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Penicillin: Reversible Inhibition of Forespore Septum Development in *Bacillus megaterium* Cells

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Benzylpenicillin inhibits the development of the forespore septum in sporulating *Bacillus megaterium* cells. The inhibitory effect is a function of the duration of exposure to the antibiotic and is completely reversible by penicillinase. Under the incubation conditions employed, less than 20% of the covalently bound antibiotic is released from the cells. The penicillin which remains bound to the cells after treatment with penicillinase may be necessary but is not sufficient for the effect; unbound antibiotic in the sporulation medium is also required.

In addition to their bactericidal effects on susceptible organisms, penicillins at concentrations similar to those required to prevent vegetative cell division can inhibit bacterial spore formation. The latter process has been described as a modified or atypical cell division (14) which involves an orderly sequence of seven well-defined morphological stages (14). Stages II and IV of bacterial sporulation include the formation of the forespore septum and the spore cortex, respectively. Penicillin, cycloserine, bacitracin, and other antibiotics which prevent vegetative cell division by inhibiting the synthesis of functional vegetative cell wall peptidoglycan can prevent development of either the forespore septum (15) or spore cortex (12, 21), depending upon the time of addition of the antibiotic to the sporulating culture. Two intervals of enhanced specific penicillin-binding capacity are observed in sporulating bacterial cultures (17). The first is seen during the formation of the primordial cell wall or forespore septum (stage II); the second is coincident with the formation of the spore cortex (stage IV). Asporogenous mutants blocked prior to the development of the forespore septum (stage II) fail to exhibit either interval of enhanced specific penicillin binding capacity. Similarly, asporogenous mutants blocked after stage II, but before stage IV, exhibit only the first peak of enhanced specific binding capacity (22).

The above observations, when combined with those presented below, suggest that penicillins inhibit bacterial forespore septum formation by a mechanism similar to that believed to be responsible for their bactericidal effect: inhibition of the terminal stages of functional cell wall peptidoglycan synthesis (24). Considerable experimental evidence indicates that penicillins irreversibly inhibit both glycopeptide transpeptidase and D-alanine carboxypeptidase activity (24). Inhibition of the latter enzyme appears neither necessary nor sufficient to cause vegetative cell death (2). Several penicillin-binding proteins have been identified in detergent extracts of vegetative *Bacillus subtilis* cells (3, 4, 24). The same proteins are also apparently present in sporulating *B. subtilis* cells, although their quantities differ somewhat from that of their vegetative counterparts (1).

Reasonable correlation has been observed between the concentration of penicillins required to saturate rapidly growing bacterial cells and that required for cell death (6, 8-11, 23). These investigations suggested that bound antibiotic is responsible for the bactericidal effect (compare [4]). Despite the obvious importance of the penicillin target proteins in bacterial sporulation, the role of covalently bound antibiotic in inhibiting this process has received little attention. Although penicillins irreversibly inhibit glycopeptide transpeptidases, the biological effect of penicillin exposure is reversible. Addition of penicillinase to a susceptible bacterial culture exposed to a lethal concentration of the antibiotic prevents cell death (6). The reversibility of penicillin inhibition of bacterial forespore septum formation has not been examined. Additional information concerning the reversibility of the biological effects of penicillins may prove useful in the identification and purification of the target protein(s) responsible for these effects. The development of covalent affinity chromatography techniques (3) permits convenient examination of these proteins which react to form a covalent bond

with penicillin. Identification of the proteins primarily responsible for the biological effects is thus of critical importance. Whereas no single, clear alteration of bacterial morphology is conveniently correlated with cell death, the formation of the forespore septum can readily be identified and quantitated. For these reasons, the inhibitory effect of penicillin on the formation of the forespore septum in sporulating *B. megaterium* cultures and the reversibility of the effect were examined.

MATERIALS AND METHODS

Organism and growth conditions. *B. megaterium* cells (ATCC 19213) were grown in the synthetic sucrose medium of Kolodziej and Slepecky (16). The culture (300 ml) was shaken at 30 C in 2-liter Fernbach flasks on a New Brunswick gyratory shaker. At appropriate intervals, 10-ml samples were removed and added to 100-ml Erlenmeyer flasks containing either benzylpenicillin (50 μg/ml, final concentration) or an equivalent volume of water. Unlabeled potassium benzylpenicillin and [^{14}C]benzylpenicillin were obtained from Sigma and Amersham Searle, respectively. The cells were shaken at 30 C, and portions were removed for septum counting by the staining procedure of Gordon and Murrell (13).

Effect of penicillinase on forespore septum formation. Cells were grown as indicated above. At appropriate intervals, 10-ml samples were removed from the Fernbach flasks and added to 100-ml Erlenmeyer flasks containing either penicillin or water. Penicillinase (20,000 U) was added to individual flasks after 10, 20, 30, or 60 min. Samples were removed from each flask at appropriate intervals, and forespore septa were counted. A non-penicillinase-treated culture and a culture to which only penicillinase was added were included as controls.

Effects of penicillinase on the binding of [^{14}C]benzylpenicillin to sporulating B. megaterium cells. Cells were grown as described above. When approximately 5% of the cells contained forespore septa, the cells were harvested and washed with buffer containing: tris(hydroxymethyl)aminomethane, 0.01 M; succinate, 0.04 M; and magnesium acetate, 0.01 M; adjusted to pH 7.2. Washed cells were suspended in a volume of buffer (ml) four times their wet weight (in grams), and the suspension was divided into two equal fractions. One was preincubated for 5 min at 0 C with unlabeled benzylpenicillin (25 mg/ml), and the other was untreated. Thereafter, both samples were incubated with [^{14}C]benzylpenicillin (10 μg/ml, final concentration) for 5 min at 0 C. Both samples were washed to remove unbound radioactive antibiotic and resuspended in buffer at 0 C. Samples were removed for determination of [^{14}C]benzylpenicillin bound to the cells (17). The cells were incubated with radioactive antibiotic at 0 C to inhibit further sporulation development. Independent studies indicate that both sporulating and vegetative *B. subtilis* cells bind the same quantity of radioactive antibiotic at 0, 30, or 37 C. (T. E. Hamilton and P. J. Lawrence, manuscript in preparation). The culture labeled with [^{14}C]benzylpenicillin was then divided into two equal fractions. Penicillinase (20,000 U) was added to one sample, and the other was untreated. The cells pretreated with unlabeled penicillin, those treated with [^{14}C]benzylpenicillin alone, and the fraction treated with both [^{14}C]benzylpenicillin and penicillinase were shaken at 30 C. After, 5, 10, 20, and 30 min, 200-μliter samples were removed, the cells were washed three times with the above buffer, and the quantity of [^{14}C]benzylpenicillin bound to the cells was determined (17). All determinations were performed in triplicate. Corrections were made for the quantity of penicillin bound nonspecifically to the cells by subtracting from the total [^{14}C]benzylpenicillin bound to the cells that quantity of radioactive antibiotic bound in the presence of unlabeled benzylpenicillin (25 mg/ml). Protein was determined by the method of Lowry et al. (20).

RESULTS

Effect of benzylpenicillin on forespore septum formation in B. megaterium cells. Under the growth conditions employed, forespore septa were distinguishable after approximately 10 h of incubation at 30 C. Forespore septation continued until approximately 85% of the cells in the culture contained clearly recognized forespore septa (Fig. 1). Thereafter, cells containing forespore septa decreased in number as the sporulation process continued and forespores, sporangia, and mature spores were formed. If benzylpenicillin at a final concentration of 50 μg/ml, were added to the culture at 10 h, subsequent forespore septum formation was completely prevented (Fig. 1) as were all later stages of sporulation (data not presented).

Penicillin at lower concentrations also prevented forespore septum formation, but in the initial experiments a comparatively high concentration of antibiotic was utilized to prevent hydrolysis of penicillin by cell-bound β-lactamase activity over the interval of study (T. E. Hamilton and P. J. Lawrence, unpublished data). The degree of inhibition of forespore septum formation was a function of the duration of penicillin exposure (Fig. 2). Addition of penicillin to the culture at 10 h completely inhibited subsequent forespore septum formation for at least 4 h (after which the experiments were terminated). A 14-h culture which had been exposed to penicillin for the last 3 h of growth produced far fewer forespore septum containing cells than an untreated culture. A similar inhibition of forespore septum formation by benzylpenicillin was seen in 13-, 12-, and 11-h cultures during the entire period of incubation in the presence of antibiotic (Fig. 2).

Reversibility of penicillin inhibition of bacterial forespore septum formation. The culture was incubated at 30 C until approximately

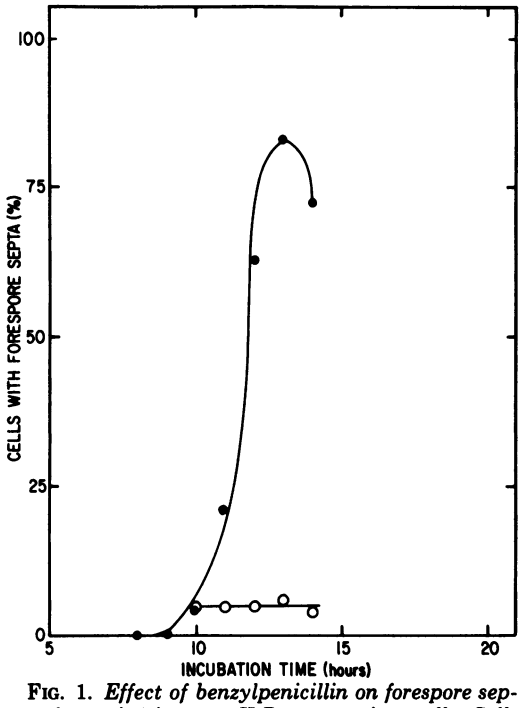

FIG. 1. *Effect of benzylpenicillin on forespore septum formation in stage II B. megaterium cells. Cells were grown and exposed to benzylpenicillin (50 μg/ml) as described in Materials and Methods. Samples (10 ml) were removed at the indicated times, stained, and forespore septa counted. Symbols: ●, forespore septa in untreated cells; ○, forespore septa in cells exposed to benzylpenicillin.*

10% of the cells contained forespore septa (10 h). Several 10-ml samples were removed and added to individual Erlenmeyer flasks containing unlabeled benzylpenicillin (50 μg/ml, final concentration). Control flasks containing no antibiotic, or penicillinase (20,000 U) in the absence of antibiotic, were also utilized. As described previously (Fig. 1), penicillin completely inhibited the formation of forespore septa, but penicillinase alone had no effect on the formation of this structure (Fig. 3). The addition of penicillinase to the antibiotic-treated cells, however, at 10, 20, 30, or 60 min after introduction of the antibiotic, completely mitigated the penicillin inhibition of forespore septum formation. The rate of forespore septum formation after the addition of penicillinase to the antibiotic-treated cultures was at least as great as that of the control cultures (Table 1).

Reversibility of the binding of [^{14}C]benzylpenicillin to stage II B. megaterium cells. The chemistry of binding of [^{14}C]benzylpenicillin to sporulating *B. megaterium* cells (17) is similar to that observed with vegetative *B. subtilis* cells or their membrane fragments (18, 19). Stage II *B. megaterium* cells were incubated with [^{14}C]benzylpenicillin at a final antibiotic concentration of 10 μg/ml at 0 C. The same amount of radioactive antibiotic is covalently bound to sporulating cells at 0 as at 30 C (T. E. Hamilton and P. J. Lawrence, manuscript in preparation). When the treated cells were washed free of unbound antibiotic and incubated at 0 C, no significant release of bound radioactive antibiotic was detected (Fig. 4). At 30 C, less than 20% of the bound antibiotic was removed from the cells over a 30-min interval. The kinetics of antibiotic removal was not significantly different in the presence of penicillinase (Fig. 4). The quantity of the radioactive antibiotic which remains bound to the cells (>80%) at 30 C after a 30-min incubation may be necessary to inhibit forespore septum

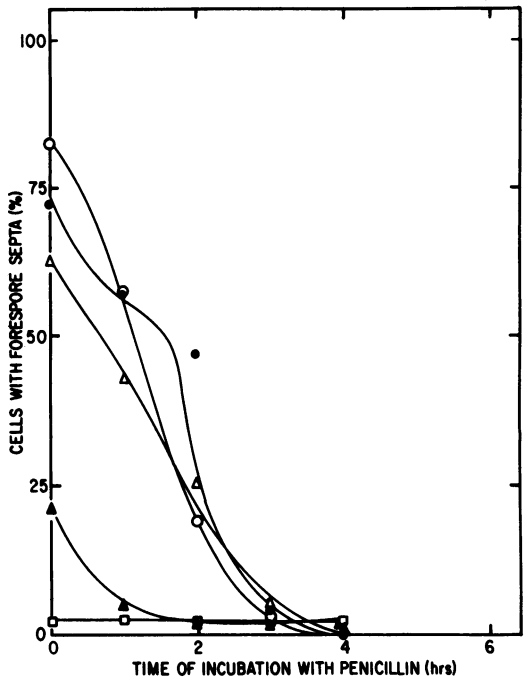

FIG. 2. *Effect of duration of penicillin exposure on forespore septum development in B. megaterium cells. Cells were grown as described in Materials and Methods. At the times designated below, 10-ml samples were added to flasks containing benzylpenicillin (50 μg/ml) and grown in the presence of the antibiotic for the indicated interval. Symbols: ●, 14-h cells, penicillin added to separate samples at 10, 11, 12, and 13 h; ○, 13-h cells, penicillin added to separate samples at 9, 10, 11, and 12 h; △, 12-h cells, penicillin added to separate samples at 8, 9, 10, and 11 h; ▲, 11-h cells, penicillin added to separate samples at 7, 8, 9, and 10 h; and □, 10-h cells, penicillin added at 6, 7, 8, and 9 h. Samples were removed from each flask at hourly intervals, and the number of cells containing forespore septa was determined.*

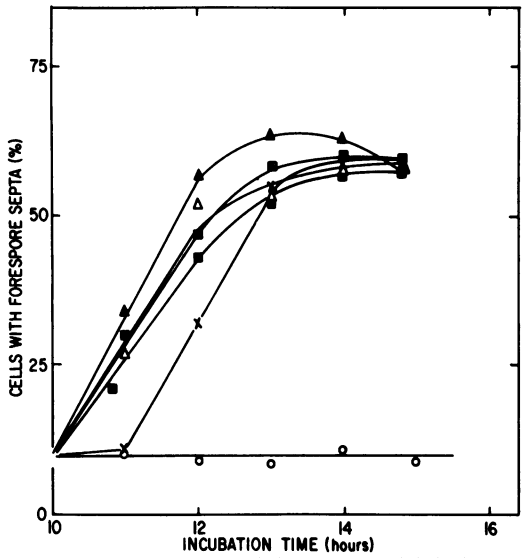

FIG. 3. *Effect of penicillinase on penicillin inhibition of forespore septum formation in stage II B. megaterium cells. Cells were incubated as described in Materials and Methods for 10 h. Samples were then removed, and the incubation continued:* ●, *with no additions;* ○, *with benzylpenicillin; or* □, *with the addition of penicillinase alone. Benzylpenicillin (50 μg/ml) was present in all of the remaining flasks. To these flasks, penicillinase (20,000 U) was added at the following times:* △, *10 min;* ▲, *20 min;* ■, *30 min; and* ×, *60 min. Samples were removed after a 10-h incubation and at hourly intervals thereafter for determination of forespore septa.*

formation, but it is clearly insufficient to cause this effect. Removal of 20% of the covalently bound antibiotic permits forespore septum development at a rate equal to that of the control culture (Fig. 3, Table 1). Several vegetative bacilli have been shown to contain multiple penicillin-binding proteins (3, 4, 24), and the penicillin-binding proteins of vegetative *B. subtilis* cells appear qualitatively the same as that of their sporulating counterparts (1).

The data presented in Fig. 4 do not indicate whether the penicillin released from sporulating cells is removed from a single protein or from several proteins. Excess, unbound antibiotic may be required to exert the biological effect for a number of reasons. Conceivably, excess, unbound antibiotic in the media is needed to re-penicilloylate the protein(s) from which penicillin is lost during the incubation period. Alternatively, the free antibiotic may be required to penicilloylate target proteins synthesized as the sporulation process occurs. Finally, the antibiotic may interact with its target protein in a reversible manner. The available data do not distinguish between these alternatives.

DISCUSSION

Several lines of experimental evidence suggest that functional cell wall peptidoglycan biosynthesis is required for the formation of forespore septa although the septum itself apparently contains no cell wall material (14). (i) Antibiotics known to prevent vegetative cell division by inhibiting specific enzymes in the synthesis of peptidoglycan (cycloserine, baci-

TABLE. 1. *Effect of penicillinase on the rate of forespore septum formation in cells exposed to benzylpenicillin*[a]

Addition	Rate of forespore septum synthesis[b]
	10.5
Penicillinase	10.5
Benzylpenicillin[c]	0
Benzylpenicillin and penicillinase at:	
10 min	10.8
20 min	22.8
30 min	10.8
60 min[d]	11.1

[a] The procedures are the same as those described in the legend to Fig. 3.

[b] Rate of forespore septum synthesis: (percent cells with forespore septa at 11 h − percent cells with forespore septa at 10 h)/(total incubation time − incubation time after the addition of penicillinase).

[c] Rate of forespore septum synthesis: (percent cells with forespore septa at 11 h − percent cells with forespore septa at 10 h)/(60 min).

[d] Rate of forespore septum formation: (percent cells with forespore septa at 12 h − percent cells with forespore septa at 11 h)/(60 min).

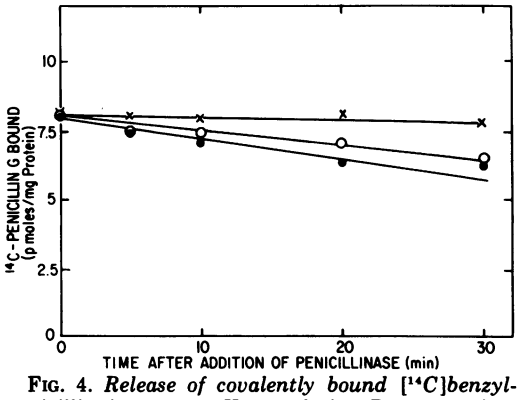

FIG. 4. *Release of covalently bound [^{14}C]benzylpenicillin from stage II sporulating B. megaterium cells. Stage II cells were harvested, washed, and treated with [^{14}C]benzylpenicillin as described in Materials and Methods. The cells were washed free of excess, unbound penicillin and incubated as follows:* ×, *0 C;* ●, *30 C, no additions; and* ○, *30 C, penicillinase (20,000 U).*

tracin, and penicillin) inhibit formation of forespore septa (15). (ii) Five proteins known to react covalently with penicillins are present in both vegetative and sporulating cells, although the quantities of the individual components differ slightly (1, 3, 4, 24). (iii) While the specific penicillin-binding capacity of a nonsporulating organism (*S. aureus*) remains constant in the post-logarithmic phase of growth, that of sporulating bacilli exhibits two intervals of enhanced specific penicillin-binding capacity; the first increase corresponds to the formation of the forespore septum, but the second is coincident with the formation of the spore cortex (17). Asporogenous mutants blocked prior to stage II show neither interval of enhanced specific binding capacity (22). (iv) The intracellular concentration of a nucleotide precursor of peptidoglycan biosynthesis (uridine diphosphate-*N*-acetylglucosamine) rises suddenly in wild-type sporulating *B. subtilis* cells at the time of forespore septum formation. The increase is not observed in asporogenous mutants blocked prior to formation of the forespore septum, but is seen in a Stage II mutant blocked after formation of this structure (5).

The data presented indicate that unbound penicillin in the sporulation medium is required for penicillin inhibition of forespore septum formation (Fig. 3, Table 1). In this respect, the effect of penicillin on forespore septum formation is similar to that observed in its inhibition of vegetative cell division; the destruction of unbound antibiotic results in a reversal of the biological effect (6). After treatment with penicillinase, less than 20% of the [^{14}C]benzylpenicillin covalently bound to stage II cells is removed during a 30-min incubation period at 30 C (Fig. 4). Nonetheless, the rate of forespore septum formation of cells exposed to penicillin for 30 min prior to the addition of penicillinase is essentially the same as that of untreated cells (Table 1). Whereas more than 80% of the penicillin remains bound to the cells under these conditions, bound antibiotic is clearly not sufficient to inhibit forespore septum synthesis. The penicillin released from the cells may be lost by one or several of the bacterial penicillin-binding proteins thus far identified (1, 3, 4, 24). Some of these proteins are present in comparatively small amounts (1, 3, 4, 24), but may be of critical biological importance. To explain the failure of covalently bound antibiotic alone to kill logarithmically growing bacteria, it has been suggested that rapid synthesis of new penicillin target proteins permits resumption of normal growth (6). In vegetatively growing *B. subtilis* cells, the total penicillin-binding capacity increases approximately 135% over a 30-min period (T. E. Hamilton and P. J. Lawrence, manuscript in preparation), whereas the increase in specific binding capacity of a stage II sporulating culture is less than 15% over the same interval (19). It is also possible that bound antibiotic is required, but is itself not sufficient to exert either biological effect. The role of excess, unbound penicillin on vegetative bacterial cell division and on forespore septum formation remains to be clarified.

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