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Effect of Cephapirin on Formation of D-Alanine Carboxypeptidase in Growing *Bacillus subtilis* Cells

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Cephapirin was utilized to examine the interaction of β-lactam antibiotics with growing *Bacillus subtilis* cells and the biological effects simultaneously produced. Saturation binding and quantitative cell death were observed at the cephapirin concentration of 0.1 μg/ml. Cephapirin bound to all penicillin-binding proteins except the D-alanine carboxypeptidase. A specific [^{14}C]benzylpenicillin-binding assay was developed for the D-alanine carboxypeptidase. At the lowest saturating concentration of antibiotic (0.1 μg/ml), cephapirin inhibited formation of the D-alanine carboxypeptidase. Upon incubation with cephapirin, 18% of the membranous D-alanine carboxypeptidase was released into the media. The data suggest that β-lactam antibiotics may affect the formation of bacterial cytoplasmic membranes in addition to their effect on cell wall synthesis.

The binding of β-lactam antibiotics to bacterial cells, membrane fragments, etc. has been extensively examined (for reviews, see ref. 6, 13). Several penicillin-sensitive enzymes are known (1, 3, 13, 17), and direct labeling experiments or affinity chromatography procedures have shown several penicillin-binding proteins (PBP) in *Bacillus subtilis, Bacillus stearothermophilus, Bacillus cereus, Staphylococcus aureus,* and *Escherichia coli* (3, 5, 15, 16). Of these PBP, only the D-alanine carboxypeptidase has been identified (3).

Whereas several PBP bind penicillin irreversibly, the biological effects of the antibiotic are largely reversible. Penicillin inhibition of bacterial forespore septum formation (9) was abolished through removal of excess antibiotic by enzymatic hydrolysis, although comparatively little antibiotic was released from the cells (10). Also, Rogers observed that penicillin-treated *S. aureus* cells, washed free of unbound antibiotic, survived while subsequently releasing only 10% of the bound radioactive benzylpenicillin (14). Cooper suggested that rapid replacement of PBP by growing cells was responsible for cell survival under these conditions (6). However, this premise has remained untested, since most of the penicillin-binding studies have utilized nongrowing cells (3, 6). A similar phenomenon was observed with growing *B. subtilis* cells, but larger quantities of [^{14}C]benzylpenicillin were released from the "rescued" cells (P. J. Lawrence, manuscript in preparation).

A procedure was recently developed which permitted determination of the [^{14}C]benzylpenicillin- and [^{14}C]cephapirin-binding capacities of rapidly growing *B. subtilis* cells (8). Although the correlation between binding and cell death was not examined, both benzylpenicillin and cephapirin unexpectedly prevented formation of binding proteins in growing cells while minimally inhibiting the normal increase in cellular protein content (8). One member of the multicomponent PBP in *B. subtilis* is the D-alanine carboxypeptidase. Although penicillin inhibition of this enzyme is neither necessary nor sufficient to cause cell death (3), it provides a convenient probe for examining more closely the mechanism(s) by which β-lactam antibiotics inhibit formation of PBP. This study, therefore, will examine the effects of a penicillinase-resistant cephalosporin, cephapirin, on the formation of the D-alanine carboxypeptidase in rapidly growing *B. subtilis* cells.

MATERIALS AND METHODS

Materials. [^{14}C]benzylpenicillin (144 μCi/mg) was purchased from Amersham-Corp., and benzylpenicillin came from Sigma Chemical Co. [^{14}C]cephapirin (1.72 μCi/mg) and cephapirin were generously provided by Bristol Laboratories.

Viability studies. The organism (*B. subtilis* Marburg) and the growth conditions were as described earlier (8) with one modification. After the cell density reached approximately 5 × 10^7 cells per ml, an equal volume of fresh medium at 37°C was added, and

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bacteria were allowed to adapt for 6 min in all experiments before cephapirin was added. Viability studies were performed as described (T. E. Hamilton, Ph.D. Thesis, University of Utah, Salt Lake City, 1975).

Antibiotic binding studies. (i) Binding of [^{14}C]cephapirin to *B. subtilis* cells. Binding of cephapirin to *B. subtilis* in dilute suspensions at 4 or 37°C was measured as described (8).

(ii) Identification of the [^{14}C]benzylpenicillin-binding components of growing *B. subtilis* cells. Growing cultures of *B. subtilis* were diluted with fresh medium and after 6 min were exposed to [^{14}C]benzylpenicillin (final concentration, 0.7 μg/ml) for 15 min at 37°C. The cells were washed and treated with lysozyme (15). Protoplasts produced were lysed in hypotonic buffer, washed, and dissolved in sodium dodecyl sulfate by agitation for 10 min at 37°C. The solubilized particulate proteins (approximately 100 μg) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide slab gels (7), and radioactive proteins were detected by fluorography (4). For comparison of the radioactive protein bands, densitometer tracings were made from X-ray film with an Ortec 4310 densitometer.

(iii) Binding of [^{14}C]benzylpenicillin to the cellular *B. subtilis* D-alanine carboxypeptidase. Cultures of *B. subtilis* were grown, harvested, washed, and suspended in a volume of buffer 1/50 that of the original culture. Samples (0.5 ml) were mixed with 50 μl of benzylpenicillin (100 mg/ml) for determination of nonspecific binding or with 50 μl of water for experimental determinations. Cephapirin (final concentration 20 μg/ml) was added to saturate the cephapirin-binding proteins, and the mixtures were incubated for 5 min at 0°C. Varying concentrations of [^{14}C]benzylpenicillin were next added to all samples. After a 5-min incubation at 37°C, 50 μl of benzylpenicillin (100 μg/ml) was added to each sample. The cells were incubated at 4°C for 5 min and washed with 95% ethanol, and bound antibiotic was determined. From the total [^{14}C]benzylpenicillin bound, that bound in the presence of excess benzylpenicillin was subtracted to correct for nonspecific binding. Radioactivity was determined by counting 1 ml of bacterial protein suspension in 10 ml of Aquasol.

(iv) Binding of [^{14}C]benzylpenicillin to D-alanine carboxypeptidase released from growing *B. subtilis* cells. Growth media from which cells were removed were concentrated 30-fold by pressure dialysis. The [^{14}C]benzylpenicillin-binding capacity of the D-alanine carboxypeptidase was then determined as described above.

Effect of cephapirin on the formation of penicillin-binding D-alanine carboxypeptidase in rapidly growing *B. subtilis* cells. Cells were grown and adapted to fresh media, and either water (control) or cephapirin (0.1 μg/ml) was added. Samples (300 ml) were taken at the appropriate times, harvested by centrifugation, washed, and suspended in 10 ml of TSM (8) buffer. Growth medium was concentrated, and the [^{14}C]benzylpenicillin-binding capacities of the D-alanine carboxypeptidase in cells and concentrated growth medium were determined. Membranes were isolated by high-speed centrifugation of cell lysates prepared from cephapirin-treated and control cells (12). Protein contents were determined (11) and used in the D-alanine carboxypeptidase specific binding capacity calculations.

RESULTS

Binding of [^{14}C]cephapirin to *B. subtilis* cells. In a previous study, dilute cell suspensions of *B. subtilis* exhibited saturation with [^{14}C]benzylpenicillin at 0.05 μg/ml (8). Similar saturation has been found at 0.1 μg of [^{14}C]cephapirin per ml with both dilute and concentrated suspensions of *B. subtilis* (Fig. 1). Cephapirin-binding capacities varied between 11 and 16 pmol of antibiotic per mg of protein, depending on the culture employed. Binding of [^{14}C]cephapirin was determined at 4°C for control cultures (grown at 37°C without antibiotic and subsequently chilled) and 37°C for experimental cultures (grown at 37°C with antibiotic). To compare data gathered under the two conditions, it was necessary to establish that antibiotic binding was the same at both temperatures. At bactericidal concentrations (0.1 to 0.5 μg/ml), dilute suspensions of *B. subtilis* cells bound equivalent amounts of [^{14}C]cephapirin at 4 and 37°C. Benzylpenicillin (0.5 μg/ml) prevented the binding of [^{14}C]cephapirin at either temperature. Cultures incubated with [^{14}C]cephapirin (0.5 μg/ml) at 4°C exhibited no additional binding when the

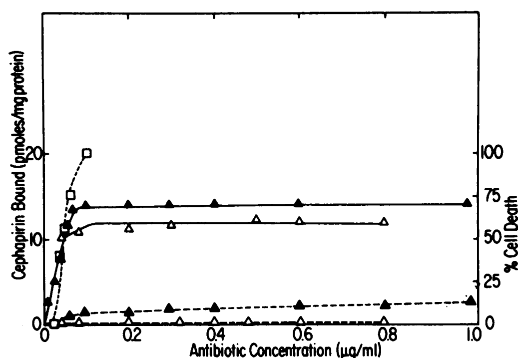

FIG. 1. *Effect of antibiotic concentration on saturation of B. subtilis cells and cell death. Growing cells were diluted 1:2 with fresh medium, and their specific [^{14}C]cephapirin-binding capacities were determined in dilute culture or concentrated suspensions (see the text). (△——△) Specific [^{14}C]cephapirin-binding capacity of a dilute B. subtilis culture; (△---△) nonspecific binding of [^{14}C]cephapirin to a dilute culture of B. subtilis cells; (▲——▲) specific [^{14}C]cephapirin-binding capacity of 250-fold-concentrated suspensions of B. subtilis cells; (▲---▲) nonspecific binding of [^{14}C]cephapirin to concentrated suspensions of B. subtilis cells; (□---□) percent cell death. Viability studies were performed as described in the text.*

temperature was raised to 37°C. Also, less than 10% of the [^{14}C]cephapirin was lost from a culture at 4°C when the temperature was raised to 37°C for 10 min. When cells were labeled with [^{14}C]cephapirin at 37°C, no additional antibiotic was bound when the temperature was decreased to 4°C. These data indicate that [^{14}C]cephapirin binds to the same sites at 4 and 37°C. Binding of cephapirin at 4°C was extremely rapid. At a concentration of 0.1 μg/ml, over 80% of the antibiotic was bound to the cells within 1 min and remained bound over a 30-min interval.

Relationship between binding of [^{14}C]cephapirin and cell viability. An experimental procedure was developed with which [^{14}C]cephapirin binding to growing *B. subtilis* and cell viability were simultaneously monitored. The correlation between binding and death could then be examined. The minimal antibiotic concentration required for saturation (0.1 μg/ml) killed all the cells in 30 min, whereas half that concentration killed only 50% of the cells (Fig. 1). The cells were judged killed both by cell viability (colony formation in the absence of antibiotic) and by microscopic examination. In subsequent experiments this saturating concentration of antibiotic was used, and experiments were terminated after 18 min of exposure to cephapirin, at which time the cells still appeared normal by the above criteria. Consequently, antibiotic saturation and cell death were examined under the same conditions of cell culture and antibiotic treatment.

Identity of the binding proteins. The number and relative abundance of bacterial PBP have been determined by labeling cytoplasmic membranes with [^{14}C]benzylpenicillin (5, 15, 16). However, similar experiments have not been performed with growing cells. To identify the PBP in vivo, growing *B. subtilis* cells were diluted with fresh medium and incubated with a saturating concentration of [^{14}C]benzylpenicillin (0.7 μg/ml). After 10 min the cells were harvested, washed, and converted into protoplasts, which were lysed and dissolved in sodium dodecyl sulfate. Labeled proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Densitometry of the resulting X-ray film revealed a multicomponent labeling pattern similar to that found by labeling previously prepared membranes of *B. subtilis* (3). Four components were clearly labeled (Fig. 2). Of these, component IV was present in the largest amount and had an electrophoretic mobility similar to that of the D-alanine carboxypeptidase (3). At 0.5 μg/ml, cephapirin did not prevent binding of [^{14}C]benzylpenicillin to this component; however, it did prevent binding of penicillin to components I, II, and III. This differential effect was observed at cephapirin concentrations up to 25 μg/ml. These data demonstrate that cephapirin, like cephalothin (3), does not interact with the D-alanine caboxypeptidase. In addition, cephapirin does not inhibit the D-alanine carboxypeptidase activity in *B. subtilis* (8). The low specific activity of [^{14}C]cephapirin in this study precluded analogous fluorographic detection of cephapirin-binding proteins.

Formation and distribution of the penicillin-binding D-alanine carboxypeptidase. Concentrated *B. subtilis* cells were preincubated with cephapirin (20 μg/ml), and binding of [^{14}C]benzylpenicillin to the D-alanine carboxypeptidase was monitored. Saturation binding occurred at 1.5 μg of benzylpenicillin per ml (Fig. 3). When membranes from cells treated in this manner were analyzed by electrophoresis and fluorography, the only radioactive protein had an electrophoretic mobility similar to the D-alanine carboxypeptidase (data not shown). When *B. subtilis* cells were grown in the absence of antibiotic, the penicillin-binding D-alanine carboxypeptidase increased along with cell mass (Fig. 4). After a slight increase, the specific binding capacity of untreated cells remained constant. However, when cephapirin was added to

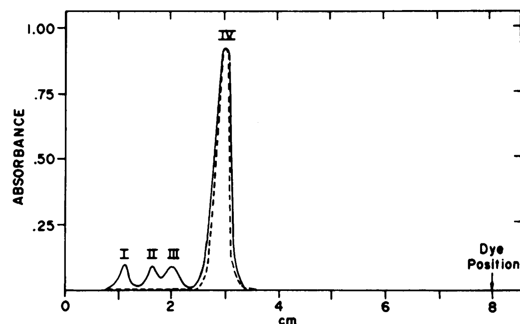

FIG. 2. *Competition of unlabeled cephapirin for the [^{14}C]benzylpenicillin-binding proteins of growing B. subtilis cells. A dilute culture (20 ml) of rapidly growing B. subtilis cells was diluted 1:2 with fresh growth medium at 37°C. [^{14}C]benzylpenicillin (0.7 μg/ml) was added to the culture, which was permitted to grow for 15 min at 37°C. These cells were harvested, washed, solubilized, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography as described. (———) Binding of [^{14}C]benzylpenicillin to untreated cells; (----) binding of [^{14}C]benzylpenicillin to cells from the same culture, which had previously been treated with unlabeled cephapirin (0.5 μg/ml). The densitometer scan was normalized by adjusting the absorbance of peak IV to 1.0.*

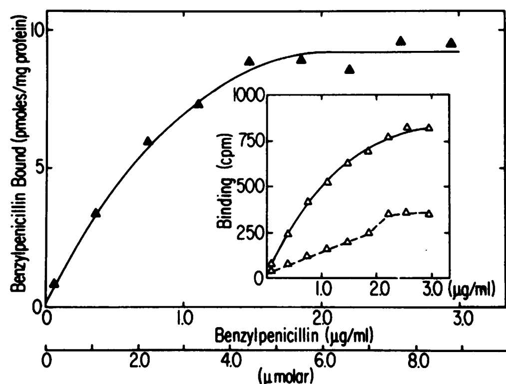

FIG. 3. *Binding of [^{14}C]benzylpenicillin to the D-alanine carboxypeptidase of B. subtilis cells. B. subtilis cells were grown as described, harvested, washed, and exposed to unlabeled cephapirin and [^{14}C]benzylpenicillin. (▲——▲) Specific cellular [^{14}C]benzylpenicillin-binding capacity of the D-alanine carboxypeptidase; (△——△) total cellular binding capacity of [^{14}C]benzylpenicillin; (△----△) binding capacity of the B. subtilis cell suspension in the presence of excess unlabeled benzylpenicillin.*

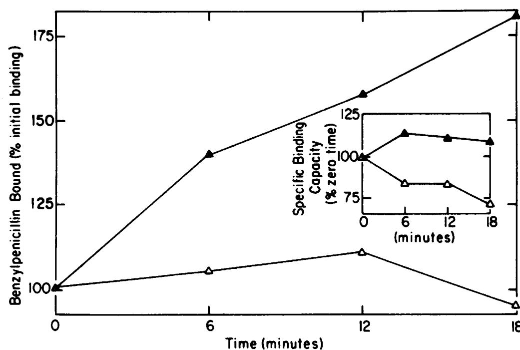

FIG. 4. *Effect of cephapirin on formation of penicillin-binding D-alanine carboxypeptidase in growing B. subtilis cultures. B. subtilis cells were grown as described, diluted 1:2 with fresh medium, and allowed to grow for an additional 6 min to compensate for mixing effects, etc. The culture was divided into two portions; to one was added water (control), and to the other, unlabeled cephapirin (0.1 µg/ml). At the indicated time intervals, 300-ml samples were harvested and washed, and the [^{14}C]benzylpenicillin-binding D-alanine carboxypeptidase was determined. (▲) Binding of [^{14}C]benzylpenicillin to the D-alanine carboxypeptidase of untreated cells; (△) binding of [^{14}C]benzylpenicillin to B. subtilis cells exposed to unlabeled cephapirin (0.1 µg/ml) throughout the growth interval. Specific binding capacities (inset) were obtained by dividing quantities of [^{14}C]benzylpenicillin bound specifically to the D-alanine carboxypeptidase by the membrane protein content (12).*

a similar culture, the D-alanine carboxypeptidase content remained essentially unchanged and the specific binding capacity of the treated culture decreased substantially. Specific binding capacities were based on membrane protein data; it has been shown (8) that cephapirin exerts a minimal effect on total cellular protein content under similar conditions. Thus, a lethal concentration of cephapirin prevented formation of the D-alanine carboxypeptidase.

The D-alanine carboxypeptidase resides on the outer surface of the cytoplasmic membrane (18). To determine if the loss of this PBP from the membrane (Fig. 4) resulted from release of enzyme into the growth media, the latter were concentrated by pressure dialysis and subjected to the penicillin-binding D-alanine carboxypeptidase assay. In untreated cultures, 99% of the enzyme was in the cellular fraction (Table 1). In cephapirin-treated cultures, however, 82% of the recovered penicillin-binding D-alanine carboxypeptidase was cell bound while 18% was released into the growth media. The sum of cellular and freed enzyme was only 67% of that found in the absence of antibiotic. Identical results were obtained if proteins in the growth medium were

TABLE 1. *Effect of cephapirin on the distribution of functional [^{14}C]penicillin-binding D-alanine carboxypeptidase in rapidly growing B. subtilis cells*[a]

Fraction	pmol of [^{14}C]benzylpenicillin bound (% of total)		Specific binding capacity, pmol/mg of membrane protein (% of control)	
	Control	Cephapirin treated	Control	Cephapirin treated
Cellular	251.1 (99)	140.3 (82)	193.2 (100)	116.9 (60)
Media	3.3 (1)	29.8 (18)		
Total	254.4 (100)	170.1 (100)		

[a] *B. subtilis* cells were grown to a density of 5 × 10^7 cells per ml and diluted 1:2 with fresh media, and the resulting culture was divided. After a 6-min equilibration period at 37°C, either cephapirin (0.1 µg/ml) or water (control) was added to each culture, and both were grown for 18 min. After cells and media were separated by centrifugation, the media were concentrated by pressure dialysis. The total penicillin-binding D-alanine carboxypeptidase of both fractions was determined as described. The specific binding capacity of the membrane-bound D-alanine carboxypeptidase was calculated by dividing the quantity of enzyme recovered in the cellular fraction by the amount of protein found in membranes isolated from control and antibiotic-treated cells.

concentrated by ammonium sulfate precipitation. The release of enzyme from the membrane was not a result of the surfactant properties of cephalosporins, since both cephapirin and cephalothin (0.1 to 1.0 μg/ml) failed to extract D-alanine carboxypeptidase from intact cells.

DISCUSSION

Cephalothin and other cephalosporins have been utilized in binding competition experiments with [^{14}C]benzylpenicillin to study the role of bacterial PBP (3). However, direct binding studies have not been possible previously because radioactive cephalothin was not available. In the present study, [^{14}C]cephapirin was utilized to examine the interaction of this antibiotic with growing *B. subtilis* cells and the biological effects simultaneously produced. Antibiotic binding under conditions where cell death was monitored permitted a more definitive examination of the physiological relevance of bound antibiotic. The lowest concentration of cephapirin required to saturate cellular binding components (0.1 μg/ml) was sufficient to kill all of the cells in 30 min (Fig. 1).

A previous study identified five [^{14}C]benzylpenicillin-binding components in membranes isolated from *B. subtilis* (2). In the present investigation, however, four binding components were detected when growing *B. subtilis* cells were incubated with [^{14}C]benzylpenicillin (Fig. 2). It was not possible to discern which component was not labeled in whole cells because of the low resolution inherent in the technique. Cephapirin, like cephalothin (3), reacted with all the PBP with the exception of the D-alanine carboxypeptidase. Cephapirin affects membranous D-alanine carboxypeptidase not only by inhibiting formation of membrane-bound enzyme but also by causing a release of this enzyme from the membrane (Fig. 4, Table 1).

Of significance is the observation that cephapirin inhibits formation of the D-alanine carboxypeptidase (Fig. 4) without binding to the enzyme (Fig. 2), affecting its enzymatic capacity, or altering total cellular protein content (8). Thus, β-lactam antibiotics, in addition to their documented effects on cell wall formation, may have a significant effect on cytoplasmic membrane formation in growing *B. subtilis* cells prior to observed cell death. These effects on membrane formation in growing vegetative cells may be analogous to inhibition of forespore septum formation in sporulating bacteria, an effect also exerted by other inhibitors of peptidoglycan biosynthesis (9, 10).

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