A Mutant of *Streptococcus pneumoniae* That Exhibits Thermosensitive Penicillin Tolerance and the Paradoxical Effect

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(Received 28 July 1986; revised 7 January 1987)

Mutants of *Streptococcus pneumoniae* that contain active autolysin and yet cannot be induced to lyse during treatment with penicillin (Lyt+ Tol+ mutants) have been described. We have now shown that these mutants are temperature dependent (32 °C); at 37 °C these bacteria underwent penicillin-induced lysis. In addition, mutants at the lysis-permissive temperature showed the so-called 'paradoxical response' to penicillin. Temperature shift experiments indicated that the change from tolerant to lytic response or vice versa is a fast process. No differences were detected in autolysin specific activity or in the kinetics of inhibition of protein, peptidoglycan and teichoic acid syntheses in cells treated with penicillin at 32 and 37 °C. The results of genetic crosses indicated that the thermosensitivity of penicillin-induced autolysis in the Lyt+ Tol+ mutants is not a property of the autolytic enzyme itself. The observations suggest that the thermosensitive process in the mutants represents either a step(s) in autolysin regulation or involves some difference in the structure of the cell walls produced at 32 °C versus 37 °C.

**INTRODUCTION**

One of the pharmacologically most useful aspects of beta-lactam antibiotics is their ability to induce loss of viability and lysis of many bacteria, including pneumococci (Tomasz, 1979). Earlier work has established that lysis involves the rapid deregulation of autolytic enzymes (in the case of pneumococci an N-acetylmuramoyl-L-alanine amidase) as a result of the perturbation of cell wall synthesis (Tomasz, 1981). The detailed mechanism of how and why inhibition of wall synthesis provokes destructive autolysin activity in so many strains of bacteria has remained an enigma. This mechanism(s) is of particular significance in view of the body of evidence in the literature describing clinical isolates of pathogenic bacteria which are penicillin tolerant, i.e., in which the lytic and/or killing action of the antibiotic is suppressed (for a recent review, see Handwerger & Tomasz, 1985).

There have been three types of bacterial mutants or isolates described in which growth inhibition by penicillin is not followed by lysis of bacteria: (i) laboratory mutants of pneumococci and other bacteria (for a review, see Tomasz, 1981) and naturally occurring strains (Horne & Tomasz, 1977) that are autolysin defective; (ii) isolates that show the so-called 'paradoxical effect', i.e. bacteria in which the lytic/cidal effects of penicillin have a concentration optimum (Eagle & Musselman, 1948); and (iii) laboratory mutants (Williamson & Tomasz, 1980) and clinical isolates (Liu & Tomasz, 1985), in which penicillin does not induce lysis in spite of the presence of normal amounts of autolysin and lysis-sensitive cell wall substrate. The mechanism of the second and third classes of tolerance are unknown. We have used the Lyt+ Tol+ mutants of pneumococci (Williamson & Tomasz, 1980) which are in the third class to investigate possible mechanisms of autolysin regulation.

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**Abbreviation**: PBP, penicillin-binding protein.

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In this paper we describe (i) some recently observed novel features of the Lyt+ Tol+ mutants (e.g. the thermosensitivity of penicillin-induced autolysis), and (ii) genetic experiments probing the biochemical basis of thermosensitive antibiotic tolerance in these mutants.

**METHODS**

**Bacterial strains.** *Streptococcus pneumoniae* strain R6 is a derivative of Rockefeller University strain R36A. Lyt-4 is a lysis-defective (Lyt-) laboratory strain with low specific autolytic enzyme activity. P345 and P349 are mutants of R6 induced by treatment with nitrosoguanidine and selected at 32 °C for tolerance to benzylpenicillin despite wild-type autolytic enzyme levels (Williamson & Tomasz, 1980). P349 (Lyt+) was constructed using DNA from a lysis-defective mutant DOC4. The selection of the Lyt- phenotype in the transformants was by the deoxycholate-agar method (Jiang & Tomasz, 1981).

**Growth conditions.** Strains were grown in a casein-based semi-synthetic medium at pH 8.0 (C medium; Lacks & Hotchkiss, 1960) supplemented with 0.1% (w/v) yeast extract (Difco). This medium is referred to as C + Y. Biosynthetic labelling of cells was accomplished in a chemically defined medium referred to as CdmY (Tomasz, 1964). Culture growth was monitored by following the light scattering of suspensions using a Coleman nephelometer (Coleman Instruments, Oak Brook, Ill., USA). Culture stocks frozen in C + Y containing 10% (v/v) glycerol, were kept at −70 °C.

**Antibiotics and reagents.** Benzylpenicillin was purchased from Eli Lilly, USA. [methyl-3H]Choline chloride [80 Ci mmol−1 (2-96 TBq mmol−1)], L-[alanine-2,3-3H]phenylalanine [21 Ci mmol−1 (0-78 TBq mmol−1)] and L-[4,5-3H(N)]lysine [90-8 Ci mmol−1 (3-36 TBq mmol−1)] were purchased from New England Nuclear. All other chemicals were reagent grade commercially available products.

**Susceptibility testing.** The MIC of benzylpenicillin for each strain was determined by the tube dilution technique. MIC values were the same at 32 °C and 37 °C for the strains tested.

Viability counts of organisms before and during benzylpenicillin treatment were determined by dilution of cultures in C medium. Penicillinase was added to give a final concentration of 100 units ml−1 if appropriate. The organisms were plated in triplicate in 0.8% (w/v) agar containing C + Y medium. Colony forming units (c.f.u.) were counted after 36 h incubation at 37 °C.

**Assay for degradation of cell wall peptidoglycan.** The method used was adapted from Park & Hancock (1960), as described by Liu & Tomasz (1985). In short, exponential-phase bacteria were labelled with L-[4,5-3H(N)]lysine followed by growth in non-radiolabelled medium for one and a half generations. Samples (10 ml) were distributed into prewarmed tubes containing 0, 2, 5, 10, 20 or 50 × MIC of benzylpenicillin. Growth and/or lysis of the cultures was monitored and 200 µl samples were removed into 30 µl ice-cold 50% (w/v) trichloroacetic acid (TCA). The samples were chilled for 30 min on ice then heated in a boiling water bath for 30 min and processed for the determination of cell wall associated label (Liu & Tomasz, 1985).

**Assay for autolytic enzyme specific activity.** The activity in extracts obtained by method A of Tomasz & Westphal (1971) was measured by the procedure of Hölttje & Tomasz (1975) with the enzyme in limiting amounts. Protein determinations on the autolysin preparations were done following the method of Lowry. One unit of autolysin specific activity was defined as 5 × 10⁸ c.p.m. [methyl-3H]choline label solubilized (µg protein)−1 under standard assay conditions (Hölttje & Tomasz, 1975). Percentage specific activity relative to the Lyt+ R6 laboratory reference strain was also calculated.

**Assay for rate of protein, peptidoglycan and teichoic acid syntheses.** Bacteria were grown in the semi-synthetic (CdmY) medium modified to contain low concentrations of phenylalanine (5 µg instead of 100 µg ml−1) and lysine (10 µg instead of 100 µg ml−1). Portions (200–500 µl) of exponentially growing cultures (in the cell concentration range 1–3 × 10⁸ c.f.u. ml−1) were removed periodically into borosilicate tubes containing 5 µCi (185 kBq) amounts of either L-[alanine-2,3-3H]phenylalanine (for labelling proteins), L-[4,5-3H(N)]lysine (for labelling the peptidoglycan) or [methyl-3H]choline chloride (for labelling teichoic acids). After 5 min incubation at 37 or 32 °C the tubes were immersed into a dry-ice-acetone bath, to freeze the samples and stop further incorporation of the isotopes. After the end of the pulse-labelling experiments, the samples were thawed; the phenylalanine-labelled and the choline-labelled samples were treated with 10% TCA (final concentration) at 0 °C for 15 min and then the extracted suspensions were collected on glass fibre filter disks (GFA, Schleicher and Schuell); these were washed with 10% (w/v) TCA three times, dried at 100 °C and counted in a toluene based scintillation cocktail using a scintillation spectrometer (Nuclear Chicago model I). The samples labelled with radioactive lysine were put through the extraction procedure described for the labelling of cell wall peptidoglycan (see above).

**RESULTS**

**Thermosensitivity of penicillin-induced autolysins and a paradoxical dependence of lysis on the concentration of penicillin**

The penicillin response of the mutant (P345) and parental (R6) cells at two different growth temperatures and at several concentrations of penicillin (expressed in multiples of the MIC) is
Thermosensitive penicillin tolerance

Fig. 1. Effect of various concentrations of benzylpenicillin on *S. pneumoniae* R6 (a, c) and P345 (b, d) at 37 °C (a, b) and 32 °C (c, d). Data are from a single experiment representative of a number (6–8) of separate runs. The MIC value for both strains was 0.006 μg ml⁻¹ at either temperature. ○, No drug; ●, 2 × MIC; □, 5 × MIC; ■, 10 × MIC, △, 20 × MIC, ▲, 50 × MIC; ▽, 100 × MIC.

shown in Fig. 1. The response of the parental culture both at 37 and 32 °C to all the penicillin concentrations shown was lysis which commenced, after some delay, following the addition of the antibiotic. The mutant response was quite different at the two temperatures. Addition of the same concentrations of penicillin to the mutant P345 grown at 32 °C caused virtually no lysis within 6 h of the addition of the antibiotic. In occasional experiments (as in the one illustrated in Fig. 1) autolysis was induced at 32 °C also but only within a narrow range of penicillin concentrations, the optimum being 5–6 × the MIC value. While treatment of mutant cultures grown at 37 °C caused lysis, the onset of lysis was substantially delayed, during which time the cultures stopped growing. The rate of loss of viability of the mutant cultures exposed to 20 × the MIC of penicillin at the two temperatures roughly paralleled the lytic behaviour of the cultures: there was extensive and rapid loss of viability at 37 °C while viability loss was slower and limited at 32 °C (Fig. 2).

At 37 °C high concentrations of penicillin were not the most effective in causing lysis. Concentrations higher than the lysis-inducing optimum (20 × MIC) caused gradually less lysis until none was detectable at 100–200 × MIC. The Concentration optimum for lysis in the mutant culture seems to represent the so-called 'paradoxical response' to penicillin (Eagle & Musselman, 1948). As shown in Table 1, this is more readily apparent when the data in Fig. 1 are analysed and expressed in terms of 'lysis rates' (K) and 'lysis delay times' (Tₙ) (defined in Table 1). The penicillin-induced disintegration of cell structure (lysis) at 37 °C measured by the decrease in light scattering was paralleled by degradation of the cell wall material, as measured by the biochemical assay: there was much less extensive wall degradation at 32 °C than at 37 °C and at 37 °C there was less wall degradation during treatment with 200 × the MIC of penicillin than during a similar exposure to 5 × or 50 × the MIC (Table 2).

The sharply different penicillin response at the two growth temperatures prompted us to examine the rate with which the tolerant versus lytic response changed when cultures were shifted from one growth temperature to another. Fig. 3 shows that such a temperature shift, in either direction, caused a virtually instant shift to the antibiotic response characteristic of the particular temperature (tolerance at 32 °C; lysis at 37 °C).
Fig. 2. Viability of *S. pneumoniae* P345 following benzylpenicillin addition (arrowed) at 37 °C (a) and 32 °C (b). Data are from a single experiment in which viability at each time was determined by serial dilution and triplicate plating. ○, No drug; △, 20 × MIC.

Table 1. *Lysis delay times* (*T*<sub>d</sub>) and *lysis rates* (*K*) for wild-type (R6) and mutant (P345) cultures of *S. pneumoniae* following exposure to benzylpenicillin

*T*<sub>d</sub> is the interval of time from the maximum nephelocolorimeter reading until the reading has declined to about 80% of the maximum. Lysis rates are expressed as the first order rate constant *K* = ln(*N*<sub>0</sub>/*N*<sub>120</sub>) × min<sup>-1</sup>, where *N*<sub>0</sub> represents the maximum nephelocolorimeter reading and *N*<sub>120</sub> represents the reading after a further 120 min incubation. Maximum lysis rates of the mutant strain were observed at penicillin concentrations of 20 × MIC (at 37 °C) or, occasionally, at 5 × MIC (at 32 °C) (see *K* values in **bold** type. ND, Not done.

<table>
<thead>
<tr>
<th>Penicillin concn</th>
<th>R6</th>
<th>37 °C</th>
<th>32 °C</th>
<th>P345</th>
<th>37 °C</th>
<th>32 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 × MIC</td>
<td>180</td>
<td>102</td>
<td>2:2</td>
<td>220</td>
<td>1:4</td>
<td>90</td>
</tr>
<tr>
<td>10 × MIC</td>
<td>30</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20 × MIC</td>
<td>25</td>
<td>42</td>
<td>9:1</td>
<td>120</td>
<td>3:4</td>
<td>240</td>
</tr>
<tr>
<td>50 × MIC</td>
<td>15</td>
<td>22</td>
<td>10:3</td>
<td>210</td>
<td>0:8</td>
<td>240</td>
</tr>
<tr>
<td>100 × MIC</td>
<td>15</td>
<td>15:6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 2. *Rate of cell wall degradation in strain P345 during treatment with penicillin at 32 °C or 37 °C*

The initial peptidoglycan-associated radioactivity was 2:1 × 10<sup>4</sup> c.p.m. (in the culture grown at 37 °C) and 1:4 × 10<sup>4</sup> c.p.m. (in the culture grown at 32 °C) per 200 μl of culture. Data are expressed as a percentage of the initial cell wall associated radioactivity released from hot SDS-insoluble form and are corrected for radioactivity that was released in the control (no penicillin) cultures. Controls lost 7:5% (32 °C) and 20% (37 °C) of radioactivity during 4 h incubation.

<table>
<thead>
<tr>
<th>Duration of exposure to penicillin (h)</th>
<th>Penicillin concn</th>
<th>32 °C</th>
<th>37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>3:5</td>
<td>2:5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2:5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>3:5</td>
<td>17:5</td>
<td>22:5</td>
</tr>
<tr>
<td>4</td>
<td>6:5</td>
<td>21:5</td>
<td>14:5</td>
</tr>
</tbody>
</table>

* Penicillin concentrations are expressed in multiples of MIC units.
**Table 3. Specific activity of autolytic enzyme in pneumococcal strains**

Units of autolysin activity were as defined in the Methods. Values in parentheses are percentages relative to the wild-type (R6) reference strain.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Grown at 37 °C</th>
<th>Grown at 32 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6</td>
<td>476 (100)</td>
<td>552 (100)</td>
</tr>
<tr>
<td>P345</td>
<td>652 (137)</td>
<td>482 (92)</td>
</tr>
<tr>
<td>P349</td>
<td>692 (145)</td>
<td>656 (126)</td>
</tr>
<tr>
<td>P349 Lyt⁻</td>
<td>3.8 (0.8)</td>
<td>6.8 (1.3)</td>
</tr>
</tbody>
</table>

Three types of experiments were done in order to obtain some clues about the nature of the thermosensitive process in the mutant. First, the autolysin activity was assayed in cultures grown at the lysis-preventing versus lysis-permissive temperatures. No significant differences in total autolysin specific activities were found (Table 3).

Next, the possibility was tested that after penicillin addition mutant cultures may shut-down cellular polymer syntheses at different rates depending on the growth temperature. However, no differences paralleling the striking differences in biological response were obtained. A possible exception was a slight delay in the shut-off time in the rate of protein synthesis in cultures grown at 32 °C (Fig. 4).

To test the possibility that the thermosensitive difference in penicillin-induced autolysis reflected some abnormality of the autolytic enzyme in the mutant bacteria, the autolysin gene of mutant P349 was replaced by the Lyt⁻ gene of an autolysin-defective mutant (DOC 4), which codes for a highly temperature-sensitive form of the pneumococcal autolytic amidase (Garcia et al., 1986). In contrast to the Lyt⁺ Tol⁺ mutants P345 and P349, the autolysis defective mutant DOC 4 has only very low specific activity of autolysin (about 0-1–1% of the wild-type cells); it does not lyse with deoxycholate or penicillin when grown at 37 °C, and undergoes only a slight amount of slow lysis when treated with penicillin at 30 °C (Garcia et al., 1986). It was expected that if the thermosensitivity of penicillin-induced lysis in P345 and P349 is related to an abnormality of the autolysin in these bacteria, then genetic transformants in which this enzyme was replaced with the low specific activity autolysin of the DOC 4 mutant would exhibit properties characteristic of the DOC 4 mutant. This was, however, not the case. The Lyt⁻ property of the DOC 4 mutant was introduced into competent cells of P349, and cells no longer capable of lysing with deoxycholate were selected (Jiang & Tomasz, 1981). These transformants were then tested for their response to penicillin. In contrast to the behaviour of the DNA donor cells, the transformants did not show any detectable lysis during treatment with penicillin at...
Fig. 4. Effect of penicillin on the rates of biosynthesis of protein (a), peptidoglycan (b) and teichoic acid (c). Incorporation of radioactively labelled precursors into macromolecular cell components was determined during 5 min exposures of 400 μl samples at intervals following addition of benzylpenicillin (20 × MIC). ——, 37 °C; ---, 32 °C. Data are from single experiments in which samples were taken in triplicate at each time. (a) [3H]Phenylalanine incorporation into protein; initial values were 5877 c.p.m. per sample (37 °C) and 8808 c.p.m. per sample (32 °C). (b) [3H]Lysine incorporation into peptidoglycan; initial values were 338647 c.p.m. per sample (37 °C) and 242661 c.p.m. per sample (32 °C). (c) [3H]Choline incorporation into teichoic acid; initial values were 12398 c.p.m. per sample (37 °C) and 9579 c.p.m. per sample (32 °C).

Fig. 5. Change in culture turbidity of S. pneumoniae P349 Lyt− treated with penicillin (20 × MIC) in the presence (●) and absence (○) of exogenous pneumococcal autolysin (see Methods) at 37 °C (----) and 32 °C (---). Penicillin and autolysin [about 10⁸ cell equivalents (ml culture)⁻¹] were added to the cultures about 1 h before the 0 time point on the Figure.

30 °C. In addition, when the cultures of transformants grown at 32 and 37 °C were challenged with penicillin in the presence of wild-type autolysin added to the growth medium, only the cells grown at 37 °C lysed (Fig. 5). Autolysin alone added to either culture had no effect on growth. Thus, in terms of this sensitivity to exogenous autolysin, the properties of the Lyt− transformant of P349 paralleled those of the original (Lyt+ Tol+) mutant culture (Williamson & Tomasz, 1980).

DISCUSSION

In the original interpretation of the phenotype of the P345 (Lyt+ Tol+) mutant of the pneumococcus, it was proposed that penicillin tolerance was the result of a block introduced into the hypothetical pathway which leads from an inhibited penicillin-binding protein (PBP) to an activated autolytic enzyme (Williamson & Tomasz, 1980). The mutant, isolated at 32 °C, had normal autolytic enzyme activity, an apparently normal set of PBPs, an unchanged penicillin
susceptibility (MIC value), and it could not be sensitized to the lytic action of exogenous autolysin when grown at 32 °C. The mutant also had no drastic change in the susceptibility of cell walls to autolytic degradation since it could be induced to autolyse by treatment with detergents or vancomycin. The outstanding feature of the mutant was the inability of penicillin to induce culture lysis.

The experiments described in this paper reveal further, initially unrecognized, features of this mutant. Apparently, the penicillin tolerance of this bacterium is restricted to the lower (32 °C) temperature, at which the mutants were selected for, while at 37 °C mutant cultures undergo loss of viability and cell lysis accompanied by wall degradation. In addition, these lytic processes at 37 °C show a peculiar ('paradoxical') dependence on the antibiotic concentration, in which lysis rates declined above an optimum concentration of penicillin with complete inhibition of lysis at high (100 × MIC) concentrations of the antibiotic. A somewhat similar autolysis induced only within a narrow range of penicillin concentrations (4–6 × MIC) was also observed on occasions (twice in eight experiments) with cultures grown at 32 °C.

Analysis of the lysis rates revealed that the dominant feature of the mutant's response to penicillin at both temperatures was the presence of a prolonged lag period after onset of growth inhibition and before the onset of detectable lysis (at 37 °C). Since the relative order and kinetics in the shut-down of protein and wall syntheses was not very different at the two temperatures, neither this delay nor the mechanism of thermosensitive lysis is likely to involve major differences in the coordination of wall synthesis and the synthesis of other biopolymers. It was conceivable, for instance, that at the lysis-preventive temperature (32 °C) the addition of penicillin might have led to a more rapid inhibition of protein synthesis than cell wall synthesis, thus initiating the known 'phenotypic tolerance' of such bacteria (Hobby, 1944). No such preferential shut-off in protein synthesis was observed: the rate of protein synthesis declined somewhat more slowly at 32 °C than at 37 °C, following addition of penicillin.

The mechanism of thermosensitivity is not yet understood. Whatever process it involves, the shift from lysis-prone to primarily lysis-permissive phenotype can occur with great rapidity during shift from one growth temperature to another. The genetic experiments described make it unlikely that the peculiar (thermosensitive) lytic process is related to some peculiarity of the autolytic enzyme present in the mutant bacteria. Two findings suggest that the thermosensitive process may involve either autolysin control or autolysin susceptibility of the cell wall. Clearly, the rate of wall degradation has the same temperature requirements and antibiotic concentration optima of cellular lysis as whole cells, implying that some aspect of autolytic activity is involved. Most interestingly, when the endogenous autolysin was eliminated from the system (in the experiments with the P349 Lyt− transformant) the mutant bacteria exhibited a temperature dependence of the effectiveness of penicillin to 'sensitize' such cells to exogenous autolysin, since autolysin added to the medium could only lyse the cells grown and treated with penicillin at 37 °C. These experiments together suggest that the thermosensitive process responsible for the 'tolerant' or 'lysis-prone' phenotype of the mutants is either production of some autolytic inhibitor or some alteration of wall structure (at 32 °C) that makes attachment of autolysin molecules difficult.

The temperature sensitivity of pneumococcal Lyt+ Tol+ mutants introduces a new experimental handle to dissect the molecular events in the triggering of autolytic activity in penicillin-treated cells. The same feature of the mutants will also be useful in the elucidation of the mechanism of the 'paradoxical effect'. These are the first laboratory mutants which exhibit this trait which exists in numerous clinical isolates of pathogenic bacteria.

REFERENCES


tolerance among clinical isolates of bacteria. Reviews of Infectious Diseases 7, 368–386.