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A *Bacillus cereus* Cytolytic Determinant, Cereolysin AB, Which Comprises the Phospholipase C and Sphingomyelinase Genes: Nucleotide Sequence and Genetic Linkage

MICHAEL S. GILMORE,[1]* ARMANDO L. CRUZ-RODZ,[1] MICHAELA LEIMEISTER-WÄCHTER,[2] JÜRGEN KREFT,[2] AND WERNER GOEBEL[2]

Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, P.O. Box 26901, Oklahoma City, Oklahoma 73190,[1] and Institut für Genetik und Mikrobiologie der Universität Würzburg, D-8700 Würzburg, 11 Röntgenring, Federal Republic of Germany[2]

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A cloned cytolytic determinant from the genome of *Bacillus cereus* GP-4 has been characterized at the molecular level. Nucleotide sequence determination revealed the presence of two open reading frames. Both open reading frames were found by deletion and complementation analysis to be necessary for expression of the hemolytic phenotype by *Bacillus subtilis* and *Escherichia coli* hosts. The 5′ open reading frame was found to be nearly identical to a recently reported phospholipase C gene derived from a mutant *B. cereus* strain which overexpresses the respective protein, and it conferred a lecithinase-positive phenotype to the *B. subtilis* host. The 3′ open reading frame encoded a sphingomyelinase. The two tandemly encoded activities, phospholipase C and sphingomyelinase, constitute a biologically functional cytolytic determinant of *B. cereus* termed cereolysin AB.

Bacillus cereus, a common soil saprophyte, has been recognized as an opportunistic pathogen of increasing importance (reviewed in reference 41). Although food-borne gastroenteritis is the most common malady attributed to *B. cereus* (41), the most devastating is *B. cereus* endophthalmitis (1, 4, 17). *B. cereus* elaborates a variety of extracellular membrane-active enzymes and cytolytic toxins. These membrane-active proteins include a phospholipase C (34), sphingomyelinase (22), phosphatidylinositol phospholipase C (23), cereolysin (7; a cytolysin of the streptolysin O, thiol-activated class), and a second, heat stabile cytolysin about which little is known (8, 10, 37). Phospholipase C, sphingomyelinase, and cereolysin have been highly purified and used in studies of membrane structure (6, 29, 43) and in studies on the evolution of cytolysins produced by diverse genera of gram-positive bacteria (13, 38). Phospholipase C is a Zn metalloenzyme of 23,000 daltons (34) which shows a high degree of stability in chaotropic agents and surfactants (27, 28). The sphingomyelinase produced by *B. cereus* is a protein of between 41,000 and 23,300 daltons, depending on the method of analysis used (40), and requires divalent cations for activity (21).

As a first step in determining the contribution that extracellular membrane-active proteins make to the ecology and virulence of *B. cereus*, a gene bank was established. Identification of a cloned cytolytic determinant from this *B. cereus* GP-4 library has been reported (25). To gain insight into the relationship of the cloned cytolysin determinant to membrane-active proteins of *B. cereus*, nucleotide sequence and enzyme activity analyses were undertaken. The results reported here show that the cytolytic determinant cloned from *B. cereus* is composed of tandemly arranged genes for two distinct protein products, the activities of both being required to effect target cell lysis (hemolysis as tested). Moreover, the individual cytolysin components possess phospholipase C and sphingomyelinase activity, respectively. These data suggest that although the sphingomyelinase and phospholipase C of *B. cereus* have been studied in detail individually, their function in nature appears to be as a cytolytic unit representing the heat-stabile hemolysin previously observed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in these experiments are listed in Table 1. *Escherichia coli* strains were routinely cultured in 2XYT medium (31) with aeration. *Bacillus subtilis* cultures were grown in HGP broth as previously described (25). Tetracycline (Sigma Chemical Co., St. Louis, Mo.) was incorporated into liquid and solid (1.2% agar) media at concentrations of 10 μg/ml for selection of resistant *B. subtilis* and *E. coli* strains. Ampicillin (Sigma) was used at 100 μg/ml to select for recombinants cloned into the vectors pUC8, -9, -18, and -19 (31, 45). In addition, to screen for insertional inactivation of the LacZa peptide encoded by these vectors, 50 μM isopropyl β-D-thiogalactoside (IPTG; Sigma) and 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; Sigma) or 0.01% Bluo-Gal (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were included in the media.

Large-scale plasmid and M13 bacteriophage replicative-form purifications from *E. coli* cultures were performed as described previously (30). Plasmid DNA was prepared from *B. subtilis* as previously reported (25). Purification of single-stranded M13 phage DNA for sequencing templates was done as described in a previous report (14, 31). *E. coli* and *B. subtilis* strains were transformed by the $CaCl_2$ procedure (30) and by generation of protoplasts (25), respectively.

Cloning conditions and strategies for localizing the cytolysin-encoding genes. Restriction enzymes were obtained from Bethesda Research Laboratories, New England Bio-Labs, Inc. (Beverly, Mass.), and Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used as appropriate

* Corresponding author.

TABLE 1. Bacterial strains, plasmid, and sources

Strain or plasmid	Relevant properties	Source
Strain		
E. coli JM109	*recA1* F *lacI*^{q}-Z M15	J. Messing
E. coli JM110	*dam*	J. Messing
B. subtilis BR151CM1	*spoCM1*	P. Lovett
Plasmid		
pJKK3-1	Tc^r shuttle vector[a]	
pJKK3-1Hly1	Tc^r Hly^{+b}	
pMG32-1	Deletion derivative of pJKK3-1Hly1	This study
pMG8-128	pUC8 clone of CerAB determinant	This study
pMG9-128	pUC9 clone of CerAB determinant	This study
pMG8-9	Deletion derivative of pMG8-128	This study
pMG9-22	Deletion derivative of pMG9-128	This study
pCerA	*cerA* cloned into a deleted pJKK3-1	This study
pCerB	*cerB* cloned into a deleted pJKK3-1	This study
pMLW3	*cerA* cloned into pACYC184	This study
pMLW4	*cerB* expressing deletion of pMG8-128	This study

[a] See reference 26.
[b] See reference 25.

for the three buffer systems described (30). The location of the cytolysin determinant within the cloned *B. cereus*-derived DNA was defined in *B. subtilis* as follows. Plasmid pJKK3-1Hly1 was purified from JM109, which was observed to result in a modification of the vector *Cla*I recognition site but not the *Cla*I site contained within the *B. cereus*-derived insert (Fig. 1; for an explanation of *dam* methylation of select restriction enzyme recognition sites, see reference 30). This permitted linearization of pJKK3-1Hly1 near one end of the insert. The linearized pJKK3-1Hly1 was then partially digested with *Hpa*II under time-limited conditions, which yielded one to two additional cleavages per molecule. Compatible *Hpa*II and *Cla*I ends were ligated and used to transform protoplasts of *B. subtilis* as described elsewhere (25). This resulted in a nested set of deletion derivatives lacking portions of the insert (and *E. coli*-derived regions of

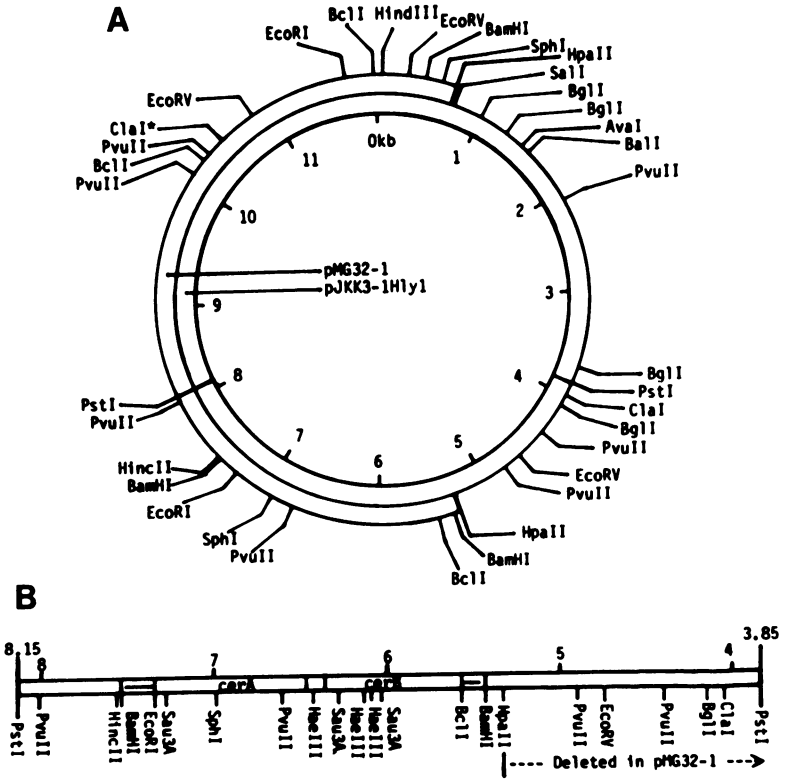


FIG. 1. (A) Expanded physical map of the shuttle vector pJKK3-1 (26) harboring the cytolysin-encoding *B. cereus* genomic insert (pJKK3-1Hyl1 [25]; inner circle). The deletion derivative pMG32-1 (outer arc) retained full cytolytic activity. *, *dam* methylase-sensitive *Cla*I recognition site; the *dam* methylase-resistant *Cla*I site used in pMG32-1 construction is located at map position 4.0. (B) Expanded map of the *B. cereus* cytolysin-encoding insert.

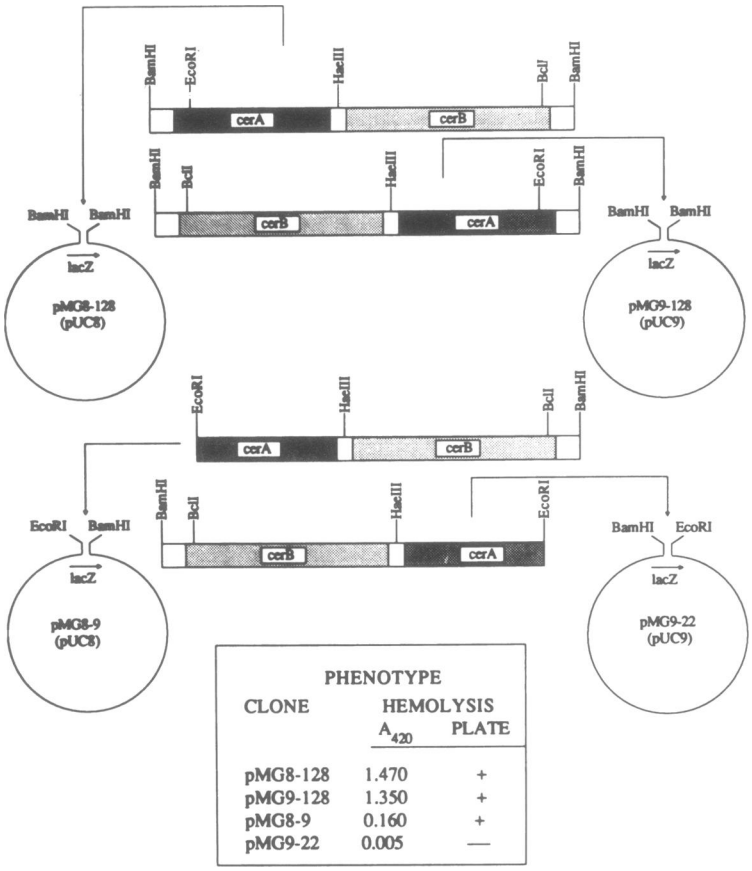


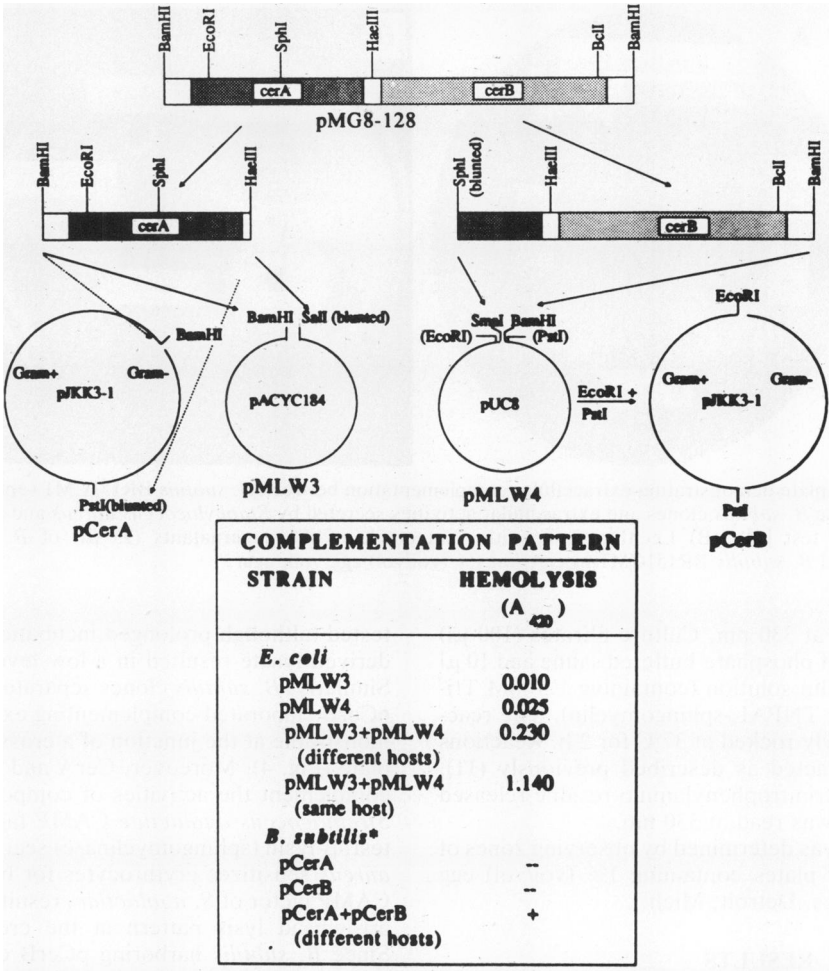
FIG. 2. Construction and deletion analysis of cereolysin AB subclones in *E. coli*.

the shuttle vector pJKK3-1 [26]) centered around the unmodified *Cla*I recognition site. The smallest derivative conferring the hemolytic phenotype to the *B. subtilis* host, pMG32-1, had a deletion spanning map positions 0.6 to 5.4 kilobase pairs (kb) (Fig. 1).

Since the 2.1-kb *Bam*HI DNA fragment contained within the original *B. cereus* insert was preserved in the deletion derivative pMG32-1, this *Bam*HI fragment was cloned directly into pUC8 (resulting in the construction pMG8-128; Fig. 2). Transformants harboring pMG8-128 were observed to be fully hemolytic, as were transformants harboring the 2.1-kb *Bam*HI fragment in the opposite orientation. Deletion of an additional 200 base pairs (bp) fusing the internal *Eco*RI site of pMG8-128 within the insert to that of the vector (yielding pMG8-9; Fig. 2) resulted in slightly hemolytic transformants. Inversion of the pMG8-9 *Eco*RI-*Bam*HI fragment by subcloning into pUC9, or filling in the *Eco*RI cohesive end in pMG8-9, resulted in loss of the hemolytic phenotype. Deletion of the 160 bp between the distal *Bam*HI and *Bcl*I sites resulted in nonhemolytic transformants in either orientation.

The observation in nucleotide sequence analyses (described below) of two complete, tandem open reading frames within regions of the *B. cereus* cytolysin determinant found to be essential for hemolytic activity suggested that the activities of two dissimilar proteins were required to effect lysis of target cells. It was therefore of interest to separately clone each open reading frame and ascertain the contribution of each to cytolysis. To provide consistent descriptive nomenclature, and since the activities of each function remained to be determined, the cytolysin was referred to as cereolysin AB, *cerA* representing the 5′ open reading frame and *cerB* representing the 3′ open reading frame. Compatible plasmids harboring *cerA* and *cerB* individually for complementation analysis in *E. coli* were constructed as follows. Construction pMG8-128 (pUC8 containing the 2.1-kb *B. cereus*-derived *Bam*HI fragment; Fig. 2) was cleaved within the vector multiple cloning site with *Sma*I and within the *cerA* open reading frame with *Sph*I. The protruding 3′ *Sph*I terminus was blunted with T4 DNA polymerase, and the flush ends were ligated. Transformants containing this construction, pMLW4, including all of the *cerB* gene and only downstream portions of the *cerA* gene, were nonhemolytic, as expected. The intact *cerA* open reading frame was cloned as a *Bam*HI-*Hae*III fragment into *Bam*HI-*Sal*I-cleaved pACYC184 (30) after the *Sal*I protruding end was filled with the Klenow fragment of DNA polymerase I. This construction, pMLW3, harboring the entire *cerA* open reading frame, also failed to confer the hemolytic phenotype to *E. coli*.

To study expression in *B. subtilis*, pMLW4 (containing the *cerB* open reading frame; Fig. 3) was digested with *Eco*RI and *Pst*I within the flanking vector multiple cloning site, and the *cerB*-containing fragment was ligated to similarly digested pJKK3-1 (yielding the nonhemolytic construction pCerB). The *cerA* open reading frame was introduced as a *Bam*HI-*Hae*III fragment of pMG8-128 (as was done for pMLW3) into *Bam*HI-*Pst*I-digested pJKK3-1 after the *Pst*I end was blunted with S1 nuclease and polished with the Klenow fragment of DNA polymerase I. The resulting



COMPLEMENTATION PATTERN

STRAIN	HEMOLYSIS (A_{420})
E. coli	
pMLW3	0.010
pMLW4	0.025
pMLW3+pMLW4 (different hosts)	0.230
pMLW3+pMLW4 (same host)	1.140
B. subtilis*	
pCerA	–
pCerB	–
pCerA+pCerB (different hosts)	+

FIG. 3. Construction of subclones separately harboring the *cerA* and *cerB* open reading frames for complementation analyses in *E. coli* and *B. subtilis*. Compatible plasmids pMLW3 and pMLW4 were constructed for complementation in *E. coli*. Plasmids pCerA and pCerB were constructed for complementation analysis in *B. subtilis* by displacing portions of the shuttle vector pJKK3-1 (26) related to function in the alternate gram-negative host as shown. * Because of the efficient secretion of *cerA* and *cerB* by *B. subtilis*, complementation analyses were performed on blood agar plates (see Fig. 4).

plasmid, pCerA, conferred a nonhemolytic, egg yolk-positive phenotype to *B. subtilis*.

Nucleotide sequencing strategy. A novel strategy was used to obtain nested sets of deletion derivatives from the universal priming sites of M13mp8, -9, -18, and -19 (14, 31, 45) for nucleotide sequence determination by the chain termination method (36). A detailed description of this strategy applied to the tandem *B. cereus* cytolysin genes, as well as partial *cerA* nucleotide sequence, has been reported (14). Both strands of the cloned cytolysin determinant were sequenced, and the accuracy of the nucleotide sequence obtained was additionally confirmed by sequence determinations performed from restriction sites located throughout the open reading frames.

Assays for the activities of the cytolysin components. Hemolysin assays of *B. subtilis* culture supernatants were performed as described previously (25). Because of the observed cell association of one or both components of the *B. cereus* cytolysin when expressed by transformed *E. coli* cells, hemolysin assays of these strains were performed with whole cultures. In this case, 0.5-ml portions of *E. coli* cultures were transferred to microcentrifuge tubes containing 0.5 ml of 4.0% washed human erythrocytes in phosphate-buffered saline plus 100 μg of chloramphenicol per ml to block additional protein synthesis. The hemolysis reaction was incubated at 37°C for 1 h, and the reaction supernatant was cleared by 30 s of centrifugation in an Eppendorf microcentrifuge. A 0.8-ml portion of the cleared supernatant was transferred to a semimicro-cuvette, and hemoglobin release was measured at 420 nm as described previously (25). For determination of hemolysin activities in cell lysates, cells from 10 ml of an overnight culture were collected by centrifugation, suspended in 1.0 ml of phosphate-buffered saline, and sonicated with a Sonifier (Branson Sonic Power Co., Danbury, Conn.) and microprobe at maximum output 10 times for 10 s each on ice. The hemolysin assays were conducted by mixing 0.1 ml of 10× phosphate-buffered saline, 0.1 ml of 10% washed human erythrocytes, 0.1 ml of *E. coli* lysate, and 0.7 ml of distilled water. Hemoglobin release was measured at 420 nm as described previously (25).

Sphingomyelinase activity was assayed as described elsewhere (11). Briefly, culture filtrates derived from *B. subtilis* or *B. cereus* were assayed for the ability to hydrolyze the sphingomyelin analog *N*-ω-trinitrophenylaminolaurylsphingosylphosphoryl choline (TNPAL-sphingomyelin; Sigma)

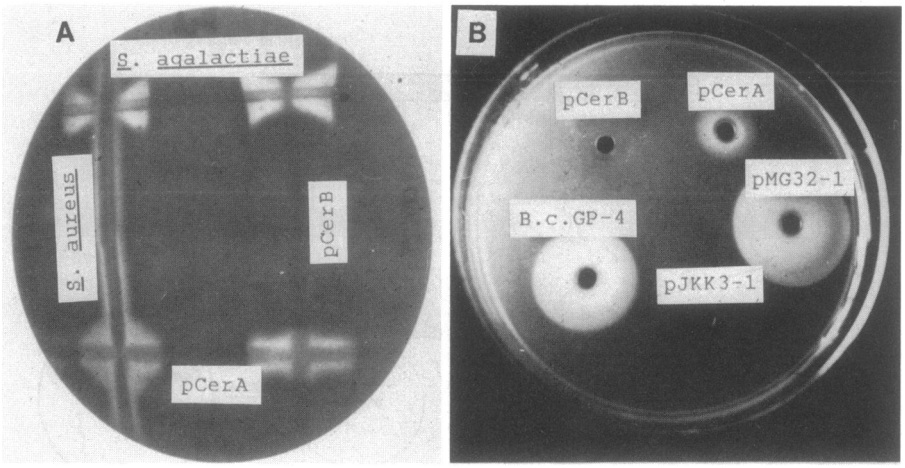


FIG. 4. (A) Blood agar plate demonstrating extracellular complementation between *B. subtilis* BR151CM1 separately harboring *cerA* and *cerB* genes and between the *B. subtilis* clones and extracellular activities secreted by *Staphylococcus aureus* and *S. agalactiae* (components of the diagnostic CAMP test [2]). (B) Lecithinase activities detectable in the supernatants (25 μl) of *B. cereus* GP-4, *B. subtilis* BR151CM1(pMG32-1), and *B. subtilis* BR151CM1(pCerA) on 1% (vol/vol) egg yolk agar.

spectrophotometrically at 330 nm. Culture filtrates (100 μl) were mixed with 90 μl of phosphate-buffered saline and 10 μl of TNPAL-sphingomyelin solution (containing 1.5 mM Triton X-100 and 0.3 mM TNPAL-sphingomyelin). The reaction mixtures were slowly rocked at 37°C for 2 h. Reactions were stopped and extracted as described previously (11), and absorbance of the trinitrophenylamino residue released into the organic phase was read at 330 nm.

Lecithinase activity was determined by observing zones of turbidity on HGP agar plates containing 1% (vol/vol) egg yolk (Difco Laboratories, Detroit, Mich.).

RESULTS

Expanded physical maps of pJKK3-1Hly1 (25) and the fully hemolytic deletion derivative pMG32-1 are presented in Fig. 1. Plasmid pMG32-1 suffered a deletion encompassing approximately 1.6 kb of the original *B. cereus* insert and most of the pBR322-derived portion of the shuttle vector (26). Direct cloning of the 2.1-kb *Bam*HI DNA fragment preserved in pMG32-1 into pUC8 and pUC9 (31) (yielding constructions pMG8-128 and pMG9-128, respectively) resulted in hemolytic *E. coli* transformants possessing the cytolysin insert in both orientations relative to the vector *lac* promoter (Fig. 2). Deletion of the 200-bp *Bam*HI-*Eco*RI fragment contained in pMG8-128 as described above resulted in a construction (pMG8-9) conferring a low level of hemolysin expression to *E. coli*. The observation that subsequent filling in of the pMG8-9 *Eco*RI cohesive ends abrogated this expression (and that expression of this 1.9-kb *Eco*RI-*Bam*HI fragment did not occur in the opposite orientation in pUC9 [pMG9-22]) suggested that reading of the cytolytic determinant through the *B. cereus*-derived *Eco*RI site occurred in the same frame and direction as did reading through the pUC8 vector *Eco*RI site. The distal terminus of the cytolysin determinant was defined by deleting the 160-bp *Bcl*I-*Bam*HI fragment at the opposite end of the insert. Such deletion derivatives were nonhemolytic in either orientation.

Separately cloned *cerA* (pMLW3) and *cerB* (pMLW4) genes were capable of *trans* complementation on separate, compatible vectors in *E. coli* within the same host or when the lysates of separate *E. coli* hosts were mixed (Fig. 3). Neither clone was significantly hemolytic over the period tested (although prolonged incubation of the *cerB* pMLW4-derived lysate resulted in a low level of erythrocyte lysis). Similarly, *B. subtilis* clones separately harboring pCerA and pCerB elaborated complementing extracellular activities demonstrable at the junction of a cross-streak on a blood agar plate (Fig. 4). Moreover, CerA and CerB were observed to complement the activities of components of the diagnostic *Streptococcus agalactiae* CAMP factor test. In the CAMP test, β-lysin (sphingomyelinase) secreted by *Staphylococcus aureus* sensitizes erythrocytes for binding and lysis by the CAMP factor of *S. agalactiae*, resulting in a typical inverted arrowhead lysis pattern at the cross-streak junction (2). Since *B. subtilis* harboring pCerB could replace *Staphylococcus aureus* in the CAMP test and *B. subtilis* harboring pCerA could replace *S. agalactiae* in effecting target cell lysis, it was deduced that CerB possesses a sphingomyelinase-type activity. CerA, although similar to the CAMP factor in lysing sphingomyelinase-sensitized target cells, was clearly distinct from the CAMP factor in its ability to confer a lecithinase-positive (egg yolk-positive) phenotype to *B. subtilis*, suggestive of phospholipase C activity (Fig. 4) (16). No enzymatic activity has been ascribed to the CAMP factor (2) (derived from lecithinase-negative *S. agalactiae*). To directly test culture supernatants derived from *B. subtilis* harboring pCerB for the ability to hydrolyze sphingomyelin, the TNPAL-sphingomyelin assay (11) described above was used. CerB culture supernatants contained higher levels of sphingomyelinase activity than did supernatants derived

TABLE 2. Sphingomyelinase activity conferred by the cloned CerB determinant

Culture supernatant	A_{330}
B. cereus GP-4	0.208
	0.216
B. subtilis	
BR151CM1(pJKK3-1)	−0.006
	−0.001
BR151CM1(pCerA)	0.002
	0.000
BR151CM1(pCerB)	0.409
	0.388
S. aureus sphingomyelinase (1 U)	0.466

from the natural producer *B. cereus* (Table 2). In contrast, culture supernatants derived from cloning vector pJKK3-1- or pCerA-harboring *B. subtilis* strains were devoid of detectable sphingomyelinase activity.

Nucleotide sequence. The results of nucleotide sequence determination confirmed the presence of two closely spaced genes (Fig. 5). Interestingly, the putative ribosome-binding site for *cerA* was found to comprise a potential 5-base loop flanked by a perfectly complementary 8-bp stem (bases −19 through +1; Fig. 5). The *cerA* and *cerB* genes are flanked by additional regions potentially capable of forming stable secondary structures. Upstream from the *cerA* gene are two sets of extended inverted repeat sequences (encompassed within bases −190 through −67) in addition to that flanking the putative ribosome-binding site. An inverted repeat involving 15 of 17 bases of a potential stem structure is observable in the intergenic region (bases 859 through 898). Downstream from the *cerB* gene (bases 1935 through 1964), a perfect 12-bp inverted repeat which appears similar in structure to procaryotic *rho*-independent terminators (18) can be discerned. The use of a valine GTG initiator starting translation of the *cerB* gene is unusual but not unprecedented in either gram-negative (e.g., *lacI* [32]) or gram-positive (e.g., *spoVG* and *0.3 kb* [33]) genes.

Examination of the *cerA* open reading frame and inferred amino acid sequence revealed that amino acids 39 through 65 (corresponding to bases 115 through 192) appear in an order identical to that reported for the phospholipase C of *B. cereus* (34). Attempts to determine the amino-terminal amino acid sequence of commercially prepared *B. cereus* sphingomyelinase (Boehringer Mannheim), using an Applied Biosystems 470-A gas-phase amino acid sequencer, for comparison with the amino acid sequence inferred from *cerB* were unsuccessful. The amino terminus of sphingomyelinase appeared to be blocked even after repeated ammonium sulfate precipitation and exhaustive dialysis.

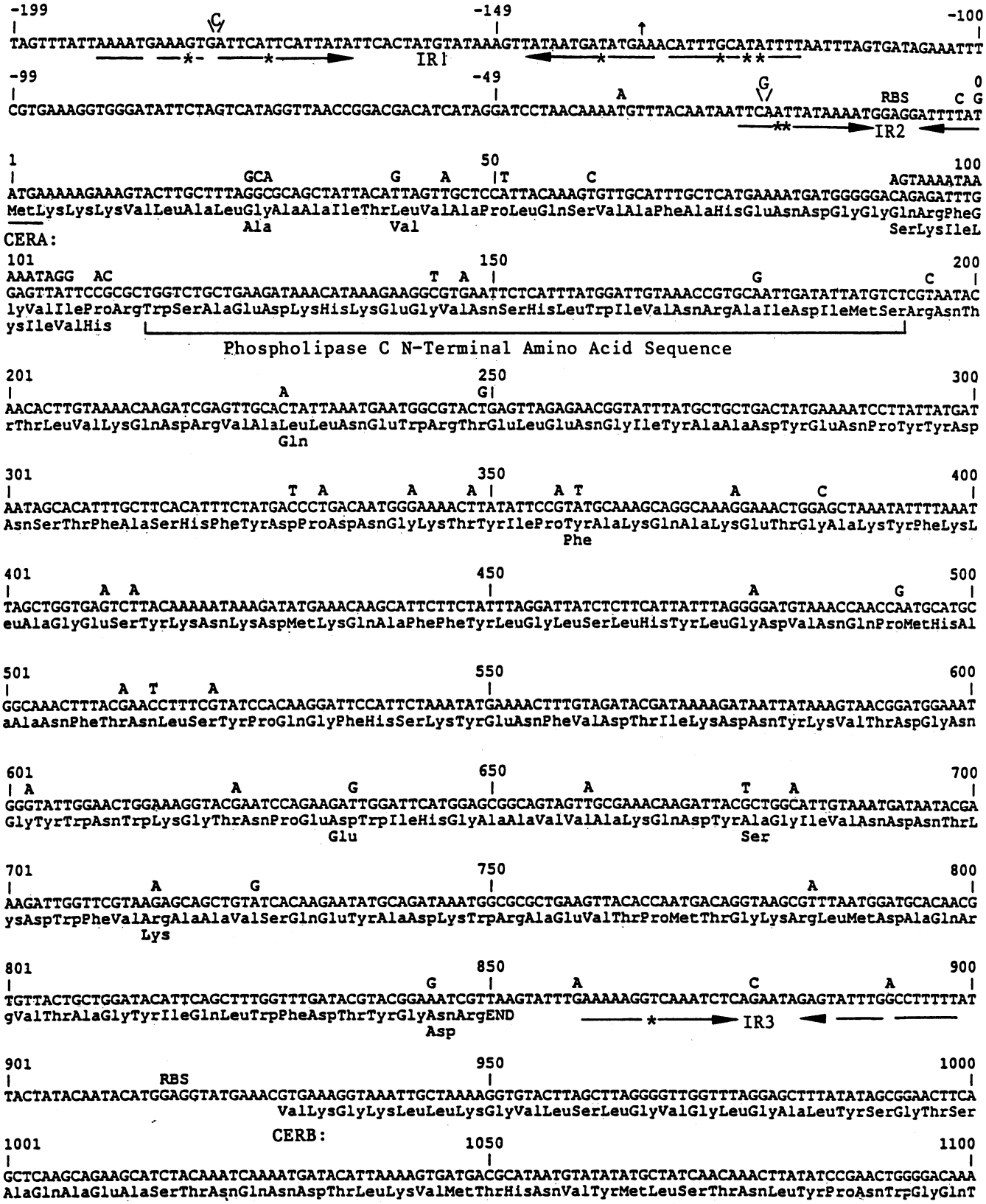
Comparison of the nucleotide sequence of *cerA* with that reported for the *B. cereus* phospholipase C gene. The nucleotide sequence of a phospholipase C gene, derived from *B. cereus* SE-1, a nitrosoguanidine-induced mutant selected for high-level phospholipase C production, was recently independently reported (24). A comparison of the wild-type *cerA* sequence with the overproducing *plc* sequence revealed nucleotide sequence regions which putatively play a role in regulating wild-type *cerA* expression. Nucleotide sequence differences throughout the structural gene region corresponding to mature phospholipase C were largely silent third-codon position changes or resulted in conserved amino acid substitutions (Fig. 5). Greater divergence could be observed in the amino-terminal region, which appeared to be removed posttranslationally. Divergence in this amino-terminal leader region may affect the efficiency of CerA-phospholipase C processing or may reflect evolutionary mutagenic drift which occurred in an enzymatically nonfunctional region of the primary translation product. Subtle nucleotide sequence differences surrounding the putative ribosome-binding sites of *cerA* and *plc*, however, may play the greatest role in regulating *cerA*/*plc* expression. A perfect 8-bp inverted repeat flanks the *cerA* ribosome-binding site (Fig. 6), whereas substitutions corresponding to nucleotide positions 0 and −2 reported for *plc* would be predicted to destabilize this stem-loop structure (no significant secondary structure in this region of *plc* was reported [24]). The involvement of such a stem-loop structure occurring on the *cerA* mRNA in regulating *cerA* expression is supported by (i) previous observations on the posttranscriptional regulation of phospholipase C synthesis (42) and (ii) the involvement of ribosome-binding site-initiator codon-obscuring secondary structures in the posttranscriptional regulation of other genes from gram-positive bacteria (15, 19).

DISCUSSION

In contrast to the well-characterized thiol-activated (streptolysin O-type) cytolysin of *B. cereus* (7), a second hemolytic activity of *B. cereus* has been consistently observed but never physically defined. Early reports of a second cytolytic activity n *B. cereus* culture filtrates described a hemolysin inseparable by DEAE-cellulose chromatography from an egg yolk turbidity-causing peak (10, 37). The phospholipase C of *B. cereus* causes the turbidity on egg yolk agar (37) but lacks hemolytic activity (43). Subsequent reports of a second cytolytic activity in *B. cereus* culture filtrates described a hemolytic activity which eluted from a Sephadex G-100 column at a position corresponding to a higher molecular weight (29,000 to 31,000) than did the peak for phospholipase C activity (8). The level of residual phospholipase C activity in this larger fraction, or the degree of resolution of the two fractions, was not reported. It was observed, however, that lysis of erythrocytes by the 29,000- to 31,000-molecular-weight cytolysin appeared to occur in two steps (in addition to adsorption), and an activation step was postulated (8).

These discrepancies in the nature of a second *B. cereus* cytolysin (distinct from the thiol-activated cytolysin) can be explained by the results reported here. Evidence is presented that a cloned cytolytic determinant from *B. cereus*, initially selected because of its ability to confer a hemolytic phenotype to *E. coli* and *B. subtilis* (25), is composed of tandem genes for phospholipase (*cerA*) and sphingomyelinase (*cerB*). The first gene in this tandem pair, *cerA*, shows inferred amino acid sequence identity with the 26 amino-terminal amino acids of *B. cereus* phospholipase C (34), shows nucleotide sequence identity with a mutant gene conferring high-level phospholipase C expression in *B. cereus* (24), and confers a lecithinase-positive phenotype to its host, an activity attributable to phospholipase C (16). The second gene of the pair, *cerB*, encodes readily detected sphingomyelin-hydrolyzing activity, which establishes the identity of its product as a sphingomyelinase. The genes for both enzymes encode highly hydrophobic amino termini following strong positive charges that are similar in structure to previously reported signal peptides (35). The activities of both cloned gene products can be demonstrated in the culture fluid of *B. subtilis*. Aside from putative loss of the signal peptide, little can be concluded about the processing of CerB-sphingomyelinase because of the method-dependent variance in reported molecular weight for the mature enzyme (40). However, lacking a 26-amino-acid signal peptide (and any additional processing steps), a maximum molecular weight for CerB-sphingomyelinase of approximately 39,000 can be deduced.

Reported isoelectric points are 6.8 for phospholipase C (3) and 5.6 for sphingomyelinase (22). Because of the relative similarities in isoelectric points and the alkaline buffer systems used, it is conceivable that significant coelution or peak overlap of phospholipase C and sphingomyelinase activities from DEAE-cellulose columns occurred in earlier studies on the cytolysins of *B. cereus* (10, 37). Such overlap would result in the hemolytic peak with egg yolk turbidity activity observed (10, 37). Similarly, a Sephadex G-100 fraction containing peak cereolysin AB activity would be expected to be broad and occur at a point intermediate to the molecular


IR1
IR2
IR3
CERA:
CERB:
Phospholipase C N-Terminal Amino Acid Sequence
RBS
END

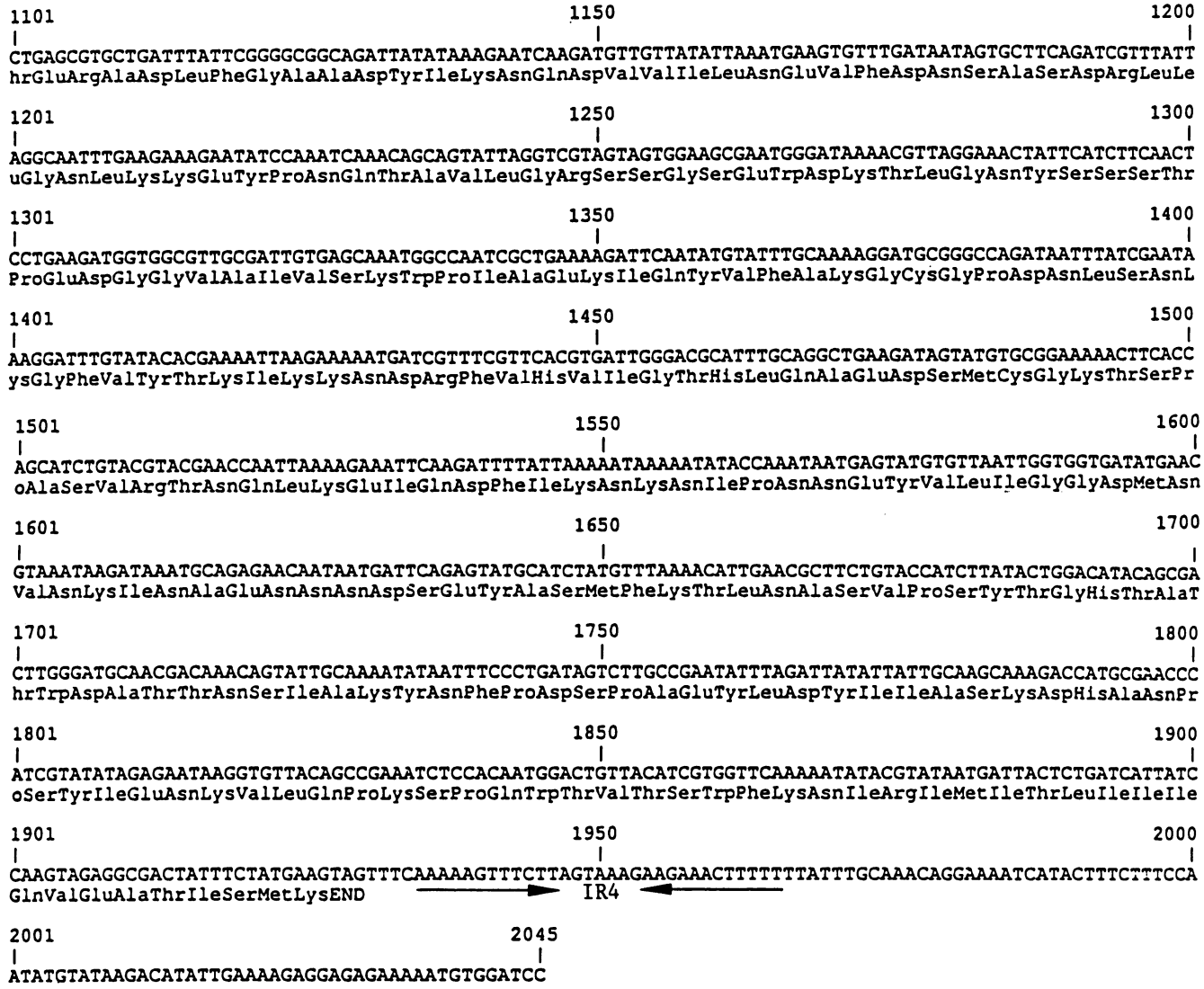


FIG. 5. Complete nucleotide sequence of the cloned cereolysin AB determinant. Arrows indicate the locations of inverted repeats IR1, IR2, IR3, and IR4 (unpaired bases are indicated by space; mismatched bases are indicated by asterisks). Differences between the *cerA* sequence and that reported for *plc* (24) are indicated immediately above the *cerA* nucleotide sequence (\/, insertions; ↑, deletions; base substitutions are as shown). Differences in the inferred amino acid sequence of Plc are indicated immediately below the CerA amino acid sequence. The position of amino acid sequence homology with that published for *B. cereus* phospholipase C (34) is shown beginning at nucleotide position 115. Putative ribosome-binding sites upstream from the initiator codons for *cerA* and *cerB* are designated RBS. The high A+T content of the *cerA*- and *cerB*-flanking regions precludes speculation on the location of putative promoter sequences.

weights of CerB-sphingomyelinase and CerA-phospholipase C, as was observed (8).

The relative activities of *B. cereus* phospholipase C and sphingomyelinase on erythrocyte membranes have been studied in detail. *B. cereus* phospholipase C was found to be unable to hydrolyze phosphatidylcholine within the membranes of intact human erythrocytes (43). However, the phosphatidylcholine content of nonsealed erythrocyte ghosts was nearly quantitatively hydrolyzed (43). It was further observed that the combination of *Staphylococcus aureus* sphingomyelinase and *B. cereus* phospholipase C acted synergistically, resulting in total lysis of an erythrocyte preparation within 60 min; neither enzyme alone effected greater than 2% lysis of the erythrocyte preparation over 180 min (43). These investigators concluded that although phosphatidylcholine (and other glycerophospholipid substrates for *B. cereus* phospholipase C) is abundant in the outer leaf of the erythrocyte membrane, it is inaccessible to phospholipase C before gross reorganization of the lipid (as induced by sphingomyelinase treatment) (43). *B. cereus* phospholipase C apparently is effective in lysing human erythrocytes after prolonged storage and ATP depletion (29), conditions which resulted in spherochinocyte formation presumably involving lipid rearrangement in the membrane outer leaf. Similar observations have been made on the activity of *B. cereus* phospholipase C toward erythrocytes from other mammalian (6), avian, and amphibian (12) sources. *B. cereus* sphingomyelinase appears to be very similar to the staphylococcal counterpart in its activity and response to divalent cations; i.e., Mg^{2+} is required for hydrolysis of sphingomyelin, and both enzymes are activated by Mn^{2+} and Co^{2+} (21).

The combined observations of synergism between the activities of *B. cereus* phospholipase C and sphingomyelinase in lysing target cells and the close genetic linkage of the

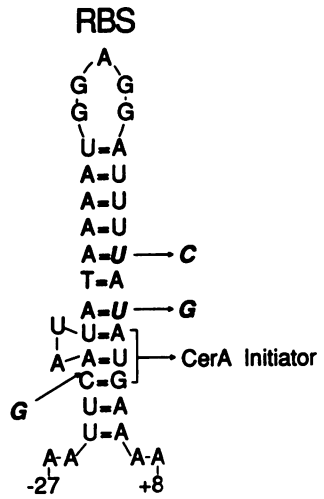


FIG. 6. Potential stem-loop structure surrounding the *cerA* ribosome-binding site formed by IR2 (Fig. 5). Insertion of a G between nucleotides −22 and −23 and the base substitutions shown at positions −2 and 0 were observed in the corresponding sequence of *plc* (24).

two genes suggest that the two activities function naturally in concert as an effective cytolysin. Such a deduction is supported by the natural occurrence of the *Clostridium perfringens* α-toxin, which possesses both sphingomyelinase and phospholipase C activities (39). Biologically, the clostridial α-toxin is hemolytic, necrotic, and lethal (39). Necrotic and lethal toxin activities have been reported to occur in *B. cereus* culture supernatants; however, their identities remain ill defined (41), leaving open their relationship to cereolysin AB. The question of how such toxins can be necrotic without being cytolytic has previously been raised (41). Since the clostridial α-toxin is about 43,000 daltons in mass (44) (nearly the sum of the reported sizes for the mature cereolysin AB phospholipase C [34] and sphingomyelinase [40] components) and possesses both cereolysin AB activities and metal-binding properties, it is interesting to speculate that the two cytolytic determinants are evolutionarily related; speculatively, an ancestral cereolysin AB-type determinant may have given rise to the clostridial α-toxin as the result of a deletion of the intergenic spacer and additional flanking sequences not required for enzymatic activity. The evolution of analogous bifunctional fusion proteins has been described in gram-positive bacteria (9), as have immunologically cross-reactive toxins produced by streptococci and staphylococci (20). Moreover, *B. cereus* and *C. perfringens* are known to produce related cytolysins of the thiol-activated class (13, 38). Further, immunological identity has recently been reported between such diverse membrane-active enzymes as the *B. cereus* phospholipase C and a phospholipase C derived from a human monocytic cell line (5).

In summary, a cytolytic determinant (cereolysin AB) has been cloned from the genome of *B. cereus* and characterized as comprising linked phospholipase C (*cerA*) and sphingomyelinase (*cerB*) genes. The designation cereolysin AB has been selected to identify its constituent activities as a biologically functional, two-component cytolysin. It is proposed that the thiol-activated cytolysin produced by *B. cereus* be designated cereolysin O (rather than simply cereolysin) to provide clear distinction between the two cytolytic activities elaborated by *B. cereus* and to provide consistency with the nomenclature used in reference to most other thiol-activated cytolysins of gram-positive origin (13, 38).

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