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Role of the Pneumococcal Autolysin (Murein Hydrolase) in the Release of Progeny Bacteriophage and in the Bacteriophage-Induced Lysis of the Host Cells

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The pneumococcal bacteriophage Dp-1 seems to require the activity of the *N*-acetylmuramic acid-L-alanine amidase of the host bacterium for the liberation of phage progeny into the medium. This conclusion is based on a series of observations indicating that the exit of progeny phage particles is prevented by conditions that specifically inhibit the activity of the pneumococcal autolysin. These inhibitory conditions are as follows: (i) growth of the bacteria on ethanolamine-containing medium; (ii) growth of the cells at pH values that inhibit penicillin-induced lysis of pneumococcal cultures and lysis in the stationary phase of growth; (iii) addition of trypsin or the autolysin-inhibitory pneumococcal Forssman antigen (lipoteichoic acid) to the growth medium before lysis; (iv) infection of an autolysin-defective pneumococcal mutant at a multiplicity of infection less than 10 (treatment of such infected mutant bacteria with wild-type autolysin from without can liberate the entrapped progeny phage particles); (v) release of phage particles and culture lysis can also be inhibited by the addition of chloramphenicol to infected cultures just before the time at which lysis would normally occur. Bacteria infected with Dp-1 under conditions nonpermissive for culture lysis and phage release secrete into the growth medium a substantial portion of their cellular Forssman antigen in the form of a macromolecular complex that has autolysin-inhibitory activity. We suggest that a phage product may trigger the bacterial autolysin by a mechanism similar to that operating during treatment of pneumococci with penicillin (Tomasz and Waks, 1975).

In a previous communication, we noted the resistance of ethanolamine-grown pneumococci to infection by the newly isolated bacteriophage Dp-1 (11). On the basis of what is known about the biochemical defects in ethanolamine-grown cells (12, 18), it has been suggested that Dp-1 may require the activity of the host autolytic enzyme, an *N*-acetylmuramic acid-L-alanine amidase, for a step in the phage infection cycle (11). In this report, we follow up this observation and describe several lines of evidence indicating that the activity of host autolysin is essential for the exit of progeny bacteriophage from the infected bacteria.

MATERIALS AND METHODS

Growth of bacterial cultures. Wild-type *Diplococcus pneumoniae* strain R36A and a lysis-defective mutant derivative (10 "cwl-1") with greatly lowered levels of the *N*-acetylmuramic acid-L-alanine amidase (5) were used in most of the experiments. Wild-type bacteria were grown in a casein hydrolysate synthetic medium (C-medium; 14) buffered with potassium phosphate (4). The same C-medium supplemented with 0.1% yeast extract (Difco) was used for the culturing of the autolysin-defective mutant.

The sharp pH dependence of some of the phenomena to be described in this paper made it necessary to monitor changes in the pH of cultures as a function of bacterial growth. With a buffer concentration of 0.05 M, the pH values of cultures were found to change in a characteristic manner during bacterial growth (Fig. 1). At a cell concentration of 5×10^7 viable units/ml, the pH of culture containing pH 6.6 buffers had shifted to about 5.8 and the pH had further dropped to 4.5 to 4.8 in the stationary phase of growth. The pH of culture with pH 8 buffers had dropped to about 6.9 when the cell concentration reached 5×10^7 viable units/ml (corresponding to the light-scattering value of $N = 200$ on the Coleman nephocolorimeter used to monitor bacterial growth) (14), and the pH value stabilized at 6.5 to 6.6 in the stationary phase of growth. All the lytic phenomena examined, and the release of progeny phage as well, had pH optima at about pH 6.8 to 7.0; this corresponded to the pH of cultures grown to a cell concentration of 5×10^7 to 1.5×10^8 viable units/ml (i.e., nephocolorimeter values of $N = 200$ to 600) in C-medium with 0.05 M, pH 8 potassium phosphate buffer (medium permissive for lysis). Cultures

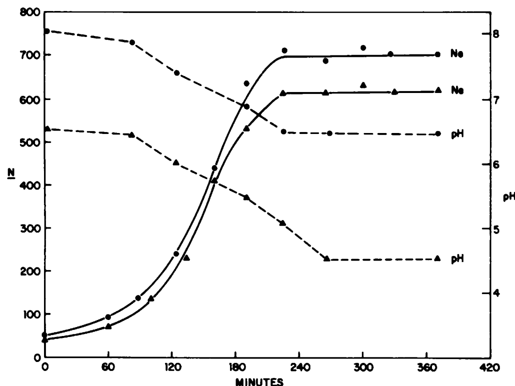

FIG. 1. *Change in the pH of pneumococcal cultures during growth. Two pneumococcal cultures were grown in C-media, one with 0.05 M, pH 8 potassium phosphate buffer and the other with 0.05 M, pH 6.6 buffer added. The cultures were incubated at 37°C; bacterial growth was monitored by the use of a nephocolorimeter (14) and is expressed in arbitrary N (nephelos) values. Periodically, 1-ml samples of each culture were removed for the determination of pH. Symbols: (▲) pH 6.6 culture; (●) pH 8 culture; (——) growth (Ne); (- - -) pH.*

grown to the same cell concentration range in C-medium with 0.05 M, pH 6.6 potassium phosphate buffer (medium nonpermissive for lysis) had pH values betweeen 5.2 and 5.8 and exhibited inhibition in phage release and bacterial lysis. Virtually all the culture lysis and phage release experiments were done with pneumococcal cultures at a cell concentration of 5×10^7 to 2×10^8 viable cells/ml.

An increase in the buffer concentration (to 0.1 M) of the C-medium resulted in the inhibition of bacterial growth. Media with a more powerful buffering capacity could be constructed by using a chemically defined enriched medium (20) from which the normal sodium chloride and sodium acetate components had been omitted and in which the buffer concentrations had been raised to 0.1 or 0.15 M. In such a medium, the initial pH values were found to change much less during culture growth (from pH 8 to 7.6 and from pH 6.6 to a minimum of 6.2). The pH optimum of 6.8 to 7.0 for lysis and phage release was confirmed in these better-buffered media.

The pH measurements were done with a Beckman automatic pH meter equipped with glass microelectrodes. Bacterial growth was monitored by determining the light scattering of suspensions, using a Coleman nephocolorimeter calibrated by viable titers of the pneumococci (14).

Preparation and assay of phage Dp-1. A modification of the previously described methods was used for the preparation of purified phage stocks (11). Bacterial cultures growing in the C-medium were infected at a cell concentration of 1.7×10^7 viable units of bacteria per ml with phage at a multiplicity of infection (MOI) of 0.02. The infected culture was allowed to grow to a cell titer of 1.2×10^8 cells/ml. An equal volume of fresh, prewarmed growth medium was added, and incubation continued until complete lysis. Sodium chloride (final concentration, 0.5 M) was added to the lysate, and debris was removed by low-speed centrifugation ($5,000 \times g$ at 4°C for 15 min). To the supernatant solution, polyethyleneglycol 6000 was added to a final concentration of 12% (wt/vol), and the precipitation of phage was allowed to proceed at 4°C for 36 to 48 h. The precipitate was collected by centrifugation ($5,000 \times g$, 15 min), and the pellet was resuspended in buffer containing 0.5 M NaCl, 10 mM Tris-hydrochloride (pH 7.5), and 10 mM $MgCl_2$ (NTM) at 1% of the original volume of the polyethyleneglycol-treated suspension. The phage was layered on top of a three-step CsCl gradient containing 1, 0.75, and 0.45 g of CsCl in 5 ml of NTM. After centrifugation at 26,000 rpm in an SW27.1 rotor for 210 min, the virus band was isolated and ultracentrifuged at 35,000 rpm in an SW50.1 rotor overnight. The phage was collected and dialyzed overnight against NTM containing 10 mM 2-mercaptoethanol. Phage stocks were kept at −70°C in NTM containing mercaptoethanol.

Assay of the phage was done according to the procedure described previously (11).

Assay of autolysis. The bacterial suspensions were incubated in the appropriate buffers at 37°C, and at intervals the turbidities were determined by using a Zeiss spectrophotometer.

Assay of radioactive material secreted into the medium. Radioactive choline ([*methyl*-^{3}H]choline; 2 Ci/mmol, 2 μCi and 5 μg/ml of medium) was added to the medium of exponentially growing pneumococci at a cell concentration of about 5×10^7 viable units/ml. Incorporation of the isotope into total macromolecular material secreted into the medium was determined by a published procedure (17), the essential features of which are as follows: 100-μl portions of the suspension were removed and treated with cold 10% trichloroacetic acid for 15 min; the precipitated material was collected onto glass fiber filter disks (GFA, Whatman) under vacuum. After drying at 100°C for 10 min, the disks were transferred to scintillation vials, and the amounts of radioactivity were determined by using toluene-based cocktail with 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(-5-phenyloxazolyl)-benzene (POPOP) and a scintillation spectrometer (Mark II, Nuclear-Chicago).

Material secreted into the medium was first separated from the bacteria by centrifugation: 500-μl portions of the cultures were centrifuged for 10 min in an Eppendorf microcentrifuge (Brinkmann Co.), and 100-μl portions of the supernatant solutions were assayed as described above.

Electron microscopy. Infected bacteria were fixed, dehydrated, embedded, and stained according to a previously published procedure (16). Thin sections were cut by an automatic Porter-Blum microtome using a diamond knife, and the sections were examined with a Hitachi H-1 electron microscope at 75-kV accelerating voltage.

RESULTS

Growth media permissive and nonpermissive for culture lysis and phage release. The

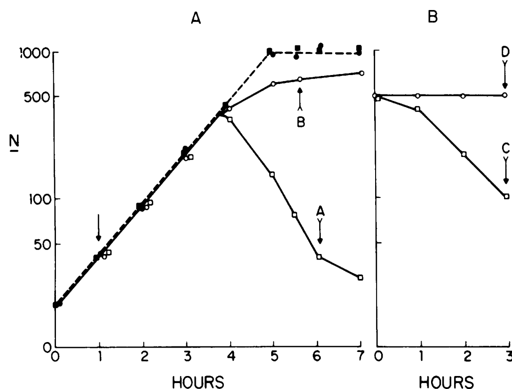

FIG. 2. *Growth conditions permissive and nonpermissive for phage-induced lysis of pneumococci. Two pneumococcal cultures, one with an initial pH of 6.6 and the other with a pH of 8, were grown at 37°C. At the time indicated by the arrow, each culture was halved; one of each pair of cultures was infected with Dp-1 at an MOI of 0.3. The concentration of bacteria was 10^7 cells per ml at the time of infection. The uninfected cultures served as controls. Growth and lysis of the cultures was followed by nephelometry during subsequent incubation at 37°C. Symbols: (○, ●) Bacteria under conditions nonpermissive for lysis (i.e., initial pH = 6.6); (□, ■) Culture growing in permissive media (i.e., initial pH = 8.0); (—— with ○ or □) Dp-1 infected cultures; (- - - with ● or ■) control (uninfected) bacteria. Part B shows the effect of postincubation on the infected bacterial culture, grown initially under nonpermissive conditions. A portion of the culture, illustrated with the open circle and solid line symbols in (A), was centrifuged, and the cells were resuspended in two kinds of fresh growth media: one at a pH permissive for lysis (□) and the other at a nonpermissive pH (○). The lysis of the two cultures was monitored. Samples were removed to titrate extracellular phage at the times indicated by the winged arrows and letters A through D. Bacteria were removed by centrifugation at 10,000 × g for 10 min, and the supernatants were used for the phage assay.*

release of progeny phage and phage-induced culture lysis were found to be completely inhibited if the infected bacteria were grown in C-medium containing pH 6.6 phosphate buffer (0.05 M). In contrast, bacteria cultured in the same medium with pH 8 buffer underwent normal lysis (Fig. 2).

Simple additional experiments using different buffers and different pH values have revealed the following. (i) In the cultures showing lysis inhibition, over 99% of the bacteriophage was intracellular at a time when virtually all progeny phage had already been released into the medium in the lysed culture. (ii) The culture with lysis inhibition was fully infected and contained normal phage particles. This conclusion was based on the fact that over 80% of the bacteria contained phage particles as demonstrated by electron microscopy of thin-sectioned cells (Fig. 3). In addition, a normal phage titer could be liberated from the lysis-inhibited bacteria by brief incubation with 0.1% deoxycholate (Table 1). (iii) For lysis and phage release to occur, the pH of the culture had to be in the range of 6.8 to 7.0 at the end of the phage cycle, which, in the experiment illustrated in Fig. 2, occurred at a bacterial concentration of about 10^8 viable units/ml (N = 400). Culture lysis and phage release were inhibited at pH values higher than 7.6 and lower than 6.0, whereas the development of intracellular phage appeared to be independent of pH. (iv) The lysis of the host bacteria and the release of bacteriophage could be reinitiated by simply transferring the infected cells from the nonpermissive to the permissive growth medium (Fig. 2B).

Inhibition of culture lysis and phage release by trypsin. Lysis of the phage-infected bacteria (growing at a pH permissive for lysis) could be completely prevented by a timely addition of trypsin to the growth medium (Fig. 4). The trypsin-treated bacteria contained a normal crop of intracellular phage (see insert in Fig. 4).

Inhibition of culture lysis and phage release by chloramphenicol. A pneumococcal culture growing in C-medium with permissive pH for lysis was infected with phage at a cell concentration of 5 × 10^7 bacteria/ml (MOI = 0.3) and was incubated at 37°C up to within 30 min of the time at which usual lysis would occur. Chloramphenicol (100 μg/ml) was added to a portion of the culture, and incubation was continued. The chloramphenicol-treated culture showed complete inhibition of lysis (Fig. 5), and over 99% of the bacteriophage had remained intracellular in these cells (not shown).

Nature of the process essential for phage release. The information summarized in Fig. 6 suggests that the mechanism of progeny Dp-1 phage release involves the triggering of the host autolytic enzyme. The evidence for this conclusion is as follows. (i) Progeny phage could be released from bacteria infected at nonpermissive pH by transferring the infected cells into simple buffers; the phage would be released and the culture lysed, provided that the pH of the buffer was between 6.8 and 7.0. A practically complete inhibition of lysis and phage release was observed in buffers at pH values of 8.0 and 5.8. (ii) Uninfected (control) pneumococci from either the exponential or early stationary phase of growth would undergo spontaneous lysis upon incubation in simple buffers of pH 6.8 to 7.0. Lysis was inhibited at pH values below 6.2 or above 7.8. (iii) Similarly to phage-induced

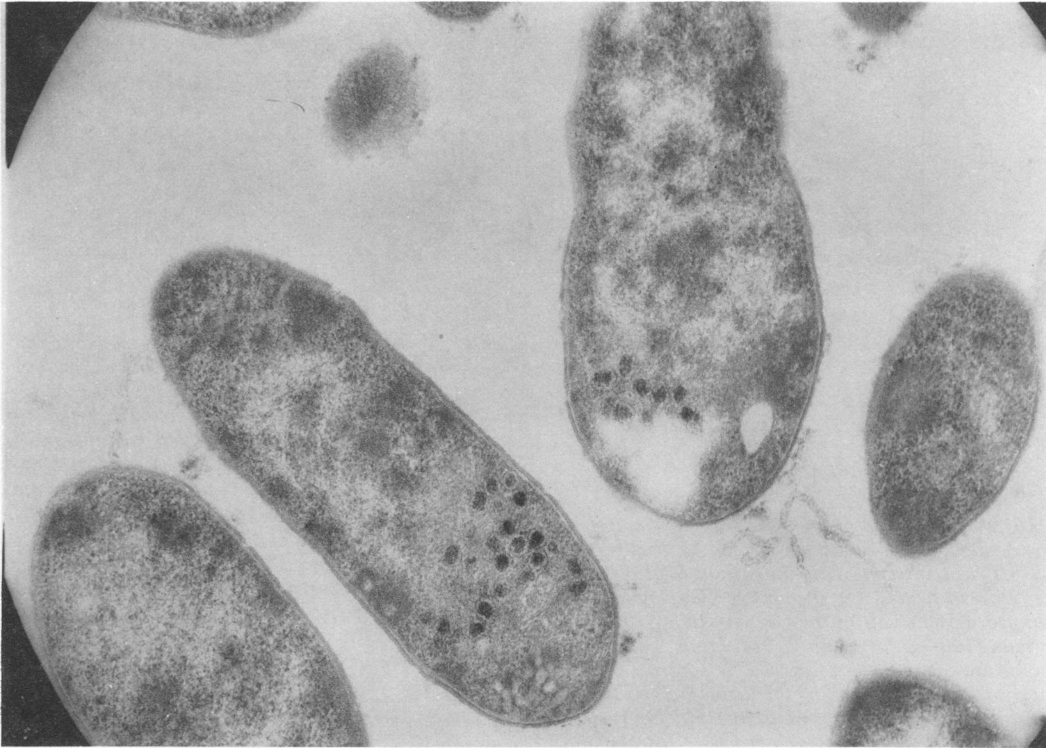

FIG. 3. *Intracellular phage in cells infected under conditions nonpermissive for lysis and for phage release. A pneumococcal culture grown at the nonpermissive pH was infected at an MOI of 1 as described in the legend to Fig. 2. Bacteria were removed for the preparation of cytological specimens at a time corresponding to that of point B in Fig. 2. The preparation of electron microscopic specimens was done according to a published procedure (11). Magnification: ×48,000.*

TABLE 1. *Effect of culture pH on the liberation of progeny phage*

pH of the medium of the infected culture	Time of assay for extracellular phage[a]	Titer of phage (PFU/ml)
Permissive for lysis (i.e., initial pH = 8.0)	Point A	2×10^8
Nonpermissive for lysis (i.e., initial pH = 6.6)	Point B	3×10^5
Nonpermissive for lysis		
Shifted to permisive pH	Point C	5×10^8
Retained at nonpermissive pH	Point D	3×10^5
Lysis induced by deoxycholate, added at point B in Fig. 2		9×10^7

[a] Points refers to Fig. 2.

lysis, spontaneous culture lysis in buffers could be prevented by a brief (15 min) pretreatment of the bacteria (still in growth medium) with chloramphenicol before transfer to the lysis buffer. Addition of chloramphenicol later, after the bacteria have been transferred to the buffer, was ineffective. (iv) Similarly to phage-induced lysis, spontaneous lysis was also inhibited by the addition of either trypsin or glucose to the lysis buffer.

Further evidence for the involvement of the host autolytic system in phage release was provided by comparative experiments in which the effects of culture pH, chloramphenicol, and trypsin addition were determined on penicillin-induced lysis and spontaneous culture lysis in the stationary phase of growth.

Both culture lysis and penicillin-induced lysis were inhibited by the addition of trypsin to the medium or by growth of the cells in a medium nonpermissive for phage-induced lysis (Fig. 7). Penicillin-induced lysis was also blocked by chloramphenicol.

Abnormal lysis of an autolysin-defective mutant pneumococcus by Dp-1. In contrast to the wild-type pneumococci, the autolysin-defective mutant strain of pneumococcus could only yield productive infections if the MOI was at least 1 (Fig. 8). The nature of this requirement is obscure, and experiments are in progress to provide some insight into this phenomenon.

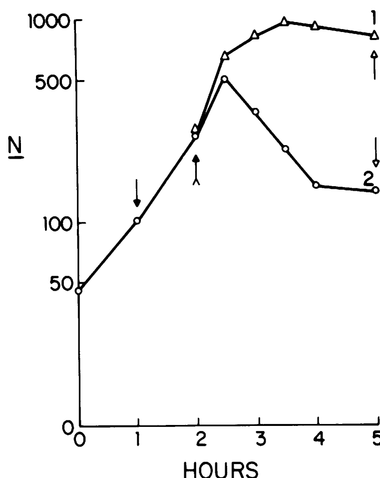

FIG. 4. *Inhibition of phage-induced lysis by trypsin. A pneumococcal culture, grown at lysis-permissive pH, was infected with Dp-1 at an MOI of 1.0, at the time indicated by the arrow. One hour later (winged arrow) half of the culture (tube 1) received trypsin (100 µg per ml; Worthington 2× crystallized); the other (tube 2) served as control. Growth and lysis were measured by the usual method. Extracellular phage was assayed at the 5th hour of the experiment (empty-headed arrows). Intracellular phage was determined by first washing the bacteria free of trypsin by three cycles of centrifugation. The bacteria were resuspended in C-medium at pH 8 and were lysed by the addition of a drop of 5% deoxycholate solution. Phage could not be assayed in the supernatant of the trypsin-treated culture because of the known trypsin sensitivity of Dp-1 (11) (*). Phage titers (PFU per milliliter) were as follows: extracellular, tube 1, 0; intracellular, tube 1, 10^8; extracellular, tube 2, 1.2×10^8.*

Nevertheless, it seems that the lack of phage production in mutant cultures infected at an MOI lower than 1 has to do with the mechanism of phage release. This conclusion is based on the observation (illustrated in Fig. 9) that mutant cultures infected at an MOI of 0.1 would be induced to lyse and release progeny phage by treatment with wild-type autolysin at the end of the infection cycle.

Release of lipoteichoic acid (Forssman antigen) containing macromolecules into the medium during phage infection in the absence of lysis. Pneumococci were grown in C-medium with pH 8 phosphate (0.05 M) with radioactive choline ([*methyl*-^{3}H]choline) in order to label the cell wall teichoic acid and the Forssman antigen (pneumococcal lipoteichoic acid; 1) of this bacterium. Half of the culture was infected with Dp-1 at an MOI of 1.0 at a cell concentration of 5×10^7 bacteria/ml. Both control and infected cultures were then resuspended in isotope-free growth medium at low pH (5.8) to prevent lysis, and the possible release of macromolecular radioactive material to the medium was monitored. About 20% of the macromolecular isotope was released from the infected bacteria by the time (60 min) of incubation at which a parallel infected culture incubating at the pH optimal for lysis had started the phage-induced lytic process (Fig. 10).

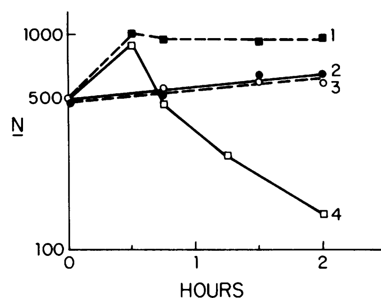

FIG. 5. *Inhibition of phage-induced lysis by chloramphenicol. Pneumococci were grown in lysis-permissive C-medium and infected with Dp-1 as described in the legend to Fig. 2. About 1 h before the expected time for lysis, the infected culture and a noninfected (control) culture were centrifuged and resuspended in fresh, lysis-permissive medium. Chloramphenicol (100 µg/ml) was added to half of the infected and to half of the control culture. Growth and lysis were followed by nephelometry. Symbols: (——) Infected cultures with (○, curve 2) or without chloramphenicol (□, curve 4); (- - -) uninfected control bacteria with (●, curve 3) or without chloramphenicol (■, curve 1).*

Preliminary experiments (not documented) indicated that the choline-containing macromolecules closely resembled those released during penicillin treatment of pneumococci (17); they had autolysin-inhibitory activity and showed a similar behavior during sedimentation in sucrose gradient and during sodium dodecyl sulfate-gel electrophoresis.

DISCUSSION

Some unusual properties of phage Dp-1 suggesting a possible need of host autolysin activity at some stage in the infection cycle have already been noted in an earlier communication (11). The experimental results described in this paper indicate that the autolytic enzyme of the host pneumococcus is required specifically for the release of progeny virus particles from the bacteria at the end of the phage infection.

Evidence for this suggestion comes from the observation that experimental conditions that specifically inhibit the activity of the pneumococcal *N*-acetylmuramic acid-L-alanine amidase would also block the release of progeny

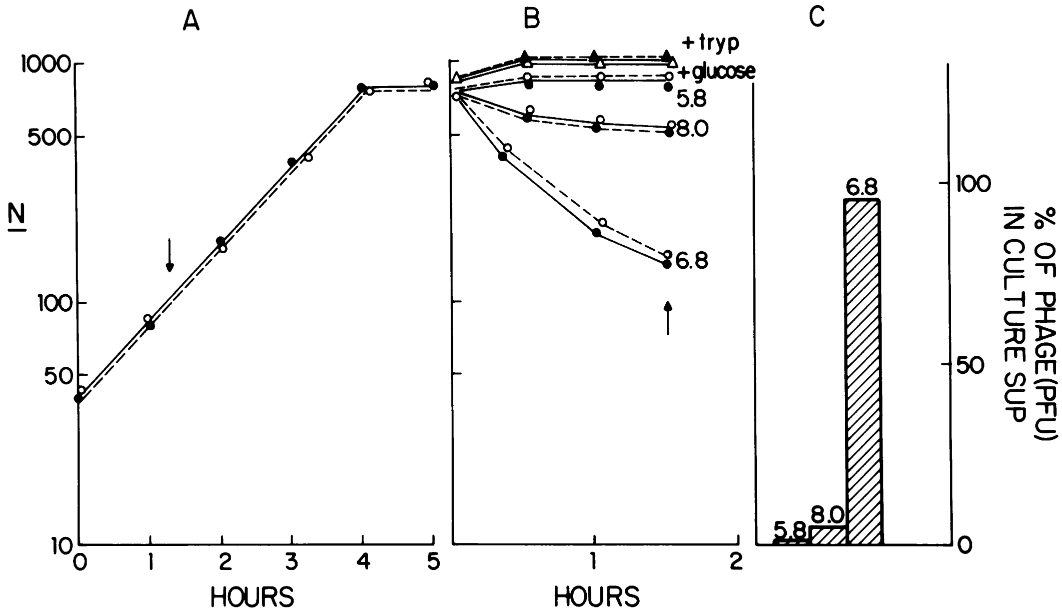

FIG. 6. *Inhibition of phage release by inhibitors of cellular autolysis. Two pneumococcal cultures were grown in C-medium with a pH nonpermissive for lysis. One of the cultures was infected with Dp-1 (MOI = 0.03) at a cell concentration of 2.5 × 10⁷ bacteria/ml (arrow), and the growth of the infected and control cultures was monitored by nephelometry (panel A). Panel B depicts the next phase of the experiment. After 5 h of growth, both cultures were centrifuged, and the bacteria were resuspended in a series of 0.1 M Tris buffers at pH 5.8, 6.8, and 8.0, and incubated at 37°C. Only small degree of lysis was observed at pH 8, lysis was completely blocked at pH 5.8, whereas normal lysis occurred at pH 6.8. The rates of lysis of infected (——) and noninfected suspensions (- - -) were identical. Two additional portions of the infected and control bacteria suspended in the buffer optimal for lysis (pH 6.8) received either glucose (2 mg/ml; △) or trypsin (100 μg/ml; ▲). Lysis was inhibited in both the infected and the control suspensions. Panel C shows the titers of extracellular phage assayed after 1.5 h of incubation of the three buffer suspensions (see arrow in panel B).*

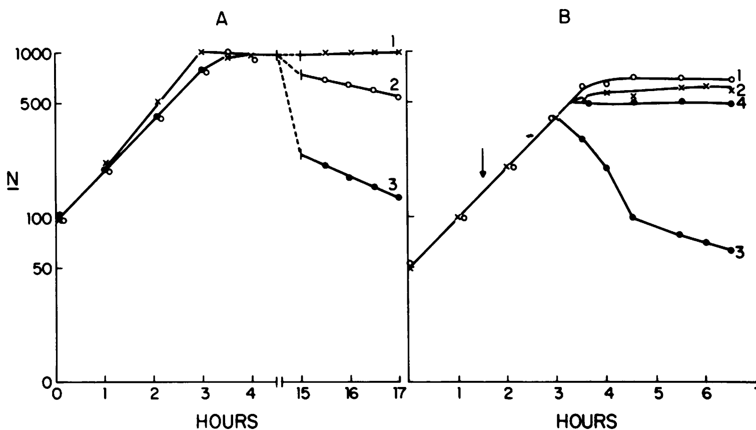

FIG. 7. *Lysis of pneumococci in the stationary phase of growth and during penicillin treatment—effects of pH and trypsin in the medium. (A) Three pneumococcal cultures were grown in C-media, cultures 1 and 3 at the initial pH of 8 and culture 2 at the initial pH of 6.6. Culture 1 also received trypsin (100 μg/ml). Growth and lysis of the bacteria were followed by the usual procedure (see text) during prolonged incubation at 37°C. (B) Four pneumococcal test tube cultures were grown; tubes 1, 3, and 4 in lysis-permissive C-medium (initial pH 8) and tube 2 in C-medium with a lysis-nonpermissive pH (initial pH 6.6). All four tubes received benzylpenicillin (0.1 μg/ml) at the time indicated by the arrow. In addition, tube 1 also received trypsin (100 μg/ml), and chloramphenicol (100 μg/ml) was added to tube 4. Growth and lysis were determined by the usual procedure (see text).*

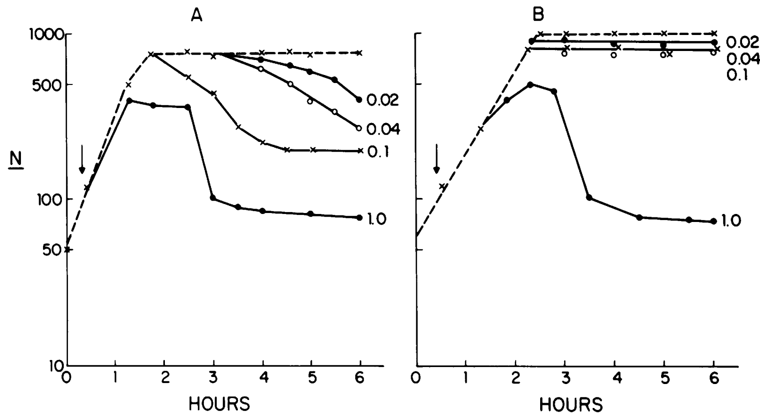

FIG. 8. *Phage-induced lysis of wild-type and autolysin-defective cultures as a function of the multiplicity of phage used for infection. Both the wild-type (A) and mutant (B) cultures were grown in lysis-permissive medium. Infection was done at the time indicated by the arrows at the MOI values indicated by the numbers on the curve. A noninfected control was also included in each experiment (- - -). Growth and lysis were followed by the usual method (see text).*

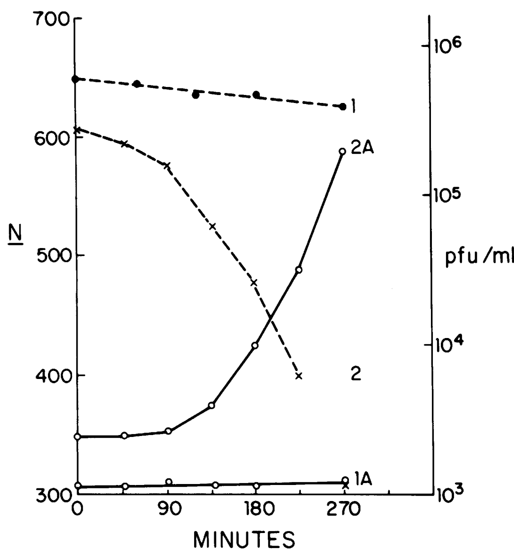

FIG. 9. *Release of progeny bacteriophage entrapped in the lysis-defective mutant bacteria by treatment with wild-type autolysin from without. A culture of mutant bacteria infected at an MOI of 0.1 was allowed to grow to a stationary phase (see Fig. 8). The infected cells were then centrifuged and resuspended in 0.1 M Tris buffer (pH 7.0); to half of the suspension (tube 2) wild-type autolysin was added (100 μg of protein per ml, corresponding to about 10^9 cell equivalents of crude autolysin); tube 1 served as a control. The figure shows the changes in the light scattering of the autolysin treated (- - -, 2) and control (- - -, 1) suspensions during incubation at 37°C. The appearance of extracellular phage was also assayed (——, 1A and 2A) in the two suspensions by methods described earlier (11).*

phage. Phage release could be prevented by transferring the infected bacteria just before the normal time of culture lysis to fresh growth media or buffer with pH values suboptimal for the activity of the host autolysin (i.e., pH above 7.5 or below 6.0) (8). Addition of trypsin or chloramphenicol to the medium just before the normal lysis time would also typically inhibit penicillin-induced and stationary-phase lysis of pneumococci; the key role of the *N*-acetylmuramic acid-L-alanine amidase in these latter processes has been documented (15, 19).

None of the conditions described as inhibitory for phage release seem to have any effect on the intracellular phases of phage infection, since normal titers of the bacteriophage particles entrapped in the host cells could be liberated by triggering the host autolytic system (e.g., by incubation in buffer, by treatment of the bacteria with wild-type autolysin from without, or by simply transferring the infected bacteria to fresh growth medium with a pH permissive for culture lysis). It was reported earlier that ethanolamine-grown pneumococci showed considerable resistance to Dp-1 infection (11); also, lysis of Dp-1-infected cells could be blocked by the addition of Forssman antigen (6), a choline-containing pneumococcal lipopolysaccharide (or "lipoteichoic acid"). The biochemical basis of lysis inhibition under both of these conditions is known to be the highly specific inhibition of the activity of the pneumococcal *N*-acetylmuramic acid-L-alanine amidase (7).

The possibility that the escape of phage progeny and the lysis of the host cells are catalyzed

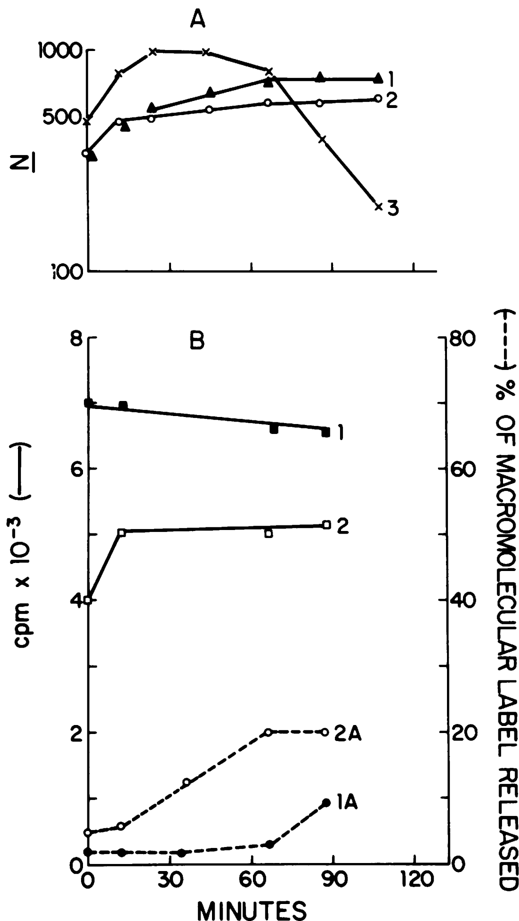

FIG. 10. *Leakage of macromolecular choline-labeled material into the culture medium of pneumococci infected with Dp-1 in lysis-nonpermissive conditions. A pneumococcal culture (40 ml) in C-medium (pH 8) was grown for three generations in the presence of radioactive choline ([methyl-^{3}H]choline, 2 μCi and 5 μg per ml). After this labeling period, at a cell concentration of 5 × 10^7 bacteria/ml, half of the culture (20 ml) received Dp-1 at an MOI of 1.0. After 20 min, excess virus was neutralized by antiserum, and both infected and control cultures were transferred to fresh, isotope-free growth media (C-medium) with a lysis-nonpermissive pH (6.0). A portion of the infected culture was transferred to fresh lysis-permissive medium (tube 3). The control (tube 1) and infected (tube 2) bacteria were incubated at 37°C. Growth was followed by the usual nephelometric procedure (see panel A). (B) In addition, samples were removed to quantitate the total macromolecular (i.e., precipitable by cold trichloroacetic acid) radioactivity in the suspensions (—, 1, control; —, 2, infected culture). At the same time, samples were also centrifuged and the fraction of macromolecular radioactivity that had leaked to the outside medium was determined by a procedure described in detail in the text (- - -, 1A, control; - - -, 2A, infected culture).*

by a phage-coded lytic enzyme cannot definitely be ruled out. However, these observations together make this unlikely. No cell wall hydrolyzing activity could be demonstrated by incubating radioactively labeled cell walls with purified Dp-1 preparations for prolonged periods of time (unpublished observation). The lysis of the autolysin-defective mutant pneumococci after infection at a multiplicity of 1 does not necessarily imply the production of phage-specific lytic enzyme, since these mutant bacteria are known to contain 5 to 10% of the wild-type autolytic amidase activity (5), which may be sufficient to catalyze phage release and cell lysis.

We would like to suggest that a phage product, synthesized at the end of Dp-1 replication, can trigger the activity of the pneumococcal autolytic *N*-acetylmuramic acid-L-alanine amidase, which in turn results in the lysis of the host bacteria and in the release of progeny bacteriophage. This mechanism would be closely analogous to the one suggested for the mechanism of bacterial lysis induced by penicillin and other cell wall inhibitory antibiotics (17).

The release of a choline-containing autolysin inhibitor into the medium during both penicillin treatment and phage replication further supports the analogy between penicillin-induced and phage-induced cell lysis. It is conceivable that some viral product formed late during infection inhibits the synthesis of bacterial peptidoglycan, which leads to the labilization of membrane-located autolysin inhibitor and to the triggering of latent autolysin activity. Alternatively, some phage component may destabilize the autolysin-inhibitory lipoteichoic acid complex in a more direct manner.

The validity of the pneumococcal findings for other bacteriophage systems remains to be tested. It is possible that the triggering of host autolysin activity is related to some unusual structural component of Dp-1. The sensitivity of Dp-1 to trypsin and organic solvents has already been noted (11), and more recent observations suggest that this bacteriophage may contain lipid material (Lopez et al., manuscript in preparation).

Although it is generally accepted that the lysis of T4-infected *Escherichia coli* is catalyzed by the phage lysozyme (13), it is interesting to note that a possible participation of host murein hydrolases in phage-induced lysis has been considered in the case of the *S* ("spackle") mutants of coliphage T4 (2). It has been suggested that the phage mutation affects the cell wall of the infected host in such a manner that the wall becomes sensitized to the activity of bacterial lytic enzymes (2).

The use of host enzymes for the synthesis of phage-specific polymers has been described in a number of instances, e.g., in the cases of the DNA and RNA polymerases of *Bacillus subtilis* infected with phage SPP1 (21) and in the case of f2-infected *E. coli* (3). The use of host autolysin for the release of progeny phage, as suggested in this paper, may represent still another example of the exploitation of bacterial host enzymes by a bacterial virus. The close analogies observed between penicillin-induced and Dp-1-induced lysis of pneumococci suggest that a detailed study of the mechanism of phage-induced lysis may yield information concerning the cellular control of the activity of murein hydrolases.

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LITERATURE CITED

1. **Briles, E. B., and A. Tomasz.** 1973. Pneumococcal Forssman antigen, a choline-containing lipoteichoic acid. J. Biol. Chem. 248:6394-6397.
2. **Emrich, J.** 1968. Lysis of T-4-infected bacteria in the absence of lysozyme. Virology 35:158-165.
3. **Fedoroff, N.** 1975. Replicase of the phage f2, p. 235-258. *In* N. D. Zinder (ed.), RNA phages. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
4. **Gomori, G.** 1955. Preparation of buffers for use in enzyme studies, p. 138-148. *In* S. P. Colowick and N. D. Kaplan (ed.), Methods in enzymology, vol 1. Academic Press Inc., New York.
5. **Höltje, V. J., and A. Tomasz.** 1974. Teichoic acid phosphorylcholine esterase: a novel enzyme activity in pneumococcus. J. Biol. Chem. 249:7032-7034.
6. **Höltje, V. J., and A. Tomasz.** 1975. Biological effects of lipoteichoic acids. J. Bacteriol. 124:1023-1027.
7. **Höltje, V. J., and A. Tomasz.** 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in pneumococcus. Proc. Natl. Acad. Sci. U.S.A. 72:1690-1694.
8. **Höltje, V. J., and A. Tomasz.** 1976. Purification of the pneumococcal N-acetylmuramic acid-L-alanine amidase. J. Biol. Chem. In press.
9. **Kamen, R. I.** 1975. Structure and function of the Q RNA replicase, p. 203-234. *In* N. D. Zinder (ed.), RNA phages. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
10. **Lacks, S.** 1970. Mutants of *Diplococcus pneumoniae* that lack deoxyribonucleases and other activities possibly pertinent to genetic transformation. J. Bacteriol. 101:373-381.
11. **McDonnell, M., C. Ronda-Lain, and A. Tomasz.** 1975. Diplophage: a bacteriophage of *Diplococcus pneumoniae*. Virology 63:577-582.
12. **Mosser, J. L., and A. Tomasz.** 1970. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J. Biol. Chem. 245:287-298.
13. **Mukai, F., G. Streisinger, and B. Miller.** 1967. The mechanism of lysis in phage T-4-infected clels. Virology 33:398-402.
14. **Tomasz, A.** 1970. Cellular metabolism in genetic transformation of pneumococci: requirement for protein synthesis during induction of competence. J. Bacteriol. 101:860-871.
15. **Tomasz, A.** 1974. The role of autolysins in cell death, p. 439-448. *In* M. R. J. Salton and A. Tomasz (ed.), Mode of action of antibiotics on microbial walls and membranes. (New York Academy of Sciences) Ann. N. Y. Acad. Sci. 235:439-448.
16. **Tomasz, A., J. D. Jamieson, and E. Ottolenghi.** 1964. The fine structure of *Diplococcus pneumoniae*. J. Cell Biol. 22:453-467.
17. **Tomasz, A., and S. Waks.** 1975. Mechanism of action of penicillin-triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. Proc. Natl. Acad. Sci. U.S.A. 72:4162-4166.
18. **Tomasz, A., and M. Westphal.** 1971. Abnormal autolytic enzyme in a pneumococcus with altered teichoic acid composition. Proc. Natl. Acad. Sci. U.S.A. 68:2627-2630.
19. **Tomasz, A., M. Westphal, E. B. Briles, and P. Fletcher.** 1975. On the physiological functions of teichoic acids. J. Supramol. Biol. 3:1-16.
20. **Tomasz, A., E. Zanati, and R. Ziegler.** 1971. DNA uptake during genetic transformation and the growing zone of the cell envelop. Proc. Natl. Acad. Sci. U.S.A. 68:1848-1852.
21. **Trautner, T. A., and H. C. Spatz.** 1973. Transfection in *B. subtilis*. Curr. Top. Microbiol. Immunol. 62:61-88.