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# Antibiotic-Tolerant Mutants of *Streptococcus pneumoniae* That Are Not Deficient in Autolytic Activity

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Several mutants of *Streptococcus pneumoniae* were isolated that appeared tolerant, to varying extents, to the lytic and bactericidal effects of some antibiotics that inhibit peptidoglycan synthesis, but were not deficient in autolytic activity. The method used to select the mutants was based on the survival of tolerant mutants during treatment with either bacitracin, benzylpenicillin, D-cycloserine plus β-chloro-D-alanine, or vancomycin. Most (60 to 80%) of the surviving isolates were found to be deficient in autolytic activity, and these were rejected. The smaller proportion that had wild-type sensitivity to deoxycholate-induced lysis was studied further with respect to tolerance to the other antibiotics used in the selection procedures. Two of these mutants (selected by treatment with benzylpenicillin) were tolerant to either benzylpenicillin or D-cycloserine plus β-chloro-D-alanine, but were supersusceptible, in terms of initiation of lysis, to either bacitracin or vancomycin. The minimal inhibitory concentration values of several antibiotics for these two mutants were identical to those for the wild-type strain. Moreover, the interaction of radioactive benzylpenicillin with the penicillin-binding proteins, examined in whole organisms, also appeared the same as previously found for either wild-type or autolytic-deficient strains of *S. pneumoniae*.

It has generally been found that mutants deficient in autolytic activity (Lyt$^-$) are resistant to the lytic (and, to various degrees, the bactericidal) effects of antibiotics that inhibit peptidoglycan synthesis (1, 2, 5, 6, 9-11, 14). The organisms remained as sensitive as the wild-type to the growth-inhibitory effect of these compounds, and the term "antibiotic tolerance" was proposed for this phenomenon (14). These observations clearly implicated the activity of bacterial autolytic enzymes in the irreversible antimicrobial effects of cell wall inhibition in the particular microorganisms. It was less clear exactly how and why inhibition of cell wall synthesis should lead to the uncontrolled activity of autolytic enzymes. In one model originally detailed by Weidel and Pelzer (20), it was proposed that a carefully balanced activity of synthetic and hydrolytic enzymes was part of the bacterial cell wall-synthesizing mechanism and that selective interference with the synthetic enzymes (e.g., by penicillin) would uncouple this balanced activity, leading to net cell wall degradation (and bacterial lysis). Although this mechanism may indeed operate in some bacteria, it was less easy to apply this model to pneumococci, in which the autolytic activity responsible for antibiotic-induced lysis was shown to be an *N*-acetylmuramyl-L-alanine amidase (14), an enzyme not likely to be directly involved with cell wall assembly. In addition, indirect evidence suggested that the activity of this enzyme may be suppressed during most of the cell cycle by a specific inhibitor (Forssman antigen) (4) which seems to be liberated into the growth medium during treatment of the bacteria with cell wall inhibitors (17). For this and several other reasons, it has been proposed that, in pneumococci, inhibition of cell wall synthesis sets up a sequence of events that in some way releases the negative control of this enzyme (12, 13, 15, 17). However, the number and nature of steps leading from the inhibited cell wall synthetic reaction(s) to the terminal event of autolytic cell wall degradation has remained obscure. The purpose of this communication is to describe a new approach with the potential of resolving these steps. Mutants blocked in any one of these steps would be expected to show tolerant response to cell wall inhibitors even if the bacteria contained potentially active autolytic enzyme. Since autolysin-defective (Lyt$^-$) mutants are known to be tolerant to all cell wall inhibitors, tolerance caused by secondary mutations in the chain of autolysin-decontrolling steps would be masked by the tolerant phenotype of the Lyt$^-$ cell. One way to bypass this problem would be to isolate mutants that are tolerant to a class (or classes) of peptidoglycan synthesis-inhibiting antibiotics but nevertheless have full autolytic activity. These mutants would therefore have a Tol$^+$ Lyt$^+$ phenotype as opposed to the previously isolated

autolysis-deficient mutants that are $Tol^+$ $Lyt^-$. The wild-type strains would be $Tol^-$ $Lyt^+$ with this nomenclature.

The present communication reports the isolation of such a class of mutants in *Streptococcus pneumoniae* and some of their characteristics.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *S. pneumoniae* R6, a derivative of the Rockefeller University strain R36A, and DOC3, an autolysis-deficient mutant derived from R6 and selected as a deoxycholate-resistant isolate by S. Zighelboim of this department, were used throughout these studies. Bacteria were grown without aeration at 32°C in C medium (8) at an initial pH of 8.0, supplemented with yeast extract (0.1%; Difco Laboratories). Growth was monitored with a Coleman nephelometer.

The organisms were also grown on plates containing C medium solidified with 1% agar and plates of tryptic soy medium containing defibrinated sheep blood.

**Viability counts.** The number of viable cells was determined by the standard procedure for counting colonies (in triplicate) on blood agar plates. Organisms were suitably diluted in 50 mM sodium phosphate buffer, pH 7.0; 0.1-ml volumes of each sample were spread onto the plates and incubated for 36 h at 32°C.

**Mutagenic procedure.** Exponential-phase R6 cells were incubated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NNG, 200 μg/ml) at 37°C for 40 min. The organisms were recovered by filtration on a membrane filter (0.45 μm, 2.5-cm diameter), washed with medium, and suspended in fresh medium for growth of survivors.

**Antibiotics and reagents.** Ampicillin was obtained from Bristol Laboratories, Syracuse, N.Y., bacitracin from Mann Research Laboratories, Becton, Dickinson & Co., New York, N.Y., benzylpenicillin from Eli Lilly & Co., Indianapolis, Ind., and β-chloro-D-alanine from Vega Biochemicals, Tucson, Ariz. D-Cycloserine and vancomycin were purchased from Sigma Chemical Co., St. Louis, Mo., methicillin was supplied by Beecham Laboratories, Piscataway, N.J., and nafcillin was supplied by Wyeth Laboratories, Inc., Philadelphia, Pa. *para*-[$^3$H]benzylpenicillin (ethylpiperidinium salt; 31 Ci/mmol) was the generous gift of E. O. Stapley, Merck, Sharp & Dohme, Rahway, N.J. [*methyl*-$^3$H]choline chloride, (82 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass.

**Susceptibility tests.** The minimal inhibitory concentration (MIC) of the various antibiotics was determined by twofold serial dilution in C medium. Exponential-phase organisms (approximately $6 \times 10^4$ colony-forming units/ml) were inoculated into tubes containing the medium (1 ml) and incubated for 16 h at 32°C. The lowest concentration to prevent visible growth of the bacteria was recorded as the MIC.

**Selection of tolerant, autolytic mutants.** Exponential-phase organisms (previously mutagenized with NNG, approximately $2.5 \times 10^5$ colony-forming units/ml) were spread onto C medium plates containing either bacitracin (15 μg/ml), D-cycloserine (50 μg/ml) plus β-chloro-D-alanine (25 μg/ml), benzylpenicillin (0.1 μg/ml), or vancomycin (2 μg/ml) and incubated at 32°C for 6 h. Surviving bacteria were obtained by replica plating with sterile velvet onto blood agar plates, followed by incubation at 32°C for 40 h. The clones were rechecked for tolerance by streaking exponential-phase organisms onto more C medium plates containing the respective antibiotics and further replica plating after incubation at 32°C for 6 h.

The ability of the tolerant mutants to autolyze was checked by covering single colonies of each isolate on blood agar plates with a 1-μl volume of 1 M Tris-hydrochloride buffer, pH 7.0, containing 1% sodium deoxycholate, followed by incubation at 32°C for 5 min. Under these conditions, wild-type, autolytic colonies disappeared rapidly, leaving a residual zone of hemolysis, and colonies of autolysis-deficient mutants remained unchanged. As a further verification of the autolytic activity of those mutants tentatively ascribed as $Tol^+$ $Lyt^+$, sodium deoxycholate (0.1%, final concentration) was added to samples of exponential-phase organisms in C medium at 32°C and examined for lysis. All of the $Lyt^+$ strains lysed within 5 min.

**Assay for autolytic activity.** The activity in extracts obtained by method B of Tomasz and Westphal (18) was measured at 32°C by the procedure of Holtje and Tomasz (4), with the enzyme in limiting amounts. Triton X-100 was added to some of the samples to ensure no inhibition by the endogenous inhibitor, lipoteichoic acid.

**Assay for release of wall material.** The method used was adapted from that described by Kitano and Tomasz (7). Exponential-phase bacteria were grown for five generations in C medium containing 1 μCi of [$^3$H]choline per ml at 32°C, collected by centrifugation ($12,000 \times g$ for 2 min), transferred to isotope-free medium, and incubated further to allow another generation of growth. Samples (2 ml) of the organisms were then distributed into tubes containing either benzylpenicillin (0.1 μg/ml), vancomycin (2 μg/ml), or no antibiotic and incubated for a further 10 min. The organisms were immediately chilled in ice, recovered by centrifugation ($12,000 \times g$ for 2 min), washed with ice-cold 50 mM sodium phosphate buffer, pH 7.0 (2 ml), and finally suspended in 2 ml of either buffer or C medium at 32°C. At intervals throughout subsequent incubation, portions (200 μl) were removed into microcentrifuge tubes containing 20 μl of 38% formaldehyde and 20 μl of 0.5% bovine serum albumin and centrifuged at $12,000 \times g$ for 10 min. The amount of radioactivity remaining in the supernatant was then measured in 4-ml volumes of Ready Solv (Beckman Instruments, Inc., Fullerton, Calif.) scintillation fluid, using a Nuclear-Chicago Mark II spectrometer and counting for 4 min. The total radioactivity incorporated into the bacteria was determined after the addition of (i) Triton X-100 (0.1%, final concentration) to 200-μl portions of the autolytic strains or (ii) detergent plus 10 μl of a crude wild-type autolysin preparation to the autolysis-deficient strain, DOC3. Complete lysis and solubilization of the radioactivity occurred within 10 min under these conditions.

**Chromatography of released wall material.** Samples (2 ml) of the supernatants obtained from the organisms treated with the antibiotics were dialyzed against distilled water and lyophilized. The material was dissolved in distilled water (400 μl) and chromatographed on a column (0.9 by 55 cm) of agarose (Bio-

Gel A5-m) as described by Hakenbeck et al. (3).

**Labeling, separation, and detection of penicillin-binding proteins (PBPs).** Exponential-phase organisms (1 ml, about $8 \times 10^7$ cells/ml) were incubated in C medium with various amounts of [$^3$H]benzylpenicillin (50 times less to 1,500 times more than the MIC) at 32°C for 10 min, an excess of unlabeled benzylpenicillin (1.7 mM) was added, and the samples were immediately chilled in ice. The bacteria were recovered by centrifugation (1,100 × *g* for 2 min at 2°C) and suspended in 50 mM sodium phosphate buffer, pH 7.0 (50 μl), containing 1.7 mM benzylpenicillin and 1% Sarkosyl NL-97. Lysis of the mutant DOC3 was achieved by adding a small amount of a crude R6 autolysin preparation to the incubation mixture. After 5 min of incubation at 37°C, complete lysis of the organisms occurred and the penicillin-binding proteins were inactivated. The lysates were then prepared for slab gel polyacrylamide electrophoresis. The techniques used for discontinuous gel electrophoresis, staining, and detection of the PBPs by fluorography have been described earlier (21), as was the method used to measure the relative intensities of the bands.

## RESULTS

With the selection techniques described in Materials and Methods, no resistant clones were isolated and the frequencies of occurrence of tolerant strains for each antibiotic were: bacitracin, $6.9 \times 10^{-5}$; cycloserine plus β-chloro-D-alanine, $1.1 \times 10^{-4}$; benzylpenicillin, $9.1 \times 10^{-5}$; and vancomycin, $2.8 \times 10^{-5}$. The majority of the phenotypically tolerant mutants were also found to be deficient in autolytic activity as judged by sensitivity to detergent-induced lysis. The numbers of Tol+ Lyt+ mutants identified and their percentage (in parentheses) of the tolerant mutants were: bacitracin, 21 (24%); cycloserine plus β-chloro-D-alanine, 46 (37%); benzylpenicillin, 19 (17%); and vancomycin, 14 (40%).

When the 100 mutants were tested for susceptibility to the particular antibiotic used for the selection of each, a range of lysis rates was observed. In all cases, these antibiotic-induced lysis rates were less than those exhibited by the wild-type strain. In particular, most of the mutants isolated after treatment with cycloserine plus β-chloro-D-alanine or benzylpenicillin had relatively low rates of lysis (less than 20% that of the wild type, as measured by the maximum rates of loss of turbidity), whereas only two mutants of those selected with bacitracin or vancomycin (B1.23, V1.28) had rates less than half that of the wild type. These latter two mutants, together with those from the other selection procedures which had the lowest rates of antibiotic-induced lysis (all of which underwent spontaneous lysis in the stationary phase of growth overnight), were then screened against the other antibiotics to check for cross-tolerance since the autolysis-deficient mutants of *S. pneumoniae* are tolerant to several types of antibiotics (17). All of the mutants that were selected by treatment with cycloserine plus β-chloro-D-alanine were found to be equally tolerant to lysis by benzylpenicillin, but most were as susceptible as the wild type to bacitracin and vancomycin. A similar result was observed with the mutants selected with benzylpenicillin in that these were cross-tolerant to cycloserine plus β-chloro-D-alanine but susceptible to bacitracin and vancomycin. However, three of these penicillin-selected mutants (P2.13, P3.45, and P3.49) appeared to be supersusceptible to bacitracin and vancomycin in that initiation of lysis after addition of these antibiotics was observed at a faster rate than in the wild type. The response of one mutant from each selection procedure is shown in Fig. 1, together with the responses of the wild-type strain and an autolysis-deficient strain, DOC3. This latter strain showed complete resistance to lysis induction by any of the cell wall synthesis inhibitors tested, including vancomycin and bacitracin (data not shown). The potential for autolysis was still present in the other mutants after inhibition of growth with the appropriate antibiotics, since addition of Nonidet P-40 (0.1%, final concentration) after 2 h of incubation resulted in complete lysis of the organisms within 5 min, whether some degree of antibiotic-induced lysis was apparent or not. In contrast, the addition of detergent to the autolysis-deficient strain under the same conditions had no apparent effect. The mutants selected by treatment with bacitracin and vancomycin (B1.23 and V1.28) only had maximal rates of lysis about 30 to 40% that of the wild type after addition of each antibiotic. Two mutants (P3.45 and P3.49) were chosen for further study since they exhibited the greatest differences between responses to the antibiotics tested. Mutant P3.45 behaved in a manner identical to P3.49 in the tests illustrated in Fig. 1 (data not shown). The autolytic activity of these mutants appeared identical to that of the wild type when extracts were obtained from the organisms (Table 1).

**Effect of benzylpenicillin on viability.** Addition of the antibiotic (at 20× MIC) to the wild-type strain resulted in rapid loss of viability (Fig. 2), whereas the rates of killing of the Tol+ Lyt− mutant, DOC3, and the two Tol+ Lyt+ mutants, P3.45 and P3.49, were some five- to sixfold less.

**Susceptibility of the mutants to various antibiotics.** The MIC values of bacitracin (3.1 μg/ml), benzylpenicillin (0.006 μg/ml), cycloserine plus β-chloro-D-alanine (10/5 μg/ml), and vancomycin (0.4 μg/ml) were found to be the same for the two Tol+ Lyt+ mutants as for the wild-type strain. Ampicillin (0.025 μg/ml), methicillin (0.2 μg/ml), and nafcillin (0.025 μg/ml)

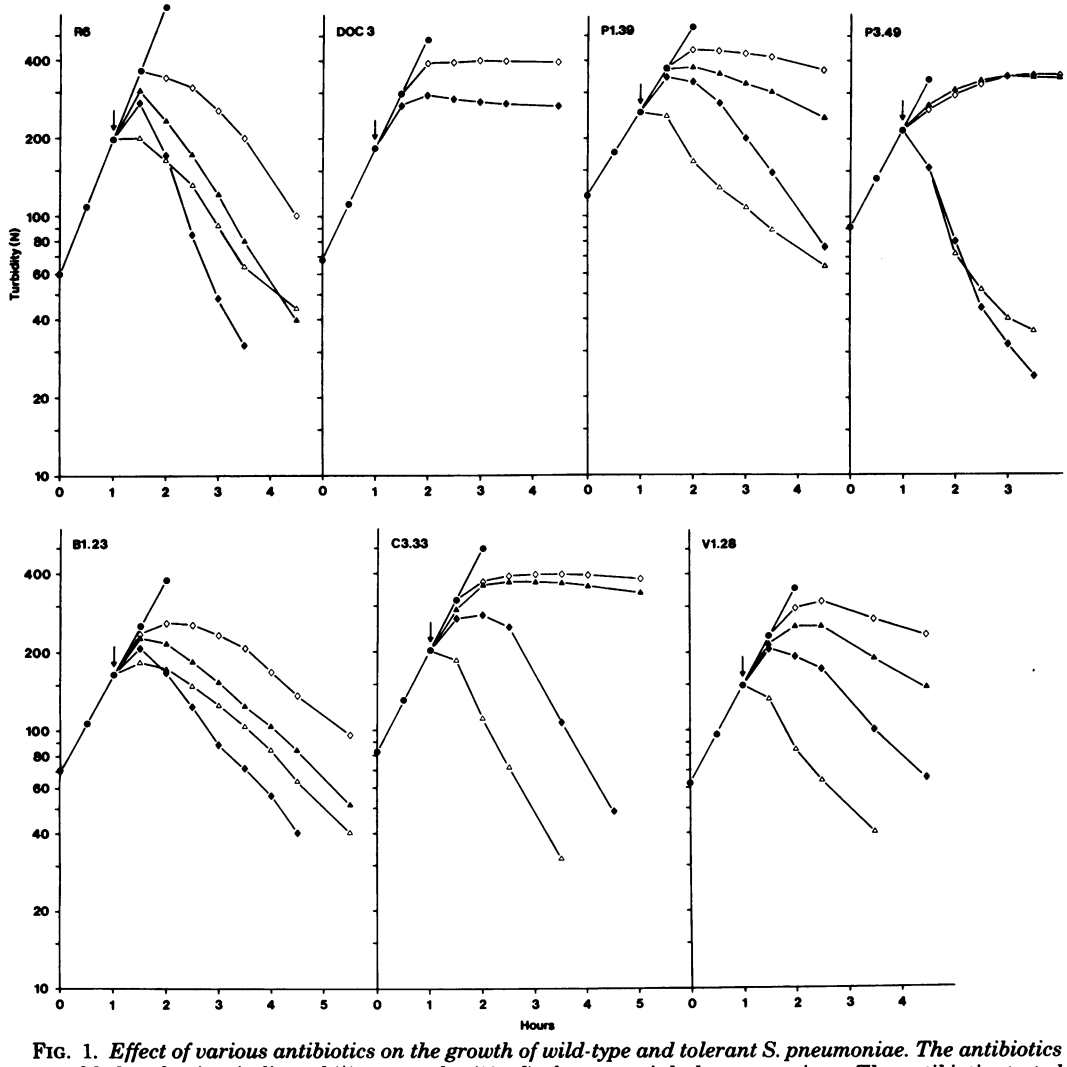


FIG. 1. *Effect of various antibiotics on the growth of wild-type and tolerant S. pneumoniae. The antibiotics were added at the time indicated (↓) to samples (10 ml) of exponential-phase organisms. The antibiotics tested and the concentrations used (in multiples of their MIC values) were: bacitracin (5×, Δ), benzylpenicillin (20×, ◇), D-cycloserine plus β-chloro-D-alanine (5×, ▲), and vancomycin (5×, ◆).*

TABLE 1. *Autolytic activity of wall extracts from tolerant and wild-type strains*[a]

| Strain | Protein concn (mg/ml) | Activity (U/ml)[b] | |
|---|---|---|---|
| | | Buffer | With Triton X-100 (0.1%) |
| R6 | 1.87 | 19.0 | 35.2 |
| DOC3 | 1.61 | 0.45 | 0.52 |
| P3.45 | 1.97 | 18.0 | 38.6 |
| P3.49 | 2.12 | 20.8 | 40.4 |

[a] Extracts, prepared by method B of Tomasz and Westphal (18), were obtained from organisms in late exponential phase (approximately 2 × $10^8$ colony-forming units/ml).

[b] Units as defined by Holtje and Tomasz (4).

were also found to have the same MIC values for the three strains.

**Sensitization to exogenous autolytic enzyme.** Previous studies with pneumococci (12, 19) have shown that autolysis-deficient mutants become sensitized to wild-type autolysin after treatment with a variety of cell wall inhibitors, including benzylpenicillin and vancomycin. Such sensitization was observed with the mutant DOC3 (Fig. 3), although much faster lysis occurred after treatment with vancomycin than with benzylpenicillin. The addition of the enzyme after a longer incubation with benzylpenicillin also appeared to promote faster lysis. This effect was not seen with the vancomycin-treated

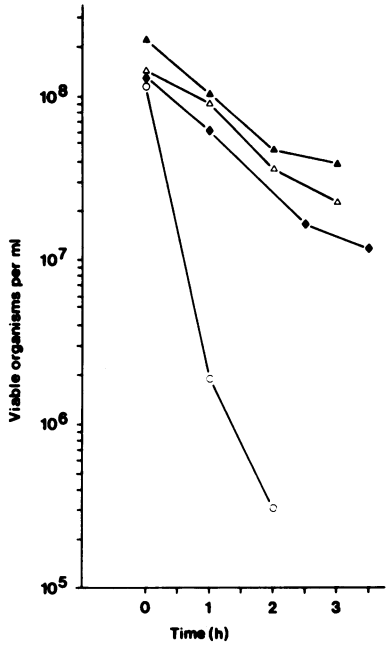


FIG. 2. *Effect of benzylpenicillin on the viability of wild-type and mutant strains. Benzylpenicillin (20 × MIC) was added to samples (10 ml) of exponential-phase organisms, and the loss of viability was determined by serial dilutions onto blood agar plates. Microscopic observation indicated no clumping or chaining in the cultures. Strains: wild-type, R6, (○); Tol+ Lyt−, DOC3 (◆); Tol+ Lyt+, P3.45 (△); Tol+ Lyt+, P3.49 (▲).*

bacteria, suggesting that the organisms were being lysed at the maximal rate after 1.5 h of incubation with the antibiotic. The two Tol+ Lyt+ mutants differed in their susceptibility to the added autolytic enzyme after treatment with benzylpenicillin (Fig. 3). Mutant P3.49 was also more susceptible to the enzyme after extended incubation with the antibiotic. The addition of enzyme alone had no effect on the growth rate or appearance of the untreated organisms, as was the case for the previously isolated Tol+ Lyt− mutants (16).

**Susceptibility to vancomycin after addition of benzylpenicillin.** Since vancomycin induced rapid lysis of the two Tol+ Lyt+ mutants, the effect of this antibiotic added to samples of each strain after addition of benzylpenicillin (to which they were tolerant) was examined to determine if the latter would antagonize the bacteriolytic effect of vancomycin. The addition of vancomycin at different times after benzylpenicillin resulted in decreasing rates of lysis of the organisms (Fig. 4). However, the bacteria were still capable of autolysis since the addition of Nonidet P-40 (0.1%, final concentration) caused a rapid drop in turbidity of each sample. The decrease in susceptibility to vancomycin was not merely due to the increase in density of the cultures because the rates of lysis of vancomycin-treated bacteria were essentially the same when the antibiotic was added to exponential-phase organisms at turbidities between 100 and 800 (data not shown).

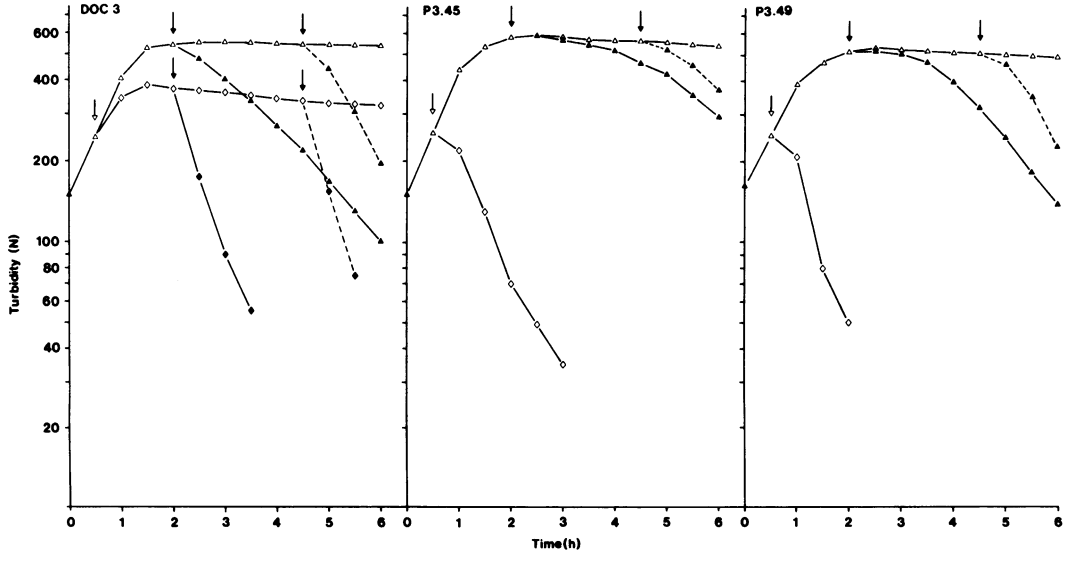


FIG. 3. *Sensitization to exogenous autolytic enzyme. Samples (10 ml) of exponential-phase bacteria were treated with either benzylpenicillin (20 × MIC, △) or vancomycin (5 × MIC, ◇) at the time indicated (▽). After 90 or 240 min of incubation, 15 μl (4.4 mg/ml, 12 U/mg) of a wild-type autolysin preparation (16) was added as indicated (▼) to the samples.*

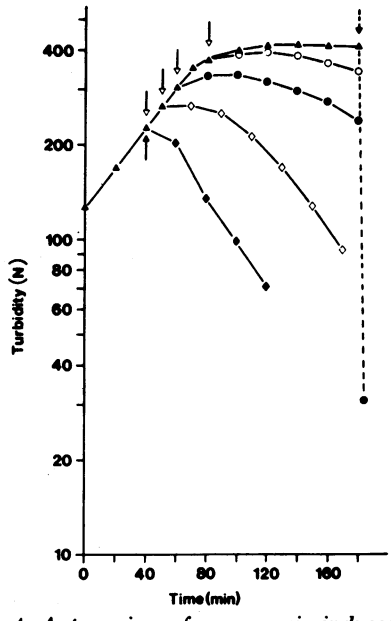


FIG. 4. *Antagonism of vancomycin-induced lysis by benzylpenicillin. Benzylpenicillin (20 × MIC) was added to exponential-phase P3.45 as indicated (▲, ▲). Vancomycin (5 × MIC) was added simultaneously to one sample (◆), and then after 10 (◇), 20 (●), and 40 (○) min of incubation. Nonidet P-40 (0.1%, final concentration) was added to three of the samples (▲, ●, ○) as indicated (▼).*

**Release of choline-containing material during incubation of antibiotic-treated mutants in growth medium.** Cultures of wild-type pneumococci and mutants DOC3, P3.45, and P3.49 were prelabeled with [$^3$H]choline during growth. After one generation of subsequent growth in isotope-free medium (chase), portions of the cultures received short treatment (10 min) with either benzylpenicillin (at 20× MIC) or vancomycin (at 5× MIC). The antibiotics were removed, the bacteria were resuspended in fresh growth medium, and the release of radioactivity into the supernatants was followed during incubation at 32°C (Fig. 5). In the case of wild-type bacteria, pretreatment with either antibiotic caused substantial loss of radioactivity from the cells (Fig. 5a). Comparable loss was also observed in the Tol$^+$ Lyt$^+$ mutants which received prior vancomycin treatment (Fig. 5c,d). In contrast, only a small fraction of cell-associated radioactivity (12 to 18% of total) was released from the DOC3 mutant pretreated with the two antibiotics (Fig. 5b), as in the case of the Tol$^+$ Lyt$^+$ mutants after the preexposure to penicillin (Fig. 5c,d).

**Analysis of the released wall material.** The material released from strains DOC3 and P3.45, treated with either benzylpenicillin or vancomycin for 120 min in medium, was separated into two peaks (I and II) by chromatography on Bio-Gel A5-m (3; see Materials and Methods). In each case, the amount of radioactive label in the first peak, the great majority of which corresponded to lipoteichoic acid (LTA; 4), was between 13 and 18% of the total incorporated into the whole organisms. Peak I accounted for 67 to 75% of the radioactivity released by either benzylpenicillin- or vancomycin-treated DOC3 and benzylpenicillin-treated P3.45. However, peak I contained only some 28% of the radioactivity released by the vancomycin-treated P3.45. Thus, the bulk of material released in this latter case was not LTA but presumably peptidoglycan-associated teichoic acid solubilized by activity of the autolytic amidase (3).

**Release of choline-containing material during incubation of antibiotic-treated mutants in buffer.** The design of this experiment was identical to that described in the previous section except that the cells were suspended in buffer solution, i.e., in the absence of nutrients (Fig. 6). As with cells resuspended in growth medium, wild-type cells pretreated with either antibiotic and Tol$^+$ Lyt$^+$ mutants pretreated with vancomycin were found to release large amounts of the cell-associated radioactivity during incubation in buffer. However, virtually no release of radioactivity was observed in the case

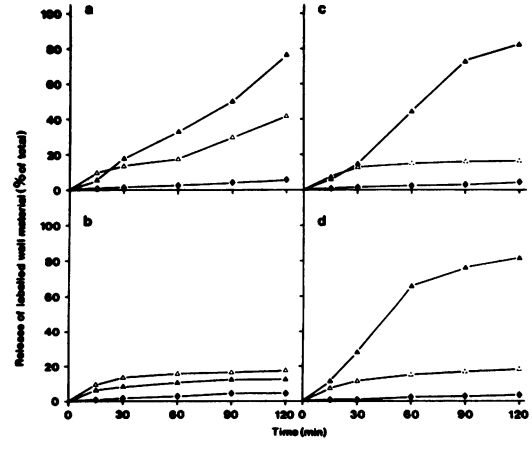


FIG. 5. *Release of labeled choline-containing material by growing organisms. Exponential-phase bacteria were treated with either benzylpenicillin (20 × MIC, △) or vancomycin (5 × MIC, ▲), washed, and suspended in fresh medium, and the percentage release of labeled material was determined as described in Materials and Methods. Control samples (◆) received no antibiotics. (a) Wild-type, Tol$^-$ Lyt$^+$, R6; (b) Tol$^+$ Lyt$^-$, DOC3; (c) Tol$^+$ Lyt$^+$, P3.45; (d) Tol$^+$ Lyt$^+$, P3.49. The 100% values of total radioactivity in the samples of the lysed organisms were 6,000 to 8,000 cpm.*

of antibiotic-treated DOC3 and the penicillin-treated Tol+ Lyt+ mutants.

**PBPs of Tol+ Lyt+ mutants.** Since the Tol+ Lyt+ mutants appeared tolerant to benzylpenicillin, the accessibility and binding affinities of the PBPs in the two mutants were compared with those from the wild-type and Tol+ Lyt− strains, using a labeling technique in which live bacteria in growth medium are exposed to [$^3$H]penicillin (21). No detectable differences between any of the strains were found (Fig. 7), and the amounts of benzylpenicillin needed to half-saturate and fully saturate the individual PBPs were also the same in each case.

## DISCUSSION

The general antibiotic tolerance (to all cell wall inhibitors) of pneumococcal mutants selected for autolytic deficiency is understandable if one assumes that autolytic activity represents the common terminal stage of "inhibitory pathways" leading from inhibited cell wall synthetic reactions to the inactivated (lysed) bacterial cell.

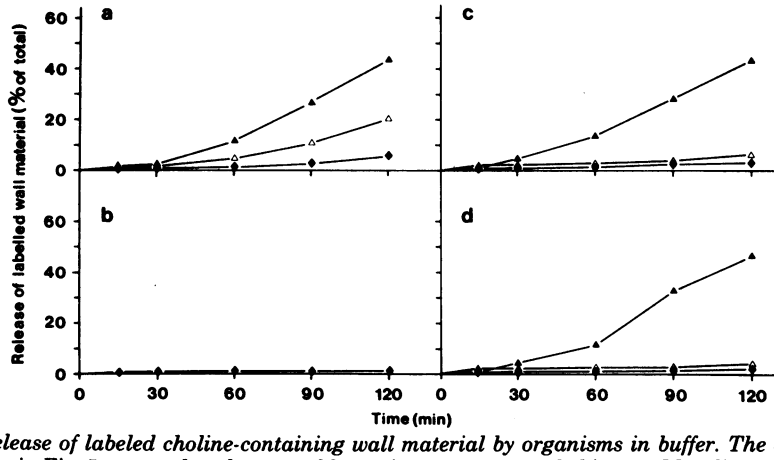


FIG. 6. *Release of labeled choline-containing wall material by organisms in buffer. The details were the same as those in Fig. 5, except that the treated bacteria were resuspended in 50 mM sodium phosphate buffer, pH 7.0. The Tol+ Lyt− mutant, DOC3, did not release any more material after treatment with the antibiotics than did the control sample, and only the latter is indicated.*

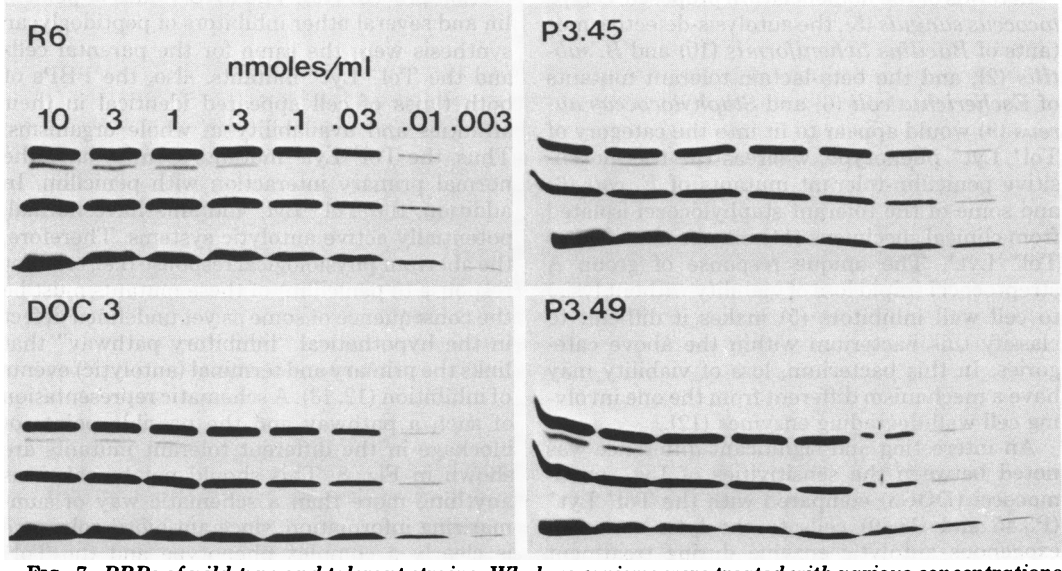


FIG. 7. *PBPs of wild-type and tolerant strains. Whole organisms were treated with various concentrations of [$^3$H]benzylpenicillin in growth medium at 32°C for 10 min (21), and the PBPs were detected by fluorography after separation of the proteins in the lysates by gel electrophoresis. The concentrations used in each case are indicated above the PBPs of R6. The PBP2 complex was not resolved into 2A and 2B (21) in this particular experiment.*

However, when the primary selection is for survival during antibiotic treatment, bacterial mutants of an additional and novel type may also be obtained, as in the case described in this report. These Tol$^+$ Lyt$^+$ mutants have normal wild-type levels of autolytic activity and, like wild-type pneumococci, undergo lysis upon exposure to detergents and during the stationary phase of growth. On the other hand, the Tol$^+$ Lyt$^+$ mutants do not lyse and do not degrade their cell walls during exposure to some cell wall inhibitors, and they exhibit a greatly reduced rate of loss of viability in response to the antibiotic treatment. Furthermore, the antibiotic tolerance of the Tol$^+$ Lyt$^+$ mutants exhibits a pattern of drug specificity, since the penicillin-selected and cycloserine-selected Tol$^+$ Lyt$^+$ mutants retained sensitivity to lysis by vancomycin and bacitracin whereas they showed cross-tolerance to the other antibiotic (i.e., mutants selected for penicillin tolerance would not lyse after cycloserine treatment and vice versa). On the other hand, tolerant mutants selected for survival during vancomycin or bacitracin treatment were all cross-tolerant to penicillin and cycloserine. However, the vancomycin- and bacitracin-selected mutants were not as markedly tolerant to antibiotic-induced lysis as were the penicillin- or cycloserine-selected mutants. One factor possibly contributing to these observations is the fact that vancomycin and bacitracin are more effective inducers of the lysis of even the wild-type pneumococci.

In terms of the nomenclature proposed, *Streptococcus sanguis* (5), the autolysis-defective mutants of *Bacillus licheniformis* (10) and *B. subtilis* (2), and the beta-lactam-tolerant mutants of *Escherichia coli* (6) and *Staphylococcus aureus* (9) would appear to fit into the category of Tol$^+$ Lyt$^-$ phenotype, whereas the thermosensitive penicillin-tolerant mutants of *E. coli* (6) and some of the tolerant staphylococci isolated from clinical specimens (11) may be classified as Tol$^+$ Lyt$^+$. The unique response of group A streptococci (rapid loss of viability without lysis) to cell wall inhibitors (5) makes it difficult to classify this bacterium within the above categories. In this bacterium, loss of viability may have a mechanism different from the one involving cell wall-degrading enzymes (12).

An interesting and significant difference was noted between the sensitivities of Lyt$^-$ pneumococci (DOC3) compared with the Tol$^+$ Lyt$^+$ (P3.45 and P3.49) cells to the lytic action of exogenous autolytic enzyme during treatment with penicillin. This finding suggests that the mechanism preventing lysis of the Tol$^+$ Lyt$^+$ cells by endogenous autolysin may be the same as that preventing sensitization to exogenous enzyme. Virtually nothing is known about the biochemical nature of this protective mechanism except that sensitization is improved with extended penicillin treatment of both the Lyt$^-$ and the Tol$^+$ Lyt$^+$ bacteria. The opposite type of effect was observed in studies with the effect of penicillin pretreatment on vancomycin-induced lysis. The experiment illustrated in Fig. 4 indicates that sensitivity of the Tol$^+$ Lyt$^+$ cells to vancomycin-induced lysis gradually decreases with increasing pretreatment of the cells with penicillin. This is presumably a reflection of a decrease in the potential for activation of the endogenous autolytic autolytic enzyme by some penicillin-induced change within the cells. Activation of the autolytic enzyme by detergent is not affected by this change, and, as was discussed above, the pretreatment with penicillin actually improves sensitivity of the cells to the exogenous autolytic enzyme.

The loss of large fractions of cell-associated choline label observed (both in media and in buffer) after antibiotic treatment of wild-type cells and vancomycin-treated Tol$^+$ Lyt$^+$ mutants is presumably a reflection of the autolytic cell wall degradation of these bacteria. The less extensive release of label observed in DOC3 seems to be due to secretion of LTA (19). This is probably also true for the penicillin-treated Tol$^+$ Lyt$^+$ cells. The lack of release of label during postincubation of both types of Tol$^+$ cells in buffer confirms the previously observed requirement of continued metabolic activity in the secretion process (19). The MIC values of penicillin and several other inhibitors of peptidoglycan synthesis were the same for the parental cells and the Tol$^+$ Lyt$^+$ mutants; also, the PBPs of both types of cell appeared identical in their affinities and availability in whole organisms. Thus, the Tol$^+$ Lyt$^+$ mutants seem to have the normal primary interaction with penicillin. In addition, the Tol$^+$ Lyt$^+$ mutants have normal, potentially active autolytic systems. Therefore, the aberrant physiological response (i.e., tolerant response to penicillin) of these mutants must be the consequence of some as yet undefined defect in the hypothetical "inhibitory pathway" that links the primary and terminal (autolytic) events of inhibition (12, 13). A schematic representation of such a pathway and the possible points of blockage in the different tolerant mutants are shown in Fig. 8. This should not be taken as anything more than a schematic way of summarizing information, since antibiotic tolerance is clearly a complex phenotype and the Tol$^+$ mutants may contain more than one mutation affecting the antibiotic response of the bacteria. It is likely that steps in the "inhibitory pathway" constitute a "pathway of control" of autolytic

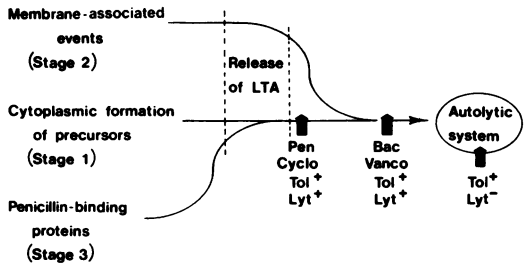


FIG. 8. *Schematic representation of the hypothetical "inhibitory pathway" of S. pneumoniae, summarizing the relationship of the various tolerant mutants to one another and, within the context of our model, of how inhibitors of cell wall synthesis cause triggering of cell wall degradation (12). Inhibition of events at any one of the stages of wall synthesis (left side) initiates some metabolic events that eventually lead to a common terminal stage: the decontrolling of pneumococcal autolysin activity. In the Tol+ Lyt− cells, the defect is in this terminal step (see arrow at right-hand end of drawing). Such mutants are tolerant to all cell wall inhibitors. In contrast, the mutants described in this paper are lysed (and killed) by only some cell wall inhibitors, and a potentially activatable autolytic system is present in each of the mutants. We propose that these mutants are blocked at some step within a hypothetical "inhibitory pathway" that "connects" the inhibited cell wall synthetic reaction with the triggering of autolytic activity. Mutants selected for survival during treatments with either penicillin or cycloserine are cross-tolerant to both drugs but still lysed by vancomycin and bacitracin. In contrast, mutants tolerant to bacitracin or vancomycin are also tolerant to penicillin and cycloserine. On the basis of these observations, we indicated the mutational block in the penicillin- and cycloserine-tolerant mutants as being to the "left" of the block in the bacitracin- and vancomycin-tolerant mutants (i.e., farther away from the autolytic system). Since antibiotic-induced release of choline-labeled macromolecules (Forssman antigen) still occurs in all the tolerant mutants (Lyt+ and Lyt− as well), we put this secretion phenomenon to the left of the blocks of both the vancomycin- and penicillin-tolerant mutants.*

activity in the normally growing bacteria. We hope that the isolation of the Tol+ Lyt+ mutants will be helpful in identifying the biochemical nature of such an important pathway.

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