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# Three *Bacillus cereus* Bacteriophage Endolysins Are Unrelated but Reveal High Homology to Cell Wall Hydrolases from Different Bacilli

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**The *ply* genes encoding the endolysin proteins from *Bacillus cereus* phages Bastille, TP21, and 12826 were identified, cloned, and sequenced. The endolysins could be overproduced in *Escherichia coli* (up to 20% of total cellular protein), and the recombinant proteins were purified by a two-step chromatographical procedure. All three enzymes induced rapid and specific lysis of viable cells of several *Bacillus* species, with highest activity on *B. cereus* and *B. thuringiensis*. Ply12 and Ply21 were experimentally shown to be *N*-acetylmuramoyl-L-alanine amidases (EC 3.5.1.28). No apparent holin genes were found adjacent to the *ply* genes. However, Ply21 may be endowed with a signal peptide which could play a role in timing of cell lysis by the cytoplasmic phage endolysin. The individual lytic enzymes (PlyBa, 41.1 kDa; Ply21, 29.5 kDa, Ply12, 27.7 kDa) show remarkable heterogeneity, i.e., their amino acid sequences reveal only little homology. The N-terminal part of Ply21 was found to be almost identical to the catalytic domains of a *Bacillus* sp. cell wall hydrolase (CwlSP) and an autolysin of *B. subtilis* (CwlA). The C terminus of PlyBa contains a 77-amino-acid sequence repeat which is also homologous to the binding domain of CwlSP. Ply12 shows homology to the major autolysins from *B. subtilis* and *E. coli*. Comparison with database sequences indicated a modular organization of the phage lysis proteins where the enzymatic activity is located in the N-terminal region and the C-termini are responsible for specific recognition and binding of *Bacillus* peptidoglycan. We speculate that the close relationship of the phage enzymes and cell wall autolysins is based upon horizontal gene transfer among different *Bacillus* phages and their hosts.**

Numerous bacteriophages for the genus *Bacillus* have been isolated, and at present, they are grouped into 33 phage species. With one exception, all of them belong to the tailed phages (1). Lytic activity is not necessarily restricted to a single species, especially with respect to the phages used for typing strains of *Bacillus cereus* and *Bacillus thuringiensis* (2, 35). *B. cereus* is genotypically closely related to *B. thuringiensis*, and it has been suggested that they be grouped into a single species (4). In contrast to the well-studied phages infecting *B. subtilis* (29, 40), no information is available on the molecular biology and relationships of viruses for the *B. cereus-B. thuringiensis* group.

At the end of their multiplication cycle, most bacteriophages are released from the cells through the action of endogenous cell wall hydrolases, termed endolysins or phage lysins (for a review, see reference 38). Several genes encoding lysins from phages infecting both gram-negative and gram-positive hosts have been cloned and sequenced. In some cases, the catalytic mechanisms have been determined, which place the phage lysins into three distinct groups: amidases, muramidases (glycosidases and transglycosylases), and endopeptidases (38). Recently, a fourth type of enzyme was described (L-alanoyl-D-glutamate peptidase) (18). It is interesting to note that phage lysins, in some cases, may be related to the autolytic enzymes of their hosts (23). However, the latter should not be confused with endolysins encoded by defective prophages, which are common to many bacterial genera (19, 37, 42). A more detailed understanding of phage lytic mechanisms is needed to reveal the (possibly phylogenetic) relationship of phage-encoded lysins and the autolysins of their bacterial hosts.

In this paper, we describe the cloning and analysis of the endolysin genes from three different *B. cereus* phages (12826, TP21, and Bastille) and present evidence for a relationship of the phage enzymes to autolysins from other bacilli. We also aimed to determine the specific substrate bonds cleaved in *B. cereus* cell walls.

## MATERIALS AND METHODS

**Organisms, plasmids, and culture conditions.** All bacterial strains, phages, and plasmids used throughout the cloning procedures are listed in Table 1.

Twenty-three phages were initially tested for their lytic activity on 17 *B. cereus* strains. The three phages investigated here were then selected on the basis of their different host range, individual morphology, and ease of propagation.

*Escherichia coli* JM109(DE3) was used in recombinant DNA work and overexpression of cloned lysin genes, and plasmid pSP72 was the basis for library construction, gene expression, and nucleotide sequencing.

Bacilli were grown in standard plate count medium at 30°C. Phages were propagated, concentrated by polyethylene-glycol precipitation, and subsequently purified by ultracentrifugation on stepped CsCl gradients as previously described (41). *E. coli* was grown in standard Luria-Bertani medium (24) at 37°C. For selection of plasmid-bearing cells, ampicillin was added at a concentration of 100 μg/ml.

**DNA manipulations and cloning procedures.** Phage DNAs were extracted and purified according to standard methods (24). For construction of expression libraries in *E. coli*, the DNAs were partially digested with restriction endonuclease *Tsp*509I (New England Biolabs). Following electrophoresis in low-melting-point agarose (FMC Bioproducts), fragments in the range of 1,500 to 3,000 bp were recovered by β-Agarase digestion (Boehringer) and concentrated by ultrafiltration in a microcentrifuge (Microcon 100, Amicon). DNA fragments were then ligated (T4 DNA ligase, Boehringer) into pSP72, which had been linearized with *Eco*RI (Boehringer) and treated with shrimp alkaline phosphatase (U.S. Biochemicals). All enzymatic manipulations were carried out according to the suppliers' instructions.

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TABLE 1. Bacterial strains, bacteriophages, and plasmids used

| Strain, phage, or plasmid | Relevant characteristic(s) | Source or reference |
|---|---|---|
| Strains | | |
| *B. cereus*[a] | | |
| HER1399 | Host for phages Bastille and TP21 | ATCC 13472 |
| WS2453 | Host for phage 12826 | ATCC 12826 |
| *E. coli* | | |
| JM109(DE3) | K-12 *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*$^q$ *Z*ΔM15] T7 RNA polymerase under *lacUV5* control | Promega, 30 |
| Phages | | |
| 12826 | Wild type; host WS2453, not classified | ATCC 12826-B1 |
| Bastille | Wild type; host HER1399, A1 morphology, ca. 127 kbp | HER, 9 |
| TP21 | Wild type; host HER1399, B1 morphology, ca. 34 kbp | HER, 8, and this study |
| Plasmids | | |
| pSP72 | Cloning and expression vector, T7 gene 10 promoter upstream of multiple cloning site, Ap$^r$ | Promega, 32 |
| pBCPL12 | 1,586-bp *Tsp*509I fragment from 12826 cloned into *Eco*RI site of pSP72 | This study |
| pBCPLba | 1,851-bp *Tsp*509I fragment from Bastille inserted into the *Eco*RI site of pSP72 | This study |
| pBCPL21 | 2,148-bp *Tsp*509I fragment from TP21 ligated into the *Eco*RI site of pSP72 | This study |

[a] HER, Félix d' Hérelle Reference Center for Bacterial Viruses, Québec, Canada; WS, Weihenstephaner Sammlung, Freising, Germany.

Ligation reactions were electrotransformed (Gene-Pulser, Bio-Rad) into the *E. coli* host, which was followed by selection of plasmid-bearing cells on antibiotic plates. Lysin-expressing colonies were identified as previously described (18), by replica plating on isopropyl-β-D-thiogalactopyranoside (IPTG)-containing agar plates, followed by chloroform treatment and overlay with a concentrated suspension of *B. cereus* cells in 0.4% soft agar. After a 30-min incubation at room temperature, clear zones of lysis could be observed around clones expressing the bacteriophage lysins.

Plasmids were analyzed by restriction endonuclease digestion and Southern hybridization to digoxigenin-labeled phage DNAs as previously described (14). All recombinant plasmids constructed in this study are listed in Table 1.

**DNA sequencing and computer analysis.** Large-scale plasmid preparations were prepared with the aid of ion-exchange chromatography columns (Qiagen). The nucleotide sequences of phage-derived plasmid inserts were determined by primer walking with synthetic oligonucleotides with initial primers complementary to the T7 and SP6 promoter sequences present on pSP72. Sequenase version 2.0 (U.S. Biochemicals) and α-$^{35}$S-dATP (Amersham) were used in all sequencing reactions. DNA sequences and proteins translated from putative open reading frames (ORFs) were analyzed employing the DNASIS/PROSIS program (Hitachi).

**Overexpression and purification of *ply* gene products.** Batch culture overexpression of the cloned endolysins was carried out as published earlier (16, 17, 31) by induction of log-phase cells (volume, 250 ml; optical density at 600 nm ($OD_{600}$), approximately 0.5) with IPTG (0.5 mM), which was followed by further incubation for 4 h. Samples were taken immediately before and at various time points after induction for analysis of total cell proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). For large-scale production, bacteria were harvested 4 h after induction by centrifugation (10,000 × *g*, 10 min, 4°C). Crude cell extracts were prepared by single passage of the resuspended cells (in a 1/100 volume of 50 mM TrisCl, pH 7.5) through a French press at 100 MPa (SLM Aminco). Debris was removed by centrifugation (20,000 × *g*, 10°C, 20 min), and the supernatants containing the phage lysins were cleared by filtration (pore size, 0.22 μm) and stored at −20°C.

The crude lysates (3 to 5 ml) were then loaded onto a preparative gel filtration column (HiLoad 16/60, Superdex 75 prep-grade, Pharmacia). The elution buffer used was 50 mM TrisCl, pH 7.5. Fractions were assayed for cell wall hydrolyzing activity using a photometric assay as described earlier (18). Protein content of active fractions was analyzed by SDS-PAGE. The partially purified endolysin suspensions were then concentrated by spin ultrafiltration (Centriprep, 10-kDa cut-off; Amicon) and subjected to anion-exchange chromatography (Mono-Q HR 5/5 or Resource-Q; Pharmacia). Lytic enzymes were eluted from the resin by a salt gradient (0 to 1.0 M NaCl). Again, fractions were assayed for lytic activity and analyzed by SDS-PAGE.

**Lytic activity and substrate specificity.** A total of 24 strains of *B. cereus*, 12 strains of other *Bacillus* spp. (see Fig. 5), and 40 bacterial strains of other (mostly gram-positive) genera were selected from the Institute's culture collection and tested for sensitivity against the recombinant phage lysins. The strains from other genera were *Aeromonas hydrophila*, *Alcaligenes faecalis*, *Arthrobacter* spp., *Brevibacterium* spp., *Brochothrix thermosphacta*, *Carnobacterium* spp., *Cellulomonas fimi*, *Clostridium tyrobutyricum*, *Corynebacterium* spp., *Curtobacterium citreum*, *Enterobacter cloacae*, *Enterococcus faecalis*, *E. coli*, *Jonesia denitrificans*, *Kurthia zopfii*, *Lactobacillus casei*, *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Listeria* spp., *Micrococcus* spp., *Nocardia asteroides*, *Oerskovia turbata*, *Pediococcus damnosus*, *Proteus mirabilis*, *Propionibacterium freudenreichii*, *Pseudomonas* spp., *Rhodococcus equi*, *Salmonella enteritidis*, *Serratia marcescens*, *Staphylococcus aureus*, and *Streptococcus* spp. Assays were carried out photometrically with 900 μl of cell suspension and 100 μl of partially purified endolysin suspension. Controls had no lysin added.

Identification of the specific substrate bonds cleaved in *B. cereus* cell walls was carried out as described earlier (6, 18) after complete digestion (30 min, 25°C) of purified cell walls of the respective host strain with the three different lysins. Amino groups released during enzyme hydrolysis were labeled with 1-fluoro-2,4-dinitrobenzene. Following acid hydrolysis of total amino bonds (6 N HCl, 18 h, 110°C), dinitrophenyl (DNP)-labeled amino acids (DNP derivatives) were identified by reversed-phase high-performance liquid chromatography (100-C-18 column, Eurosphere) with purified DNP-labeled amino acids (Sigma) as standards. The loading buffer used was 70 mM sodium citrate, pH 2.2. Buffer A was 30% methanol–50 mM acetic acid, and labeled amino acids were eluted by using a linear gradient up to 95% methanol–50 mM acetic acid. The DNP derivatives were detected at a 360-nm wavelength.

**Nucleotide sequence accession numbers.** The DNA sequences reported in this paper will appear in the EMBL, GenBank, and DDBJ databases under the accession numbers Y11476, Y11477, and Y11478.

## RESULTS

**Cloning and nucleotide sequencing of endolysin genes.** DNA purified from *B. cereus* phages Bastille, 12826, and TP21 was shotgun cloned into the inducible expression vector pSP72 and transformed into *E. coli* JM109(DE3). Following IPTG induction and chloroform treatment, lysin-expressing colonies of the *E. coli* library could be readily identified by their lytic phenotype when overlaid with a lawn of *B. cereus* cells. Plasmid inserts of positive clones were recovered by restriction enzyme digestion, and their identity was verified by Southern hybridization to labeled phage DNAs. Nucleotide sequencing of phage-derived DNA fragments and subsequent computer analysis allowed the identification of several ORFs (Fig. 1). Presumptive endolysin-encoding genes were designated as *ply12*, *ply21*, and *plyBa*. Identity of the genes was confirmed by comparing the predicted sizes of their products with those observed in SDS-PAGE analysis of overexpressed lytic enzymes (see below). Insert sizes are given in Table 1, and Table 2 presents basic characteristics of the three endolysin genes and their products.

The *ply* genes are preceded by ribosome binding sites of

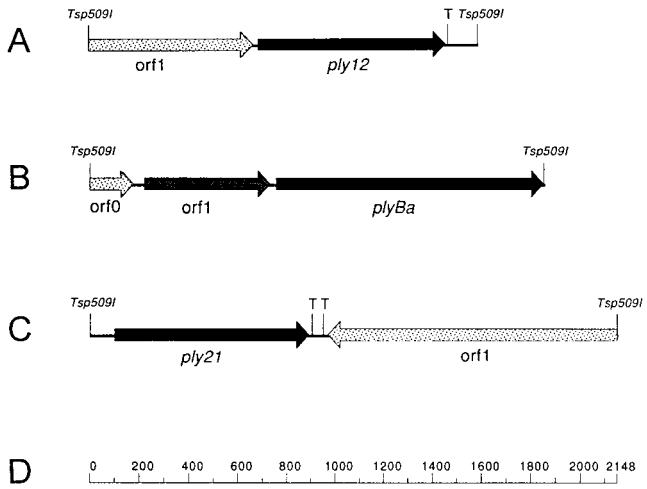


FIG. 1. Locations and orientation of the identified ORF on the genetic maps of the cloned phage DNA fragments. The *ply* genes and other identified ORFs (solid arrows, complete ORF; stippled arrows, incomplete ORFs) are drawn to scale, which is shown in base pairs in panel D. Probable transcription termination structures are indicated by the letter T.

perfect (*ply12* and *plyBa*) or near-perfect (*ply21*) complementarity to the 3′ end of *Bacillus* 16S rRNA (3′-UCUUUCCUC CACU· · ·-5′) (20). Inverted sequence repeats capable of forming stem-loop structures were identified downstream of *ply12* (ΔG, −65.5 kJ/mol) and *ply21* (ΔG, −171.3 kJ/mol). The latter sequence was predicted to form a very stable double hairpin, which may function as a dual transcription terminator since it is located between the downstream ends of two transcriptional units (Fig. 1). Interestingly, a nearly identical sequence is present downstream of the *Bacillus* sp. cell wall hydrolase CwlSP (22), which is highly homologous to Ply21 (see below). Possible secondary structures were also predicted for the immediate upstream regions (encompassing the ribosome binding sites) of *plyBa* (ΔG, −12.6 kJ/mol) and *ply21* (ΔG, −97.0 kJ/mol). Interestingly, in both predicted structures the putative ribosome binding sites are presented on a loop, which may have significance in expression of the genes (15).

**Relationships of the three phage lysins.** The overall DNA sequence similarity of the three endolysin genes is very low. Alignment of deduced amino acid (aa) sequences (Fig. 2) also revealed no significant relationships, except for some small patches of similarity in the N-terminal regions of Ply12 and Ply21 and in the C termini of all three enzymes.

**Homologies of Ply21.** We found excellent aa sequence homology between the N-terminal-to-central region of Ply21 and three other lytic enzymes associated with the genus *Bacillus* (Fig. 3): (i) 80% identity and 92% similarity in a 154-aa overlap with the cell wall hydrolase CwlSP from an unidentified *Bacillus* isolate (22); (ii) 61% identity and 84% similarity in a 140-aa overlap with the *B. subtilis* autolysin CwlA (11); (iii) 41% identity and 75% similarity in a 141-aa overlap to the lytic amidase from the defective *B. subtilis* phage PBSX (19).

For both CwlA and CwlSP, a potential signal cleavage site was proposed after $Ala_{39}$ (Fig. 3) (11, 22). The identical sequence is present in Ply21. Also, an amidase motif as described by Lazarevic et al. (13) was found at two positions within the sequence of Ply21.

The putative protein translated from the ORF1 facing *ply21* probably belongs to the "resolvase" family of site-specific recombinases, since it shows striking sequence similarity to the invertase Pin from *E. coli* (21) (43% identity and 86% similarity in a 70-aa overlap) and other related enzymes like the Cin invertases from phages P7 and P1.

**Homologies of PlyBa.** Figure 3 shows that the lytic enzyme from phage Bastille contains a C-terminal homologous repetition of 77 aa (domains C I and C II; 42% identity and 77% similarity in a 69-aa overlap). Moreover, this repeat is highly homologous to the C terminus of CwlSP (53% identity and 81% similarity in a 75-aa overlap). A short sequence (KVVTY) is conserved in both C-terminal repeats of PlyBa and the corresponding domain of CwlSP. However, the significance of this motif is yet unclear.

**Homologies of Ply12.** Comparison of amino acid sequences revealed some homologies of the central region of Ply12 to the C terminus of CwlB (37% identity and 72% similarity in a 93-aa overlap), which is the major autolysin of *B. subtilis* (12), and to a C-terminal domain of the *E. coli* autolytic enzyme AmiA (33, 34) (30% identity and 85% similarity in a 54-aa overlap). Both CwlB and AmiA are *N*-acetylmuramoyl-L-alanine amidases.

**Overexpression in *E. coli* and chromatographical purification of *ply* gene products.** The total protein content of the plasmid host *E. coli* JM109(DE3), carrying one of the three plasmids, was analyzed by SDS-PAGE at various times (0, 1, and 4 h) after IPTG-induced protein synthesis. Each of the three clones was shown to overproduce a single individual protein and the sizes of these proteins correlated with those deduced from the nucleotide sequence of the three *ply* genes. The yield of recombinant PlyBa protein was approximately 20% of total cellular protein (Fig. 4, lane B). Following preparative gel filtration chromatography of the crude cell lysate, partially purified fractions of high specific activity were obtained (Fig. 4, lane C). The enzyme could be further purified by anion-exchange chromatography, which yielded lysin preparations of more than 90% homogeneity (Fig. 4, lane D). Overexpression of Ply21 and Ply12 was somewhat less effective (8 to 10% of total protein), and two-step chromatography yielded fractions of approximately 70 to 80% purity (data not shown).

**Enzymatic activity and specificity of the phage murein hydrolases.** The three recombinant enzymes showed a high degree of specificity towards cells of *B. cereus* and related species.

TABLE 2. Characteristics of the three *B. cereus* bacteriophage endolysin genes and their encoded proteins

| Phage | Gene characteristic | | | Gene product characteristic | | | |
|---|---|---|---|---|---|---|---|
| | Gene (bp) | RBS[a] | Start/stop codon | Protein | No. of amino acids | Molecular mass (kDa) | Predicted pI |
| 12826 | *ply12* (774) | AAGGAGG (7) | ATG/TAG | Ply12 | 257 | 27.7 | 6.1 |
| TP21 | *ply21* (792) | AGAGAGG (8) | ATG/TAA | Ply21 | 263 | 29.5 | 8.5 |
| Bastille | *plyBa* (1,095) | AAGGAGG (8) | ATG/TAA | PlyBa | 364 | 41.1 | 5.6 |

[a] RBS, ribosome binding site (distance to start codon in nucleotides is given in parentheses).


FIG. 2. Alignment of the complete aa sequences of Ply12, Ply21, and PlyBa. Identical aa residues and conservative replacements are indicated by vertical lines and colons, respectively. A few gaps have been introduced to optimize alignment. Homologous regions are shown in boldface letters.

Figure 5 shows the exogenous hydrolytic activity of Ply21 on intact, living cells of several bacilli. The results obtained with PlyBa and Ply12 were similar. All of the 24 tested *B. cereus* strains were sensitive to the phage enzymes, while *B. subtilis* cells were almost resistant to lysis. Also, lytic activity seems to be generally restricted to the genus *Bacillus*, since none of the other tested bacterial cultures were susceptible, i.e., they did not show a substantial decrease in OD after addition of the phage lysins.

***ply12* and *ply21* encode *N*-acetylmuramoyl-L-alanine amidases.** Purified cell walls were digested with the individual recombinant enzymes. Bond specificity of the hydrolases could be determined by labeling and subsequent detection of newly released amino termini from the specific amino acids occurring in the peptide subunits of the *B. cereus* peptidoglycan. Both Ply12 and Ply21 were shown to cleave the peptide chain between the acetyl group of the *N*-acetyl-muramic acid and the L-alanine (Fig. 6B), as deduced from the marked increase of DNP-labeled L-alanine in the derivatized digests (Fig. 6A). In contrast, the results obtained for PlyBa did not permit positive identification of the substrate bond cleaved. Although the enzyme very effectively hydrolyzed the substrate cell walls, only minor increases in DNP-labeled L-alanine and D-glutamate were observed. Therefore, a different enzymatic specificity cannot be excluded, such as a lysozyme (glycosidase) or other muramidase (transglycosylase) activity.

## DISCUSSION

The prevalent system in cell lysis by tailed phages is based upon a peptidoglycan-hydrolyzing enzyme (endolysin) and a nonspecific, membrane pore-forming protein (holin) which allows access of the cognate endolysin to its murein substrate (38). Other investigated *Bacillus* phages, such as $\phi$29 and PZA, possess holin genes upstream of their lysin genes (28). However, no potential holin-encoding genes could be identified upstream of the endolysin genes investigated here, since none of the other identified ORFs was predicted to encode a small hydrophobic membrane protein showing at least some of the characteristic properties of a holin (39). Apparent lack of a holin was also reported for some other phages, such as *Listeria* phage A511 (18). Several explanations are possible, including (i) the holin genes may not be directly associated with the lysins with respect to their location on the phage genomes and (ii) alternative, nonholin mechanisms are employed for endolysin export. As pointed out by Young and Bläsi (39), all endolysins characterized so far are apparently synthesized without a signal sequence. Therefore, it is interesting that Ply21 may have an N-terminal signal sequence, as suggested by the sequence identities to the respective domains in *Bacillus* autolysins (Fig. 3). However, whether this predicted leader or signal peptide is actually significant in maturation or, in particular, in membrane permeation of the amidase needs further clarification.

So far, the available data suggest that many lytic enzymes of phages or bacteria may be composites of specifically adapted modules, encoding substrate recognition or hydrolysis domains, respectively. A modular organization was shown or proposed for several other lytic enzymes where the catalytic activity is (almost always) located in the N-terminal region while the C-terminal part contains the substrate recognition and/or binding domains (5, 10, 11, 19, 22, 25, 36). The N-terminal 60% of the CwlSP protein reflects the corresponding domain of Ply21, and the remaining C-terminal region (approximately 40%) is highly similar to the repetitive domains in the C terminus of PlyBa. Moreover, all three of the enzymes homologous to the N terminus of Ply21 are *N*-acetylmuramoyl-L-alanine amidases

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PBSX  (1)   MVNIIQDFIPVGANN-RPGYAMTPLYITVHNTANTAVGADAAAHARYLKNPDTTTSWHFTVDDTEIYQHLPLNENGWHAGDG-NGSGNRA (89)
CwlA  (1)   MAIKVVKNLVSKSKYGLKCPNPMKAEYITIHNTAN---DASAANEISYMKNNSSSTSFHFAVDDKQVIQGIPTNRNAWHTGDGTNGTGNRK (88)
Ply21 (1)   MQIKQMLVPEYKYELLCPNPMTPTEITLHNTYN---DAPAINERNNVANNSQGTSFHVVVDDKEAIQLIPFNRNAWHAGDGGSGRGNRH (86)
CwlSP (1)   MEIKQMLVPVSRYSVLCPYEMNPTEITFHNTYN---DAPAINERNNVANNSTGTSFHIAVDDKEAIQLIPFNRNAWHAGDGTNGRGNRH (86)
                                                ↑

PBSX  (90)  SIGIEIC--ENADGDFAKATANAQWLIKTLMAEHNISLANVVPHKYWSGKECPRKLL--DT--WDSFKAGIGGGGSQTYVVKQG (174→203)
CwlA  (89)  SIGVEICYSKSGGVRYKAAEKLAIKFVAQLLKERGWGIDRVRKHQDWNGKYCPHRILSEGR--WIQVKTAIEAELKKLGGKTNSSKASVAKK (178)
Ply21 (87)  SIGVEICYSKSGGPRYEQAVRNAIIVIRQLMDQFNIPIDRVKTHQERNGKYCPHRMLAEGRVGWFKQQLVSGDYVPPTPIPQPEPQLPSGQY (178)
CwlSP (87)  SIGVEICYSQSGGARYRKAELNAVEVIAQLMIQFDIPISKVKTHQERNGKYCPHRMLDEGRVQWFKNQCANRASSIKNSNKTQETGKV (174)

PBSX  (204) SAPSAAEKPELYPLPDGIIQLTTPYTSGEHVFQVQRALAALYFYPDKGAVNNGIDGVYGPKTADAVARFQSVNGLTADGIYGPATKEKIAAQLS (297)
CwlA  (179) KTTNTSSKKTSYALPSGIFKVKSPMMRGEKVTQIQKALAALYFYPDKGAKNNGIDGVYGPKTADAIRRFQSMYGLTQDGIYGPKTKAKLEALLK (272)
Ply21 (179) DSSWFTKESGTFTLNTTINLRTAPFSNAPLIATLSKGQQVSYDGYGIELDGHVWIRQPRANGTYGYMATGESANGKRVDYWGSFK (263)
CwlSP (175) EIIVNKFNKVVTYEFGTALVPEMLGMMDALGYESRIISYGDKQGLVRFETAYRQGNELDKATAWLDAKGLKYFYTKE (251)
PlyBa (211) ETLVPALNKVVTYEVGTNLIPEIQDKLAFLGYEARINFTGLGDGLVSIETSHQVGAELDKLTAWLDERGWAYYYTSS (287) [Domain C I]
PlyBa (288) KEGYNGKSKVVTYDMGTNKIPELSNVLAYQGMQTAIVFTGKGDGLIRLESTPLDESRLQNFKNILEAQKIAYYMYSE (364) [Domain C II]
PlyBa       --------KVVTY--GTN-IPE----LA--G----I-FTG-GDGL---E--------L------L-----AYY--S-       [Consensus]
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FIG. 3. Homology of the primary aa sequence of Ply21 to cell wall hydrolases (autolysins) from *B. subtilis* (CwlA), *Bacillus* sp. (CwlSP), and the lytic enzyme from a defective *B. subtilis* phage (PBSX). Homologous domains are shown in boldface letters. Identical residues and conservative replacements are indicated by vertical lines and colons, respectively. The proposed cleavage site for processing of CwlSP and CwlA is marked by an arrow. Proposed amidase motifs (13) are underlined. The C-terminal portion of the PlyBa enzyme contains two homologous repeats (C I and C II), which show significant sequence similarity to the C-terminal region of CwlSP. The consensus sequence for the repetitive PlyBa domains is also given.

(EC 3.5.1.28) and exhibit reported amidase motifs in identical locations (Fig. 3). Not only did these observations already suggest the probable catalytic function of Ply21, but they also support our conclusion that the N terminus of Ply21 contains the enzymatically active site. Consequently, the C-terminal re-

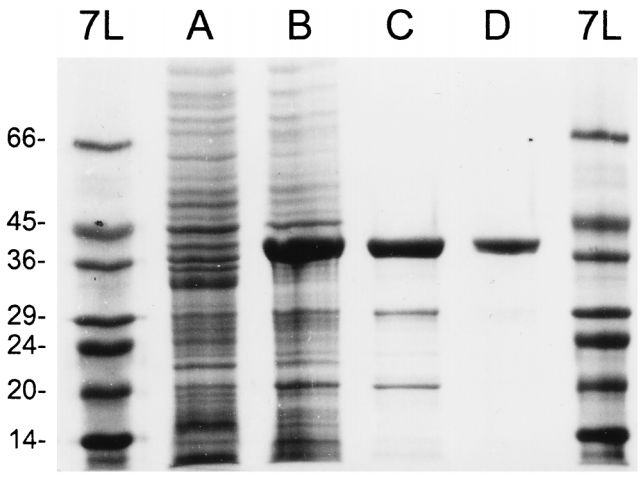


FIG. 4. Chromatographical purification of the recombinant PlyBa endolysin demonstrated by SDS-PAGE. Lanes 7L, molecular mass markers (indicated in kilodaltons); lane A, cell extract of *E. coli* JM109(DE3)(pBCPLba) before induction with IPTG; lane B, cell extract after induction and 4-h incubation; lane C, partially purified PlyBa following gel filtration on a preparative Superdex 75 column; lane D, purified enzyme from active Mono-Q fractions.

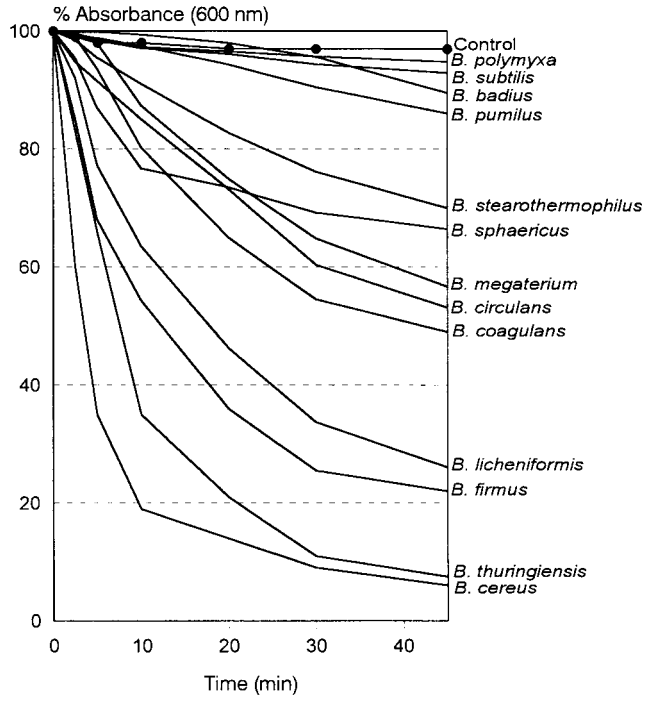


FIG. 5. Lysis of viable cells of 12 different *Bacillus* species by the recombinant *B. cereus* phage endolysin Ply21.

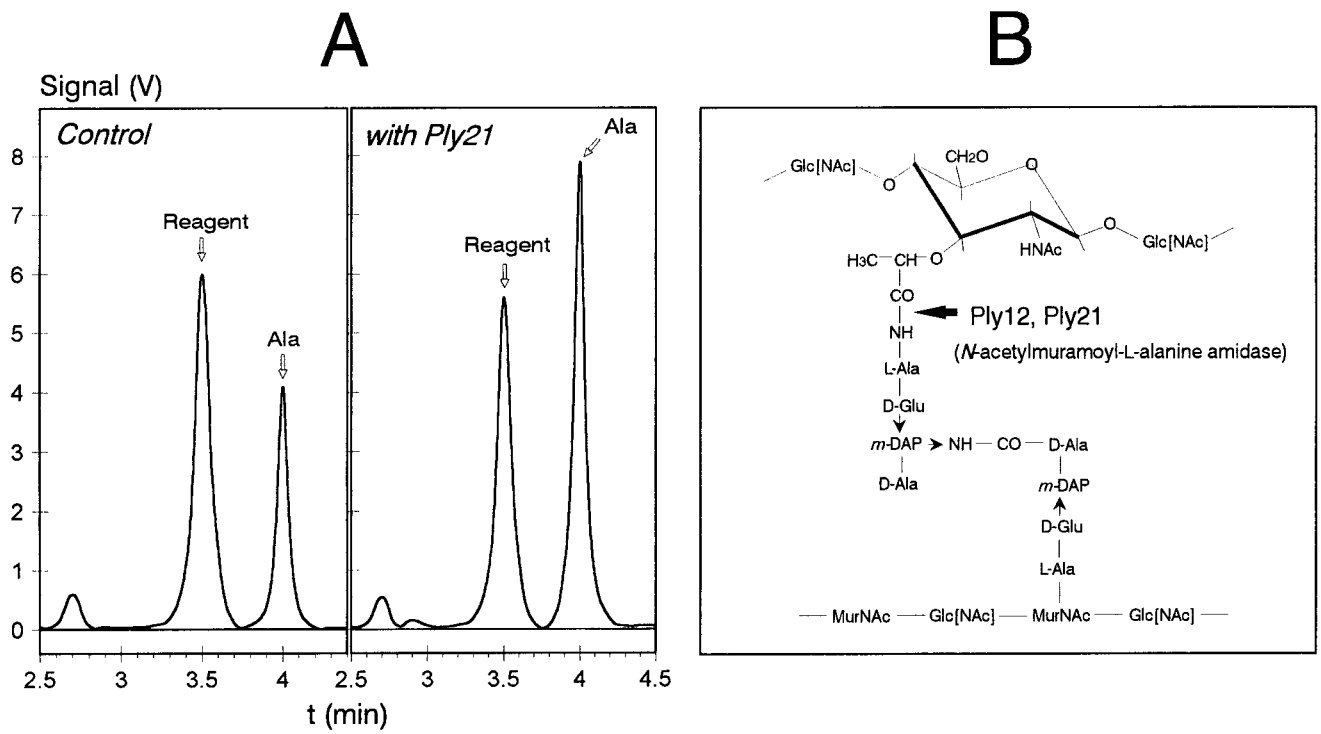


FIG. 6. (A) Results of reversed-phase high-performance liquid chromatography analysis of DNP-labeled amino groups after predigestion of *B. cereus* cell walls with Ply21. Note the marked increase in DNP-alanine after release by Ply21. The reagent peak comes from the reaction of DNP with the buffer substance (Tris) that was used. (B) Specific substrate bond cleaved by endolysins Ply12 and Ply21, which classifies these enzymes as *N*-acetylmuramoyl-L-alanine amidases. The *Bacillus* peptidoglycan A1γ variation (26) is schematically shown. Glc[Nac], *N*-acetyl-D-glucosamine (which is mostly deacetylated in *B. cereus* cell walls [3]); MurNac, *N*-acetyl-D-muramic acid; *m*-DAP, *meso*-diaminopimelic acid.

gion may be responsible for specific binding to the *Bacillus* peptidoglycan. This is in line with our finding that Ply21 did not lyse *Micrococcus luteus* cells, whereas CwlSP can do so (22). This difference could be explained by the enzymes' completely different C-terminal (binding) domains.

A similar structure can be proposed for Ply12, whose N terminus reveals convincing homology to the C terminus of the major *B. subtilis* amidase, CwlB. Since the catalytic region of CwlB is located in the C-terminal region (which is unusual) (12), it is very likely that the Ply12 N terminus determines the enzymes' amidase activity.

The C terminus of PlyBa shows two sequence repeats which are similar to the C-terminal (binding) domain of the CwlSP amidase, and we therefore suppose that these modules are involved in substrate recognition. On the basis of similarity, PlyBa should be able to bind to *M. luteus* peptidoglycan. However, the enzyme could not lyse these cells, which may be attributed to a differential catalytic mechanism unable to hydrolyze the respective bonds in *M. luteus* murein. This corresponds to our result that PlyBa is not an amidase.

The N-terminal-to-central region of Ply21 is almost identical to that of CwlSP, an autolytic amidase from an unidentified *Bacillus* species (22). It should be noted that these authors provide a list of 23 biochemical properties of the *Bacillus* strain in question, and all but one of them (butanediol fermentation is negative) exactly match the biochemical profile of *B. cereus* (27). Therefore, it seems quite possible that CwlSP actually is a *B. cereus* autolysin. It would help to explain the strong similarity of the two enzymes if one assumes that phage lysin and autolysin could be phylogenetically related.

The three *B. cereus* phages are unable to produce plaques on lawns of *B. subtilis* cells, and their endolysins do not permit efficient lysis of *B. subtilis* cell walls. Nevertheless, these enzymes show substantial similarity to specific domains of *B. subtilis* autolysins. It is tempting to speculate that these genes (i.e., genetic modules) were exchanged through horizontal gene transfer, as suggested for the tail fiber genes of some enterobacterial phages (7).

The lytic spectrum of the cloned enzymes seems to be restricted to peptidoglycan types of the genus *Bacillus*, with highest activity on cell walls of the *B. cereus-B. thuringiensis* group. *B. subtilis* cells were resistant to lysis, despite the close relationship of its own autolysins to the cloned phage enzymes. The question arises whether this specificity could be due to (i) unique linkages cleaved only in *Bacillus* cell walls, (ii) specific enzyme activation by components present exclusively in or on *Bacillus* cell walls (which is the case for some pneumococcal phage lysins that depend on choline-containing teichoic acids for activation [5, 23]), or (iii) specificity in substrate recognition and cell wall binding. The first possibility can be excluded since the directly cross-linked A1γ variation of *Bacillus* peptidoglycan is very common (26). It cannot be excluded that *Bacillus* cell walls contain unique factors capable of endolysin activation. However, the more likely prospect concerns the specific recognition of *Bacillus* cell walls with a preference for the highly deacetylated *B. cereus* peptidoglycan (3), which is insensitive to the action of lysozyme. This speculation is supported by the exclusive, individual C termini (proposed binding domains) of Ply12 and Ply21, which show no homology to the other *Bacillus* lytic enzymes.

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