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Cloning and Expression of the CAMP Factor of Group B Streptococci in *Escherichia coli*

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The genetic determinant of the CAMP factor from a strain of group B streptococcus (GBS; *Streptococcus agalactiae*) was cloned in *Escherichia coli*. Total cell DNA from the GBS strain R268 was used to construct a gene bank with bacteriophage λ EMBL4 in the *E. coli* K-12 strain LE392. Recombinant phage plaques were detected by immunoblots by using a specific antiserum raised against purified CAMP factor. Two hybrid phages showing expression of CAMP factor were identified. Subcloning the CAMP gene (*cfb*) into the high-copy-number vector pUC8 resulted in highly unstable plasmids. Therefore, subcloning was performed with the low-copy-number vector pLG339 resulting in the stable recombinant plasmids pCO61 and pCO62 which lead to expression of CAMP protein first identified by colony immunoblotting. Western blot (immunoblot) analysis revealed a similar CAMP protein pattern in λ EMBL4 recombinant phage lysates (molecular weight, 22,000 to 24,000) as compared to that obtained from a GBS culture supernatant (molecular weight, 22,000 to 26,000) but a different CAMP protein pattern (molecular weight, 20,000 to 23,000) from lysates of *E. coli* carrying pCO61 or pCO62. To study the excretion of the CAMP protein we performed a semi-quantitative dot blot analysis of proteins recovered from cell fractions and supernatants of the *E. coli* recombinant clones. In contrast to GBS R268, where the CAMP factor is readily excreted, the CAMP protein is not excreted in *E. coli* clones containing pCO61 and pCO62 but is found associated with the cell fractions.

A lytic phenomenon exhibited by group B streptococci (GBS; *Streptococcus agalactiae*) on sheep erythrocytes was first described by Christie, Atkins, and Munch-Petersen in 1944 (3). These authors had found that GBS induced a distinct zone of complete hemolysis when grown near the diffusion zone of the beta-hemolysin (beta-toxin) of *Staphylococcus aureus*. Later on, this phenomenon was called the CAMP reaction and was a useful diagnostic test for the presumptive identification of GBS.

Bernheimer et al. (1) characterized the CAMP fator responsible for this reaction as an extracellular protein of molecular weight 23,500 and also determined some physical properties and the amino acid composition. They described its mode of action as an interaction with cell membranes pretreated with the staphylococcal beta-toxin (a sphingomyelinase) leading to instability and subsequent lysis of erythrocyte membranes. Conversion of membrane sphingomyelin to ceramide by a sphingomyelinase was considered to be a prerequisite for cell lysis induced by the CAMP factor.

Fehrenbach and co-workers (4, 20), using liposomes as artificial membranes, demonstrated that the action of the CAMP factor on membranes is dependent on the ratio of cholesterol to phospholipid and that sphingomyelin may be replaced by other phospholipids in this reaction. They essentially confirmed the findings of Bernheimer et al. (1), reporting a similar amino acid composition and a molecular weight of 25,000 (9).

Some other bacterial species, in particular other streptococci and *Listeria* species, show reactions similar to the CAMP phenomenon of GBS when they are grown on blood agar in the vicinity of bacteria which produce a phospholipase (5, 18). It has not yet been investigated whether a structural relation of these CAMP factor-like substances to the GBS CAMP factor exists.

The CAMP factor has long been considered to be of no relevance for pathogenicity. In 1981, Skalka and Smola (17) reported that a partially purified CAMP factor preparation was lethal for experimental animals, especially for rabbits, when injected intravenously. In a recent study, unspecific binding of the CAMP factor to immunoglobulins was detected by Jürgens et al. (11). These experiments suggested a protein A-like binding of the CAMP factor to the Fc sites of immunoglobulins; the GBS CAMP factor was therefore designated protein B. Additionally, injections of purified CAMP factor resulted in a rise in pathogenicity of sublethal doses of GBS given to mice intraperitoneally. From these data, the CAMP factor can be considered a potential virulence factor.

The aim of the work described here was to clone the genetic determinant of the GBS CAMP protein in *Escherichia coli* to obtain data on its molecular and functional structure. The cloned CAMP factor should enable us to study this substance further under two main aspects: to investigate its mode of action in vitro and in vivo in the absence of other factors from its original host and to determine base sequence homologies with the genetic determinants of similar products from other bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. The bacterial strains, plasmids, and bacteriophage used are listed in Table 1. The GBS R268, a completely nonhemolytic, CAMP-positive, tetracycline-resistant type III/R strain, isolated from a case of neonatal sepsis, served as the source of the CAMP factor determinant. *E. coli* K-12 strains LE392, BHB2688, and BHB2690 and the λ phage EMBL4 were a gift from P. Starlinger, Cologne, Federal Republic of Germany. Plasmid pLG339 was a gift from N. F. Fairweather, Beckenham, United Kingdom.

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TABLE 1. Bacterial strains, plasmids, and phages used

Bacterial strain, phage, or plasmid	Property	Reference or source
Bacteria		
S. agalactiae R268	CAMP$^+$ Hly^{-a} Tet^r, type III/R	This laboratory
Staphylococcus aureus FDA196E	Beta-toxin$^+$	ATCC 13565
E. coli		
MC1000	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK strA*	2
LE392	F$^-$ *hsdR514* (r_K^- m_K^-) *supE44 supF58* Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* λ$^-$	15
Q358	*hsdR hsdM*$^+$ *supF*, φ80^r	12
Q359	*hsdR hsdM*$^+$ *supF* φ80 P2	12
BMH71-18	Δ(*lac pro*) *thi supE* F' *lacI*^{q} *Z* Δ*M15 pro*$^+$	16
BHB2688	N205 *recA* [λ *imm*434 cIts *b2 red*3 *E*am4 *S*am7]	7
BHB2690	N205 *recA* [λ*imm*434 cIts *b2 red*3 *D*am15 *S*am7]/λ	7
Phage: λ EMBL4		6
Plasmids		
pUC8	Ap^r *lacZ'*	22
pLG339	Tc^r Km^r, low copy number	21

[a] Hly, Hemolysin.

Media. For preparation of chromosomal DNA, *S. agalactiae* R268 was grown at 37°C in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, Mich.) containing 20 mmol of D,L-threonine per liter (pH 7.2). Generally, *E. coli* strains were cultivated in LB medium (yeast extract [Difco], 5 g/liter; Tryptone [Difco], 10 g/liter; NaCl, 10 g/liter; pH 7.5) with selective agents (ampicillin, 35 mg/liter; kanamycin, 50 mg/liter) added if required. Media for preparation, titration, and maintenance of phages were used as described by Maniatis et al. (15).

Chemicals and enzymes. IPTG (isopropyl-β-D-thiogalactoside), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and lysozyme were purchased from Sigma Chemical Co., St. Louis, Mo. Seakem agarose from FMC BioProducts, Rockland, Maine, was used for DNA electrophoresis. Restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, and *Sau*3A and T4 DNA ligase were purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany. Pronase protease came from Behring Diagnostics, San Diego, Calif. Other chemicals were reagent grade substances mostly obtained from E. Merck (Darmstadt, Federal Republic of Germany).

DNA preparation. Chromosomal DNA from the GBS R268 was prepared by a modification of the method of So et al. (19). Cells were incubated for 150 min in a solution containing 6.25 g of Pronase per liter, after which they were incubated for another 2 h in lysozyme solution (1 g/liter in 10 mmol of Tris buffer [pH 8.2] per liter); lysis was achieved by adding 1% sodium dodecyl sulfate (SDS); DNA was further extracted as described previously (19). Large-scale isolation of plasmid DNA from *E. coli* was done as described by Maniatis et al. (15). The method of Ish-Horowicz and Burke (8) was used for rapid plasmid screening in *E. coli*.

DNA enzymology. Restriction endonuclease digestions were carried out in buffers recommended by Maniatis et al. (15). Incubations were performed for 2 to 6 h at 37°C; reactions were terminated by the addition of 0.25 volume of gel-loading buffer (0.07% bromphenol blue, 33% glycerol, 7% SDS, 50 mmol of Tris hydrochloride per liter, 5 mmol of EDTA per liter, 50 mmol of NaCl per liter; pH 8.0) or by heat inactivation for 15 min at 65°C. DNA ligation reactions were performed at 12°C for 12 h in ligation buffer (15).

Cloning and subcloning procedures. Chromosomal DNA (150 μg) from the GBS strain R268 was partially digested with *Sau*3A (4 U), purified by extraction with phenol, chloroform, and ether, and subsequently fractionated by centrifugation in a 10-to-60% linear sucrose gradient (15).

The phage λ EMBL4 (6) was digested with *Bam*HI and *Sal*I, and the small central fragments were removed by isopropanol precipitation (6). Phage vector arms (1 μg) were ligated with 1 μg from the 10- to 20-kilobase (kb) fraction of R268 chromosomal DNA by using T4 DNA ligase. Ligated DNA was packaged in vitro by using the packaging mix strains BHB2688 and BHB2690 (7). The content of hybrid phage was measured by plating a sample of the packaged phages on *E. coli* Q359 (P2 lysogen). For subcloning experiments, DNA from recombinant phages λ CO1 and λ CO2 and plasmids pLG339 and pUC8 was prepared as described previously (15), cut by restriction enzymes, and ligated with T4 DNA ligase.

Immunoblotting procedures. Phage suspensions were diluted and plated on *E. coli* LE392 in soft agar on LB medium so that approximately 1,000 plaques per plate were obtained. Plates were cooled down to 4°C and covered with nitrocellulose membrane filters (BA85, Schleicher & Schuell, Inc., Keene, N.H.) to absorb proteins for 2 h.

Colonies or bacterial streaks grown on LB plates were covered with nitrocellulose filters; the filters were then transferred to fresh LB plates to be incubated at 37°C for 2 h. For lysis of the bacterial cells, filters were put for 15 min onto Whatman 3M filter paper soaked with 1% SDS solution and exposed to chloroform vapor for a further 15 min. During the blocking procedure (see below), cell walls were digested with lysozyme (Sigma; 10 mg/liter) and DNA was digested with DNase I (Serva, Heidelberg, Federal Republic of Germany; 10 mg/liter).

For Western blot analysis, cellular proteins were prepared in the following way. The sediments from 1-ml overnight cultures were boiled for 5 min in 200 μl of 1% SDS solution. A 40-μl portion of the supernatant from this solution was then mixed with 40 μl of sample buffer and separated on 1.5-mm-thick 14% polyacrylamide gels (14) and transferred by electrophoresis to an Immobilon filter (Millipore Corp., Bedford, Mass.).

Dot blotting was performed on nitrocellulose membranes (Schleicher & Schuell). A 2-μl portion from each test solu-

tion was spotted onto a filter space (0.8 by 0.8 cm) and thoroughly dried.

After protein absorption, all filters were blocked by shaking them for 30 min in TBS (50 mmol of Tris hydrochloride per liter, 100 mmol of NaCl per liter; pH 8.0) containing 4% instant skim milk powder (Glücksklee Co., Hamburg, Federal Republic of Germany), washed three times in TBST (TBS plus 0.05% Tween 20), and incubated for 1 h with a 1:100 dilution of rabbit anti-CAMP factor serum (10) in TBST. After further washings and a 1-h treatment with protein A-linked horseradish peroxidase (Amersham Corp., Arlington Heights, Ill.) diluted 1:1,000, filters were again washed three times in TBST and then in TBS, and finally the substrate 4-chloro-1-naphthol was added.

Preparation of culture supernatants and cell fractions for dot blot analysis. THB (70 ml) was inoculated with *S. agalactiae* R268 or with *E. coli* MC1000 strains harboring plasmids pLG339, pCO61, or pCO62 (see below); cultures were incubated for 16 h at 37°C. Kanamycin (50 mg/liter) had been added to the *E. coli* cultures for plasmid maintenance. A 35-ml portion from each of these cultures was centrifuged for 15 min at 6,000 × *g*, and the supernatant was saved. The cell pellets were resuspended in 5 ml of phosphate-buffered saline (pH 7.4) and sonicated with a Branson sonifier for 5 min at 150 W; the cell debris was sedimented by centrifugation at 4°C and 6,000 × *g* for 15 min, and the supernatant was saved as "cytoplasmic-plus-periplasmic fraction" (accepting some contamination with membrane fragments).

RESULTS

Cloning of the CAMP factor gene in *E. coli*. The bacteriophage λ replacement vector EMBL4 was used to construct a gene bank of the GBS strain R268. Chromosomal DNA from this nonhemolytic GBS strain was partially digested with *Sau*3A and size fractionated in a sucrose gradient; from this, 10- to 20-kb DNA fragments were recovered. By using T4 DNA ligase, 1 μg of the fragments was ligated to 1 μg of *Bam*HI-generated phage vector arms. Ligated DNA was packaged in vitro, and approximately 5,000 hybrid phages were obtained and stored without amplification. These phages containing the GBS R268 gene bank were plated onto *E. coli* LE392 at a density of 1,000 PFU per plate and screened for CAMP protein by immunoblotting. Two independent phages, λ CO1 and λ CO2, showed CAMP protein in the plaque immunoblot assay. For further proof, crude recombinant phage lysates were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent Western blots (Fig. 1). A broad protein band (molecular weight, 22,000 to 24,000) corresponding to the size of the CAMP protein in the *S. agalactiae* R268 supernatant was recognized by the Western blot technique in lysates of the two recombinant phages (λ CO1 and λ CO2).

Subcloning. To subclone and identify the smallest possible DNA fragment containing the CAMP factor determinant, we performed a restriction endonuclease mapping of the two recombinant phages λ CO1 and λ CO2. Digestion of λ CO2 with *Eco*RI and *Sal*I generated four DNA fragments (Fig. 2). These fragments were isolated and subcloned into pUC8 by insertion into the *Sal*I site or between the *Sal*I and *Eco*RI sites of this plasmid vector.

Recombinant plasmids were identified by transformation into *E. coli* BMH71-18 and plating on LB agar plates containing ampicillin, X-Gal, and IPTG. β-Galactosidase-negative (white) colonies were analyzed by colony immunoblotting and rapid plasmid extraction and gel electrophoresis

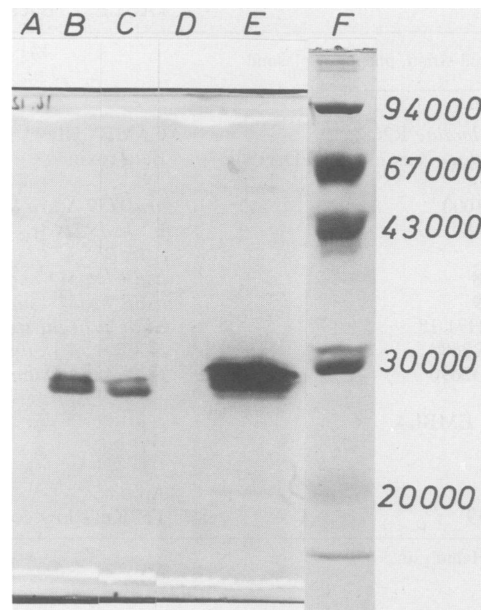

FIG. 1. Electrophoretic immunoblot (lanes A through E) of recombinant phage lysates and GBS culture supernatant. Lanes: A, λ EMBL4 lysate as negative control; B, recombinant phage λ CO2 lysate; C, recombinant phage λ CO1 lysate; D, size marker proteins; E, crude culture supernatant of GBS R268. Lane F is taken from the Coomassie blue-stained SDS-polyacrylamide gel and shows the molecular weight marker proteins of the sizes indicated on the right of the figure.

of restriction enzyme-digested plasmid DNA. We could not find any clone from these subcloning experiments expressing CAMP factor. Clones containing pUC8-derived recombinant plasmids proved to be very unstable and readily reverted to blue colonies on X-Gal plates.

To overcome these stability problems, we chose the low-copy-number vector pLG339 (21) and inserted the four fragments from λ CO2 into the *Sal*I site or between the *Sal*I and *Eco*RI sites of this plasmid (Fig. 2). Recombinant clones were screened by colony immunoblots by using rabbit

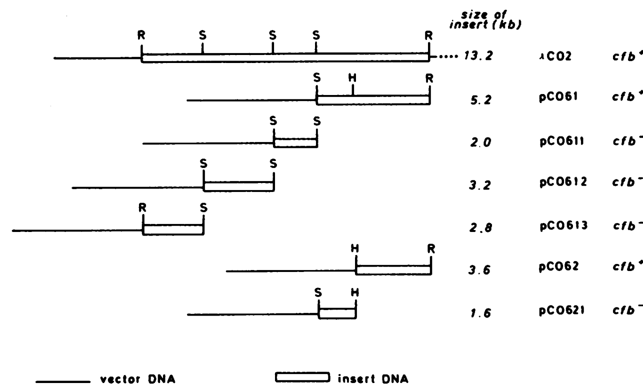

FIG. 2. Restriction enzyme maps of recombinant phage λ CO2 and the recombinant plasmids obtained by subcloning fragments from the GBS DNA insert of λ CO2 into the vector pLG339. Expression and lack of expression of CAMP protein, as detected by immunoblot procedures, are indicated by *cfb*$^+$ and *cfb*$^-$, respectively. Restriction endonuclease cleavage sites: R, *Eco*RI; S, *Sal*I; H, *Hin*dIII; sites in vector DNA are not completely depicted.

anti-CAMP serum as the source of the first antibody. Several *E. coli* MC1000 clones were found which carried a recombinant plasmid (pCO61) with a 5.2-kb insertion between the *Sal*I and *Eco*RI sites of pLG339 (Fig. 2). Recombinant plasmids pCO611, pCO612, and pCO613 (Fig. 2), containing one of the other fragments derived from λ CO2, did not confer CAMP activity to their *E. coli* host.

The recombinant plasmid pCO61 was purified and analyzed in more detail with restriction enzymes (map, Fig. 2). For further subcloning experiments, the 5.2-kb insert of pCO61 could be separated into a 3.6- and a 1.6-kb fragment by digestion with *Hin*dIII. These fragments were inserted into pLG339 linearized by two enzymes in such a way that deletions in pLG339 occurred and suitable cohesive ends resulted. For subcloning of the 3.6-kb fragment, pLG339 was digested with *Eco*RI and partially with *Hin*dIII. From the ligation of the 3.6-kb fragment with the vector thus treated, the recombinant plasmid pCO62 resulted (Fig. 2). pCO62 confers CAMP activity to its *E. coli* host as detected by colony immunoblotting. In a similar experiment with *Sal*I-plus-*Hin*dIII-digested pLG339 (resulting in a 600-base-pair deletion in the vector), insertion of the 1.6-kb fragment created the recombinant plasmid pCO621 (Fig. 2); the latter plasmid did not confer the ability to express CAMP protein to its *E. coli* host.

CAMP factor expression analyzed by Western and dot blots. *E. coli* MC1000 clones carrying pCO61 or pCO62 were found to express CAMP protein in colony immunoblots. For Western blot analysis, crude lysates of the *E. coli* cultures were prepared as described in Materials and Methods. The lysates were separated on SDS-polyacrylamide gels together with the supernatant of a GBS R268 culture as the source of reference CAMP protein.

In the Western blots, the CAMP protein band(s) obtained from *E. coli* harboring either pCO61 or pCO62 appeared to have a slightly lower molecular weight than that (or those; see discussion below) obtained from the GBS culture supernatant (Fig. 3), suggesting some differnces in expression or some processing of the CAMP protein in *E. coli*. These differences were not apparent in the Western blot analysis of recombinant phage lysates (Fig. 1).

Cultures of *S. agalactiae* R268 and *E. coli* MC1000, containing plasmids pCO61 or pCO62 with the presumed *cfb* gene or pLG339 as a negative control, were fractionated as described in Materials and Methods, and the fractions were analyzed by semi-quantitative dot immunoblot analysis. For this purpose, the fractions were spotted in 1:10, 1:50, 1:100, 1:500, and 1:1,000 dilutions onto the filters to be used for immunoblot reactions. In GBS, CAMP protein could only be detected in the supernatant (up to a 1:100 dilution), whereas in the CAMP-positive *E. coli* clones, CAMP protein could be found in the cytoplasmic-plus-periplasmic fraction (up to a 1:1,000 dilution) but not in the supernatant.

Functional activity of the cloned CAMP factor. *E. coli* clones containing the *cfb* gene, as indicated by immunoblots, were also tested for activity in a conventional CAMP test. For this purpose, the strains were streaked on 5% sheep blood agar plates perpendicular to a streak of the beta-toxin-producing *Staphylococcus aureus* ATCC 13565. *S. agalactiae* R268 and *E. coli*(pLG339) were used as positive and negative controls, respectively. After about 20 h of incubation time, *E. coli* strains carrying pCO61 or pCO62 clearly showed a weak CAMP reaction in this assay (Fig. 4).

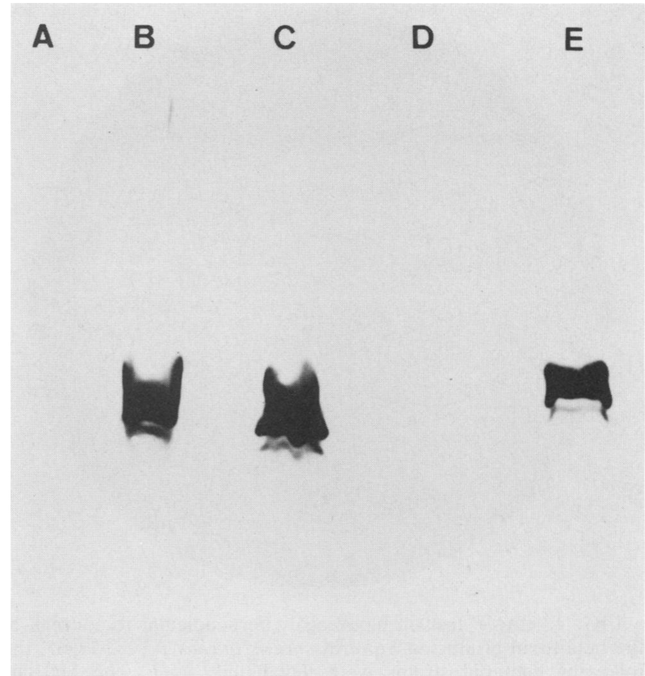

FIG. 3. Electrophoretic immunoblot of lysates of *E. coli* clones producing CAMP protein and the respective controls. Lanes: A, *E. coli* MC1000 harboring the vector plasmid pLG339 only; B, *E. coli* MC1000 containing recombinant plasmid pCO62; C, *E. coli* MC1000 harboring recombinant plasmid pCO61; D, position of the molecular weight marker proteins; E, culture supernatant of GBS R268. A slightly smaller molecular weight of the CAMP protein is apparent in the *E. coli* strains harboring recombinant plasmids (lanes B and C) than with the CAMP factor from GBS (lane E).

DISCUSSION

Since, on the basis of sound experimental data, the capsular polysaccharides have to be considered as the main virulence factors of GBS, extracellular products from this streptococcal species have gained relatively little attention as candidates for additional virulence factors. This especially applies to the CAMP factor, which is generally only known as a useful diagnostic tool. However, the CAMP factor is a potent cocytolysin for mammalian cells conditioned by staphylococcal sphingomyelinase or a phospholipase.

Bernheimer et al. (1) were the first to thoroughly investigate the nature and mode of action of the CAMP factor. They described it as a protein of molecular weight 23,500 and an isoelectric point of 8.3. In *S. agalactiae*, the CAMP protein is found in the culture supernatant in a high concentration. Bernheimer et al. also described its principle of mode of action; the CAMP factor stably binds to ceramide-containing membranes, e.g. erythrocytes, in which the membrane sphingomyelin had been converted to ceramide by a sphingomyelinase, and leads to lysis of the membranes by a hitherto unknown process. An interesting property of the GBS CAMP factor was recently added by Jürgens et al. (11); the authors showed that the CAMP protein binds to immunoglobulins via their Fc part similarly to staphylococcal protein A.

Our approach to cloning the genetic determinant of the CAMP factor (*cfb* gene) will help answer the question of whether the CAMP factor plays a role in the pathogenesis of GBS infections. Sequencing the DNA insert in pCO62 car-

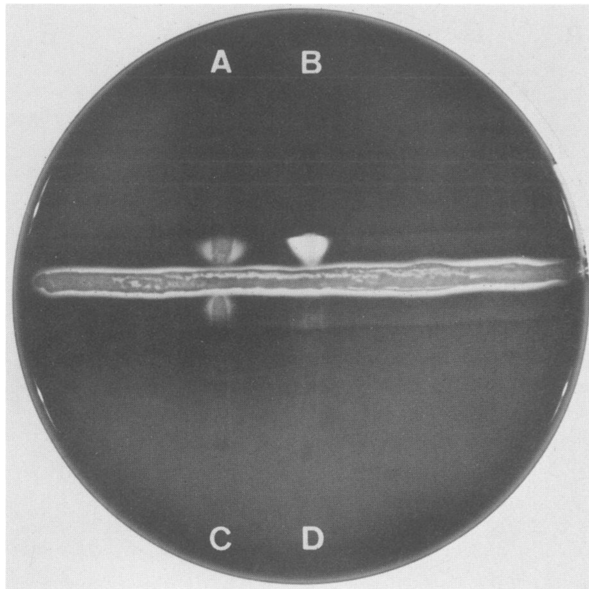

FIG. 4. CAMP test on blood agar. Perpendicular to a streak of the beta-toxin-producing *Staphylococcus aureus* ATCC 13565, the following bacterial strains were inoculated: A, *E. coli* MC1000 harboring recombinant plasmid pCO62; B, *S. agalactiae* R268; C, *E. coli* MC1000 containing recombinant plasmid pCO61; D, *E. coli* MC1000 harboring the vector pLG339.

rying the *cfb* determinant (work in progress) will give more insight into the nature of the CAMP protein than was obtained by conventional protein purification methods up to now; only the overall amino acid composition of the CAMP protein has been reported thus far (1, 9). The determination of the base sequence (and amino acid sequence from this) will put us into the position to compare the GBS CAMP factor with CAMP factor-like substances from other bacterial species on a molecular basis.

In the course of our experiments, one difficulty was encountered which might be a rather common phenomenon when cloning membrane-active polypeptides from gram-positive bacteria in *E. coli*; subcloning of the *cfb* gene in the high-copy-number plasmid vector pUC8 could not be achieved because the resulting recombinant plasmids appeared to be highly unstable. This is analogous to what Kehoe and Timmis described in their report on cloning the streptolysin O determinant from *Streptococcus pyogenes* (13).

Although CAMP protein could easily be detected in fractions prepared from the *E. coli* cells by an immunoblot technique, the *E. coli* clones had to be grown to late stationary phase on blood agar in a conventional CAMP test to release enough CAMP factor and produce a weak lysis (a comparison with GBS is shown in Fig. 4).

From the Western blot analysis depicted in Fig. 3, it may be concluded that the CAMP protein produced in *E. coli* clones harboring the *cfb* gene on a plasmid has a slightly lower molecular weight than the CAMP protein from GBS. Whether this reflects intracellular or intramembrane processing in *E. coli*, or whether another reason exists, has yet to be determined.

In the Western blots (Fig. 1 and 3), the CAMP protein always appears as a rather broad band. This could be due to the fact that, in addition to the lytic CAMP protein, four "satellite" proteins of similar molecular weight exist in GBS which show the same immune reactivity as the CAMP protein does (3a). From this and our Western blot results, it may be imagined that the satellite proteins are also genetically determined by the *cfb* gene and are expressed in *E. coli*.

The eventual determination of the exact localization and sequence of the GBS CAMP determinant will yield the information necessary for in vitro mutagenesis studies needed for assessing the function of different domains on the CAMP protein. In particular, detailed information on the domains involved in the lytic activity or in immunoglobulin binding could be an important clue to the study and understanding of the contribution of the CAMP factor to GBS pathogenicity.

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