to 5 h of growth remains constant (P. Lawrence, unpublished results). However binding decreases substantially during presporulation and early sporulation events but the decrease is not continuous (Fig. 2). After the initial decrease, two intervals of enhanced specific binding capacity are invariably seen. The first increase (9.5 h) occurs before the appearance of refractile endospores, whereas the second (14 h) occurs at the onset of refractility and is believed to represent the presence of the terminal enzymes for cortical PG biosynthesis. In sporulating *B. megaterium* cultures the initial increase in specific binding activity is concurrent with septation (21). Sporulation septa could be detected in sporulating cultures of *B. subtilis* neither with Normarski optics nor with the staining techniques of Gordon and Murrell (12), but septum formation is believed to coincide with the first peak of enhanced specific binding activity. When forespores were observed in sporulating cultures, they always appeared after the first binding increment (Fig. 5).

Penicillin binding characteristics of mutants SpoI 4AC and Spo 1AC. The SpoI 4AC and Spo 1AC markers are linked by PBS-1 mediated transduction to *hisA* (27). The mutants produce the spore-dependent protease and antibiotic present during stage O of sporogenesis but not dipicolinic acid made during stage IV (27), suggesting that these mutants are blocked after stage O but prior to stage IV of sporulation. The mutant SpoI 4AC initially simulates the wild-type binding pattern showing a decrease in specific binding capacity during the presporulation and early sporulation events (Fig. 3). However, the decrease is continuous and not followed by the periods of enhanced binding found in the wild-type organism at stage II and stage IV. In previous studies this mutant was determined by elec-

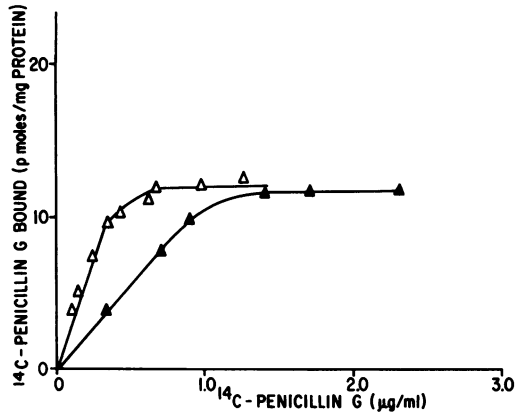

FIG. 1. *Effect of ^{14}C-penicillin G concentration on the extent of binding to Bacillus subtilis Marburg cells. Symbols: Δ, 2-h vegetative cultures; ▲, 16-h sporulating culture.*

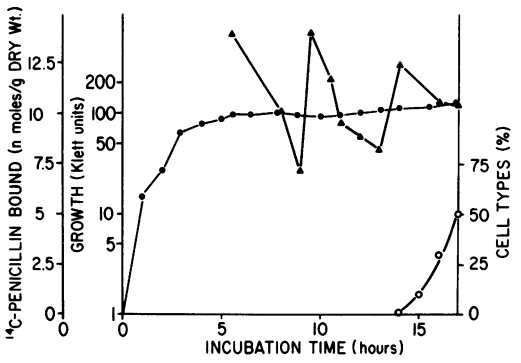

FIG. 2. *^{14}C-penicillin G-binding capacity of a sporulating wild-type B. subtilis Marburg strain. Symbols: ●, growth; ▲, penicillin binding; ○, refractile forms.*

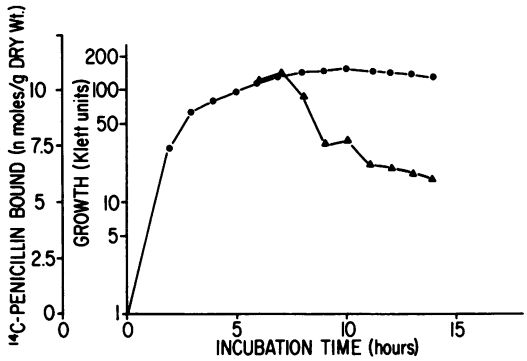

FIG. 3. *^{14}C-penicillin G-binding capacity of mutant SpoI 4AC during postlogarithmic growth. Symbols: ●, growth; ▲, penicillin binding.*

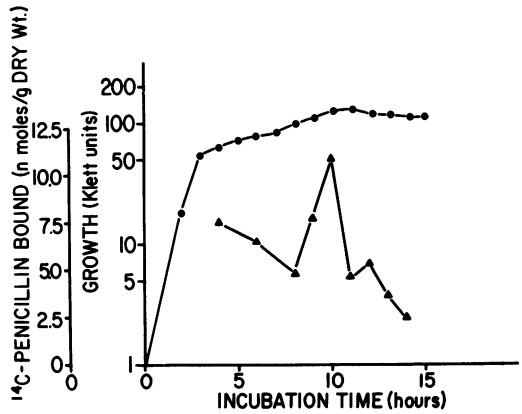

FIG. 4. *^{14}C-penicillin G-binding capacity of mutant Spo 1AC during postlogarithmic growth. Symbols: ●, growth; ▲, penicillin binding.*

tron microscopy to be morphologically blocked prior to the completion of the spore septum (Bacteriol. Proc., p. 22, 1967), confirming the results indicated by the binding data. Alternatively, Spo 1AC simulates the wild-type binding pattern in that the specific binding capacity decreases during presporulation and early sporulation events (Fig. 4) but, unlike SpoI 4AC, shows a peak of enhanced specific binding capacity at 10 h similar to that of the wild-type organism (Fig. 2). A second peak of postexponential binding is absent (Fig. 4). Both SpoI 4AC and Spo 1AC bind radioactive penicillin in the vegetative stage of growth to approximately the same extent as the wild-type organism (approximately 12 nmol/g dry wt).

Penicillin binding characteristics of mutants SpoIII 94U and SpoIII 7Z. SpoIII 94U is linked to *phe-1* and SpoIII 7Z to *lys-1* as defined by transduction with PBS-1 (16). Additional genetic analysis indicated that SpoIII 7Z cotransduced with *lys-2* at a frequency of 40% (Table 1). Both asporogenous mutants are morphologically blocked at stage III of morphogenesis (16). According to the interpretation of the penicillin binding pattern of wild-type organisms previously presented (21), sporulation mutants blocked after stage II should exhibit the first increment of enhanced specific binding capacity. The specific binding pattern of SpoIII 94U resembles that of the Spo$^+$ culture in having both peaks of enhanced binding capacity (Fig. 5). The second peak at 13 h suggests that this mutant produces the terminal enzymes for cortical PG synthesis. The biosynthesis of cortical PG should allow for the detection of the developing spore under phase contrast microscopy. As indicated in Fig. 5, mutant SpoIII 94U forms nonrefractile forespores during the interval where the second peak was observed. Mutant SpoIII 7Z exhibits

TABLE 1. *^{14}C-benzylpenicillin binding patterns of Spo$^-$ mutants during postlogarithmic growth*

Mutant genotype	Cotransduction with *lys-2* (%)[a]	Increased binding during septation	Increased binding during cortical biosynthesis
Wild type		+	+
Spo 9NA	34	–	–
SpoIII 7Z[b]	40	+	–
Spo 105NG	41	+	–
Spo 29NA	45	–	–
Spo 33NA	45	+	–
Spo 35NA	45	–	–
Spo 32NA	46	–	–
Spo 2NA	48	–	–
Spo 102NG	48	–	–
Spo 30NA	48	–	–
Spo 23NA	50	+	–
Spo 10M	50	–	–
Spo 22NA	53	–	–
Spo 34NA	53	+	–

[a] Cotransduction frequencies derived in previous studies (27).

[b] Isolated and cytologically characterized by Ionesco et al. (16) to be blocked during stage III of sporulation.

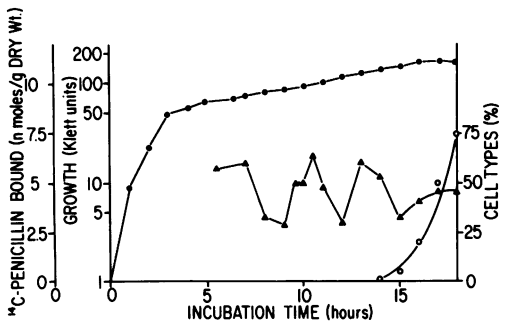

FIG. 5. *^{14}C-penicillin G-binding capacity of mutant SpoIII 94U during postlogarithmic growth. Symbols: ●, growth; ▲, penicillin binding; ○, nonrefractile forespores.*

only the first period of enhanced specific binding capacity which corresponds to that of the wild-type organism produced 9.5 h after inoculation (Fig. 2). No clear second peak was seen and no forespores could be observed in this mutant with phase contrast microscopy.

Penicillin-binding characteristics of mutants with asporogenous defects linked to lys-2. Many of the asporogenous mutations mapped in previous studies form a tight cluster linked to *lys-2* which is near the terminal end of the *B. subtilis* chromosome (27). The linkage values between some of these mutations and *lys-2* are reviewed in Table 1. To indicate the position of the SpoIII 7Z marker with respect to the other markers (Table 1), its linkage to *lys-2* was determined as described above. Since mutations in the *lys-2* region represent blocks at various stages of morphogenesis (15, 16, 27, 37), the postlogarithmic binding patterns of these mutants were examined to determine the effect of the mutations on the previously observed wild-type binding pattern (21). Table 1 summarizes the data obtained with these mutants. Five of the mutants (Spo 105NG, Spo 33NA, Spo 23NA, Spo 34NA, and Spo 7Z) exhibit a decrease in specific binding capacity during presporulation and early sporulation events. This decrease is followed by an increase in specific binding capacity at approximately 10 h. Figure 6 depicts the binding pattern observed with Spo 105NG, illustrating the behavior of a mutant showing the first peak of postlogarithmic binding. The mutants in this category exhibited neither the second interval of enhanced binding capacity seen with wild-type organism nor the presence of forespores. The remaining mutants (Table 1) exhibited neither the first nor the second increment of enhanced specific binding capacity during the postexponential phase of growth. Nonetheless, two distinct postlogarithmic binding patterns were observed. They may be classified as either (i) a decrease in specific binding capacity during the first hours of vegetative growth with no subsequent, significant peaks as shown by Spo 2NA (Fig. 7) or (ii) a relatively low, constant, specific binding capacity as indicated by mutant Spo 32NA in Fig. 8. The postlogarithmic binding pattern of the wild-type organism is included in Fig. 8 for comparison. Even in the vegetative stage of growth, Spo 32NA binds much less radioactive benzyl-

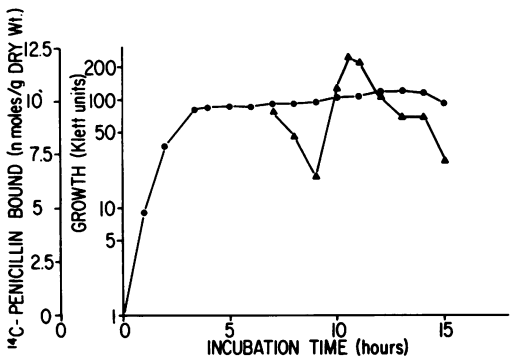

FIG. 6. *^{14}C-penicillin G-binding capacity of mutant Spo 105NG during postlogarithmic growth. Symbols: ●, growth; ▲, penicillin binding.*

penicillin than the wild-type organism, and the constant binding pattern more closely resembles the binding pattern found with *S. aureus*, a non-spore former (21). The binding patterns of Spo 102NA and Spo 29NA are similar to that of Spo 32NA. Mutants Spo 35NA and Spo 22NA behave like Spo 32NA but bind higher amounts of penicillin. The binding patterns of Spo 9NA, Spo 10M, and Spo 30NA resemble that of Spo 2NA.

A genetic map of *B. subtilis* determined from previous studies (27) depicted a number of asporogenous markers linked to *lys-2*. The map distances between some of these Spo$^-$ markers and *lys-2* can be derived from the cotransduction frequencies listed in Table 1. Spo$^-$ mutations blocked prior to stage I all map at one end of this spore linkage group except for SpoO 9V (16). The postexponential binding pattern of all the Spo$^-$ mutants represented in Table 1 were examined. Mutants Spo 105NG, SpoIII 7Z, Spo 23NA, Spo 33NA, and Spo 34NA were all blocked after the first increment of postexponential binding, whereas the remaining mutants were blocked prior to the first increment. These observations suggest that the spore genes within the linkage group next to *lys-2* are not sequentially arranged according to the order in which they are induced in sporulation.

DISCUSSION

Mature bacterial spores contain two distinct structural peptidoglycans: the germ cell wall and the spore cortex (32). The germ cell wall, synthesized by the IFSM, develops into a vegetative cell wall on germination and has a structure similar to that of vegetative cell wall PG, although *B. subtilis* spores apparently lack teichoic acid (4, 32). The germ cell wall has a lysozyme susceptibility and a PG structure characteristic of vegetative cell wall (32). Integuments of *B. sphaericus* spores contain a PG of the D-isoasparaginyl-L-lysine type similar to that of the vegetative wall (32). Pearce and Fitz-James found that a cortexless mutant of *B. cereus* still forms a germ cell wall contiguous to the IFSM and has a morphologically altered OFSM (23, 32). The structure of cortical PG has been examined in *B. subtilis* (35) and in *B. sphaericus* (31, 32). Cortical PG differs from that in the vegetative cell wall in that it contains a lactam of muramic acid not found in the latter, is substantially less cross-linked, and has a high percentage of mono- and tetrapeptide subunits (31, 32, 35). In *B. sphaericus*, the cortex is devoid of L-lysine found in the vegetative cell wall peptidoglycan, and

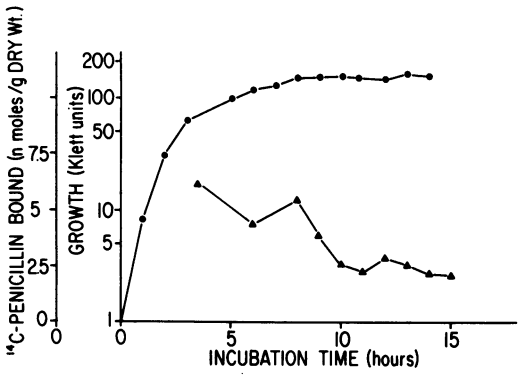

FIG. 7. *^{14}C-penicillin G-binding capacity of mutant Spo 2NA during postlogarithmic growth. Symbols: ●, growth; ▲, penicillin binding.*

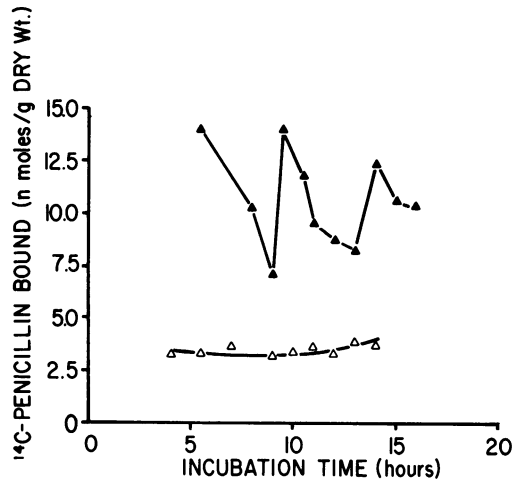

FIG. 8. *^{14}C-penicillin G-binding capacity of sporulating wild-type culture compared to that of mutant Spo 32NA during postlogarithmic growth. Symbols: ▲, binding pattern of the wild type; △, binding pattern of Spo 32NA.*

contains *meso*-Dap (31). Because of the differences found in the structures of cortical and vegetative cell wall PG, Warth and Strominger suggested that the two structures may be synthesized by different sets of enzymes (35). In *B. sphaericus*, cortex biosynthesis is accompanied by a disappearance of the L-lysine-adding enzyme, and the synthesis of the enzyme which incorporates *meso*-Dap into cortical peptidoglycan (31).

Penicillin and other antibiotics which interfere with functional PG biosynthesis can block sporulation at either of the stages requiring functional PG synthesis (11, 13, 23). If the postulates concerning the role of the IFSM and OFSM in the synthesis of the respective PG

polymers are correct (32), penicillin has access to the target enzymes located in these membranes. Thus, the quantity of radioactive benzylpenicillin bound to sporulating organisms provides an estimate of the quantity of penicillin target proteins present in the bacteria. Sporulating cultures exhibit two periods of enhanced penicillin binding in contrast to the constant level of binding shown by a nonsporulating organism during postlogarithmic growth (21). The second peak of binding is concurrent with the formation of refractile endospores. In *B. megaterium*, where forespore septa were distinguished by staining, the first peak corresponds with septation (21). In *B. subtilis* and *B. cereus* sporulation septa were not distinguishable by staining, but the first enhanced binding peak occurred at a time interval corresponding to septation. These data agree with those of Vinter who demonstrated that the incorporation of ^{14}C-Dap into a hot trichloroacetic acid-insoluble fraction of sporulating *B. cereus* cells displays a similar pattern (33, 34). The first interval of enhanced Dap incorporation was attributed to germ cell wall formation and the second to cortex biosynthesis. The present studies indicate that mutations affecting spore formation alter the specific penicillin-binding pattern seen with the wild-type strain. Several of the mutants studied failed to exhibit either peak of enhanced penicillin binding during postlogarithmic growth, indicating that they were blocked prior to the appearance of the terminal enzymes needed for germ cell wall biosynthesis. One of these mutants, SpoI 4AC (Fig. 3), was morphologically blocked prior to the completion of the spore septum (Bacteriol. Proc., p. 22, 1967). This interpretation is consistent with the finding of Chow and Takahashi (5) who found that the intracellular concentration of a cell wall precursor, uridine diphosphate-*N*-acetylglucosamine, suddenly increases in wild-type *B. subtilis* cells at the time of septum formation. The nucleotide precursor fails to increase in a mutant blocked prior to the formation of the forespore septum, but a stage II mutant blocked after septum formation shows an increase in the uridine diphosphate-*N*-acetylglucosamine level similar to that of the wild type. Similarly, mutants examined in the present studies which were morphologically blocked after stage II produce either one or both postlogarithmic peaks of enhanced penicillin binding (Table 1). A cortexless mutant of *B. cereus* exhibits the first peak of enhanced ^{14}C-Dap incorporation into PG during postlogarithmic growth but not the second (23). The activity of the enzyme which incorporates L-lysine into the PG of *B. sphaericus* cells decreased as the culture entered the stationary phase, but consistently increased 30 to 40% between swelling and cortex synthesis. Staining indicated that swelling is coincident with spore septum formation (31). Tipper and Pratt, therefore, concluded that lysine-adding enzyme activity is maximal at a stage where the germ cell wall, containing a vegetative type PG, is being formed (31).

The genetic regulation which permits asporogenous mutants to make vegetative cell wall PG but not that of the germ cell wall or cortex is poorly understood. If some genes for synthesizing enzymes needed in PG biosynthesis during vegetative growth and sporogenesis are identical, mutations within these genes or in regulators of the genes are conditional and are fully expressed only during sporulation. However, since specific binding capacities below that of the wild type were observed during the vegetative growth of most Spo$^-$ mutants examined, mutations blocking spore PG synthesis may also be affecting vegetative cell wall synthesis. It has been suggested that specific promoter sites may regulate the transcription of spore genes (8). Therefore, a defect in one of these promoter sites would be expected to have a major effect on genes during sporulation rather than during vegetative growth.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Araki, Y., A. Shimada, and N. Ishimoto. 1966. Effect of penicillin on cell wall mucopeptide synthesis in an *Escherichia coli* particulate system. Biochem. Biophys. Res. Commun. **23**:518-525.
2. Araki, Y. A., R. Shimai, A. Shimada, and N. Ishimoto. 1966. Enzymatic synthesis of cell wall mucopeptide in a particulate preparation of *Escherichia coli*. Biochem. Biophys. Res. Commun. **23**:466-472.
3. Blumberg, P. M., and J. L. Strominger. 1971. Inactivation of D-alanine carboxypeptidase by penicillins and cephalosporins is not lethal in *Bacillus subtilis*. Proc. Nat. Acad. Sci. U.S.A. **68**:2814-2817.
4. Boylen, C. W., and J. C. Ensign. 1968. Ratio of teichoic acid and peptidoglycan in cell walls of *Bacillus subtilis* following spore germination during vegetative growth. J. Bacteriol. **96**:421-427.
5. Chow, C. T., and I. Takahashi. 1972. Acid soluble nucleotides in an asporogenous mutant of *Bacillus subtilis*. J. Bacteriol. **109**:1175-1180.
6. Cooper, P. D. 1956. Site of action of radiopenicillin. Bacteriol. Rev. **20**:28-48.

7. Daniel, J. W., Jr., and M. J. Johnson. 1954. Properties of the penicillin binding component of *Micrococcus pyogenes*. J. Bacteriol. **67**:321-328.
8. Doi, R. H., and T. J. Leighton. 1972. Regulation during initiation and subsequent stages of bacterial sporulation, p. 225-232. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
9. Donnellan, E. J., E. H. Nags, and H. S. Levinson. 1964. Chemically defined synthetic media for sporulation for generation and growth of *Bacillus subtilis*. J. Bacteriol. **87**:332-336.
10. Edwards, J. R., and J. F. Park. 1969. Correlation between growth inhibition and the binding of various penicillins and cephalosporins to *Staphylococcus aureus*. J. Bacteriol. **99**:459-462.
11. Fitz-James, P. C. 1963. Spore formation in wild and mutant strains of *B. cereus* and some effects of inhibitors, p. 529-544. *In* M. J. C. Senez (ed.), Regulations chez les microorganismes. Centre National Recherche Science, Paris.
12. Gordon, R. A., and W. G. Murrell. 1967. Simple method of detecting spore system formation and synchrony of sporulation. J. Bacteriol. **93**:495-496.
13. Hitchens, A. D., and R. A. Slepecky. 1969. Antibiotic inhibition of the septation stage in sporulation of *Bacillus megaterium*. J. Bacteriol. **97**:1513-1515.
14. Hitchens, A. H., and R. A. Slepecky. 1969. Bacterial sporulation as a modified procaryotic cell division. Nature (London) **223**:804-807.
15. Hoch, J. A., and J. Spizizen. 1969. Genetic control of some early events in sporulation of *Bacillus subtilis*, p. 112-127. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
16. Ionesco, H., J. Michel, B. Cami, and P. Schaeffer. 1970. Genetics of sporulation in *Bacillus subtilis* Marburg. J. Appl. Bacteriol. **33**:13-24.
17. Izaki, K., M. Matsuhashi, and J. L. Strominger. 1966. Glycopeptide transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions. Proc. Nat. Acad. Sci. U.S.A. **55**:656-663.
18. Izaki, K., M. Matsuhashi, and J. L. Strominger. 1968. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin sensitive enzymatic reactions in strains of *Escherichia coli*. J. Biol. Chem. **243**:3180-3192.
19. Lawrence, P. J., and J. L. Strominger. 1970. The binding of radioactive penicillin to the particulate enzyme preparation of *Bacillus subtilis* and its reversal with hydroxylamine or thiols. J. Biol. Chem. **245**:3653-3659.
20. Lawrence, P. J., and J. L. Strominger. 1970. The reversible fixation of radioactive penicillin G to the D-alanine carboxypeptidase of *Bacillus subtilis*. J. Biol. Chem. **245**:3660-3666.
21. Lawrence, P. J., M. Rogolsky, and Vo Thi Hanh. 1971. Binding of radioactive benzylpenicillin to sporulating *Bacillus* cultures: chemistry and fluctuations in specific binding capacity. J. Bacteriol. **108**:662-667.
22. Maas, E. A., and M. J. Johnson. 1949. Penicillin uptake by bacterial cells. J. Bacteriol. **57**:415-422.
23. Pearce, S. M., and P. C. Fitz-James. 1971. Sporulation of a cortexless mutant of a variant of *Bacillus cereus*. J. Bacteriol. **105**:339-348.
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
25. Rogers, H. J. 1967. The inhibition of mucopeptide synthesis in relation to the irreversible fixation of the antibiotic by staphylococci. Biochem. J. **103**:90-102.
26. Rogolsky, M. 1968. Genetic mapping of a locus which regulates the production of pigment associated with spores of *Bacillus subtilis*. J. Bacteriol. **95**:2426-2427.
27. Rogolsky, M. 1969. Chromosomal regions which control sporulation in *Bacillus subtilis*. Can. J. Microbiol. **15**:787-790.
28. Spizizen, J. 1958. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc. Nat. Acad. Sci. U.S.A. **44**:1072-1078.
29. Tipper, D. J., and J. L. Strominger. 1965. Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc. Nat. Acad. Sci. U.S.A. **54**:1133-1141.
30. Tipper, D. J., and J. L. Strominger. 1968. Inhibition of cross-linking by penicillins and cephalosporins: studies in *Staphylococcus aureus in vivo*. J. Biol. Chem. **243**:3169-3179.
31. Tipper, D. J., and I. Pratt. 1970. Cell wall polymers of *Bacillus sphaericus* 9602. J. Bacteriol. **103**:305-317.
32. Tipper, D. J., and J. J. Gauthier. 1972. Structure of the bacterial endospore, p. 3-12. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
33. Vinter, V. 1965. The fate of preexisting diaminopimelic acid-containing structures during germination and postgerminative development of bacterial spores. Folia Microbiol. (Praha) **10**:280-288.
34. Vinter, V. 1965. Commencement of synthetic activities of germinating bacterial spores and changes in vulnerability of cells during outgrowth, p. 25-37. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.
35. Warth, A. D., and J. L. Strominger. 1969. Structure of the peptidoglycan of bacterial spores: occurrence of the lactam of muramic acid. Proc. Nat. Acad. Sci. U.S.A. **64**:528-535.
36. Wise, E. M., and J. F. Park. 1965. Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. Proc. Nat. Acad. Sci. U.S.A. **54**:75-81.
37. Young, F. F., and G. A. Wilson. 1972. Genetics of *Bacillus subtilis* and other gram-positive sporulating bacilli, p. 77-106. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.