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Characterization of the Pneumococcal Bacteriophage HB-3 Amidase: Cloning and Expression in *Escherichia coli*

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HB-3, a temperate bacteriophage of *Streptococcus pneumoniae*, synthesizes its own murein hydrolase activity when multiplying on cultures of pneumococcus. The enzyme (HBL) was purified and biochemically characterized as an *N*-acetylmuramoyl-L-alanine amidase of 36,000 daltons, and a 2.1-kilobase *DraI* fragment containing the lysin gene (*hbl*) was cloned and expressed in *Escherichia coli*. Our results demonstrated that the primary product of the *hbl* gene is a form with low enzyme activity that can be converted to a more active form under conditions similar to those previously described for the major pneumococcal autolysin (E. García, J. L. García, C. Ronda, P. García, and R. López, Mol. Gen. Genet. 201:225–230, 1985). The phage-encoded amidase requires the presence of choline in the teichoic acids of the pneumococcal cell walls for in vivo and in vitro activity. Comparative biochemical and immunological tests of the phage-encoded and host amidases revealed a remarkable similarity between these enzymes, although analyses of their N-terminal amino acid sequences allowed us to conclude that the amidases are similar but not identical. This appears to be the first description of the cloning of a phage-encoded amidase in gram-positive bacteria.

The study of cell wall lytic enzymes has provided useful information on mechanisms of enzyme action, protein structure, and evolution (14). Nevertheless, only a few examples of lytic enzymes purified from bacteriophages infecting gram-positive bacteria are known, and consequently, the primary sequences of only two phage lysins have been published (4, 11).

We have recently characterized several enzymes that degrade the cell wall of *Streptococcus pneumoniae*. These lytic enzymes have been isolated either from the host cells or from bacteriophage-infected pneumococci. The genes coding for the muramidases of bacteriophages Cp-1, Cp-7, and Cp-9 as well as for the host amidase have been cloned and sequenced (4, 8; P. García, J. L. García, E. García, J. M. Sánchez-Puelles, and R. López, Gene, in press), and the comparative study of their sequences has provided valuable information relative to the peculiar modular organization of these enzymes. On the other hand, we have also reported the isolation of a murein hydrolase induced by bacteriophage Dp-1 in *S. pneumoniae* that was purified and characterized as an amidase, similar but not identical to the host one (10). The detailed knowledge of a phage-encoded amidase would have been of relevant interest for our current investigations on the relationships between lytic enzymes encoded by pneumococcus and its phages. Unfortunately, our efforts to isolate the gene coding for the Dp-1-induced amidase have been unsuccessful so far, and we decided to investigate other pneumococcal phages to achieve this goal. HB-3, a pneumococcal bacteriophage isolated from a lysogenic strain of *S. pneumoniae* (1), provides a way to look for the presence of lytic enzymes either phage-encoded or induced by the phage when multiplying in a lytic cycle. In this study, we observed a lytic activity (HBL) in the lysates of HB-3-infected cultures of the amidase-deficient M31 strain of *S. pneumoniae*. Furthermore, we showed that the genome of HB-3 encodes for its own amidase and report the cloning and expression in *Escherichia coli* of a 2.1-kilobase *DraI* fragment of HB-3 DNA containing the lysin gene. The phage amidase was purified to electrophoretic homogeneity.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Phage growth, purification, and preparation of DNA. Bacteriophage HB-3 is a temperate phage isolated from *S. pneumoniae* by H. P. Bernheimer (1) and provided to us by S. Lacks. The pneumococcal strain R6st was grown in a slightly modified C medium (19), and at a cell concentration of 1.2×10^7 CFU/ml, the culture was infected with the HB-3 (multiplicity of infection of 0.2) and incubated at 37°C. After 2.5 h, an equal volume of the same medium was added and the incubation was continued until the culture lysed (ca. 2.5 h later). The titer of crude lysates ranged from 5×10^7 to 1×10^8 PFU/ml. After removal of cell debris, NaCl (final concentration, 0.5 M) and polyethylene glycol 6000 (final concentration, 10%) were added to the supernatant and stored at 4°C. The phage were then purified in a two-step CsCl gradient following a published procedure (5). Proteinase K-treated phage DNA was prepared as described previously (13).

Determination of susceptibility of pneumococcal cultures to DOC-induced lysis. Susceptibility of the bacteria to sodium deoxycholate (DOC)-induced lysis was tested as previously described (26). In short, 0.5-ml portions of the culture were periodically pipetted into test tubes containing 0.5 ml of potassium phosphate buffer (0.1 M, pH 8.0); after the initial light scattering value of the suspension was recorded (in a Coleman nephelometer), 50 μl of a 5% DOC solution was added, the suspension was incubated at 37°C for 5 min (to induce lysis of the susceptible portion of the population), and the light scattering value of the suspension was recorded again. The percentage values represent the degree of lysis susceptibility expressed as the fraction of initial light scattering value lost upon incubation with DOC.

Preparation of cell walls and assay for enzymatic activity. Pneumococcal cell walls labeled with [*methyl*-^{3}H]choline

TABLE 1. Bacterial strains and plasmids

Strain and plasmid	Relevant genotype or phenotype	Source or reference
Streptococcus pneumoniae		
R6	Wild type	Rockefeller University
R6st	*str*	Rockefeller University
M31	*hex-4 end-1 exo-2ΔlytA*	20
Escherichia coli JM83	*ara ΔlacproAB rspL* ϕ80 *lacZΔM15* (r^+_k m^+_k)	28
Plasmids		
pUC13	Ap^r	28
pUC18	Ap^r	28
pHL50	Ap^r *hbl*$^+$	This work
pHL51	Ap^r *hbl*$^+$	This work

(specific activity, 60 Ci/mmol), L-[4,5-^{3}H]lysine monohydrochloride (specific activity, 40 Ci/mmol), or [2-^{14}C]ethanolamine (specific activity, 44 mCi/mmol) were prepared by biosynthetic labeling of the bacteria (18). The standard assay conditions for phage-induced lytic activity (HBL) were similar to those previously described (12). One unit of enzymatic activity was defined as the amount of enzyme that catalyzed the hydrolysis (solubilization) of 1 μg of cell wall material in 10 min at 37°C (12). Pneumococcal lipoteichoic acid was prepared as previously described (3).

Purification of lytic enzyme of HB-3 (HBL). In a typical experiment, an M31 culture was grown at 37°C to a cell density of 2.5×10^7 CFU/ml, and purified HB-3 was added at a multiplicity of infection of 0.5. After 180 min, complete lysis occurred. The lysate was cooled on ice, and the cellular debris was removed by centrifugation. The supernatant (250 ml; 0.1 mg of protein per ml) containing the crude enzyme was loaded on a DEAE-cellulose column (21 by 2.5 cm) and washed with 150 ml of 50 mM Tris-maleate buffer (pH 6.5) containing 1.5 M NaCl. The enzymatic activity adsorbed to the column was eluted with the same buffer containing 2% choline. Active fractions were pooled and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (15), and proteins were visualized with Coomassie blue. This procedure yielded about 50 μg of purified HBL with a recovery of about 20%.

N-terminal amino acid sequence analyses. The N-terminal amino acid sequence of the purified HBL was determined by Edman degradation in a pulse-liquid phase protein sequencer model 477A (Applied Biosystems). The same method was used to identify the N-terminal amino acid of the purified peptides released by treatment of the purified cell walls with HBL (see below).

Southern blot hybridization. Hybridization (22) was performed by the method of Maniatis et al. (16). Radioactive probes were labeled by nick translation (16) in the presence of [α-^{32}P]dCTP. Radioactive bands were detected with Agfa Curix RP2 films and Cronex Lightning-Plus intensifying screens at −70°C.

Western blot (immunoblot) analyses. The purified proteins and crude extracts were electrophoresed in sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (16). After nonspecific binding sites were blocked with reconstituted dried skim milk, the membrane was subsequently incubated at room temperature with anti-pneumococcal amidase serum (6), peroxidase-conjugated AffiniPure goat anti-rabbit serum, and 4-chloro-1-naphthol, essentially as recommended by the suppliers.

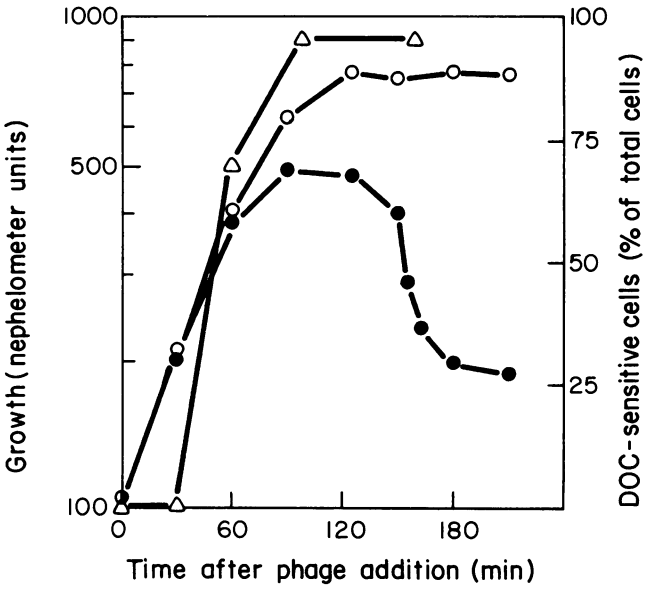

FIG. 1. HB-3-induced lysis and sensitization to DOC of cultures of the pneumococcal M31 strain. Growth (and lysis) of M31 infected (multiplicity of infection of 0.5) (●) or not (○) with HB-3 was observed by nephelometry. Susceptibility to DOC-induced lysis (△) was determined at different times as described in Materials and Methods. 100% indicates complete lysis.

Analytical methods and materials. The analyses of soluble degradation products of the cell walls have been previously described (18). Reducing groups were determined by using glucose as the standard (24). The release of the free amino groups was assayed with fluorescamine (17). Plasmid DNA was prepared by the alkaline method of Birnboim and Doly (2). *E. coli* JM83 was made competent by the rubidium chloride method (16). Restriction endonucleases, phage T4 DNA ligase, and proteinase K were purchased from New England BioLabs, Inc. (Beverly, Mass.), Boehringer Mannheim Biochemicals, Indianapolis, Ind.), or Amersham Corp. (Arlington Heights, Ill.) and were used according to the recommendations of the suppliers. Hybond membranes and the kit for nick translation were obtained from Amersham Corp. Nitrocellulose sheets were from Schleicher & Schuell, Inc. (Keene, N.H.). Peroxidase-conjugated AffiniPure goat anti-rabbit serum was purchased from Jackson Immunoresearch, and 4-chloro-1-naphthol was from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS AND DISCUSSION

Phage-induced lysis of autolysis-deficient pneumococcal strain M31. M31 is a mutant of *S. pneumoniae* deleted in the *lytA* gene coding for the major autolysin, an *N*-acetylmuramoyl-L-alanine amidase (20). As a result of this genetic defect, M31 does not lyse at the end of the stationary phase of growth when incubated at 37°C or upon the addition of detergents (e.g., DOC). Nevertheless, when M31 was infected with bacteriophage HB-3 at a multiplicity of infection higher than 0.5, the culture lysed after 3 h of incubation at 37°C (Fig. 1), and a lytic activity capable of degrading pneumococcal cell walls was found in the culture medium. Together with this finding, we observed a change in cultures

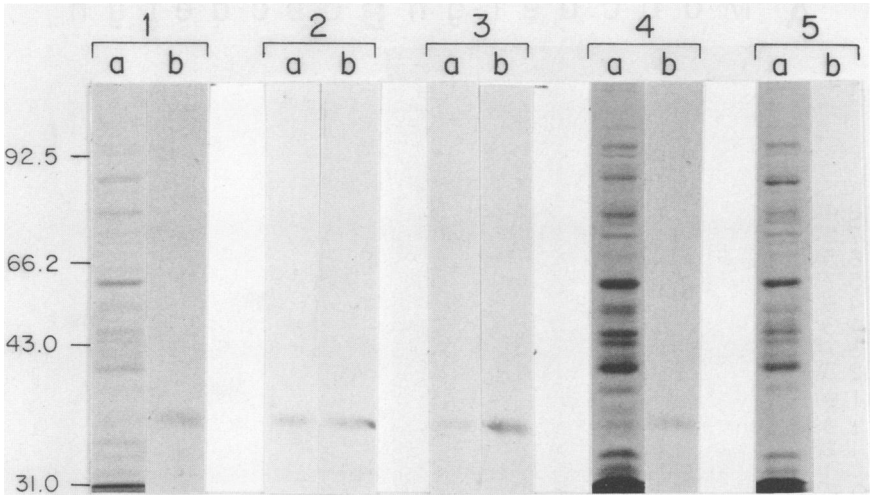

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes a, Stained with Coomassie blue. Lanes b, Immunoblot analysis. 1, Crude lysate from HB-3-infected M31; 2, purified HBL; 3, purified host amidase; 4, crude sonicated extract from R6 strain; 5, crude sonicated extract from M31 strain. The antiserum was raised by the host amidase. The positions of molecular mass standards are indicated on the left in kilodaltons.

of M31 in the lytic response to DOC (Fig. 1) since HB-3-infected cultures of this strain became rapidly sensitized to lysis by DOC. Inasmuch as detergent-induced lysis of wild-type pneumococci is known to be caused by the triggering of the amidase (25), this finding also suggested the appearance of a murein hydrolase activity in the infected cultures.

Purification, characterization, and properties of phage-induced peptidoglycan hydrolase (HBL). The peptidoglycan hydrolase activity present in HB-3-infected cultures (HBL) was purified by following a procedure similar to that developed in our laboratory for the rapid purification of both the host and Cp-1 bacteriophage-encoded lytic enzymes (see Materials and Methods). Since these enzymes show a remarkable affinity for choline and its analogs (21) (a property also shared by HBL), we took advantage of this property to purify HBL by adsorbing it to a DEAE-cellulose column, since DEAE acts as an analog of choline (Fig. 2). A single band with an apparent M_r of 36,000 was found. The most relevant biochemical properties of HBL can be summarized as follows. (i) The enzymatic activity of HBL was maximal at pH 6.0 in 20 mM phosphate buffer. (ii) The dependence of the reaction rate on substrate concentration followed Michaelis-Menten kinetics, with an apparent K_m of 0.067 g/liter. The inhibitory effect of choline and lipoteichoic acid was of a noncompetitive nature, a property shared with all the lytic enzymes of *S. pneumoniae* and its bacteriophages which require the presence of choline in the cell wall substrate for activity (7, 9, 12). (iii) Lipoteichoic acid and free choline were found to be powerful inhibitors of HBL in vitro (0.06% choline inhibited up to 50% of the activity, whereas a complete inhibition of the enzyme was observed at 0.1% choline). (iv) Analyses of the soluble degradation products of the pneumococcal cell walls radioactively labeled either with choline or lysine, determination of free amino groups and of the reducing power of cell walls digested by HBL, as well as the identification of L-alanine as the N-terminal amino acid of the purified wall peptides obtained after treatment of the cell walls with HBL, indicated that this lytic enzyme is an endo-*N*-acetylmuramoyl-L-alanine amidase (data not shown).

Taking into account the biochemical similarities between HBL and the host amidase and the fact that they cleave the same bond in the cell wall, it seemed likely that the two amidases were also immunologically related. Western blotting analyses showed that HBL did cross-react with anti-pneumococcal amidase antibodies at a titer similar to that found for the host autolysin (Fig. 2). As expected, a single band corresponding to the mobility of the host amidase also appeared when a sonicated extract of the wild-type strain R6 was used to perform the Western blotting analyses, whereas no band was observed when crude extracts of M31 were analyzed. This result demonstrated that the amidase-defective M31 strain apparently does not contain proteins immunologically related to the HBL, suggesting that this amidase is a phage-induced lysin.

Sequence homology between HBL and host amidase and their corresponding genes. The determination of the N-terminal amino acid sequence of the purified HBL in two independent analyses in which no ambiguities were found (Fig. 3) confirmed the similarity between HBL and the host amidase since 16 of 21 residues analyzed were identical and, particularly, no changes were found from Leu-12 to Val-23 in both proteins. The remarkable similarities between HBL and the pneumococcal amidase suggested that the genes coding for these enzymes were also homologous. DNA-DNA hybridization between the *lytA* gene, coding for the host amidase, and the HB-3 bacteriophage genome gave additional support to this hypothesis. Southern blot experiments showed that the *lytA* gene hybridized with several restriction fragments obtained from HB-3 DNA (Fig. 4). Single hybrid-

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Host amidase:   M E I N V S K L R T D L P Q V G V Q P Y R
                : . : . . : : : . : : : : : : : : : :
HBL        :    M D I D R N R L R T G L P Q V G V Q P Y R
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FIG. 3. Amino acid sequences of the N termini of the host amidase (8) and of HBL. Purified HBL (250 pmol) was used for the analyses. Identical amino acids are represented by double dots (:) and conservative ones by a single dot (·).

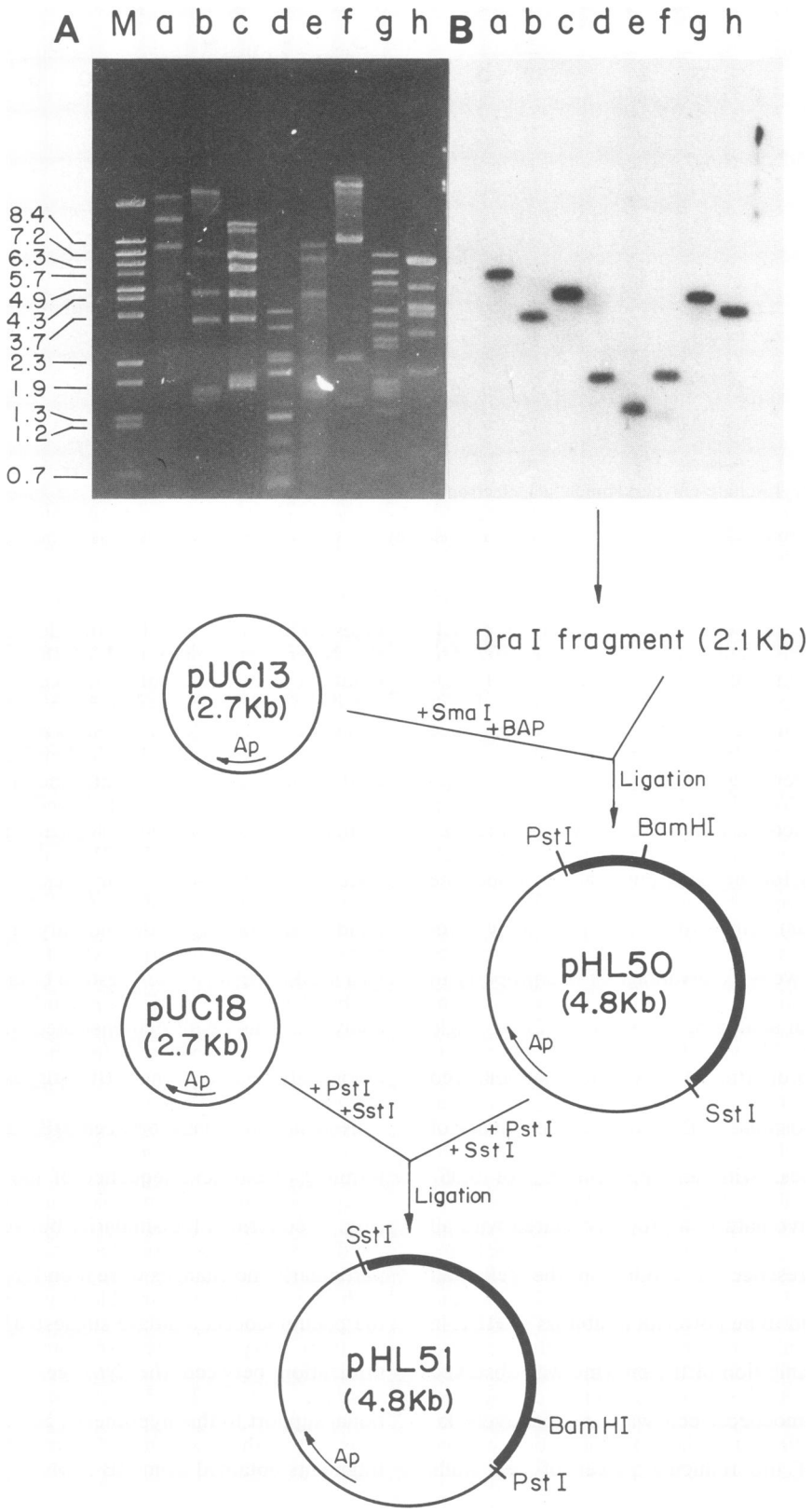

FIG. 4. Construction of plasmids containing the *hbl* gene. The ethidium bromide-stained agarose gel (A) shows the restriction pattern obtained from the digestion of HB-3 DNA with different restriction enzymes and the corresponding Southern blot analysis with the *lytA* fragment as a probe, performed at 60°C (B). Lanes: a, *Acc*I; b, *Bcl*I; c, *Bgl*II; d, *Dra*I; e, *Eco*RV; f, *Nco*I; g, *Pvu*II; h, *Cla*I; M, molecular size markers (in kilobases) from *Bst*EII-digested λ DNA are also shown on the left. Plasmids are drawn as circles with the relevant elements and restriction sites indicated. The sizes of the recombinant plasmids are given in kilobases (Kb). Heavy lines correspond to the phage DNA inserts. Ap, Ampicillin; BAP, bacterial alkaline phosphatase.

TABLE 2. Pneumococcal cell wall hydrolysis by extracts obtained from different *E. coli* strains[a]

Strain	Condition[b]	Substrate[c]	Activity (U/mg of protein)
JM83(pUC18)	No preincubation	C-cw	<10
JM83(pHL50)	No preincubation	C-cw	1,150
JM83(pHL50)	Preincubation	C-cw	4,300
JM83(pHL50)	Preincubation	EA-cw	<10
JM83(pHL51)	No preincubation	C-cw	3,050
JM83(pHL51)	Preincubation	C-cw	10,700
JM83(pHL51)	Preincubation	EA-cw	<10

[a] The cultures were grown in LB medium supplemented with isopropyl-β-D-thiogalactopyranoside (1 mM) and ampicillin (100 μg/ml) to the mid-stationary phase. Lysates were obtained by sonication as previously described (7).

[b] Incubation was at 37°C for 10 min, with or without preincubation at 4°C for 5 min.

[c] C-cw, Choline-containing cell walls; EA-cw, ethanolamine-containing cell walls.

ization bands appeared with *Dra*I, *Pvu*II, *Acc*I, *Bcl*I, *Bgl*II, and *Cla*I, and two bands appeared with *Nco*I. These results strongly suggested the presence of a lytic gene coding for the HBL enzyme in the genome of HB-3.

Cloning and expression of gene coding for HBL in *E. coli*. HB-3 DNA was digested with *Dra*I and electrophoresed on agarose, and the 2.1-kilobase fragment that hybridizes with the *lytA* gene was excised from the gel, purified as described elsewhere (27), and ligated to dephosphorylated, *Sma*I-cut pUC13. The ligation mixture was used to transform *E. coli* JM83 to ampicillin resistance, and a transformant harboring the recombinant plasmid pHL50 containing the 2.1-kilobase *Dra*I fragment of HB-3 DNA was isolated (Fig. 4). When crude sonicated extracts obtained from *E. coli* JM83(pHL50) were tested for enzymatic activity, a lytic activity that degraded choline-containing pneumococcal cell walls but not ethanolamine-containing ones was found (Table 2). These results confirmed the absolute requirement for activity of the phage-encoded enzyme for the presence of choline in the teichoic acids of the cell walls as already demonstrated for HBL purified from pneumococci. Furthermore, HBL synthesized in *E. coli* required a brief period of incubation at low temperature (5 min at 4°C) in the presence of choline to achieve full activity, a property that had been reported to be exclusive of the host amidase (25). These results indicated that a low-specific-activity form of HBL is the primary translation product of the *hbl* gene of phage HB-3. To investigate whether the *hbl* gene is expressed utilizing its own promoter, we constructed pHL51 with the same insert as pHL50 but in the opposite direction (Table 2). Similarly, crude extract from *E. coli* JM83 harboring pHL51 also maintained full enzymatic activity, suggesting that the *hbl* gene is expressed from its own promoter. To further ascertain that the enzyme expressed in *E. coli* was identical to that purified from pneumococci (see above), we did a Western blot analysis using purified proteins and crude extracts obtained from *E. coli* JM83(pHL50) and *E. coli*(pHL51). A single band with the same mobility as that of the purified HBL appeared in the lanes corresponding to extracts obtained from *E. coli* JM83 carrying pHL50 or pHL51, whereas no band was detected in crude extracts prepared from JM83(pUC18) (Fig. 5).

A modular organization for the lytic enzymes of *S. pneumoniae* and its bacteriophages has been previously proposed on the basis of the comparative analysis of the primary

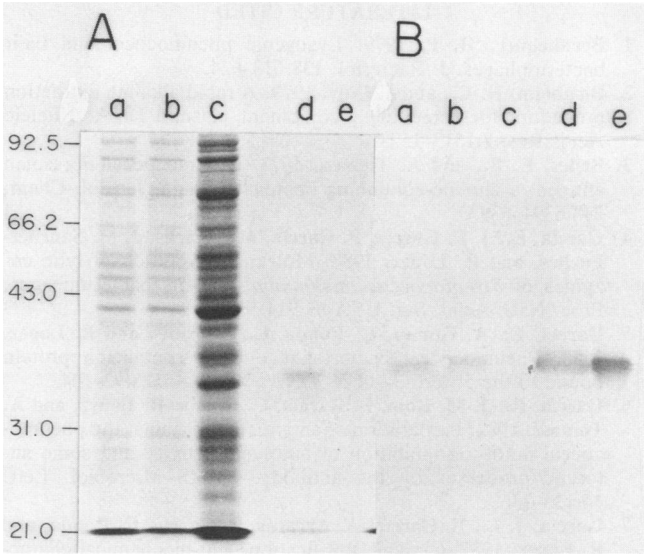

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (A) Stained with Coomassie blue. (B) Immunoblot analysis. Lanes: a, Crude extract from *E. coli* JM83(pHL51); b, crude extract from *E. coli* JM83(pHL50); c, crude extract from JM83(pUC18); d, purified HBL; e, purified host amidase. The antiserum was raised by the host amidase. Numbers on left show molecular size in kilodaltons.

structures of the host amidase and the phage muramidases (4). The presence of homologous sequences between phages and their host have been suggested to promote site-specific recombination (23), and consequently, they should serve as potential tools for evolution of the bacterial as well as phage genomes; e.g., the presence of this gene in HB-3 should allow the successful infection of amidase-deficient strains of pneumococcus, although a correct evaluation of the occurrence of these nonlytic strains in nature is still lacking. We report here, for the first time, the construction of plasmids containing a phage gene coding for an amidase that has been cloned and expressed in a heterologous system, apparently utilizing its own promoter. This experimental approach should facilitate the synthesis of this enzyme in high amounts, providing new and valuable information on the molecular characteristics of the phage-encoded amidase. Our results strongly suggest the existence of extensive DNA homologies between the *lytA* and *hbl* genes, and the biochemical and immunological analyses described here revealed that both amidases are similar but not identical. The availability of a cloned phage gene coding for an amidase that shares many properties with the host amidase might be of general interest to provide new insights on the modular organization of the wall lytic enzymes of pneumococcus and its phages and would be of great help to achieve a better understanding of the peculiar virus-host relationship.

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