Evidence for Participation of Autolysins in Bactericidal Action of Oxacillin on Staphylococcus aureus

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A comparison of the autolytic enzyme activity in Staphylococcus aureus strains that differ markedly in their rates of lysis and killing after exposure to oxacillin has been made. Log-phase cells of the clinical isolate that is tolerant to oxacillin inhibition were found to contain a level of autolytic enzyme activity comparable to that in a sensitive strain. This autolysin from log-phase cells was recovered after a single freeze-thaw cycle and assayed by using both native and penicillin (un-cross-linked) mureins. These same assays, however, revealed a significant difference in autolysin activity extractable from the two strains if the cells were inhibited by oxacillin. Under these conditions, the S. aureus strain that is susceptible to the killing and lytic effects of oxacillin had considerably more activity on penicillin murein than did the tolerant organism. These results provide evidence that hydrolytic enzymes on the cell surface are required to augment the wall damage initiated by oxacillin and other β-lactam antibiotics to produce a bactericidal effect.

Interest in the mechanism(s) by which antibiotics kill bacteria has a very practical basis since the patient with diminished host defenses must rely almost entirely on drugs to eradicate infecting organisms. Bacteriostatic antibiotics are not satisfactory in such cases (8).

Most mechanisms of action studies with the β-lactam group of antibiotics have been concerned with the nature of the lesion in cell wall synthesis incurred through penicillin action and, more recently, with the mechanism of inhibition by penicillins of various carboxypeptidases and transpeptidases which are presumed to be involved in peptidoglycan synthesis (1, 3, 8, 15, 18, 19). These latter studies have been directed toward an elucidation of the specific molecular mechanism whereby penicillins block the cross-linking of peptidoglycan in growing bacteria. Regardless of the eventual answer to the interesting question of how the penicillins act mechanistically, an inhibition of growth and cell wall synthesis is not clearly related to a bactericidal response by the cell. Rogers (11) in studies with Staphylococcus aureus adequately demonstrated that the inhibition of growth and inhibition of cell wall synthesis by penicillins is reversible (and not followed by death of the cells) unless they have grown for a sufficient time in the antibiotic. In this same study, it was confirmed that chloramphenicol not only antagonizes the bactericidal effects of penicillins, but that it simultaneously blocks the lysis of cells growing in the presence of penicillins. These results, and some later studies with Bacillus licheniformis and B. subtilis (12), have led to the hypothesis that penicillins and other antibiotics that inhibit the biosynthesis of a structurally functional cell wall eventually cause cell death because the autolysins indigenous to the cell surface hydrolyze more peptidoglycan than the cell can produce biosynthetically. The wall then becomes too weak to restrain the osmotic forces within the cell, and the result is either a subtle leakage of cytoplasmic constituents or at the extreme there is lysis (11, 12).

By far the most complete biochemical support for the thesis that autolysins are responsible for antibiotic-induced lysis (and death) has been obtained with Diplococcus pneumoniae (17). Tomasz and associates have demonstrated that the replacement of choline by ethanolamine in the wall teichoic acid rendered the cells tolerant to penicillin, cycloserine, phosphonmycin, and other inhibitors of cell wall synthesis. This means the cells were affected in a static fashion since growth was inhibited, but the cells were poorly killed and weakly lytic. Thus, modification of wall teichoic acid is sufficient to suppress "normal" autolysin activity and to render cells tolerant to otherwise lethal antibiotics.

Our recent isolation of an S. aureus strain with an enhanced ability to survive oxacillin in-
hilation afforded an attractive opportunity to determine if autolysins were involved in the bactericidal effect of oxacillin and to establish the biological basis for β-lactam tolerance in staphylococci. This paper describes the lytic effect of oxacillin on tolerant and susceptible strains of *S. aureus* and compares the autolysin activity toward native and un-cross-linked murein in these strains.

**MATERIALS AND METHODS**

**Organisms.** The *S. aureus* strains used in this study are recent clinical isolates that have been found from preliminary experiments to differ in their bactericidal response to penicillinase-resistant derivatives of penicillin. These organisms have been designated *S. aureus* Evans, Jackson, and Lovett. Each strain is sensitive to oxacillin in terms of the minimal inhibitory concentration (0.4 to 1.6 μg/ml), but *S. aureus* Evans resists the killing effect of these β-lactam derivatives.

All cultures were grown on Trypticase soy media at 37 C. Stock cultures were stored on Trypticase soy agar slants at 4 C.

**Autolysin extraction from *S. aureus* strains.** Two procedures were used to extract autolytic enzymes from the staphylococcal strains. The freeze-thaw procedure was essentially that reported by Huff (4). Log-phase cells of the appropriate strain were produced by inoculating Trypticase soy broth with cells from an overnight culture so as to give an absorbance at 540 nm (A440) of 0.1. This was incubated with shaking in a water bath, and the cells were harvested by centrifugation at an A440 of 0.70. The resulting cell pellet was washed twice with 0.01 M potassium phosphate buffer (pH 7.0) by centrifugation. The cells were then suspended in the desired volume of this buffer and placed in a refrigerator freezer (−20 C). The freeze-thaw extract of autolysins was obtained by thawing the cells at room temperature, removing the cells by centrifugation (10,000 x g for 10 min) and saving the supernatant solution.

A variation of this procedure involved the addition of oxacillin (10 μg/ml) to the growing culture described above when the A440 reached 0.70. After an additional 90-min incubation, the cells were harvested and subjected to the freeze-thaw procedure. This time period was chosen after preliminary experiments indicated that under these conditions the culture absorbance increased to about 1.4 and there was no detectable lysis of the cells. The amount of protein released from the cells by freezing and thawing was routinely found to be about 1 μg per ml per optical density unit in both log-phase and oxacillin-inhibited cells. Protein was measured by the procedure of Lowry et al. (6). A second freeze-thaw cycle released only about 10% as much autolysin as the initial cycle.

Another procedure used to obtain autolytic enzymes involved an extraction of either log-phase or oxacillin-inhibited cells with 3 M LiCl (7). Washed-cell pellets were simply suspended in the desired volume of 3 M LiCl (4 C) and stirred for 10 min, and the cells were removed by centrifugation (15,000 x g, 20 min). The resulting supernatant solution was dialyzed overnight against 1,000 volumes of 0.01 M potassium phosphate buffer (pH 7.0) to remove the salt. Each of the autolysin extracts could be stored at −70 C for at least 2 weeks with little loss in activity.

**Preparation of radioactive mureins.** The autolysin assays employed murein from *S. aureus* Evans and Jackson that was labeled with either L-[14C]lysine or [3H]glycine. Actively growing cells (A440 = 0.40) were suspended in peptone-yeast extract broth (16) containing 0.05 μCi of L-[14C]lysine per ml (27 mCi/mmol, Mallinkrodt) and grown to an A440 of 0.80. The cells were then harvested by centrifugation and washed three times with distilled water. Cell walls were prepared by the procedure of Schleiffer and Kandler (13). The cells were suspended in 10% trichloroacetic acid and incubated for 20 min at 95 C. The extracted cells were then washed repeatedly (about five times) with distilled water and suspended in 0.01 M potassium phosphate buffer (pH 7.0). Trypsin (100 μg/ml) was next added, and the suspension was incubated for 2 h at 37 C. The cell walls were then washed repeatedly with distilled water. Murein prepared in this fashion, referred to here as “log walls,” lacked any residual autolysin activity and routinely contained about 10,000 counts per min per mg (dry weight) under the counting conditions described below.

The other substrate used was “penicillin” or un-cross-linked murein, and was prepared by harvesting log-phase cells from peptone-yeast extract broth at an A440 of 0.70 and suspending them in a medium that permits cell wall synthesis without growth. This medium contained: glucose, 0.1 M; CL-alanine, 1.5 mM; L-glutamic acid, 0.003 M; glycine, 0.003 M; L-lysine, 0.006 M; MgCl2, 0.003 M; MnCl2, 0.0003 M; uracil, 30 μg/ml; nicotinamide, 3 μg/ml; thiamine, 3 μg/ml; and potassium phosphate buffer 0.24 M (pH 7.3). Other additions were oxacillin (25 μg/ml) and [2-14C]glycine at 0.4 μCi/ml (specific activity = 9.4 Ci/mmol, New England Nuclear). After a 90-min incubation with shaking at 37 C, the cells were harvested, and murein was isolated as described above. The resulting cell wall preparation usually had about 40,000 counts per min per mg (dry weight).

**Radioactivity assay.** The assay for autolysin activity used in these studies was based on the release of radioactivity from staphylococcal mureins that were labeled during either log growth or during oxacillin inhibition. Preliminary studies with these extracts indicated that maximum activity was obtained at 37 C in pH 7.0 potassium phosphate buffer and that Mg2+ stimulated wall hydrolisis (4). The usual incubation mixture contained: 0.4 ml of labeled murein (350 to 400 μg [dry weight]); 70 μlitters of MgCl2 (0.8 M); 10 μlitters of KPO4 (1 M at pH 7.0); autolysin extract (25 to 75 μg of protein); and distilled water, to give 1.0 ml total volume. The assay was initiated by adding the autolysin to tubes prewarmed to 37 C. At time zero, and suitable intervals thereafter, a 0.2-ml sample was removed and added to 0.3-ml of ice-cold, distilled water. The samples were centrifuged immediately, the supernatant solutions were transferred to
counting vials, and radioactivity was determined as previously described (10) with a Beckman LS-230 scintillation counter.

RESULTS

Oxacillin-induced lysis of susceptible and tolerant strains of staphylococci. S. aureus Evans, a clinical isolate that resists the killing effect of oxacillin, is also resistant to the lytic effect of this drug. The data from the lytic responses of S. aureus Evans were compared with the Lovett strain, another clinical isolate that is fully sensitive to the bactericidal action of oxacillin (Fig. 1). Actively growing cultures of the two strains were exposed to 5 μg of oxacillin, per ml and the turbidity was followed spectrophotometrically. The absorbance of both cultures increased during the 1st h of growth in oxacillin, but the susceptible strain then underwent a rapid and progressive lysis whereas the tolerant strain did not (Fig. 1). Only a slow decline in culture turbidity was observed upon continued incubation of S. aureus Evans. These results were not appreciably affected by conditions that slowed growth (such as a lower temperature), by higher oxacillin concentrations (lysis was retarded in both strains), or by using other penicillinase-resistant β-lactams such as methicillin, nafcillin, or cloxacinil.

Comparison of autolysis of whole cells of susceptible and tolerant staphylococci. Since S. aureus Evans did not lyse as readily as the other staphylococci when inhibited by oxacillin, we first examined the possibility that this strain possessed some general autolysin deficiency which permitted the organism to escape the lytic (and lethal) consequences of oxacillin inhibition of growth. This was done by growing S. aureus Evans and a susceptible strain (the Jackson strain) to the log phase of growth and by suspending the cells from each to an A40 of 0.75 in 0.01 M potassium phosphate buffer (pH 6.5, 7.0, and 7.5). The turbidity of the cell suspensions was then measured during an incubation with shaking at 37 C. The results shown in Fig. 2 reveal that the tolerant strain is not deficient in “native” autolysis potential (Fig. 2). At least, log-phase cells of this strain lysed about as readily as susceptible strains at each of the three pH levels tested.

Comparison of extractable autolysin activity from S. aureus Evans and Jackson. The indication that log-phase cells of susceptible and tolerant staphylococci did not exhibit significantly different autolytic activity was confirmed with assays of autolytic activity which could be extracted with LiCl or by a freeze-thaw cycle. There was little difference in the ability of either the freeze-thaw or LiCl extracts to release radioactivity from native, log-phase cell walls

![Graph](http://aac.asm.org/)
(Table 1). Both extracts, from both organisms, were more active toward the murein from the Jackson strain, and the extracts from the Jackson strain had more activity against the Jackson murein than the Evans extracts had for the Evans murein. These differences, however, appeared insufficient to account for the diminished lytic response by the tolerant strain after oxacillin inhibition.

Comparison of autolytic activity from \textit{S. aureus} Evans and Jackson with penicillin murein. Since the autolysin activity from the tolerant strain of \textit{S. aureus} did not appear to be significantly less than that from a susceptible strain when native mureins were used as substrate, we next compared the activity of freeze-thaw extracts with penicillin mureins. Presumably, un-cross-linked murein is the substrate for any autolysin(s) involved in the lytic and bacte- ricidal events which are secondary to the effect of these antibiotics on peptidoglycan synthesis. However, log-phase cultures of the two strains have comparable autolysin activity toward penicillin mureins (Table 2). The data in Table 2 were obtained with freeze-thaw extracts, but a similar relationship was found with LiCl extracts from the two strains.

Autolysin activity in oxacillin-inhibited cells of \textit{S. aureus} Evans and Jackson. Since log-phase cultures of the susceptible and tolerant strains had comparable autolytic activity toward both native and penicillin mureins, we next extracted cells of the two strains that had been incubated for 90 min in oxacillin to determine if there was a significant difference in the amount of autolytic activity associated with the two strains after inhibition was established. Of the two \textit{S. aureus} strains the tolerant strain has slightly more activity toward its own penicillin murein during the log phase of growth, but considerable less autolysin activity was detectable in extracts from inhibited cultures (Fig. 3). Therefore, based on the autolysin activity in freeze-thaw extracts, the ability to hydrolyze un-cross-linked murein decreases (relative to log-phase cells) after inhibition in the tolerant strain and increases measurably after oxacillin inhibition of the susceptible strain.

Since the extracts from the two strains were made after a 90-min incubation with oxacillin, we next examined the kinetics of appearance of autolysin activity after the addition of antibiotic to growing cultures of the two strains. As

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Source of extract & Source of murein & Sp act* (counts per min per h per mg of protein) & \\
\hline
 & & Freeze-thaw & LiCl extract & \\
\hline
Evans & Evans & 5,000 & 63,000 & \\
 & Jackson & 13,000 & 193,000 & \\
Jackson & Evans & 2,000 & 76,000 & \\
 & Jackson & 8,000 & 142,000 & \\
\hline
\end{tabular}
\caption{Comparison of autolysin activity extractable from log-phase cells of \textit{S. aureus} strains}
\end{table}

*The levels of activity indicated represent the average values obtained from at least three separate experiments.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Source of extract & Source of murein & Sp act* (counts per min per h per mg of protein) & \\
\hline
 & & Expt 1 & Expt 2 & Expt 3 & \\
\hline
Evans & Evans & 17,000 & 13,000 & 17,000 & \\
 & Jackson & 24,000 & 13,000 & 22,000 & \\
Jackson & Evans & 11,000 & 10,000 & 9,000 & \\
 & Jackson & 16,000 & 13,000 & 6,000 & \\
\hline
\end{tabular}
\caption{Comparison of autolysin activity for penicillin mureins in extracts from \textit{S. aureus} strains}
\end{table}

*The specific activities were calculated from initial rates of release of [\textsuperscript{3}H]glycine from un-cross-linked cell walls, and each experiment involved separate enzyme and cell wall preparations.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Comparative representation of the autolysin activity in log-phase and oxacillin-inhibited cultures of \textit{S. aureus} Evans and Jackson. The activity of freeze-thaw extracts from log-phase cultures or oxacillin-inhibited cultures of the susceptible and tolerant strains for un-crosslinked murein is depicted. The specific activity for each extract (counts per min released per h per \textmu g of protein) is the mean of at least three separate experiments.}
\end{figure}
Likewise, the initial response to oxacillin in the Evans and Jackson strains is identical. After 30 to 60 min, however, the Jackson strain begins to lyse, and the turbidity of the Evans culture stabilizes.

Comparison of the freeze-thaw extractable autolysin in the cultures indicated that both the susceptible and tolerant strains had the same low autolytic activity toward penicillin murein during exponential growth (Fig 4b). After the addition of oxacillin, however, there was a rapid, fivefold increase in activity in the susceptible strain and about a twofold increase in extracts from the tolerant organism. This new level of autolysin activity was maintained for about 30 min in both cultures and subsequently declined. The rate of loss of autolysis activity was somewhat greater in the Jackson strain, but this is perhaps attributable to the fact that this culture had begun to lyse. Support for this conclusion is provided by a consideration of the cultural differences in the cells extracted for the assays depicted (Fig. 3 and 4b). The cell suspensions used to obtain extracts for Fig. 3 were heavy (A$\text{$_{540}$}$ = 1.4), and there was little lysis of the cells during the 90-min incubation in oxacillin. In the experiment described in Fig. 4, however, the culture turbidity was less, and lysis was evident in the susceptible strain. Presumably, this would account for the greater difference in autolysin activity in inhibited cultures of the Evans and Jackson strains which was observed previously (Fig. 3).

**DISCUSSION**

The results of this investigation suggest that *S. aureus* Evans manifests a drug tolerance that is similar to that described by Tomasz for certain pneumococci (15). In both instances, $\beta$-lactam antibiotics were shown to be effective in terms of an ability to inhibit cell growth, but had markedly reduced bactericidal activity. Even large increases in the antibiotic concentration do not overcome tolerance. In our experiments with staphylococci, this presumably means that the strains can be equally susceptible to the inhibitory effects of oxacillin on the enzyme(s) involved in cross-linking strands of peptidoglycan (17), but differ significantly in those secondary events that follow the defect in cell wall synthesis and determine whether the cells survive or are killed.

The most apparent physiological characteristic of tolerance in both pneumococci and staphylococci is an ability to resist the bacteriolytic consequences of growth in the presence of oxacillin. The exponential growth rate of the Evans and Jackson strains is the same.
known cell wall inhibitors. This observation has focused attention on the mechanisms by which autolytic enzymes could participate in the killing process in susceptible bacteria. Numerous studies have been made of autolytic enzymes from staphylococci. Unlike some organisms that appear to produce only a single autolytic enzyme, the *S. aureus* strains that have been examined possess glycosidases, amidases, glucosaminidases, and various peptidases. Some of the autolysins are extracellular (3), some are bound to the cell wall (14), some are intracellular (13), and some are both wall-associated and soluble (4). Thus, the specifics of the relationship among antibiotic action, autolytic activity, and cell death will not be easily revealed. However, our results with staphylococci do indicate that a general deficiency in autolysin activity is not required for β-lactam tolerance. Log-phase cells of the staphylococcal strains that we examined possessed comparable activity for both native and un-cross-linked mureins. The critical difference in our strains appeared in the period immediately after the addition of oxacillin to growing cells. The amount of extractable autolysin from both susceptible and tolerant strains rapidly increases after adding oxacillin, but the level is considerably elevated in the susceptible organism. Assuming that a freeze-thaw cycle releases enzyme from the surface of the two strains equally well, then the diminished lysis by *S. aureus* Evans could well be attributable to a lower amount of autolytic enzyme with access to the damaged murein (Fig. 4b).

The reason for a diminished autolysin level after oxacillin inhibition of the tolerant strain is not known. One possibility which has not been examined is that cytoplasmic autolysin(s) is not transported through the membrane as readily in the tolerant strain as in the susceptible strains. This hypothesis could predict an intracellular accumulation of autolysin. Singer et al. (14) reported that *S. aureus* H contains an intracellular *N*-acetylmuramyl-α-alanine amidase of unusually high molecular weight, which has its greatest activity on penicillin murein.

An alternative explanation of β-lactam tolerance in *S. aureus* could result from a demonstration of structural (or compositional) differences in the cell walls that either reduce autolysin activity on damaged murein or lessen the amount of autolysin that can localize at appropriate wall sites. This possibility is related to the pneumococcal tolerance that occurs upon substituting ethanolamine for choline in the wall teichoic acid (15). A modification of this nature is presumably sufficient to affect either the amount of autolysin that can associate with the cell wall or the activity of localized enzyme(s).

Additional studies will be required to resolve these and other potential explanations for antibiotic tolerance in *S. aureus*.

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