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Two Bactericidal Targets for Penicillin in Pneumococci: Autolysis-Dependent and Autolysis-Independent Killing Mechanisms

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It has been assumed that penicillin (and also other cell wall inhibitors) kill pneumococci predominantly by triggering their major autolytic enzyme (an *N*-acetylmuramoyl-L-alanine amidase; referred to as amidase), resulting in massive cell wall degradation. Three types of experiments suggest that only part of this killing is due to cell lysis by amidase. (i) Suppression of penicillin-induced lysis by specific inhibitors of amidase protected pneumococci only marginally from killing in spite of prolonged exposure to concentrations of penicillin that were 10×, 20×, or 100× greater than the MIC. (ii) Mutants from which the amidase was completely eliminated by plasmid insertion or deletion (Lyt^-) were still killed, albeit at a slower rate than the wild-type Lyt^+ strains (3 to 4 log units instead of 4 to 5 log units per 6 h, i.e., about 1 log unit slower than the wild type; $P < 0.001$). (iii) A new mutation (*cid*), which was not related to the amidase gene, further reduced killing of mutants lacking amidase to 1 log unit per 6 h ($Lyt^- Cid^-$ phenotype). Reintroduction of the amidase gene into $Lyt^- Cid^-$ cells partially restored penicillin-induced lysis but increased only slightly the rate of killing (from 1 log unit per 6 h in $Lyt^- Cid^-$ cells to 2 log units per 6 h in $Lyt^+ Cid^-$ cells). We conclude that penicillin kills pneumococci by two distinct mechanisms: one that involves the triggering of the amidase (about 1 log unit of killing per 6 h) and another, amidase-independent mechanism that is responsible for 3 to 4 log units of killing per 6 h. Triggering of the amidase activity in situ in growing bacteria was significantly reduced in $Lyt^+ Cid^-$ cells, indicating that there is some regulatory interaction between the *cid* gene product and the amidase.

Many bacteria, including pneumococci, undergo rapid loss of viability accompanied by triggering of the activity of autolytic wall-degrading enzymes and culture lysis when they are exposed to penicillin (or other cell wall inhibitors, such as vancomycin or D-cycloserine). The concurrence of cell death and lysis in these bacteria has led to the widely held belief that the cause of cell death is the suicidal (unregulated) activity of autolytic enzymes. On the other hand, it is also known that in certain species of bacteria penicillin can have powerful bactericidal activity without accompanying autolysis (10, 13, 18), suggesting that mechanisms other than cell lysis may exist in bacteria for the killing effect of penicillin.

In order to clarify the relationship between penicillin-induced lysis and killing in pneumococci, we performed a series of experiments using autolysis-inhibiting agents and pneumococcal autolysin mutants carrying a variety of defects in the structural determinant (*lytA*) of the major autolysin (an *N*-acetylmuramoyl-L-alanine amidase; referred to as amidase). We also characterized in detail a recently described (19) new type of mutation (*cid*) that drastically reduces penicillin-induced killing in both wild-type (Lyt^+) cells and Lyt^- mutants of pneumococci. In addition, in Lyt^+ cells the *cid* marker was found to reduce the triggering of amidase activity during penicillin treatment. While the nature of the *cid* gene product is unknown, a likely site for its activity is the plasma membrane.

(Some of the findings described in this report have been presented in a preliminary form at the 88th Annual Meeting of the American Society for Microbiology, 8 to 13 May 1988, Miami Beach, Fla. [P. Moreillon and A. Tomasz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, A61, p. 11].)

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MATERIALS AND METHODS

Strains of pneumococci and growth of the microorganisms. The strains of *Streptococcus pneumoniae* used in this study are described in Table 1. The microorganisms were grown in casein-based semisynthetic medium at pH 8 (14) supplemented with 0.1% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) and are referred to as C+Y. Growth of the cultures was monitored at an optical density of 620 nm (OD_{620}) with a spectrophotometer (Sequoia-Turner, Mountainville, Calif.). Stocks of the cultures were frozen in C+Y containing 10% glycerol and were stored at −70°C.

Antibiotics and reagents. Benzylpenicillin and vancomycin were obtained from Eli Lilly & Co. (Indianapolis, Ind.); and L-[4,5-^{3}H(N)]lysine (97.5 Ci/mmol), L-[alanine-2,3-^{3}H]phenylalanine (21.0 Ci/mmol), and [5-^{3}H]uridine (25.8 Ci/mmol) were obtained from Dupont, NEN Research Products (Boston, Mass.). Antibodies against the pneumococcal autolytic amidase were prepared as described previously (2). All other chemicals were reagent grade, commercially available products.

Susceptibility testing and rates of autolysis and killing of the microorganisms. MICs were determined by the tube dilution method in C+Y by using 1-ml volumes per tube and 10^5 CFU/ml as inocula. Rates of autolysis were measured in 10-ml cultures of exponentially growing *S. pneumoniae* (OD_{620} = 0.2 to 0.3, corresponding to 0.5×10^8 to 1×10^8 CFU/ml) that received various concentrations of the antibiotics (ranging from 10× to 100× the MIC). Control cultures, which were used for the evaluation of stationary-phase lysis, received no antibiotic. Autolysis rates were expressed as the first-order rate constant K, where $K = \ln (A_0/A_{120}) \times \text{min}^{-1}$ and where A_0 represents the peak OD_{620} reading and A_{120} represents the reading after a further 120 min of incubation (15). To determine the effect of antibiotic treatment on the viable counts of bacteria, 100-μl portions of the cultures were removed after various times of exposure, serially

TABLE 1. *S. pneumoniae* strains used in this study

Phenotype and strain	*lytA* gene	Relevant phenotype		Origin or reference
		Autolytic status	Killing by penicillin[a]	
Lyt$^+$ (wild type)				
R6x	Normal	Lyt$^+$	4–5	23
Lyt$^-$ mutants				
Lyt-4-4	Point mutation	Lyt$^-$	3–4	4
M31	Deletion	Lyt$^-$	3–4	22
RUP-24	Plasmid insert, inactivation	Lyt$^-$	3–4	20
Cid$^-$ mutant				
Cid-1	Normal	Lyt$^+$	1	19
Lyt$^-$ Cid$^-$ recombinants				
Lyt 4-4 × Cid-1	Point mutation	Lyt$^-$	1	This work
RUP-24 × Cid-1	Plasmid insert, inactivation	Lyt$^-$	1	This work
Lyt$^+$ Cid$^-$ recombinants				
Lyt$^-$ Cid$^-$ × R6x	Normal	Lyt$^+$	1–2	This work

[a] Loss of $\log_{10}$ CFU after 6 h of treatment with a 20× MIC of penicillin.

diluted in semisynthetic medium supplemented with 100 U of penicillinase per ml (Becton Dickinson, Cockeysville, Md.), and plated onto trypic soy agar plates (Difco) containing 3% defibrinated sheep blood. The CFU was counted after 36 h of incubation at 37°C.

Inhibition of pneumococcal autolysis by specific inhibitors of amidase. Cultures of the lysis-prone (Lyt$^+$) wild-type strain R6x (see Table 1) were grown to the early exponential phase (about 0.5×10^8 CFU/ml) and treated with the following autolysin-inhibitory agents: (i) a high concentration of choline (10 mg/ml) (1, 9), (ii) antiserum prepared against the autolytic amidase (2) (100 μl of serum per ml), and (iii) trypsin (16) (100 μg/ml). Incubation continued for an additional mass doubling time, when the cultures received 0.1 μg of penicillin per ml (10× the MIC). The OD and viable titers were then followed as described above.

Genetic transformation assay. DNA was prepared by the method of Marmur (17). Competent cells were prepared by a previously published procedure (24). Competent cells (about 5×10^7) were incubated with 0.1 to 1 μg of transforming DNA per ml at 30°C for 30 min, followed by the addition of DNase (final concentration, 0.1 μg/ml) and an expression time of 90 min at 37°C. Transformants were scored either for their lytic response to deoxycholate by a membrane assay (Millipore Corp., Bedford, Mass.) described previously (3) or for their ability to resist killing by penicillin (Cid$^-$ transformants; see below) or for streptomycin resistance.

Transformation of the *cid* determinant. High-molecular-weight DNA prepared from a pneumococcal mutant (Cid-1; see Table 1) that was highly resistant to the killing effect of penicillin (Cid$^-$ phenotype) was used to transfer the kill resistance marker (the *cid* marker) into kill-sensitive (Cid$^+$ phenotype) competent cells with defective or inactivated *lytA* genes (strains Lyt-4-4 and RUP-24, Lyt$^-$ phenotype; see Table 1). As a control, competent cells were treated with DNA from the lysis-prone and highly kill-sensitive wild-type strain R6x carrying a streptomycin resistance (St^r) marker (strain R6xSt). Cid$^-$ transformants were selected by two methods, first in liquid medium and second on agar plates.

(i) Selection in liquid medium. Pneumococcal cultures that were treated with either Cid-1 or R6xSt DNA were exposed to consecutive enrichment cycles of treatment with 20× the MIC of penicillin for 6 h, followed by outgrowth of the survivors in drug-free medium. Such consecutive enrichment cycles select for Cid$^-$ cells (19).

(ii) Selection on agar plates. Selection on agar plates was aimed at the determination of the frequency of transformation of the *cid* marker. Ten thousand CFU from cultures treated with either Cid-1 or R6xSt DNA was plated onto C+Y agar plates (12 ml of 1.5% agar and 250 U of catalase per ml) and were subsequently overlaid with 8 ml of the same medium and incubated for 5 h at 37°C, in order to allow each cell to form a microcolony. Then, a solution of penicillin (1 ml of a 10-μg/ml preparation) was overlaid and allowed to seep into the agar, and the incubation was continued for 12 h, after which time penicillinase (1 ml of a 10^5-U/ml solution) was spread onto the agar plates, in order to inactivate the antibiotic. Bacteria that survived penicillin treatment were counted after an additional 48 h of incubation at 37°C.

Rates of protein and RNA synthesis. Strains of pneumococci were grown at 37°C in 100-ml volumes of a chemically defined medium, Cden (A. Tomasz, Bacteriol. Proc., p. 29, 1964), from which the normal phenylalanine and uracil components were omitted; this medium was supplemented with 0.1% (wt/vol) yeast extract (Difco). At an OD_{620} of 0.2 to 0.3 (corresponding to about 10^8 CFU/ml), 20× the MIC of penicillin was added to the cultures and incubation was continued. The OD and viable titers were followed as described above, and rates of synthesis of protein and RNA were determined by pulse-labeling the cells with [^{3}H]phenylalanine or uridine, as follows. At several times before and after the addition of penicillin, 0.950-ml samples of the cultures were pipetted into prewarmed (37°C) borosilicate tubes (12 by 75 mm) containing 50 μl of either [^{3}H]phenylalanine (5 μCi and 0.04 μg) or [^{3}H]uridine (5 μCi and 0.044 μg), incubated for 5 min at 37°C, and rapidly frozen in dry ice. Eventually, the samples were thawed, treated for 30 min with 10% (wt/vol; final concentration) ice-cold trichloroacetic acid to precipitate proteins and RNA, and filtered through either 0.45-μm-pore-size filters (Millipore) (for proteins) or glass microfiber filters (Whatman, Inc., Clifton, N.J.) (for RNA). The filters were rinsed extensively with a 10 mg/100 ml solution of cold phenylalanine or uridine, dried, and transferred to a toluene-based PPO (2,5-phenyloxazole)–POPOP [1,4-bis(5-phenyloxazolyl)benzene] scintillator for counting. All ^{3}H-labeling experiments were performed in triplicate.

Autolysin-specific activity. The autolytic activity in crude extracts of pneumococci was measured by a previously published method (12, 19). Protein determination was done by a bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.).

Preparation of enzyme extracts. Bacterial cultures (1 liter) were harvested at the middle of the exponential growth phase (about 5×10^8 CFU/ml). Pellets were suspended in 5 ml of 10 mM Tris hydrochloride (pH 6.8) containing 10 mM $MgCl_2$ and DNase I and RNase I, each at 100 μg/ml; the suspension was mixed with an equal volume of glass beads (diameter, 100 μm; Thomas Scientific, Swedesboro, N.J.) and stirred vigorously by vortexing (Scientific Industries Inc., Bohemia, N.Y.) at 4°C for 10 to 15 min. The detergent Brij 35 and a LiCl solution were added to give 1% and 1.0 M final concentrations, respectively. The debris of broken cells

and glass beads was removed by centrifugation (10,000 × *g* for 10 min), and the supernatant was further clarified by ultracentrifugation at 40,000 × *g* for 1 h. The supernatant was then dialyzed overnight at 4°C against 10 mM Tris hydrochloride (pH 7.0) containing 0.1% Brij 35, and small batches containing 1.0 mg of protein per ml were stored frozen at −70°C. Cell walls that were labeled biosynthetically with radioactive lysine were prepared by a previously published procedure (5). Purified wall preparations (specific radioactivity of walls, 2.3×10^5 cpm/660 μg) were suspended at a concentration of 660 μg/ml in distilled water and stored frozen at −20°C.

Measurement of the cell wall-hydrolyzing capacity of cell extracts. Extracts of pneumococci from which the genetic determinant of the major autolysin (amidase) was deleted contained another enzymatic activity capable of a slow hydrolysis of cell walls (21, 22). This activity was quantitated in the following manner. A total of 50 μl of enzyme extract (protein concentration, 1 mg/ml) was mixed with 90 μl of buffer (10 mM Tris hydrochloride [pH 6.8]), 10 μl of Brij 35 (5% solution), and 20 μl of [^{3}H]lysine-labeled cell walls (specific activity, 2.3×10^5 cpm; 660 μg/ml); and the suspension was incubated for several hours at either 30 or 37°C. The reaction was stopped by the addition of 20 μl of formaldehyde and 20 μl of 1% bovine serum albumin. After centrifugation at 10,000 × *g* for 10 min, the radioactivity that was released to the supernatant was measured by liquid scintillation counting. For the removal of cell wall teichoic acids, cell walls (3.6 mg) were suspended in 2 ml of 49% hydrofluoric acid (HF), and the suspension was stirred slowly at 0°C for 24 h. The HF was removed by centrifugation of the cell walls and extensive washing of the pellet with distilled water. The pellet (cell wall peptidoglycan free of teichoic acid) was dried (recovery, 1.6 mg) and subsequently suspended in distilled water and stored at −20°C. This treatment quantitatively removed teichoic acid components of the cell wall, as indicated by chemical analysis for galactosamine, phosphorus, and choline.

Statistical analysis. Differences between means were calculated by the Student *t* test.

RESULTS

Effect of autolysis-inhibiting agents on penicillin-induced killing of pneumococci. Figure 1 shows that while inhibitory concentrations of each of the autolysis-inhibiting agents (i.e., choline, trypsin, or anti-amidase serum) prevented culture lysis (as measured by the decline in OD_{620}), there was practically no protection from the loss of viability in the presence of lysis-inhibiting concentrations of choline. Identical results were also obtained when trypsin or anti-amidase serum was used in place of choline (data not shown).

Rate of penicillin-induced killing in pneumococcal mutants defective in amidase activity. Cultures of Lyt$^-$ mutants Lyt-4-4, M31, and RUP-24 (Table 1) in the exponential phase of growth received penicillin (10×, 20×, or 100× the MIC), and the effect of the drug on the OD_{620} and the viable titer of the cultures was followed. None of the mutant cultures showed significant penicillin-induced lysis, yet substantial loss of viability continued in all three mutants, albeit at a slower rate than that in wild-type (Lyt$^+$) cells. Figure 2 shows the number of surviving CFU in cultures of isogenic Lyt$^+$ (R6x) and Lyt$^-$ (RUP-24) strains (Table 1) that received 100× the MIC of penicillin at a cell concentration of 10^8 CFU/ml (corresponding to an OD_{620} of 0.25) and were followed for 6 h. The number of survivors in RUP-24 (Lyt$^-$) cultures was $2.8 \times 10^4 \pm 1.3 \times 10^4$ (mean ± standard deviation of 19 determinations in three separate experiments). In comparison, in R6x (Lyt$^+$) cultures the number of survivors was $2.1 \times 10^3 \pm 1 \times 10^3$ (mean ± standard deviation of 17 determinations in three separate experiments), which was significantly lower than the numbers of survivors in Lyt$^-$ cells ($P < 0.001$). Similar results were obtained after exposure of the same isogenic pair of pneumococci to 10× or 20× the MIC of penicillin or when the Lyt$^-$ mutants Lyt-4-4 and M31 (Table 1) were used in place of RUP-24. Thus, suppression of the major autolytic amidase by either structural mutations or inactivation of the *lytA* gene resulted in a statistically significant reduction of penicillin-induced killing of pneumococci (3 to 4 log units of killing per 6 h compared with 4 to 5 log units of killing per 6 h in the parental Lyt$^+$ strain; $P < 0.001$). However, loss of viability

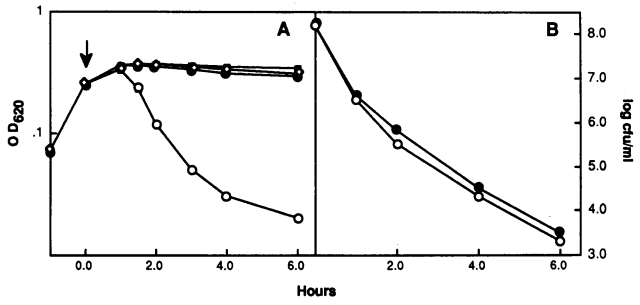

FIG. 1. Effect of various specific inhibitors of the autolytic enzyme amidase on penicillin-induced lysis (A) and loss of viability (B) of the lysis-prone (Lyt$^+$) and highly kill-sensitive wild-type strain R6x. Cultures (10 ml) of R6x in the early exponential phase of growth (about 5×10^7 CFU/ml) received high concentrations of choline (●), amidase antiserum (■), or trypsin (◇) and were incubated for an additional doubling time, when they were treated with 10× the MIC of penicillin (arrow). Control cultures (○) received only penicillin treatment. The OD_{620} of the cultures and bacterial survival were followed for 6 h.

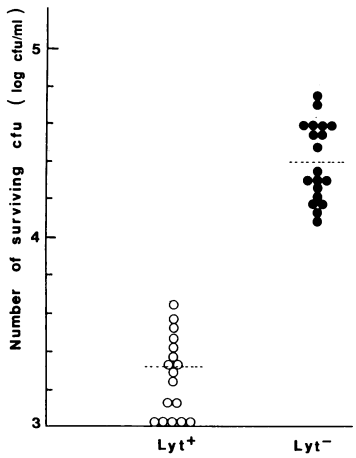

FIG. 2. Number of surviving CFU of the Lyt$^+$ wild-type strain R6x (○) and the Lyt$^-$ strain RUP-24 containing an insertionally inactivated amidase gene (●) after 6 h of treatment with 100× the MIC of penicillin. Cultures (10 ml) of strains in the exponential phase of growth (10^8 CFU/ml) were treated with penicillin for 6 h, and the numbers of surviving CFU were determined. Horizontal dashed lines represent the mean values of the number of survivors. Means ± standard deviations of the surviving CFU of strains R6x and RUP-24 were statistically different ($P < 0.001$).

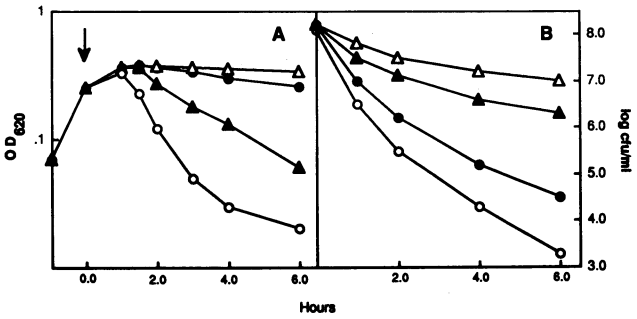

FIG. 3. Penicillin-induced lysis (A) and loss of viability (B) of the Lyt$^+$ wild-type strain R6x (○), the Lyt$^-$ strain RUP-24 containing an insertionally inactivated amidase gene (●), the kill-resistant mutant Cid-1 (△), and a Lyt$^+$ Cid$^-$ recombinant (▲). Cultures (10 ml) of strains in the exponential phase of growth (10^8 CFU/ml) were treated with 20× the MIC of penicillin (arrow), and both the OD_{620} and bacterial survival were followed for 6 h.

continued at a considerable rate in all Lyt$^-$ mutants (3 to 4 log units per 6 h) whether the lytic defect was due to a point mutation, insertional inactivation, or a complete deletion of the *lytA* gene. This finding shows that the residual killing of Lyt$^-$ pneumococci by penicillin does not involve the autolytic amidase but some other as yet undefined mechanism(s).

Mutants with drastically reduced sensitivity to the bactericidal effect of penicillin. Recently, we reported the isolation of a new class of pneumococcal mutants (called Cid$^-$, for bactericidal, throughout) that were able to resist both lysis and killing induced by penicillin treatment even though they contained quantitatively and qualitatively normal autolytic amidase and cell walls normally susceptible to digestion by amidase (19). Figure 3 shows the lytic and killing effects of 20× the MIC of penicillin on one such mutant (Cid-1). Cid-1 did not lyse and lost about 1 log unit of viable titer during 6 h of penicillin treatment. In comparison, the parent strain R6x was rapidly lysed and lost 4 to 5 log units during the same period, and cultures of autolysin-deficient (Lyt$^-$) cells lost 3 to 4 log units although they were not lysed by penicillin. The ability of the Cid$^-$ mutants to survive penicillin treatment was first thought to be the consequence of defective lysis during antibiotic treatment (19). However, the continued penicillin-induced killing of Lyt$^-$ mutants completely lacking amidase activity clearly excludes this explanation. The kill resistance observed in the Cid-1 mutant (Fig. 3) must be the result of some alteration in a second, as yet undefined, bactericidal target.

To better define this second target, we transferred the *cid* mutation to Lyt$^-$ (and Cid$^+$) cells by genetic transformation.

Genetic transformation of the *cid* determinant. (i) Selection in liquid medium. Cultures of Lyt$^-$ pneumococci sensitive to the killing action of penicillin (i.e., Lyt$^-$ Cid$^+$ strains) were treated with transforming DNA prepared from the kill-resistant mutant Cid-1. Such cultures were quantitatively converted to the Cid$^-$ phenotype (1 log unit of killing per 6 h of penicillin treatment; see Fig. 3) after three to four enrichment cycles, whereas those treated with the control R6xSt DNA (rate of transformation to St^r of 1%) were still rapidly killed after six to nine cycles of penicillin treatment (3 to 4 log units of killing per 6 h). Table 2 shows the properties of five such Cid$^-$ transformants that were randomly picked from the plates of two crosses in which either Lyt-4-4 or RUP-24 was used as the recipient. All the transformants showed the reduced bactericidal sensitivity of the DNA donor, while they retained the Lyt$^-$ phenotype of the recipients.

(ii) Selection on agar plates. Selection on agar plates allowed an approximate estimation of the frequency of transformation of the *cid* marker. In a typical experiment, 11 of 10^4 (0.1%) CFU treated with Cid-1 DNA survived the penicillin treatment. Upon testing the bactericidal sensitivities of these colonies in liquid culture, 10 of them showed the typical Cid$^-$ phenotype. No survivors were detected among the 10^4 bacteria treated with R6xSt DNA (transformation rate to St^r of 1%). These observations suggest a frequency of transformation of about 0.1% for the *cid* marker.

Lack of linkage between the *cid* and the *lytA* markers during genetic transformation. It has already been shown (19) that

TABLE 2. Transformation of the *cid* determinant

DNA donor × DNA recipient and transformant no.	Lysis with:		Killing by penicillin (log_{10} CFU[a])	Autolytic sp act (%)	Assigned phenotype
	Deoxycholate (0.1%)	Penicillin (K [10^3][b])			
Cid-1 × Lyt-4-4					
1	−	0.4	1	0.1–1	Lyt$^-$ Cid$^-$
2	−	0.4	1	ND[c]	Lyt$^-$ Cid$^-$
3	−	0.4	1	ND	Lyt$^-$ Cid$^-$
4	−	0.4	1	ND	Lyt$^-$ Cid$^-$
5	−	0.4	1	ND	Lyt$^-$ Cid$^-$
Cid-1 × RUP-24					
1	−	0.4	1	ND	Lyt$^-$ Cid$^-$
2	−	0.4	1	ND	Lyt$^-$ Cid$^-$
3	−	0.4	1	ND	Lyt$^-$ Cid$^-$
4	−	0.4	1	ND	Lyt$^-$ Cid$^-$
5	−	0.4	1	ND	Lyt$^-$ Cid$^-$
Cid-1 (donor)	+	0.5	1	100	Lyt$^+$ Cid$^-$
Lyt-4-4 (recipient)	−	0.4	3–4	0.1–1	Lyt$^-$ Cid$^+$
RUP-24 (recipient)	−	0.4	3–4	<0.1	Lyt$^-$ Cid$^+$

[a] Loss of log_{10} CFU after 6 h.
[b] $K = \ln (A_0/A_{120}) \times min^{-1}$, where A_0 represents the peak OD_{620} and A_{120} represents the OD_{620} after an additional 120 min of incubation.
[c] ND, Not determined.

TABLE 3. Reintroduction of the *lytA* gene into a Lyt$^-$ Cid$^-$ transformant

DNA donor × DNA recipient and transformant no.	Lysis with:		Killing by penicillin ($\log_{10}$ CFU[a])	Autolytic sp act (%)	Assigned phenotype
	Deoxycholate (0.1%)	Penicillin (K [10^3][b])			
R6x × Lyt$^-$ Cid$^-$					
1	+	6	1–2	118	Lyt$^+$ Cid$^-$
2	+	9	1–2	115	Lyt$^+$ Cid$^-$
3	+	7	1–2	140	Lyt$^+$ Cid$^-$
4	+	6	1–2	ND[c]	Lyt$^+$ Cid$^-$
5	+	6	1–2	ND	Lyt$^+$ Cid$^-$
6	+	7	1–2	ND	Lyt$^+$ Cid$^-$
R6x (donor)	+	20	4–5	100	Lyt$^+$ Cid$^+$
Lyt$^-$ Cid$^-$ (recipient)	−	0.4	1	0.1–1	Lyt$^-$ Cid$^-$

[a] See footnote *a* to Table 2.
[b] See footnote *b* to Table 2.
[c] ND, Not determined.

the *lytA* and *cid* markers are genetically distinct. We showed here that these markers are also not linked during genetic transformation. In some of the transformation experiments described in the previous section, the cultures treated with Cid-1 DNA were scored not only for the acquisition of the *cid* marker (see above) but also for the acquisition of the *lytA* gene (also present in Cid-1 DNA) as well. Lyt$^+$ transformants were recognized by the membrane assay (Millipore) (3). The transformation rate of the *lytA* marker was about 0.1%. In one typical experiment, none of 116 individual Lyt$^+$ transformants acquired the *cid* marker.

Introduction of the *lytA* gene into Lyt$^-$ Cid$^-$ transformants. The original Cid$^-$ mutants were selected after extensive exposures to high concentrations of penicillin (19). Since such treatment might have selected for more than one mutation, it was not clear whether the two alterations detected in these mutants (resistance to lysis and reduced killing by penicillin) were both reflecting the effects of a single *cid* mutation. To examine the influence of the *cid* mutation on penicillin-induced lysis, the Lyt$^-$ Cid$^-$ cells described in Table 2 were transformed to the Lyt$^+$ phenotype with DNA of R6x (Cid$^+$ Lyt$^+$ phenotype; Table 1), and their lytic and bactericidal responses to penicillin treatment were tested. The rate of transformation to the Lyt$^+$ phenotype was 0.1% (as determined by the membrane assay [Millipore] [3]).

Table 3 shows the properties of six such Lyt$^+$ transformants. All of them were lysed by deoxycholate and contained normal (wild-type) levels of amidase activity. However, both their rates of lysis and killing by penicillin were intermediate between the values typical of the DNA recipient and the DNA donor strains (Fig. 3).

Exploratory experiments into the nature of the amidase-independent killing mechanism. (i) Sensitivity of Cid$^-$ cells to killing by D-cycloserine and vancomycin. The reduced sensitivity of Cid$^-$ cells to the killing effect of penicillin was not restricted to beta-lactam antibiotics. Treatment of growing cultures of a Lyt$^-$ Cid$^-$ transformant for 6 h with 20× or 100× the MIC of D-cycloserine–β-chloro-D-alanine (MIC, 25/12.5 μg/ml) resulted in a loss of viability of ≤1 log unit, whereas control cultures (Lyt$^-$ Cid$^+$) lost 3 log units of viability during the same length of time. Essentially similar results were obtained with vancomycin.

(ii) The kinetics of growth inhibition after the addition of penicillin. Nongrowing bacteria, including pneumococci, are known to be resistant to the killing effect of cell wall inhibitors (phenotypic tolerance [8, 10, 26]). A conceivable mechanism for the kill resistance of Cid$^-$ cells would be a uniquely rapid inhibition of protein synthesis in these mutants after the addition of penicillin. We used isogenic pairs of Lyt$^-$ Cid$^-$ and Lyt$^-$ Cid$^+$ cultures to compare the rates with which Cid$^-$ and Cid$^+$ cells slowed growth rates (as measured by the increase in OD) and rates of protein and RNA synthesis after the addition of penicillin. Figure 4 shows that the rates of growth and of protein and RNA synthesis slowed in the two cultures at virtually identical initial rates. The differences in the rates of protein and RNA synthesis after longer times of penicillin incubation presumably reflected the very substantial difference in the numbers of live bacteria in the Cid$^-$ cultures versus those in the Cid$^+$ cultures (Fig. 4A).

(iii) Specific activity of a second wall-acting hydrolase in *cid* mutants. Pneumococci contain, in addition to the major autolysin amidase, a second enzyme which also appears to introduce breaks in the glycan chain of the cell walls (21). A penicillin-induced abnormal activity of such an enzyme could contribute to the killing effect of this antibiotic. We compared the specific activities of this second cell wall-acting hydrolase in crude extracts prepared from Cid$^-$ and Cid$^+$ bacteria. No significant differences were obtained (Table 4).

DISCUSSION

The experimental results described here demonstrate the existence of two distinct targets or mechanisms for the killing effect of penicillin (as well as of other cell wall inhibitors such as vancomycin and D-cycloserine) on pneumococci. These two targets could be identified by a careful quantitation of the susceptibilities of wild-type pneumococci (Lyt$^+$ Cid$^+$) and two kinds of pneumococcal mutants, autolysin defective (Lyt$^-$ Cid$^+$) and Lyt$^-$ Cid$^-$ cells, to the bactericidal effect of penicillin. Under the conditions of our assays (exposure to 20× the MIC of penicillin for 6 h), each one of these strains lost viable titers at characteristic, highly reproducible rates. Wild-type (Lyt$^+$ Cid$^+$) cells lost 4 to 5 log units of viable counts per 6 h, Lyt$^-$ Cid$^+$ mutants lost 3 to 4 log units of viable counts per 6 h (a protection of 1 log unit that resulted from inactivation of the *lytA* gene), and Lyt$^-$ Cid$^-$ cells lost only 1 log unit of viable titer per 6 h. Reintroduction of the *lytA* gene into either Lyt$^-$ Cid$^+$ or Lyt$^-$ Cid$^-$ strains caused an increase in the rate of killing by 1 log unit per 6 h. These data indicate that in wild-type strains, triggering of amidase activity is responsible for about

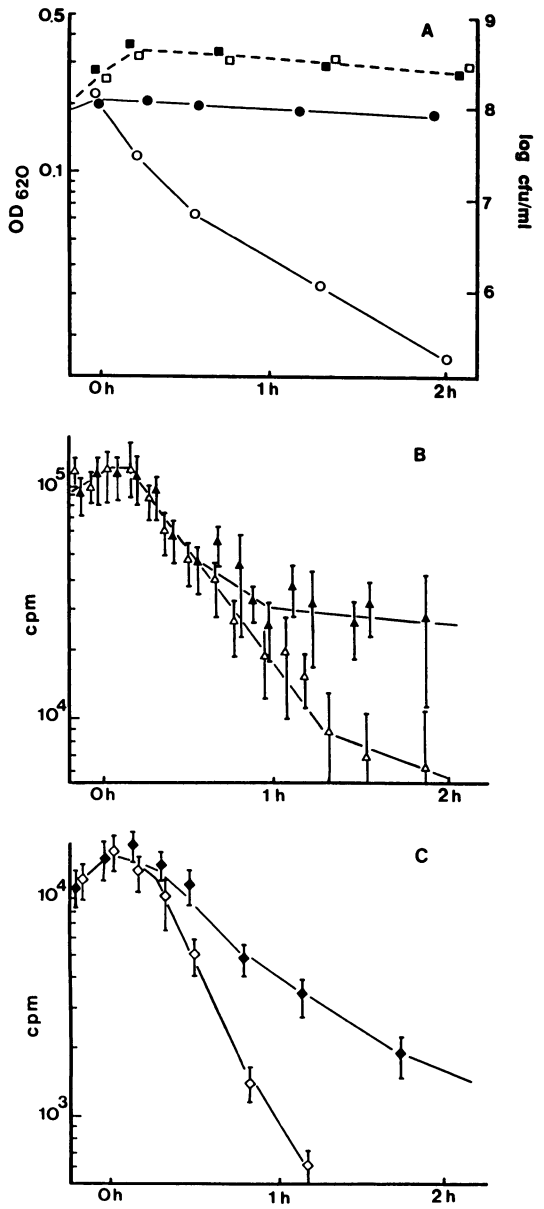

FIG. 4. Kinetics of growth inhibition and loss of viability after the addition of penicillin. (A) Cultures of isogenic pairs of Lyt$^-$ Cid$^+$ (open symbols) and Lyt$^-$ Cid$^-$ (closed symbols) pneumococci in the exponential phase of growth received 20× the MIC of penicillin at 0 h, and the effect of antibiotic treatment on the cultures' OD_{620} (dashed lines) and loss of viability (solid lines) was monitored. Panels B and C show the effect of penicillin treatment on the rates of protein (B) and RNA synthesis (C), as determined by pulse-labeling samples of the cultures for 5 min with either [^{3}H]phenylalanine (△, ▲) or [^{3}H]uridine (◇, ◆), followed by measurement of the number of counts per minute incorporated by the cells in trichloroacetic acid-precipitable macromolecules during this period of time (see text). Each dot in panels B and C represents the mean ± standard deviation of three determinations.

1 log unit of viability loss, while the rest of the killing (3 to 4 log units) occurs by a second, amidase-independent mechanism. This second bactericidal target has been identified in genetic terms. The *cid* marker is distinct from the genetic determinant of the autolytic amidase (*lytA*), and it shows no genetic linkage to *lytA* in genetic transformation. The protective effect of the *cid* mutation is fully expressed in pneumococci that completely lack the amidase.

TABLE 4. Specific cell wall-hydrolyzing activities in extracts of isogenic Cid$^+$ and Cid$^-$ pneumococci

Enzyme source[a]	Cell wall substrate[b]	Cell wall material released (%) at[c]: 37°C	30°C
Lyt$^-$ Cid$^+$	Complete wall	1.6	3.6
Lyt$^-$ Cid$^-$	Complete wall	1.5	3.5
Lyt$^-$ Cid$^+$	HF-stripped wall[d]		8.12
Lyt$^-$ Cid$^-$	HF-stripped wall[d]		7.85

[a] Crude extracts were prepared as described in the text. Protein concentrations were 1 mg/ml for Lyt$^-$ Cid$^+$ for pneumococci and 1.1 mg/ml for Lyt$^-$ Cid$^-$ pneumococci.

[b] Cell walls that were biosynthetically labeled with radioactive lysine were prepared from a Lyt$^+$ Cid$^+$ strain (R6x) by a previously published method (12).

[c] Hydrolysis of cell walls was measured after 18 h of incubation, as described in the text.

[d] Cell walls were treated with HF to remove teichoic acid (see text).

The biochemical mechanism of the greatly reduced bactericidal sensitivity to penicillin (i.e., penicillin tolerance) of the Cid$^-$ cells is not known. The increased tolerance of the mutant is not restricted to beta-lactam antibiotics, and there is no evidence for any alteration in penicillin-binding proteins (19). The *cid* mutation did not seem to involve an unusual hypersensitivity (or early shutoff) of protein synthesis to penicillin either. Cid$^+$ and Cid$^-$ bacteria also contained comparable amounts of both the amidase as well as the second murein hydrolase.

A clue concerning the nature of the *cid* gene product may be provided by the experiment for which the results are illustrated in Table 3 (see also Fig. 3), which shows that the penicillin-induced activity of amidase in situ was substantially reduced in the Cid$^-$ cells. While it is conceivable that this effect was due to some sophisticated change in the cell wall structure of Cid$^-$ cells, this is unlikely since Lyt$^+$ Cid$^-$ cells were lysed normally by deoxycholate and since amidase can rapidly and completely degrade cell walls prepared from Lyt$^-$ Cid$^-$ cells. We favor an alternative model, in which the *cid* gene product operates at the level of the plasma membrane, controlling the access of autolytic enzymes to the pneumococcal cell wall and providing a mechanism of transport of other surface-related macromolecules. We propose that the *cid* protein performs such a physiological function at some low copy number per cell, which is under precise control, and a substantial increase in the copy number of this protein would be toxic for the bacterium.

We assume that the toxicity of a high concentration of the Cid protein involves some injury (increased selective permeability?) to the plasma membrane, the integrity of which is essential for the survival of the bacteria. The same injury would allow the release of large amounts, unregulated forms, or both of the autolytic enzymes to the cell wall. The death of completely amidase-deficient cells may be caused by the membrane injury per se, or it may be mediated by breaks (nicks) introduced into the cell wall by some wall-acting hydrolase. This model does not specify the number or nature of factors that participate in the killing of autolysin-defective pneumococci. The model is formally analogous to the mechanism proposed for the triggering of autolytic activity by penicillin (25) and is also similar to models proposed for the mechanism of cell lysis induced by the bacteriophages lambda, Φx174, and MS_2 (6, 7, 11). The suggested role of the

cid gene product in penicillin-induced lysis would be analogous to the postulated role of the S protein in phage lambda in the disintegration of the host cell at the end of a lytic cycle.

The observations described in this report demonstrate that the *cid* system plays a major role in the loss of viability of pneumococci treated with cell wall inhibitors. To envision the mechanism of this autolysis-independent death, we propose that the *cid* gene is under the negative control of a repressor which is composed of cell wall material (cell wall precursors or cell wall metabolites). We cannot speculate about the precise chemical nature of this repressor, except to suggest that its production is inhibited by the same antibiotics that inhibit cell wall synthesis. We do not yet know whether the *cid* mutation, which allows such a dramatic resistance to antibiotic-induced killing, is in the structural gene of the Cid protein or in some regulatory element. It is possible that the well-known resistance of dormant (nongrowing) bacteria to the killing effect of cell wall inhibitors is also mediated by the *cid* system; it may be related to the inhibition of the overproduction of the Cid protein rather than to a halt in the increase in cytoplasmic mass, as it is sometimes assumed (25).

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