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The Murein Hydrolase of the Bacteriophage ϕ3626 Dual Lysis System Is Active against All Tested *Clostridium perfringens* Strains

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Clostridium perfringens **commonly occurs in food and feed, can produce an enterotoxin frequently implicated in food-borne disease, and has a substantial negative impact on the poultry industry. As a step towards new approaches for control of this organism, we investigated the cell wall lysis system of *C. perfringens* bacteriophage ϕ3626, whose dual lysis gene cassette consists of a holin gene and an endolysin gene. Hol3626 has two membrane-spanning domains (MSDs) and is a group II holin. A positively charged beta turn between the two MSDs suggests that both the amino terminus and the carboxy terminus of Hol3626 might be located outside the cell membrane, a very unusual holin topology. Holin function was experimentally demonstrated by using the ability of the holin to complement a deletion of the heterologous phage λ *S* holin in λΔSthf. The endolysin gene *ply3626* was cloned in *Escherichia coli*. However, protein synthesis occurred only when bacteria were supplemented with rare $tRNA^{Arg}$ and $tRNA^{Ile}$ genes. Formation of inclusion bodies could be avoided by drastically lowering the expression level. Amino-terminal modification by a six-histidine tag did not affect enzyme activity and enabled purification by metal chelate affinity chromatography. Ply3626 has an N-terminal amidase domain and a unique C-terminal portion, which might be responsible for the specific lytic range of the enzyme. All 48 tested strains of *C. perfringens* were sensitive to the murein hydrolase, whereas other clostridia and bacteria belonging to other genera were generally not affected. This highly specific activity towards *C. perfringens* might be useful for novel biocontrol measures in food, feed, and complex microbial communities.**

Clostridium perfringens is the causative agent of diseases such as gas gangrene or necrotic enteritis (NE) in humans and animals, and due to its ability to form an enterotoxin it also is a common source of food poisoning (23, 29, 33). In its surveillance reports the Centers for Disease Control ranks *C. perfringens* among the top five most common causes of food-borne disease in the United States (2, 26). *C. perfringens* is also responsible for severe economic losses in poultry production, mainly by causing NE. Even subclinical infections with *C. perfringens* seem to be correlated with impaired production performance (increased feed conversion and retarded growth rate) in broiler flocks (9, 20). Antibiotics are commonly used prophylactically for the control of NE in poultry feed, but in some cases development of resistance by *C. perfringens* strains has been described (3). However, it has been emphasized previously that use of antibiotics as growth promoters does have an impact on the development of cross-resistance of pathogenic bacteria against therapeutically used antibiotics (38, 39). Therefore, there is a need for development of alternatives to the antibiotics used in feed in order to control diseases such as NE in poultry.

Specifically acting murein hydrolases from bacteriophages that infect *C. perfringens* might be such an alternative. At the end of their lytic life cycle, bacteriophages release their progeny by lysis of the host cell. Most commonly, phages do this by consecutively using two proteins encoded by dual lysis genes (40). First, small hydrophobic proteins termed holins disrupt the cell membrane and form holes, through which the endolysins then can pass (41). The endolysins are mureolytic enzymes, which directly target bonds in the peptidoglycan (40). The result of their activity is the destruction of the cell and the release of the newly produced virions. Besides lysis from within, endolysins from phages of gram-positive hosts are also able to quickly lyse the bacteria when they are applied exogenously (18). Recently, the potential for using streptococcal phage lysins to control pathogenic bacteria in situ has been demonstrated (12, 25).

In this study, we characterized the dual lysis system of *C. perfringens* bacteriophage ϕ3626, and we cloned and expressed the genes in *Escherichia coli*. Experimental evidence was obtained which demonstrated the function of Hol3626. We were able to produce and purify recombinant Ply3626 endolysin, and we found that the endolysin specifically lyses *C. perfringens* cells.

MATERIALS AND METHODS

Organisms, phages, plasmids, and culture conditions. Bacterial strains, phages, and plasmids used in this study are listed in Table 1. The endolysin gene *ply3626* was cloned into pQE-30 and pSP72 and was overexpressed in *E. coli* strains JM109 and JM109(DE3), respectively. *E. coli* LE392 was used for plating λ phage and for generation of lysogens.

Luria-Bertani medium was used for propagation of *E. coli* at 37°C. For production of recombinant endolysin the temperature was reduced to 21°C, and for propagation of lysogens the temperature was reduced to 30°C. For growth of LE392, 0.2% maltose and 10 mM $MgSO_4$ were added. Plasmid-bearing cells were selected by using 100 μg of ampicillin ml^{-1} and/or 10 μg of chloramphenicol ml^{-1}, when appropriate. Lysogens were selected on media containing 30 μg of ampicillin ml^{-1}.

Lysis genes of ϕ3626. The complete nucleotide sequence of *C. perfringens* bacteriophage ϕ3626 has recently become available (43). The Husar analysis package (version 4.0; http://genome.dkfz-heidelberg.de) was used for analysis of the amino acid sequences; TmHMM was used to predict probabilities for trans-

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TABLE 1. Bacterial strains, bacteriophages, and plasmids used in this study

Strain, phage, or plasmid	Genotype or relevant properties	Source or reference
Bacterial strains		
E. coli DH5αMCR	F$^-$ *mcrA* Δ(mrr-*hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 supE44* λ$^-$ *thi-1 gyrA96 relA1*	Invitrogen
E. coli JM109	*recA1 endA1 gyrA96* thi *hsdR17* (r_{K^-} m_{K^+}) *supE44 relA1* Δ(lac-*proAB*) [F′ *traD36 proAB lacI*^{q} ZΔM15]	Laboratory stock
E. coli JM109(DE3)	JM109 with λDE3	Promega
E. coli LE392	*hsdR514*(r_{K^-} m_{K^+}) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* (phage host; permissive for λgt11)	Promega
C. perfringens NCTC 3110	Type B strain, propagation strain for ϕ3626, used as standard in the lysis assay	NCTC[a]
Phages		
ϕ3626	Isolated from *C. perfringens* ATCC 3626	43
λΔSthf	In-frame deletion of *S*, single *Eco*RI site, cIts857, Amp^r	36
λΔSthf::*S*	λ *S* inserted into *Eco*RI site of λΔSthf	36
λΔSthf::*hol3626*	*hol3626* cloned into *Eco*RI site of λΔSthf	This study
Plasmids		
pACYC-IRL10	Additional copies of *ileX, argU,* and *leuW*, Cm^r	42
pQE-30	3.4-kb coning and expression vector, T5 promoter/lac operator element; 5′ six-His tag coding sequence; Amp^r	Qiagen
pSP72	2.4-kb cloning and expression vector; T7 promoter, Amp^r	Promega
pHPL3626	*ply3626* cloned into *Bam*HI-*Sal*I sites of pQE-30	This study
pHPL3626ΔC	5′ fragment (540 bp) of *ply3626* cloned into pQE-30	This study
pSPply3626	*ply3626* cloned into the *Eco*RI site of pSP72	This study

[a] NCTC, National Collection of Type Cultures, London, United Kingdom.

membrane domains of proteins (30), and HMMscan (with the Pfam database, release 5; http://pfam.wustl.edu) was used to scan for protein families (4). Both algorithms are based on the Hidden Markov model.

Cloning and functional analysis of the holin. To study the function of the putative holin, a specialized vector system (λΔSthf) was used (36). Briefly, *hol3626* was amplified from purified phage DNA by PCR by using the following primers: hol3626-up (5′-ATCAGAATTC**TTA**ATTTTCTTTTTTATTAAATCC TTCTTT-3′) and hol3626-down (5′-ATCAGAATTC**ATG**TTTAAATTTATAC CAGAAGTAATAAGT-3′) (*Eco*RI sites are underlined, and start and stop codons are indicated by boldface type). The products were cloned into the *Eco*RI site of λΔSthf and were packaged by using a λ DNA packaging extract (Stratagene). A preliminary test for holin function was a test to determine the ability to form plaques on *E. coli* LE392. Single plaques were then isolated, and the identity of *hol3626* was verified by sequencing (36). Purified λΔSthf::*hol3626* was then used to lysogenize LE392. Growth and the lysis kinetics of the lysogenic cells in liquid cultures were monitored by measuring optical density at 600 nm (OD_{600}). Lytic development was induced by a temperature shift from 30 to 42°C for 20 min, followed by incubation at 37°C for 120 min. Lysogens carrying native λ *S* (λΔSthf::*S*) served as a positive control, and lysogens carrying λΔSthf without holin served as a negative control.

Cloning of *ply3626*. For cloning *ply3626* into pSP72, primers PlyEcoStart (5′-ATCAGAATTC*GAGGAG*AAATTACT**ATG**AAGATAGCAGAAAGAGGC GGTCATAAT-3′) and PlyEcoStop (5′-ATCAGAATTC**TTA**TATTCTTTCTA AATATTTAGCTGTAAA-3′) (*Eco*RI sites are underlined, start and stop codons are indicated by boldface type, and a ribosome binding site is indicated by italics) were used for amplification of the endolysin gene, and purified phage DNA was used as the template. The purified PCR product was digested with *Eco*RI (Roche) and ligated (T4 DNA ligase; Roche) into the *Eco*RI site of pSP72, and reaction mixtures were transformed into *E. coli* JM109(DE3). For cloning into pQE-30, primers PlyBamStart (5′-TCTAGGATCC**ATG**AA GATA GCAGAAAGAGGCGGTCATAAT-3′) and PlySalStop (5′-ATAAGTGTCGA C**TTA**TATTCTTTCTAAATATTTAGCTGTAAA-3′) were used. A truncated Ply 5′ fragment encoding only the N-terminal 180 amino acids of Ply3626 was amplified by using PlyBamStart and PlySalMid (5′-AAGTGTCGAC**TTA**TTG TCCCCCAGCAACTTTACAAACTCC-3′), the latter of which is complementary to nucleotides 513 to 540 of *ply3626* and is equipped with a stop codon. PCR products were digested with *Bam*HI and *Sal*I (MBI Fermentas) and ligated into pQE-30, and plasmids were transformed into *E. coli* JM109(pACYC-IRL10). Correct insertion and the integrity of the cloned fragments were checked by nucleotide sequencing.

Production and purification of Ply3626. Strain JM109(DE3)(pPly3626) failed to produce enzymatically active protein upon induction of protein synthesis by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM). In order to avoid inclusion bodies, the incubation temperature of strain JM109(pACYC-IRL10)(pHPL3626) was reduced to 21°C, and no IPTG was added. Growth was monitored photometrically (OD_{600}) for 20 h. At regular intervals, samples of the growing cultures were taken, and total protein synthesis was documented by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). At a final OD_{600} of approximately 1.0, cells were harvested by centrifugation at 8,000 × *g* for 15 min at 4°C, and pellets were stored at −20°C overnight. After thawing, cells were resuspended in 0.02 volume of phosphate-buffered saline (pH 8.0) or in Ni-nitrilotriacetic acid (NTA) buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole; pH 8.0). Crude extracts were prepared by single passage through a French pressure cell (SLM Aminco) at 100 MPa. Debris was removed by centrifugation at 30,000 × *g* and 10°C for 30 min, and supernatants containing the soluble enzyme were cleared by filtration (pore size, 0.22 μm) and finally stored at −20°C.

The six-His-tagged endolysin was purified by using immobilized metal chelate affinity chromatography, as previously described (17). Individual fractions were checked by SDS-PAGE and assayed for lytic activity. Protein concentrations were determined by a modified Bradford assay (Nanoquant; Roth, Karlsruhe, Germany), and buffer exchange and concentration of the proteins were performed by using centrifugal concentrators (Fugisep Maxi; 10-kDa cutoff; Intersep Systems).

Lytic activity and substrate specificity. A total of 48 *C. perfringens* strains, 30 strains of various other *Clostridium* species (*C. absonum*, *C. barati*, *C. beijerinckii*, *C. bifermentans*, *C. botulinum*, *C. butyricum*, *C. difficile*, *C. fallax*, *C. novyi*, *C. pasteurianum*, *C. sporogenes*, *C. tertium*, *C. tetani*, *C. tetanomorphum*, and *C. tyrobutyricum*), and 34 bacterial strains belonging to other genera (*Bacillus cereus*, *Bacillus megaterium*, *Bacillus polymyxa*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bifidobacterium gallicum*, *Bifidobacterium gallinarum*, *Bifidobacterium ruminantium*, *Brochothrix thermosphacta*, *Campylobacter jejuni*, *Enterobacter cloacae*, *Enterobacter gallinarum*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Lactobacillus aviarius*, *Lactobacillus plantarum*, *Lactobacillus ruminis*, *Lactococcus lactis*, *Leuconostoc carnosum*, *Leuconostoc citreum*, *Leuconostoc mesenteroides*, *Listeria innocua*, *Listeria ivanovii*, *Listeria monocytogenes*, *Pediococcus pentosaceus*, *Salmonella enterica*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus gallinarum*, *Streptococcus pyogenes*, and *Streptococcus thermophilus*) were selected and tested for sensitivity to the recombinant phage endolysin. Cultures were grown to the early stationary growth phase (16 to 20 h), and cells were harvested from 1-ml aliquots by centrifugation and resuspended in phosphate-buffered saline (pH 7.5) to an optical density of approximately 1.0. Cell suspensions (180 μl) were used directly as substrates for the lysis assays and were mixed with 20 μl (1:10, vol/vol) of crude extract of JM109(pACYC-IRL10)(pHPL3626) in sterile, uncoated polystyrene microplates. The subsequent decrease in optical density was monitored with a

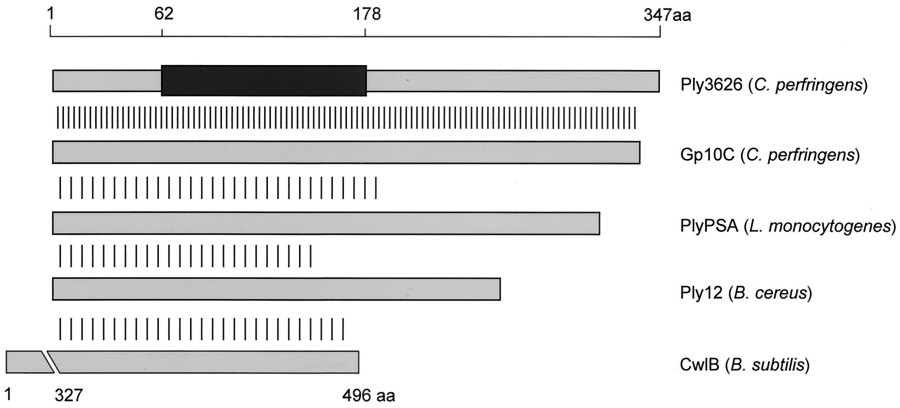

FIG. 1. Alignment of the amino acid sequences of Ply3626 and the gene product of open reading frame 10C (Gp10C) from *C. perfringens* and three amidases having other origins. The sizes of the polypeptides (in amino acids [aa]) are indicated by the scale bar at the top. The solid box indicates the *N*-acetylmuramoyl-L-alanine amidase domain identified by HMMscan (see text). The vertical bars indicate the extents and degrees of similarity among the different enzymes.

Victor 1420 plate reader (Perkin-Elmer) at 37°C. Cell extract from JM109 (pACYC-IRL10) was used as a negative control. In order to confirm that the recombinant enzyme is the lytic principle in assays in which lysis was observed, the experiments were repeated with affinity-purified HPL3626 (195 μl of cells mixed with 5 μl of enzyme preparation). The activity of the C-terminally truncated endolysin protein was tested by using cell extracts of JM109(pACYC-IRL) (pHPL3626ΔC).

RESULTS

Ply3626 is a peptidoglycan hydrolase. The endolysin gene was tentatively identified by its localization at the distal end of a late gene region and its genetic organization with the proposed holin gene in a dual lysis gene cassette (43). This genetic organization seems to occur in many viruses belonging to the *Siphoviridae* family (21). An HMMscan analysis revealed an *N*-acetylmuramoyl-L-alanine amidase domain in a 116-amino-acid N-terminal portion of Ply3626 (Fig. 1). This assignment was further supported by sequence alignments, which also indicated the relatedness of the Ply3626 N-terminal region to amidases, particularly the PlyPSA enzyme from *L. monocytogenes* phage PSA (E. Sattelberger, M. Zimmer, R. Calendar, R. Inman, and M. J. Loessner, submitted for publication), the *B. subtilis* autolysin CwlB (10), and the *B. cereus* phage 12862 endolysin (16). Interestingly, there are extended similarities (72 to 75% over 265 to 346 amino acids) to hypothetical proteins having unknown functions of *C. perfringens* and *C. perfringens* plasmid pIP404 (8, 22, 28).

Ply3626 can be synthesized only in *E. coli* supplemented with rare tRNA genes. Our first attempt to produce functional recombinant Ply3626 was based on a combination of *E. coli* JM109(DE3) and plasmid pSP72, which have repeatedly been shown to be useful for this purpose (14, 16, 19). However, cloning of *ply3626* did not yield the desired enzymatically active product. In fact, SDS-PAGE revealed that no appreciable amount of recombinant protein of the expected size was synthesized (results not shown). We then used a pQE-30 backbone and modified the N terminus of Ply3626 by adding a six-histidine tag (yielding HPL3626), in order to allow concentration of enzyme from dilute cell extracts. However, still no lytic activity was detected (results not shown). One reasonable explanation was that the failure to express low-G+C-content genes having a *C. perfringens* origin in an *E. coli* background might have been due to different codon usage. In fact, *ply3626* contains 12 copies of the isoleucine codon ATA (4.8% of the codons in *C. perfringens*) and 16 copies of the arginine codon AGA (2.7% in *C. perfringens*). In contrast, these codons are rarely used in *E. coli* (0.7 and 0.4%, respectively) (http://www.kazusa.or.jp/codon). Thus, our next approach was designed so that proper tRNA genes were substituted in order to efficiently express *ply3626* in *E. coli*. These genes were provided on plasmid pACYC-IRL10 (42) encoding tRNAs for isoleucine, arginine, and leucine. Therefore, pHPL3626 was introduced into *E. coli* JM109(pACYC-IRL10). However, induction with IPTG resulted in rapid formation of insoluble inclusion bodies. These bodies largely contained inactive Ply3626 enzyme, and our attempts to resolubilize the polypeptides to obtain an active form were unsuccessful (results not shown). This phenomenon could be avoided by culturing the bacteria at room temperature (21°C) without IPTG. Synthesis of the protein was monitored over time (Fig. 2), and the results revealed that approximately 20 h of incubation was required for sufficient protein production. Under these conditions, background expression from the strong T5 promoter on pQE-30 was apparently sufficient for appreciable gene expression. Crude cell extracts were then prepared, and HPL3626 was purified by Ni-NTA affinity chromatography (Fig. 2). A total yield of approximately 1.3 mg of purified enzyme per liter of culture was obtained. The predicted masses were 38.8 kDa for native Ply3626 and 40.2 kDa for HPL3626, which correlate very well with the experimentally determined sizes (Fig. 2).

Ply3626 lyses only *C. perfringens* cell walls. To test sensitivity to enzymatic lysis, bacterial cell suspensions were exposed to extracts of *E. coli*(pACYC-IRL10)(pHPL3626). The rapid decrease in the optical density of a cell suspension of *C. perfringens* NCTC 3110 (the standard propagation strain for ϕ3626) is shown in Fig. 3. Moreover, we found that HPL3626 was able to lyse all 48 tested strains of *C. perfringens*, although variation in the sensitivities of the different strains was observed (Fig. 3). Most of the other bacteria were not affected by the enzyme. The single exception was a strain of *C. fallax*, which also displayed sensitivity. Nevertheless, the lytic activity of Ply3626 seems

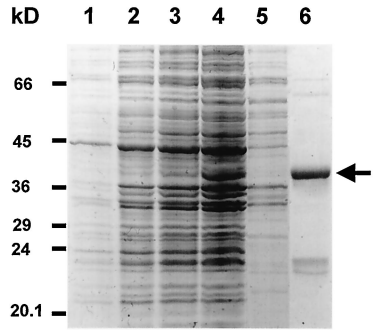

FIG. 2. Synthesis of recombinant HPL3626 endolysin in *E. coli* JM109(pACYC-IRL10)(pHPL3626) over time and results of protein purification. Lanes 1 to 4, samples taken at different times during growth (0, 8, 14, and 20 h); lane 5, wash fraction from Ni-NTA affinity chromatography; lane 6, purified HPL3626 (M_r, 40,200) (arrow) after elution and buffer exchange. kD, kilodaltons.

to be generally restricted to *C. perfringens.* Identical results were obtained with purified HPL3626, which again demonstrated the nature of the lytic activity. In contrast, the C-terminally truncated form HPL3626ΔC was found to be inactive, indicating the crucial role of the C terminus for enzyme function.

***hol3626* encodes the membrane pore-forming holin.** The putative holin gene was also initially identified by its association with the endolysin gene (i.e., its location in a lysis cassette). The deduced amino acid sequence showed some similarity to the sequence of a putative holin of *B. subtilis* phage ϕ105 (accession number AB016282) (50% similarity over 105 amino acids), whereas a dual start motif (41) is not present in *hol3626*. Bioinformatics revealed that Hol3626 is a polypeptide with two highly hydrophobic amino acid sequence regions, and the results of TmHMM analysis strongly suggested that these regions function as membrane-spanning domains (MSDs) (Fig. 4B).

If the positive-inside rule (35) was used, the fact that the positively charged amino acids were located in the putative beta-turn region between the two MSDs (coordinates 33 to 46) (Fig. 4A) suggests that this domain is located on the inside of the cytoplasmic membrane when the polypeptide is inserted into the membrane (Fig. 4C). This suggests that both the N terminus and the C terminus are most likely located on the outside of the membrane (Fig. 4C).

For functional analysis of the holin in an established background, *hol3626* was cloned into a λΔSthf background (36). Recombinant λΔSthf::*hol3626* was able to form normal-size plaques on *E. coli* LE392, apparently due to the ability of *hol3626* to support R-mediated lysis in *E. coli*. Thermal induction of the lytic cycle of λΔSthf::*hol3626* in lysogenized LE392 resulted in lysis kinetics relatively similar to those of λΔSthf::*S*, with a slight delay in lysis onset of approximately 15 min (Fig. 4D). Together, these results clearly demonstrated that the *hol3626* gene product is the phage holin.

DISCUSSION

New approaches are needed for control of anaerobic sporeformers such as *C. perfringens*, which produces an enterotoxin frequently implicated in food-borne disease and has a strong impact on the poultry industry (23, 29, 33). As a first step, we investigated the cell wall-lytic system of *C. perfringens* phage ϕ3626. This report is the first description of lysis functions of a clostridial virus. The genes are localized at the 3′ end of the late gene region next to the lysogeny module, in a dual gene cluster. These findings are consistent with the findings for several other bacteriophages belonging to the order *Caudovirales* (21, 37).

We were able to experimentally demonstrate the function of the proposed holin and endolysin gene products. Holins are proteins which are thought to insert themselves into the cytoplasmic membrane and subsequently oligomerize to form unspecific lesions that permit the cognate endolysins to access the murein (41). The ϕ3626 *hol* gene does not contain a dual start motif, which has an important role in S-mediated lysis timing and has been found in many other holin genes (41). However, expression in a λ background showed that Hol3636 can substitute for S in R-mediated cell lysis, not only indicating that the clostridial phage holin is fully functional in *E. coli* but also suggesting that it contains its own intrinsic timing function. Moreover, we speculate that its mode of action might be substantially different from that of S. The primary structure of Hol3626 features two MSDs, which means that it is a novel protein among the class II holins (41). However, as mentioned above, the positive-inside rule (35) suggests that the charged domain between the MSDs might be located in the cytoplasm, which would mean that the termini are located outside (Fig. 4C). If this is true, Hol3626 has an unusual and novel topology among the extremely diverse family of holin proteins (37, 41), which certainly warrants further investigation.

Located immediately downstream of the holin gene, *ply* encodes the ϕ3626 endolysin, which likely functions as an amidase. In contrast to the N terminus, the Ply3626 C terminus is unrelated to the C termini of other lytic enzymes. It is known that bacterial peptidoglycan hydrolases, particularly those encoded by phages, display a modular domain structure. They usually have at least one enzymatically active domain, linked to a corresponding cell wall binding domain, which targets the

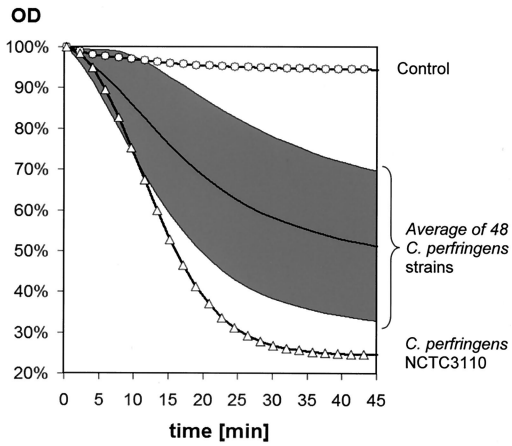

FIG. 3. Lysis of *C. perfringens* cells by exogeneously added recombinant HPL3626. See the text for an explanation. The percent decrease in optical density (OD) (*y* axis) over time (*x* axis) following addition of the enzyme to standardized cell suspensions is shown. Symbols: △, lysis response of phage host *C. perfringens* NCTC 3110; ○, negative control (no enzyme added). The average cell lysis for 48 different *C. perfringens* strains is indicated by a line, and the standard deviations of the assays are indicated by the shaded area.

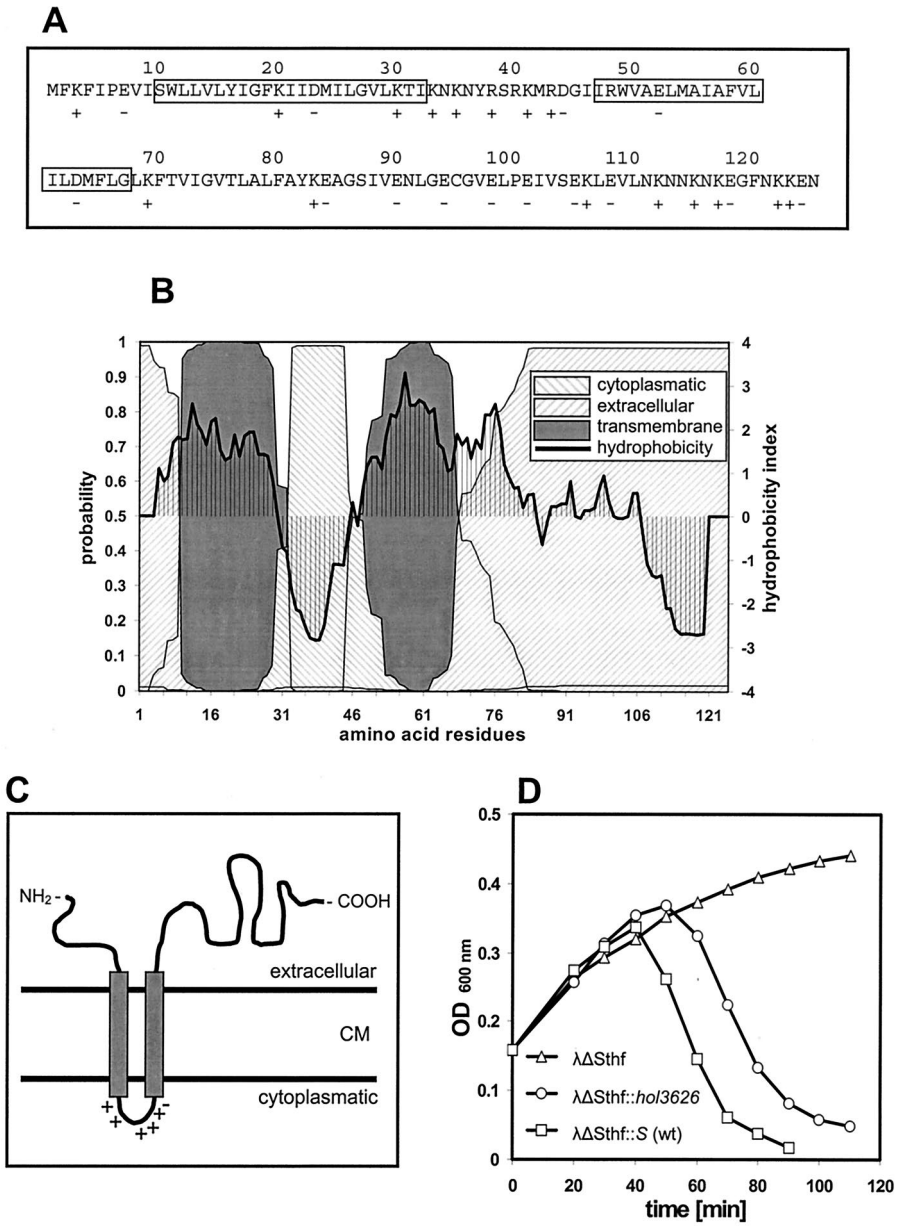

FIG. 4. Analysis of Hol3626. (A) Amino acid sequence of Hol3626. Charged amino acid residues are indicated by plus and minus signs, and sequences with a high probability for transmembrane domains (as determined by TmHMM [see text]) are enclosed in boxes. (B) Predicted probability for MSDs (left *y* axis, dark grey areas) and probable cytoplasmic and extracellular regions of the polypeptide (diagonal stripes). A hydrophobicity plot (right *y* axis) (11) was overlaid (bold line); hydrophobic regions are in the positive area, and hydrophilic regions are in the negative area. (C) Putative topological model for Hol3626 when it is inserted into the cell membrane (CM) (see text). The grey bars indicate the membrane-spanning regions. (D) Lysis profiles following thermal induction of *E. coli* LE392 lysogenized with λΔSthf::*hol3626* (□), λΔSthf::*S* (○), and λΔSthf (control without holin) (△).

enzyme to its site of action (6, 15). It has been demonstrated that such binding domains provide recognition specificity for the hydrolases (1, 15). Since Ply3626 exhibits a highly evolved specificity for *C. perfringens* cell walls, it is reasonable to assume that its unique C terminus harbors a cell wall binding domain which directs the enzyme to a specific receptor found exclusively on *C. perfringens* cells. Loss of lytic activity in the C-terminally truncated HPL3626ΔC mutant (containing the isolated amidase module) also indicated the crucial role of the C terminus in lysis. Our hypothesis is supported by the similar structures and functions of two *Listeria* phage endolysins (15). However, it is in contrast to the findings obtained for the multidomain murein hydrolases of *S. aureus* phages, where up to 75% of the enzymes could be deleted, which exhibit increasing lytic activity (13, 14, 24).

The gene encoding Ply3626 was cloned and expressed in *E. coli*. Efficient production of native and modified (six-His-tagged) endolysin was possible only under conditions that compensated for the unusual codon composition of *ply3626*, due to a very low G+C content. An *E. coli* vector containing addi-

tional copies of the rarely used tRNA genes *ileX* and *argU* under control of a constitutive promoter recently became available (42). Although our difficulties were similar to the difficulties encountered by other researchers attempting expression of clostridial proteins in *E. coli* (7, 8, 42), the next hurdle in production of these enzymes (i.e., formation of insoluble inclusion bodies after induction) was unexpected. At least in our hands, this problem is rare with highly soluble, charged proteins such as cytoplasmic endolysins. Decreasing the expression level by omitting IPTG and reducing the growth temperature apparently promoted correct protein folding and solubility and therefore prevented or minimized formation of inclusion bodies. Nevertheless, further research should help us investigate the molecular and biochemical properties of Ply3626, particularly its stability and aggregation behavior.

HPL3626 exhibited stringent substrate specificity for *C. perfringens* cells, although some variation in lysis sensitivity was observed among the different strains. Besides *C. perfringens*, only *C. fallax* was found to be sensitive to the murein hydrolase. The latter organism is also considered a pathogen associated with cases of gas gangrene (33), albeit at a lower frequency. Most of the clostridial species tested in our study belong to a phylogenetically closely related 16S ribosomal DNA cluster, cluster I (32), which includes more than one-half of the pathogenic species (31). It is interesting that *C. perfringens* and some strains of *C. fallax* have the same type of peptidoglycan (A3γ), which has an LL-diaminopimelic acid-glycine bridge to the terminal alanine of the opposite peptide chain instead of directly cross-linked *meso*-diaminopimelic acid, which is found in most other clostridia (27). The peptide bridges are close to the bond that is cleaved by the amidase and, besides the cell wall binding domain, are very likely to affect enzyme substrate specificity.

The phage ϕ3626 endolysin is active on *C. perfringens* cells when it is added exogenously, because of the direct accessibility of the cell wall peptidoglycan from the outside. The high sensitivity to lytic enzymes under otherwise nondisruptive conditions is a feature of most gram-positive bacteria and is particularly useful when native or recombinant phage lytic enzymes are applied (12, 13, 14, 16, 19, 25). The highly specific action of Ply3626 on *C. perfringens* cells forms the basis for several potential applications of this enzyme, preferably as a recombinant product. One of the foremost goals for future development is to evaluate its usefulness as a novel antimicrobial additive for the control and prevention of growth of *C. perfringens* in poultry intestines. In such applications, it is very important not to affect organisms other than the target bacteria in order to leave the natural microbial communities undisturbed. Based on the present data, Ply3626 may fulfill this requirement. Application as a novel biopreservative in food items such as raw chicken or turkey may also be considered. The enzyme could be directly added to food or feed (12, 25), or, alternatively, it could be produced by recombinant bacteria. *L. lactis* has been used to produce and secret a *Listeria* phage amidase endolysin, which remained fully active and was able to kill *L. monocytogenes* cells in medium (5).

Our lysis assays were performed under standard conditions (physiological pH and ionic strength), which worked fine and made sense in the context of the applications proposed for the enzyme. However, a detailed biochemical characterization will be required at a stage when enzyme production may be scaled up and optimal conditions for storage and specific activity need to be determined.

The use of the bacteriophage itself in a therapeutic approach to control *C. perfringens* may be also be considered as a possible way to harness the specificity of a bacterial virus (34). However, due to its very narrow host range, ϕ3626 would affect only approximately 22% of *C. perfringens* strains, and the lytic range of another *C. perfringens* phage is also only 8% (43). These relatively narrow host ranges would require pretherapeutic testing or pooling of a larger number of different phages. Moreover, the likely appearance of resistance and lysogenization would limit the killing potential of native, unmodified phage. On the other hand, development of resistance against the activity of bacteriophage endolysins has not been reported. This finding has been linked to the necessity of the phage not to be trapped inside the host cell and thereby targeting unique and highly conserved bonds in the cell wall (12).

The results of this study are certainly promising. However, the potential use of Ply3626 for the prevention or biological control of the pathogen *C. perfringens* requires further study; in particular, the function of the enzyme in a food environment and its potential function and effects in the intestines of infected poultry must be studied.

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