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Camelysin Is a Novel Surface Metalloproteinase from *Bacillus cereus*

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Bacillus cereus **frequently causes food poisoning or nosocomial diseases. Vegetative cells express the novel surface metalloproteinase camelysin (casein-cleaving metalloproteinase) during exponential growth on complex, peptide-rich media. Camelysin is strongly bound to the cell surface and can be solubilized only by detergents or butanol. Camelysin spontaneously migrates from the surface of intact bacterial cells to preformed liposomes. The complete sequence of the camelysin-encoding gene, *calY*, was determined by reverse PCR on the basis of the N-terminal sequence and some internal tryptic cleavage peptides. The *calY* gene codes for a polypeptide of 21.569 kDa with a putative signal peptide of 27 amino acids (2.513 kDa) preceding the mature protein (19.056 kDa). Although the predicted amino acid sequence of CalY does not exhibit a typical metalloprotease consensus sequence, high-pressure liquid chromatography-purified camelysin contains one zinc ion per protein molecule. Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry and tryptic peptide mass fingerprinting confirmed the identity of this zinc-binding protein as CalY. Disruption of the *calY* gene results in a strong decrease in the cell-bound proteolytic activity on various substrates.**

Bacillus cereus, a gram-positive, ubiquitous soil bacterial species, is closely related to *Bacillus anthracis* and *Bacillus thuringiensis* (20, 25, 27, 48). There are remarkable morphological and biochemical similarities between these species, for example, in the structures of their rRNAs and their cell wall composition (59). The main divergence between the species is the occurrence of different toxins, causing a variable spectrum of disease symptoms (20). *B. cereus* was described as a food-poisoning organism, causing illness due to production of a heat-stable emetic toxin (4, 43) and diarrheal enterotoxins (20, 23).

B. cereus strains, when detected in clinical specimens, were earlier mistaken for accidentally occurring contaminating germs. However, during the last decade they have been identified to an increasing extent as pathogenic agents themselves (5, 32, 57). *B. cereus* can be the cause of severe, even lethal infections such as sepsis, pneumonia, meningitis, endocarditis, or wound infections, especially for patients in an immunocompromised state. Additionally, *B. cereus* is of great importance as the common pathogen for the highly fulminant posttraumatic endophthalmitis (7, 12).

B. cereus strains secrete a wide spectrum of extracellular virulence factors and exoenzymes (18, 20, 32), including an ADP-ribosylating enzyme, phospholipase C, enterotoxins, subtilisin-like proteases, and neutral metalloproteases (bacillolysin) with high homology to thermolysin. Expression of those exoproteins is under the control of the PlcR regulator in the stationary growth phase (3, 18). Hitherto, the participation of the different pathogenic factors and their interactions in nongastrointestinal infections caused by *B. cereus* have not been well-understood, and they remain a subject of intensive investigations.

During the search for bacterial surface proteases, a highly active, cell envelope-bound azocaseinolytic activity was detected in *B. cereus*. The protease was purified in its detergent form. It was homogeneous in mass spectrometry (MS), with a molecular mass of 19,073.1 ± 15 Da, and was called casein-cleaving metalloproteinase or camelysin (16, 17). The protein differs from known extra- and intracellular *Bacillus* proteinases in its N-terminal sequence, substrate specificity, and inhibition pattern. Camelysin preferentially cleaves peptide bonds in front of aliphatic hydrophobic amino acids and hydrophilic amino acid residues, avoiding bulky aromatic residues in the P_1' position, and is therefore almost completely unable to release chromogenic and fluorogenic groups from this position (17). Camelysin belongs to the neutral metalloproteases, showing their typical strong inhibition by metal chelators (16), but it is insensitive to phosphoramidon or zincov, which are the strongest inhibitors of neutral metalloproteinases of the thermolysin-type (clan MA) (47).

Bacterial surface proteases were detected and characterized for some gram-positive species (8, 40) and gram-negative species (26, 54), often playing a role as important virulence factors through interactions with the host defense and blood coagulation systems and by destruction of extracellular matrix proteins. Significant examples are the cell wall-anchored, complement C5a-cleaving peptidase from group B streptococci (8), the plasminogen-activating outer membrane proteases (OmpT) (54), and some members of the serine protease autotransporters (26, 58) in gram-negative bacteria. Camelysin from *B. cereus* could have a similar significant role in host-pathogen interactions, because it cleaves serum protease inhibitors, collagen type I, fibrin, and fibrinogen and causes a partial activation of human plasminogen (17).

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This study describes the novel cell surface-bound metalloprotease, CalY, that might be involved in pathogenicity of *Bacillus* species such as *B. cereus* and *B. anthracis*.

MATERIALS AND METHODS

Chemicals. Fractogel TSK butyl 650 (M) and calibration proteins VIII for the sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (ovalbumin, 42.7 kDa; glutamate dehydrogenase, 56 kDa; ovotransferrin, 78 kDa; phosphorylase *b*, 97.4 kDa; and β-galactosidase, 116.3 kDa) were obtained from Merck (Darmstadt, Germany), sulfobetain SB-12 (*N*-dodecyl-*N*,*N'*-dimethyl-3-ammonio-1-propanesulfonate) was obtained from Serva (Heidelberg, Germany), and peroxidase-labeled anti-rabbit immunoglobulin G was obtained from Boehringer (Mannheim, Germany). Complete and incomplete Freund's adjuvants as well as the fluorogenic protease substrates were manufactured by Calbiochem (Bad Soden, Germany). *Escherichia* phospholipids were obtained from Avanti Polar Lipids (Alabaster, Ala.). All other chemicals were of the highest purity available.

Restriction endonucleases and DNA-modifying enzymes were purchased from Roche or MBI Fermentas (St. Leon-Roth, Germany). The Expand high-fidelity PCR system was from Roche (Mannheim, Germany). DNA fragments and PCR products were purified after electrophoresis from agarose gels with QIAquick spin columns (Qiagen, Hilden, Germany). DNA sequencing was performed with the dRhodamine termination cycle sequencing ready reaction kit from Perkin-Elmer (Colonia, Germany) and with the SequiTherm EXCEL II Long-Read DNA sequencing kit-ALF (Epicentre Technologies, Madison, Wis.). The GPS-1 priming system was from New England Biolabs (Frankfurt/Main, Germany).

Bacterial strains and media. *E. coli* strain XLI Blue (Stratagene, Amsterdam, The Netherlands) was used for initial cloning. Plasmid pBS Bluescribe SK (pBSK) was from Stratagene, and plasmid pGEM-T Easy was from Promega (Mannheim, Germany). *Bacillus* suicide integration vector pBGSC6 was from the Bacillus Genetic Stock Center (Ohio State University) (BGSC no. ECE22).

B. cereus (strain DSM 14729) was cultivated in a laboratory fermentor (Biostat S; Braun, Melsungen, Germany) in a yeast extract medium (1% yeast extract) to the mid-logarithmic phase as described in detail recently (17). Freshly harvested cells were washed several times with an excess of saline (0.5%, wt/vol), adjusted to a dry weight of 100 mg/ml in Tris-HCl buffer (pH 7.5, 50 mM) (buffer A), and solubilized by treatment with an equal volume of 8% (wt/vol) sulfobetain SB-12 at room temperature for 1 h with shaking (100 rpm). This solubilized material was used for the further purification (17).

In order to determine the protease expression profile, cultures were cultivated with shaking (150 rpm, 32°C) in yeast extract medium, glucose-yeast extract medium (0.1% yeast extract, 1% glucose), and glucose medium (1% glucose). Samples were taken hourly, and the proteolytic activities of intact cells and of the medium were determined.

E. coli strains (XLI Blue; Stratagene) were cultivated on Luria-Bertani (LB) plates or in LB broth containing the appropriate antibiotic (49).

Cloning of the camelysin-coding *calY* gene. Chromosomal DNA was isolated from *B. cereus* as described elsewhere (41). Degenerate primers were generated from the four previously sequenced peptides of mature camelysin (17): peptide 1 (proximal to the N terminus, NNTFAAGTLDL), peptide 2 (near the N terminus, TLNPKTLVD), peptide 3 (internal, GDNAGEDFGK), and peptide 4 (internal, FLWNWDK). These sequences were reverse translated to primers T1 (AAYAAYACITTYGCIGCIGGIACITTRGAYYT), T2 (GAYYTIACIYTIA AYCCIAARACIYTIGTIGA), P2 (YTTRTCCCARTTCCAIARRAA), and P1 (TTICCRAARAARTCYTCICCIGCRTTRTCICC). PCR was performed with primer combinations T1-P1 and T2-P2. The resulting amplicons were cloned into the pGEM T-Easy vector for sequencing.

Extension fragments with known DNA sequences were obtained by PCR. In short, size-selected genomic DNA fragments were ligated to a plasmid vector (pBSK), and the ligation mixture was used as template in a PCR, where the region between the vector and the already known region of the target gene was amplified. For that purpose, one primer homologous to the vector was kept constant and primers in opposite directions derived from the known part of the target gene were used in order to obtain both the unknown upstream and the downstream sequences. Total *B. cereus* DNA (10 μg) was digested with *Hin*dIII overnight at 37°C. The digested DNA was purified from buffer and uncut DNA by using Qiagen spin columns and ligated into the *Hin*dIII site of pBSK Bluescript vector. The ligation mixture was used as a template for inverse PCR (36) with a T7 promoter primer (GTAATACGACTCACTATAGGGC) and an up primer (TTAACGAACCGCTGTTTTGTAATAAGAACT) or a down primer (TGCAAAAGGTGATAATGCTGGTGAAG) generated from sequences T1 and P1, respectively. The resulting amplicons were cloned into the pGEM T-Easy vector for sequencing.

DNA and RNA techniques. Recombinant DNA methods, including restriction endonuclease digestion, ligation, and transformation, were performed according to standard protocols (49). Plasmid DNA was purified by using the Spin miniprep kit (Qiagen) according to the manufacturer's instructions.

Plasmid DNA was sequenced by the dideoxy chain termination method (50) at the University of Halle Biology Department sequencing core facility. For sequencing into downstream regions of the 243-bp T1-P1 fragment, the GPS priming system was used according to the manufacturer's instructions. DNA sequence analysis was performed with Clone Manager 4.0 (Scientific & Educational Software).

RNA was prepared as described by Grosse et al. (22). The transcriptional start point was determined by primer extension analysis as described by Grass et al. (21).

Generation of a *calY* disruption mutant. An internal region of *calY* was PCR amplified with primers ATA$\underline{\text{CTGCAG}}_{398}$CAGCTGGGACGTTAGACCTT ACAT (forward) and TCT$\underline{\text{CTGCAG}}_{719}$GCTGCTAATCCACCCTTTTCTCC (reverse) (*Pst*I sites are underlined and numbers indicate the position in *calY*) and cloned into plasmid pBGSC6. The disruption plasmid pBGSC6::*calY'* was introduced into *B. cereus* (strain DSM 14729) by electroporation (Gene Pulser; Bio-Rad) according to the protocol of Xue et al. (60) for *Bacillus subtilis*. After homologous recombination, *calY*::*cat* transformants were selected on LB plates containing chloramphenicol (20 μg/ml) and further cultivated in the presence of antibiotic.

Transfer of CalY from bacterial cells to liposomes. To obtain liposomes, 20 mg of *E. coli* phospholipids was dissolved in chloroform, dried at 35°C on a rotary evaporator, and resuspended with vigorous shaking in 2 ml of buffer A (50 mM Tris-HCl, pH 7.5). Liposomes were extruded to an average diameter of 200 nm by 20 passages through polycarbonate filters with defined pores (Liposofast Basic; Avestin Inc., Ottawa, Canada). A volume of 0.5 ml of these liposomes was added to the same volume of freshly harvested *B. cereus* cells, which had been cultivated to the exponential phase of growth, washed several times with buffer A, and adjusted to 100 mg (dry weight)/ml. The liposome-cell mixture was shaken for 1 h at 200 rpm and 22°C. Cells and liposomes were separated again by sucrose gradient centrifugation. Gradients (0 to 30% [wt/wt] sucrose in buffer A) were formed by using a High-Load system (Pharmacia, Freiburg, Germany), loaded with the cell-liposome mixture, and centrifuged until the sedimentation equilibrium was reached (80,000 × *g*, 18 h, SW 28 rotor [Beckman, Munich, Germany]). Since the bacterial cells formed a pellet at the bottom, the gradient was fractionated (1-ml fractions) from the top to the bottom and the density, protein concentration, phosphorus content (13), and proteolytic activity of each fraction were determined. As a negative control, a similar procedure was performed in parallel with *B. cereus* cells mixed with 0.5 ml of buffer A instead of liposomes.

Density gradients for cell envelope separation. Washed cell envelopes, obtained by cell disruption with glass beads as described previously in detail (16), were adjusted to a protein concentration of 20 mg/ml. They were fractionated with a continuous sucrose gradient (0 to 60% sucrose, dissolved in buffer A). Fractions (1.5 ml) were taken from the bottom and analyzed for their density, diaminopimelic acid content (39) and activities of succinate dehydrogenase, NADH dehydrogenase (29), and protease (17).

Generation of antibodies. Rabbit antisera specific for flagellin or camelysin were raised in rabbits by repeated subcutaneous injection of flagellin or camelysin in Freund's adjuvant. Camelysin was purified as described previously (17). Flagellin was extracted with guanidine hydrochloride according to the procedure for surface proteins from the outer side of intact cells (51), dialyzed against distilled water, and separated by semipreparative SDS electrophoresis. The band corresponding to flagellin (determined by N-terminal sequencing) was excised and electroeluted (Electro-Eluter; Bio-Rad, Munich, Germany).

SDS electrophoresis. Gradient fractions were analyzed by SDS electrophoresis on discontinuous Tris-glycine gels (10%) (33) after delipidation (46). Gels were blotted on polyvinylidene difluoride membranes and stained with colloidal gold according to the instructions of the manufacturer (Bio-Rad). Alternatively, Western blotting was performed with polyclonal antibodies against camelysin or flagellin (dilution of 1:1,000) by a standard method with peroxidase-labeled anti-rabbit immunoglobulin G. The blots were scanned and quantified with the software Phoretix 1D quantifier, version 4 (Phoretix International, Newcastle upon Tyne, United Kingdom).

Determination of proteolytic activity. Proteolytic activity was measured with azocasein as the substrate (10) as proteolytic units (PU) (1 PU = 1 nmol of azocasein cleaved per s at 37°C). N-protected substrates of the AMC (aminomethylcoumaride) type with more than one amino acid (SLLVTM [Suc-Leu-

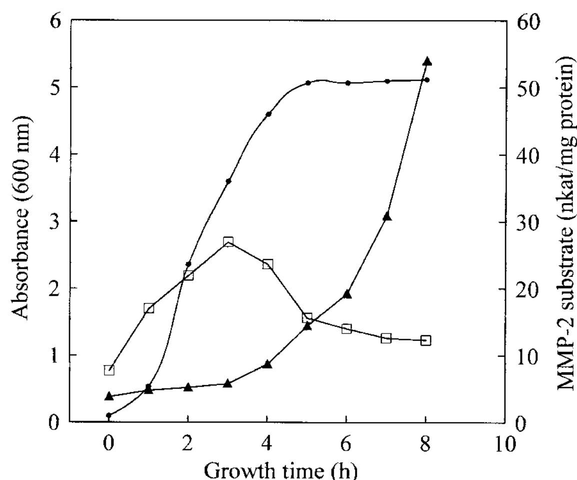

FIG. 1. Expression of cell-bound camelysin during growth on yeast extract medium. Bacteria were cultivated in a laboratory fermentor. Aliquots of the culture (100 ml) were taken hourly, medium and bacteria were separated by centrifugation, and the bacteria were washed several times with saline. The proteolytic activity was determined with the MMP-2 substrate and calculated as specific proteolytic activity (nanokatals per milligram of protein). ●, absorbance of the culture at 600 nm; □, cell-bound activity; ▲, proteolytic activity in the medium.

Leu-Val-Tyr-AMC]) were tested in a coupled assay with an excess of aminopeptidase M (λ_{ex}, 365 nm; λ_{em}, 440 nm) (2, 17). Cleavage of the internally quenched Mca [(7-methoxycoumarin-4-yl)acetyl] substrate of matrix metalloprotease 2 (MMP-2) {Mca-Pro-Leu-Ala-norvaline-Dpa [*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2) was also measured (λ_{ex}, 328 nm; λ_{em}, 393 nm) (17, 30). The protein content was determined by the bicinchoninic acid microassay according to the instructions of the manufacturer (Perbio Science, Bonn, Germany).

Determination of bound zinc atoms and peptide mass fingerprint. Camelysin, purified as described recently (17), was loaded onto a reverse-phase column (125- by 2-mm Nucleosil 500-5 C_3 PPN; Macherey-Nagel, Düren, Germany) and eluted at a flow rate of 0.2 ml/min with a gradient ranging from 0 to 60% eluent B (eluent A, 0.1% trifluoroacetic acid [TFA] in water; eluent B, 0.08% TFA in acetonitrile) for 40 min (HP1100 high-pressure liquid chromatography [HPLC] system; Agilent Technologies, Böblingen, Germany). The major protein peak at 27.2 min was collected, and the molecular mass of the protein was determined by matrix-assisted laser desorption–ionization time-of-flight (MALDI-TOF) MS (matrix, sinapinic acid [REFLEX II; Bruker Daltonik GmbH, Bremen, Germany]). Its zinc content was analyzed by graphite furnace atomic absorption spectroscopy (AAS5 Solid; Analytik Jena AG, Jena, Germany) after pyrolysis (500°C) at a wavelength of 213.9 nm. Additionally, the protein fraction was dried, dissolved in 50 mM ammonium bicarbonate, and cleaved with trypsin overnight at 37°C (protease/protein ratio, 1:10 by mass). An aliquot of the tryptic digest (1 μl) was spotted onto a thin layer of α-cyano-4-hydroxycinnamic acid matrix, washed three times with 0.1% TFA, and characterized by MALDI-TOF MS. The masses of the cleavage peptides were calculated with the Peptide Mass software from the ExPASy server (http://us.expasy.org/tools/peptide-mass.html) on the basis of the possible cleavage sites for trypsin in the camelysin sequence.

Nucleotide sequence accession number. The nucleotide sequence reported in this article has been assigned GenBank accession no. AJ514407.

RESULTS

Influence of cultivation conditions on camelysin expression. Cell envelopes of *B. cereus* contain a novel neutral metalloproteinase (CalY), which was most highly expressed in the logarithmic phase during growth on complex media and declined rapidly during the transition to the stationary phase (Fig. 1). The amount of proteolytic activity measured with azocasein

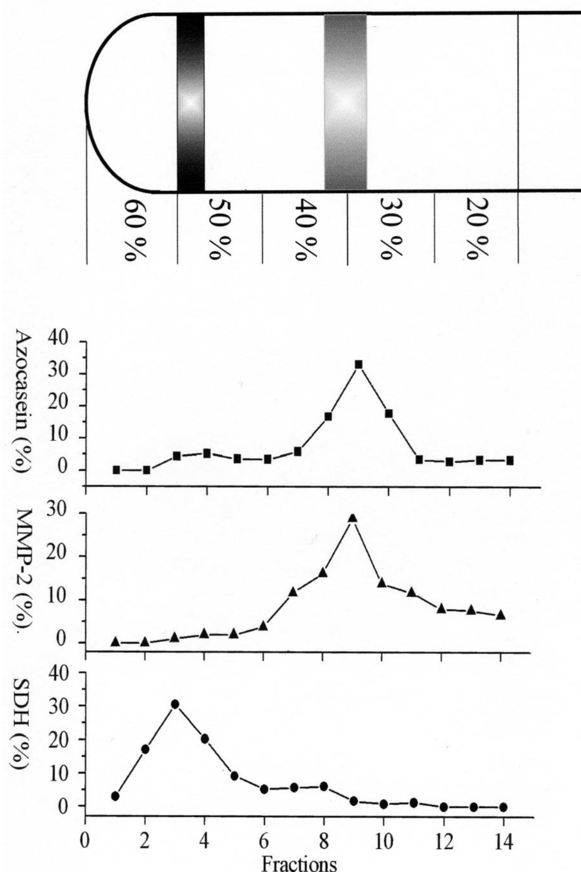

FIG. 2. Density gradient centrifugation of isolated cell envelopes. Two clearly separated bands were obtained in the sucrose density gradient. The lighter band contained the main portion of the proteolytic activity against azocasein and the MMP-2 substrate, and the heavier band contained the cell wall (determination of the diaminopimelic acid) colocalized with the cytoplasmic membranes, identified by the determination of the marker enzymes NADH dehydrogenase (not shown) and succinate dehydrogenase (SDH). The percent distributions of the various enzymatic activities were calculated and plotted.

and the specific synthetic substrate (MMP-2 substrate) depends on the growth phase and the medium composition. Only in the presence of peptides in the medium was the proteolytic activity detectable. In a synthetic medium, containing 1% glucose only, the novel protease could not be detected. A decrease of the peptide content from 1 to 0.1% yeast extract reduced the cell-bound activity in the mid-logarithmic phase from 27 ± 3 to 2.3 ± 0.4 nkat/mg of protein (n = 4; MMP-2 substrate).

Localization of camelysin. Density gradient preparation of isolated cell envelopes yielded two bands with different proteolytic activities (Fig. 2). Determination of diaminopimelinic acid and presence of the marker enzymes NADH dehydrogenase and succinate dehydrogenase identified the band appearing at a higher density as the bacterial cell wall attached to the cytoplasmic membrane.

The other band (lower density) contained the main portion of the proteolytic activity. The major protein thereof was isolated, and its N-terminal sequence was determined as MRIN TNINSMRTQEYMRQNQ, which was completely identical to

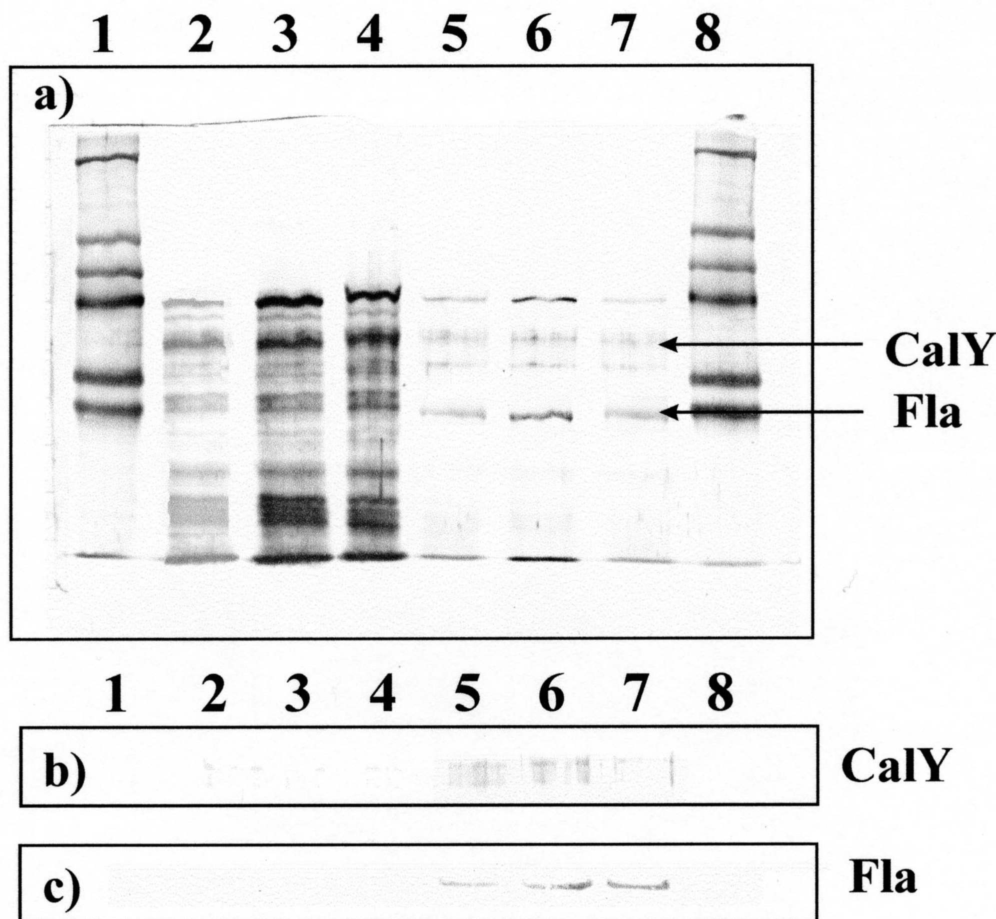

FIG. 3. Localization of camelysin from *B. cereus* and flagellin in the density gradient fractions by SDS electrophoresis and immunoblotting with polyclonal antibodies. (a) Gold staining. Lanes 1 and 8, calibration proteins; lanes 2 to 4, fractions 2 to 4 of the density gradient (heavier band); lanes 5 to 7, fractions 8 to 10 of the density gradient (lighter band). (b) Immunoblot against camelysin. (c) Immunoblot against flagellin.

the N terminus of the S-flagellins FlaA and FlaB of *B. thuringiensis* (35). Fractions of this density gradient band were electrophoretically separated and blotted onto polyvinylidene difluoride membranes. Immunoblot analysis with polyclonal rabbit antibodies against flagellin or camelysin demonstrated the presence of flagellin and camelysin in the lighter band (Fig. 3). Thus, the proteins of the lighter band appeared to consist of surface proteins of the cell envelope, and camelysin was identified as a surface protein of *B. cereus*.

Attachment of camelysin to the cell envelope. When a membrane protein is attached to the cell envelope through a single amino acid sequence (type I membrane protein), a soluble protease-form of the protein in question can be released into the supernatant by proteolytic cleavage of the anchor region and used for further purification, avoiding all of the disturbing effects of detergents. Of course, the proteolytic cleavage can also occur within the catalytic domain, and the biological activity of the membrane enzyme is partially or totally lost (52). We tested various proteases for their effects on the enzymatic activity of camelysin and its release into the supernatant (Table 1). The proteases were then inactivated by class-specific serine or cysteine protease inhibitors. The proteases chymotrypsin and ficin caused a decrease of the casein-cleaving metalloproteinase activity, demonstrating that camelysin is surface exposed and was cleaved within the active region, The other proteases showed only minimal effects. Short amino acid stalks embedded in a membrane are often poorly accessible to proteolytic cleavage (52); therefore, the protease treatment was combined with organic solvents (butanol and toluol), which disturb the membrane integrity. This treatment improved the accessibility of camelysin but also resulted in decreased activity. This demonstrated that the surface protease camelysin is bound to the cell envelope neither through a defined domain nor by a short anchor sequence.

Treatment of the cell envelopes with murein hydrolases and washing with high-ionic-strength buffer did not result in the release of camelysin. However, the enzyme could be extracted with butanol. It is noteworthy that the long-term stability of camelysin was reduced by the butanol exposure. Therefore, butanol extraction is not suitable as an extraction step at the beginning of camelysin purification. Among a spectrum of different detergents, only the zwitterionic detergent sulfobetain SB-12 could solubilize camelysin effectively while preserving its protease activity. Thus, it can concluded that camelysin is not

TABLE 1. Release of proteolytic activity from the cell envelope by cell wall-lytic enzymes, proteases, or organic solvents, determined with azocasein as the substrate

Treatment	Proteolytic activity released into supernatant (% of residual activity)[a]	Residual activity[a,b] (% of activity before treatment)
Wash with high-ionic-strength buffer (3 M NaCl in buffer A)	7.8 ± 0.6	100 ± 6.5
Cell wall-lytic enzymes (1 mg/10 mg of cell envelope protein)		
Lysostaphin	8.9 ± 0.5	100 ± 7.4
Lysozyme	9.0 ± 0.3	100 ± 6.8
Mutanolysin	8.8 ± 0.5	100 ± 7.1
Proteinases (1 mg/100 mg of cell envelope protein)		
Chymotrypsin	12.0 ± 0.5	35.0 ± 1.5
Ficin	11.5 ± 0.4	23.5 ± 1.3
Papain	26.4 ± 0.9	66.2 ± 2.6
Proteinase K	12.5 ± 0.6	60.0 ± 2.7
Subtilisin	6.1 ± 0.3	64.2 ± 2.7
Trypsin	11.0 ± 0.5	80.0 ± 4.2
Organic solvents alone or with trypsin		
Butanol extraction[c]	93.0 ± 4.5	89.1 ± 4.5
Butanol + trypsin	44.2 ± 2.8	10.6 ± 0.4
Toluol + trypsin[d]	23.0 ± 1.2	95.3 ± 5.2
Solubilization with detergents		
Deoxycholate (1% wt/vol)	35.1 ± 2.4	126.5 ± 7.1
Sulfobetain SB-12 (1%, wt/vol)	42.0 ± 2.8	158.3 ± 8.2
Sulfobetain SB-12 (4%, wt/vol)	79.6 ± 3.8	180.8 ± 8.5
Triton X-100 (1%, wt/vol)	16.5 ± 0.9	140.4 ± 7.8

[a] Means and standard errors from three independent experiments.
[b] Sum of activities in supernatant and sediment after treatment.
[c] From reference 19.
[d] From reference 52.

covalently bound to the cell wall but is bound by means of hydrophobic forces.

Transition to liposomes. Some surface-located proteins with hydrophobic properties like those of camelysin can be transferred from their original location to artificial membranes (liposomes or phospholipid mono- and bilayers). Therefore, transfer of camelysin from the cell envelope to liposomes was investigated. Highly active proteolytic liposomes were obtained under the high-osmolarity conditions of a sucrose gradient (Fig. 4). These liposomes were able to cleave azocasein, SLLVTM, and the MMP-2 substrate, while the negative control (spontaneously shed cell envelope proteins, no liposomes) exhibited only low proteolytic activity. Camelysin is capable of spontaneous transfer into membrane bilayers and to be reconstituted as an active protease. This yielded additional evidence that camelysin is a hydrophobic protease which is attached to the cell envelope of *B. cereus* by hydrophobic interactions; to our knowledge, this is a novel property for *Bacillus* cell envelope proteins.

Cloning of the gene for a novel protease, *calY*, from *B. cereus*. To gain more insight into the primary structure and function of CalY, the *calY* gene was cloned and sequenced. Degenerate primers were designed from the amino acid sequence of the N-terminal peptide of CalY (PIR protein database accession number S78771) and those of internal tryptic cleavage peptides. Independent PCRs with various primer pairs yielded a 243-bp DNA fragment (T1P1) and a smaller, 178-bp fragment (T2P2) that was completely encoded within T1P1. These fragments were cloned and sequenced, and new primers for the final cloning step were designed from the sequence of fragment T1P1.

By using these primers in inverse PCRs, a 1,050-bp DNA fragment was amplified from chromosomal DNA of *B. cereus* (Fig. 5). The DNA sequence of this fragment contained an open reading frame that encompassed the N-terminal sequences of CalY and of internal peptide fragments. Thus, full-length *calY* had probably been cloned.

Properties of the *calY* gene and the predicted CalY protease. Upstream of the *calY* reading frame, a potential leader and a potential Shine-Dalgarno sequence were found (Fig. 5). Primer extension analysis identified an mRNA 5′ end for *calY* 113 nucleotides upstream of the GTG start codon. A promoter consensus −10 region (TATAAT) was present in the DNA sequence upstream of this transcriptional start point. Therefore, transcription may be initiated by a sigma 70-dependent RNA polymerase. However, no consensus −35 region was present, and the −10 consensus was only 9 nucleotides apart from the transcriptional start point. (Fig. 5). Downstream of the mature *calY* reading frame, two potential *rho* independent terminators were located, which may terminate transcription of *calY*.

The deduced amino acid sequence of CalY contained a typical signal peptide for translocation consisting of basic amino acid residues in the N-terminal part, a long hydrophobic stretch, and some Gly residues in the C-terminal part (45). The predicted cleavage site of the leader peptidase was between

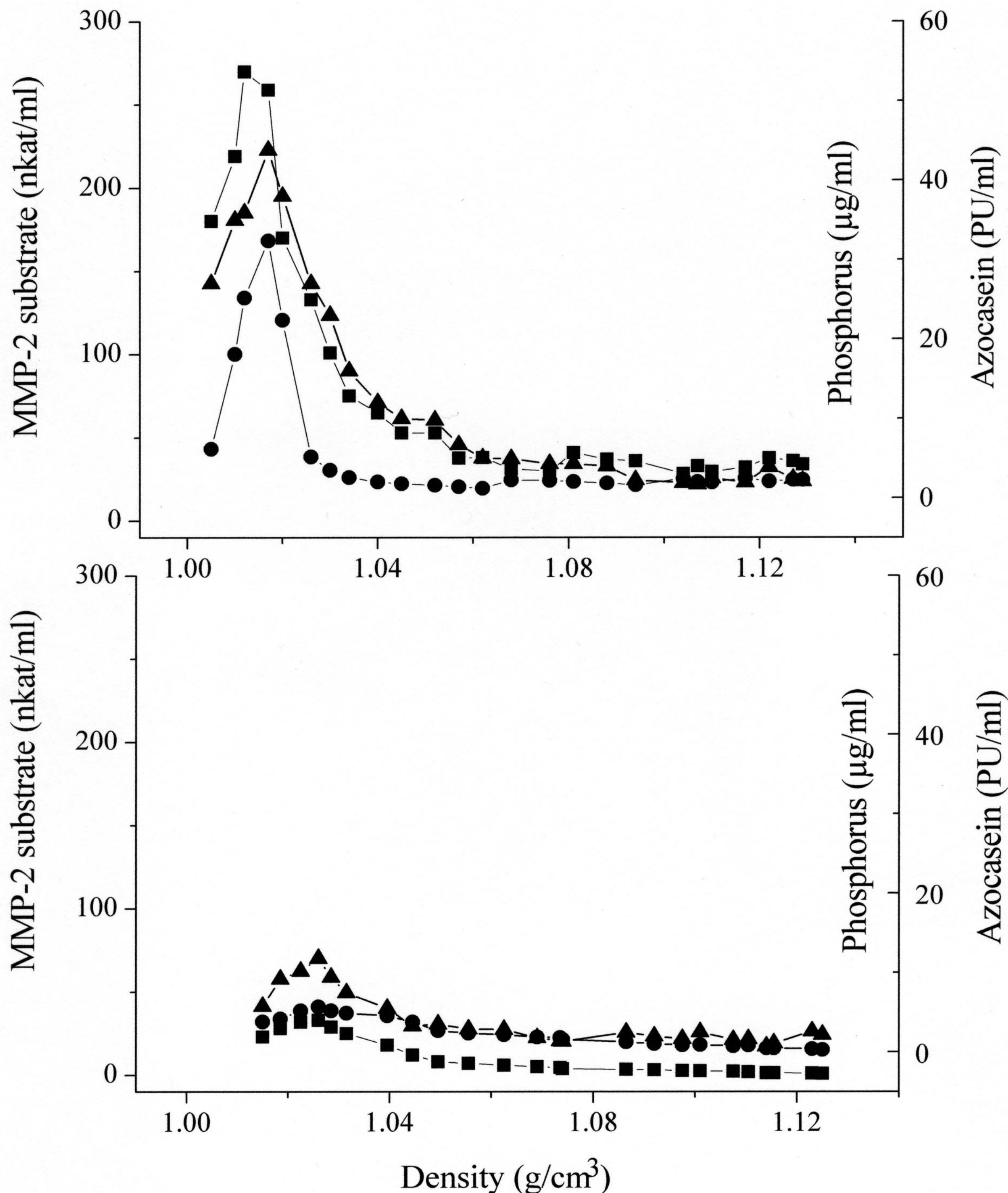

FIG. 4. Transfer of camelysin from intact bacterial cells to preformed liposomes. (a) Incubation of bacterial cells together with liposomes and subsequent separation by density gradient centrifugation. (b) Incubation of bacterial cells with buffer and subsequent density gradient centrifugation under the same conditions (blank). ●, azocasein cleavage; ■, cleavage of MMP-2 substrate; ▲, phosphorus content.

positions 27 and 28. The N terminus of the mature protein was recently determined by Edman degradation (17). Downstream of a potential ribosomal binding site, the start of the translation might be encoded by the alternative start codon GTG (Fig. 5). Similar initiation codons for aliphatic hydrophobic amino acids are sometimes used for other *B. cereus* proteins to code for Met (27).

A hypothetical protein encoded on the *B. anthracis* genome (BA_1819; accession no. NP_655179) (http://ftp.tigr.org/tdb/mdbcomplete.html) was almost completely identical (94% identity) to the sequence for the mature camelysin (not shown). Similarities to other known proteins obtained from the SIB BLAST Network Service (http://us.expasy.org/tools/blast/) encompassed those for the spore coat-associated protein from

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1     CTTTGTTTTT CTTTTTTCTT ATAGTATTAA ATGATTTGAG TGTGAAAAAA GTTATAAATT
61    ATCATCATTT TTTTCTTGAA AAGTCTAAAT AATATTGAGA AATAAAAATA ACTGAAAATA
121   TTAATAAATA TGTGTTGTGT TTATATAGGG TTGTTCGTTA TAATGAACAT AAGGTTTTTA
181   AAAAAGAACA CATATTAGCT AAGCTAATAT AGTTTTCTTT ACATCTCTTA TTGAAAAATA

241   AGTGATAAAA ATATAAAAAA AAGCTAGGGG GAATTGATTG TGAGTCTGAA AAAGAAATTA
                                                   V  S  L  K  K  K  L

301   GGTATGGGAG TTGCATCAGC AGCATTGGGG TTAGCTTTAA TTGGTGGAGG AACATTTGCA
        G  M  G  V  A  S  A  A  L  G  L  A  L  I  G  G  G  T  F  A

361   TTCTTTAGCG ATAAAGAAGT ATCAAACAAT ACATTTGCAG CTGGGACGTT AGACCTTACA
        F  F  S  D  K  E  V  S  N  N  T  F  A  A  G  T  L  D  L  T

421   TTAAACCCTA AGACGCTTGT AGATATTAAA GATTTAAAAC CAGGGGATTC TGTTAAGAAA
        L  N  P  K  T  L  V  D  I  K  D  L  K  P  G  D  S  V  K  K

481   GAGTTCTTAT TACAAAACAG CGGTTCGTTA ACTATTAAAG ATGTTAAATT AGCAACAAAG
        E  F  L  L  Q  N  S  G  S  L  T  I  K  D  V  K  L  A  T  K

541   TACACTGTTA AAGATGCAAA AGGTGATAAT GCTGGTGAAG ACTTTGGTAA GCACGTTAAA
        Y  T  V  K  D  A  K  G  D  N  A  G  E  D  F  G  K  H  V  K

601   GTAAAATTCC TTTGGAACTG GGATAAACAA AGTGAGCCTG TATACGAAAC AACTTTAGCA
        V  K  F  L  W  N  W  D  K  Q  S  E  P  V  Y  E  T  T  L  A

661   GACTTACAAA AAGTTGATCC AGATCTTTTA GCTAAAGACA TCTTTGCTCC TGAGTGGGGA
        D  L  Q  K  V  D  P  D  L  L  A  K  D  I  F  A  P  E  W  G

721   GAAAAGGGTG GATTAGCAGC TGGTACAGAG GATTATCTAT GGGTACAATT TGAATTTGTA
        E  K  G  G  L  A  A  G  T  E  D  Y  L  W  V  Q  F  E  F  V

781   GATAATGGAA AAGATCAAAA TATCTTCCAA GGTGATACAT TGAATTTAGA ATGGACATTC
        D  N  G  K  D  Q  N  I  F  Q  G  D  T  L  N  L  E  W  T  F

841   AATGCTAACC AAGAAGCTGG GGAAGAAAAA TAATAAAAAA AGCGGGTATC CCCCGCTTTT
        N  A  N  Q  E  A  G  E  E  K  -  -
                                       <------------------     ----------

901   TTTATAAAGA AAAAGAAGTG CTACTTGTAA GCACTTCTTT CTATTTATTA TTTTGCTTCC
      ----->       <------------         ------------->

961   ACTTTGTAAA TTCAAGAAAT TCACGAAATT GTTCTTTGGA GACACCAGAG TTCATTGCAT
1021  CTTTAACGAG TTGTGCCCAA TCGGAGTCCA
```

FIG. 5. Nucleotide sequence of the *calY* region. The amino acid sequences of the N terminus (double lines) and of the tryptic cleavage peptides (single line) of the mature camelysin are underlined. Asterisks indicate stop codons. The transcription start, identified by primer extension (bp 168), is in boldface. Arrows indicate inverted repeats.

Bacillus halodurans (Q9KB07; 31% identity) and the sporulation-inhibiting protein from *B. subtilis* (COTN_BACSU; SwissProt entry P54507; 28% identity).

The mature camelysin was predicted to be a protein of 170 amino acid residues with a molecular mass of 19.056 kDa and a pI of 4.56 (http://www.expasy.ch/cgi-bin/protparam). Except for the signal peptide, no further transmembrane sequences were present in the mature protein, but six alternating stretches of medium hydrophobicity were found (data not shown). The amino acid sequence did not contain a metalloprotease consensus sequence of the HEXXH type (or its inverted derivative) and showed no homology to any known protease.

Peptide mass fingerprint and zinc determination. Purified camelysin contained Zn^{2+} ions in an equimolar ratio as shown by reverse-phase HPLC-MS and graphite furnace AAS of the HPLC fraction containing the protein (data not shown). A peptide mass fingerprint of the Zn^{2+}-containing protein cor-

TABLE 2. Peptide mass fingerprint of the Zn^{2+}-containing HPLC peak[a]

Mass (Da)	Position	Peptide sequence
3,011.365	145–170	DQNIFQGDTLNLEWTFNANQEAGEEK
2,629.314	1–24	FFSDKEVSNNTFAAGTLDLTLNPK
2,416.145	123–144	GGLAAGTEDYLWVQFEFVDNGK
2,005.024	6–24	EVSNNTFAAGTLDLTLNPK
1,791.985	41–56	EFLLQNSGSLTIKDVK
1,756.021	25–40	TLVDIKDLKPGDSVKK
1,721.859	90–104	QSEPVYETTLADLQK
1,577.890	40–53	KEFLLQNSGSLTIK
1,449.795	41–53	EFLLQNSGSLTIK
1,235.657	81–89	VKFKWNWDK
1,191.568	113–122	DIFAPEWGEK
1,008.494	83–89	FLWNWDK
870.493	105–112	VDPDLLAK

[a] Camelysin was eluted from a reverse-phase column by an acetonitrile gradient. The mass and the zinc content of the main protein peak were determined. The zinc-containing peak was digested with trypsin, and the cleavage peptides were identified by MALDI-TOF MS and analyzed with the Peptide Mass software.

responded well to the amino acid sequence determined for camelysin (Table 2). Therefore, camelysin was identified as a zinc metalloprotease.

Phenotype of mutant strains containing a disrupted *calY* gene. To verify the camelysin-encoding gene, a disruption plasmid for the *calY* gene was generated and introduced into the chromosome of *B. cereus*, yielding a *calY*::*cat* insertion mutant. Cells from the *B. cereus* wild-type strain and from the disruption mutant were grown in 1% yeast extract medium to the mid-logarithmic phase. The bacteria were washed several times to remove loosely bound extracellular components, adjusted at the same density (dry weight), and tested for their proteolytic activity with various substrates. The enzymatic activity (nanokatals per milliliter) was divided by the protein content, yielding the specific proteolytic activity (nanokatals per milligram of protein or PU per milligram of protein). The cell-bound activity of the mutant strain was significantly decreased to a residual activity of 14 to 18% for the azocasein, SLLVTM, and MMP-2 substrates (Fig. 6). This final evidence clearly demonstrated that the camelysin activity was indeed encoded by the *calY* gene. Moreover, it could be shown that camelysin is not essential for growth of *B. cereus* under the conditions tested.

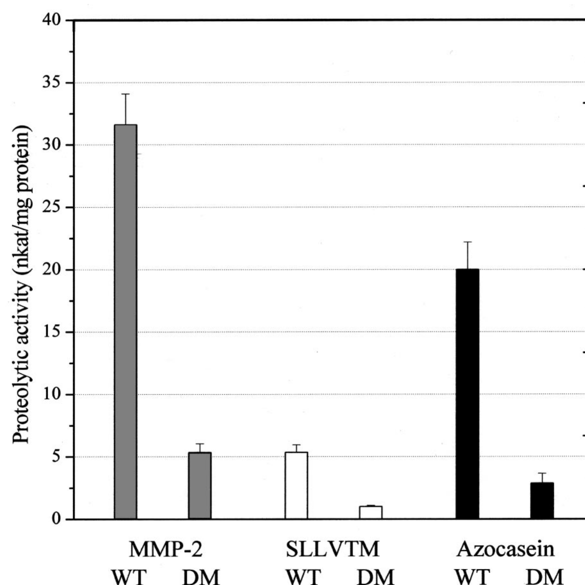

FIG. 6. Effect of a *calY* disruption mutation on the cell-bound proteolytic activity of *Bacillus cereus*. Bacteria were cultivated at 32°C on yeast medium with shaking (150 rpm) to the mid-logarithmic phase. Medium and bacteria were separated by centrifugation, and the cells were washed repeatedly. Activities with the MMP-2 and SLLVTM substrates are in nanokatals per milligram of protein; activity with azocasein is in PU per milligram of protein. WT, wild-type strain; DM, *calY* disruption mutant strain. Error bars indicate standard errors of the means.

DISCUSSION

Surface proteins of gram-positive bacteria have been described to be attached to the cell envelope by five major mechanisms (i) by covalent linkage to the cell wall, (ii) noncovalently to peptidoglycan by SLH domains (S-layer proteins), (iii) by electrostatic interactions with cell wall components (often teichoic acids), (iv) by lipoprotein modification, or (v) by a transmembrane stretch of hydrophobic amino acids (44, 51, 53). Most of these binding mechanisms are characterized by specific structural features that can be identified in the amino acid sequence of the proteins (11). Surface proteins of *Bacillus* species are reported to interact by electrostatic contacts with the murein layer, often over tandem repeat domains (44, 55). Electrostatically bound surface proteins should be released by washing procedures with high-salt medium and by cell wall-lytic enzymes. However, this was ineffective for camelysin (Table 1). A covalent linkage to cell envelope components can also be excluded, since the molecular mass determined for the purified protein (19,073.1 ± 15 Da) corresponds well with the molecular mass predicted from the gene sequence (19,056 Da). Therefore, carbohydrate or lipid modifications are unlikely. Additionally, the typical recognition domain for the lipoprotein signal peptidase (56) is missing in the primary sequence of camelysin.

In camelysin, only the signal peptide corresponds in its hydrophobicity and length to a transmembrane α-helical segment. The DAS transmembrane prediction server (http://www.sbc.su.sse/-miklos/DAS/tmdas.cgi) identified six domains of alternating hydrophobicity over the residual sequence of the mature protein (data not shown) that were below the limit for transmembrane sequences but could be regions for hydrophobic intra- and intermolecular interactions. Camelysin behaves as a hydrophobic protein. It is strongly bound to the cell envelope and can be solubilized only with high concentrations of zwitterionic detergent and by organic solvents (Table 1). The protease is enriched in the detergent phase as determined by phase separation experiments (15), a typical property of hydrophobic proteins (9). The protease is not necessarily bound to the cell envelope by hydrophobic α-helical segments. There are other possible means of binding to the cell envelope, for example amphipathic α-helices, complex β-sheet structures, facing of hydrophobic sites to the acyl chains of the membrane leaflet, or hydrophobic interactions with other cell envelope proteins.

The protease is freely accessible to its protein and synthetic

peptide substrates without disrupting bacterial cells (16, 17), and it can be solubilized by detergents from outside intact cells (17). This clearly indicated a location of camelysin at the outside of the cell envelope. The transfer of camelysin from intact bacterial cells to preformed liposomes is in this context the most convincing argument favoring camelysin localization at the outside of the cell envelope (Fig. 4). Only a surface-located protein can switch from the bacterial cell envelope to liposomes. A comparable transition of surface proteins to liposomes was described only for outer membrane porins from pathogenic gram-negative bacteria (37). To our knowledge, no similar phenomenon has yet been described for cell envelope proteins of gram-positive bacteria. A surface-located protease can interact with host defense proteins and proteins of the extracellular matrix at a high local concentration without a dilution effect and therefore can facilitate bacterial colonization and invasion into host tissues.

To date, the only *Bacillus*-surface protease characterized is the serine protease WprA (formerly CWBP52). WprA was isolated from vegetative cells of a *B. subtilis* strain. The *wprA* gene was shown to encode a 96-kDa polypeptide with an N-terminal consensus signal sequence. The protein is processed by removal of the middle portion of the precursor protein into two previously described cell wall-binding proteins, CWBP23 and -52, both of which are attached to the cell wall by electrostatic forces and extractable by high salt concentrations (40). Interestingly, *wprA* is expressed during the exponential growth phase, similar to our data for *calY* of *B. cereus.* These patterns differ from that of the secreted, well-characterized proteases from gram-positive bacteria, whose gene expression is generally induced during the stationary phase. Early expression of cell envelope proteases might indicate their involvement in exoprotein degradation during bacterial attachment and colonization before secreted proteases are synthesized for host invasion. Usually pathogenic bacteria synthesize adhesion and colonization factors early in growth prior to secretion of virulence factors that are up-regulated by quorum-sensing mechanisms (14, 42). The significant induction effect caused by peptides from the growth medium on camelysin expression in the cell envelope might mimic gastrointestinal conditions in vitro.

In an early work of Mäkinen and Mäkinen (38), an extracellular, collagenolytic metalloprotease from a periodontitis-causing *B. cereus* strain was purified and its substrate specificity was characterized. Unfortunately, the primary structure of this protease was not determined (38). Kotiranta et al. (31) presumed that a surface-bound collagenase mediates the strong adhesion of *B. cereus* cells to host proteins of the extracellular matrix during the logarithmic growth phase. Beecher et al. (7) suggest the involvement of a hypothetical collagenase in post-traumatic endophthalmitis by means of cleaving the collagen of the lens capsule, facilitating the penetration of the invading bacteria. Jackson et al. (28) used a long-term 2-furanaryloyl-Leu-Gly-Pro-Ala assay for intact cells and demonstrated the presence of a cell envelope-bound collagenolytic activity for several strains of *B. cereus*. The collagenolytic enzymes were classified as metalloproteases, but they were not further purified and characterized. However, it is likely that this cell envelope-bound collagenolytic activity and camelysin are identical enzymes, because CalY can also cleave collagen type I and cleaves FALGPA at a low rate (17).

Within the amino acid sequence of camelysin, no typical zinc binding motif was found, but the HPLC-purified protein contained zinc, and a peptide mass fingerprint clearly showed the identity of this zinc-binding protein as camelysin. The reduced proteolytic activity of the disruption mutant confirms that this purified and sequenced protein is indeed a protease. Lack of the HEXXH consensus motif does not automatically exclude membership of CalY in the zinc metalloprotease family. According to Barrett (6), His, Glu, Asp, and Arg are possible zinc ligands. The sequence motif HEXXH is only common for the metallopeptidase clans MA and MB. In proteases belonging to these clans, the two His residues are regarded as zinc ligands, and Glu is regarded as the catalytic residue. In clan MB, the third zinc ligand is the C-terminal His (or Asp) in the motif His-Glu-Xaa-Xaa-His-Xaa-Xaa-Gly-Xaa-Xaa-His/Asp. There are other known metalloproteases without the HEXXH consensus motif. Examples of this growing and heterologous group are the *O*-sialo-glycoendopeptidase (EC 3.4.24.57) (1), the β-lytic metalloendopeptidase (EC 3.4.24.32) (34), and lysostaphin (EC 3.4.24.75) from *Staphylococcus capitis* and *Staphylococcus staphylolyticus* (24). The absent HEXXH motif and the lack of similarity to other proteases exclude camelysin from known protease families. Since a camelysin-like protease is also encoded by the genome of *B. anthracis*, camelysin might be the first example of a novel metalloprotease family.

The occurrence of a *calY* gene in the genome of the pathogenic bacterium *B. anthracis* indicates that CalY-like proteases might be pathogenicity factors (48). Thus, with CalY, a novel cell surface-bound metalloprotease which might be involved in pathogenicity of *Bacillus* species such as *B. cereus* and *B. anthracis* was identified and characterized. Further studies with cell culture and animal models will provide insight as to whether camelysin is a virulence factor.

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