

CapE, a 47-Amino-Acid Peptide, Is Necessary for *Bacillus anthracis* Polyglutamate Capsule Synthesis

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Polyglutamate is found in various bacteria, but displays different functions depending on the species and their environment. Here, we describe a minimal polyglutamate synthesis system in *Bacillus anthracis*. In addition to the three genes previously described as sufficient for polyglutamate synthesis, this system includes a small open reading frame, *capE*, belonging to the *cap* operon. The polyglutamate system's requirement for the five *cap* genes, for capsulation and anchoring, was assayed in nonpolar mutants. The *capA*, *capB*, *capC*, and *capE* genes are all necessary and are sufficient for polyglutamate synthesis by *B. anthracis*. *capD* is required for polyglutamate anchoring to the peptidoglycan. The 47-amino-acid peptide encoded by *capE* is localized in the *B. anthracis* membrane. It is not a regulator and it is required for polyglutamate synthesis, suggesting that it has a structural role in polyglutamate synthesis. CapE appears to interact with CapA. *Bacillus subtilis ywtC* is similar to *capE* and we named it *pgsE*. Genes similar to *capE* or *pgsE* were found in *B. subtilis* natto, *Bacillus licheniformis*, and *Staphylococcus epidermidis*, species that produce polyglutamate. All the bacterial polyglutamate synthesis systems analyzed show a similar genetic organization and, we suggest, the same protein requirements.

Poly- γ -glutamate (PGA) is a natural polymer. It has diverse functions that vary according to the species and their environment. For example, because it is anionic, PGA has a high affinity for metal ions (27). *Planococcus halophilus*, *Sporosarcina halophila*, and probably the halophilic archaeon *Natrialba asiatica* produce PGA that allows them to resist high salt concentrations (16, 17). PGA is also produced in the nematocyst of the common freshwater polyp *Hydra vulgaris* that belongs to the cnidarian species. PGA is also very poorly immunogenic, and bacilli, such as *Bacillus anthracis*, that are covered by a capsule of PGA do not elicit anticapsule antibodies in the host (4, 14, 32, 34). When present, bacterial capsules are the outermost structure of the bacterial surface. Capsules can prevent desiccation and can also promote adherence. They have mainly been studied because many capsulated bacteria are pathogenic and the capsule is involved in the first contact between the bacterium and the host. However, very little is known about PGA synthesis.

B. anthracis, the etiological agent of anthrax, is a gram-positive sporeforming bacterium that expresses two major virulence factors, toxins and capsule. Anthrax disease is a lethal infection that involves both toxemia and septicemia. The toxin genes are carried on a 186-kb plasmid, pXO1, and the capsule synthetic operon (*capBCAD*) is carried on a 90-kb plasmid, pXO2. The *B. anthracis* capsule is composed of poly- γ -D-glutamate and is covalently linked to the peptidoglycan (4). Three *B. anthracis* genes, *capB*, *capC*, and *capA*, were shown to be necessary and sufficient to drive the production of polyglutamate in *Escherichia coli* (24, 25). However, Makino et al. reported that polyglutamate production was weak in *E. coli*.

Moreover, these three genes are not sufficient to promote PGA synthesis in *Bacillus subtilis* (24). Therefore, there may be an essential element that is absent in *B. subtilis*.

CapB, CapC, and CapA have not been studied in *B. anthracis*. Their functions have been predicted or deduced by similarity to the corresponding proteins in *B. subtilis*. Other gram-positive bacteria, including bacilli, also produce PGA: over the last 10 years, genes similar to *B. anthracis capB*, *capC*, and *capA* have been found in *Bacillus subtilis* 168 IFO3336 (named *ywsC*, *ywtA*, and *ywtB*, respectively), *Staphylococcus epidermidis* ATCC 12228 (*capB*, *capC*, and *capA*), *Bacillus licheniformis* ATCC 14580, and *B. subtilis* natto IFO16449 (*pgsB*, *pgsC*, *pgsAA*, and *pgsA* for both) genomes (1, 12, 22, 31, 45). PGA synthesis systems have been studied in *B. licheniformis* (18, 19, 43), and more recently in *B. subtilis* (1, 44). However, the PGAs in these species are composed of D- and L-glutamates whereas the *B. anthracis* PGA is composed of the D-isomer only. Furthermore, the *B. anthracis* PGA is anchored, whereas the *B. subtilis* and some *B. licheniformis* PGAs are secreted or released into the culture supernatant.

A *B. licheniformis* membranous enzymatic complex was shown to be involved in PGA synthesis using L-glutamate as substrate and ATP as the energy source, but its components were not described (23, 43). YwsC, YwtA, and YwtB in *B. subtilis* 168 and the similar PgsB, PgsC, and PgsA in *B. subtilis* natto may also be organized in complexes (3, 44). It is assumed that PgsB, PgsC, and PgsA have similar roles to CapB, CapC, and CapA, respectively. CapB/PgsB harbors a Walker sequence and appears to be the polymerase, able to use ATP (10, 44). Ashuishi et al. reported that PgsB, PgsC, and PgsA together had a higher ATPase activity than either the isolated proteins or the PgsB PgsC pair of proteins, and therefore the three proteins might be organized in a complex (3). PgsB and PgsC together possess activity, albeit lower than that of the

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TABLE 1. Plasmids and *B. anthracis* strains used in this study

Plasmid or strain	Relevant characteristics or genotype	Source or reference
Conjugative plasmids		
pAT18	Conjugative plasmid, replicative in <i>B. anthracis</i> , 20 to 50 copies per cell	41
pAT28	Conjugative plasmid, replicative in <i>B. anthracis</i> , 20 to 50 copies per cell	40
pAT113	Conjugative suicide plasmid used for gene inactivation in <i>B. anthracis</i>	9
p113capA	pAT113 carrying the inactivated <i>capA</i> gene	This work
p113capB	pAT113 carrying the inactivated <i>capB</i> gene	This work
p113capC	pAT113 carrying the inactivated <i>capC</i> gene	This work
pDSP20	pAT113 carrying the inactivated <i>capD</i> gene	4
p113capE	pAT113 carrying the inactivated <i>capE</i> gene	This work
pPPA40	pAT18 carrying <i>Ppag</i>	This work
pPAGE	pPPA40 carrying <i>capE</i>	This work
pPAG55	pPPA40 carrying <i>B. subtilis ywtD</i>	This work
pPGSA	pPPA40 carrying <i>B. subtilis ywtB</i>	This work
pPGSAE	pPPA40 carrying <i>B. subtilis ywtB</i> and <i>ywtD</i>	This work
pPGSA1	pPPA40 carrying <i>B. licheniformis ywtB</i>	This work
pPGSE1	pPPA40 carrying <i>B. licheniformis ywtD</i>	This work
pPGSAE1	pPPA40 carrying <i>B. licheniformis ywtB</i> and <i>ywtD</i>	This work
pMini20	pAT28 carrying <i>Pcap</i> , <i>capB</i> , <i>capC</i> , <i>capA</i> , <i>capD</i> , and <i>capE</i>	This work
pMini21	pAT28 carrying <i>Pcap</i> , <i>capB</i> , <i>capC</i> , and <i>capA</i>	This work
<i>B. anthracis</i> strains		
9131	pXO1 ⁻ , pXO2 ⁻	9
RPG1	pXO1 ⁺ (<i>cya lef</i>), pXO2 ⁺	13
RTC10	$\Delta capB$ derivative of RPG1	This work
RTC20	$\Delta capC$ derivative of RPG1	This work
RTC30	$\Delta capA$ derivative of RPG1	This work
RTC40	$\Delta capD$ derivative of RPG1	4
RTC50	$\Delta capE$ derivative of RPG1	This work

three proteins, suggesting that PgsB and PgsC form a tight complex (3).

Contradictory data have been published. PgsA may be necessary for PGA synthesis (3), or PgsA may be required to improve PGA production, indicating that PgsB and PgsC could have the polymerase role (44). The complex must also transport the substrate or the PGA through the plasma membrane, and PgsC (YwtB) has similarity with a transport protein (44). In *B. anthracis*, a fourth protein encoded by the *capBCAD* operon, CapD, belongs to the γ -glutamyl-transpeptidase family. It is involved in anchoring of the capsule (4), and it may well catalyze the covalent anchoring of PGA to the peptidoglycan.

Here, we defined a minimum PGA-synthesizing system in *B. anthracis*. Our data indicate that *capB*, *capC*, and *capA* are not sufficient to produce PGA in *B. anthracis*. A previously undescribed open reading frame (ORF), *capE*, encoding a 47-amino-acid peptide, is necessary for PGA synthesis. The protein encoded, CapE, appears to interact with CapA. A genetic analysis suggests that PGA synthesis in other bacteria also requires a similar gene.

MATERIALS AND METHODS

Bacterial strain, cloning vectors, and culture conditions. *Escherichia coli* TG1 (33) was used as a host for derivatives of pUC19 (New England Laboratories), pAT113, pAT18 and pAT28 (40, 41, 42), pGemT-easy (Promega), pSPCH + 1, pSPCH + 2 (28), and pBACP1 (4, 11). HB101(pRK24) was used for mating experiments (39). Two *B. anthracis* strains were used during the study, 9131 (pXO1⁻ and pXO2⁻) and RPG1 (pXO1⁺, pXO2⁺, Tox⁻) (9, 13). *B. anthracis* strains were grown in brain-heart infusion (BHI; Difco Laboratories). To observe capsule, *B. anthracis* was grown in R-medium with 0.6% sodium bicarbonate (35) or on CAP medium under a 5% CO₂ atmosphere (11). Antibiotics were used as previously described (37).

DNA manipulation. Plasmid extraction, endonuclease digestion, ligation and agarose and polyacrylamide gel electrophoreses were carried out as described by Maniatis et al. (26). pXO2 was purified by a method derived from the classical alkaline purification method. The lysis step is carried out in 20% sucrose and the protoplast formation is followed by optical microscopy. PCR amplifications were carried out with long-range high fidelity Taq DNA polymerase according to the manufacturer's instructions (Roche).

Genetic constructions. The *capB*, *capA*, *capC*, and *capE* genes were inactivated with non polar spectinomycin cassettes (28). A BglII and PstI fragment from pBACP1 was inserted into pUC19 digested with BamHI and PstI giving pUCBC10. pUCBC20 was constructed by replacing XbaI-HincII fragment of pUCBC10 with a HincII fragment of pSPCH+2 containing a nonpolar spectinomycin cassette. The SphI-SacI fragment of pUCBC20 was inserted into pAT113 digested with the same endonucleases, giving p113capB.

A DNA fragment was amplified by PCR with oligonucleotides capBinterne (GGTGGTGTTCGACGTGAAGGAGGAGCATTATGTCAGAAGAATTCTTACGAAAATTGATTACATGGTCTTCCC) and capA2a (TCCCCGGGCA TTTTGATACACAATATTTTTTACATCTTTGAAAT) using *B. anthracis* pXO2 as the template. The fragment was inserted into pUC19 digested with SmaI and HincII giving pUCCA10. pUCCA20 was constructed by replacing the StuI/EcoNI fragment with a HincII fragment of pSPCH+1 containing a nonpolar spectinomycin cassette. The SphI-SacI fragment of pUCCA20 was inserted into pAT113 digested with the same endonucleases, giving p113capA.

The *capC* open reading frame was amplified by PCR with oligonucleotides capCs (CGCGGATCCATGATCTTCATAAATAGGTATATGTACAGTGTTT TTTG) and capCa (TCCCCGGGTTAAAATAAGTAATAAATATTCATGAT TGCAAATG) using *B. anthracis* pXO2 as the template. This fragment was inserted into pUC19 digested with SmaI giving pCAPC10. A HincII fragment of pSPH+2 was inserted into pCAPC10 digested by SnaBI to give pCAPC20. The BamHI-SmaI fragment of pUCCA20 was inserted into pAT113 digested with the same endonucleases, giving p113capC.

An SphI-NheI fragment of pBACP1 was inserted into pUC19 digested by SmaI giving pUCE10. A HincII fragment of pSPCH+1 was inserted into pUCE10 digested by SnaBI giving pUCE20. The KpnI-SphI fragment of pUCE20 was inserted into pAT113 digested with the same endonucleases, giving p113capE.

To overexpress CapA2, a peptide contained within CapA (amino acid residues 46 to 191), we inserted the corresponding DNA into an expression vector. A DNA

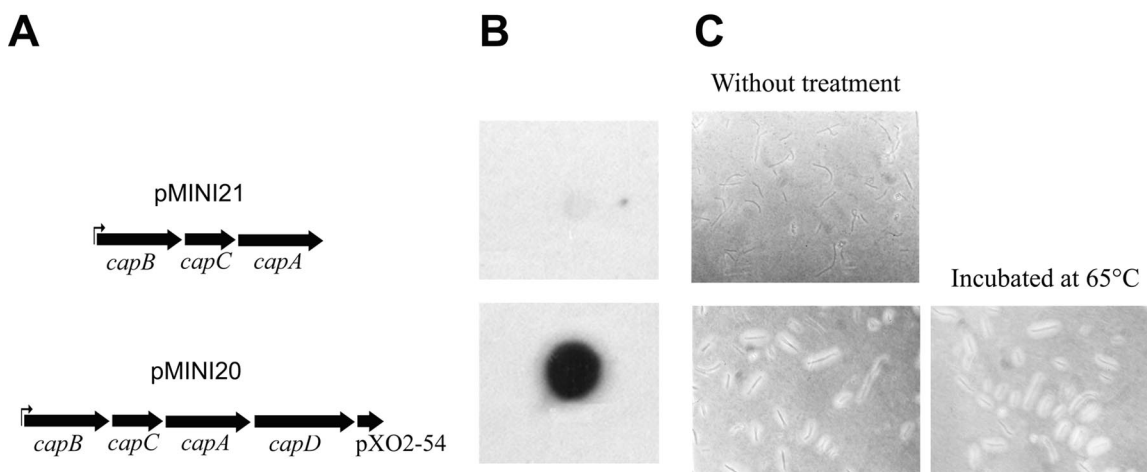


FIG. 1. The minimal synthesis and anchoring system includes a small ORF, *capE*. Bacilli containing pMINI20 or pMINI21 were tested for PGA production. (A) Schematic representation of the plasmid content (pMINI21 and pMINI20). (B) PGA was detected by dot blotting using anti-PGA specific antibodies. (C) PGA around the bacilli was visualized by light microscopy with India ink without further treatment. Covalent anchoring of capsule was tested by visualizing the PGA after heat treating the bacteria at 65°C.

fragment was amplified with oligonucleotides *capA*2s (CGCGGATCCGAAGCAGTAGCACCAGTAAACATCGTGAGAACGAAAATT) and *capA*2a using *B. anthracis* pXO2 as the template. This fragment was inserted into pGEMT-easy and moved to pQE30 digested by BamHI-SmaI, giving rise to pQCapA2.

Plasmids containing *capE* or *ywI*C were constructed, using a replicative plasmid containing the *pag* promoter named pPPA40 (constructed as described below). First, an integrative plasmid harboring a NdeI cloning site 3' to the *pag* promoter was constructed. To do so, an NdeI site was introduced overlapping the *pag* ATG codon by directed mutagenesis of the 6-kb BamHI fragment contained in pACP1 (5). The 3.4-kb XhoI-BamHI fragment was transferred to the replicative vector pAT28, giving pACP50. pPPA40 was constructed by inserting the SphI 2-kb fragment of pACP50 into pAT18 digested by SphI.

All open reading frames were amplified with *capEs* (CATATGGTTAAAAAGTTTTGGATGGATTATGCC) and HindIII-48a (AAGCTTTAGGGGTAGCCTGTAGATAATCACTAATC) for *capE*, and with 55s (CATATGAAA TTTGTCAAAGCATCTGGCCGTTTG) and 55a (AAGCTTTTATTGGCCGTTTACCGGTTCTTCTGCTGTC). All fragments were inserted into pGemT-easy and moved into pPPA40 by NdeI and SacI digestion, giving rise to pPAGE and pPAG55, respectively. The same strategy was used to obtain *ywI*B, *ywI*B-*ywI*C, *pgsA* and *pgsA*-*pgsE*. The following oligonucleotides were used, harboring NdeI and EcoRI sites (in uppercase letters): *pgsAls* (CATATGaaaaaacaactgaa ctttcaggaaaaactgc), *pgsAla* (GAATTCcatttggctcaccactcgtttttattttttcage), *pgsEls* (CATATGaaattgtcagagccatttggccctgc), *pgEla* (GAATTCtattgctgttttctgtttg atctcgtc), *pgsAs* (CATATGatgaaaaagaactgaccttcatgaaaagctgtaaagc), *pgsAa* (GAATTCtagattttgttctcactatgatcaaatcaaacgtc), 55s, and 55a.

The SacI fragment of pBACP1 was inserted into pAT28 digested with SacI giving rise to pMINI10. The SpeI fragment of pMINI10 was deleted, giving pMINI20. The Bsu36I fragment of pMINI20 was deleted, giving rise to pMINI21 (Table 1).

All recombinant plasmids were transferred from *E. coli* to *B. anthracis* by a heterogramic conjugation procedure (39).

RNA extraction and RT-PCR. Total RNA extraction from *B. anthracis* RTC50 was adapted from the protocol described by Guilloard et al. for *B. subtilis* and using the Trizol reagent (Invitrogen) (15). The quality of RNA preparations was analyzed on an RNA NanoLabChip (Agilent Technologies). Five μ g of RNA was reverse transcribed using an Invitrogen M-MLV reverse transcriptase kit and random hexamers from Amersham, according to the manufacturer's instructions. Ten ng of cDNA was then amplified by reaction with the Expand high-fidelity PCR system from Roche. Negative controls included PCR amplification using 10 ng of RNA without a reverse transcription step. Reverse transcription (RT)-PCR was performed with *capD*-RT2s (GGGGAAACGATCGGCATTGGGTCACCAGGTGG), *capDa*, *capEs*, *capE*-internea (AAGCTTGTTCGGAACGTTTAAAGGTCCCAATTGTTAC), and *capEa*.

Phenotypic studies. To visualize *B. anthracis* capsule, bacilli were incubated in the presence of India ink. The samples were then observed by light microscopy

at a magnification of $\times 400$. To observe capsule attachment, bacilli were incubated at 65°C for 30 min and then similarly examined by light microscopy (4).

Cell fraction separation. Pellets from *B. anthracis* liquid culture were sonicated and the soluble fraction (corresponding to the cytoplasm) and insoluble fraction (corresponding to the cell wall) were separated. After solubilization in 2% Triton X-100, membrane and insoluble fractions (corresponding mostly to peptidoglycan) were further separated, by ultracentrifugation (20,000 $\times g$ for 1 h) and tested as described previously (4); 10 μ g of each fraction were loaded on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) for analysis.

Antibody production. CapA2 (a peptide contained in CapA, amino-acid residues 46 to 191) was overexpressed and purified under denaturing conditions as described in Candela and Fouet (4). CapA2 serum was obtained by subcutaneously injecting 10 μ g of CapA2 in Freund incomplete adjuvant into OF1 mice (Charles River) four times at 2-week intervals. CapB and CapE antibodies were raised by Neosystem (Strasbourg, France).

Immunodetection. Dot blotting for PGA was performed as described in Candela and Fouet (4). Briefly, bacteria were loaded directly onto nitrocellulose membranes and these were probed with specific anti-PGA antibodies. The following antibodies were used for Western blotting: anti-CapD antibodies (mouse) diluted 1:1,000 (4), CapA2 antibodies (mouse) diluted 1:1,000, CapB antibodies (rabbit) diluted 1:10,000 and CapE antibodies (rabbit) diluted 1:5,000. Antibody binding was revealed with anti-mouse, or anti-rabbit, peroxidase-conjugated secondary antibodies. Blots were developed using the ECL Western blotting analysis system (Amersham).

Virulence assays. OF/1 outbred mice (6 to 8 weeks old, females; Charles River, Arbrèsle, France), were used. The 50% lethal dose (LD₅₀) was determined by subcutaneous injection of spores of the *capE* mutant or parental strain, as described previously (30).

Bioinformatic. We used TopPred as membranous domain prediction program (6). Sequences were aligned using ClustalW and Boxshade programs (38). The similarity (identical plus accepted replacement amino acid residues) scores were deduced from the Boxshade figure.

RESULTS

In *B. anthracis* CapB, CapC, and CapA require an additional small peptide to synthesize PGA. In *E. coli*, *capB*, *capC*, and *capA* are necessary and sufficient for a weak PGA synthesis, but are not sufficient in *B. subtilis* (24). We tested if *capB*, *capC*, and *capA* are necessary and sufficient for PGA synthesis in *B. anthracis*. We constructed a replicative multicopy plasmid, pMINI21, carrying *capB*, *capC* and *capA* genes preceded

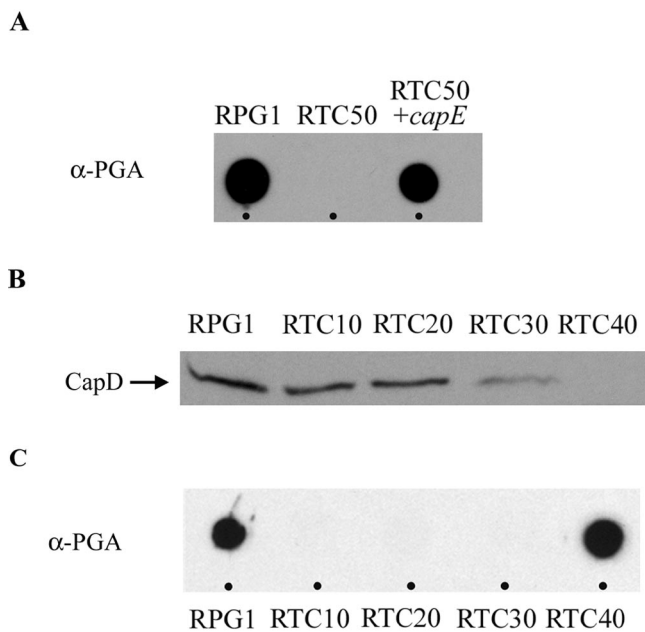


FIG. 2. *capA*, *capB*, *capC*, and *capE* are each necessary for PGA synthesis. (A and C) PGA was detected by dot blot. Bacteria were loaded directly on a nitrocellulose membrane and the membranes were probed with specific anti-PGA antibodies. (A) Strains RPG1, RTC50 (*capE*), and RTC50 complemented with *capE*. (B) Testing for CapD in RPG1, RTC10 (*capB*), RTC20 (*capC*), RTC30 (*capA*) and RTC40 (*capD*) strains. The same quantity of each sample was loaded. The Western blot membrane was probed with anti-CapD antibodies. (C) Strains RPG1, RTC10, RTC20, RTC30, and RTC40.

by the *cap* operon promoter (Fig. 1A) and introduced it into strain 9131 (pXO1⁻, pXO2⁻). No PGA synthesis was revealed by dot blotting (Fig. 1B). The absence of PGA was confirmed by optical microscopy (capsule can be easily observed under the light microscope as a white halo in presence of India ink [8]) (Fig. 1C). CapB, CapC, and CapA were therefore, as in *B. subtilis*, not sufficient for PGA production in *B. anthracis*.

The *cap* locus sequence on pXO2 was examined thoroughly. It includes a small ORF just downstream of *capD*. This ORF is described in the pXO2 sequence annotation as ORF pXO2-54 (ID AAF13659) (accession number AF188935) (29). pXO2-54 is 144 base pairs long, coding a 47-amino-acid peptide. A new plasmid was constructed, pMINI20, containing *capB*, *capC*, *capA*, *capD* and ORF pXO2-54, and was transferred into strain 9131 (Fig. 1A). Bacilli containing pMINI20 synthesized PGA (Fig. 1B and C). Strain 9131 contains no plasmids, and the transcriptional activating elements required for *cap* transcription, AcpA, AcpB and AtxA, are absent (7). Nevertheless, our results indicated that there was sufficient transcriptional readthrough for expression of the *cap* genes on a multicopy plasmid to obtain capsulated bacteria.

CapD is involved in the anchoring, not in the synthesis, of PGA (4). We also tested if CapB, CapC, CapA, CapD, and the product of ORF pXO2-54 are sufficient for the covalent anchoring of the capsule to the peptidoglycan (4). The PGA was not released from the 9131 bacteria containing pMINI20 by heat treatment (Fig. 1C). The capsule was therefore covalently anchored to the peptidoglycan as in a wild-type strain (4). The

plasmid harboring *capB*, *capC*, *capA*, *capD*, and pXO2-54 therefore defines a system that is sufficient for synthesis and anchoring of *B. anthracis* PGA.

CapB, CapC, CapA, and CapE are necessary for PGA synthesis. We studied the involvement of pXO2-54 in PGA synthesis when on its natural genetic element, pXO2. We inactivated this ORF in the RPG1 (pXO1⁺, pXO2⁺, Tox⁻) strain and PGA production was tested by dot blot. The parental strain, RPG1, produced PGA whereas the mutant strain, RTC50 (Table 1), did not (Fig. 2A). This is consistent with the results obtained in strains 9131(pMINI21) and 9131(pMINI20) (Fig. 1). We named the pXO2-54 ORF *capE*. The PGA-producing phenotype was restored to strain RTC50 by complementation with a plasmid containing a wild-type *capE* (Fig. 2A). *capE* is therefore necessary for PGA synthesis in *B. anthracis*.

To test whether *capB*, *capC*, *capA*, and *capD* genes are all necessary for PGA synthesis, nonpolar mutants of each of these genes (see below) were constructed in the RPG1 genetic background, giving RTC10, RTC20, RTC30, and RTC40, respectively (Table 1). The presence of CapD, product of the most downstream of the four genes, was assayed by loading the same quantity of the insoluble fraction proteins (see Materials and Methods) on SDS-PAGE and it was revealed with anti-CapD antibodies (Fig. 2B). CapD was found in the parental strain and in the *capB*, *capC*, and, although in a lower abundance, in the *capA* mutants, indicating that all mutants are nonpolar. The corresponding strains were assayed for PGA synthesis by dot blotting (Fig. 2C). The *capD* mutant produced PGA, indicating that the PGA synthesis complex is active in that mutant. All the other three nonpolar *cap* mutants were deficient for PGA synthesis (Fig. 2C). All nondeleted genes are expressed; thus, the absence of PGA in the mutants confirmed that CapB, CapC, and CapA, as well as CapE (Fig. 2A), are necessary for PGA synthesis.

***capE* is the fifth ORF of the *cap* operon and encodes a membranous protein.** Many small peptides are regulators. We therefore tested for the presence of CapB, CapA, and CapD in the *capE*-deficient strain RTC50 by Western blotting (Fig. 3A). We did not test for CapC because antibodies directed against it were not obtained. Proteins CapB, CapA, and CapD were found in similar amounts in strains RTC50 and RPG1. Therefore CapE is not required for the synthesis of these proteins, and it is not a regulator of the *cap* operon. Moreover, the membranous localization of both CapB and CapA was the same in a *capE* mutant strain and in a wild-type strain (data not shown).

Only 14 nucleotides separate the *capD* stop codon from the *capE* translation initiation codon. An RT-PCR experiment was performed to test if *capE* belongs to the *cap* operon. Because capsule interferes with RNA extraction, RNAs were extracted from RTC50 and not RPG1. mRNA for the *capD* gene and that for the cassette harboring *capE* were detected (Fig. 3B, lanes 1, 2, and 3). Moreover, RT-PCR with the fourth pair of oligonucleotides indicated that the transcript encompasses the end of *capD* and the beginning of *capE* (Fig. 3A, lane 4). Therefore, the *capE* gene belongs to the *cap* operon.

The predicted amino acid sequence of CapE suggests that it may be associated with membranes. This was tested. The crude bacterial extract was separated into three fractions: the cyto-

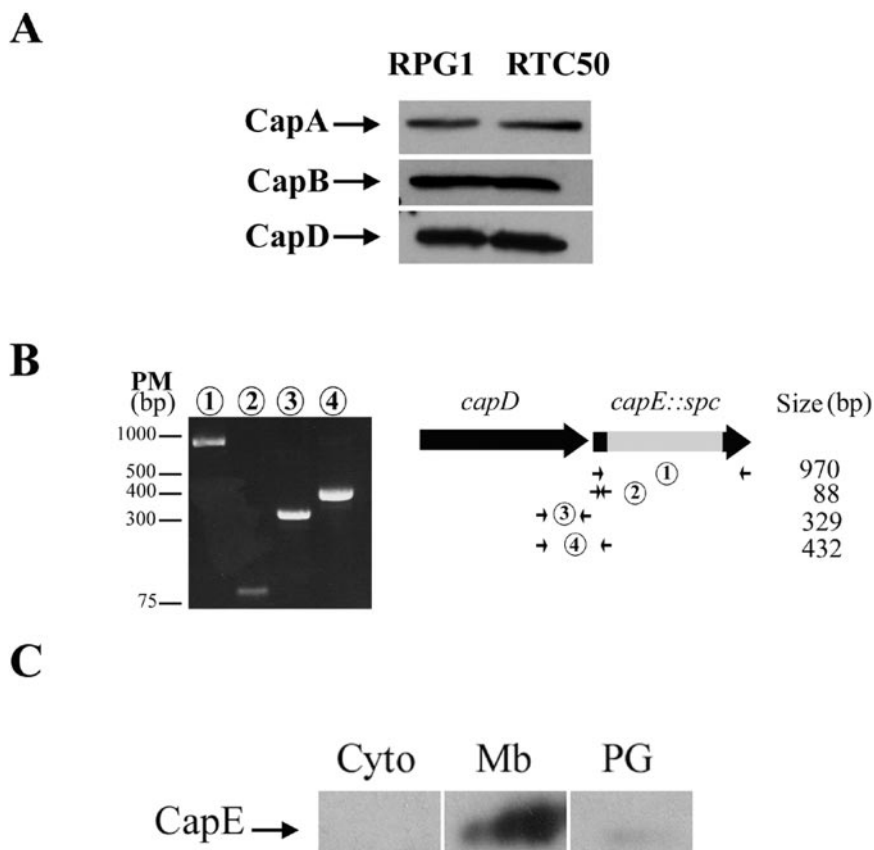


FIG. 3. CapE, the product of the 5th gene of the *cap* operon, is a membranous peptide. (A) Strains RPG1 and RTC50 were tested for CapB, CapA, and CapD. The same quantities of protein were loaded for each sample. CapB, CapA, and CapD were revealed by the corresponding specific antibodies. (B) RT-PCR was carried out on RNA from strain RTC50 using *capEs/capEa* (1), *capEs/capEinternea* (2), *capD-RT2s/capDa* (3), and *capD-RT2s/capEinternea* (4) primers. (C) Localization of the CapE peptide: cytoplasm (Cyto), membranous (Mb), and peptidoglycan (PG)-enriched fractions were separated. CapE was revealed by Western blot with specific anti-CapE antibodies.

plasmic protein fraction, the membranous protein fraction, and the peptidoglycan-associated protein fraction (4). Only one specific band was found in the membranous protein fraction corresponding to the size of CapE. This band is slightly smeared because of Triton X-100 contained in the sample that disturbed the membranous protein fraction migration (Fig. 3C). Therefore, CapE is a membranous peptide.

The LD₅₀ of the *capE* mutant was determined. The LD₅₀ of RPG1 was 5×10^5 spores/mouse, whereas no mice were killed when 8×10^8 spores of strain RTC50 were injected. The *capE* mutant strain is therefore avirulent, as expected for a nontoxicogenic and noncapsulated strain (46).

CapE and CapA functionally interact. The polyglutamate synthetic (*pgs*) operon has been described to be responsible for the PGA synthesis in *B. subtilis*, and the *yw(s/t)* operon in *B. licheniformis* may have the same function, on the basis of sequence similarity. In silico analysis of these operons, *pgsB*, *pgsC*, and *pgsA* in *B. subtilis* and *ywsC*, *ywtA*, and *ywtB* in *B. licheniformis*, shows that they are both followed by a small ORF, called *ywtC* (Fig. 4A) (22, 45). *ywtC* encodes a 55-amino-acid peptide and we investigated whether it has the same function as CapE. We therefore complemented RTC50, a *capE* mutant strain, with the *B. subtilis* and *B. licheniformis*

ywtC genes. There was no PGA production (Table 2): *YwtC*, on its own, does not fulfill CapE function.

We compared the Cap/Pgs/Yw(s/t) protein sequences, deduced from the available DNA sequences, for *B. subtilis*, *B. licheniformis*, and *B. anthracis*. CapB/PgsB/YwsC and CapC/PgsC/YwtA were all very similar (above 80% similarity). Interestingly, CapA/PgsA/YwtB were more divergent, with large

TABLE 2. *pgsA* and *pgsE* are necessary to complement a *capE* mutant strain^a

Complementing gene	RTC50 ($\Delta capE$)		RTC30 ($\Delta capA$)	
	Light microscopy	Petri dishes	Light microscopy	Petri dishes
<i>capE</i>	+	+	ND	ND
<i>ywtC</i>	-	-	ND	ND
<i>ywtB</i> or <i>pgsA</i>	-	-	+	+
<i>ywtB-ywtC</i> or <i>pgsA-ywtC</i>	+	-	+	+

^a Colonies on petri dishes were smooth (+) or rough (-), indicative of capsulation. The presence of PGA surrounding the bacteria was also determined by light microscopy in the presence of India ink. Capsulated bacteria present a white halo (+); others do not (-). The *ywtC* and *pgsA* genes are from *B. subtilis*, and the *ywtC* and *ywtB* genes are from *B. licheniformis*. ND, not done.

confirmed by the analysis of sequence divergence between the CapA and CapE proteins and their equivalents (Fig. 4A).

All previous studies with the *B. subtilis* *pgs* operon included *ywtC* (also called *pgsE*) (3, 44). The minimal region of *B. subtilis* genome required to synthesize PGA in *E. coli* was found by screening a plasmid library (3). One plasmid in 10,000 was able to direct PGA synthesis in *E. coli* (3). These authors described a DNA fragment composed of about 3 kb containing the *pgsB*, *pgsC*, and *pgsAA* genes. However, *pgsE* is also present on this fragment. This result is strongly in favor of *pgsE* being necessary for PGA synthesis. Independently, Urushibata et al. directly cloned the four genes and obtained PGA synthesis in *B. subtilis* (44). They suggested that these genes form an operon but did not discuss the role of *ywtC* (also called *pgsE*). Both studies are in agreement with our finding that *ywtC* (also called *pgsE*), like *capE* in *B. anthracis*, is indispensable for PGA synthesis. Also consistent with our study, the minimal system defined for *E. coli* by Makino et al. did not function in *B. subtilis*, showing that an element was missing (24). Moreover, the PGA synthesis was weak in *E. coli* and in *B. anthracis*, probably due to the absence of the same element (24). This element was *capE*. It is not excluded that when the CapB, CapC, and CapA proteins are overproduced, rare complexes can be formed in the bacterium even in absence of CapE. They would lead to some PGA synthesis, which can be detected in *E. coli* and *B. anthracis* boiled extracts, as described by Makino et al. (24). However, our results showed that the PGA synthesis yield is insufficient to give rise to properly capsulated bacilli.

CapE is a very small peptide, 47 amino acids. Small peptides are generally involved in regulation. However, this is not the case for CapE, our results suggesting that CapE has a structural protein-interaction role. CapE is involved in the synthesis function, and probably interacts with CapA. Indeed, the phenotype of the *capE* mutant was only complemented in the presence of both *pgsAA* and *pgsE*. Whereas PgsAA fully replaces CapA in RTC30, PgsAA and PgsE did not fully complement RTC50: the strain was less capsulated than the wild type. This suggested that PgsE may also interact, but not as efficiently as CapE, with one or the other two components of the enzymatic complex, probably CapB and/or CapC. Indeed, CapE may need to interact with the entire complex and not only with CapA.

We propose two possible roles for CapE. It could interact functionally with CapA. Alternatively, through its interaction with CapB, CapC, and CapA, it could contribute to the formation of the complex. However, CapE is not involved in stabilization or in membrane localization of CapB and CapA. *pgsE* was unsuccessfully used for complementation. PgsE interacting with at least PgsAA may have the same function as CapE, and by extrapolation, this may also be the case for *B. licheniformis* PgsE and *S. epidermidis* YwtC.

The replicative plasmid pMINI20 harbors all elements required to produce PGA in a strain devoid of pXO1 and pXO2. Moreover, the PGA produced is anchored to the peptidoglycan. This demonstrated that there is no enzyme encoded by pXO2, other than *capD*, involved in PGA anchoring, reinforcing the suggestion that CapD alone is responsible for catalysis of this anchoring. Differences between bacteria in which PGA is not anchored and those that harbor a PGA that is anchored to the peptidoglycan, constituting a capsule, appear therefore

to be due to a single gene belonging to the *pgs* and *cap* operons. In the absence of a γ -glutamyltranspeptidase, encoded by *capD*, PGA is secreted. Noteworthy, the *pgs* operons encode a γ -glutamyl-hydrolase. This is the case for *B. subtilis* natto and *B. licheniformis* ATCC 14580, which possess the γ -glutamyl-hydrolase genes *pgsS* and *ywtD*, respectively (2, 36). The only well-characterized example of PGA-capsulated bacteria is *B. anthracis*, which possesses CapD, a γ -glutamyltranspeptidase. *S. epidermidis* CapD is predicted to be a glutamyltranspeptidase precursor (12). It is very different from *B. anthracis* CapD (less than 35% similarity). PGA anchoring in *S. epidermidis* has not been studied, but PGA was observed on the surface by immunoscanning electron microscopy (20). This result is in agreement with PGA anchoring involving a glutamyltranspeptidase.

The virulence of the *B. anthracis* RPG1 parental strain is due to its ability to multiply within the host, causing septicemia. The *capE* mutant strain was fully avirulent in mice. The requirement for the small 47-amino-acid CapE peptide in *B. anthracis* capsule synthesis is absolute, and therefore it is a potential therapeutic target for the control of anthrax septicemia.

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