Demonstration of a Capsule Plasmid in *Bacillus anthracis*

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Virulent and certain avirulent strains of *Bacillus anthracis* harbor a plasmid, designated pXO2, which is involved in the synthesis of capsules. Two classes of rough, noncapsulated (Cap⁻) variants were isolated from the capsule-producing (Cap⁺) Pasteur vaccine strains ATCC 6602 and ATCC 4229. One class was cured of pXO2, and the other class still carried it. Reversion to Cap⁺ was demonstrable only in rough variants which had retained pXO2. Proof that pXO2 is involved in capsule synthesis came from experiments in which the plasmid was transferred by CP-51-mediated transduction and by a mating system in which plasmid transfer is mediated by a *Bacillus thuringiensis* fertility plasmid, pXO12. Cells of *Bacillus cereus* and a previously noncapsulated (pXO2⁻) strain of *B. anthracis* produced capsules after the acquisition of pXO2.

Bacillus anthracis requires two virulence factors to cause disease. One of these is a toxin composed of three different proteins known as edema factor, lethal factor, and protective antigen (2, 10, 17). Mikesell et al. (7) and Robillard et al. (N. J. Robillard, T. M. Koehler, R. Murray, and C. B. Thorne, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, H54, p. 115) demonstrated that a 114-megadalton plasmid, formerly referred to as pBA1 but now designated pXO1, is associated with toxin production; Vodkin and Leppla (18) showed by cloning experiments that pXO1 carries the structural genes for protective antigen. The other virulence factor is a capsule composed of D-glutamyl polypeptide (6, 11, 16). When virulent strains of *B. anthracis* are grown on media containing serum or bicarbonate or both and incubated in a CO_2-enriched atmosphere, they produce capsules and the colonies appear mucoid. In the absence of serum or bicarbonate, they fail to produce capsules and the colonies appear rough. Thus, colonies of mutants which cannot make capsules can be easily distinguished from colonies of the capsulated parental cells and are readily isolated by selection of rough sectors or outgrowth from mucoid colonies grown on medium containing bicarbonate.

It was reported that the heat-attenuated Pasteur vaccine strains of *B. anthracis*, which form capsules but are avirulent because they are unable to produce toxin, were devoid of plasmid DNA (7). However, it has been shown recently that the Pasteur vaccine strains ATCC 6602 and ATCC 4229 do contain a capsule plasmid which has been designated pXO2 (B. D. Green, L. Battisti, and C. B. Thorne, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H99, p. 124) and that the plasmid is present in all capsulated strains of *B. anthracis* examined (B. E. Ivins and C. B. Thorne, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H100, p. 124). Details of this work are presented here.

MATERIALS AND METHODS

Organisms. The organisms used in this study are listed in Table 1.

Media. NBY medium (pH 6.8) contained 8 g of nutrient broth (Difco Laboratories, Detroit, Mich.) and 3 g of yeast extract (Difco) per liter. For capsule production, NBY medium was supplemented with $NaHCO_3$ (sterilized by

filtration of a 9% solution) at a final concentration of 0.7% (wt/vol) and with horse serum (GIBCO Laboratories, Grand Island, N.Y.) at a final concentration of 10% (vol/vol). BHI medium contained 37 g of brain heart infusion (Difco) per liter. L broth contained 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and 10 g of NaCl per liter with the pH adjusted to 7.0. R medium has been described previously (8). Phage assay medium contained the following (in grams per liter): nutrient broth (Difco), 8; $CaCl_2 \cdot 2H_2O$, 0.15; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.05; and NaCl, 5. The pH was adjusted to 6.0 with HCl. For solid medium, 15 g of agar was added per liter. Soft agar contained 5 g of agar per liter.

Immunoassay agar plates contained 12 ml of R agar plus 2 ml of antiserum prepared in goats by immunization with viable spores of the Sterne strain of *B. anthracis*.

Capsule production. The ability of *B. anthracis* and *B. cereus* to produce capsules was determined by growing cells on R agar or on NBY agar containing bicarbonate and serum. Plates were incubated in the presence of 5 or 20% CO_2 at 37°C for 24 to 48 h.

Toxin assays. Lethal factor and edema activities were assayed as described previously (7).

Detection of plasmid DNA. Plasmid DNA was extracted by a modification of the procedure described by Kado and Liu (3). Cells for plasmid extraction were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth. Cultures were incubated for 16 h at 37°C on a rotary shaker (100 to 160 rpm). Cells from 25 ml of culture were collected by centrifugation at 10,000 rpm in a Sorvall SS34 rotor for 10 min at 15°C and suspended in 1 ml of E buffer (0.04 M Tris-hydroxide [Sigma Chemical Co., St. Louis, Mo.], 0.002 M EDTA [tetrasodium salt; Sigma], 15% sucrose [pH 7.9]) by gentle vortexing. Cells were lysed by adding 1 ml of the suspension to 2 ml of lysis buffer prepared by adding 3 g of sodium dodecyl sulfate and 5 ml of 3 N NaOH to 100 ml of 15% (wt/vol) sucrose in 0.05 M Tris-hydroxide. The tubes were rapidly inverted 20 times to mix the cells and buffer and were then held in a 60°C water bath for 30 min. Pronase (0.5 ml; Calbiochem-Behring, La Jolla, Calif.) solution (2 mg/ml in 2 M Tris [pH 7.0]) was added, and the tubes were mixed as described above and incubated in a 37°C water bath for 20 min. The lysate was extracted with 6 ml of phenol-chloroform (1:1, vol/vol) by inverting the tubes 40 times. The

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TABLE 1. Strains used in this study

Strain[a]	Relevant characteristics[b]	Relevant plasmid(s)	Origin or reference
B. anthracis			
Ames	Virulent, Cap$^+$ Tox$^+$	pXO1, pXO2	USDA[c]
Buffalo	Virulent, Cap$^+$ Tox$^+$	pXO1, pXO2	R. A. Packer
Colorado	Virulent, Cap$^+$ Tox$^+$	pXO1, pXO2	A. McChesney
NH	Virulent, Cap$^+$ Tox$^+$	pXO1, pXO2	USAMRIID[d]
Vollum 1B	Virulent, Cap$^+$ Tox$^+$	pXO1, pXO2	USAMRIID
Vollum 1B-1	Avirulent, Cap$^+$ Tox$^-$	pXO2	This study
Vollum 1B VNR-1	Avirulent, Cap$^-$ Tox$^+$	pXO1	This study
Weybridge (Sterne)	Avirulent, Cap$^-$ Tox$^+$	pXO1	MRE[e]
Weybridge UM44	Ind$^-$	pXO1	UV[f] of Weybridge
Weybridge A	Colony variant of Weybridge	pXO1	C. B. Thorne
Weybridge A UM23C1	Ura$^-$, cured of pXO1	None	C. B. Thorne
Weybridge A UM23C1 td10	Ura$^-$ Cap$^+$	pXO2	This study
4229 (Pasteur)	Avirulent, Cap$^+$ Tox$^-$	pXO2	ATCC[g]
4229 UM12	Nal^r Cap$^+$	pXO2	UV of 4229
4229 UM12 tr299-3	Nal^r Cap$^+$ Tc^r Cry$^+$	pXO2, pXO12, pBC16	This study
6602 (Pasteur)	Avirulent, Cap$^+$ Tox$^-$	pXO2	ATCC
6602 R1	Cap$^-$ Tox$^-$	None	This study
B. cereus			
569	Wild type		NRRL[h]
569 UM20	Ant$^-$		UV of 569
569 UM20-1	Ant$^-$ Str^r		UV of UM20
569 UM20-1 td10	Ant$^-$ Str^r Cap$^+$	pXO2	This study
569 UM20-1 tr202-1	Ant$^-$ Str^r Tc^r Cry$^+$	pXO12, pBC16	This study
569 UM20-1 tr305-1	Ant$^-$ Str^r Tc^r Cry$^+$ Cap$^+$	pXO2, pXO12, pBC16	This study
569 UM20-1 tr305-5	Ant$^-$ Str^r Tc^r Cry$^+$ Cap$^+$	pXO2, pXO12, pBC16	This study
11950	Carries bacteriophage Wα		ATCC
B. thuringiensis subsp. *thuringiensis* 4042A UM8 td2	Ade$^-$ Cry$^+$ Tc^r	pXO12, pBC16	1
B. thuringiensis subsp. *thompsoni* 4060			NRRL

[a] In these strain designations, tr denotes a transcipient strain, and td denotes a transductant obtained by CP-51-mediated transfer of a plasmid.
[b] Abbreviations: Ade, adenine; Ant, anthranilic acid; Cap, synthesis of capsule; Cry, synthesis of parasporal crystal; Ind, indole; Nal^r, nalidixic acid resistant; gtr^r, streptomycin resistant; Tc^r, pBC16-encoded tetracycline resistant; Tox, synthesis of toxin; Ura, uracil.
[c] USDA, U.S. Department of Agriculture, Ames, Iowa.
[d] USAMRIID, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md.
[e] MRE, Microbiological Research Establishment, Porton, England.
[f] UV, Mutagenesis by UV light (14).
[g] ATCC, American Type Culture Collection, Rockville, Md.
[h] NRRL, Northern Regional Research Laboratory, Peoria, Ill.

emulsions were separated by centrifugation at 10,000 rpm for 10 min at 15°C, and the aqueous phase was removed for electrophoresis.

Extracts (40 µl) were mixed with 10 µl of tracking dye (0.25% bromphenol blue, 15% Ficoll), and samples (40 µl) were applied to horizontal 0.7% agarose gels prepared and run in Tris-borate buffer (0.089 M Tris-hydroxide, 0.089 M boric acid, 0.0025 M EDTA [pH 8.2 to 8.3]). Electrophoresis was carried out at 70 V for 90 to 120 min at room temperature. Gels were stained with ethidium bromide (0.5 µg/ml in Tris-borate buffer).

Plasmid curing. Strains cured of pXO2 were found among spontaneous rough variants (see below) or isolated from cultures treated with novobiocin. For novobiocin treatment, 10 to 100 CFU of *B. anthracis* was inoculated into 250-ml flasks containing 50 ml of L broth plus novobiocin (1 µg/ml) and incubated for 3 days at 37°C with gentle shaking. Cultures were diluted, and 0.1-ml samples were spread on plates of immunoassay agar and incubated at 37°C for 24 h in 5% CO_2. Rough colonies of noncapsulated cells were isolated and tested for the loss of pXO2. Strains were cured of pXO1 by serial passage at 43°C (7, 15).

Isolation of spontaneous rough mutants of *B. anthracis* 6602. Cells were plated for single colonies on NBY agar containing bicarbonate and serum and incubated at 37°C in 20% CO_2. After several days, rough outgrowth appeared at the edge of some of the mucoid colonies. The rough outgrowth was picked and purified by streaking on fresh plates of the same medium.

Propagation and assay of bacteriophages. Bacteriophage Wα (4) was obtained from *B. cereus* W (ATCC 11950). It was propagated on *B. anthracis* 6602 R1 (pXO2$^-$) in soft overlays of phage assay agar incubated at 37°C for 17 to 20 h and was assayed against the same strain in soft overlays of phage assay agar incubated at 30°C. Bacteriophages CP-51 and CP-54 were propagated and assayed by methods described previously (9, 13, 14). *B. thuringiensis* subsp. *thompsoni* 4060 was the host for the propagation of CP-54. Phage lysates were routinely treated with DNase (50 µg/ml) to inactivate bacterial DNA, and they were confirmed to be free of bacterial contamination by plating samples on NBY agar.

Isolation of Cap$^+$ revertants of rough mutants. To demonstrate reversion, ca. 10^5 spores of a rough mutant were

spread with 10^8 PFU of phage Wα on NBY agar containing bicarbonate and serum. The plates were incubated at 37°C in 20% CO_2 for 2 days and examined for mucoid colonies.

Transduction of pXO2. Bacteriophage CP-51 was propagated on *B. anthracis* 6602 and assayed on *B. cereus* 569. Recipient cells for transduction were grown in 250-ml Erlenmeyer flasks containing 25 ml of L broth (for *B. cereus*) or BHI broth with 0.5% (wt/vol) glycerol (for *B. anthracis*) and were incubated at 37°C on a rotary shaker at 250 rpm. Cells from a 10% (vol/vol) transfer of a 16-h culture were grown for 5 h. Cells (0.1 ml containing ca. 10^8 CFU) and phage (0.1 ml containing ca. 5×10^9 PFU) were spread together on NBY agar containing bicarbonate. Plates were incubated at 37°C in 20% CO_2. After 3 h, 0.1 ml of phage CP-54 (3×10^9 PFU) was spread on the transduction plates to lyse noncapsulated cells and to allow the selection of capsulated transductants. Incubation in CO_2 was continued for 36 to 48 h.

Transfer of plasmids by mating. Matings were performed as described by Battisti et al. (1).

RESULTS

Plasmid analysis of *B. anthracis* strains. A number of virulent and avirulent strains of *B. anthracis* were analyzed for plasmid content. All virulent strains examined, which included NH, Ames, Colorado, Buffalo, and Vollum 1B, carried two plasmids, as shown in Fig. 1, lane 1, for Vollum 1B. In addition to pXO1, they contained a second, smaller plasmid which has been designated pXO2. Strains could be cured of pXO2 by growing them in the presence of novobiocin. A total of 3,000 to 5,000 colonies each of strains NH, Ames, and Vollum 1B obtained from cultures treated with novobiocin were screened for capsule formation and protective antigen synthesis on immunoassay agar. All colonies produced protective antigen, as evidenced by the halos

FIG. 2. Colonies of a *B. anthracis* Pasteur vaccine strain grown on bicarbonate agar in 20% CO_2. Left, Strain 6602 (wild type). Right, Strain 6602 R1 (rough variant of 6602).

surrounding them. However, ca. 1% of the colonies were rough (nonmucoid), indicating a failure to produce capsules. Two rough colonies of each strain were subcultured and examined for plasmid content and the capacity to synthesize toxin and capsules. These strains were designated Vollum 1B VNR-1 and VNR-2, NH NNR-1 and NNR-2, and Ames ANR-1 and ANR-2. They produced biologically active toxin, failed to produce capsules in 5 or 20% CO_2, and contained only one plasmid, pXO1 (Fig. 1, lane 5). They were identical to the toxinogenic noncapsulated Weybridge (Sterne) veterinary vaccine strain with respect to phenotype and plasmid content (Fig. 1, lane 2).

The growth of toxinogenic strains of *B. anthracis* at 43°C has been shown to result in the elimination of the toxin plasmid, pXO1 (7, 15). After virulent strains, including NH, Ames, and Vollum 1B, were cultured at 43°C, variants which were cured of pXO1 but still carried pXO2 were selected on immunoassay agar. Such variants, represented by a Pasteur vaccine strain (ATCC 6602) and Vollum 1B-1 in Fig. 1, lanes 3 and 4, produced no toxin. They synthesized capsules on R agar during growth in 20% CO_2 but not in 5% CO_2.

Isolation of rough (noncapsulated) variants of the Pasteur vaccine strains ATCC 6602 and ATCC 4229. When capsulated *B. anthracis* strains are grown on agar for several days under conditions conducive to capsule formation, mucoid colonies frequently have areas of rough outgrowth. Such areas of rough outgrowth have been shown to yield reverting and nonreverting noncapsulated mutants (5, 12). To demonstrate further a correlation between capsule formation and the presence of pXO2, we isolated spontaneous rough variants of *B. anthracis* 6602 and 4229. Figures 2 and 3 show the differences in colony and cell morphology between the wild-type mucoid strain and a rough variant of strain 6602 grown under conditions required for capsule formation. Strains 6602 and 4229, like virulent strains of *B. anthracis*, failed to produce capsules when grown in air (in the absence of bicarbonate and serum) and were indistinguishable in both colony and cell morphology from rough variants grown in either air or CO_2. Upon analysis of several rough variants for plasmid content, two classes were found. Some of them were cured of pXO2 and contained no detectable plasmid DNA (see Fig. 4, lane 3), and others still carried pXO2.

We reasoned that if pXO2 is involved in the formation of capsules, it should be possible to demonstrate reversion to
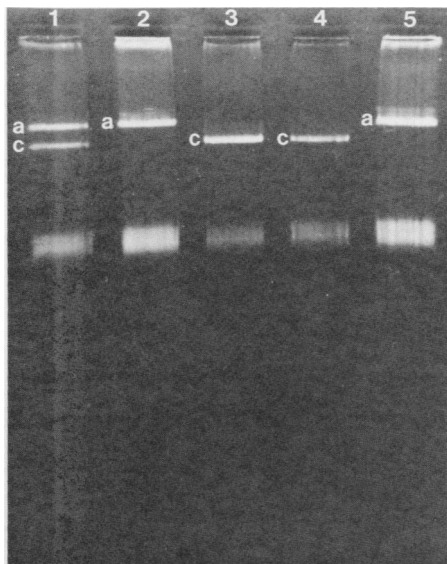

FIG. 1. Agarose gel electrophoresis of plasmid DNA from *B. anthracis* strains. Lanes: 1, Vollum 1B (virulent); 2, Sterne (avirulent); 3, Pasteur vaccine strain ATCC 6602 (avirulent); 4, Vollum 1B-1 (avirulent); 5, Vollum 1B VNR-1 (avirulent). Plasmids are labeled as follows: a, pXO1 (114 megadaltons); c, pXO2 (ca. 60 megadaltons). The large diffuse bands in all lanes is chromosomal DNA.
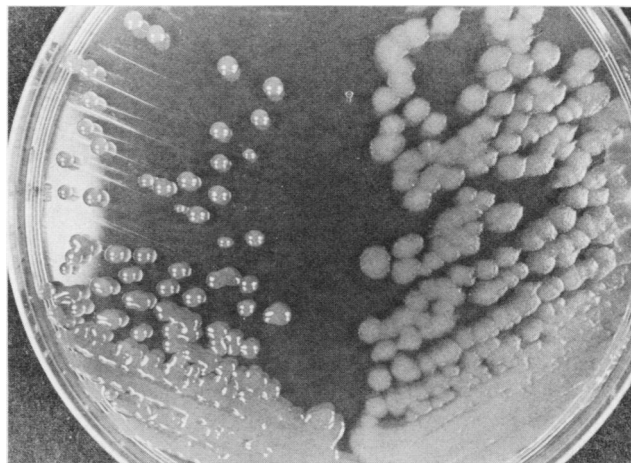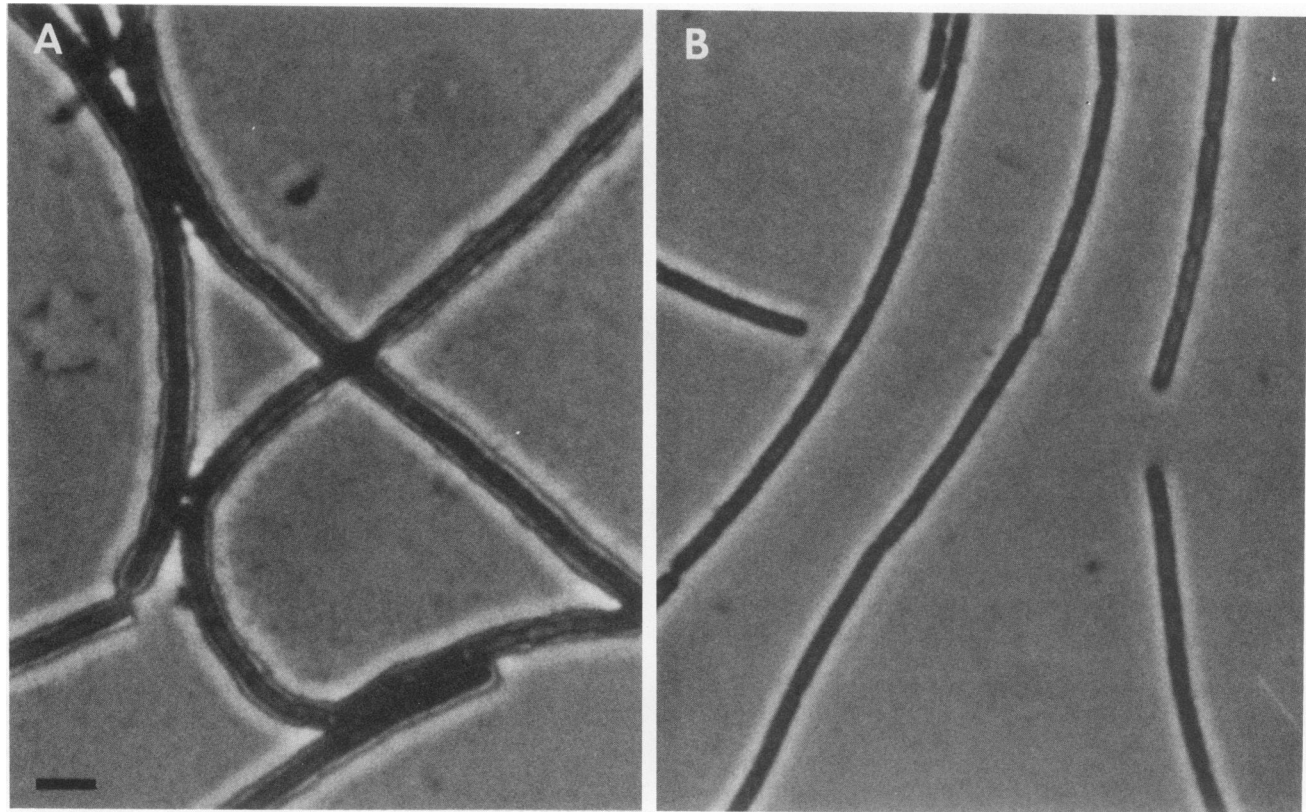
FIG. 3. Phase-contrast photomicrograph of cells of a *B. anthracis* Pasteur vaccine strain grown on bicarbonate agar in 20% CO_2. (A) Strain 6602 (wild type). (B) Strain 6602 R1 (rough variant of 6602). Bar, 2.0 μm.

Cap$^+$ among rough variants which retain pXO2 but not among rough variants which are cured of the plasmid. Both classes of rough variants of strain 6602 were tested for reversion to Cap$^+$ by being exposed to bacteriophage Wα. The use of this phage, which can lyse noncapsulated cells but not capsulated cells, affords a convenient means of detecting small numbers of Cap$^+$ revertants in populations of Cap$^-$ cells (5). As predicted, no Cap$^+$ revertants were found among three independently isolated pXO2$^-$ variants, whereas Cap$^+$ revertants were found in each of three independently isolated rough strains which had retained pXO2.

Transduction of pXO2 with phage CP-51. Phage CP-51, propagated on *B. anthracis* 6002, was used to transduce pXO2 into *B. cereus* 569 UM20-1 Ant$^-$ Str^r and *B. anthracis* Weybridge A UM23C1 (pXO1$^-$, pXO2$^-$) Ura$^-$. Phage CP-54, which is active on both *B. cereus* and *B. anthracis*, lyses noncapsulated cells but does not adsorb to capsulated cells (C. B. Thorne, unpublished data). Its application to transduction plates thus allowed the selection of Cap$^+$ transductants from a large population of Cap$^-$ cells. The frequency of Cap$^+$ transductants was ca. 10^{-8}/PFU. Plasmid analysis, after clonal purification of Cap$^+$ transductants, revealed the presence of pXO2, which migrated in agarose gels at the same rate as pXO2 from strain 6602 (Fig. 4). The pXO2$^+$ *B. cereus* and *B. anthracis* transductants retained their respective auxotrophic markers, allowing positive identification. They produced capsules when grown in 20% CO_2 on agar containing bicarbonate. Under such conditions, the mucoid colonies could not be distinguished from those of encapsulated cells of strain 6602. In the absence of bi-
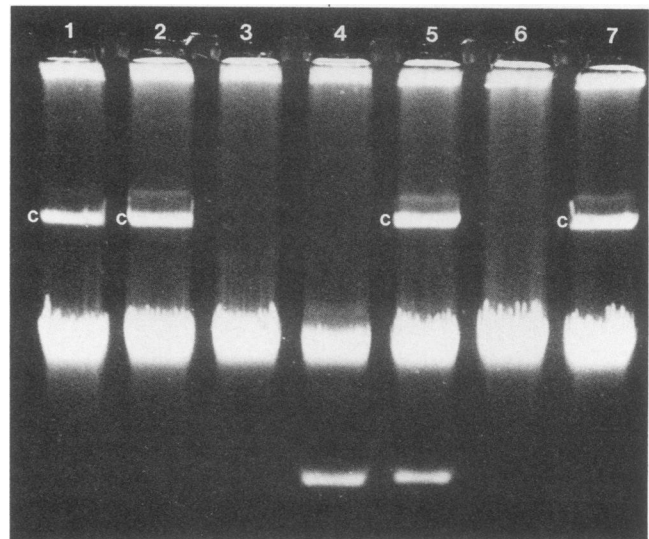

FIG. 4. Agarose gel electrophoresis of plasmid DNA demonstrating transduction of pXO2. Lanes: 1, *B. anthracis* 6602, Cap$^+$; 2, *B. anthracis* 4229, Cap$^+$; 3, *B. anthracis* 6602 R1, a Cap$^-$ pXO2$^-$ strain; 4, *B. cereus* 569 UM20-1, a recipient; 5, *B. cereus* 569 UM20-1 td10, a Cap$^+$ transductant; 6, *B. anthracis* Weybridge UM23C1, Cap$^-$, a recipient; 7, *B. anthracis* Weybridge UM23C1 td10, a Cap$^+$ transductant. c, pXO2. The faint bands above pXO2 are alternative forms of the plasmid.

carbonate and CO_2, the colonies were rough and could not be distinguished from colonies of pXO2⁻ strains.

The size of pXO2, estimated to have a molecular mass of about 60 megadaltons based on its rate of migration in agarose gels, is probably very close to the maximum size of DNA that can be packaged by CP-51. Previously, a value of ca. 60 megadaltons was found for the molecular mass of CP-51 DNA (19).

Transfer of pXO2 to *B. cereus* by mating. To transfer pXO2 to *B. cereus*, we also made use of the *Bacillus* mating system in which plasmid transfer is mediated by the *B. thuringiensis* fertility plasmid, pXO12 (1). pXO12 was transferred from *B. thuringiensis* subsp. *thuringiensis* 4042A UM8 td2(pXO12, pBC16) to *B. cereus* 569 UM20-1 Str^r. Tc^r transcipients of *B. cereus* were selected on L agar containing streptomycin (200 μg/ml) and tetracycline (25 μg/ml) and screened by phase microscopy for the presence of parasporal crystals, a phenotypic characteristic of pXO12⁺ cells (1). A *B. cereus* transcipient, 569 UM20-1 tr202-1, which inherited both pXO12 and pBC16, was then mated with *B. anthracis* 4229 UM12 Nal^r, and Tc^r transcipients were selected on NBY agar containing tetracycline (5 μg/ml) and nalidixic acid (30 μg/ml). Approximately 50% of the Tc^r transcipients also inherited the fertility plasmid, pXO12. One of these, *B. anthracis* 4229 UM12 tr299-3(pXO2, pXO12, pBC16), was used as the donor for transferring pXO2 to *B. cereus* 569 UM20-1. Mating mixtures were plated on NBY agar supplemented with bicarbonate, horse serum, streptomycin, and tetracycline. The plates were incubated in 20% CO_2. After 2 or 3 h, 0.1 ml of phage CP-54 (10^8 PFU) was spread on some of the plates to lyse Cap⁻ Tc^r transcipients and to allow the selection of Cap⁺ Tc^r transcipients. Other plates

FIG. 6. Colonies of *B. cereus* grown on bicarbonate agar in 20% CO_2. Left, *B. cereus* 569 UM20-1, Cap⁻. Right, *B. cereus* 569 UM20-1 tr305-1, a Cap⁺ transcipient carrying pXO2.

were left untreated so that the total number of Tc^r transcipients could be scored. Incubation was continued for 24 h.

Mating mixtures yielded from 10^4 to 10^5 Tc^r transcipients per ml, and an average of 1 Tc^r colony out of 500 was mucoid and produced Cap⁺ cells on NBY agar plates containing bicarbonate and serum and incubated in CO_2. Plasmid analysis confirmed the presence of pXO2 along with pXO12 and pBC16 (Fig. 5). The Ant⁻ marker of the original *B. cereus* recipient was retained, and sporulating cells contained parasporal crystals characteristic of pXO12⁺ cells. Capsule production by a *B. cereus* pXO2⁺ transcipient is shown in Fig. 6 and 7. With respect to capsule formation, *B. cereus* cells that received pXO2 by mating could not be distinguished from those that received pXO2 by transduction. As with *B. anthracis* 6602 and 4229, capsules were produced by *B. cereus* only when cells carrying pXO2 were grown in CO_2.

DISCUSSION

The results presented here demonstrate that plasmid pXO2 is involved in the formation of capsules by *B. anthracis*. All capsule-producing strains, both virulent and avirulent, examined thus far have been shown to harbor pXO2. Proof that pXO2 is involved in capsule synthesis was provided by experiments in which the plasmid was transferred by CP-51-mediated transduction or by the recently developed mating system for transferring plasmids among strains of *B. anthracis*, *B. cereus*, and *B. thuringiensis* (1). *B. cereus* 569, which is normally noncapsulated, produced capsules after the acquisition of pXO2 by either transduction or mating. Similarly, a noncapsulated strain of *B. anthracis* acquired the Cap⁺ phenotype when pXO2 was transferred to it by transduction.

Novobiocin was determined to cure strains of pXO2 selectively when both pXO1 and pXO2 were present. Approximately 1% of the colonies grown from novobiocin-treated cultures were cured of pXO2, but none of several thousand colonies was observed to be cured of the toxin plasmid, pXO1. All strains cured of pXO2 failed to produce capsules under conditions which normally promote capsule synthesis in pXO2⁺ strains.

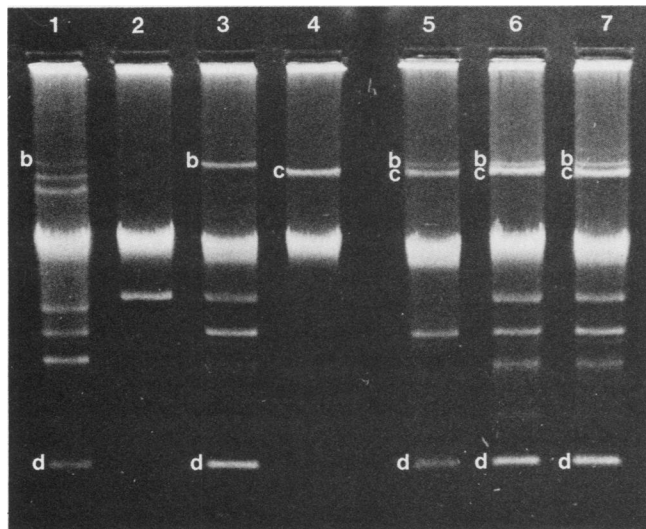When spontaneous rough variants were isolated from the

FIG. 5. Agarose gel electrophoresis of plasmid DNA demonstrating transfer of pXO2 and pXO12 by mating. Lanes: 1, *B. thuringiensis* subsp. *thuringiensis* 4042A UM8 td2; 2, *B. cereus* 569 UM20-1; 3, *B. cereus* 569 UM20-1 tr202-1, a Cry⁺ Tc^r transcipient derived from mating 4042A UM8 td2 (lane 1) with 569 UM20-1 (lane 2); 4, *B. anthracis* 4229 UM12, Cap⁺; 5, *B. anthracis* 4229 UM12 tr299-3, a Cry⁺ Cap⁺ transcipient derived from mating 569 UM20-1 tr202-1 (lane 3) with 4229 UM12 (lane 4); 6 and 7, *B. cereus* 569 UM20-1 tr305-1 and tr-305-5, respectively, Cry⁺ Cap⁺ transcipients derived from mating 4229 UM12 tr299-3 (lane 5) with 569 UM20-1 (lane 2). Plasmids are labeled as follows: b, pXO12 (size not determined); c, pXO2 (ca. 60 megadaltons); d, pBC16 (2.8 megadaltons).
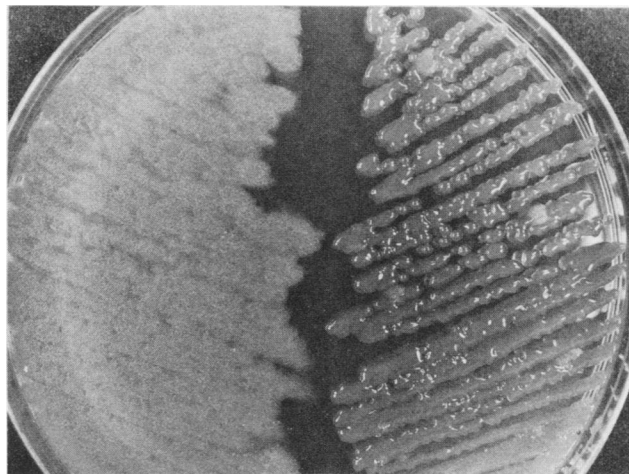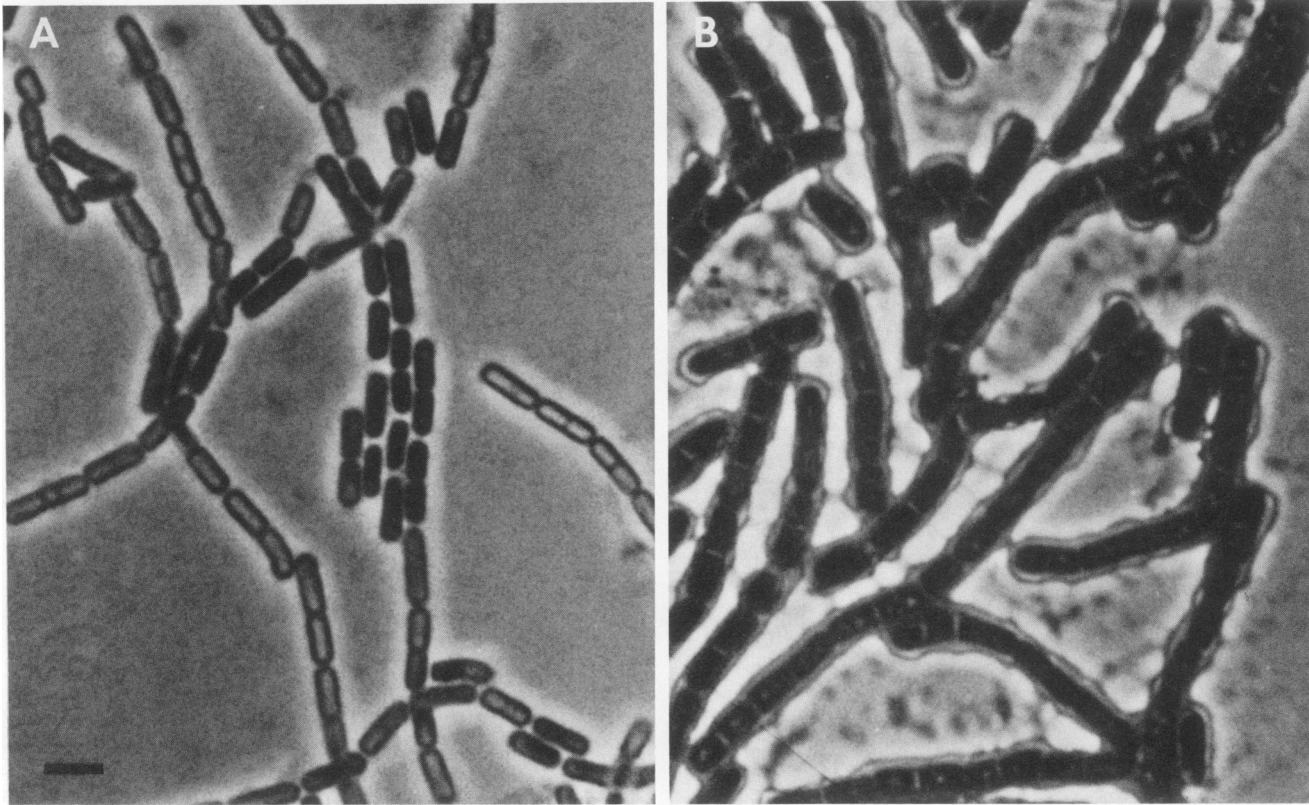
FIG. 7. Phase-contrast photomicrograph of cells of *B. cereus* grown on bicarbonate agar in 20% CO_2. (A) *B. cereus* 569 UM20-1, Cap⁻. (B) *B. cereus* 569 UM20-1 tr305-1, a Cap⁺ transcipient carrying pXO2. Bar, 2.0 μm.

avirulent Pasteur vaccine strains of *B. anthracis*, both pXO2⁺ and pXO2⁻ types were found. Rough variants that retained pXO2 were able to revert to Cap⁺, but reversion of those cured of the plasmid could not be demonstrated. This observation explains earlier reports by Thorne (12) and Meynell (5) that some noncapsulated variants of *B. anthracis* were stable but that others were able to revert to Cap⁺. This also explains why the avirulent Weybridge (Sterne) vaccine strain, which produces toxin but not capsules, has not been observed to revert to the Cap⁺ virulent type. The Sterne strain carries pXO1 but not pXO2 and is therefore unable to revert to Cap⁺.

In view of the demonstration that rough variants which still carry pXO2 are able to revert to Cap⁺, it will be interesting to determine whether the mutations that engender the rough phenotype are chromosome or plasmid borne. We plan to test this by transferring pXO2 from rough pXO2⁺ variants to strains that have been cured of the plasmid.

ACKNOWLEDGMENTS

ADDENDUM

After this manuscript was submitted for publication, the following paper, which confirms the presence of a capsule plasmid in *B. anthracis*, appeared: I. Uchida, T. Sekizake, K. Hashimoto, and N. Terakado, J. Gen. Microbiol. 131:363–367, 1985.

LITERATURE CITED

1. Battisti, L., B. D. Green, and C. B. Thorne. 1985. Mating system for transfer of plasmids among *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. J. Bacteriol. 162:543–550.
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. Kado, C. I., and S.-T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365–1373.
4. McCloy, E. W. 1951. Studies on a lysogenic *Bacillus* strain. I. A bacteriophage specific for *Bacillus anthracis*. J. Hyg. 49:114–125.
5. Meynell, E. W. 1963. Reverting and non-reverting rough variants of *Bacillus anthracis*. J. Gen. Microbiol. 32:55–60.
6. Meynell, G. G., and E. Meynell. 1966. The biosynthesis of poly D-glutamic acid, the capsular material of *Bacillus anthracis*. J. Gen. Microbiol. 43:119–138.
7. Mikesell, P., B. E. Ivins, J. D. Ristroph, and T. M. Dreier. 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. 39:371–376.
8. Ristroph, J. D., and B. E. Ivins. 1983. Elaboration of *Bacillus anthracis* antigens in a new defined culture medium. Infect. Immun. 39:483–486.
9. Ruhfel, R. E., N. J. Robillard, and C. B. Thorne. 1984. Interspecies transduction of plasmids among *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis*. J. Bacteriol. 157:708–711.
10. Stanley, J. L., K. Sargeant, and H. Smith. 1960. Purification of anthrax toxin factors I and II of the anthrax toxin produced *in vivo*. J. Gen. Microbiol. 22:206–218.
11. Thorne, C. B. 1956. Capsule formation and glutamyl polypeptide synthesis by *Bacillus anthracis* and *Bacillus subtilis*. Symp. Soc. Gen. Microbiol. 6:68–80.
12. Thorne, C. B. 1960. Biochemical properties of virulent and

avirulent strains of *Bacillus anthracis*. Ann. N.Y. Acad. Sci. **88:**1024–1033.

13. **Thorne, C. B.** 1968. Transducing bacteriophage for *Bacillus cereus*. J. Virol. **2:**657–662.

14. **Thorne, C. B.** 1978. Transduction in *Bacillus thuringiensis*. Appl. Environ. Microbiol. **35:**1109–1115.

15. **Thorne, C. B.** 1985. Genetics of *Bacillus anthracis*, p. 56–62. *In* L. Leive (ed.), Microbiology—1985. American Society for Microbiology, Washington, D.C.

16. **Thorne, C. B., C. G. Gómez, and R. D. Housewright.** 1952. Synthesis of glutamic acid and glutamyl polypeptide by *Bacillus anthracis*. II. The effect of carbon dioxide on peptide production on solid media. J. Bacteriol. **63:**363–368.

17. **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.

18. **Vodkin, M. H., and S. H. Leppla.** 1983. Cloning of the protective antigen gene of *Bacillus anthracis*. Cell **34:**693–697.

19. **Yelton, D. B., and C. B. Thorne.** 1971. Comparison of *Bacillus cereus* bacteriophages CP-51 and CP-53. J. Virol. **8:**242–253.