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Cloning and Expression in *Escherichia coli* and *Bacillus subtilis* of the Hemolysin (Cereolysin) Determinant from *Bacillus cereus*

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From a cosmid gene bank of *Bacillus cereus* GP4 in *Escherichia coli* we isolated clones which, after several days of incubation, formed hemolysis zones on erythrocyte agar plates. These clones contained recombinant cosmids with *B. cereus* DNA insertions of varying lengths which shared some common restriction fragments. The smallest insertion was recloned as a *Pst*I fragment into pJKK3-1, a shuttle vector which replicates in *Bacillus subtilis* and *E. coli*. When this recombinant plasmid (pJKK3-1 *hly-1*) was transformed into *E. coli*, it caused hemolysis on erythrocyte agar plates, but in liquid assays no external or internal hemolytic activity could be detected with the *E. coli* transformants. *B. subtilis* carrying the same plasmid exhibited hemolytic activity at levels comparable to those of the *B. cereus* donor strain. The hemolysin produced in *B. subtilis* seemed to be indistinguishable from cereolysin in its sensitivity to cholesterol, activation by dithiothreitol, and inactivation by antibodies raised against cereolysin. When the recombinant DNA carrying the cereolysin gene was used as a probe in hybridization experiments with chromosomal DNA from a streptolysin O-producing strain of *Streptococcus pyogenes* or from listeriolysin-producing strains of *Listeria monocytogenes*, no positive hybridization signals were obtained. These data suggest that the genes for these three SH-activated cytolysins do not have extended sequence homology.

Extracellular proteins with hemolytic (cytolytic) activity are produced by a large variety of gram-positive and gram-negative bacteria (for a review, see reference 23). Since the production of these proteins is often associated with pathogenic strains, they have been considered factors that contribute to virulence (13, 27, 28).

Recently, we succeeded in isolating by recombinant DNA techniques the genetic determinants for hemolysin synthesis in *Escherichia coli* (1, 12) and showed that other gram-negative bacteria seem to carry related genes involved in hemolysin production (C. Hughes, unpublished data).

The biochemical characteristics of the hemolysins from the most important pathogenic gram-positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes, Listeria monocytogenes*, etc.) have been studied quite extensively, but little is known about the genetics of these compounds. Among the hemolysins from gram-positive bacteria, the thiol- or SH-activated hemolysins (cytolysins) comprise a group of proteins that possess similar biological properties (3). This group includes streptolysin O (produced by *Streptococcus pyogenes*), θ toxin (*Clostridium perfringens*), pneumolysin (*Streptococcus pneumoniae*), listeriolysin (*L. monocytogenes*), cereolysin (*Bacillus cereus*), and others. Although the molecular weights and probably also the amino acid compositions of these hemolysins are different (3, 23), they apparently cross-react in neutralization and immunodiffusion tests (3, 21). In addition, they share common biochemical properties, including activation by thiol-reducing agents, such as cysteine, 2-mercaptoethanol, dithiothreitol, and sodium thioglycolate, and inactivation by sterols, particularly cholesterol, which is the presumptive cellular receptor for these hemolysins. The mechanisms of action of these SH-activated hemolysins, which lyse a variety of cells in addition to erythrocytes and are therefore better called cytolysins, also seem to be similar (23).

To learn more about the genetics of this interesting group of extracellular bacterial cytolysins and to study their relationships at the DNA level, we first cloned the cereolysin deter-

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype and/or phenotype	Source or reference
Strains		
E. coli HB101	F⁻ *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* λ⁻	H. W. Boyer
E. coli 5K	*thr thia hsdR hsdM* Sm^r	S. Glover
E. coli DP29B	Δ*lac pro thiA nalA SuII pepB*	D. Gho
E. coli K-12 N205	λ *imm434 c*Its *b2 red3 Eam4 Sam7*/λ	7
E. coli K-12 N205	λ *imm434 c*Its *b2 red3 Dam15 Sam7*/λ	7
B. subtilis BR151CM1	*trpC2 metB10 lys3 spoCM1*	P. Lovett
B. cereus GP4	*hly*⁺ Ap^r	This study
L. monocytogenes NCTC 10527	Listeriolysin producer	H. Seeliger and J. Donker-Voet
L. monocytogenes ATCC 19119	Listeriolysin producer	H. Seeliger and J. Donker-Voet
Streptococcus pyogenes 203U	Streptolysin O producer	J. van Embden
Plasmids		
pJC74	Ap^r λ *cos*	6
pJC74 *hly-1*	Ap^r λ *cos hly*⁺	This study
pJKK3-1	Tc^r	14
pJKK3-1 *hly-1*	Tc^r *hly*⁺	This study
pJKK3-1 *hly-2*	Tc^r *hly*⁺	This study

minant by the cosmid cloning technique in *E. coli* and, after subcloning on a bifunctional vector, introduced the determinant into *Bacillus subtilis*. We found that cereolysin is poorly expressed in *E. coli*, but that synthesis and transport across the cytoplasmic membrane take place quite efficiently in *B. subtilis*. Using the recombinant plasmid carrying the cereolysin gene(s) as a hybridization probe, we showed that neither the gene(s) for streptolysin O from *Streptococcus pyogenes* nor the listeriolysin gene(s) from *L. monocytogenes* cross-hybridizes with the cereolysin gene(s).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and buffers. We used Penassay broth (Difco Laboratories, Detroit, Mich.) and HGP (10 g of peptone from casein per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter, 5 g of glucose per liter, pH 7.0). Erythrocyte agar contained (per liter) 10 g of Bacto-Peptone (Oxoid Ltd., London, England), 10 g of meat extract (Difco), 5 g of NaCl, 10 g of Bacto-Agar (Difco), and 50 ml of washed human erythrocytes. Liquid assays for hemolysin were performed with supernatants from cultures grown in TGNC medium (25). SMMP medium and DM3 plates for regeneration of *B. subtilis* protoplasts have been described previously (4). TES buffer (30 mM Tris-hydrochloride [pH 8.0], 50 mM NaCl, 5 mM EDTA [disodium salt]), TE buffer (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA), and 1× SSC (150 mM NaCl, 15 mM trisodium citrate, pH 7.0) were used as buffers for DNA solutions.

If not otherwise stated, all chemicals were obtained from E. Merck AG, Darmstadt, Germany. Ampicillin was a gift from Bayer, Leverkusen, Germany, and tetracycline was purchased from Boehringer, Mannheim, Germany. Sodium dodecyl sulfate, ethidium bromide, polyethylene glycol 6000, and pancreatic RNase were obtained from Serva, Heidelberg, Germany. α-^{32}P-labeled deoxyribonucleotides were obtained from Amersham-Buchler, Braunschweig, Germany.

Preparation of DNA. Chromosomal DNAs from the strains studied were obtained by the method of Marmur (15), with the following modifications. The cells were treated with 1 mg of lysozyme per ml in TES buffer containing 20% (wt/vol) sucrose for 30 min at 37°C before sodium dodecylsulfate was added. Extraction with chloroform-isoamyl alcohol was done only twice. The DNA was then centrifuged for 42 h at 42,000 rpm and 20°C in a CsCl density gradient (density, 1.710 g/cm^3) in 1× SSC by using a 60Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.). The fractions containing chromosomal DNA were pooled and dialyzed against TE buffer. Plasmid DNAs from *E. coli* and *B. subtilis* were prepared by previously described procedures (2, 16).

Digestion with restriction enzymes. Digestion with restriction enzymes was done as suggested by the manufacturers (New England Biolabs, Beverly, Mass., and Boehringer). Electrophoresis of DNA fragments was performed on vertical 1% agarose gels as described previously (17).

Cosmid cloning system. Recombinant DNA was packaged in vitro as described previously (7), except that NZ (O. Aldag, Hamburg, Germany) was used as the culture medium.

Construction of hybrid plasmids and transformation. The in vitro construction of hybrid plasmids by using T4 DNA ligase (5), transformation of *E. coli* (11), and formation of *B. subtilis* protoplasts (4) have been described previously. Transformants of *E. coli* were selected on HPG plates and erythrocyte agar plates containing 100 μg of ampicillin per ml and 15 μg of tetracycline per ml, respectively. Transformants of *B. subtilis* were selected on DM3 plates containing 15 μg of tetracycline per ml.

Nick translation. Nick translation for labeling plas-

mid DNA with ^{32}P and bidirectional transfer of DNA to nitrocellulose filters (Schleicher & Schüll, Dassel, Germany) were performed by previously described procedures (10, 22), except that the agarose gel containing DNA was treated twice at room temperature for 15 min with 0.25 M HCl, washed three times with distilled water, and treated twice for 15 min at room temperature with 0.5 M NaOH–1.5 M NaCl and three times for 30 min at room temperature with 1 M ammonium acetate–0.02 M NaOH before the DNA was transferred to filters.

Hybridization. Hybridization of DNA transferred to nitrocellulose filters with nick-translated, ^{32}P-labeled, plasmid DNA probes (10) was done in a solution containing 70% (vol/vol) formamide, 6.5× Denhardt solution (2% polyvinylpyrollidone 360, 2% Ficoll 400, 3% bovine serum albumin), 6.6× SSC, 25 mM sodium phosphate (pH 6.5), and 0.1 mg of sonicated salmon sperm DNA per ml. Prehybridization (2 h) and hybridization (48 h) of the filters were at 42°C. The filters were washed three times at room temperature with 2× SSC containing 0.1% sodium dodecylsulfate and three times at 50°C (or 37°C) with 0.1× SSC containing 0.1% sodium dodecyl sulfate, dried, and exposed at −70°C to Cronex X-ray film (Du Pont Co., Wilmington, Del.).

Liquid assay for hemolysin activity. The liquid assay for hemolysin activity was performed as previously described (25), except that calcium chloride was omitted from the reaction mixture. The amount of hemoglobin released was measured by determining the absorbance of the supernatant at 420 nm.

RESULTS

Cloning of the hemolysin determinant from *B. cereus* GP4 in *E. coli*. Initial experiments showed that *B. cereus* GP4, a natural isolate that produced large amounts of cereolysin, contained no plasmid DNA. To isolate the gene(s) involved in cereolysin synthesis, chromosomal DNA from this strain was partially digested with restriction endonuclease *Sau*3A and ligated to *Bam*HI-linearized cosmid vector pJC74 (6). The ligated DNA was packaged in vitro into λ heads (7), and *E. coli* HB101 was infected with the packaged DNA mixture. Ampicillin-resistant (Ap^r) clones were selected on HGP agar plates supplemented with 100 μg of ampicillin per ml and screened for hemolysis on erythrocyte agar plates containing 100 μg of ampicillin per ml. Of 4,650 clones tested, we detected 6 which showed small hemolytic zones around the colonies after 2 days of incubation at 37°C (Fig. 1). Plasmid DNAs were isolated from these clones, digested with *Pst*I, and analyzed on agarose gels. All six clones contained recombinant cosmid pJC74 with an insertion in the *Bam*HI site; the size of the insertion varied between 5.0 and 19 kilobases (kb). A restriction analysis by *Pst*I-*Eco*RI double digestion revealed that some fragments were common in all recombinant cosmids isolated from the hemolytic clones (data not shown). The recombinant cosmids with larger insertions were very unstable (i.e., lost hemolytic activity) even in the presence of ampicillin. The recombinant cosmid with the smallest insertion, designated pJC74 *hly-1*, proved to be relatively stable and was chosen for further analysis.

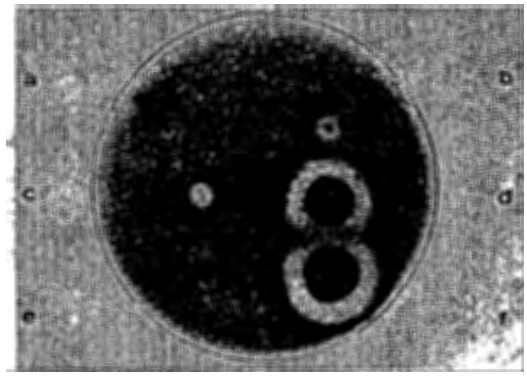

FIG. 1. Hemolytic activity on erythrocyte agar. The *E. coli* strains were inoculated on day 1. After overnight incubation of this plate at 37°C, the *Bacillus* strains were inoculated, and the plate was again incubated overnight at 37°C. Spot a, *E. coli* HB101; spot b, *E. coli* HB101 (pJC74 *hly-1*); spot c, *E. coli* HB101 (pJKK3-1*hly*-1); spot d, *B. cereus* GP4; spot e, *B. subtilis* BR151CM1; spot f, *B. subtilis* BR151CM1 (pJKK3-1 *hly-1*).

To demonstrate that the inserted DNA in pJC74 *hly-1* originated from *B. cereus* GP4, chromosomal DNA from this strain was digested with *Eco*RI, and the resulting fragments were separated on an agarose gel and blotted onto nitrocellulose paper (22). *Pst*I-cleaved pJC74 *hly-1* DNA was treated in the same way. pJC74 and pJC74 *hly-1* DNAs were labeled with ^{32}P by nick translation (10) and hybridized with *Eco*RI-digested chromosomal DNA from *B. cereus* GP4 and with *Pst*I-digested pJC74 *hly-1* DNA as a control. Figure 2 shows that the labeled pJC74 *hly-1* DNA hybridized with only one *Eco*RI fragment of *B. cereus* GP4 DNA and with the *Pst*I fragments of pJC74 *hly-1*. The labeled vector pJC74 DNA alone hybridized with pJC74 *hly-1* DNA but not with *B. cereus* GP4 chromosomal DNA. This clearly showed the *B. cereus* GP4 origin of the inserted DNA in pJC74 *hly-1*. Similar results were obtained with recombinant DNAs containing larger insertions in pJC74 (data not shown).

Despite the fact that *E. coli* HB101 containing pJC74 *hly-1* reproducibly produced hemolysis zones around growing colonies on erythrocyte agar plates (Fig. 1), no hemolytic activity could be found in the culture supernatant or in cell-free extracts from this strain by using the liquid assay described above.

Recloning the hemolysin determinant by using a bifunctional *E. coli-B. subtilis* vector. Assuming that expression and secretion of the cloned hemolysin determinant from *B. cereus* GP4 were

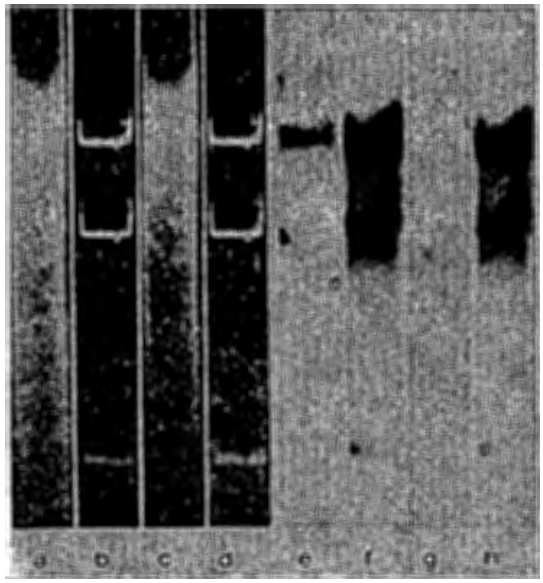

FIG. 2. Gel electrophoresis and hybridization of chromosomal DNA from *B. cereus* GP4. Separation was done on 1% agarose gels, and the DNA was stained with ethidium bromide (5 μg/ml) and visualized under UV light. Lanes a and c, *B. cereus* GP4 DNA digested with *Eco*RI; lanes b and d, pJC74 *hly-1* digested with *Pst*I; lanes e and f, autoradiogram after hybridization with ^{32}P-labeled pJC74 *hly-1* DNA; lanes g and h, autoradiogram after hybridization with pJC74 DNA.

more efficient in *B. subtilis*, we recloned the *Pst*I fragment from pJC74 *hly-1* containing the insertion with the presumed hemolysin gene(s) into plasmid pJKK3-1. This bifunctional vector replicates in both *E. coli* and *B. subtilis* and confers tetracycline resistance upon both hosts (14). To do this, pJC74 *hly-1* DNA was completely digested with *Pst*I and ligated with T4 DNA ligase to *Pst*I-linearized pJKK3-1. *E. coli* HB101 was transformed with the ligation mixture, and several tetracycline-resistant (Tc^r), hemolytic (Hly$^+$) transformants were obtained. Plasmid DNAs were isolated from these transformants and analyzed by restriction analysis with *Pst*I. We found that with one exception all Tc^r Hly$^+$ clones contained pJKK3-1 with an insertion that was the same size as the *Pst*I fragment from the original cosmid, pJC74 *hly-1* (Fig. 3).

We found one clone which contained a smaller plasmid with a deletion about 1 kb long within this *Pst*I fragment (Fig. 3, lane c). This deletion obviously did not affect the hemolysin determinant since *E. coli* and *B. subtilis* transformed with this plasmid produced normal hemolysis zones on erythrocyte agar.

Hybridization of ^{32}P-labeled pJC74 *hly-1* DNA with *Pst*I-digested DNAs of the deleted and undeleted plasmids (designated pJKK3-1 *hly-1* and pJKK3-1 *hly-2*, respectively) showed strong hybridization with the inserted fragments (Fig. 3). The hybridization obtained with vector pJKK3-1 DNA alone was caused by homologous sequences in the two vectors (i.e., the complete beta-lactamase gene of pJC74 and the partially deleted beta-lactamase gene of pJKK3-1) (14).

In supernatants from *E. coli* HB101, *E. coli* 5K, and the peptidase-negative mutant *E. coli* DP29B transformed with pJKK3-1 *hly-1* or pJKK3-1 *hly-2* hemolysin activity was not detected by liquid assays, although all strains produced detectable hemolytic zones around the growing colonies after 2 days of incubation at 37°C.

Plasmid pJKK3-1 *hly-1* was used to transform protoplasts of *B. subtilis* BR151CM1 to tetracycline resistance. All Tc^r transformants were *Hly*$^+$, producing large hemolysis zones around the colonies after 1 day of incubation at 37°C that were comparable in size to the hemolysis zone of the *B. cereus* GP4 donor strain (Fig. 1). An analysis of plasmid DNA from *B. subtilis* BR151CM1 showed that no further deletion of the recombinant plasmid occurred in this host. The same result was obtained with recombinant plasmid pJKK3-1 *hly-2*, which contained an undeleted *Pst*I fragment of pJC74 *hly-1*, as shown above.

The cloning and recloning procedures are outlined in Fig. 4. pJKK3-1 *hly-1* was analyzed with several restriction enzymes, and a detailed restriction map is shown in Fig. 5.

As determined by gel filtration, the molecular weight of the cereolysin from original strain *B.*

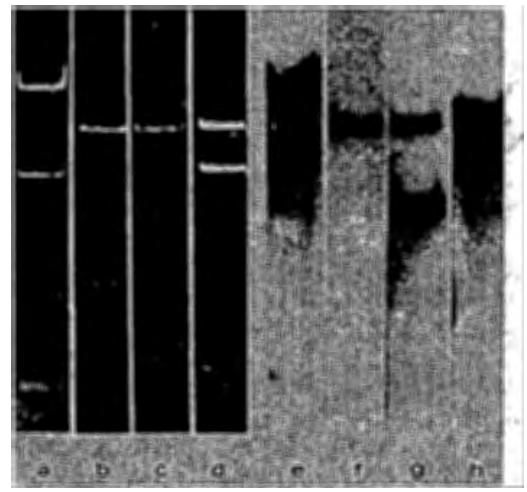

FIG. 3. Gel electrophoresis and hybridization of recombinant plasmids. The conditions used were the same as those described in the legend to Fig. 2. All of the plasmids were digested with *Pst*I. Lane a, pJC74 *hly-1*; lane b, pJKK3-1; lane c, pJKK3-1 *hly-1*; lane d, pJKK3-1 *hly-2*; lanes e through h, autoradiograms after hybridization with ^{32}P-labeled pJC74 *hly-1* DNA.

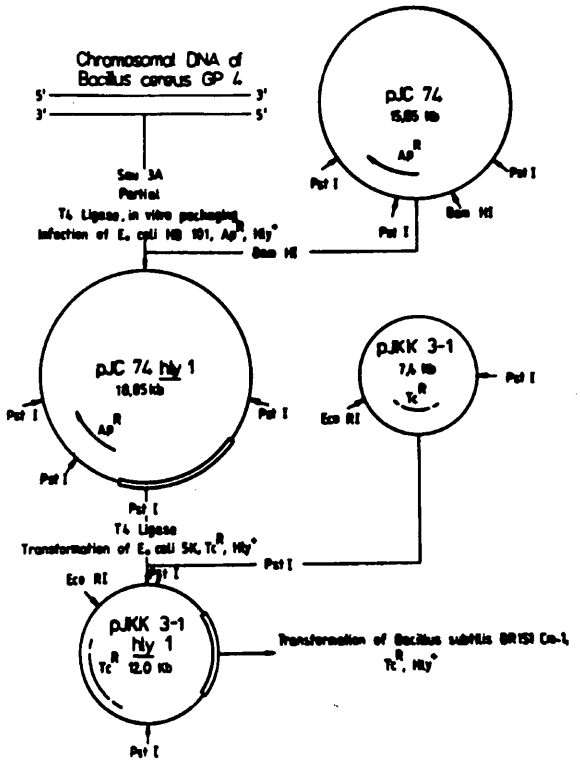

FIG. 4. Schematic presentation of the cloning and recloning of the hemolysin determinant from *B. cereus* GP4.

cereus GP4 is about 70,000 (Härtlein, unpublished data), a value somewhat higher than the value reported previously (23). A protein of this size requires a coding capacity of about 1,900 base pairs, which suggests that the cloned fragment (4.0 kb) contains additional DNA from *B. cereus* GP4. A comparison of the restriction maps of the fragments from pJC74 *hly-1* inserted into pJKK3-1, yielding pJKK3-1 *hly-1* and pJKK3-1 *hly-2*, with the map of pJC74 alone showed that only about 1 kb of the vector pJC74 was also still present on the *Pst*I fragment. The original size of this *Pst*I fragment from vector pJC74 was 2.8 kb; this showed that a deletion(s) of the recombinant cosmid occurred in *E. coli*, leaving intact the two *Pst*I sites flanking the cloned DNA.

Biochemical characterization of the hemolysin (cereolysin) expressed by pJKK3-1 *hly-1*. In contrast to *E. coli* carrying pJKK3-1 *hly-1*, the *B. subtilis* BR151CM1 host carrying the same plasmid produced large amounts of hemolysin (cereolysin) which was secreted into the medium. This hemolytic activity could be demonstrated in the culture supernatants by using liquid assays. Figure 6a shows that the total amount of hemolysin synthesized and secreted by *B. subtilis* BR151CM1 (pJKK3-1 *hly-1*) almost equaled the amount produced by the donor strain, *B. cereus* GP4. To determine whether the hemolysin produced by *B. subtilis* BR151CM1(pJKK3-1 *hly-1*) was identical to the cereolysin synthesized by *B. cereus* GP4, we performed a number of tests.

Cereolysin is inhibited by cholesterol (8), and Fig. 6b shows that incubation of culture supernatants with increasing amounts of cholesterol before the assay for hemolytic activity inhibited the hemolysin produced by *B. subtilis* BR151CM1 (pJKK3-1 *hly-1*) to the same extent that it inhibited the cereolysin produced by *B. cereus* GP4. Another property of cereolysin (and related toxins) is its activation by SH reagents (23), and Fig. 6c shows that the addition of dithiothreitol to a culture supernatant of *B. subtilis* BR151CM1(pJKK3-1 *hly-1*) stimulated hemolytic activity to the same extent that it stimulated the hemolytic activity of the *B. cereus* GP4 supernatant. Finally, the culture supernatants of both strains were preincubated with antiserum raised against cereolysin from *B. cereus* GP4. A liquid assay of these culture supernatants showed that preincubation with specific antiserum inhibited hemolysis by both strains at the same dilution of antiserum (Fig. 6d). The inhibition observed with 1,000-times-greater concentrations of serum from nonimmunized animals was due to the cholesterol inevitably present in such sera.

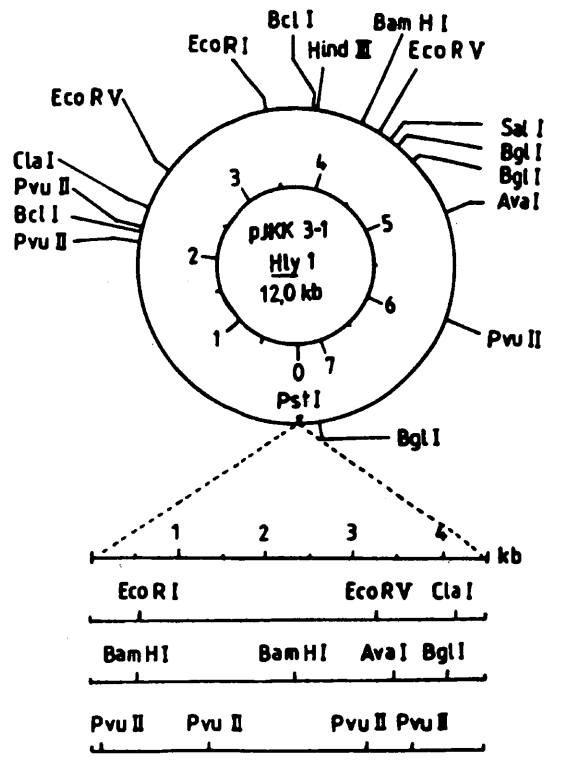

FIG. 5. Restriction map of pJKK3-1 *hly-1*. The *Pst*I fragment derived from pJC74 *hly-1* is shown in linear form.

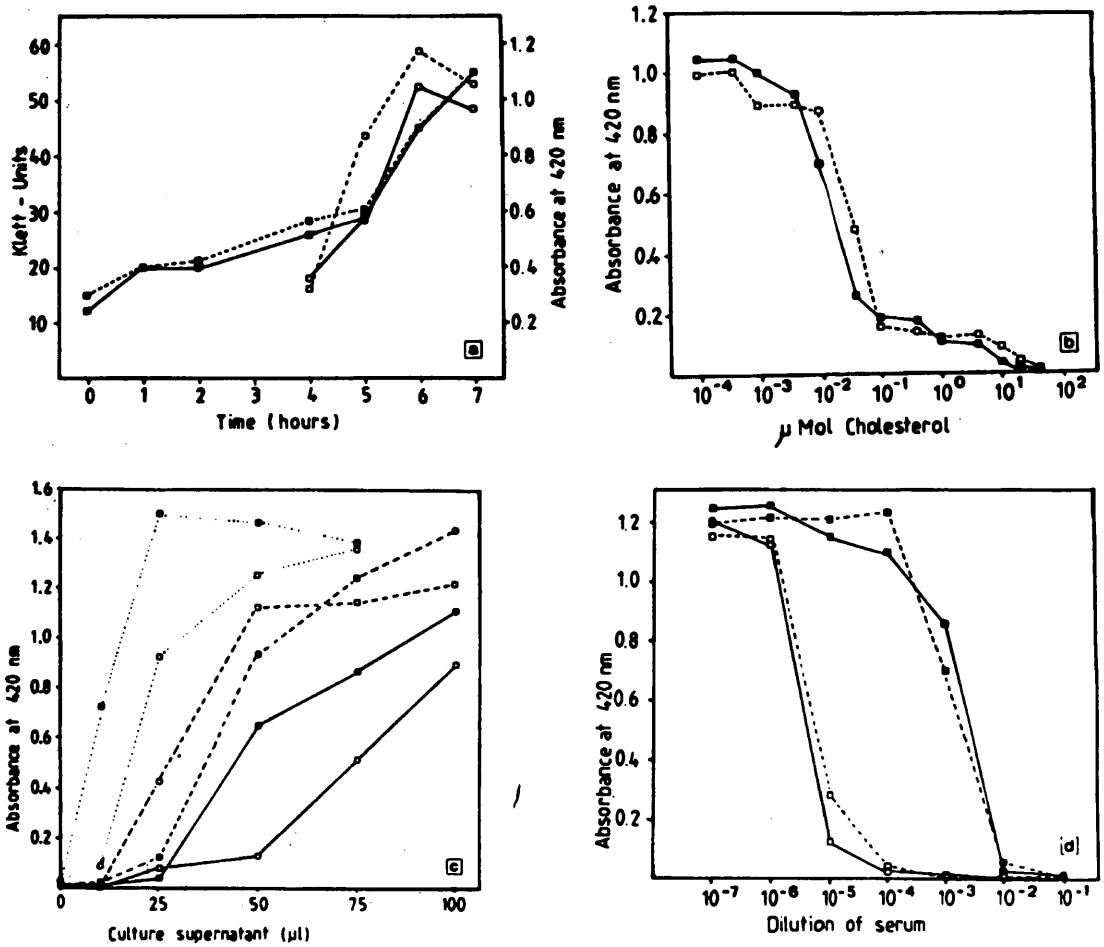

FIG. 6. Comparison of hemolysin from *B. subtilis* BR151CM1 (pJKK3-1 *hly-1*) and cereolysin from *B. cereus* GP4. (a) Growth curves and hemolytic activities in culture supernatants. Symbols: ■- - -■, *B. subtilis* growth curve; ■——■, *B. cereus* growth curve; □- - -□, *B. subtilis* hemolytic activity; □——□, *B. cereus* hemolytic activity. (b) Inhibition of hemolytic activity after preincubation for 10 min at room temperature of culture supernatant with increasing amounts of cholesterol. Symbols: □, *B. subtilis*; ■, *B. cereus*. (c) Activation of hemolytic activity after preincubation for 10 min at room temperature of culture supernatants with two different concentrations of dithiothreitol. Symbols: □——□, *B. subtilis*, no dithiothreitol; □- - -□, *B. subtilis*, 0.5 mM dithiothreitol; □ · · · · □, *B. subtilis*, 1.25 mM dithiothreitol; ■——■, *B. cereus*, no dithiothreitol; ■- - -■, *B. cereus*, 0.5 mM dithiothreitol; ■ · · · · ■, *B. cereus*, 1.25 mM dithiothreitol. (d) Neutralization of hemolytic activity after preincubation for 10 min at room temperature of culture supernatants with increasing amounts of normal serum or antiserum raised against cereolysin. Symbols: ■- - -■, *B. subtilis*, normal serum; □- - -□, *B. subtilis*, antiserum; ■——■, *B. cereus* normal serum; □——□, *B. cereus*, antiserum.

Taken together, the results described above show that the hemolysin synthesized by *B. subtilis* BR151CM1 carrying pJKK3-1 *hly-1* has biochemical properties that are similar to those of authentic cereolysin from *B. cereus* GP4.

Lack of homology between the cloned cereolysin gene(s) and the genes for streptolysin O and listeriolysin. To determine whether there is detectable DNA homology between the cloned cereolysin gene(s) from *B. cereus* GP4 and the genes for streptolysin O and listeriolysin, chromosomal DNAs from *Streptococcus pyogenes* 203U and *L. monocytogenes* NCTC 10527 and ATCC 19119 (producers of streptolysin O and listeriolysin, respectively) were digested with *Eco*RI and electrophoresed in an agarose gel, and the fragments were transferred to nitrocellulose filters. *Pst*I-digested pJC74 *hly-1* DNA served as a control, and ^{32}P-labeled pJC74 *hly-1* DNA was used as a probe and hybridized to the filter-bound DNA fragments. Figure 7 shows that under standard conditions (hybridization at 42°C, washing of filters at 50°C) there was no hybridization of *Streptococcus* or *Listeria* chromosomal DNA with the cereolysin gene probe. Similar results were obtained when hybridization was carried out under less stringent conditions (data not shown).

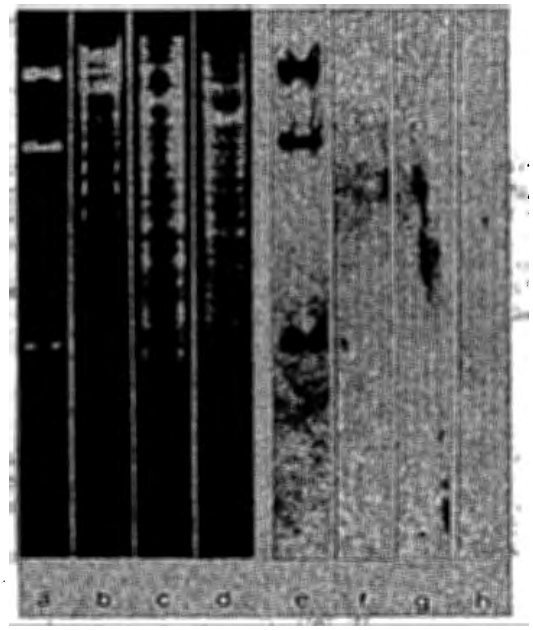

FIG. 7. Gel electrophoresis and hybridization of chromosomal DNAs from *Streptococcus pyogenes* and *L. monocytogenes*. The conditions used were the same as those described in the legend to Fig. 2. Lane a, pJC74 *hly-1* digested with *Pst*I; lane b, *Streptococcus pyogenes* 203U DNA digested with *Eco*RI; lane c, *L. monocytogenes* NCTC 10527 DNA digested with *Eco*RI; lane d, *L. monocytogenes* ATCC 19119 DNA digested with *Eco*RI; lanes e through h, autoradiograms after hybridization with ^{32}P-labeled pJC74 *hly-1* DNA.

DISCUSSION

The recombinant DNA technique has been a useful tool for studying the contribution to virulence of properties which are more frequently encountered in pathogenic bacterial strains than in their nonpathogenic counterparts. By using this approach the importance of heat-stable and heat-labile enterotoxins and the importance of the adhesion pili of enterotoxic *E. coli* strains in causing diarrhea have been clearly shown (9, 18, 24, 26). Similarly hemolysin genes cloned from various virulent *E. coli* strains that cause infections of the urinary tract have been shown to increase virulence when they are introduced on recombinant plasmids into suitable *E. coli* strains (28; J. Hacker, C. Hughes, and W. Goebel, submitted for publication).

Hemolysin formation is also frequently found in pathogenic gram-positive bacteria, and the involvement in pathogenicity of the extracellular hemolysins, which often exhibit rather general cytolytic activity, has been claimed (23). However, the studies performed with hemolytic strains and their nonhemolytic counterparts suffer from a lack of knowledge of the events which lead to the loss of the hemolytic phenotype (J. Hacker, S. Knapp, and W. Goebel, submitted for publication). The isolation of the genes that determine these properties is probably the most unambiguous way of clarifying the involvement of these genes in pathogenicity. In addition, such investigations offer the opportunity to study the relationships of genes that determine similar phenotypes in different bacteria and hence the molecular basis for the possible spread of a gene among these bacteria (10, 19).

The thiol- or SH-activated hemolysins (cytolysins) produced by various gram-positive bacteria (23) seem to be particularly well suited for such studies since they are synthesized mainly by pathogenic species and share several common biochemical properties, which suggests that the genes for these extracellular toxins are derived from a common ancestral genetic determinant. One member of this group of cytolysins is cereolysin, which is produced by most *B. cereus* strains.

We cloned the genetic determinant for the synthesis of cereolysin in *E. coli* by inserting chromosomal fragments of *B. cereus* GP4 into cosmid vector pJC74. We isolated four hemolytic *E. coli* clones, and hybridization studies showed that all four contained recombinant cosmids with *B. cereus* DNA insertion having sizes ranging from 5 to 19 kb. Since the size of a packageable recombinant cosmid is around 45 kb (7), the insertion of a pJC74 recombinant cosmid should be around 30 kb long. Therefore, the considerably smaller sizes of the hemolytic recombinant cosmids which we obtained suggest that deletion events occurred in *E. coli* after transfer.

Cereolysin is readily secreted by its natural producer, *B. cereus*. The weak hemolytic activity expressed by the new gram-negative host *E. coli* and the retarded secretion by this host suggest that in addition to a probably drastically decreased expression of the cereolysin determinant, transport of cereolysin or processing of cereolysin or both are also impaired in the new host.

Recloning of the inserted *B. cereus* DNA fragment from the hemolytic recombinant cosmid pJC74 *hly-1* in the shuttle vector pJKK3-1, which replicates in both *E. coli* and *B. subtilis*, was performed to give more insight into this problem. The recombinant plasmids obtained, pJK3-1 *hly-1* and pJKK3-1 *hly-2*, exhibited the same phenotype in *E. coli* as pJC74 *hly-1*. So far we have been unable to detect hemolytic activity in supernatants from logarithmic or stationary phase cultures, in ultrasonic extracts, or in osmotic shock fluids (periplasmic hemolysin) obtained from these *E. coli* strains. This result is not unexpected, as it has been shown previously that extracellular hemolysin from *E. coli* requires a specific transport system for secretion

through the outer membrane (20). The late appearance of hemolytic activity in the *E. coli* strains which we manipulated on erythrocyte agar may indicate that the cereolysin in *E. coli* cannot pass through the outer membrane but is only released from lysed cells. Whether the cereolysin is released from the periplasm (indicating transport of cereolysin in *E. coli* across the inner membrane) or from the cytoplasm cannot be deduced from our results. In contrast to *E. coli*, normal, "quick" hemolysis was observed after a short growth period of *B. subtilis* carrying pJKK3-1 *hly-1* or pJKK3-1 *hly-2*. In culture supernatants hemolytic activity comparable to that of donor strain *B. cereus*, GP4 could be shown by liquid assays. No increased secretion of cereolysin was observed in *B. subtilis* compared with *B. cereus*, although the cereolysin determinant was cloned on multicopy (about 20 copies per cell) plasmid pJKK3-1 in *B. subtilis*. On the other hand, hybridization of pJC74 *hly-1* with only one fragment of *Eco*RI-cleaved chromosomal DNA from *B. cereus* GP4 showed that the cereolysin gene presumably is present as only a single copy in the donor strain. This suggests that the cereolysin gene(s) is less well expressed in the new host or that its expression is regulated similarly in both hosts. Alternatively or in addition, limited secretion into the medium or enhanced degradation of cereolysin in *B. subtilis* may be responsible for this effect.

The hemolysin secreted by *B. subtilis* carrying pJKK3-1 *hly-1* is indistinguishable from cereolysin by the following criteria: (i) sensitivity to cholesterol, which is assumed to be the receptor (or part of it) for all SH-activated cytolysins; (ii) activation by dithiothreitol; and (iii) neutralization by antibodies raised against purified cereolysin from *B. cereus* donor strain GP4.

Hybridization experiments performed with the cloned cereolysin gene(s) to demonstrate homology with genes of two other SH-activated cytolysins, streptolysin O and listeriolysin, were negative. We observed hydridization with neither chromosomal DNAs isolated from two strains of *L. monocytogenes* nor chromosomal DNA from *Streptococcus pyogenes* 203U regardless of whether stringent or relaxed conditions were used for hybridization. If the genes for these SH-activated cytolysins are derived from a common ancestral gene, as suggested by their similar biochemical properties and cross-immunity (3, 21), at least the cereolysin gene must have diverged from the two other cytolysin genes a long time ago. This suggests that the cytolysin determinant is probably not located on a mobile element which can spread among gram-positive bacteria. Nucleotide sequence analyses of the cloned genes will probably answer this and other questions concerning the genetics of cereolysin and other SH-activated cytolysins from gram-positive bacteria.

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