Extracellular Proteases Increase Tolerance of *Bacillus subtilis* to Nafcillin

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Mutants of *Bacillus subtilis* capable of secreting high amounts of protease were highly tolerant to the lethal and lytic effects of nafcillin. Protease-deficient mutants were more susceptible. However, when subtilisin was added exogenously to a protease-deficient strain, the organism assumed the characteristics of nafcillin tolerance. Similarly, when phenylmethylsulfonyl fluoride, a serine protease inhibitor, was added to the tolerant strains, they became susceptible to nafcillin-induced lysis. The effects of nafcillin on *B. subtilis* were studied with both viability determinations and assay of cellular lysis. The minimum inhibitory concentrations of nafcillin tended to be higher for the protease hyperproducing strains, but these values could be reduced by the protease inhibitor. No loss of antibiotic activity was observed when nafcillin was incubated with either subtilisin or trypsin. Furthermore, protease and autolysin from *B. subtilis* were not modified by nafcillin. The results showed that extracellular proteases could render *B. subtilis* relatively tolerant to the killing and lytic effects of a cell wall antibiotic. The proteases were probably acting on the autolysins of the organism, thereby increasing tolerance to nafcillin.

Autolytic enzymes have been implicated in several growth and division events in bacteria. Formation of a septum and subsequent cell separation appear to depend on a functional autolysin (6, 18). Additional manifestations of autolytic activity in bacterial cells may be the ability to take up exogenous DNA (transformation) (16) and the turnover of cell wall components. Moreover, lysis in energy-depleted *Bacillus subtilis* is a result of poorly regulated autolytic activity (10).

In several different kinds of microorganisms, autolysis activity appears to be necessary for the killing and lytic effects induced by cell wall antibiotics such as penicillin (7, 11, 14). Other workers suggest that the susceptibility of bacteria to penicillin is unrelated to the level of autolysin (3). To clearly distinguish among penicillin-induced cell killing, cellular lysis, cell wall turnover, and autolysis levels, a system is required in which the various parameters can be monitored and modified. We found that the addition of proteases and protease inhibitors had a marked effect on the rate of turnover of peptidoglycan in *B. subtilis* (9). Turnover is an expression of autolytic activity but does not necessarily reflect absolute levels of autolysins (9). In the present study, we showed that proteases had considerable influence on the killing and lytic effects induced by nafcillin in *B. subtilis*. The results suggested that high levels of protease render the bacterium tolerant to cell wall antibiotics.


**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** All strains used in this study were derivatives of *B. subtilis* 168 and have been described in detail previously (9, 10, 15). The bacteria were maintained on tryptose-blood agar (Difco Laboratories, Detroit, Mich.) plates at 4°C.

Growth conditions have been outlined in an earlier publication (9). Briefly, overnight starter cultures were used to inoculate 20 ml of fresh growth medium (0.1 ml per 10 ml of fresh medium). The minimal-salts medium of Spizizen (19), without citrate, supplemented with 0.02% (wt/vol) casein hydrolysate and 0.5% (wt/vol) glycerol was used unless otherwise specified. Amino acid requirements for each auxotroph were met by supplementing the medium with 50 μg of the appropriate amino acid(s) per ml. All cultures were incubated in a 37°C gyratory shaker at 200 rpm.

**Autolysis assays.** Cellular dissolution resulting from the addition of nafcillin (final concentration, 1.0 μg/ml) to exponentially growing cells was monitored by measuring the decrease in culture turbidity on a Klett-
RESULTS

Susceptibility to nafcillin by strains of \textit{B. subtilis} having mutations in protease production. In an earlier study, we observed that extracellular proteases could modify cell wall turnover and autolytic activity in \textit{B. subtilis} (9). The evidence implicating autolysins in the mechanism of action of penicillin and our observations of protease inhibition of autolytic activity (9) led us to investigate the effects of extracellular proteases on cell wall antibiotic susceptibility in \textit{B. subtilis}. Evidence suggesting a critical role for autolytic enzymes in the lytic and killing action of penicillin has been presented for \textit{Escherichia coli} (7, 11), \textit{Streptococcus pneumoniae} (22, 24), and \textit{B. subtilis} (14).

Nafcillin was chosen for use in this study because it is not inactivated by the penicillinase produced by \textit{B. subtilis} (2). The genotypes of the mutants in protease production and the levels of extracellular proteases produced by each strain have been described previously (9). Preliminary experiments were performed to measure the lysis resulting from nafcillin treatment of exponential cultures of a protease hyperproducing strain, QB136, and a protease-deficient strain, SR22 (Fig. 1). Strain SR22 exhibited a rapid rate of lysis beginning a short time after the addition of nafcillin, whereas strain QB136 showed a reduction in the rate of lysis and did not begin to lyse exponentially until 3 h after the addition of the antibiotic. Having observed a significant difference in lysis between these strains, we measured the rates of lysis for several other protease hyperproducing mutants and the parental wild type, strain 168 (Table 1). The rate of lysis of strain SR22 in the presence of nafcillin was increased 20.3% as compared with strain 168, which produces normal levels (9) of extracellular proteases. In contrast, the protease hyperproducing strains hpr12, QB136, and hpr10 showed decreases of 60, 70, and 71%, respectively, when compared with the wild type. If protease alone can modulate tolerance to nafcillin-induced lysis in \textit{B. subtilis}, it should be possible to inhibit proteolytic activity and stimulate the lytic effects of the antibiotic. The serine protease inhibitor PMSF was added to cultures of strains QB136 and hpr10. After attainment of exponential growth, the cells were subjected to nafcillin. The results (Table 1) showed that PMSF enhanced susceptibility of the cells to the lytic effects induced by nafcillin. The times between cell lysis and the addition of nafcillin were also significantly shortened by PMSF.
TABLE 1. Nafcillin-induced lysis of *B. subtilis* protease mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protease activity</th>
<th>ΔTc</th>
<th>k/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>168</td>
<td>1.0</td>
<td>1.25</td>
<td>0.69</td>
</tr>
<tr>
<td>SR22</td>
<td>0.0</td>
<td>0.94</td>
<td>0.83</td>
</tr>
<tr>
<td>SR22 + subtilisin</td>
<td>1.75</td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td>(100 μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR22 + PMSF (0.5 mM)</td>
<td>1.00</td>
<td></td>
<td>0.88</td>
</tr>
<tr>
<td>hpr12</td>
<td>4.7</td>
<td>3.50</td>
<td>0.28</td>
</tr>
<tr>
<td>QB136</td>
<td>19.4</td>
<td>3.02</td>
<td>0.21</td>
</tr>
<tr>
<td>QB136 + PMSF (0.5 mM)</td>
<td>2.54</td>
<td></td>
<td>0.46</td>
</tr>
<tr>
<td>hpr10</td>
<td>5.4</td>
<td>3.27</td>
<td>0.20</td>
</tr>
<tr>
<td>hpr10 + PMSF (0.5 mM)</td>
<td>2.06</td>
<td></td>
<td>0.57</td>
</tr>
</tbody>
</table>

a Nafcillin (final concentration, 1 μg/ml or 25 μg/gal equivalents for strain SR22) was added to exponential-phase cells in minimal medium without citrate, supplemented with 0.02% (wt/vol) hydrolyzed casein, 0.5% (wt/vol) glycerol, and appropriate amino acids. Cellular lysis was measured by monitoring culture turbidity with a Klett-Summerson colorimeter. All cultures were incubated at 37°C and aerated continuously. Results are the averages of two to six experiments.

b Protease activity is taken from reference 9. The values shown are relative to strain 168 and reflect total protease activity on a hide powder azure substrate.

c ΔTc, Time period (hours) between nafcillin addition and the beginning of cell lysis.

d k/h = ln (initial Klett unit/final Klett unit)/time. Values represent first-order rates of cell lysis.

In Fig. 2, the effects of inhibition of protease activity on nafcillin-induced lysis in *B. subtilis* hpr10 are shown. Nafcillin was added to an exponential culture of strain hpr10 (a protease hyperproducing strain) after 90 min. The culture was split, and PMSF was added to one-half of the cells. Lysis was then monitored by light scattering. The results clearly showed that PMSF reversed the effects of extracellular protease on the susceptibility of *B. subtilis* to nafcillin. Inhibition of proteolytic activity enhances the lytic effects induced by the antibiotic. In control experiments, it was established that PMSF had no effects on exponential growth (as determined by mass increase by use of Klett readings). The protease inhibitor does, however, increase the rates of cell wall turnover by protease hyperproducing mutants of *B. subtilis* (9). The extracellular proteases may reduce the rates of lysis of *B. subtilis* resulting from treatment with nafcillin (Table 1).

Modification of nafcillin-induced lysis of strain SR22 by the addition of exogenous subtilisin. In an earlier investigation (9), it was shown that the addition of subtilisin, an extracellular protease produced by *Bacillus amyloliquefaciens*, to exponential cultures of strain SR22 caused a significant and immediate decrease in the rate of cell wall turnover. It was reasoned that the addition of subtilisin to exponential cultures of strain SR22 may also reduce the rate of lysis induced by nafcillin. The prediction that exogenously added subtilisin could modify the rate of nafcillin-induced lysis was confirmed (Fig. 3). As the concentration of subtilisin was increased, the observed rate of lysis of the protease-deficient strain, SR22, decreased by a factor of 4. At the highest concentration of subtilisin used (100 μg/ml), the rate of nafcillin-induced lysis of strain SR22 was identical to those observed for the protease hyperproducing strains. As an additional control, a 10-μg/ml solution of nafcillin was incubated with 100-μg/ml solutions of trypsin or subtilisin at 37°C in minimal salts. Samples were removed at intervals, diluted, and mixed with exponential cultures of *B. subtilis* SR22. There was no time-dependent (up to 4 h) decrease (compared with untreated nafcillin) in the activity of the antibiotic. Therefore, we concluded that protease does not have a direct effect on nafcillin.

Nafcillin-induced viability loss in strains of *B. subtilis* having mutations in protease production. Unregulated autolytic activity appears to be an
event which follows the initiation of the lethal action of penicillin (11, 14, 24). However, a positive correlation between impaired autolytic activity and resistance to viability loss after penicillin treatment has been observed in several gram-positive and gram-negative species of bacteria (7, 11, 14, 24). These observations led Tomasz and Waks to suggest that the autolysis activity may be the cause of cell death as well as lysis in pneumococci (24). Similar claims have been made for the effects of penicillin on E. coli (11, 12). To characterize the relationship of autolytic enzymes to penicillin-induced killing of B. subtilis, viability loss in the presence of nafcillin for strain QB136 (a protease hyperproducer) and strain SR22 (protease-deficient) was compared (Fig. 4 and 5). The turbidities of the cultures of strains SR22 and QB136 were adjusted to give similar numbers of CFU per milliliter. This was done by trial and error. Strain SR22 tends to form only a few cells in a chain, whereas strain QB136 forms several units of cells in a single chain (9). Strain SR22 exhibited a rapid rate of killing \( k_{\text{viability/h}} = 1.8 \) after the addition of nafcillin \( 1.0 \mu g/ml \). In contrast, the rate of viability loss in strain QB136 was significantly decreased \( k_{\text{viability/h}} = 0.57 \). The data suggest that the presence of a high level of extracellular protease induces an increased tolerance against the killing effects of nafcillin in B. subtilis.

Protease levels and MIC of nafcillin. One prediction that follows as a result of the experiments described above is that extracellular protease should increase the concentration of nafcillin required to inhibit growth of B. subtilis. Results from experiments to test this prediction are shown in Table 2. For strain SR22, the minimal inhibitory concentration (MIC) was found to be \( 0.04 \mu g \) of nafcillin per ml. The addition of subtilisin increased the MIC to \( 0.10 \mu g/ml \). The protease-deficient strain TH41 also exhibited a low MIC compared with that of wild-type strain 168 and the protease hyperproducing cells. Strains hpr10, hpr12, and QB136 all had several-fold higher MIC values than strain SR22. When PMSF was added to a growing culture of strain hpr10, the MIC was decreased from 0.75 to 0.50, showing that decreased proteolysis results in decreased MIC values for a single strain. In control experiments (data not shown), we found that nafcillin concentrations equivalent to 50 times the MIC only slightly increased the rates of lysis for strain QB136, hpr10, or hpr12. At these concentrations of nafcillin, the effects of PMSF were not observed. The massive concentrations of nafcillin may have effects on the multiple targets (2) in B. subtilis.

Does nafcillin modify the activities of the autolysins or proteases? Some of the results de-
scribed above could be explained if the autolysins or proteases were altered by nafcillin. Studies were carried out to determine whether nafcillin could either increase or decrease the in vitro activities of B. subtilis autolysin and protease preparations. Nafcillin did not reduce the rate of autolysis of a cell wall preparation (Table 3). The studies were performed at a hydrogen ion concentration which corresponds to that of the minimal medium (pH 7.2). Furthermore, the incubation of subtilisin or of extracellular proteases from B. subtilis QB136 with nafcillin did not result in a decrease in proteolytic activity (Table 3). We concluded that nafcillin has no effect on autolysis of cell walls of B. subtilis or on the proteolytic activity of the culture supernatant of a Bacillus protease mutant.

### DISCUSSION

B. subtilis can be rendered tolerant to the killing and lytic effects of a cell wall antibiotic by extracellular proteases. The order of susceptibility to nafcillin is protease-deficient mutants > wild type > protease hyperproducing strains. In addition, a serine protease inhibitor (PMSF) partially reverses the tolerance to nafcillin exhibited by the protease hyperproducing mutants. Furthermore, the addition of subtilisin to a protease-deficient mutant markedly enhances tolerance of the mutant to nafcillin. These results suggest that the lytic susceptibility of B. subtilis to a cell wall antibiotic is inversely related to the level of extracellular protease in the growth medium.

The exact mechanism by which protease acts to induce tolerance to nafcillin in B. subtilis is uncertain. Because proteases decrease the rates of cell wall turnover in B. subtilis (9) and because turnover is directly related to autolytic activity, we suggest that the resistance is a result of autolysin inactivation by proteolysis.

In exponentially growing cells of B. subtilis, autolytic activity is expressed only on the external cell surface (9, 18). Autolysins secreted by the organism are regulated by the energized

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**TABLE 2. MICs of nafcillin for B. subtilis protease mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR22</td>
<td>0.04</td>
</tr>
<tr>
<td>SR22 + subtilisin (100 µg/ml)</td>
<td>0.10</td>
</tr>
<tr>
<td>TH41</td>
<td>0.05</td>
</tr>
<tr>
<td>168</td>
<td>0.20</td>
</tr>
<tr>
<td>hpr10</td>
<td>0.75</td>
</tr>
<tr>
<td>hpr10 + PMSF (0.5 mM)</td>
<td>0.50</td>
</tr>
<tr>
<td>hpr12</td>
<td>0.75</td>
</tr>
<tr>
<td>QB136</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* The MIC is defined as that amount of nafcillin which inhibits growth in the minimal medium for at least 1 h without detectable lysis. Mass increase was measured on the Klett colorimeter.

**TABLE 3. Autolysin and protease of B. subtilis are refractory to the effects of nafcillin**

<table>
<thead>
<tr>
<th>Type of activity</th>
<th>Effect of following concn (µg/ml) of nafcillin:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Autolysis of B. subtilis SR22 cell walls&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00</td>
</tr>
<tr>
<td>Proteolysis of subtilisin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00</td>
</tr>
<tr>
<td>Extracellular B. subtilis QB136 proteases&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.00</td>
</tr>
</tbody>
</table>

<sup>a</sup> Autolysis was in 50 mM sodium phosphate (pH 7.2). Data are normalized to the walls in the absence of nafcillin.<br>
<sup>b</sup> Subtilisin concentration was 50 µg/ml. Solvent was 50 mM sodium phosphate (pH 7.2). Hide powder azure was the substrate. Assays were conducted as described previously (9).<br>
<sup>c</sup> Extracellular protease activity was determined as described previously (9).
tolerant to compared with inhibited by (21). Teolysis to the implicate by cells, are always higher. or markedly sensitive membrane (10), could block proteolysis synthetase of Bacillus for that. Other of cell walls can induced lysis LTA may be blocked. Lopez mutants deficient is responsible for penicillin-induced cell death in group A streptococci (8). Chatterjee et al. (3) have also observed a disparity between the lytic and killing events in a penicillin-inhibited, autolysin-deficient strain of Staphylococcus aureus H. They showed that the Lyt™ mutant exhibited a rate of viability loss identical to that observed for the wild-type strain. Expression of an apparent increased autolytic activity leading to cell lysis was not observed for the mutant or the wild type. These results indicated that the regulatory mechanisms controlling the secondary events may not be universal for all susceptible microorganisms. Nevertheless, our results for B. subtilis are in agreement with the proposal made by Tomasz that uncontrolled autolytic activity induced by penicillin is responsible for cell death and lysis.

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LITERATURE CITED


