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Molecular Characterization and Protein Analysis of the *cap* Region, Which Is Essential for Encapsulation in *Bacillus anthracis*

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By using genetic complementation tests with various in vitro-constructed mutants with mutations in the *cap* region (which is essential for encapsulation in *Bacillus anthracis*), we identified three cistrons, *capB*, *capC*, and *capA*, in this order of arrangement. Minicell analysis revealed that these cistrons produce proteins of 44, 16, and 46 kilodaltons, respectively. The complete nucleotide sequence of 3,244 base pairs covering the whole *cap* region was determined and revealed the existence of the three open reading frames of *capB* (397 amino acid residues; molecular weight, 44,872), *capC* (149 amino acid residues; molecular weight, 16,522), and *capA* (411 amino acid residues; molecular weight, 46,420) arranged in the order predicted by complementation tests. These three cistrons were all transcribed in the same direction from promoters unique to each cistron. Judging from the predicted amino acid sequence of the three proteins and from their localization and their sensitivity to various physicochemical treatments, they appeared to be membrane-associated enzymes mediating the polymerization of D-glutamic acid via the membrane. Capsular peptides immunologically identical to that of *B. anthracis* were found in *B. subtilis*, *B. megaterium*, and *B. licheniformis*, but no sequence homologous to the *cap* region was found in any of these bacilli other than *B. anthracis*. Using strains of *B. anthracis* with or without insertional inactivation of the *cap* region, we found that the capsule of *B. anthracis* conferred strong resistance to phagocytosis upon the bacterial host.

Bacillus anthracis is the causative agent of anthrax. This is primarily a disease of domestic and wild animals, but in humans exposed to diseased animals the infection manifests as a skin infection or rarely a pulmonary infection or septicemia. Two major virulence factors of *B. anthracis*, a capsule and a tripartite toxin consisting of protective antigen, edema factor, and lethal factor, are known (2, 36, 37, 43). The former is encoded by the 60-megadalton (MDa) plasmid (9, 46), and the latter is encoded by the 110-MDa plasmid (27).

The role of the capsule as a virulence factor has been investigated rather extensively for some bacterial species; these systems include the K1 antigen of *Escherichia coli* (6), the capsule of *Neisseria meningtidis* (21), and the capsule of *Franciscella tulafensis* (30). In these bacteria the capsule confers serum resistance or resistance to phagocytosis. A similar biological role of the capsule is generally assumed in *B. anthracis*, but no reliable evidence has yet been reported. The capsule of *B. anthracis* is unique in that it is composed of a homopolypeptide of D-glutamic acid (11). Other bacilli, such as *B. subtilis* (4), *B. megaterium* (10), *B. licheniformis* (44), *Sporosarcina halophilia* (18), and *Planococcus halophilus* (18), also have a capsule consisting of a homopolypeptide of D- or L-glutamic acid. The immunological and genetic relatedness among these polyglutamate capsules has not been determined. D-Glutamyl transpeptidase in *B. subtilis* (*natto*) (12) and membrane-associated glutamylpolypeptide synthetase in *B. licheniformis* (44) have been reported to play a part in the synthesis of these polyglutamate capsules. However, thus far, nothing is known about the synthetic pathway of capsule formation in *B. anthracis*.

We have made a restriction enzyme cleavage map of pTE702, a 60-MDa plasmid essential for the encapsulation in *B. anthracis* (45), cloned the genetic determinants required for encapsulation (*cap* region) in *E. coli*, and transformed the cloned *cap* region back into *B. anthracis* cured of the 110- and 60-MDa plasmids to examine the expression in the original host bacteria (23, 24). *B. anthracis* and *E. coli* but not *B. subtilis* were found to express an immunologically identical capsular peptide on the bacterial surface upon introduction of the cloned *cap* region.

In the present study the *cap* region was further characterized by cistron analysis, nucleotide sequence determination, minicell product analysis, promoter search, and localization of the products under various experimental conditions. These studies have identified three cistrons, *capB*, *capC*, and *capA*, in this order of arrangement and with a promoter unique to each cistron. Although the capsules of *B. anthracis* and other bacilli were found to be immunologically identical, no genetic homology with the *cap* region was detected in bacilli other than *B. anthracis*.

MATERIALS AND METHODS

Bacterial strains and vector plasmids used. Bacterial strains and representative plasmids are listed in Table 1. pCAP1, pCAP2, and pTPCAP1 were used as parental plasmids containing the intact *cap* region (24).

Construction of plasmids. Deletions 5-5, 5-6, 5-9, 5-3, and 5-10 were constructed by BAL31 treatment of *Bgl*II-digested pCAP2 and *Sal*I linker insertion. Deletions 2-3, 2-22, 2-46, and 2-33 were constructed by BAL31 treatment of *Sal*I-digested pCAP1 and *Bgl*II linker insertion. A *Bgl*II fragment of deletion 2-22 was inserted in the *Bam*HI site of pMYSH6003 to obtain pTPCAP1.

To construct the pCAP and pTPCAP series of plasmid mutants, we used the following three methods: (i) filling in of *Hin*dIII, *Xba*I, *Bam*HI, and *Bst*EII sites with the Klenow fragment; (ii) insertion of a *Bgl*II linker into each of the

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TABLE 1. Bacterial strains and representative plasmids used

Strain or plasmid	Relevant characteristic[a]	Reference or source
E. coli		
MC1061	*hsr lac leu*	7
C600 recA	*recA*	This laboratory
CC118	*phoA*	26
M2124	Minicell-producing cell	39
B. anthracis		
TE702	Spo^- Cap^+ Tox^-	24
TE703	As TE702 but cured of the Cap plasmid	24
TE7021	As TE703 but containing pTE7021	24
Shikan	Virulent strain	45
B. subtilis NIAH801	Cap^+	Product *natto*
B. megaterium NIAH368	Cap^+	Human stool
B. licheniformis NIAH227	Cap^+	Human stool
Plasmids		
pHY300PLK	Ap^r Tc^r pACYC177 derivative	14
pMY6003	Ap^r Tp^r pBR322 derivative	22
pBR322	Ap^r Tp^r	3
pCAP1	*cap* region cloned into pHY300PLK	24
pCAP2	Self-cloning of pCAP1 with *Bgl*II	24
pTPCAP1	*cap* region cloned into pMY6003	This study

[a] Abbreviations: Spo^-, asporogenous; Cap^+, encapsulated; Cap^-, nonencapsulated; Tox^-, non-toxigenic; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Tp^r, trimethoprim resistance.

*Hinc*II, *Pvu*II, and *Aat*I sites; and (iii) deletion of four nucleotides from the *Pst*I site by using the Klenow fragment.

pCAPA and pCAPB were constructed by partial digestion of pCAP2 and pCAP1 with *Pst*I, respectively. pCAPC was constructed by inserting the *Bam*HI-*Bgl*II fragment of deletion 2-33 into the *Bam*HI site of pHY300PLK.

Minicell analysis. Minicells were isolated and labeled with [^{35}S]methionine (Amersham Corp., Amersham, United Kingdom) by the method of Thompson and Achtman (39). For fractionation of the protein products, sodium dodecyl sulfate–12.5% polyacrylamide gels were used.

Nucleotide sequencing. The dideoxynucleotide procedure of Sanger et al. was used for nucleotide sequencing (31). Single-stranded pUC118 DNA was purified and used for sequencing by the Kilo-sequence deletion kit and 7-DEAZA sequencing kit (Takara Shuzo, Co., Kyoto, Japan).

Cell fractionation and trypsin treatment. Labeled minicells were treated with lysozyme (15) at 4°C, and spheroplast and periplasmic preparations were isolated by microcentrifugation at 15,000 × *g* for 30 min at 4°C. The spheroplast preparation was sonically disrupted in the presence of phenylmethylsulfonyl fluoride. Then, membrane and cytoplasmic fractions were isolated by centrifugation at 70,000 rpm for 45 min in a TLA-100.2 rotor (Beckman Instruments, Inc., Fullerton, Calif.). For trypsin digestion experiments for topological mapping (34), spheroplasts were lysed by sonication after freezing and thawing. Intact and lysed spheroplasts were reacted with trypsin in 3 mM EDTA buffer (pH 7.5) at 37°C for 30 min. The reaction was terminated by addition of an excess amount of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.), and proteins were precipitated with 5% trichloroacetic acid. Portions were analyzed on sodium dodecyl sulfate–12.5% polyacrylamide gels.

Dot hybridization. Total DNAs from *B. anthracis*, *B. megaterium*, *B. subtilis*, and *B. licheniformis* were prepared by the method of Johnson (16), except that the lysozyme concentration was 10 mg/ml and the treatment was carried out for 90 min at 37°C for *B. anthracis* and 30 min for other strains. To label the probes with [^{35}P]dCTP (Amersham Corp.), we used a random-priming labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Total DNAs were transferred to nitrocellulose filters by the method of Maniatis et al. (25). Hybridization was carried out in the presence of 50% formamide at 42°C.

Tn*phoA* insertion experiments. Tn*phoA* (26) was transposed to a suicide plasmid, pCHR81 (32), to construct pCHR71::Tn*phoA*. pCHR71::Tn*phoA* was conjugally introduced into *E. coli* MC1061 containing pCAP1 at 30°C. A Km^r Tp^r transconjugant was spread on an agar plate to yield about 500 separate colonies at 30°C. Each of these isolates was grown at 30°C in L broth containing ampicillin (50 μg/ml) and kanamycin (50 μg/ml), spread on an agar plate containing kanamycin (400 μg/ml), and then grown at 42°C. Cells on the plate were collected, and plasmid DNA was isolated and used to transform *E. coli* CC118. Transformants were selected on an agar plate supplemented with 5-bromo-4-chloro-3-indolyl phosphate (XP; 40 μg/ml) (Sigma) and kanamycin (50 μg/ml) at 37°C. Blue and light-blue colonies were selected and analyzed.

Phagocytosis experiments. Phagocytosis experiments were performed mainly by the method described by Beachey et al. (1). Fresh human blood was heparinized, and erythrocytes were removed by sedimentation in 2% (wt/vol) dextran at 37°C for 40 min. Buffy-coat leukocytes were recovered by centrifugation (400 x *g* for 10 min) and suspended at 56°C for 30 min in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. Then, 20 μl of bacterial cells was mixed with 200 μl of leukocytes, and the mixture was incubated for 30 min at 37°C with gentle shaking. Smears were examined microscopically.

Other procedures. Media, reagents, isolation of plasmid DNA, and transformation were as described previously (24).

RESULTS

Genetic and molecular identification of the *cap* region on the Cap plasmid. An approximately 6.2-kilobase fragment of the

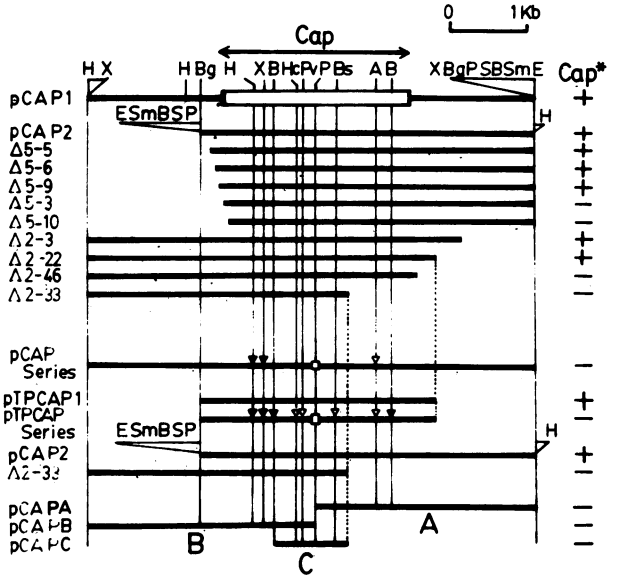

FIG. 1. Physical and genetic map of the *cap* region. In the pCAP and pTPCAP series, pHY300PLK and pMY6003, respectively, were used as vector plasmids. A multicloning site of pHY300PLK is shown at both termini of pCAP1 and pCAP2. Cap* indicates the ability to produce the capsule; positive and negative reactions are judged by reactions with anti-Cap serum, respectively (24). Symbols: ▼, 4-bp insertion; ▽, *Bgl*II linker insertion; □, 4-bp deletion; △, deletion or frameshift mutation; ——, regions present on these plasmids (interruption in each line indicates the deletion); A, B, C, *capA*, *capB*, and *capC*; H, *Hin*dIII; Bg, *Bgl*II; X, *Xba*I; B, *Bam*HI; Hc, *Hin*cII; Pv, *Pvu*II; P, *Pst*I; Bs, *Bst*EII; A, *Aat*I; S, *Sal*I; Sm, *Sma*I; E, *Eco*RI.

cap region of the Cap plasmid, pTE702, which is required for encapsulation in *B. anthracis* TE702, had been cloned in *E. coli* and shown to phenotypically express a capsule substance immunologically identical to that of wild-type *B. anthracis* TE702 (24). To identify and localize the minimum essential region more precisely, various deletion mutants were made by exonucleolytic cleavage with BAL 31 and frameshift mutants were made by in vitro site-specific mutagenesis at the specific restriction sites (Fig. 1). It was identified as an approximately 3.0-kilobase-pair region on pCAP1 (Fig. 1). To analyze the genetic organization of the minimum essential region thus identified, we performed cistron analysis in *E. coli* C600 (*recA*) by complementation between the mutants obtained above. These mutants consist of pCAP series and pTPCAP series mutually compatible with one another. The former were mutants of pCAP1 cloned into pHY300PLK, and the latter were derivatives of pTPCAP1 cloned into pMY6003. The complementation tests scored by the immunological reaction to anti-Cap serum resulted in at least three cistrons, which were designated according to the order of arrangement as *capB*, *capC*, and *capA* (the order A, B, and C is based on the molecular weight of the products in minicells and also on the sizes of the open reading frames) (Table 2). To identify the region coding for the three cistrons, pCAPA, pCAPB, and pCAPC (Fig. 1) were constructed by using the vector pHY300PLK and examined for their ability to restore the Cap^+ phenotype when coexisting with one of the mutants of the pTPCAP series. The results agreed with those of the complementation tests and showed that pCAPA, pCAPB, and pCAPC contain *capA*, *capB*, and *capC*, respectively (Table 2). This does not necessarily show that each of the cistrons has its own promoter, because it has been suggested that a promoter activity exists in both directions at the multicloning sites of the vector pHY300PLK.

Identification in minicells of proteins encoded by the Cap region. The products encoded by the *cap* region were analyzed in the *E. coli* minicell system (Fig. 2). Four major proteins, CapA (ca. 46 kDa), CapB (ca. 44 kDa), CapB′ (ca. 20 kDa), and CapC (ca. 16 kDa), were produced by the intact *cap* region of pTPCAP1 (Fig. 2A, lane 1). The site-specific mutagenesis of the *cap* region at the *Hin*dIII site abolished the production of CapB (Fig. 2A, lane 2). Mutation at the *Xba*I, *Bam*HI, *Hin*cII, or *Pvu*II site resulted in the loss of CapB and CapB′ (Fig. 2A, lanes 3 to 6). Mutation at the *Pst*I or *Bst*EII site inactivated the coding capacity of CapC (Fig. 2A, lanes 7 and 8). Finally, mutation at the *Aat*I site abolished the production of CapA (Fig. 2A, lane 9). Among BAL 31 deletion mutants from the right of pCAP2 with respect to the direction shown in Fig. 1, deletion 2-22 produced the whole intact protein products, whereas deletion 2-46 failed to produce CapA (Fig. 2B, lanes 1 and 2). Similarly, deletion 5-9 was intact with respect to the four protein products, but deletions 5-3 and 5-10 did not produce CapB (Fig. 2B, lanes 3 to 5). pCAPA, pCAPB, and pCAPC were found to encode CapA, CapB plus CapB′, and CapC, respectively (Fig. 2C, lanes 3 to 5). All these results are consistent with the conclusion that cistrons *capA*, *capB*, and

TABLE 2. Complementation and rescue tests

pCAP series	pTPCAP series[a]								
	capB					*capC*		*capA*	
	▼*Hin*dIII	▼*Xba*I	▼*Bam*HI	▽*Hin*cII	▽*Pvu*II	□*Pst*I	▽*Bst*EII	▽*Aat*I	▼*Bam*HI
▼*Hin*dIII	−	−	−	−	−	+	+	+	+
▼*Xba*I	−	−	−	−	−	+	+	+	+
□*Pst*I	+	+	+	+	+	−	−	+	+
▽*Aat*I	+	+	+	+	+	+	+	−	−
△2-46	+	NT	NT	NT	NT	+	NT	−	−
△2-33	+	NT	NT	NT	NT	+	NT	−	−
△5-10	−	−	−	−	−	+	NT	+	NT
pCAPA	−	−	−	−	−	−	−	+	+
pCAPB	+	+	+	+	+	−	−	−	−
pCAPC	−	−	−	−	−	+	+	−	−

[a] Symbols: +, Cap^+; −, Cap^-; NT, not tested. The symbols just before the name of each restriction enzyme represent the nature of in vitro-constructed mutants (Fig. 1).

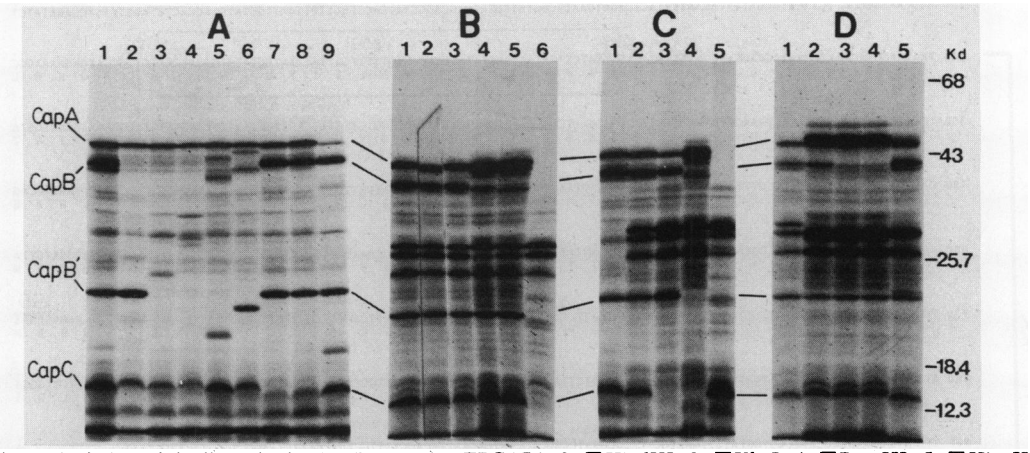

FIG. 2. Protein analysis by minicell methods. (A) Lanes: 1, pTPCAP1; 2, ▼*Hin*dIII; 3, ▼*Xba*I; 4, ▼*Bam*HI; 5, ▽*Hin*cII; 6, ▽*Pvu*II; 7, □*Pst*I; 8, ▽*Bst*EII; 9, ▽*Aat*I. For the significance of each symbol just before the name of each restriction enzyme, see Fig. 1. (B) Lanes: 1, 2-22; 2, 2-46; 3, 5-9; 4, 5-3; 5, 5-10; 6, pHY300PLK. These deletion mutants are derivatives cloned to pHY300PLK. (C) Lanes: 1, pTPCAP1; 2, pCAP1; 3, pCAPB; 4, pCAPA; 5, pCAPC. (D) Lanes: 1, pCAP1; 2, pBRCAP 5-5; 3, pBRCAP 5-6; 4, pBRCAP 5-9; 5, pBRCAP1. These deletion mutants are derivatives cloned to pBRCAP1. Protein products were fractionated on sodium dodecyl sulfate–12.5% polyacrylamide gels. Major proteins, CapA, CapB, CapB′, and CapC, are shown on the left side. Positions of marker proteins are shown on the right side. Kd, Kilodaltons.

capC encode CapA, CapB plus CapB′, and CapC, respectively.

Complete nucleotide sequence of the *cap* region. The nucleotide sequence of the 3,244 base pairs (bp) covering the whole intact *cap* region and the vicinity of the mutation endpoints of some deletion mutants was determined (Fig. 3). Three open reading frames corresponding to the products CapB (44 kDa), CapC (16 kDa), and CapA (46 kDa) were found in this order and in the same direction. No open reading frame capable of coding for a protein larger than 8 kD was found in the opposite direction. Each coding frame was finally identified on the basis of the following findings. There were three possible translational initiation codons for CapB in frame, the ATG codon at the nucleotide 81 (abbreviated as ATG-81), ATG-282, and GTG-309. Since deletion mutants 5-5, 5-6, and 5-9 were Cap$^+$ but 5-10 lacked the product CapB (Fig. 2B, lanes 3 to 5), ATG-282 was most likely to be the initiation codon for CapB. Thus, CapB contains 397 amino acid residues and has a molecular weight of 44,872. The production of CapB′ was abolished by site-specific mutagenesis at any one of the *Xba*I, *Bam*HI, *Hin*cII, or *Pvu*II sites but not at the *Hin*dIII site (Fig. 2A, lanes 2 to 6), indicating that the translational initiation codon for CapB′ is GTG-849 in frame with CapB. CapB′ consists of 208 amino acid residues and has a molecular weight of 23,349. Since CapB′ mutants are always CapB mutants also but the reverse is not the case, the contribution of CapB′ to encapsulation cannot easily be decided. However, since the normal production of CapB′ always accompanies the normal production of CapB, the physiological function of CapB′ may at this stage be considered together with that of CapB. Since CapC disappeared from the minicell products when the *Pst*I site was deleted, the translational initiation codon for CapC appears to be ATG-1490. It consists of 149 amino acid residues and has a molecular weight of 16,522. The translational initiation codon for CapA may be either ATG-1951 or ATG-2134. However, since CapA is appreciably larger than CapB in the minicell analysis, ATG-1951 seems to be more probable. Hence, CapA contains 411 amino acid residues and has a molecular weight of 46,420. For each of the open reading frames described above, one possible ribosome-binding site (Shine-Dalgarno sequence) (35) was found at 5 to 10 bp upstream of the translational initiation codons described above.

Deletion mutant 5-9, with a deletion 274 bp from the *Bgl*II site, leaving only 8 bp up to the translational initiation codon, was found to be Cap$^+$. This is probably due to readthrough from the vector. To identify the promoter unique to the *capB* cistron, we constructed deletion mutants with the vector derived from pBR322 rather than pHY300PLK by exchanging the fragment covering the CapB-coding region. Since a *Sal*I linker had been inserted at the site of deletion endpoints of the pHY300PLK-cloned deletions 5-5, 5-6, 5-9, 5-3, and 5-10, the *Sal*I-*Aat*I fragment of each deletion mutant was exchanged with the *Sal*I-*Aat*I fragment of pBRCAP1 having the intact *cap* region cloned into pBR322 (Fig. 4). Thus, a series of pBRCAP-cloned deletion mutants with the same code number as those of the original pHY300PLK-cloned deletion mutants were obtained. pBRCAP deletion mutant 5-5 was Cap$^+$ like its parental pHY300PLK deletion mutant, 5-5. However, pBRCAP deletion mutants 5-6, 5-9, 5-3, and 5-10 were Cap$^-$, despite the previous finding that the original pHY300PLK deletion mutants 5-6 and 5-9 were Cap$^+$ (Fig. 1). In the minicell analysis, pBRCAP deletion 5-5 produced intact CapA, CapB, and CapC, whereas pBRCAP deletions 5-6 and 5-9 produced intact CapA and CapC but not CapB (Fig. 2D). These observations indicate that the promoter unique to *capB* exists between nucleotides 135 and 239. Since CapA and CapC were phenotypically expressed in minicell analysis of pBRCAP deletions 5-6 and 5-9 in the absence of the promoter unique to *capB*, it seems reasonable to assume that they have their own promoter(s). The putative promoter sequences for each of the three cistrons *capB*, *capC*, and *capA* are shown in Fig. 3. To confirm that these sequences are in fact active as the promoter, the *Bst*EII-*Aat*I fragment, the *Bgl*II-*Hin*dIII fragment, and the *Pvu*II-*Pst*I fragment, containing the putative promoter sequence of *capB*, *capC*, and *capA*, respectively, were each cloned to an expression vector pKK232-8 (5) in the direction of transcription for the chloramphenicol resistance gene. Insertion of either of these sequences increased the MIC of chloramphenicol by about

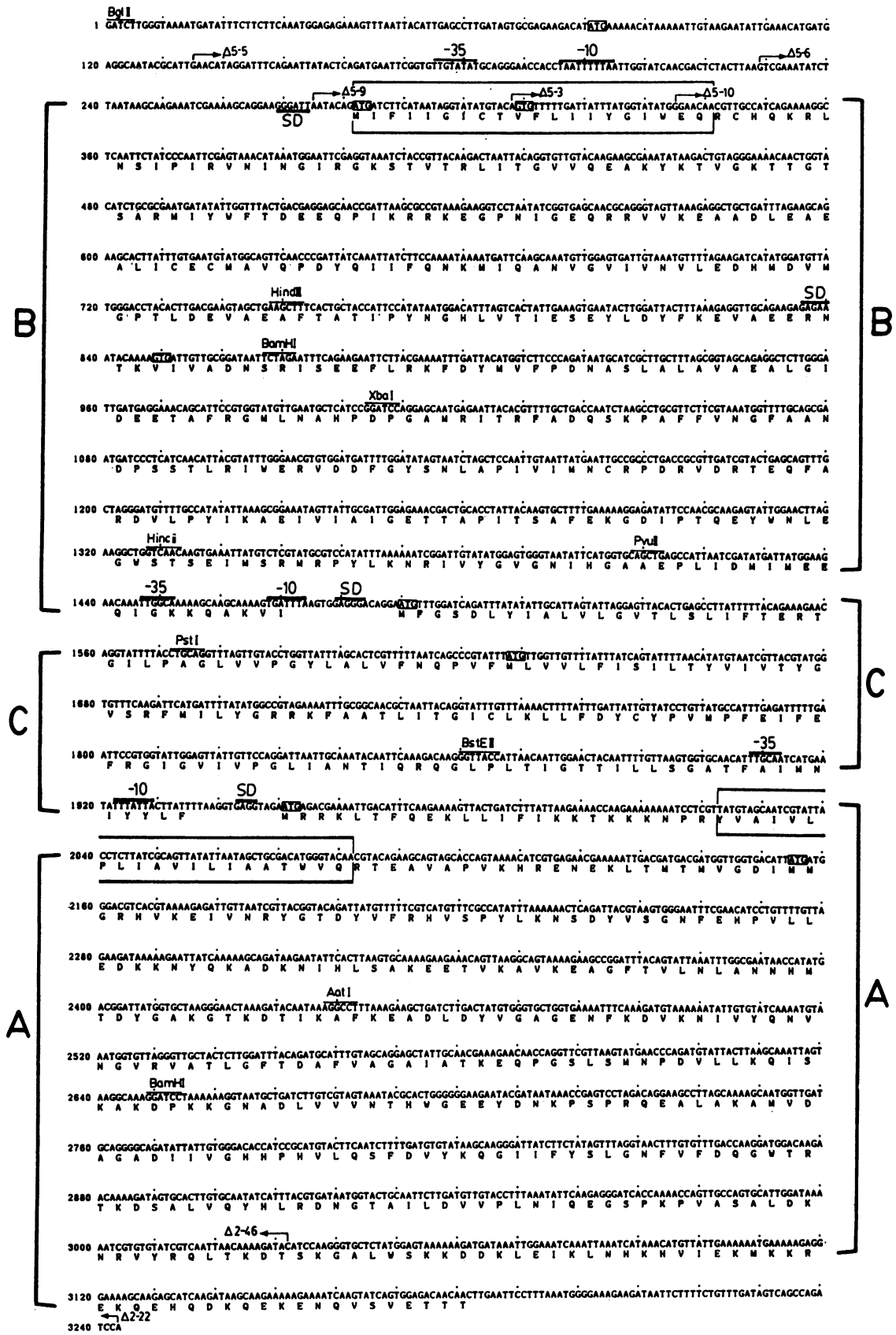

FIG. 3. Nucleotide sequence of the *cap* region. The sequence is numbered starting from the *Bgl*II site of the *cap* region. Possible translational initiation codons are enclosed in small boxes. Possible promoter sequences are shown as −35 and −10, and SD is the possible Shine-Dalgarno sequence (35). Right-directed arrows above the sequence point to the region of DNA remaining undeleted in the denoted deletion mutants (described in the legend to Fig. 1). Hydrophobic amino acid sequences in CapA and CapB are boxed. A, B, and C are the open reading frames of CapA, CapB, and CapC products, respectively.

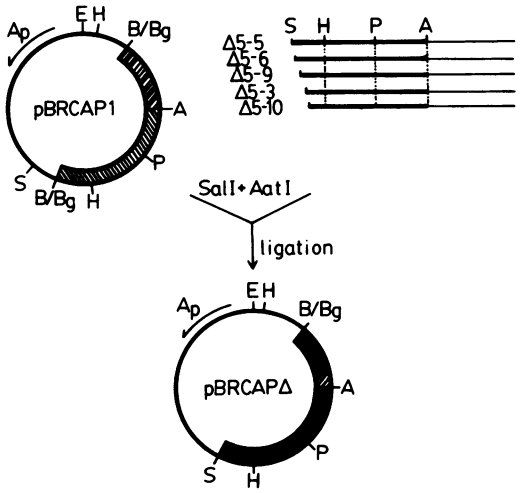

FIG. 4. Construction of pBRCAP1-derived deletions.

4- to 16-fold in *E. coli*. This indicates that the sequences contain a promoter sequence active in *E. coli*. However, the extent of the increase in MIC was not as high as that of the *E. coli*-derived promoter. The introduction of a functional *E. coli* promoter upstream from that of the *B. anthracis cap* region highly potentiated the promoter activity of the latter (data not shown).

Localization of CapA, CapB and CapC proteins in *E. coli*. To determine the location of these proteins, we fractionated the cytoplasmic, membrane, and periplasmic fractions of minicells (Fig. 5A). All the CapA, CapB, and CapC fractions were found in the membrane fraction, as opposed to β-lactamase, which was found in the periplasmic fraction. When the spheroplast preparation was treated with trypsin, all three proteins were unaffected and remained intact, whereas the trypsin treatment following lysis of the spheroplasts by sonication resulted in the loss of the CapA and the CapB proteins (Fig. 5B). These results indicate that all three proteins are membrane-associated proteins.

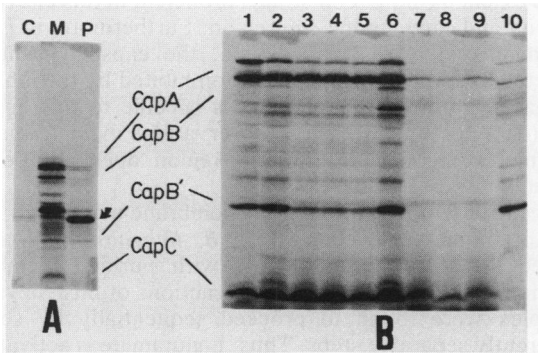

FIG. 5. Fractionation of minicells containing the *cap* region and its trypsin digestion. (A) Fractionation of minicells containing pBRCAP1. C, cytoplasmic fraction; M, membrane fraction; P, periplasmic fraction. An arrow shows the mature β-lactamase protein. (B) Trypsin treatment of spheroplast preparations. Lane 1, intact minicell containing pTPCAP1; Lane 10, periplasmic fraction. The intact (lanes 2 to 5) and the lysed (lanes 6 to 9) spheroplasts derived from minicells containing pTPCAP1 were treated with trypsin at concentrations of 0 (lanes 2 and 6), 5 (lanes 3 and 7), 20 (lanes 4 and 8), or 50 (lanes 5 and 9) μg/ml. Protein products were fractionated on sodium dodecyl sulfate–12.5% polyacrylamide gels.

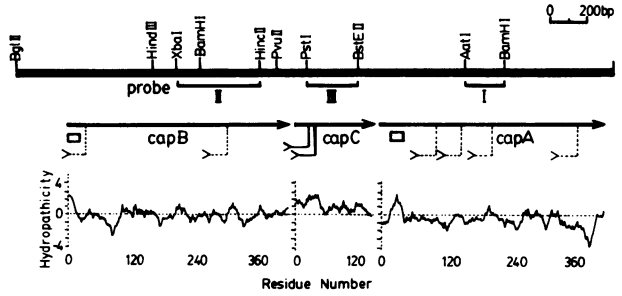

FIG. 6. Protein map of the *cap* region. A hydropathicity plot of each protein was generated by using the program of Kyte and Doolittle (20); window = 20 was used. The value of each position was plotted against the residue number of each protein. Arrows with solid lines at the middle are the open reading frames of the three proteins CapA, CapB, and CapC. Open boxes in CapA and CapB proteins shown near the N terminus are hydrophobic amino acid sequences. Tn*phoA* insertion sites are shown as solid and broken lines below the arrows of open reading frames, and the direction of *phoA* transcription is shown as an arrow; solid and broken lines represent blue and light-blue colonies on XP plates, respectively. Probes I, II, and III were used for dot hybridization experiments (shown in text and in Fig. 7).

Distribution of the sequence homologous to the *cap* region in other bacilli. In addition to *B. anthracis*, *B. subtilis*, *B. megaterium*, and *B. licheniformis* are known to have a capsule consisting of glutamyl polypeptide. To identify the capsular component and its genetic determinant among these bacilli, *B. anthracis* TE702, TE703 (as TE702 but cured of the Cap plasmid), and Shikan (a virulent Cap$^+$ strain), *B. subtilis* NIAH801, *B. megaterium* NIAH368, and *B. licheniformis* NIAH227 were used to prepare the antigen for Ouchterlony precipitation assays against the antibody raised to *B. anthracis* TE702 capsular antigen. Except for the Cap$^-$ TE703 strain, all produced fused precipitation lines (see Fig. 7A), indicating that they produce an immunologically identical polypeptide. On the other hand, dot hybridization was performed with the six strains described above by using three probes, probes I, II and III (Fig. 6 and 7B). Only the Cap$^+$ strains of *B. anthracis*, TE702 and Shikan, were homologous to the probes, indicating that the genetic determinant for the capsular polypeptide in three bacilli other than *B. anthracis* is not homologous to the Cap region.

The *cap* region confers resistance to phagocytosis. Using a pair of Cap$^+$ and Cap$^-$ strains, we examined the biological

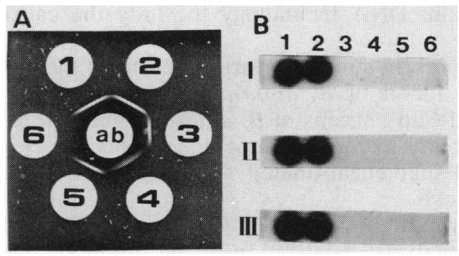

FIG. 7. Distribution of the capsular antigen and the sequence homology to the *cap* region in various bacilli. (A) Ouchterlony assay with anti-Cap serum raised by the capsule antigen from *B. anthracis* TE702 (24). Antigens were isolated by a 20-min autoclaving of the cell suspension grown on NBY agar plates (24) in the presence of 20% CO_2. (B) Dot hybridization with probes I, II, and III (Fig. 6). About 20 μg of total DNA per spot was used. Dots: 1, *B. anthracis* TE702; 2, *B. anthracis* Shikan; 3, *B. subtilis*; 4, *B. megaterium*; 5, *B. licheniformis*; 6, *B. anthracis* TE703.

TABLE 3. Phagocytosis of *B. anthracis* Cap$^+$ and Cap$^-$ strains

Bacterial cells[a]	Treatment[b]	% Phagocytosis[c]
TE702	Untreated	4
TE7021	Untreated	98
TE702	Autoclaved[d]	37
TE7021	Autoclaved[d]	97
TE702	Pronase[e]	55
TE7021	Pronase[e]	93
TE702	Anti-Cap serum[f]	38
TE7021	Anti-Cap serum[f]	96

[a] *B. anthracis* TE702 is Cap$^+$ and contains an intact capsule plasmid pTE702. TE7021 is Cap$^-$ and contains a pTE7021 plasmid with an insertion mutation in the *cap* region.

[b] Because *B. anthracis* cells were linked together, they were slightly sonicated and separated from each other.

[c] Expressed as number of neutrophiles per 100 counted cells containing one or more bacterial cells.

[d] Sonicated cells were autoclaved at 121°C for 30 min and washed in phosphate-buffered saline.

[e] Sonicated cells were treated with pronase (100 μg/ml) at 37°C for 30 min and washed in phosphate-buffered saline.

[f] Sonicated cells were incubated with phagocytic cells in the presence of anti-Cap serum.

role of the capsule. TE702 is a strain of *B. anthracis* that is avirulent as a result of the loss of the toxin plasmid, but it carries the intact capsule plasmid pTE702 and hence is Cap$^+$. TE7021 (24) is a Cap$^-$ derivative of TE702 containing pTE7021 (an insertion mutant in the *cap* region of pTE702). TE7021 but not TE702 was efficiently phagocytosed in the absence of complement and specific antibody (Table 3). Treatments more or less removing the capsular substance from the bacterial surface, such as autoclaving and pronase digestion and the addition of anti-Cap serum to the reaction mixture, slightly but consistently decreased the ability of the Cap$^+$ bacteria to resist phagocytosis.

DISCUSSION

Although a considerable amount of information is available concerning the biosynthesis of bacterial surface structures including capsular polysaccharides (47), lipopolysaccharides (29), peptidoglycan (13), and membrane mannans (33), relatively little is known about the molecular mechanism of the synthesis of the poly-D-glutamyl capsule of *B. anthracis*. We demonstrated in a previous study (24) that the cloned *cap* region was phenotypically expressed in *E. coli* and produced a capsular substance immunologically identical with that of *B. anthracis*. This permitted us to adopt recombinant DNA technology to study the capsule of *B. anthracis* in *E. coli*.

In the present study, to clarify the role of the capsule as a virulence factor of *B. anthracis*, we constructed isogenic Cap$^+$ and Cap$^-$ strains of *B. anthracis* and compared them for the ability to resist phagocytosis. This has clearly demonstrated that encapsulated strains of *B. anthracis* resist phagocytosis.

Furthermore, in the present study, complementation tests, minicell analysis, localization of the products, and nucleotide sequence determination for the *cap* region performed in *E. coli* have identified three cistrons, *capA*, *capB*, and *capC*. Each of these has a respective unique promoter but the same direction of transcription. The hydropathicity of the products, CapA, CapB, and CapC, was plotted (Fig. 6) on the basis of the nucleotide sequences obtained (Fig. 3). CapC was shown to be a hydrophobic protein, strongly suggesting that it is a membrane protein (19). CapA has a hydrophobic segment from Try-25 to somewhere around Gln-44, and the segment is surrounded by Arg. The N-terminal 20 amino acids of CapB are also hydrophobic, and amino acid 21 is Arg (Fig. 3 and 6), suggesting that the N-terminal 20-amino-acid segment is associated with the membrane and the other portion is in the cytoplasmic space. In addition, minicell analysis has revealed that all the CapA, CapB, and CapC fractions are in the membrane fraction (Fig. 5). These results are consistent with the hypothesis that all three proteins are membrane associated; that the hydrophobic segment of CapA and CapB, consisting of 20 amino acids (boxed in Fig. 3), is integrated into the membrane, with the other portion remaining in the cytoplasmic space; and, finally, that CapC is hydrophobic and integrated into the membrane so that its eight trypsin-susceptible sites are protected from digestion by trypsin.

The capsular substance of *B. anthracis*, *B. subtilis*, *B. megaterium*, and *B. licheniformis* was found to be immunologically identical (Fig. 7A). However, the genetic determinant for the synthesis of the capsule substance seems to be distinctive between *B. anthracis* and other bacilli, because no sequence was found in bacilli other than *B. anthracis* that was homologous to any probes derived from the *cap* region (Fig. 7B). Since functionally identical enzymes derived from different species of bacteria may be encoded by genetic determinants completely nonhomologous with one another (17), this observation may not be exceptional.

On the other hand, the lack of homology may reflect the difference in the synthetic pathways. There have been several reports on the enzymatic pathways leading to the biosynthesis of the poly-D-glutamate capsule in bacilli (12, 40, 42, 44). Although the synthetic pathway of capsule formation in *B. anthracis* remains obscure, the capsule of this organism is composed of only a homopolypeptide of D-glutamic acid (11). In contrast, glutamyl polypeptide produced by *B. subtilis* (*natto*) contains both D- and L-glutamic acid (41), and it was reported that γ-glutamyltranspeptidase might participate in the synthesis of capsular substance in *B. subtilis* (12). However, *E. coli* strains (38) defective in γ-glutamyltranspeptidase activity could not be complemented by the *cap* region of *B. anthracis* with respect to this enzymatic defect, but they synthesized the capsule to the same extent as did *E. coli* strains proficient in this enzymatic activity and carrying the *cap* region. Furthermore, in *E. coli* strains defective in this enzyme, the capsular synthesis directed by the *cap* region was not inhibited by the inhibitor 6-diazo-5-oxo-norleucine, which is specific to this enzyme (28) (data not shown). These observations indicate that the enzyme(s) encoded by the *cap* region does not possess γ-glutamyltranspeptidase activity.

In contrast, the existence of membrane-associated polyglutamyl synthetase complex in *B. licheniformis* was reported (8, 44). In experiments with purified membrane fractions of *B. licheniformis*, the reactions of this enzymatic complex were found to proceed sequentially by several apparently separable steps. Thus, L-glutamate is activated in the presence of ATP to form the activated intermediate L-glutamyl AMP. Subsequently, the stereochemical inversion of the L to the D form occurs independently of the activation step. A protein-bound thioester mediates racemization and the subsequent transfer to growing polymeric chains. Several experimental observations have also been reported which support the involvement of an endogeneous acceptor of the N terminus of growing nascent chains onto which sequential addition of activated glutamyl residues occurs (8).

Our preliminary data have revealed that *E. coli* carrying the *cap* region incorporates appreciably higher L-[^{14}C]Glu levels than *E. coli* without the *cap* region does (data not shown). This indicates that L-Glu may be the direct precursor of the capsule encoded by the *cap* region, as is the case in *B. licheniformis*.

Moreover, since the three proteins, CapA, CapB, and CapC, encoded by the *cap* region are all membrane associated (Fig. 5), it is conceivable that the *cap* region codes for a membrane-associated polyglutamyl synthetase complex similar to that in *B. licheniformis*. In *B. anthracis*, the capsular polymer could not be detected in any in vitro-constructed Cap$^-$ mutants (Fig. 1), even after lysis by sonication (data not shown). That is, without all of the three products from the three cistrons, the capsule substance cannot be synthesized at all. This is not compatible with the possibility that the polymerized capsule is synthesized in the cytoplasm and transported to the bacterial surface but suggests that polymerization of glutamate occurs in the membrane. Also, in this respect, the synthetic pathway of the capsular substance encoded by the *cap* region in *B. anthracis* seems to resemble that in *B. licheniformis* (8).

The three proteins in *B. anthracis* were found to be associated with the membrane (Fig. 5). CapC is a hydrophobic protein and seems to exist in membrane-integrated form (Fig. 6). The insertion of Tn*phoA* into the *capC* cistron resulted in a mutant producing a blue colony on an XP plate, which was used for scoring alkaline phosphatase activity, whereas the insertion into *capB* and *capA* resulted in light-blue mutants in the same assay (Fig. 6). These observations indicate that the membrane protein CapC exists neither in the inner membrane nor in the periplasmic space but in the outer membrane in *E. coli*. CapC, but not the two other proteins, was in fact detected in the outer membrane fraction (data not shown). Although these results were obtained with *E. coli*, CapC, among these three proteins encoded by the *cap* region, may exist on the outermost surface in *B. anthracis* also. Thus, if the synthetic pathway in *B. anthracis* highly resembles that in *B. licheniformis*, CapC may be the most suitable candidate for the endogeneous acceptor of glutamyl residues.

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LITERATURE CITED

1. **Beachey, E. H., and I. Ofek.** 1976. Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. J. Exp. Med. **143**:759–771.
2. **Beall, F. A., M. J. Taylor, and C. B. Thorne.** 1962. Rapid lethal effect in rats of a third component found upon fractionating the toxin of *Bacillus anthracis*. J. Bacteriol. **83**:1274–1280.
3. **Bolivar, F., R. L. Rodriquez, P. J. Green, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
4. **Bovarnick, M.** 1942. The formation of extracellular D-glutamic acid polypeptide by *Bacillus subtilis*. J. Biol. Chem. **145**: 415–424.
5. **Brosius, J.** 1984. Plasmid vectors for the selection of promoters. Gene **27**:151–160.
6. **Cabello, F. C.** 1979. Determinants of pathogenicity of *E. coli* K1, p. 155–160. *In* K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North Holland Publishing Co., Amsterdam.
7. **Casadaban, M., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *E. coli*. J. Mol. Biol. **138**:179–207.
8. **Gardner, J. M., and F. A. Troy.** 1979. Chemistry and biosynthesis of the poly (γ-D-glutamyl) capsule in *Bacillus licheniformis*. J. Biol. Chem. **254**:6262–6269.
9. **Green, B. D., L. Battisti, T. M. Koehler, C. B. Thorne, and B. E. Ivins.** 1985. Demonstration of a capsule plasmid in *Bacillus anthracis*. Infect. Immun. **49**:291–297.
10. **Guex-Holzer, S., and J. Tomcsik.** 1956. The isolation and chemical nature of capsular and cell-wall haptens in a *Bacillus* species. J. Gen. Microbiol. **14**:14–25.
11. **Hanby, W. E., and H. N. Rydon.** 1946. The capsule substance of *Bacillus anthracis*. Biochem. J. **40**:297–309.
12. **Hara, T., and S. Ueda.** 1982. Regulation of polyglutamate production in *Bacillus subtilis* (*natto*): transformation of high PGA productivity. Agric. Biol. Chem. **46**:2275–2281.
13. **Higashi, Y., J. L. Strominger, and C. C. Sweeley.** 1967. Structure of a lipid intermediate in cell wall peptidoglycan synthesis: a derivative of a C_{55} isoprenoid alcohol. Proc. Natl. Acad. Sci. USA **57**:1978–1984.
14. **Isiwa, H., and N. Tsuchida.** 1984. New shuttle vectors for *Escherichia coli* and *Bucillus subtilis*. 1. Construction and characterization of plasmid pHY460 with twelve unique cloning sites. Gene **32**:129–134.
15. **Ito, K., T. Sato, and T. Yura.** 1977. Synthesis and assembly of the membrane proteins in *E. coli*. Cell **11**:551–559.
16. **Johnson, J. L.** 1981. DNA isolation, p. 452–453. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Kreig, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
17. **Jones, B. D., and H. L. T. Mobley.** 1987. Genetic and biochemical diversity of ureases of *Proteus*, *Providencia*, and *Morganella* species isolated from urinary tract infection. Infect. Immun. **55**:2198–2203.
18. **Kandler, O., H. Konig, J. Wiegel, and D. Claus.** 1983. Occurrence of poly-γ-D-glutamic acid and poly-γ-L-glutamine in the genera *Xanthobacter*, *Flexithrix*, *Sporosarcina* and *Planococcus*. Syst. Appl. Microbiol. **4**:34–41.
19. **Klein, P., M. Kanehira, and C. DeLisi.** 1985. The detection and classification of membrane-spanning proteins. Biochim. Biophys. Acta **815**:468–476.
20. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
21. **Liu, T. Y., E. C. Gotschlich, E. K. Jonssen, and J. R. Wysocki.** 1979. Studies on the meningococcal polysaccharides. I. Composition and chemical properties of the group A polysaccharide. J. Biol. Chem. **246**:2849–2857.
22. **Makino, S., C. Sasakawa, K. Kamata, T. Kurata, and M. Yoshikawa.** 1986. A genetic determinant required for continuous reinfection of adjacent cells on large plasmid in *S. flexneri* 2a. Cell **46**:551–555.
23. **Makino, S., C. Sasakawa, I. Uchida, N. Terakado, and M. Yoshikawa.** 1987. Transformation of cloning vector, pUB110, into *Bacillus anthracis*. FEMS Microbiol. Lett. **44**:45–48.
24. **Makino, S., C. Sasakawa, I. Uchida, N. Terakado, and M. Yoshikawa.** 1988. Cloning and CO_2-dependent expression of the genetic region for encapsulation from *Bacillus anthracis*. Mol. Microbiol. **2**:371–376.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**: 8129–8133.
27. **Mikesell, P., B. E. Ivins, J. D. Ristoph, and T. M. Dreier.** 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. **39**:371–376.
28. **Nakayama, R., H. Kumagai, and T. Tochikura.** 1984. Leakage of glutathione from bacterial cells caused by inhibition of γ-glutamyltranspeptidase. Appl. Environ. Microbiol. **47**:653–657.
29. **Osborn, M. J.** 1969. Structure and biosynthesis of the bacterial cell wall. Annu. Rev. Biochem. **38**:501–538.
30. **Sandstrom, G., S. Lofgren, and A. Tarnvik.** 1988. A capsule-

deficient mutant of *Francisella tularensis* LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. Infect. Immun. **56**:1194–1202.

31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
32. **Sasakawa, C., and M. Yoshikawa.** 1987. A series of Tn*5* variants with various drug-resistance markers and suicide vector for transposon mutagenesis. Gene **56**:283–288.
33. **Scher, M., and W. J. Lennarz.** 1969. Studies on the biosynthesis of mannan in *Micrococcus lysodeikticus*. I. Characterization of mannan-^{14}C formed enzymatically from mannosyl-1-phosphoryl-undecaprenol. J. Biol. Chem. **244**:2777–2789.
34. **Shiba, K., K. Ito, T. Yura, and D. P. Cerretti.** 1984. A defined mutation in the protein export gene within the *spc* ribosomal protein operon of *Escherichia coli*: isolation and characterization of a new temperature-sensitive *secY* mutant. EMBO J. **3**:631–635.
35. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**:1342–1346.
36. **Stanley, J. L., K. Sargeant, and H. Smith.** 1960. Purification of anthrax toxin factors I and II of the anthrax toxin produced *in vitro*. J. Gen. Microbiol. **22**:206–218.
37. **Sterne, M.** 1937. Variation in *Bacillus anthracis*. Onderstepoort J. Vet. Sci. Anim. Ind. **8**:279–349.
38. **Suzuki, H., H. Kumagai, and T. Tochikura.** 1987. Isolation, genetic mapping, and characterization of *Escherichia coli* K-12 mutants lacking γ-glutamyltranspeptidase. J. Bacteriol. **169**: 3926–3931.
39. **Thompson, R., and M. Achtman.** 1978. The control region of the F sex factor DNA transfer cistrons: restriction mapping and DNA cloning. Mol. Gen. Genet. **165**:295–304.
40. **Thorne, C. B., C. G. Gomez, and R. D. Housewright.** 1955. Transamination of D-amino acids by *Bacillus subtilis*. J. Bacteriol. **69**:357–362.
41. **Thorne, C. B., and C. G. Leonard.** 1958. Isolation of D- and L-glutamyl polypeptides from culture filtrates of *Bacillus subtilis*. J. Biol. Chem. **233**:1109–1112.
42. **Thorne, C. B., and D. M. Molnar.** 1955. D-Amino acid transamination in *Bacillus anthracis*. J. Bacteriol. **70**:420–426.
43. **Thorne, C. B., D. M. Molnar, and R. E. Strange.** 1960. Production of toxin in vitro by *Bacillus anthracis* and its separation into two components. J. Bacteriol. **79**:450–455.
44. **Troy, F. A.** 1973. Chemistry and biosynthesis of the poly(γ-D-glutamyl) capsule in *Bacillus licheniformis*. J. Biol. Chem. **248**:305–315.
45. **Uchida, I., K. Hashimoto, S. Makino, C. Sasakawa, M. Yoshikawa, and N. Terakado.** 1987. Restriction map of a capsule plasmid of *Bacillus anthracis*. Plasmid **18**:178–181.
46. **Uchida, I., T. Sekizaki, K. Hashimoto, and N. Terakado.** 1985. Association of the encapsulation of *Bacillus anthracis* with a 60 megadalton plasmid. J. Gen. Microbiol. **131**:363–367.
47. **Vijay, I. K., and F. A. Troy.** 1975. Properties of membrane-associated sialyltransferase of *Escherichia coli*. J. Biol. Chem. **250**:164–170.