Mutants of *Streptococcus pneumoniae* That Contain a Temperature-sensitive Autolysin

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Two mutants of *Streptococcus pneumoniae* deficient in autolysin activity produced a protein that showed immunological identity with the N-acetyl-muramyl-L-alanyl-amidase present in the wild-type strain, when tested with antiserum obtained against this enzyme. The protein was produced by the mutant cultures grown either at 37 °C or at 30 °C, although only the cell extracts obtained at 30 °C showed significant cell wall hydrolysing activity. In contrast to the lysis resistance of these bacteria grown at 37 °C, mutant cultures grown at 30 °C exhibited significant degrees of autolysis when treated with detergent or cell wall inhibitors. Extracts of the mutant cultures contained a cell wall hydrolysing activity that was rapidly inactivated during incubation at 37 °C.

**INTRODUCTION**

A mutant of *Streptococcus pneumoniae* that did not autolyse when treated with deoxycholate at 37 °C was first described in 1970 (Lacks, 1970). Several mutants showing similar characteristics have been isolated since then after different mutagenic treatments (Jiang & Tomasz, 1981; Zighelboim, 1980). These mutants, designated as deficient in autolytic activity (Lyt\(^-\)), are resistant to the lytic and, in varying degrees, to the bactericidal effects of antibiotics that inhibit peptidoglycan synthesis (Horne & Tomasz, 1977; Kitano & Tomasz, 1979; Rogers & Forsberg, 1971; Tomasz, 1979, 1983), although they remain as sensitive as the wild-type cells to the growth inhibitory effects of the same antibiotics. In addition, cultures of at least one of these mutants (cw1) are also resistant to infection by bacteriophage Dp-1 under certain experimental conditions that allow normal lysis of the parental strain (Ronda-Lain *et al.*, 1977).

Using antiserum specific against the subunit (E-form) of the pneumococcal amidase isolated from wild-type cells (Garcia *et al.*, 1982), we have examined extracts of the autolysin defective mutants of pneumococci for the possible presence of a protein that may be immunologically related to the wild-type E-form autolysin.

**METHODS**

*Bacterial strains and growth conditions. S. pneumoniae* R6 is a derivative of the Rockefeller University strain R36A. Strains cw1 (obtained from S. Lacks, Brookhaven National Laboratory) and DOC-3 (isolated by S. Zighelboim at Rockefeller University) are autolysin-defective mutants derived from R6. Bacteria were grown without aeration in C medium (Lacks & Hotchkiss, 1960) supplemented with 0.15% (w/v) yeast extract (Difco) at an initial pH of 8.0 (C + Y medium). Growth was monitored with a Coleman nephelometer. The bacteria were also grown on the surface of plates containing C + Y medium, catalase (250 units ml\(^-1\); Boehringer-Mannheim) and solidified with 1% (w/v) agar.

*Viable counts.* The number of viable cells was determined by counting the number of colonies from appropriate dilutions of culture (in triplicate) spread on the surface of agar plates of C + Y medium.
Antibiotics and reagents. Benzylpenicillin was from Eli Lilly, [methyl-3H]choline chloride (60 Ci mmol⁻¹; 220 GBq mmol⁻¹) and L-[4,5-3H]lysine monohydrochloride (40 Ci mmol⁻¹; 1480 GBq mmol⁻¹) were purchased from Amersham.

Assay for autolytic activity. The activity in extracts obtained by the method of Tomasz & Westphal (1971) was measured using purified cell walls labelled by [3H]choline or lysine, as previously described (Holtje & Tomasz, 1976; Tomasz & Westphal, 1971). In short, 10 µl [3H]choline labelled cell walls (0.15 mg dry weight, 10⁶ c.p.m. ml⁻¹) was mixed with 100 µl bacterial lysate (obtained as described in Results) and 150 µl 0.02 M-potassium phosphate buffer, pH 6.9, and the mixture was incubated, unless otherwise stated, for 15 min at 37 °C or 30 °C. The reaction was stopped by adding 20 µl 35% (v/v) formaldehyde and 20 µl 4% (w/v) bovine serum albumin (Armour fraction IV; Sigma). Samples were centrifuged in a microcentrifuge at 10000 g for 10 min. The radioactivity in 200 µl portions of the supernates was quantified using Ready Solv (Beckman) scintillation fluid in an Intertechnique Scintillation Counter.

Testing of detergent-induced lysis. Samples (1 ml) of cultures were mixed with 100 µl buffer (potassium phosphate, 1 M, pH 8.0) and 50 µl Triton X-100 (4%, v/v, solution). The suspension was incubated at 37 °C for 10 to 30 min and was inspected for lysis visually.

Preparation of antisera and pneumococcal amidase. Anti-Dp-1 and anti-E sera were prepared as previously reported (Garcia et al., 1982; López et al., 1977). Immunodiffusion tests were done by the Ouchterlony technique (Ouchterlony & Nilsson, 1978). Purified pneumococcal amidase (E-form) was obtained and purified as previously described (Holtje & Tomasz, 1976).

RESULTS

Immunological detection of autolysin in Lyt- mutant. When crude extracts of strain cw1 were tested using an antiserum obtained against R36A (Garcia et al., 1982), this antiserum recognized a protein that showed immunological identity with the pneumococcal amidase present in R36A (Fig. 1). This protein was present in extracts obtained from strain cw1 incubated either at 37 °C or at 30 °C. Identical results were obtained with extracts obtained from strain cw1 incubated either at 37 °C or at 30 °C. Identical results were obtained with extracts of strain DOC-3 (not shown).

Stationary phase lysis of autolysin-defective mutants at low-temperature. The biological activity of the protein recognized in strain cw1 by the anti-E serum was investigated under different experimental conditions. When incubated at 30 °C strain cw1 started to lyse after 7 h incubation (Fig. 2) in remarkable contrast to what was found when incubation was done at 37 °C, as previously described (Tomasz, 1983; Tomasz et al., 1970). Furthermore, the lysis at 30 °C was blocked when the culture was incubated with anti-E serum added to the growth medium. Addition of nonspecific antiserum (anti-Dp-1 serum) did not prevent autolysis in the stationary phase at 30 °C.

Cultures of strains cw1 and DOC-3 grown at 37 °C were completely resistant to detergent-induced lysis. The same cultures grown at 30 °C lysed upon the addition of Triton X-100 (not shown).

Susceptibility of the mutant to benzylpenicillin. Addition of the antibiotic (at 0.2 µg ml⁻¹ or 20 × MIC) to the two autolysin-deficient strains resulted in lysis when incubated at 30 °C, whereas they were resistant to lysis at 37 °C (Fig. 3). Although the rate of lysis at 30 °C was much slower than the lysis of the wild-type strain at 37 °C (Tomasz et al., 1970), it was clear that the primarily bacteriostatic response of these mutants to penicillin at 37 °C was changed to a bacteriolytic one when the bacteria were grown at 30 °C. It has been shown (Tomasz, 1979; Tomasz et al., 1970) that, in pneumococci in which the amidase activity was suppressed (i.e. strain cw1), the rate of loss of viability was slower than in the wild-type strain. When the mutants were incubated with penicillin (at 0.04 µg ml⁻¹ or 4 × MIC) at 30 °C, the rate of loss of viability increased substantially over that observed in cultures exposed to penicillin at 37 °C (Fig. 4).

Temperature sensitivity of the cell wall hydrolysing activity in mutant extracts. Two cultures of strain cw1 were grown, one at 37 °C and the other at 30 °C, to a concentration of 2 × 10⁸ c.f.u. ml⁻¹, and crude autolytic extracts were prepared from them, as described in the legend to Fig. 1. Portions (100 µl each) were distributed into a series of test tubes containing cell walls labelled with radioactive choline and the rates of cell wall hydrolysis were tested during incubation at various temperatures. Extracts prepared from cells grown at 37 °C had only poor
Fig. 1. Ouchterlony double-diffusion plate of *S. pneumoniae* extracts and anti-E serum. The experiments were done on agar-coated microscope slides with antibody in the centre well and the following additions in the peripheral wells: 1 and 3, cell extracts obtained from strain cw1 grown at 30 °C; 2 and 5, extracts obtained from strain cw1 grown at 37 °C; 4, purified amidase (E-form, 40 units); 6, extract obtained from the wild-type strain (R36A). Extracts were prepared as follows. The pellet of a 400 ml bacterial culture was resuspended in 4 ml 0.15 M-NaCl solution buffered at pH 7.0 (50 mM-potassium phosphate buffer). After the addition of 0.5 g glass beads (100 μm diameter; Serva Biochemicals), the suspension was exposed to 3 × 1 min bursts of sonication using a 1 cm probe and maximum energy output (Heat Systems Ultrasonics sonicator). The glass beads and cell debris were removed by centrifugation (10000 g, 10 min) and the supernate was used as a crude cell extract.

Fig. 2. Stationary-phase lysis of strain cw1 of *S. pneumoniae* incubated at 30 °C. A culture of strain cw1 grown in C + Y medium was incubated at 30 °C with no additions (C) or in the presence of anti-E serum (B) or anti-Dp-1 serum (D). A control culture of strain cw1 was grown at 37 °C (A, A). Each point represents the mean of five determinations.

Fig. 3. Effect of benzylpenicillin on strains cw1 and DOC-3 of *S. pneumoniae* grown at 30 °C and 37 °C. Cultures of strain cw1 (∆, ▲) or DOC-3 (○, ■) were grown in C + Y medium at 37 °C (solid line; A, B) or 30 °C (dashed line; C, D). At the time the cultures reached a concentration of 5 × 10⁹ c.f.u. ml⁻¹ they received 0.1 U benzylpenicillin ml⁻¹ (arrows). The growth was followed by nephelometry. Each point represents the mean of eight determinations.

hydrolytic activity, irrespective of the temperature at which the assay was run (lines D, E, F and H, Fig. 5). In contrast, extracts of the cells grown at 30 °C contained significant amounts of cell wall hydrolysing activity, provided that the enzymic assay was done at 24 °C or 30 °C (lines A and B, Fig. 5). These findings suggested that the autolytic activity in the mutant extract was thermolabile. Extracts prepared from the two mutant cultures (strains cw1 and DOC-3) and the wild-type bacteria and purified autolysin were preincubated in buffer at various temperatures for 30 min and residual enzyme activities were determined. Such a preincubation of the mutant extracts (but not the wild-type cell autolysins) at 37 °C led to a loss of over 75% of enzyme activities (data not shown).

The specific autolysin activities of mutant extracts prepared from cells grown at 30 °C represented about 0.5 to 1.0% of the specific activity of wild-type cells. Preincubation of mutant or wild-type extracts with antiserum against wild-type enzyme resulted in a virtually complete
inhibition of enzyme activities. Purified Forssman antigen also inhibited the mutant enzyme activity. Pneumococcal cell walls containing enthanolamine in place of choline (Tomasz & Westphal, 1971) were not hydrolysed by mutant extracts.

When cultures of strain cw1 grown at 37 °C were shifted to 30 °C, a rapid increase in autolytic activity could be observed in cell extracts within minutes of the shift to the lower temperature. Temperature shift in the opposite direction resulted in an eventual decline in autolytic activity of cell extracts (data not shown).

**DISCUSSION**

The results presented here show that two mutants of *S. pneumoniae*, which have been described as autolysin-deficient (Jiang & Tomasz, 1981; Lacks, 1970), contain a protein that is recognized by an antiserum produced against the pneumococcal autolytic amidase present in the wild-type strain. The autolytic enzyme produced by the mutant cells is much more temperature sensitive than the wild-type enzyme, and the rapid inactivation of the mutant autolysin at 37 °C explains the low specific autolysin activity of extracts prepared from such bacteria. The simplest interpretation of our data is that the two autolysin-defective mutants contain a thermolabile form of the wild-type autolytic amidase. This conclusion is supported by the immunological cross-reactivity and also by the inhibition of the mutant enzyme activity by reagents that specifically block the wild-type enzyme (antiserum, Forssman antigen; no activity with ethanolamine-containing cell wall substrate).
Temperature-sensitive autolysin

It is interesting that, in parallel with the relative stability of the mutant enzyme at 30 °C, cultures of the mutant bacteria regain a considerable degree of autolytic potential when grown at this temperature. Thus, strain cw1 cultures were lysed by Triton X-100, autolyzed in the stationary phase of growth and responded to penicillin treatment by increased loss of viability and lysis. These findings further support the proposed role of autolytic activity in the penicillin-induced killing and lysis of pneumococci (Rogers & Forsberg, 1971; Tomasz, 1979; Tomasz et al., 1970).

It has been previously suggested (Seto & Tomasz, 1975) that the pneumococcal autolysin plays an important role during genetic transformation in this species. Since the competent cells are incubated at 30 °C for 15 min during the addition of the donor DNA, the rapid synthesis of an active form of the amidase in these mutants when incubated at 30 °C could explain why these strains remained fully transformable (Lacks, 1970).

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REFERENCES


