PlyC: A multimeric bacteriophage lysin

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Lysins are murein hydrolases produced by bacteriophage that act on the bacterial host cell wall to release progeny phage. When added extrinsically in their purified form, these enzymes produce total lysis of susceptible Gram-positive bacteria within seconds, suggesting a unique antimicrobial strategy. All known Gram-positive lysins are produced as a single polypeptide containing a catalytic activity domain, which cleaves one of the four major peptidoglycan bonds, and a cell-wall-binding domain, which may bind a species-specific carbohydrate epitope in the cell wall. Here, we have cloned and expressed a unique lysin from the streptococcal bacteriophage C_1, termed PlyC. Molecular characterization of the *plyC* operon reveals that PlyC is, surprisingly, composed of two separate gene products, PlyCA and PlyCB. Based on biochemical and biophysical studies, the catalytically active PlyC holoenzyme is composed of eight PlyCB subunits for each PlyCA. Inhibitor studies predicted the presence of an active-site cysteine, and bioinformatic analysis revealed a cysteine, histidine-dependent amidohydrolase/peptidase domain within PlyCA. Point mutagenesis confirmed that PlyCA is responsible for the observed catalytic activity, and Cys-333 and His-420 are the active-site residues. PlyCB was found to self-assemble into an octamer, and this complex alone was able to direct streptococcal cell-wall-specific binding. Similar to no other proteins in sequence databases, PlyC defines a previously uncharacterized structural family of cell-wall hydrolases.

multimeric protein | cell-wall hydrolase | *Streptococcus*

Bacteriophage cell-wall hydrolases, or lysins, have recently been exploited for their bacteriolytic activity as an alternative to antibiotic therapy (1, 2). During a bacteriophage (or phage) infection cycle within a host organism, phage-directed proteins, called holins, are produced to perforate the bacterial membrane, allowing the accumulating cytoplasmic lysins access to the cell wall (3). The released lysins cleave covalent bonds in the peptidoglycan, resulting in lysis of the bacterial cell and liberation of progeny phage. Appreciably, exogenous addition of purified lysins to susceptible Gram-positive bacteria also produces complete lysis in the absence of bacteriophage (4, 5).

All Gram-positive cell-wall-hydrolyzing lysin family members described thus far are composed of a single polypeptide that has a modular design consisting of a well conserved catalytic domain and a cell-wall-binding domain. The catalytic domain is represented by one of four families of peptidoglycan hydrolases: *N*-acetylglucosaminidases, *N*-acetylmuramidases (lysozymes), *N*-acetylmuramoyl-L-alanine amidases, and endopeptidases (6). In contrast, the cell-wall-binding domains are notably divergent and can distinguish discrete epitopes present within the cell wall, typically carbohydrates or teichoic acids, giving rise to the species- or strain-specific activity of a particular lysin. Accordingly, it is possible to combine the catalytic domain of one lysin and the cell-wall-binding domain of a second lysin to make a chimeric protein with altered specificity or activity (7).

The streptococcal C_1 bacteriophage lysin, now called PlyC for "phage lysin from C_1," was first described in 1957, when C_1 phage lysates were found to rapidly lyse cultures of groups A and C streptococci, despite the fact that the C_1 phage does not infect group A streptococci (8). Consequently, this enzyme has been used as a molecular tool for decades to isolate cell-wall-linked proteins and extract DNA from group A streptococci (9, 10). More recently, we have shown that the bacteriolytic properties of PlyC can protect mice from streptococcal challenge, suggesting a therapeutic use of the enzyme (11). Significantly, PlyC is the most potent bacteriophage-derived enzyme studied to date. Whereas microgram or milligram quantities of most phage lysins can effect a multiple-log drop of target bacteria within minutes, PlyC requires only nanogram quantities. For instance, we have shown that 10 ng of PlyC is sufficient to sterilize 10^7 group A streptococci seconds after contact (11). Despite 50 years of work with PlyC, little else is known about the enzyme itself. Given the potential therapeutic implications of this powerful enzyme, we present here the cloning, elucidation of the catalytic and cell-wall-binding domains, and a proposed structural model, based on biochemical and biophysical characterization of PlyC.

Results and Discussion

PlyC Consists of a Heavy Chain and a Light Chain. PlyC purified from C_1 bacteriophage lysates behaves as a homogeneous protein on native-gel electrophoresis (Fig. 1*A*). Moreover, this protein band is responsible for the lytic activity, as observed by formation of a clearing zone on an overlay of streptococci-embedded agarose (Fig. 1*B*). However, SDS/PAGE analysis of the same material on a 4–20% gradient gel revealed the presence of two bands (Fig. 1*C*), an ≈50-kDa heavy chain, termed PlyCA, and an ≈8-kDa light chain, termed PlyCB, neither of which displayed lytic activity on overlay (data not shown). N-terminal sequencing of the PlyC heavy and light chains resulted in two unique sequences (SKKYTQQQYE and SKINVNVENV, respectively), and sequencing of the native band resulted in a dual sequence, which corresponds exactly to both chains.

Cloning and Sequencing the PlyC Gene(s). To identify the gene(s) responsible for PlyC activity, we partially digested the C_1 bacteriophage genome with Tsp509I and ligated the fragments into a pBAD24 vector. Screening the resulting *Escherichia coli* expression library revealed a single clone (pBAD24::*plyC*) that encoded lytic activity toward group A streptococci. The purified, recombinant PlyC had identical properties on column chromatography and native and SDS/PAGE as compared with PlyC purified from phage lysates (data not shown). The *plyC* clone contained a 2.2-kb insert comprising three putative ORFs in addition to ≈100 bp of a noncoding sequence flanking the 5′ and 3′ ends of the insert (Fig. 2*A*). These genes correspond to ORFs 9, 10, and 11 of the recently sequenced C_1 phage genome (12).

The first gene of the 2.2-kb insert, hereafter referred to as *plyCB*, encodes a 72-aa polypeptide that matches the N-terminal sequence of PlyCB. The 7.858-kDa predicted mass of the *plyCB*

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Abbreviations: CHAP, cysteine, histidine-dependent amidohydrolase/peptidase; LS, light-scattering; RI, refractive index; SEC, size-exclusion chromatography.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY212251).

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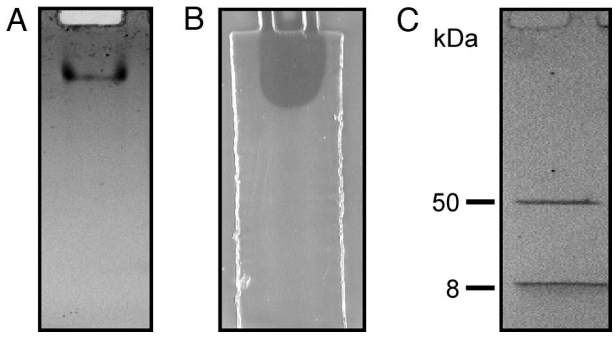

Fig. 1. Elucidation of subunits for PlyC. (*A*) Native PAGE of purified PlyC shows a single, homogeneous band. (*B*) An unstained native PAGE identical to *A* was placed on agarose that had been embedded with group A streptococci and was allowed to incubate for 2 h. The clearing zone on the agarose indicated that the PlyC activity corresponds to the single band on the native PAGE. (*C*) An SDS/PAGE of the purified PlyC used in *A* demonstrates that PlyC is composed of at least two subunits. The 50-kDa heavy chain is designated PlyCA, and the 8-kDa light chain is designated PlyCB. N-terminal sequencing of the native PAGE gave a double sequence, which corresponded to the two chains sequenced in the SDS/PAGE (see *Results and Discussion* for details).

gene product approximates the observed size of PlyCB by SDS/PAGE (Fig. 1*C*). A position-specific iterative (PSI)-BLAST search revealed no significant matches for this protein in any database.

The second gene of the *plyC* clone encodes a putative 105-aa protein, which had significant homology (*E* value better than threshold) to 31 endonucleases, most of them belonging to the "HNH" endonuclease family, such as those for *Streptococcus agalactiae* prophage λSa2 (ANN00738), *Lactococcus* phage bIL170 (AAC27227), and Vibriophage VpV262 (AAM28379). HNH endonucleases are known to embed themselves within group I introns and confer mobility to the host intervening

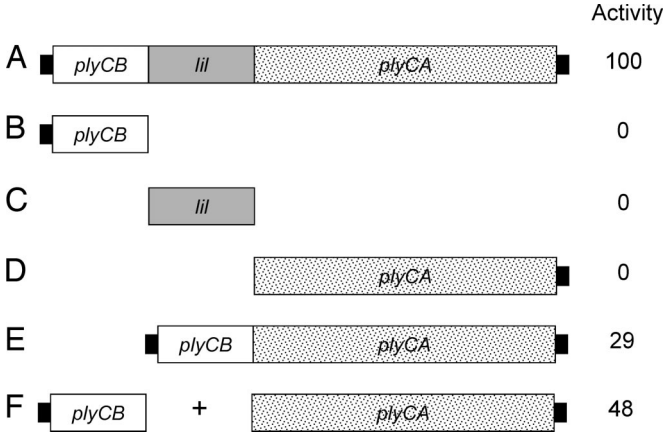

Fig. 2. Gene structure and analysis of the *plyC* gene(s). (*A*) A *plyC* clone displaying PlyC activity was found to contain 2.2 kb of sequence comprising three ORFs and ≈100 bp of untranslated region on both the 5′ and 3′ ends. ORF1 contains the sequence for the PlyC light-chain, PlyCB and is called *plyCB*. ORF2 is positioned between the genes for the two chains of PlyC and, hence, is called *lil*, for lysin intergenic locus. ORF3 contains the sequence for the PlyC heavy-chain, PlyCA and is called *plyCA*. See *Materials and Methods* for details about the activity measurement. (*B*) Expression of *plyCB* by itself contained no activity. (*C*) Expression of *lil* by itself contained no activity. (*D*) Expression of *plyCA* by itself yielded no activity. (*E*) Expression of a Δ*lil* construct, which contained full-length *plyCB* and *plyCA*, possessed attenuated lysin activity compared with the *plyC* parental clone in *A*. (*F*) A double transformant, containing *plyCB* in pBAD33 (Cm^+) and *plyCA* in pBAD24 (Amp^+), yielded half the lysin activity of the *plyC* parental clone.

sequence (13). Because of the unique position of the putative endonuclease gene between *plyCB* and the third ORF, we chose to call this region *lil*, for lysin intergenic locus.

The third gene of the 2.2-kb insert, hereafter referred to as *plyCA*, encodes a 465-aa polypeptide with a predicted size of 50.333 kDa, matching both the size and N-terminal sequence of PlyCA. A PSI-BLAST search indicated moderate homology to putative minor structural proteins from the *Streptococcus thermophilus* phage Sfi11 (AAC34413) and *S. agalactiae* prophage λSA03 (ABA46334) as well as a putative tail protein of the *Streptococcus pyogenes* prophage 315.5 (AAM79918). Although the PSI-BLAST search did not detect strong homology to known phage endolysins, it should be noted that phage tail proteins often contain lytic domains. For example, the T4 phage tail protein gp5 contains a lysozyme domain (14).

Analysis of the *plyC* Operon. To determine the minimal region necessary for lytic activity, several *plyC* derivatives were constructed and evaluated for their ability to lyse group A streptococci. Individually expressed, none of the genes was sufficient for activity (Fig. 2 *B*, *C*, and *D*). A Δ*lil* expression construct containing full-length *plyCB* and *plyCA* did, however, encode activity (Fig. 2*E*), albeit less than a third that of the full-length clone. As an alternate to the Δ*lil* construct, we created an *E. coli* clone containing *plyC* genes on separate plasmids [pBAD33::*plyCB* (Cm^r) and pBAD24::*plyCA* (Amp^r)]. When induced, an active enzyme was produced (Fig. 2*F*). All truncations of *plyCB* or *plyCA* ablated enzymatic activity (data not shown).

Although the above data suggest that *lil* is not necessary for lytic activity, we felt that this region warranted further investigation because of its position between *plyCB* and *plyCA* and its homology to known homing HNH endonucleases associated with introns. A group I intron is known to interrupt the lysin gene of the staphylococcal phage K (15), and active introns have been observed in half of 62 tested *S. thermophilus* bacteriophage lysins (16). However, RT-PCR and Northern blot analysis both indicate that intron splicing does not take place in the *plyC* operon (data not shown). At present, the function of *lil*, if any, remains unknown.

A Model for the Proposed Structure of PlyC. In 1971, it was suggested that the mass of the C_1 streptococcal lysin (PlyC) was 101–105 kDa, based on gel-filtration and sedimentation analysis of a purified preparation (17), which was confirmed in 2001 with a highly pure preparation (11). To further corroborate the mass of native PlyC, we used a noncleavable cross-linker, bis(sulfosuccinimidyl) suberate (BS^3), which reacts with primary amines and the ε amine of lysine. SDS/PAGE analysis on a 4–20% gradient gel of the cross-linked PlyC indicated that the holoenzyme enzyme is ≈120 kDa (Fig. 3). Considering the genetic and biochemical data of a 50-kDa heavy chain and an 8-kDa light chain, a simple 1:1 heterodimer model of the two chains does not rationalize the apparent 100–120 kDa mass of the native enzyme. However, none of these analytical methods had the precision needed to determine exact stoichiometric ratios of PlyCA and PlyCB.

Ultimately, we determined the exact mass for the PlyC holoenzyme by size-exclusion chromatography (SEC) coupled with in-line laser light-scattering (LS), UV, and refractive index (RI) measurements. The amount of light scattered is directly proportional to the product of the weight-average molar mass and the concentration of the macromolecule, which is measured by RI. Furthermore, LS provides the absolute molecular mass of a protein in solution and does not depend on the Stokes radius, which causes variability in results from traditional gel-filtration analysis. As seen in Fig. 4, the PlyC holoenzyme eluted as a monodispersed peak at 13.7 ml. The data from 20 independent

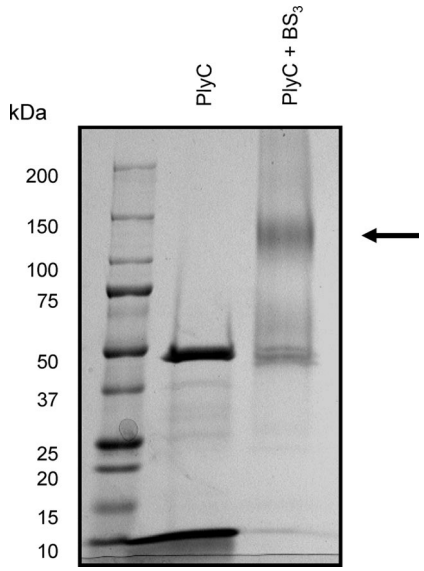

Fig. 3. Cross-linking of PlyC. SDS/PAGE of PlyC shows the presence of the 50-kDa PlyCA and the 8-kDa PlyCB subunits. However, SDS/PAGE of PlyC that had been pretreated with the noncleavable cross-linker BS^3 showed a band at ≈120 kDa (arrow). See *Materials and Methods* for details.

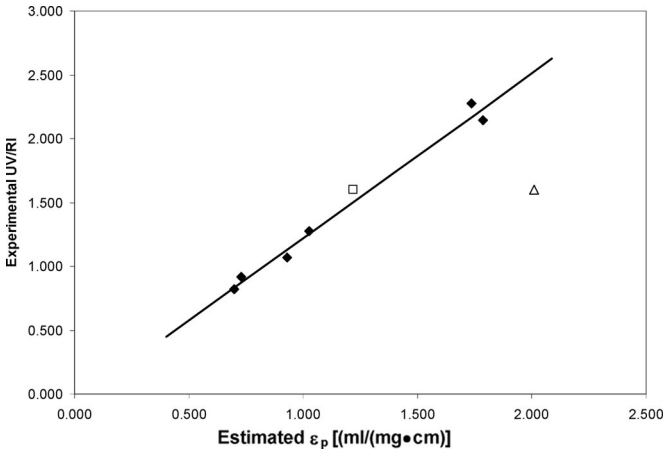

Fig. 5. Extinction-coefficient analysis of two models of PlyC. Polypeptide-extinction coefficients, ε_p, were estimated for six standard proteins and two theoretical models of PlyC (8 PlyCB:1PlyCA and 2 PlyCB:2 PlyCA), based on the number of tryptophans, tyrosines, and cysteines, and plotted against the experimentally determined UV/RI ratios for all proteins (see Table 1). Linear regression analysis was performed on standard proteins ($R^2 = 0.9878$). ◆,standard proteins; □, 8 PlyCB:1 PlyCA model; △, 2 PlyCB:2 PlyCA model.

injections at various concentrations (100–300 μg) confirms that the PlyC holoenzyme is 114.0 kDa with a standard deviation of ± 0.4 kDa (Table 2, which is published as supporting information on the PNAS web site). Based the known masses of 50.366 kDa for PlyCA and 7.858 kDa for PlyCB, the SEC-LS/UV/RI data predicts 8.0 ± 0.005 PlyCB subunits for each PlyCA (Table 2). No other stoichiometric ratio of PlyCB and PlyCA falls within the standard deviation of the experimentally determined 114.0-kDa holoenzyme. Nonetheless, we evaluated a 2 PlyCB/2 PlyCA model, which, at 116.5 kDa, was the ratio that gave the closest mass outside of the standard deviation. To differentiate between the 8:1 and 2:2 stoichiometric models, we used signals from the UV and RI detectors to calculate the (UV)/(RI) ratio, which is directly proportional to the polypeptide-extinction coefficient (ε_p) at 280 nm of a 1 mg/ml solution at a 1-cm path length. Because PlyCA contains 13 tryptophan, 28 tyrosine, and 7 cysteine residues, and PlyCB contains only 2 tyrosine residues, the two models would differ significantly in their ε_p at 280 nm. Thus, a plot of estimated ε_p vs. experimentally determined (UV)/(RI) for standard proteins will yield a linear relationship (Fig. 5). Any significant deviation from the line indicates that the experimentally determined and theoretically determined extinction coefficients do not match. As can be seen in Fig. 5 and Table 1, the stoichiometric assumption of the 8:1 model does fit the linear regression of the standard proteins, but the 2:2 assumption does not.

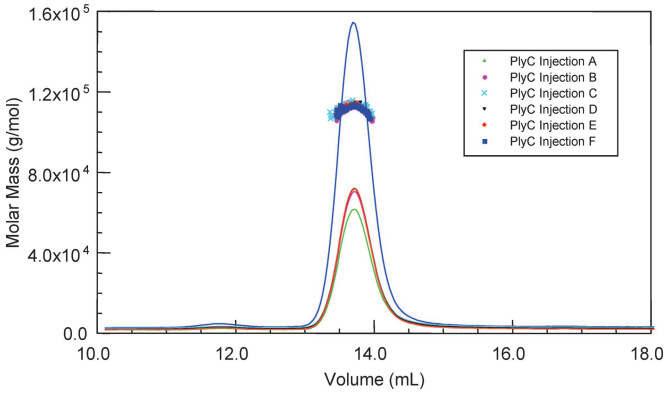

Fig. 4. Determination of PlyC molecular mass. Purified PlyC was applied to an S-200 SEC column, and the eluant was analyzed by UV, RI, and multiangle LS detectors. The solid curves correspond to the RI signal, and the points represent the molecular mass of the eluting species at each second derived from the RI and LS measurements. This figure shows six independent injections at multiple concentrations (100–300 μg); however, a total of 20 independent injections were used for mass determination (see Table 2).

PlyCA Contains a Functional Cysteine, Histidine-Dependent Amidohydrolase/Peptidase (CHAP) Domain. As stated above, neither PlyCA nor PlyCB showed homology to lysin or lysin-like proteins in BLAST or PSI-BLAST searches. However, screening the Pfam database of conserved protein domains (www.sanger.ac.uk/Software/Pfam) revealed the presence of a CHAP domain within PlyCA. The recently described CHAP domain is common in many amidase enzymes, including some peptidoglycan hydrolases (18–20). Significantly, PlyC has been shown to have amidase activity, because it is able to cleave the amide bond between *N*-acetyl muramic acid and L-alanine in the streptococcal peptidoglycan (21). To test for an active-site cysteine, we used several thiol-reactive agents. An oxidizing compound, dithiodipyridine, and two sulfhydryl alkylating reagents, ethylmaleimide and iodoacetamide, significantly diminished PlyC enzymatic activity (Fig. 6*A*). When an excess of reducing agent was added (10 mM DTT), activity was partially restored to the oxidized PlyC but not the alkylated PlyC. As a result, an active-site cysteine residue is implicated.

Alignment of PlyCA against known members of the CHAP family indicates that Cys-333 and His-420 likely constitute the active-site residues (data not shown). We therefore altered these residues by site-directed mutagenesis: Cys-333 was replaced with a serine residue (C333S) and His-420 with an alanine (H420A). As a control, we made point mutations changing three non-active-site cysteine residues (C268S, C345S, and C404S). As seen in Fig. 6*B*, both C333S and H420A mutants completely ablated lytic activity, whereas non-active-site mutations retained near-wild-type activity, confirming the presence of a CHAP catalytic domain in PlyCA.

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Table 1. Extinction-coefficient analysis for two stoichiometric models of PlyC

Protein	ε_p, ml/(mg·cm)	Experimental UV/RI	Computed UV/RI	Residual^2	Correct assumption?
Apo-ferritin	1.026	1.279	1.271	0.000	
β-Amylase	1.788	2.147	2.215	0.005	
BSA	0.700	0.821	0.867	0.002	
Carbonic anhydrase	1.737	2.273	2.152	0.015	
Ovalbumin	0.730	0.919	0.904	0.000	
Trypsin inhibitor	0.928	1.070	1.150	0.006	
PlyC (8:1 model)	1.218	1.600	1.509	0.008	Yes
PlyC (2:2 model)	2.011	1.600	2.491	0.794	No

PlyCB Contains the Binding Domain. We showed above that PlyCA contained the catalytic active site; however, PlyCA alone is not sufficient for activity (Fig. 2*D*). Therefore, we wanted to determine whether PlyCB directed the binding of PlyC to the target bacteria. Significantly, we noticed individually expressed PlyCB eluted from gel filtration at ≈60 kDa, suggesting it self-assembled into an octamer. When gel filtration was repeated with 0.1% SDS, PlyCB eluted at ≈8 kDa, the mass of the monomer. Therefore, we used Alexa Fluor, a small organic dye, to label purified PlyC, PlyC-C333S, its inactivated mutant, and the self-assembled octamer of PlyCB. Labeled PlyC not only retained binding properties, but it also retained full catalytic activity, which lysed target bacteria before they could be mounted on a microscope (data not shown). Consequently, further experiments used either PlyCB or PlyC-C333S. PlyCB and PlyC-C333S specifically labeled the target bacteria (*S. pyogenes*), but not other bacterial species (*S. agalactiae*, *Streptococcus mutans*, and *Staphylococcus aureus*) (Fig. 7), indicating that the PlyCB octamer contains the cell-wall-binding domain for PlyC.

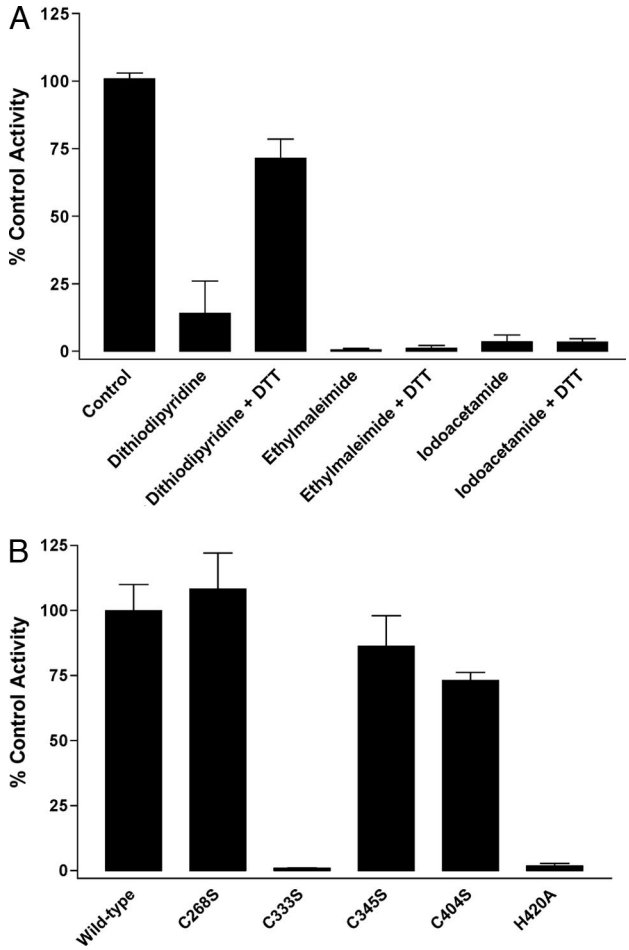

Fig. 6. Active-site determination. (*A*) PlyC (1,000 units) was incubated with PBS or inhibitors at 1 mM final concentration for 15 min before a standard lytic assay (see *Materials and Methods*). Alternatively, PlyC was incubated with an excess (10 mM) of DTT for 15 min after inhibition to assess reversibility of inhibitors. (*B*) Putative active-site mutants of PlyCA (C333S and H420) displayed no lytic activity. Conversely, non-active-site mutants (C268S, C345S, and C404S) showed near-wild-type activity.

Conclusions

All previously reported lysins derived from Gram-positive-infecting bacteriophage are composed of one or more (22) catalytic domains linked to a binding domain and are synthesized as single polypeptides. Thus, PlyC is an unusual example of a multimeric lysin composed of two distinct gene products, PlyCA and PlyCB. Based on biochemical, biophysical, and genetic data, we propose that the PlyC holoenzyme is composed of eight PlyCB subunits for each PlyCA, which further illustrates the distinctiveness of this enzyme. Additionally, we have provided evidence that PlyCA is responsible for the hydrolytic amidase activity, and PlyCB contains the cell-wall-binding domain. It is not clear how the PlyCB octamer binds to its receptor on target cells, nor is the exact identity of the receptor known. Moreover, it is unknown whether the catalytic PlyCA dissociates from PlyCB during cleavage of substrate peptidoglycan in the cell walls. However, because this enzyme is >200 times more active than other lytic enzymes, this enhanced activity may be linked to its unique structure. Although crystallographic studies and identification of additional multimeric lysins may help establish a previously uncharacterized structural class of cell-wall hydrolases, at present, PlyC remains the only known multimeric lysin.

Materials and Methods

Unless otherwise stated, all reagents were obtained from Sigma and were of the highest purity available.

Bacterial Strains, Phage, and Growth Conditions. *S. pyogenes* D471 (group A streptococcus), *Streptococcus equisimilis* 26RP66 (group C streptococcus), and the C_1 bacteriophage are part of The Rockefeller University collection and were grown and maintained as described (11, 12). *E. coli* XL-1 blue (Stratagene) was grown in Luria–Bertani broth at 37°C in a shaking incubator (250 rpm), unless otherwise stated. When needed, ampicillin (100 $\mu g \cdot ml^{-1}$) or chloramphenicol (34 $\mu g \cdot ml^{-1}$) was added to the growth medium. See Table 3, which is published as supporting information on the PNAS web site, for references to strains and plasmid constructs.

DNA Manipulation. Phage DNA was isolated as described (12), and plasmid DNA was isolated by using the QIAprep kit (Qiagen,

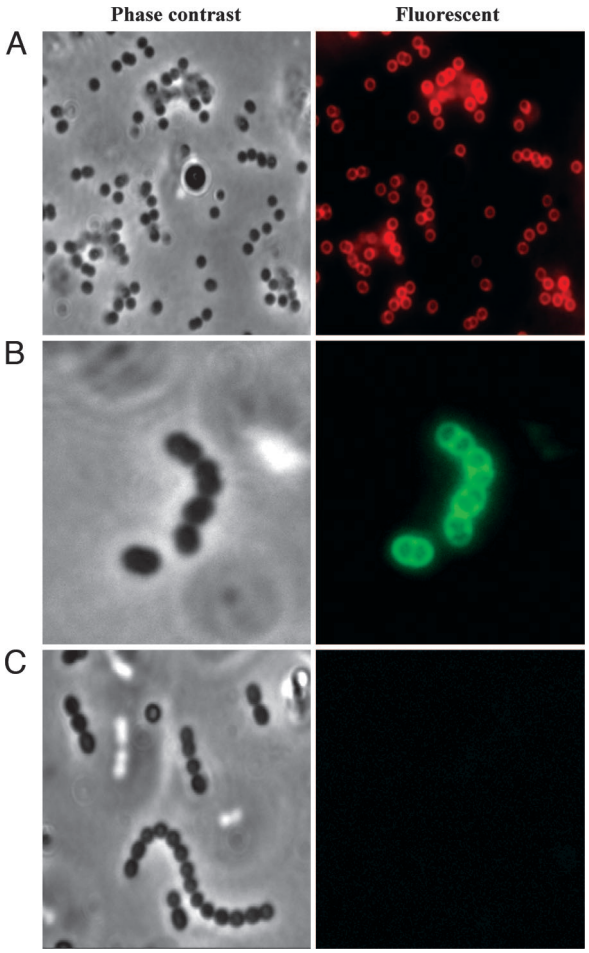

Fig. 7. PlyCB contains the cell-wall-binding domain. Fifty micrograms of labeled protein was added to 1 ml of various bacterial cells, washed two times, and viewed under both phase-contrast and fluorescent light sources. (*A*) PlyC-C333S cross-linked with Alexa Fluor 568 specifically labels *S. pyogenes*. (*B*) PlyCB labeled with Alexa Fluor 488 specifically labels *S. pyogenes;* thus PlyCB directs cell-wall-binding for PlyC. Note that staining is heaviest near the septal poles. (*C*) Bacterial species insensitive to PlyC [*S. agalactiae* (shown), *S. mutans*, and *S. aureus* (not shown)] are resistant to labeling by PlyCB-Alexa Fluor 488. All panels were viewed through a ×100 magnification oil-immersion objective lens with an additional ×2 magnifier (×200 total). Additionally, *B* was digitally zoomed ×2, for a final magnification of ×400.

Valencia, CA). DNA polymerase, restriction, and modification enzymes were all purchased from New England Biolabs and used according to the manufacturer's instructions. Oligonucleotides were obtained from Sigma-Genosys, and all PCR was performed with the Eppendorf Mastercycler.

Cloning *plyC*. A Tsp509I expression library of the C_1 bacteriophage genome was cloned into pBAD24 (23) and screened for lytic activity against group A streptococci as described (4). Plasmid DNA was prepared and sequenced at The Rockefeller University DNA Sequencing Resource Center. DNA sequence analysis and manipulations required the BLAST [National Center for Biotechnology Information (NCBI)], ORF FINDER (NCBI), and SEQMAN 5.0 (DNASTAR, Madison, WI) programs. The full-length sequence of the C_1 bacteriophage has been deposited in GenBank (accession no. AY212251) as described (11). This accession has been updated to reflect information contained in this manuscript about the C_1 bacteriophage lysin genes.

Biochemical Techniques. PlyC was induced with 0.5% arabinose overnight from XL1-blue/pBAD24::*plyC* cultures. Cells were washed in 20 mM phosphate buffer at pH 7.0 and lysed with 20% wt/vol chloroform to yield crude PlyC. Alternatively, group C streptococci were infected with the C_1 bacteriophage as previously described (11), and phage-produced PlyC was isolated from the crude lysate. Purification of the phage-derived enzyme and the recombinant enzyme, the plate assay to follow activity, and the unit definition were identical to previously described methods (11). Purified enzymes were routinely stored in PBS at 4°C and were stable for several months. Purification of the individually expressed heavy and light chains used the same column-chromatography methods as the native enzyme, but purification was followed by SDS/PAGE rather than activity assays. Native and SDS/PAGE analyses were performed according to the method of Schagger and von Jagow (24), and blotting to poly(vinylidene difluoride) membranes was according to Matsudaira (25). N-terminal sequencing was performed at The Rockefeller University Proteomics Resource Center. For inhibitor studies, PlyC (1,000 units) was incubated with inhibitors at 1 mM final concentration for 15 min before assay. Alternately, an excess of DTT (10 mM) was added for an additional 15-min incubation to observe reversibility of inhibitors. In the cross-linking experiments, we used bis(sulfosuccinimidyl) suberate (BS^3; Pierce), a water-soluble, noncleavable, homobifunctional cross-linker with a chain length of 11.4 Å and reactivity toward amino groups. A fresh stock of BS^3 (1 mg·ml^{-1}) was made in 20 mM phosphate buffer, pH 7.4, and 55 μl of this solution (5 μM final concentration) was added to 100 μg of purified PlyC in a final volume of 1 ml and allowed to react for 45 min at room temperature. The reaction was quenched by the addition of 25 mM Tris for 15 min at room temperature and analyzed by SDS/PAGE in reduced sample buffer. Alternately, PlyCB, native PlyC, and cross-linked PlyC samples were subjected to analytical gel filtration for size estimation on a Superose 12 column (Amersham Pharmacia Biosciences) calibrated with gel-filtration standards (Bio-Rad).

Expression of Individual ORFs, Deletions, and Cotransformants. Plasmid DNA was purified from XL1-blue/pBAD24::*plyC*, and individual ORFs were amplified by PCR as follows: for the light chain (*plyCB*), the primers *Start* (5′-GTA CCC GGG GAA GTA ATT TCC ATT CTT GAA-3′) and *Light-R* (5′-CCC AAG CTT TTA CTT TTT CAT AGC CTT TCT-3′); for the intergenic region (*lil*), the primers *LIL-F* (5′-GTA CCG GGG AGG AGG AAT TCA TGA TTG AGG AGT GGG TC-3′) and *LIL-R* (5′-GGG AAG CTT TTA CTC ATT AAA TAA ATT CTC CCT TTC-3′); and for the heavy chain (*plyCA*), the primers *Heavy-F* (5′-GTA CCC GGG AAA GGG AGA ATT TAT TTA ATG-3′) and *End* (5′-CCC AAG CTT TGG GTT CAA TTC AAG GGA ATA-3′). All forward primers contained a SmaI site, and all reverse primers contained a HindIII site and a stop codon. PCR products were digested by SmaI and HindIII and ligated into a SmaI/HindIII-digested pBAD24 (Amp^r) vector or a pBAD33 (Cm^r) vector. For the Δ*lil* construct, *plyCB* was PCR amplified by *Start* and *Light-R* primers, blunt ended, and ligated to pBAD24::*plyCA* that had been linearized by digestion with SmaI. A 1.6-kb PCR product using the primers *Start* and *End* confirmed the proper insert size for this deletion construct. Finally, XL1-blue cells were cotransformed with pBAD24::*plyCA* and pBAD33::*plyCB*, and cells displaying a Cm^r/Amp^r phenotype were selected for further study. Positive PCR products were obtained by using either primer set *Start*/*Light-R* or *Heavy-F*/*End*, but no product was observed by using *Start*/*End*, thus verifying that *plyCB* and *plyCA* were on separate plasmids. All constructs were induced by 0.5% arabinose for 4 h.

For quantification of PlyC activity, each construct was simultaneously grown and induced under identical conditions, and the cells were washed in PBS and adjusted to OD_{660} = 1.0, lysed in an equal volume with chloroform, and titered for activity on

group A streptococcal cells as described (11). The activity reported represents the average of triplicate data, normalized to 100% for the *plyC* parental clone. Additionally, SDS/PAGE gels were run on the crude extracts for all clones and mutants (see below) to establish that background expression profiles for each clone were equal, and any activity/inactivity was not due to different levels of protein expression between the constructs.

Site-Directed Mutagenesis. Specific mutations to *plyCA* within the *plyC* operon were made by using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The following forward primers were used: *C268S*, 5′-CGG CAC TAT CCT GAA ATC TGA TTT AAC AGA TGA CGG-3′; *C333S*, 5′-GGT AAG TAT ATT GGT GAC GGT CAA TCT TAT GCT TGG GTT GGT TGG-3′; *C345S*, 5′-GGT CAG CTA GGG TAT CTG GTT ATT CTA TTT CAT ACT CAA CAG G-3′; *C404S*, 5′-GGC GCG ATA TGG TCT GCT ACA GCA TTC TCT GGC-3′; and *H420A*, 5′-CGT TTT ATA CAG GAC AAT ACG GCG CTA CTG GTA TCA TTG AAA GC-3′. The reverse primers were the exact complements. The plasmid inserts were digested out of the resulting clones and recloned into pBAD24, and the full-length inserts were sequenced to confirm that only the desired nucleotide sequence changes had been introduced. Expression of point mutants and wild-type PlyC were carried out in 0.5% arabinose, as described above.

SEC Coupled with in-Line Laser LS, UV, and RI Measurements. The molecular mass of the native PlyC holoenzyme was determined in solution by using SEC-LS/UV/RI (E. Folta-Stogniew, Biophysics Facility, W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, CT), as described (26, 27). Briefly, purified PlyC was applied to a Superdex-200 size-exclusion column (Amersham Pharmacia) upstream of a Dawn DSP LS detector (Wyatt Technology, Santa Barbara, CA), a 996-photodiode array multiwavelength UV/VIS detector (Waters), and an Optilab DSP RI detector (Wyatt Technology). The column was equilibrated in 20 mM Hepes/150 mM NaCl/1 mM EDTA, pH 7.4, at a flow rate of 0.5 ml/min. The molecular mass, *M*, for PlyC was calculated at peak maxima from 20 independent runs of 100–300 μg per injection by using the ASTRA software (Wyatt Technology) as described (26). For experiments to differentiate stoichiometric models of similar molecular mass, the molar-extinction coefficient, in units of $M^{-1}\cdot cm^{-1}$, was calculated for standard proteins (apo-ferritin, BSA, carbonic anhydrase, ovalbumin, trypsin inhibitor, and β-amylase) as well as PlyC (8:1 model and 2:2 model) according to the method of Pace (28). Next, the polypeptide-extinction coefficient, ε_p, was determined for a 1 mg/ml solution at 1-cm path length, based on the molar-extinction coefficient and the molecular mass for each protein. Finally, the UV and RI detectors are used according to the following equation: $\varepsilon_p \propto (UV)/(RI)$. Thus, a plot of computed ε_p vs. experimentally determined UV/RI will yield a linear relationship. Both PlyC models were tested for deviation from the linear regression of standard proteins and residuals reported in Table 1. See refs. 29–32 for additional details on the background, theory, and derivation of formulas used by SEC-LS/UV/RI.

Labeling with Alexa Fluor. Five hundred micrograms each of purified PlyC, PlyC-C333S, and PlyCB was reacted with the carboxylic acid, succinimidyl ester of Alexa Fluor 568, or Alexa Fluor 488 (Molecular Probes) according to the manufacturer's instructions. Unreacted dye was removed from the labeled protein by application to a 5-ml HiTrap Desalting column (GE Healthcare), equilibrated with PBS. Bacterial cultures were grown overnight, washed in PBS, mixed with 50 μg of labeled lysin, washed two times in PBS, and mounted on a slide. An Eclipse E400 microscope with a Plan Apo ×100 objective (both from Nikon) was used, and images were obtained by using a Retiga Exi camera with Q CAPTURE PRO software (both from QImaging, Burnaby, BC, Canada).

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