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Insertional Inactivation of the Major Autolysin Gene of *Streptococcus pneumoniae*

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The *lytA* gene encoding the major pneumococcal autolysin (*N*-acetylmuramoyl-L-alanine amidase) was inactivated by inserting the 2-kilobase *Msp*I fragment of pE194 containing the staphylococcal *ermC* gene. Stable autolysis-deficient (Lyt^-) mutants and their isogenic Lyt^+ parents were used in experiments designed to test possible physiological functions of the amidase. No autolysis could be induced in the mutants grown at 37°C by deoxycholate, by incubation in stationary phase, or by treatment with penicillin. On the other hand, the Lyt^- mutants exhibited normal growth rates and yields and normal adaptive responses during shifts from one growth temperature or nutritional condition to another. There was no evidence for impeded cell separation (chain formation). Colonies of Lyt^- insertional mutants produced normal hemolytic zones on blood agar; they showed normal (high) levels of competence for genetic transformation. Lyt^- mutants were also able to produce type 3 and 6 capsular polysaccharides, and such strains showed the same degree of virulence in mice as did the isogenic Lyt^+ parent. The physiological function(s) of the amidase remains a puzzle.

Numerous observations in the literature have clearly established the essential role of bacterial murein hydrolases in autolytic phenomena, such as lysis of pneumococci by detergents (2, 12, 22) or by inhibitors of cell wall synthesis (24). On the other hand, it is not at all clear what physiological function(s) these enzymes may perform for the bacterial cells.

Mutants of *Streptococcus pneumoniae* defective in the major murein hydrolase activity (*N*-acetylmuramoyl-L-alanine amidase, or amidase) (7, 9, 12) have been described previously (4, 10, 26). They did not undergo autolysis under the same experimental conditions that caused lysis and cell wall degradation in the parental and wild-type cells, but they grew normally, showed no apparent defect in genetic transformation, and exhibited only a limited degree of chain formation. Crude extracts prepared from these mutants contained low but detectable cell wall hydrolytic activity (0.1 to 1% of that of the parental cells), and it was conceivable that this represented residual amidase activity sufficient for the performance of some presumed vital or physiologically important function(s) for the bacteria. However, more recently, a vital role for the amidase in cell growth could be conclusively ruled out by the isolation of a mutant, M31, in which the amidase gene (*lytA*) was deleted without affecting the ability of the strain to grow and divide under the usual conditions of cultivation (16). Nevertheless, *lytA* appears to be ubiquitous in pneumococcal isolates (G. Pozzi, M. Oggioni, and A. Tomasz, submitted for publication), strongly suggesting some physiologically relevant (even if nonvital) role for the amidase. The deletion mutant M31 is not an ideal candidate for testing such possible physiological functions of this enzyme, since M31 was obtained after generalized mutagenesis with nitrosoguanidine. Its deletion also involves DNA sequences adjacent to *lytA* (about 5 kilobases in excess of the 1.2-kilobase *lytA* gene). It has a slower growth rate than the parent strain, and it also shows some differences from the parental strain in protein band pattern in sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

In this paper, we describe the construction and testing of isogenic pairs of *S. pneumoniae* strains differing only in the presence or absence of a functional amidase gene. In these strains, the *lytA* coding sequence was interrupted by the

TABLE 1. Strains of *S. pneumoniae*

Strain	Relevant properties[a]	Source or reference
Recipients in transformation		
Rx1	Cps^- *lytA*	6
R6x	Cps^- *lytA*	21
A112	*cps6A lytA*	Clinical isolate
Donors in transformation		
HB565	*cps-3 str-565*	1 (spontaneous mutant of A66 resistant to high levels of streptomycin)
DP1002	*nov-1*	14
Lyt^- mutants		
RUP1	(Rx1) Cps^- *lytA*::*ermC*	This work
RUP24	(R6x) Cps^- *lytA*::*ermC*	This work
M31	(R6x) Cps^- Δ*lytA*	16
Capsulated isogenic pairs		
RUP25	(R6x) *cps-3 lytA*::*ermC*	This work
RUP26	(R6x) *cps-3 lytA*	This work
RUP20	(A112) *cps6A lytA*::*ermC*	This work
RUP21	(A112) *cps6A lytA nov-1*	This work

[a] Cps^-, Mutation in the genetic determinant(s) for production of capsular polysaccarides; *lytA*, gene encoding for the major pneumococcal autolysin (amidase); *cps-3* and *cps6A*, genetic determinant(s) for the production of type 3 and type 6A capsular polysaccarides, respectively; *lytA*::*ermC*, insertion of *ermC* into *lytA*, conferring a stable Lyt^- phenotype (see text); *nov-1* and *str-565*, chromosomal point mutations conferring resistance to novobiocin and streptomycin, respectively. (Rx1), (R6x), and (A112) indicate the genetic background of strains obtained by transformation.

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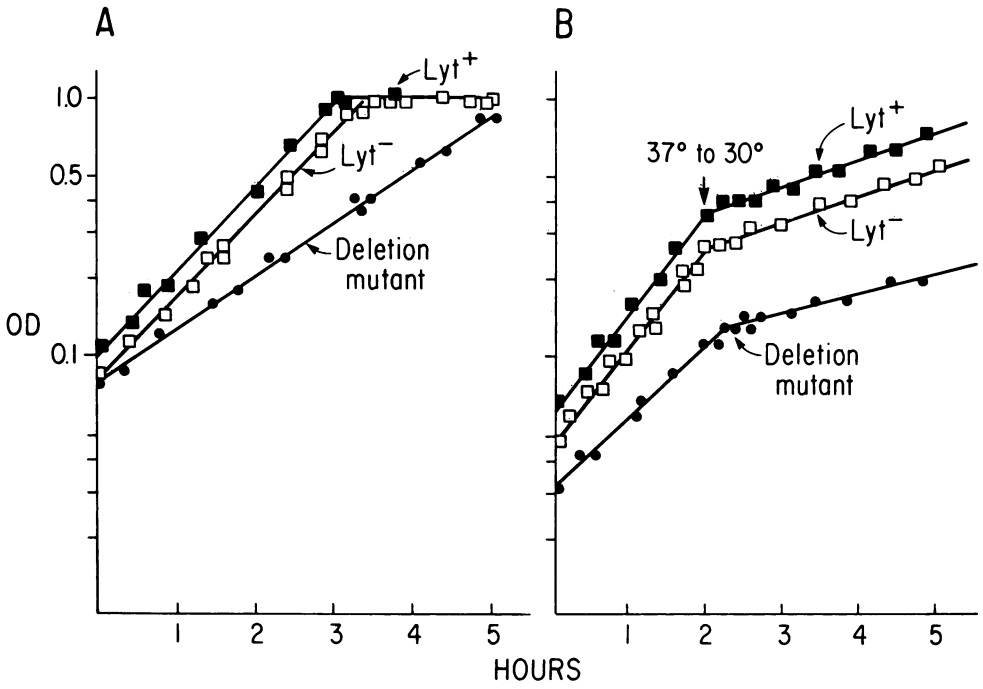

FIG. 1. Growth rate of the pneumococcal mutant with insertionally inactivated autolysin. Cultures of the pneumococcal Lyt$^-$ mutant RUP24 (*lytA*::*ermC*) and its isogenic Lyt$^+$ parent R6x were grown in the chemically defined medium (A) or in casein hydrolysate medium supplemented with yeast extract (11) (B) at 37°C. Cultures of the Lyt$^-$ deletion mutant M31 (Δ*lytA*) were also grown under the same conditions. In the experiments illustrated in panel B, growth temperature was shifted from 37 to 30°C at the time indicated by the arrow. Growth was monitored as optical density (OD) by a spectrophotometer (Sequoia-Turner Spectrophotometer, Mountainview, Calif.). The deletion mutant M31 grew with distinctly slower growth rates, particularly in the chemically defined medium, and cultures of this mutant also showed a longer delay in resumption of exponential growth upon back dilution of a stationary-phase culture into fresh medium. These and other abnormalities of M31 may be related to some function(s) deleted with the DNA in excess of the *lytA* gene.

insertion of *ermC*, a staphylococcal gene conferring resistance to erythromycin (8, 14).

Plasmid pGL80 carrying the *lytA* gene (5) was cut with *Taq*I and ligated to the 2.0-kilobase *Msp*I fragment of pE194 containing the *ermC* gene (8). The ligation mixture (DNA, 50 μg/ml) was used to transform *S. pneumoniae* Rx1 or R6x. Of 102 Em^r transformants, 97 proved to be Lyt$^-$, as judged by resistance to lysis with deoxycholate or by the blue spot assay or both (3). After growth without selection for erythromycin resistance for 50 generations, three of six transformants analyzed showed a stable Em^r Lyt$^-$ phenotype; one of these, RUP1, was used for characterizing the mutation. No amidase activity was detectable in crude extracts of RUP1 by using the standard enzymatic assay (7). The Lyt$^-$ phenotype could be transferred linked to Em^r in transformation; when RUP1 DNA was used to transform Rx1, all the Em^r transformants analyzed showed a stable Em^r Lyt$^-$ phenotype.

Properties of insertionally inactivated Lyt$^-$ mutants. (i) Growth rates and adaptive responses. The literature abounds in speculations suggesting that the activity of at least some murein hydrolases may be essential for cell wall enlargement and bacterial growth (17–19, 28), although experimental evidence for this notion is lacking (23). Cultures of the insertionally inactivated Lyt$^-$ mutants and their isogenic Lyt$^+$ parents grew with indistinguishable growth rates to comparable maximal cell concentrations (1×10^9 to 5×10^9 CFU/ml in the stationary phase) at either 37 or 30°C (doubling times, 60 and 140 min, respectively) in a chemically defined medium (27).

The rate of adjustment to steady-state doubling times was tested in the following situations: a shift from 30 to 37°C and vice versa; a shift from poor to rich medium; and a shift from the stationary to the exponential phase of growth (back dilution). No differences were observable between the strains. On the other hand, the deletion mutant M31 showed a distinctly slower growth rate in the chemically defined medium (doubling times, 85 to 90 min) (Fig. 1).

(ii) Cell separation at the end of cell division. No significant chain formation was observed in Lyt$^+$-Lyt$^-$ pairs growing in liquid culture in early or late log phase or in young colonies picked from the surface of blood agar plates (Fig. 2).

(iii) Production of hemolysin. Lyt$^-$ insertional mutants plated on the surface of blood agar produced normal alpha-hemolytic zones around the colonies indistinguishable from those surrounding colonies of Lyt$^+$ cells (unpublished observation).

(iv) Genetic transformation. Lyt$^+$-Lyt$^-$ pairs of cultures were grown according to a standard procedure used for the induction of competence (15), and DNA from strain DP1002 carrying the *nov-1* marker was used for transformation (7). No differences could be observed in the rates of acquisition or levels of competence. At 120 min after dilution of cultures into the competence medium, the Lyt$^+$ (Rx1) culture had 5.6×10^7 viable cells and 2×10^6 novobiocin-resistant transformants per ml. In the case of the Lyt$^-$ (RUP1) culture, the corresponding numbers were 5.4×10^7 and 1.8×10^6.

(v) Autolysis. Cultures of insertionally inactivated Lyt$^-$ mutants grown at 37°C did not undergo lysis in the stationary phase of growth or when treated with deoxycholate or penicillin (10× MIC). The same conditions induced some lysis when the Lyt$^-$ cultures were incubating at 30°C (unpublished observation).

(vi) Virulence of Lyt$^+$-Lyt$^-$ pairs. Isogenic (Lyt$^+$-Lyt$^-$)

	PRE-STATIONARY PHASE LIQUID CULTURES		EARLY LOG LIQUID CULTURES		YOUNG COLONIES FROM BLOOD-AGAR SURFACE	
	LYT$^+$	LYT$^-$	LYT$^+$	LYT$^-$	LYT$^+$	LYT$^-$
TOTAL COUNTED	1336	1516	726	593	351	235
SINGLES	—	—	4.2%	7.2%	—	—
DOUBLETS	—	—	90	83	—	—
SINGLES & DOUBLETS	94%	96%	—	—	88%	68%
THREES	0.9	0.5	—	—	1.1	11.0
FOURS	1.2	0.6	—	—	3.7	5
THREES & FOURS	—	—	0.8	1.8	—	—
>FOURS	3.3	1.1	3.5	7.2	6.8	14

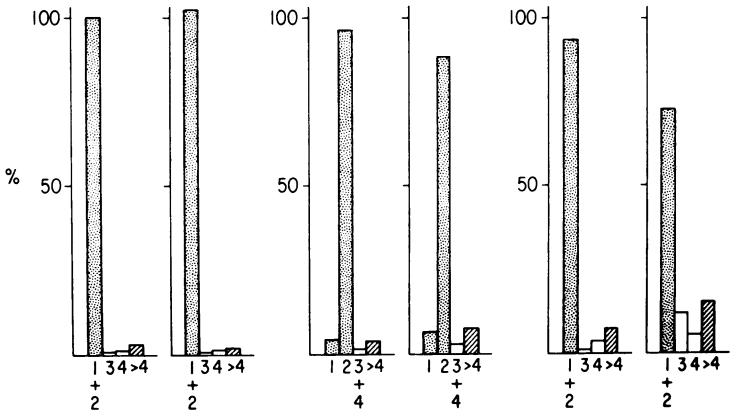

FIG. 2. Daughter cell separation at the end of cell division in the pneumococcal Lyt$^-$ mutant with insertionally inactivated autolysin. Cultures of the Lyt$^-$ RUP24 and its Lyt$^+$ parent R6x were grown in C medium supplemented with yeast extract (11). Samples were removed and examined for degree of chain formation in the early log phase (about 1×10^8 CFU/ml) and at the beginning of the stationary phase of growth (about 1×10^9 CFU/ml). Randomly picked fields were scanned by phase-contrast microscopy (Zeiss Research Microscope). Visual observation was preferred to the use of a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.), since passage of cells through the orifice of the instrument may cause artifacts (e.g., breaking up of chains). Occasionally, bacteria were first fixed with glutaraldehyde and osmium tetroxide by a previously published procedure (25). The same isogenic pair of pneumococci was also grown on the surface of tryptic soy agar blood plates, and young colonies (12 h of growth) were picked and scanned for chain formation. Bacteria that appeared to be single cells, doublets, or cells forming chains with three, four, or more than four members were registered, and their frequencies were expressed as the percentage of total cells counted.

strains were constructed from encapsulated pneumococci in the following manner. The Lyt$^-$ (Em^r) marker from RUP1 was introduced by genetic transformation into a type 6 clinical isolate (strain A112). The capacity to produce type 3 capsule was transformed into the isogenic pair of R6x (Lyt$^+$)-RUP24 (Lyt$^-$ Em^r) cells by using transforming DNA isolated from strain HB 565 as the donor of type 3 capsular determinant(s) (Table 1). No significant differences could be detected between the Lyt$^+$-Lyt$^-$ pairs in degree of virulence as determined by intraperitoneal injection into mice (Table 2). CD-1 female mice (8 weeks old) were challenged intraperitoneally with 0.4 ml of bacterial inoculum grown in tryptic soy broth supplemented with glucose (2 mg/ml) and yeast extract (0.1 mg/ml) (13) in the exponential phase of growth. In order to correct for loss of virulence during in vitro growth of pneumococci, bacterial strains were first passaged in three consecutive steps in mice in the following manner. Groups of three mice were challenged with large inocula (10^7 to 10^8) of a given strain and killed 24 h later. The spleens were aseptically removed, homogenized, suspended in tryptic soy broth supplemented with glucose and yeast extract, and incubated at 37°C for 16 to 18 h. Such a culture was then used to inoculate a second set of animals. After passage 3, groups of 10 mice were challenged with a series of inocula from each strain and survival rates of mice were monitored by daily observation.

TABLE 2. Virulence of Lyt$^+$ and Lyt$^-$ isogenic pairs

Isogenic pair	No. of dead mice/total no. of mice tested with an inoculum size (CFU) of:		
	10^4	10^5	10^6
RUP20 *cps6A* Lyt$^-$	1/10	5/10	10/10
RUP21 *cps6A* Lyt$^+$	0/10	8/10	8/10
RUP25 *cps3* Lyt$^-$	10/10	10/10	10/10
RUP26 *cps3* Lyt$^+$	4/10	8/10	10/10

In conclusion, complete and selective suppression of the activity of the major pneumococcal autolytic amidase by insertional inactivation of the *lytA* gene did not alter a number of physiological properties that could conceivably involve the activity of this enzyme. On the other hand, absence of the functional amidase gene did produce a major phenotype, inhibition of autolysis. Yet, as far as the pneumococcus itself is concerned, autolysis still represents a suicidal process, and thus the physiological function of amidase and the reasons for the apparently universal presence of the *lytA* gene in pneumococcal clinical isolates (Pozzi et al., submitted) remain a puzzle.

Exposure of the Lyt$^-$ strains of penicillin at a lower temperature (30°C) initiated a slow but definite decline in optical density which was also observable during prolonged incubation of cultures at 30°C in the stationary phase. This confirms the findings obtained with the deletion mutant M31 (16). It is conceivable that some as yet unidentified vital or physiologically (or ecologically) important function(s) of the amidase is taken over in the amidase mutants by a second murein hydrolase, perhaps in a fashion envisioned in the case of the two murein hydrolases of *Streptococcus faecium* (19). There are examples among bacteria for the availability of alternate enzymes to fulfill important physiological functions (20).

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