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GENETIC AND PHYSIOLOGICAL CONTROL OF PROTECTIVE ANTIGEN SYNTHESIS

BY BACILLUS ANTHRACIS

ANNUAL PROGRESS REPORT

CURTIS B. THORNE

DECEMBER 1984

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
Fort Detrick, Frederick, Maryland 21701

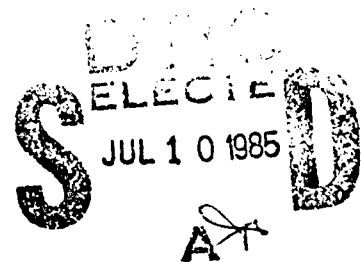
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newly discovered plasmid which encodes capsule formation in B. anthracis.

The mating system for transfer of plasmids among B. anthracis, B. cereus, and B. thuringiensis is very useful for assessing the biological significance of plasmids found in the three species. We have identified two B. thuringiensis plasmids, pX011 and pX012, which are capable of bringing about their own transfer as well as that of other plasmids. B. anthracis and B. cereus transipients that inherit either pX011 or pX012 were, in turn, effective donors. By means of the mating system we have been able to demonstrate transfer of the anthrax toxin plasmid, pX01, to B. cereus and strains of B. anthracis previously cured of the plasmid.

We have demonstrated that the avirulent Pasteur strain (ATCC 6602) of B. anthracis carries a plasmid, pX02, which encodes capsule formation. In collaboration with Dr. Bruce Ivins of USAMRIID we have shown that virulent strains of B. anthracis also contain pX02. Two classes of rough noncapsulated variants were isolated from the capsule-producing pasteur strain; those which were cured of pX02 and those which still carried the plasmid. Reversion to capsule formation was observed only in the variants that retained pX02. The Weybridge (Sterne) strain does not contain pX02 and that explains why capsule-forming revertants have not been observed with that strain.

By means of the Bacillus mating system we have transferred the capsule plasmid, pX02, to B. cereus. B. cereus transipients that acquired the plasmid produced capsules under the same conditions required for capsule synthesis by B. anthracis.

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## SUMMARY

This is a progress report (annual report) of research being carried out with Bacillus anthracis. The primary objective of the research is to gain information and to develop genetic systems that will contribute to the development of an improved vaccine for anthrax. The toxin-producing, but avirulent, Weybridge (Sterne) strain of B. anthracis is being used. Genetic and physiological factors controlling the synthesis and accumulation of protective antigen as well as the two other components of anthrax toxin are being investigated. During the past year our major effort was placed on further development and application of a mating system by means of which plasmids can be transferred among strains of B. anthracis, B. cereus, and B. thuringiensis. We also spent considerable effort on studies of a newly discovered plasmid which encodes capsule formation in B. anthracis.

The mating system for transfer of plasmids among B. anthracis, B. cereus, and B. thuringiensis is very useful for assessing the biological significance of plasmids found in the three species. The tetracycline resistance plasmid, pBC16, which we moved into B. thuringiensis by transduction, made it possible to select transipients quantitatively. We have identified two B. thuringiensis plasmids, pX011 and pX012, which are capable of bringing about their own transfer as well as that of pBC16 and other plasmids. B. anthracis and B. cereus transipients inheriting either pX011 or pX012 were, in turn, effective donors. Transipients harboring pX012 were more efficient donors than those harboring pX011; transfer frequencies ranged from  $10^{-4}$  to  $10^{-1}$  and from  $10^{-8}$  to  $10^{-5}$ , respectively. Cell-to-cell contact was necessary for plasmid transfer and the addition of deoxyribonuclease to mating mixtures had no effect. The high frequencies of transfer, along with the fact that cell-free filtrates of donor cultures were ineffective, suggested that the transfer was not phage-mediated. B. anthracis and B. cereus transipients that inherited pX012 also acquired the ability to produce parasporal crystals ( $\text{Cry}^+$ ) resembling those produced by B. thuringiensis, indicating that pX012 carries gene(s) involved in crystal formation. Transipients that inherited pX011 were  $\text{Cry}^-$ . This mating system provides an efficient method for transfer of a large range of Bacillus plasmids within and among species by a conjugation-like process.

By means of the mating system we have been able to demonstrate transfer of the anthrax toxin plasmid, pX01, to strains of B. anthracis previously cured of

the plasmid. Transcipients that inherited pX01 regained the ability to synthesize protective antigen and, in addition, they resembled the original uncured parent strain with respect to a number of other phenotypic characteristics. This provides proof that the phenotypic changes observed in cured strains were, in fact, a result of plasmid loss rather than a consequence of the selection procedure used in their isolation.

To determine whether pX01 had integrated into the bacterial chromosome of strains presumed to be cured of the plasmid, and also to explore the possibility that pX01 and the chromosome might have sequences in common, we carried out hybridization experiments using pX01 DNA as a probe. Results indicate that pX01 was completely absent from the cured strains and also that the plasmid does not share homologous sequences with the chromosome.

Very recently we have demonstrated transfer of pX01 to B. cereus at a high frequency; more than 50% of tetracycline-resistant transcipients also inherited pX01. It will be important to study the biological activity of this plasmid in B. cereus.

We have demonstrated that the avirulent Pasteur strain (ATCC 6602) of B. anthracis carries a plasmid, pX02, which encodes capsule formation. In collaboration with Dr. Bruce Ivins of USAMRIID we have shown that virulent strains of B. anthracis also contain pX02. Two classes of rough noncapsulated (Cap<sup>-</sup>) variants were isolated from the capsule-producing (Cap<sup>+</sup>) Pasteur strain; those which were cured of pX02 and those which still carried it. Reversion to Cap<sup>+</sup> was demonstrable only in rough variants which contained pX02. This explains an observation reported several years ago that some Cap<sup>-</sup> variants of B. anthracis were revertible to Cap<sup>+</sup> and others were nonrevertible. By means of the mating system discussed above, pX02 was transferred from B. anthracis to B. cereus. B. cereus transcipients which acquired pX02 produced capsules under the same conditions required for capsule synthesis by B. anthracis.

#### Foreword

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This is the fifth annual report submitted under contract No. DAMD 17-80-C-0099. Research on the contract began July 1, 1980. The contract was extended for a second year beginning July 1, 1981, for two more years on July 1, 1982, and it was finally extended a third time for a fifth year beginning August 1, 1984. The previous annual reports are dated December 1980, December 1981, December 1982, and December 1983.

During the year represented by this annual report our research concentrated largely on aspects of a mating system for the transfer of plasmids among B. anthracis, B. cereus, and B. thuringiensis. As a result we have been able to transfer B. anthracis plasmids to B. cereus and cured strains of B. anthracis. This makes it possible to better evaluate the biological significance of the plasmids in question. We have demonstrated during the past year that capsule synthesis by B. anthracis is encoded by a newly discovered plasmid, which we have designated pX02. In this report our main efforts for the past year are discussed following a general description of materials and methods. Specific procedures which themselves are results of the research are described as appropriate under individual sections.

#### MATERIALS AND METHODS

Organisms. The Weybridge (24) strain of B. anthracis was obtained from the Microbiological Research Establishment, Porton, England in 1957. It was isolated by Sterne (21) and used by the Ministry of Agriculture, Fisheries, and Food (Weybridge, England) as a living spore vaccine. Table 1 lists specific strains and mutants referred to in this report.

Media. For convenience to the reader, compositions of the various culture media commonly used in our laboratory are given below. All amounts are for one liter final volume. For preparation of solid medium, 15 grams of agar (Difco) were added per liter of the corresponding broth.

NBY broth: Nutrient broth (Difco), 8 g; Yeast extract (Difco), 3 g.

Phage assay broth: Nutrient broth (Difco), 8 g; NaCl, 5 g;  $MgSO_4 \cdot 7H_2O$ , 0.2 g;  $MnSO_4 \cdot H_2O$ , 0.05 g;  $CaCl_2 \cdot 2H_2O$ , 0.15 g. The pH was adjusted to 6.0 with HCl.

Phage assay agar: For bottom agar, 15 g of agar were added per liter of phage assay broth. For soft agar, 0.6 g of agar were added per liter.

L broth: Tryptone (Difco), 10 g; Yeast extract (Difco), 5 g; NaCl, 10 g.

The pH was adjusted to 7.0 with NaOH.

BHI broth: Brain heart infusion broth (Difco), 37 g.

BHI-glycerol broth: BHI broth with 0.5% (w/v) glycerol added aseptically.

Minimal I:  $(\text{NH}_4)_2\text{SO}_4$ , 2 g;  $\text{KH}_2\text{PO}_4$ , 6 g;  $\text{K}_2\text{HPO}_4$ , 14 g; sodium citrate, 1 g; glucose, 5 g; L-glutamic acid, 2 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.04 g;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.00025 g. The pH was adjusted to 7.0 with NaOH. The glucose and  $\text{FeCl}_3$  were sterilized separately.

Minimal IC: Minimal I with 5 g of vitamin-free Casamino acids (Difco) and 10 mg of thiamine hydrochloride.

Minimal M: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine and L-proline.

Minimal O: To Minimal I was added 10 mg of thiamine hydrochloride, 200 mg of glycine, and 40 mg of L-methionine, L-serine, L-threonine, and L-proline.

Antisera. All antisera were kindly supplied by personnel of USAMRIID.

Protective antigen assays. These were carried out by the agar diffusion method as outlined by Thorne and Belton (24).

Propagation and assay of bacteriophage CP-51. The methods described by Thorne (23) were followed. The indicator for routine assay of CP-51 was B. cereus NRRL B-569.

Propagation and assay of bacteriophage W $\alpha$ . Bacteriophage W $\alpha$  (12) was obtained from B. cereus W (ATCC 11950). It was propagated on B. anthracis 6602 R1 in soft overlays of phage assay agar incubated at 37°C for 17 to 20 hours. It was assayed against the same strain in soft overlays of phage assay agar incubated at 30°C.

Test for 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 supplemented with 0.7% (w/v) sodium bicarbonate and 10% (v/v) horse serum. Plates were incubated in the presence of 20%  $\text{CO}_2$  at 37°C for 24 to 48 hours.

Isolation of spontaneous rough mutants of B. anthracis 6602. Cells were plated for single colonies on NBY agar containing 0.7% (w/v) bicarbonate and 10%

(v/v) horse serum and incubated at 37°C in 20% CO<sub>2</sub>. After several days rough outgrowth appeared at the edge of some of the mucoid colonies. These were picked and purified by streaking on fresh plates of the same medium.

Isolation of capsulated revertants of rough mutants. To demonstrate reversion, approximately  $1 \times 10^5$  spores of a rough mutant were spread with  $1 \times 10^8$  PFU of phage W<sub>α</sub> on NBY agar containing bicarbonate and horse serum as above. The plates were incubated at 37°C in 20% CO<sub>2</sub> for two days and examined for mucoid colonies. The presence of capsules was confirmed by phase microscopy.

Procedures used in mating experiments. Cells for mating were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth and incubated at 30°C with slow shaking. Donor and recipient strains were grown separately for 8 to 10 hours from 1% (v/v) transfers of 14- to 15-hour cultures. Each culture was diluted 1:50 in BHI broth, yielding  $10^6$  to  $10^7$  cells per ml, and mating mixtures were prepared by mixing 1 ml of donor cells with 1 ml of recipient cells in 20-mm culture tubes. Control tubes contained 1 ml of BHI broth and 1 ml of donor or recipient cells. Mixtures were incubated at 30°C with slow shaking. Samples were removed at times indicated and plated on appropriate selective media for determining the numbers of donors, recipients, and transciipients. Dilutions were made in peptone diluent. Plates were incubated at 30°C and colonies were scored after 24 to 48 hours.

When mating mixtures were prepared with streptomycin-resistant recipients and tetracycline-resistant donors, tetracycline-resistant transciipients were selected on L-agar containing streptomycin (200 µg/ml) and tetracycline (5 or 25 µg/ml). If the recipients were streptomycin-sensitive, tetracycline-resistant transciipients were selected on Min 1C agar supplemented with tetracycline and the appropriate growth requirement of the auxotrophic recipient. For selecting B. cereus transciipients 25 µg of tetracycline per ml was used, but with B. anthracis the number of transciipients recovered was greater when the concentration of tetracycline was only 5 µg per ml. Once transciipients were selected with the lower concentration of tetracycline, they were then fully resistant to 25 µg per ml. When recipients were rifampicin-resistant, rifampicin (10µg/ml) was included in the selection medium.

Transfer frequency is expressed as the number of transciipients per ml divided by the number of donors per ml at the time of sampling. It should be emphasized that the use of both auxotrophic and drug-resistant strains allowed unambiguous strain selection and recognition.

Test for effect of DNase in mating mixtures. Donor cells were first incubated alone in the presence of 100  $\mu\text{g}$  of DNase per ml (Worthington Biochemical Corp.) and 0.01 M  $\text{MgSO}_4$  for 15 min at 37°C. One ml of donor and 1 ml of recipient cells were mixed together, and DNase (100  $\mu\text{g}/\text{ml}$ ) was added again after 1, 2, and 3 hours of mating.  $\text{MgSO}_4$  without DNase was added to control matings. After 4 hours of incubation samples were plated for selection of transciipients.

Test for plasmid transfer by bacteriophage in mating mixtures. To investigate the possibility of phage-mediated plasmid transfer, cell-free filtrates of donor cultures were substituted for donor cells. The supernatant fluid from a centrifuged donor culture was filtered through a Millipore HA membrane (Millipore Corp., Bedford, MA), and 1 ml of cell-free filtrate was mixed with 1 ml of recipient cells. Such mixtures were incubated and assayed for  $\text{To}^r$  transciipients as described above.

Detection of plasmid DNA. Plasmid DNA was extracted by a modification of the procedure described by Kado and Liu (9). Cells for plasmid extraction were grown in 250-ml Erlenmeyer flasks containing 25 ml of BHI broth supplemented when appropriate with tetracycline (10  $\mu\text{g}/\text{ml}$ ). With some strains better results were obtained when 0.5% (w/v) glycerol was included in the BHI broth to prevent sporulation. Cultures were incubated for 16 hours 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 resuspended in 1 ml of E buffer (0.04 M Tris-OH (Sigma), 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 gm of sodium dodecyl sulfate and 5 ml of 3 N NaOH to 100 ml of 15% (w/v) sucrose in 0.05 M Tris-OH. The tubes were rapidly inverted 20 times to mix the cells and buffer and they were then held in a 60°C water bath for 30 min. Five-tenths ml of Pronase (Calbiochem-Behring Corp., La Jolla, CA) solution (2 mg per ml in 2 M Tris at pH 7.0) was added, and the tubes were mixed as above and incubated in a 37°C water bath for 20 min. The lysate was extracted with 6 ml of phenol-chloroform (1:1, v/v) by inverting the tubes 40 times. The 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  $\mu\text{l}$ ) were mixed with 10  $\mu\text{l}$  of tracking dye (0.25% bromphenol blue, 15% ficoll) and samples (40  $\mu\text{l}$ ) were applied to horizontal 0.7% agarose

(Sigma, Type II medium EEO) gels prepared and run in Tris-borate buffer (0.089 M Tris-OH, 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 (1 µg/ml in Tris-borate buffer).

Screening colonies for protective antigen production. Colonies were picked to plates of R medium (14, 17) and incubated at 37°C in 20% CO<sub>2</sub>. Each "halo agar" plate 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. A zone of precipitate formed around PA<sup>+</sup> colonies.

## RESULTS AND DISCUSSION

### I. Renaming of B. anthracis and B. thuringiensis plasmids

When my laboratory began work on plasmids we chose the designation pBA for plasmids of B. anthracis and pUM or pBT for plasmids of B. thuringiensis. However, it came to our attention that these three designations were already in use by other laboratories. Therefore, we have now chosen the designation pXO for all plasmids named by our laboratory. This designation has been approved and officially assigned to our laboratory by the Plasmid Reference Center at Stanford University. Accordingly, the B. anthracis toxin plasmid, which was originally designated pPA1, is now named pXO1. The plasmid found in the Pasteur strain of B. anthracis (ATCC 6602) and associated with capsule formation is named pXO2. The B. thuringiensis fertility plasmid formerly referred to as pUM1 or pBT1 is now named pXO11, and the fertility plasmid formerly referred to as pUM2 or pBT2 is now named pXO12.

### II. Plasmid profiles of the Pasteur strain (ATCC 6602) and the M strain of B. anthracis

The Pasteur strain (ATCC 6602) of B. anthracis carries one large plasmid, which we have designated pXO2. The plasmid migrates more rapidly than pXO1 during electrophoresis in agarose gels, and we estimate that its molecular mass is in the order of 60 megadaltons. As discussed later in this report, we have shown that pXO2 is involved in capsule formation.

The M strain of B. anthracis, a mutant that does not require added bicarbonate for the synthesis of capsules, carries two plasmids which migrate in agarose gels at the same rates as pX01 and pX02. This observation has stimulated our interest in the role of bicarbonate in synthesis of capsule. Bicarbonate is required for the synthesis of capsules by the Pasteur strain, 6602, and by virulent strains. It will be interesting to learn whether the mutation which enables the M strain to produce capsules in the absence of bicarbonate is carried by the plasmid, pX02, or whether it is chromosomal. We should be able to determine this by transferring pX02 from the M strain to other strains of B. anthracis that have been cured of pX02. If the mutation is on the plasmid, strains infected with the mutant plasmid should not require bicarbonate for capsule production. On the other hand, if the mutation is chromosomal, strains infected with the plasmid should not have the mutant phenotype. We hope to answer this question in the near future by using the mating system to transfer pX02 from the M strain to appropriate recipients.

### III. The mating system for transfer of plasmids among strains of B. anthracis, B. cereus, and B. thuringiensis

We have continued to investigate the mating system described in Report Number Four (Annual Progress Report, December 1983). We have concentrated our studies on two fertility plasmids found in B. thuringiensis 4042A subsp. thuringiensis. The following summarizes the utility of the mating system and the current status of our understanding of the process.

Transfer of pBC16 from B. thuringiensis subsp. thuringiensis. Plasmid profiles of the two donor strains, B. thuringiensis 4042A UM8 td2 and 4042A UM8-13 td1-A, as well as B. anthracis and B. cereus recipients and transipients are shown in Fig. 1 and 2. All Tc<sup>r</sup> transipients inherited plasmid DNA which migrated at the same rate as pBC16. In addition to pBC16, most transipients also inherited various combinations of other plasmids derived from the B. thuringiensis donor. Examination of a large number of transipients has shown that (1) the two most frequently transferred B. thuringiensis plasmids were pX011 and pX012 and (2) the smaller molecular weight plasmids were transferred more or less randomly as demonstrated by their variable distribution in transipients. In matings with donor strain 4042A UM8-13 td1-A, which harbors

pX011 as well as several other unnamed plasmids, the majority of Tc<sup>r</sup> transcipts acquired pX011. Similarly, with donor cells of 4042A UM8 td2, which harbors both pX011 and pX012, the majority of Tc<sup>r</sup> transcipts inherited either pX011 or pX012. However, we have observed that in matings with the latter donor, transcipts inherited pX011 more frequently than pX012. No transcipts thus far examined have contained both of these plasmids, suggesting that there may be competition between pX011 and pX012 during transfer. All transcipts retained both the auxotrophic and streptomycin resistance markers of the recipient strain. Although spontaneous tetracycline-resistant mutants of B. cereus 569 were occasionally found at low frequencies, we have never observed such spontaneous mutants of B. anthracis.

Formation of parasporal crystals by B. anthracis and B. cereus transcipts. Phase microscopy revealed that some Tc<sup>r</sup> transcipts derived from matings in which strain 4042A UM8 td2 was the donor had also acquired the ability to produce parasporal crystals (Cry<sup>+</sup>) resembling those produced by the B. thuringiensis donor. In contrast, no Cry<sup>+</sup> transcipts were obtained from matings in which strain 4042A UM8-13 td1-A was the donor. Plasmid analysis of the two donor strains indicated that pX012 was associated with crystal production. As shown in Fig. 2, (lanes 1 and 6), the plasmid profiles of the Cry<sup>-</sup> mutant, 4042A UM8-13 td1-A, and the Cry<sup>+</sup> mutant, 4042A M8 td2, were identical except for the absence (spontaneous loss, see footnote 1 to Table 1) of pX012 from UM8-13 td1-A. This, along with the fact that there was a strict correlation between the Cry<sup>+</sup> phenotype and the presence of pX012 in transcipts, is strong evidence that pX012 is involved in crystal production. A number of reports have established that one or more plasmids are involved in parasporal crystal formation in a variety of B. thuringiensis strains (3-8, 10, 11, 15, 19, 20). Although Cry<sup>+</sup> transcipts of both B. anthracis and B. cereus were obtained from matings in which 4042A UM8 td2 was the donor, the frequency of Cry<sup>-</sup> transcipts was 10<sup>2</sup> to 10<sup>3</sup> times higher than that of Cry<sup>+</sup> transcipts. The lower frequency of Cry<sup>+</sup> transcipts is in agreement with plasmid analyses which showed that the donor, 4042A UM8 td2, transferred pX012 much less frequently than pX011.

Transfer of pBC16 and other plasmids from B. anthracis and B. cereus transcipts. In our system of labeling transcipts for identification, e.g., Weybridge UM44 tr203-1, UM44 designates the recipient from which the transcipt was derived and tr203-1 identifies a particular transcipt that

has been purified by single colony isolation. Transcipients isolated from mating mixtures in which B. thuringiensis was the donor are referred to as primary transcipients. Secondary transcipients are those derived from matings in which the donors were fertile B. cereus or B. anthracis transcipients harboring either pX011 or pX012.

Matings were performed to determine whether primary and secondary B. anthracis and B. cereus transcipients could also function as donors of pBC16 to tetracycline-sensitive B. anthracis and B. cereus recipients. Results (Table 2) show that B. anthracis and B. cereus transcipients which acquired either pX011 or pX012 were, in turn, effective donors of pBC16. Plasmid analysis confirmed the transfer of pBC16 as well as other B. thuringiensis plasmids from the primary and secondary transcipients. Transcipients which inherited only the smaller B. thuringiensis plasmids migrating below chromosomal DNA (Fig. 1, lane 8) were not fertile. Donor ability of the fertile transcipients was stably maintained during subsequent growth and sporulation. pBC16, itself, was ineffective in promoting plasmid transfer; this was evidenced by the fact that cells of B. anthracis or B. cereus into which pBC16 was introduced by transduction were completely devoid of donor activity.

As shown in Table 2, transcipients that harbored pX012 were more effective donors of pBC16 than those that harbored pX011. The data there also reflect a difference between B. cereus and B. anthracis with respect to their activity as recipients. In experiments with (pX012)<sup>+</sup> transcipients as donors, B. cereus recipients usually yielded 10- to 100-fold more transcipients than B. anthracis recipients. However, with (pX011)<sup>+</sup> transcipients as donors, recipient cells of the two species yielded approximately equal numbers of transcipients.

Tc<sup>r</sup> transcipients from both intraspecies and interspecies matings retained the auxotrophic marker of the recipient strain. Prototrophic recombinants were never found, suggesting that transfer of chromosome occurred rarely or not at all.

Evidence for plasmid mobilization by pX011 and pX012. Taking advantage of a large collection of B. anthracis and B. cereus transcipients displaying various plasmid profiles, we attempted to identify the fertility factors responsible for plasmid mobilization. Based on the fact that all transcipients harboring either pX011 or pX012 were capable of plasmid transfer, we speculated that either of these two plasmids alone could confer donor capability to host cells. Examination of plasmid content and transfer ability of numerous Tc<sup>r</sup>

transcipients confirmed that both pX011 and pX012 are fertility plasmids, each capable of promoting its own transfer as well as that of other plasmids. The random distribution of the smaller B. thuringiensis plasmids in fertile transcipients suggested that no combination of these plasmids in conjunction with either pX011 or pX012 was necessary for plasmid transfer. Furthermore, transcipients acquiring only these small B. thuringiensis plasmids (Fig. 1, lane 8) were ineffective in transferring pBC16. In contrast, transcipients inheriting only pBC16 and either pX011 or pX012 (Fig. 2, lanes 7 through 10) were capable of transferring pBC16 (Table 2).

B. anthracis and B. cereus transcipients that harbored pX012 were more fertile than the original donor, B. thuringiensis 4042A UM8 td2, with respect to transfer of pBC16. On the other hand, B. anthracis and B. cereus transcipients that inherited pX011 were less effective donors of pBC16 than either of the two B. thuringiensis donors, 4042A UM8 td2 and 4042A UM8-13 td1-A. This latter observation suggests that other factors in B. thuringiensis may contribute to the donor activity of pX011.

Time and frequency of pBC16 transfer by B. anthracis. Fig. 3 shows that the numbers of transcipients from a mating between a B. anthracis donor carrying pX012 and a B. anthracis recipient increased rapidly during the period between 2 and 6 hours and very slowly after that. The greatest relative increase (164-fold) in transcipients occurred between 2 and 4 hours of mating indicating that many independent transfer events occurred during that period. Although not included here, comparable results were obtained with B. anthracis donors carrying pX011 and with B. cereus and B. thuringiensis donors carrying either pX011 or pX012. In experiments in which mating mixtures were sampled at 0, 30, 60, 90, and 120 min, no transcipients could be detected before 120 min, suggesting that a period of growth of donor and recipient cells together was required prior to plasmid transfer. The necessity for exponential growth of donor and recipient cells together was further supported by the failure to detect transcipients from mating mixtures prepared from donors and recipients which had been grown separately for increasing periods of time (4 to 16 hours) before they were mixed.

Mechanism of transfer. To investigate the possibility of phage-mediated plasmid transfer, we tested cell-free filtrates prepared from donor cultures for the ability to convert recipients to tetracycline-resistance. No  $Tc^r$  transcipients could be detected in such experiments. To determine whether

plasmid transfer occurred by transformation, we examined the sensitivity of pBC16 transfer to DNase as described under Materials and Methods. In matings between B. anthracis donors and B. cereus recipients the number of  $Tc^r$  transipients obtained after 4 hours in the presence of DNase and  $MgSO_4$  ( $3.2 \times 10^6$  per ml) was not significantly different from the number obtained in the presence of  $MgSO_4$  alone ( $3.0 \times 10^6$  per ml). Finally, to determine whether cell-to-cell contact was necessary for plasmid transfer we conducted a mating between a B. anthracis donor and a B. cereus recipient in a U-tube. A 0.45- $\mu m$  filter (Millipore, type HA) inserted between the two strains prevented cell-to-cell contact but allowed diffusion of filterable material between the two cultures. As a control, the two cultures were also mixed together in a second U-tube without a filter. After 3 hours of incubation normal numbers of transipients were recovered from the tube without the filter, but no transipients were detected in samples from the tube containing the filter.

Discussion. The results presented here demonstrate that each of two plasmids, pX011 and pX012, indigenous to strain 4042A of B. thuringiensis subsp. thuringiensis, is capable of promoting plasmid transfer within and among strains of B. anthracis, B. cereus, and B. thuringiensis. All transfer proficient B. anthracis and B. cereus transipients thus far examined inherited either pX011 or pX012. That pX011 and pX012 can function independently of the small molecular weight plasmids of B. thuringiensis is direct proof that they are fertility plasmids capable of bringing about their own transfer as well as that of other plasmids. Transipients harboring pX012 were more effective donors than those harboring pX011, and B. cereus was generally a better recipient than B. anthracis. For example, maximum frequencies for pBC16 transfer by (pX012)<sup>+</sup> B. anthracis donors to B. anthracis and B. cereus recipients were 5% and 80%, respectively.

Plasmid pX012 was inherited less frequently than pX011 by recipients mated with the B. thuringiensis donor which carried both pX011 and pX012. However, once the two plasmids were segregated, transipients inheriting pX012 were more fertile than those inheriting pX011. These observations, together with the failure to detect transipients that acquired both pX011 and pX012, suggests that these two fertility plasmids may compete in the transfer process. An analogous competition phenomenon has been reported by Clewell (2) for streptococcal matings in which the transfer ability of the conjugative erythromycin-resistance plasmid, pAM81, is drastically reduced in the presence

of either of two other conjugative plasmids, pAMa1 and pAD1. Incompatibility is a universal trait exhibited among plasmids which are isogenic for maintenance, replication, and transfer functions, and this may explain the apparent competition between pX011 and pX012 and the spontaneous loss of pX012 from B. thuringiensis 4042A UM8-13 td1-A.

Although the mechanism of transfer is still unknown, several lines of evidence support a conjugation-like process: (1) the addition of DNase to mating mixtures did not reduce transfer frequencies; (2) donor filtrates were inactive and cell-to-cell contact was necessary; (3) the high frequencies of transfer are typical of conjugation systems; (4) the large increase in numbers of transipients between 2 and 4 hours ( $10^2$ - to  $10^5$ -fold) indicates that many independent transfer events occurred. Our results showing a requirement for cell-to-cell contact and the ineffectiveness of DNase in preventing plasmid transfer from B. anthracis donors are similar to those obtained by Gonzalez and Carlton (6) in plasmid transfer experiments with B. thuringiensis donors.

There appeared to be an essential period (2 to 4 hours) of growth of donor and recipient cells together before plasmid transfer could be detected. The requirement for growing donor and recipient cells together during the exponential phase of growth was dramatically illustrated by the drastic reduction in plasmid transfer when mating mixtures were prepared from donor and recipient cells grown separately for similar periods of time (data not shown).

In addition to transfer functions, the fertility plasmid, pX012, was found to carry information involved in parasporal crystal formation which was expressed in all three species of Bacillus tested. The evidence for this was two-fold: (1) All transipients harboring pX012 were Cry<sup>+</sup> while those harboring pX011 were Cry<sup>-</sup>, and (2) the inability of strain 4042A UM8-13 td1-A to produce parasporal crystals when infected with the converting phage TP-13 (see footnote, Table 1) correlated with the spontaneous loss of pX012.

Future work to determine the extent of homology between pX011 and pX012 may provide further insight into their apparent incompatibility and their maintenance and transfer functions. Presently, the ability to transfer a large range of plasmids makes this a useful genetic exchange system for the functional analysis of genetic determinants on plasmids of B. anthracis, B. cereus, and B. thuringiensis.

#### IV. Studies with the B. anthracis toxin plasmid, pX01

Size determination of pX01 by restriction analysis. Purified pX01 DNA, isolated from the Weybridge strain of B. anthracis, was fragmented with HindIII or EcoRI. The mobilities of the fragments during electrophoresis in agarose gels were compared with those of standard fragments (lambda DNA fragmented with the same enzymes and also gel marker DNA obtained from Bethesda Research Laboratories). Electrophoresis was carried out in 0.5% and 1.2% agarose gels. Standard curves comparing mobility with known fragment size were constructed for gel marker DNA and lambda DNA fragments. Large pX01 fragments were assigned size values from the curve constructed with data from 0.5% gels and smaller pX01 fragments were assigned size values from the curve constructed with data from 1.2% gels. The size of pX01 estimated from the HindIII digest was 171.5 kilobase pairs and the size estimated from the EcoRI digest was 177.0 kilobase pairs. The average value from the two determinations, 174.3 kilobase pairs, is in good agreement with the value of 168.4 plus or minus 7.3 kilobase pairs as estimated by Vodkin and Leppla (25) from electron microscopic measurements. Complete details of these analyses were given by Robillard (18).

Hybridization studies with pX01 as a probe. To determine whether pX01 had integrated into the bacterial chromosome of strains presumed to be cured of the plasmid and also to explore the possibility that pX01 and the chromosome might have sequences in common, we carried out experiments using the Southern hybridization technique. <sup>32</sup>P-nick translated pX01 DNA was used to probe whole DNA from the parent Weybridge strain and from three derivatives cured of pX01 by three different methods. DNA extracted from the parent strain readily hybridized to <sup>32</sup>P-labeled pX01 DNA. However, DNA from the cured strains failed to hybridize with the labeled plasmid DNA to a detectable extent. These results suggest very strongly that pX01 is completely absent from the cured strains and also that pX01 does not share homologous sequences with the chromosome. Further details of these experiments can be found in Robillard's Ph. D. dissertation (18).

Transfer of pX01 to B. cereus and cured strains of B. anthracis by mating. We have succeeded in demonstrating the transfer of pX01 from cell to cell during the mating process mediated by the B. thuringiensis plasmid, pX012. When B.

anthracis Weybridge A UM2 tr244-1, a transcient carrying the three plasmids, pX01, pX012, and pBC16, was mated with a (pX01)<sup>-</sup> strain, UM44-1-C9 Ind<sup>-</sup>Str<sup>r</sup>, two Tc<sup>r</sup>Str<sup>r</sup> transcipts out of 475 tested on halo agar for zones of precipitation were positive (PA<sup>+</sup>). These two transcipts, labeled UM44-1-C9 tr41G-1 and tr41G-2, were both shown to carry the three plasmids, pX01, pX012, and pBC16. They were confirmed to produce protective antigen in broth culture.

From a mating experiment in which UM44-1-C9 tr41G-1 was the donor and Weybridge A UM23C1-1 Ura<sup>-</sup>Str<sup>r</sup>(pX01)<sup>-</sup> was the recipient, three out of 64 Tc<sup>r</sup> transcipts tested on halo agar for protective antigen synthesis were positive. The three transcipts were labeled Weybridge A UM23C1-1 tr47G-6, -34, and -61. The first two were also Cry<sup>+</sup> and the third one was Cry<sup>-</sup>. Plasmid analysis on agarose gels revealed that lysates of each of the three transcipts had only one plasmid band which migrated more slowly than chromosomal DNA, and it was apparently larger than pX01 or pX012. This is in contrast to UM2 tr244-1 and UM44-1-C9 tr41G-1 in which both pX01 and pX012 could be demonstrated. Thus, it appeared that the newly observed high molecular weight band found in each of the three transcipts, tr47G-6, -34, and -61, represented a cointegrate or recombinant plasmid.

These three transcipts were tested for the ability to transfer pBC16 when mated with B. cereus 569 UM20-1. The results are shown in Table 3. As expected, only the two transcipts that were Cry<sup>+</sup> were able to mobilize pBC16. The numbers of Tc<sup>r</sup> transcipts obtained are typical of those observed from matings of Cry<sup>+</sup>Tc<sup>r</sup> B. anthracis donors with B. cereus recipients.

Sixty-two B. cereus transcipts from the mating in which UM23C1-1 tr47G-34 was the donor (Table 3) were tested on halo agar for protective antigen production, and at least 35 formed a zone of precipitation. (Because B. cereus is motile, the colonies spread considerably on halo agar and it is sometimes difficult to determine whether there is a zone of precipitation.) A number of PA<sup>+</sup> and PA<sup>-</sup> transcipts were examined for plasmid content, and the results are confusing. A variety of large plasmids were found among the transcipts. The majority of PA<sup>+</sup> transcipts had two plasmids above chromosomal DNA, and they appeared to correspond to pX01 and pX012. A minority of the PA<sup>+</sup> transcipts had only one band migrating above chromosomal DNA and it resembled that present in the donors. Those transcipts that were PA<sup>-</sup> had either no plasmid DNA bands above chromosomal DNA or one that migrated to the position of pX012.

These results suggest that pX01 and pX012 can form a cointegrate and/or

recombinant plasmid, such as that found in B. anthracis UM23C1-1 tr47G-34, and that it is sometimes resolved during or after transfer. The much higher frequency of PA<sup>+</sup> transcipts found in matings with the "cointegrate" donor, compared to the frequencies found in matings with donors carrying resolved pXO1 and pXO12, suggests that the resolution may occur after transfer. The fact that transcipt UM23C1-1 tr47G-61, which also apparently contained a cointegrate or recombinant plasmid, was Cry<sup>-</sup> and nonfertile (Table 3), suggests that sequences involved in the synthesis of parasporal crystal and in fertility were lost or rearranged during formation of the complex plasmid.

These results are very recent and we have not answered the many questions regarding the complex array of plasmids we are seeing in transcipts. For that reason we have not included photographs of agarose gels showing migration of the various plasmids. More precise information regarding the nature of the presumed cointegrates must await restriction analyses of the plasmids involved. In the meantime we plan to test PA<sup>+</sup> B. cereus transcipts for protective antigen production in broth culture.

Comparison of characteristics of cured and uncured strains of B. anthracis.

As reported previously (Annual Report, December 1982 and December 1983), a number of cured derivatives (pXO1)<sup>-</sup> have been isolated from several genetically marked mutants of the Weybridge strain. In addition to the loss of ability to produce protective antigen, all cured strains we have tested are similar with respect to the following characteristics: (1) Cured cells sporulate earlier and at a higher frequency than uncured cells; (2) Colonies of cured cells differ from colonies of uncured cells in their morphology; (3) Cured cells are more sensitive than uncured cells to certain bacteriophages; (4) Cured cells do not grow as well as uncured cells in certain synthetic media; and (5) Spore-negative mutants are found at a much higher frequency in cultures of cured strains than in cultures of (pXO1)<sup>+</sup> strains.

Proof that the altered characteristics listed above were, in fact, a result of plasmid loss rather than a consequence of selection procedures used in isolating cured strains could be established only by transferring pXO1 back into strains previously cured of the plasmid. This was made possible by development of the mating system discussed above. Cured strains that were reinfected with pXO1 by the mating process could not be distinguished from the uncured parent strain from which they were originally derived. This confirms conclusively that the phenotypic changes listed above, including loss of ability to produce

protective antigen, are consequences of plasmid loss.

#### V. Studies with the *B. anthracis* capsule plasmid, pX02

Noncapsulated (rough) variants of the Pasteur strain, ATCC 6602. When Cap<sup>+</sup> strains of *B. anthracis* are grown under conditions conducive to capsule formation, i.e., on agar containing bicarbonate and/or serum and incubated in 20% CO<sub>2</sub>, rough sectors or edges occur frequently among the mucoid colonies. Once it was found that strain 6602 carries the plasmid, pX02, which is associated with capsule formation, it became of interest to investigate rough variants of this strain with respect to plasmid content. Therefore, we isolated a number of noncapsulated rough variants from rough sectors or edges of mucoid colonies grown on NBY agar containing 0.7% sodium bicarbonate and 10% horse serum and incubated in approximately 20% CO<sub>2</sub>.

Plasmid analyses revealed that there were two classes of rough variants. Some of the rough variants had been cured of pX02 and contained no detectable plasmids, but other rough variants still carried pX02. We then predicted that those rough variants which retained pX02 could be demonstrated to revert to Cap<sup>+</sup>, but that reversion to Cap<sup>+</sup> could not be demonstrated in those which had been cured of the plasmid. This prediction was found to be true. Both classes of rough variants were tested for reversion by exposing them to the virulent bacteriophage, W<sub>6</sub>, which lyses rough cells but not those that are capsulated. No mucoid revertants were found among three independently isolated (pX02)<sup>-</sup> strains; however, mucoid revertants were found in each of three independently isolated rough strains that retained pX02. Presumably, rough variants that carry pX02 are rough because they carry point mutations. This observation explains the reports by Thorne (22) and Meynell (13) that some noncapsulated variants of *B. anthracis* were stable and others reverted to Cap<sup>+</sup>. Plasmid profiles of the Pasteur strain 6602 and a rough Cap<sup>-</sup> (pX02)<sup>-</sup> variant are shown in Fig. 4 (lanes 1 and 2).

In view of the above demonstration that revertible rough variants still carry pX02, it becomes of interest to determine whether the mutations that engender the rough phenotype are chromosomal or plasmid borne. It seems possible that such mutations could reside on either replicon. Now that we are able to transfer pX02 by the mating system we are in a position to test whether a particular rough variant carries a mutation on the chromosome or on the

plasmid. If the mutation is chromosomal, presumably transcipts that inherit pX02 from the rough variant would be Cap<sup>+</sup>.

Transfer of pX02 to *B. cereus* and its expression in that host. Proof that plasmid pX02 confers on its host the ability to produce capsules was obtained by transferring the plasmid to *B. cereus* 569. To do this we first put the fertility plasmid, pX012, into strain 6602 by mating the donor, *B. anthracis* Weybridge A UM23C2 tr60B-1 Ura<sup>-</sup> (pX01)<sup>-</sup>(pX012)<sup>+</sup>(pBC16)<sup>+</sup>, with strain 6602 and selecting transcipts that were Tc<sup>r</sup> and Cry<sup>+</sup>, i.e., (pBC16)<sup>+</sup> and (pX012)<sup>+</sup>, on Min 1C agar (counterselcting the Ura<sup>-</sup> donor). Such 6602 transcipts were shown to carry the three plasmids, pX02, pBC16, and pX012. One of these transcipts, 6602 tr172B-2 (Fig. 4, lane 3), was mated with *B. cereus* 569 UM20-1 Ant<sup>-</sup>Str<sup>r</sup>, and Tc<sup>r</sup> Str<sup>r</sup> transcipts were selected on NBY agar containing bicarbonate and serum plus tetracycline and streptomycin and incubated in 20% CO<sub>2</sub>. The frequency of Tc<sup>r</sup> transcipts was normal, i.e., about 6 x 10<sup>5</sup> per ml, but only one in 500 of these was mucoid. One of these, *B. cereus* 569 UM20-1 tr49G-4 (Fig. 4, lane 5), was chosen for further study. It was Cap<sup>+</sup> when grown in CO<sub>2</sub>, produced crystals when it was allowed to sporulate, and retained the Ant<sup>-</sup> and Str<sup>r</sup> markers of the original recipient. Capsule production by a *B. cereus* transcipt and comparison of the transcipt with the Pasteur strain, ATCC 6602, are demonstrated in the photographs of Figures 5, 6, 7, and 8.

The two plasmids, pX02 and pX012, separated poorly if at all when lysates of the Cap<sup>+</sup>Cry<sup>+</sup> *B. cereus* transcipt, 569 UM20-1 tr49G-4, were subjected to electrophoresis in agarose gels (Fig.4, lane 5). This is discussed below.

Further studies on transfer of the capsule plasmid, pX02. For further confirmation of the mobilization of pX02 the mating described above was repeated, i.e., *B. anthracis* 6602 tr172B-2 was the donor and *B. cereus* 569 UM20-1 Ant<sup>-</sup>Str<sup>r</sup> was the recipient. Tc<sup>r</sup>Str<sup>r</sup> transcipts were selected on NBY agar supplemented with tetracycline, streptomycin, bicarbonate, and serum and incubated in 20% CO<sub>2</sub>. Five Cap<sup>+</sup> transcipts were isolated and found to produce capsules under the same conditions required for capsule formation by *B. anthracis* strain 6602. All five retained the Ant<sup>-</sup> marker of the *B. cereus* recipient, and all five were Cry<sup>+</sup>. The plasmid content of the five Cap<sup>+</sup>Cry<sup>+</sup> transcipts and three Cap<sup>-</sup>Cry<sup>+</sup> transcipts was examined. The plasmid profiles of four of the five Cap<sup>+</sup> transcipts were identical to the profile of the donor, 569 UM20-1 tr49G-4 (Fig. 9, lane 8), i.e., above the chromosomal DNA there was a single band which migrated to the same position as pX012 (tr60G-6 in

Fig. 9, lane 3). Each of the three  $\text{Cap}^-$  transcipts also had only one band above the chromosome (tr60G-11 in Fig. 9, lane 5), and it migrated to the position of pX012 but was less intense than the corresponding band in the  $\text{Cap}^+$  transcipts. The fifth  $\text{Cap}^+$  transcipt to be analyzed (569 UM20-1 tr60G-10) was different, however, in that no band was present where pX012 migrates, but instead, a plasmid larger than either pX02 or pX012 was present (Fig. 9, lane 4). Presumably it is a cointegrate of pX02 and pX012 since the transcipt is both  $\text{Cap}^+$  and  $\text{Cry}^+$ .

If the large plasmid found in tr60G-10 is a cointegrate of pX02 and pX012, we hypothesized that if its host were used as a donor in the mating system, all transcipts that acquired the plasmid would be both  $\text{Cap}^+$  and  $\text{Cry}^+$ . Therefore, a mating was carried out in which 569 UM20-1 tr60G-10 was the donor and B. anthracis Weybridge A UM23C1-2  $\text{Ura}^- \text{Rif}^r (\text{pX01})^-$  was the recipient.  $\text{Tc}^r \text{Rif}^r$  transcipts were selected on NBY agar supplemented with rifampicin, tetracycline, bicarbonate, and serum and incubated in 20%  $\text{CO}_2$ . Approximately  $1 \times 10^4$  transcipts per ml were found after 6 hours of mating, and 12% of them were  $\text{Cap}^+$ . We examined 17  $\text{Cap}^+$  and 38  $\text{Cap}^-$  transcipts for parasporal crystal production and, as expected, all transcipts which were  $\text{Cap}^+$ , and only those which were  $\text{Cap}^+$ , produced crystals. Thus far, only one  $\text{Cap}^-$  and three  $\text{Cap}^+$  transcipts have been examined for plasmid content. All three  $\text{Cap}^+ \text{Cry}^+$  transcipts contained a plasmid (Fig. 9, lane 7) the same size as the suspected cointegrate plasmid in the donor (Fig. 9, lane 6), and the  $\text{Cap}^-$  transcipt contained no plasmid that migrated above the chromosomal DNA (not shown). These observations are consistent with the interpretation that the large plasmid found in  $\text{Cap}^+ \text{Cry}^+$  transcipts is a cointegrate or recombinant plasmid formed between pX02 and pX012.

A question still remaining is why both pX02 and pX012 can not be visualized in electrophoretic gels of  $\text{Cry}^+ \text{Cap}^+$  B. cereus transcipts such as 569 UM20-1 tr49G-4 discussed above. Let it be recalled that such transcipts appear to contain only one large plasmid migrating slower than chromosomal DNA and it migrates at the same rate as pX012. Because both pX02 and pX012 can be seen in B. anthracis 6602 tr172B-2, which was the original donor for 569 UM20-1 tr49G-4 (Fig. 9, lane 8), we thought that if 569 UM20-1 tr49G-4 were used as the donor in a mating with B. anthracis recipients, the two plasmids might be resolved in the transcipts. The mating was carried out with B. anthracis UM23C1-2  $\text{Ura}^- \text{Rif}^r (\text{pX01})^-$  as the recipient, and  $\text{Tc}^r \text{Rif}^r$  transcipts were selected on NBY

agar supplemented with rifampicin, tetracycline, bicarbonate, and serum and incubated in 20% CO<sub>2</sub>. Nine Cap<sup>+</sup> Cry<sup>+</sup> and four Cap<sup>-</sup> Cry<sup>+</sup> transciipients were examined for their plasmid content. In no instance was there evidence of both pX02 and pX012; all contained only one plasmid band above the chromosomal DNA and it migrated to the same position as pX012. Again, however, when the transciipients were Cap<sup>+</sup> Cry<sup>+</sup> (Fig.9, lane 9), the plasmid band was more intense than the corresponding band in transciipients that were Cap<sup>-</sup> Cry<sup>+</sup> (Fig. 9, lane 10). One possible explanation is that the plasmid in Cap<sup>+</sup> Cry<sup>+</sup> transciipients which gives a more intense band than the plasmid band given by Cap<sup>-</sup> Cry<sup>+</sup> transciipients is a recombinant plasmid made up mostly of pX012 but carrying capsule genes from pX02. Hopefully, we will be able to resolve some of the questions concerning these presumed cointegrate and recombinant plasmids by restriction analyses of the plasmids involved.

TABLE 1. Strains used in this study

| Strain                       | Relevant Characteristics <sup>a</sup>                             | Relevant Plasmids <sup>b</sup> | Origin/reference               |
|------------------------------|-------------------------------------------------------------------|--------------------------------|--------------------------------|
| <u>B. anthracis</u>          |                                                                   |                                |                                |
| M strain                     | Cap <sup>+</sup> in air                                           | pX01, pX02                     | Origin unknown <sup>c</sup>    |
| Pasteur 6602                 | Avirulent, Cap <sup>+</sup> Tox <sup>-</sup>                      | pX02                           | ATCC <sup>d</sup> 6602         |
| Pasteur 6602 R1              | Avirulent, Cap <sup>-</sup>                                       | None                           | This study                     |
| Pasteur 6602 tr172B-2        | Avirulent, Cap <sup>+</sup> Cry <sup>+</sup> Tc <sup>r</sup>      | pX02, pX012, pBC16             | This study                     |
| Weybridge                    | Avirulent, Cap <sup>-</sup> Tox <sup>+</sup>                      | pX01 <sup>b</sup>              | MRE <sup>e</sup>               |
| Weybridge A                  | Colonial variant of Weybridge                                     | pX01                           | C. B. Thorne                   |
| Weybridge A UM2              | Ind <sup>-</sup>                                                  | pX01                           | UV <sup>f</sup> of Weybridge A |
| Weybridge A UM2 tr244-1      | Ind <sup>-</sup> Tc <sup>r</sup> Cry <sup>+</sup>                 | pX01, pX012, pBC16             | This study                     |
| Weybridge A UM17             | Ade <sup>-</sup>                                                  | pX01                           | UV of Weybridge A              |
| Weybridge A UM17 tr57B-6     | Ade <sup>-</sup> Tc <sup>r</sup> Cry <sup>+</sup>                 | pX01, pX012, pBC16             | This study                     |
| Weybridge A UM23             | Ura <sup>-</sup>                                                  | pX01                           | UV of Weybridge A              |
| Weybridge A UM23C1           | Ura <sup>-</sup> , Cured of pX01                                  | None                           | C. B. Thorne                   |
| Weybridge A UM23C1-1         | Ura <sup>-</sup> Str <sup>r</sup>                                 | None                           | UV of A UM23C1                 |
| Weybridge A UM23C1-1 tr47G-6 | Ura <sup>-</sup> Tc <sup>r</sup> Cry <sup>+</sup> PA <sup>+</sup> | See text                       | This study                     |

TABLE 1. (continued)

|                               |                                                                                     |                    |                 |
|-------------------------------|-------------------------------------------------------------------------------------|--------------------|-----------------|
| Weybridge A UM23C1-1 tr47G-34 | Ura <sup>-</sup> Tc <sup>r</sup> Cry <sup>+</sup> PA <sup>+</sup>                   | See text           | This study      |
| Weybridge A UM23C1-1 tr47G-61 | Ura <sup>-</sup> Tc <sup>r</sup> Cry <sup>-</sup> PA <sup>+</sup>                   | See text           | This study      |
| Weybridge A UM23C1-2          | Ura <sup>-</sup> Rif <sup>r</sup>                                                   | None               | UV of A UM23C1  |
| Weybridge A UM23C1-2 tr63G-10 | Ura <sup>-</sup> Tc <sup>r</sup> Rif <sup>r</sup> Cap <sup>+</sup> Cry <sup>+</sup> | See text           | This study      |
| Weybridge A UM23C1-2 tr63G-21 | Ura <sup>-</sup> Tc <sup>r</sup> Rif <sup>r</sup> Cap <sup>-</sup> Cry <sup>+</sup> | See text           | This study      |
| Weybridge A UM23C1-2 tr66G-1  | Ura <sup>-</sup> Tc <sup>r</sup> Rif <sup>r</sup> Cap <sup>+</sup> Cry <sup>+</sup> | See text           | This study      |
| Weybridge A UM23C2            | Ura <sup>-</sup> , cured of pX01                                                    | None               | C. B. Thorne    |
| Weybridge A UM23C2 tr45B-12   | Ura <sup>-</sup> Tc <sup>r</sup> Cry <sup>-</sup>                                   | pX011, pBC16       | This study      |
| Weybridge A UM23C2 tr60B-1    | Ura <sup>-</sup> Tc <sup>r</sup> Cry <sup>+</sup>                                   | pX012, pBC16       | This study      |
| Weybridge A UM23C2 tr96B-3    | Ura <sup>-</sup> Tc <sup>r</sup> Cry <sup>-</sup>                                   | pX011, pBC16       | This study      |
| Weybridge A UM23C2 tr237-10   | Ura <sup>-</sup> Tc <sup>r</sup> Cry <sup>+</sup>                                   | pX012, pBC16       | This study      |
| Weybridge UM44                | Ind <sup>-</sup>                                                                    | pX01               | UV of Weybridge |
| Weybridge UM44-1              | Ind <sup>-</sup> Str <sup>r</sup>                                                   | pX01               | UV of UM44      |
| Weybridge UM44-1 tr84-6       | Ind <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>-</sup>                  | pX01, pX011, pBC16 | This study      |
| Weybridge UM44-1 tr84-7       | Ind <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>-</sup>                  | pX01, pX011, pBC16 | This study      |
| Weybridge UM44-1 tr203-1      | Ind <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>+</sup>                  | pX01, pX012, pBC16 | This study      |
| Weybridge UM44-1 tr203-7      | Ind <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>+</sup>                  | pX01, pX012, pBC16 | This study      |

TABLE 1 (continued)

|                             |                                                                                     |                    |                   |
|-----------------------------|-------------------------------------------------------------------------------------|--------------------|-------------------|
| Weybridge UM44-1 tr203-23   | Ind <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>+</sup>                  | pX01, pX012, pBC16 | This study        |
| Weybridge UM44-1 tr203-28   | Ind <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>-</sup>                  | pX01, pX011, pBC16 | This study        |
| Weybridge UM44-1-C9         | Ind <sup>-</sup> Str <sup>r</sup> , Cured of pX01                                   | None               | This study        |
| Weybridge UM44-1-C9 tr41G-1 | Ind <sup>-</sup> Tc <sup>r</sup> Str <sup>r</sup> Cry <sup>+</sup> PA <sup>+</sup>  | pX01, pX012, pBC16 | This study        |
| Weybridge UM44-1-C9 tr41G-2 | Ind <sup>-</sup> Tc <sup>r</sup> Str <sup>r</sup> Cry <sup>+</sup> PA <sup>+</sup>  | pX01, pX012, pBC16 | This study        |
| <u>B. cereus</u>            |                                                                                     |                    |                   |
| 569                         | wild type                                                                           |                    | NRRL <sup>g</sup> |
| 569 UM20                    | Ant <sup>-</sup>                                                                    |                    | UV of 569         |
| 569 UM20-1                  | Ant <sup>-</sup> Str <sup>r</sup>                                                   | One unnamed        | UV of UM20        |
| 569 UM20-1 tr2B-1           | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>-</sup>                  | pX011, pBC16       | This study        |
| 569 UM20-1 tr2B-3           | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>-</sup>                  | pX011, pBC16       | This study        |
| 569 UM20-1 tr2B-4           | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>-</sup>                  | pX011, pBC16       | This study        |
| 569 UM20-1 tr49G-4          | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>+</sup> Cap <sup>+</sup> | See text           | This study        |
| 569 UM20-1 tr60G-6          | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cap <sup>+</sup> Cry <sup>+</sup> | See text           | This study        |
| 569 UM20-1 tr60G-10         | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cap <sup>+</sup> Cry <sup>+</sup> | See text           | This study        |
| 569 UM20-1 tr60G-11         | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cap <sup>-</sup> Cry <sup>+</sup> | See text           | This study        |
| 569 UM20-1 tr195B-35        | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>+</sup>                  | pX012, pBC16       | This study        |

TABLE 1 (continued)

|                            |                                                                    |                                     |                           |
|----------------------------|--------------------------------------------------------------------|-------------------------------------|---------------------------|
| 569 UM20-1 tr210B-1        | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>-</sup> | pX011, pBC16                        | This study                |
| 569 UM20-1 tr251-1         | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>+</sup> | pX012, pBC16                        | This study                |
| 569 UM20-1 tr251-5         | Ant <sup>-</sup> Str <sup>r</sup> Tc <sup>r</sup> Cry <sup>-</sup> | pBC16                               | This study                |
| 11950                      | Carries bacteriophage Wα                                           |                                     | ATCC                      |
| <u>B. thuringiensis</u>    |                                                                    |                                     |                           |
| 4042A                      | subsp. <u>thuringiensis</u>                                        | pX011, pXC12                        | (1)                       |
| 4042A UM8                  | Ade <sup>-</sup> Cry <sup>+</sup>                                  | pX011, pX012                        | UV of 4042A               |
| 4042A UM8 td2 <sup>h</sup> | Ade <sup>-</sup> Cry <sup>+</sup> Tc <sup>r</sup>                  | pX011, pX012, pBC16                 | C. B. Thorne              |
| 4042A UM8-13               | Ade <sup>-</sup> Cry <sup>-</sup> Osp                              | pX011, pX012                        | C. B. Thorne <sup>i</sup> |
| 4042A UM8-13 td1           | Ade <sup>-</sup> Cry <sup>-</sup> Osp Tc <sup>r</sup>              | pX011, pX012, pBC16                 | C. B. Thorne <sup>i</sup> |
| 4042A UM8-13 td1-A         | Ade <sup>-</sup> Cry <sup>-</sup> Osp Tc <sup>r</sup>              | pX011, (pX012) <sup>-</sup> , pBC16 | C. B. Thorne <sup>i</sup> |

<sup>a</sup> Abbreviations: Ade, adenine; Ant, anthranilic acid; Cap, synthesis of capsule; Cry, synthesis of parasporal crystals; Ind, indole; Osp, oligosporogenous; PA, synthesis of protective antigen; Rif<sup>r</sup>, rifampicin-resistant; Str<sup>r</sup>, streptomycin-resistant; Tc<sup>r</sup>, pBC16-encoded tetracycline resistance; Tox, synthesis of toxin; tr, transcripient; Ura, uracil.

<sup>b</sup> pX01 encodes synthesis of anthrax toxin; pX02 carries genes involved in capsule synthesis; pX011 is a fertility plasmid from B. thuringiensis; pX012 is a fertility plasmid from B. thuringiensis and it encodes parasporal crystal formation; pBC16 encodes resistance to tetracycline.

TABLE 1 (concluded)

<sup>c</sup>The origin of the M strain is unknown. It is a mutant which produces capsules when grown in air in the absence of serum or added bicarbonate.

<sup>d</sup>ATCC, American Type Culture Collection.

<sup>e</sup>MRE, Microbiological Research Establishment, Porton, England.

<sup>f</sup>UV, Mutagenesis by UV light.

<sup>g</sup>NRRL, Agricultural Research Service, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill.

<sup>h</sup>td, Tc<sup>r</sup> transductant obtained by phage CP-51-mediated transfer of pBC16.

<sup>i</sup>Although 4042A UM8-13 was Osp and Cry<sup>-</sup>, it contained pXO12 and could be converted to Spo<sup>+</sup>Cry<sup>+</sup> by phage TP-13 (14). UM8-13 td1, a Tc<sup>r</sup> transductant of UM8-13, could also be converted to Spo<sup>+</sup>Cry<sup>+</sup>. Following frequent transfers of UM8-13 td1 on L-agar slants it was found to have lost pXO12 and, consequently, could not be converted to Cry<sup>+</sup> by TP-13. The (pXO12)<sup>-</sup> derivative is designated UM8-13 td1-A. Freeze dried preparations of UM8-13 td1 retained pXO12.

TABLE 2. Effectiveness of B. anthracis and B. cereus transplants as donors  
in the transfer of pBC16<sup>a</sup>

| Donor                                              | Tc <sup>r</sup> transplants when mated with |                        |                       |                        |
|----------------------------------------------------|---------------------------------------------|------------------------|-----------------------|------------------------|
|                                                    | <u>B. anthracis</u>                         |                        | <u>B. cereus</u>      |                        |
|                                                    | No. per ml                                  | Frequency              | No. per ml            | Frequency              |
| Primary Transplants                                |                                             |                        |                       |                        |
| <u>B. anthracis</u> Weybridge                      |                                             |                        |                       |                        |
| UM44-1 tr84-6(pX01,pX011,pBC16) Cry <sup>-</sup>   | 4.9 x 10 <sup>2</sup>                       | 1.1 x 10 <sup>-5</sup> | 1.5 x 10 <sup>2</sup> | 3.5 x 10 <sup>-6</sup> |
| UM44-1 tr203-1(pX01,pX012,pBC16) Cry <sup>+</sup>  | 2.7 x 10 <sup>5</sup>                       | 6.3 x 10 <sup>-3</sup> | 1.0 x 10 <sup>7</sup> | 2.3 x 10 <sup>-1</sup> |
| UM44-1 tr203-7(pX01,pX012,pBC16) Cry <sup>+</sup>  | 1.3 x 10 <sup>5</sup>                       | 3.0 x 10 <sup>-3</sup> | 7.5 x 10 <sup>6</sup> | 1.7 x 10 <sup>-1</sup> |
| UM44-1 tr203-23(pX01,pX012,pBC16) Cry <sup>+</sup> | 1.2 x 10 <sup>5</sup>                       | 2.8 x 10 <sup>-3</sup> | 2.6 x 10 <sup>7</sup> | 6.0 x 10 <sup>-1</sup> |
| UM44-1 tr203-28(pX01,pX011,pBC16) Cry <sup>-</sup> | 5.0 x 10 <sup>2</sup>                       | 1.2 x 10 <sup>-5</sup> | 4.2 x 10 <sup>2</sup> | 9.8 x 10 <sup>-6</sup> |
| <u>B. cereus</u> 569                               |                                             |                        |                       |                        |
| UM20-1 tr2B-1(pX011,pBC16) Cry <sup>-</sup>        | 3.5 x 10 <sup>1</sup>                       | 8.1 x 10 <sup>-7</sup> |                       |                        |
| UM20-1 tr2B-3(pX011,pBC16) Cry <sup>-</sup>        | 4.5 x 10 <sup>1</sup>                       | 1.0 x 10 <sup>-6</sup> |                       |                        |
| UM20-1 tr2B-4(pX011,pBC16) Cry <sup>-</sup>        | 2.0 x 10 <sup>1</sup>                       | 4.7 x 10 <sup>-7</sup> |                       |                        |

Table 2 (continued)

## Secondary Transcipients

B. anthracis Weybridge

|                                                                       |                       |                        |                       |                        |
|-----------------------------------------------------------------------|-----------------------|------------------------|-----------------------|------------------------|
| UM17 tr57B-6(pX01, pX012, pBC16) Cry <sup>+</sup>                     | 1.0 x 10 <sup>5</sup> | 2.3 x 10 <sup>-3</sup> | 2.1 x 10 <sup>6</sup> | 4.9 x 10 <sup>-2</sup> |
| UM23C2 tr45B-12(pX011, pBC16)<br>(pX01) <sup>-</sup> Cry <sup>-</sup> | 6.7 x 10 <sup>2</sup> | 1.6 x 10 <sup>-5</sup> | 2.7 x 10 <sup>2</sup> | 6.3 x 10 <sup>-6</sup> |
| UM23C2 tr60B-1(pX012, pBC16)<br>(pX01) <sup>-</sup> Cry <sup>+</sup>  | 8.0 x 10 <sup>4</sup> | 1.9 x 10 <sup>-3</sup> | 2.0 x 10 <sup>6</sup> | 4.7 x 10 <sup>-2</sup> |
| UM23C2 tr96B-3(pX011, pBC16)<br>(pX01) <sup>-</sup> Cry <sup>-</sup>  | 8.8 x 10 <sup>2</sup> | 2.0 x 10 <sup>-5</sup> | 5.2 x 10 <sup>2</sup> | 1.2 x 10 <sup>-5</sup> |
| UM23C2 tr237-10(pX012, pBC16)<br>(pX01) <sup>-</sup> Cry <sup>+</sup> | 7.9 x 10 <sup>5</sup> | 1.8 x 10 <sup>-2</sup> | 3.5 x 10 <sup>7</sup> | 8.1 x 10 <sup>-1</sup> |

B. cereus 569

|                                                |                       |                        |                       |                        |
|------------------------------------------------|-----------------------|------------------------|-----------------------|------------------------|
| UM20-1 tr210B-1(pX011, pBC16) Cry <sup>-</sup> | 7.1 x 10 <sup>1</sup> | 1.6 x 10 <sup>-7</sup> | 3.5 x 10 <sup>1</sup> | 8.0 x 10 <sup>-8</sup> |
| UM20-1 tr251-1(pX012, pBC16) Cry <sup>+</sup>  | 1.3 x 10 <sup>5</sup> | 3.0 x 10 <sup>-4</sup> | 8.6 x 10 <sup>5</sup> | 2.0 x 10 <sup>-3</sup> |
| UM20-1 tr251-5(pBC16) Cry <sup>-</sup>         | 0                     | 0                      | 0                     | 0                      |

<sup>a</sup>To permit selection and identification of Tc<sup>r</sup> transcipients we used B. cereus and B. anthracis recipients which had auxotrophic requirements different from those of the respective donors. Mating mixtures were incubated 20 hours. The average number of B. anthracis donors per ml was  $4.3 \times 10^7$  CFU and that of B. cereus donors was  $4.4 \times 10^8$  CFU.

TABLE 3. High frequency transfer to B. cereus of a presumed cointegrate plasmid carrying information for protective antigen synthesis<sup>a</sup>

| Mating mixture No. | Donor                                                          | Tc <sup>r</sup> transplants |                                 |
|--------------------|----------------------------------------------------------------|-----------------------------|---------------------------------|
|                    |                                                                | No. per ml                  | No. PA <sup>+</sup> /No. tested |
| 72G                | Weybridge A UM23C1-1 tr47G-6 PA <sup>+</sup> Cry <sup>+</sup>  | 1.4 x 10 <sup>6</sup>       | Not tested                      |
| 73G                | Weybridge A UM23C1-1 tr47G-34 PA <sup>+</sup> Cry <sup>+</sup> | 1.6 x 10 <sup>6</sup>       | 35/62 (56%)                     |
| 74G                | Weybridge A UM23C1-1 tr47G-61 PA <sup>+</sup> Cry <sup>-</sup> | 0                           |                                 |

<sup>a</sup>The recipient was B. cereus 569 UM20-1 Ant<sup>-</sup>Str<sup>r</sup>

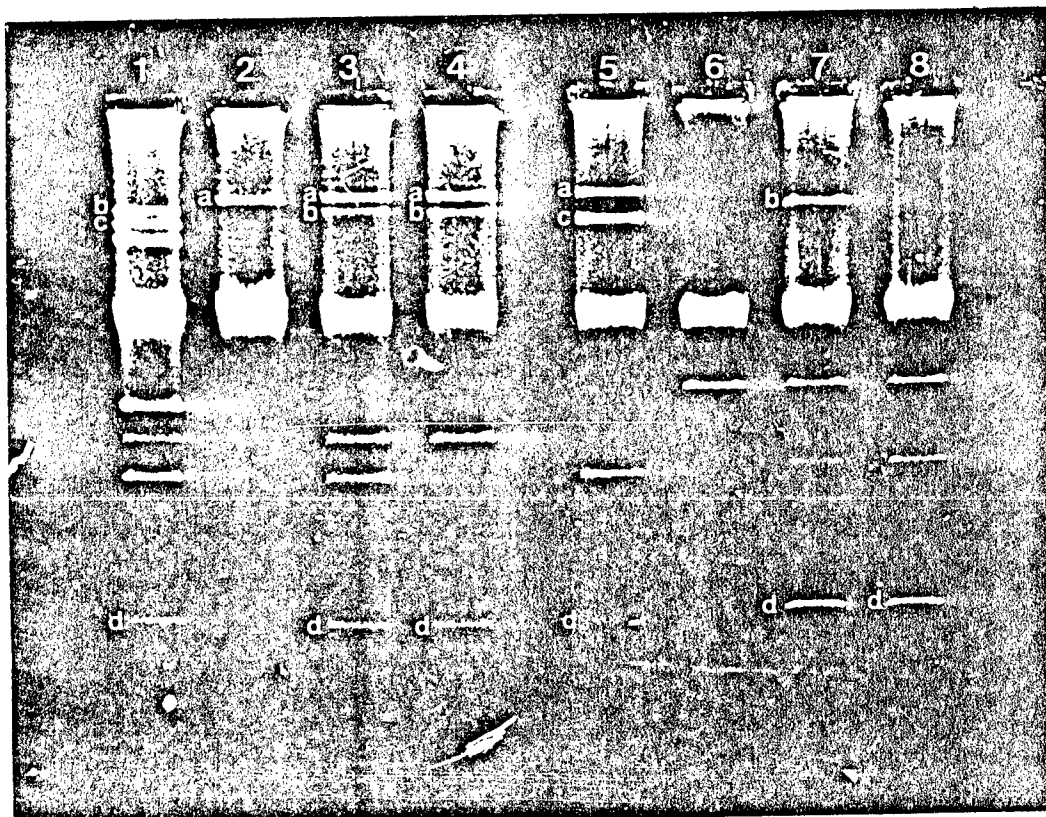


FIG. 1. Agarose gel electrophoresis of plasmid DNA from a B. thuringiensis donor and B. anthracis and B. cereus recipients and transcipts. Plasmids are labeled as follows: (a) pX01; (b) pX012; (c) pX011; (d) pBC16. The large diffuse band in all lanes is chromosomal DNA. Lanes: 1, B. thuringiensis 4042A UM8 td2, Cry<sup>+</sup> donor; 2, B. anthracis Weybridge UM44-1, recipient; 3, Weybridge UM44-1 tr203-1, Cry<sup>+</sup> transcient; 4, Weybridge UM44-1 tr203-7, Cry<sup>+</sup> transcient; 5, Weybridge UM44-1 tr203-28, Cry<sup>-</sup> transcient; 6, B. cereus 569 UM20-1, recipient; 7, 569 UM20-1 tr251-1, Cry<sup>+</sup> transcient; 8, 569 UM20-1 tr251-5, Cry<sup>-</sup> transcient.

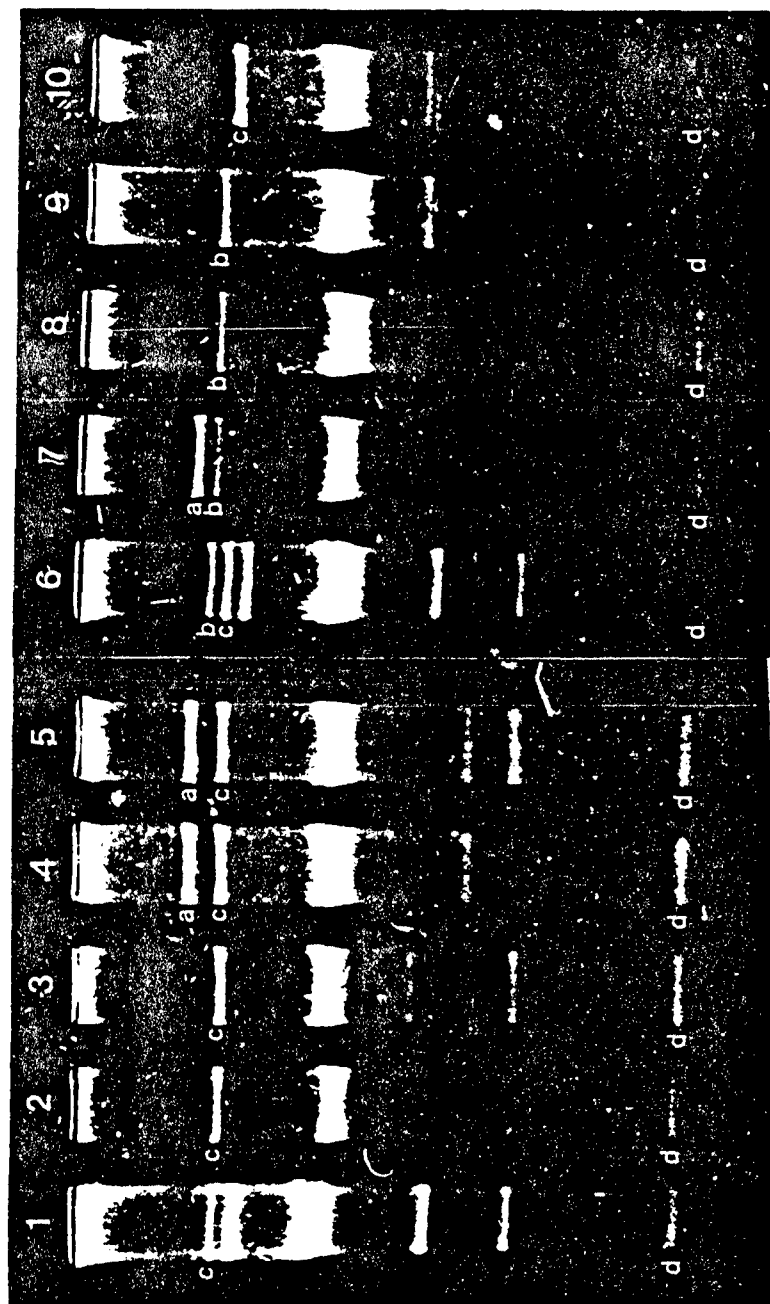


FIG. 2. Agarose gel electrophoresis of plasmid DNA from B. thuringiensis donor strains and B. anthracis and B. cereus transcipts. Plasmid designations are the same as in FIG. 1. Lanes: 1, B. thuringiensis 4042A UM8-13 td1-A, Cry<sup>-</sup> donor; 2, B. cereus 569 UM20-1 tr2B-3, Cry<sup>-</sup> transcipt; 3, 569 UM20-1 tr2B-1, Cry<sup>-</sup> transcipt; 4, B. anthracis Weybridge UM44-1 tr84-6. Cry<sup>-</sup> transcipt; 5, Weybridge UM44-1 tr84-7, Cry<sup>-</sup> transcipt; 6, B. thuringiensis 4042A M8 td2, Cry<sup>+</sup> donor; 7, B. anthracis Weybridge UM44-1 tr203-23, Cry<sup>+</sup> transcipt; 8, Weybridge A UM23C2 tr237-10, Cry<sup>+</sup> transcipt; 9, B. cereus 569 UM20-1 tr195B-35, Cry<sup>+</sup> transcipt; 10, 569 UM20-1 tr2B-4, Cry<sup>-</sup> transcipt. The very faint bands below chromosomal DNA are polymeric forms of pBC16.

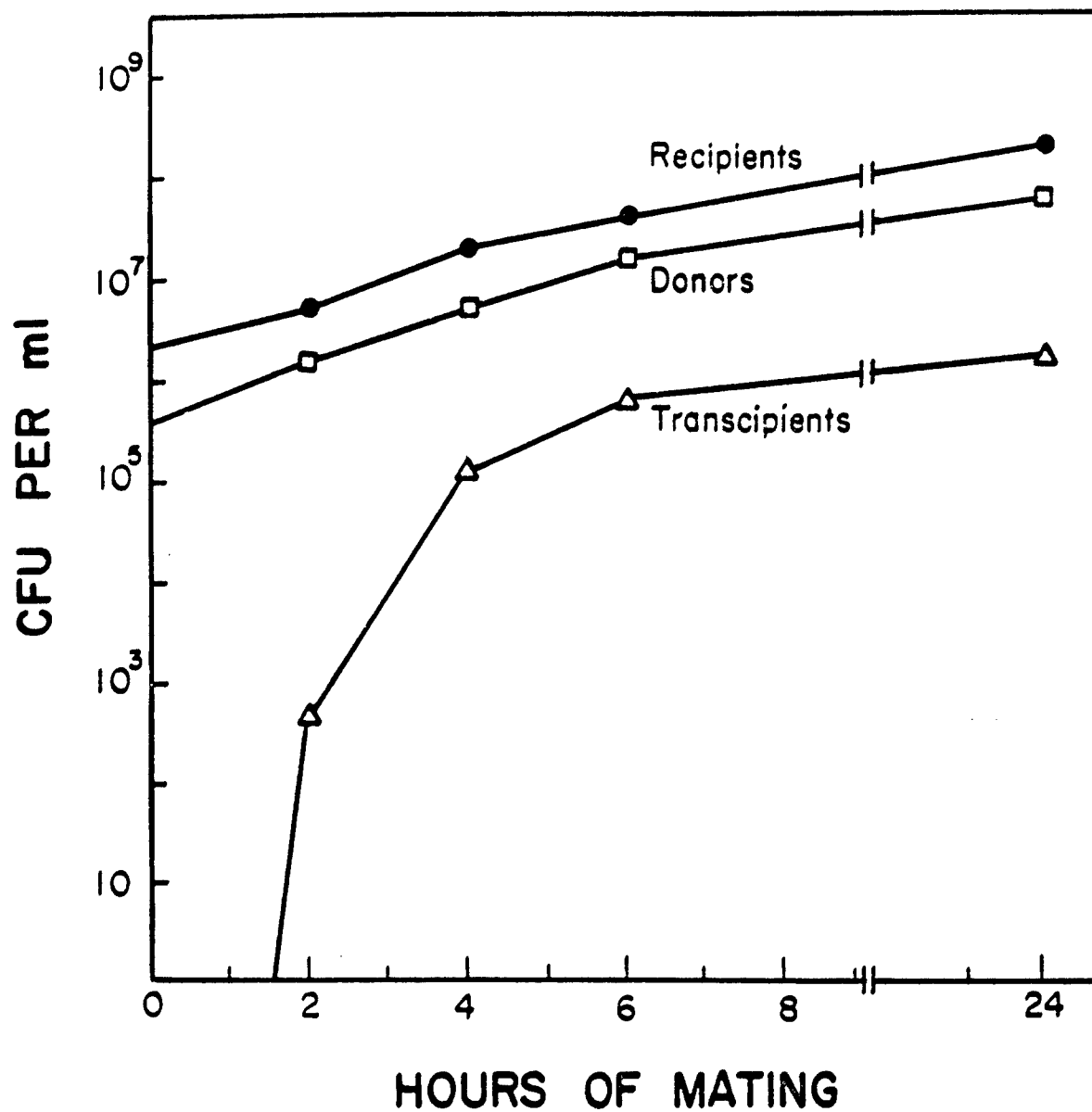


FIG. 3. Transfer of pBC16 from *B. anthracis* Weybridge A UM23C2 tr237-10(pX012, pBC16) Ura<sup>-</sup>Tc<sup>R</sup>Cry<sup>+</sup> to *B. anthracis* Weybridge UM44-1 Ind<sup>-</sup>Str<sup>R</sup>. At the indicated times samples were plated on L-agar containing tetracycline or streptomycin or both antibiotics to score donors, recipients, and transcipients, respectively.

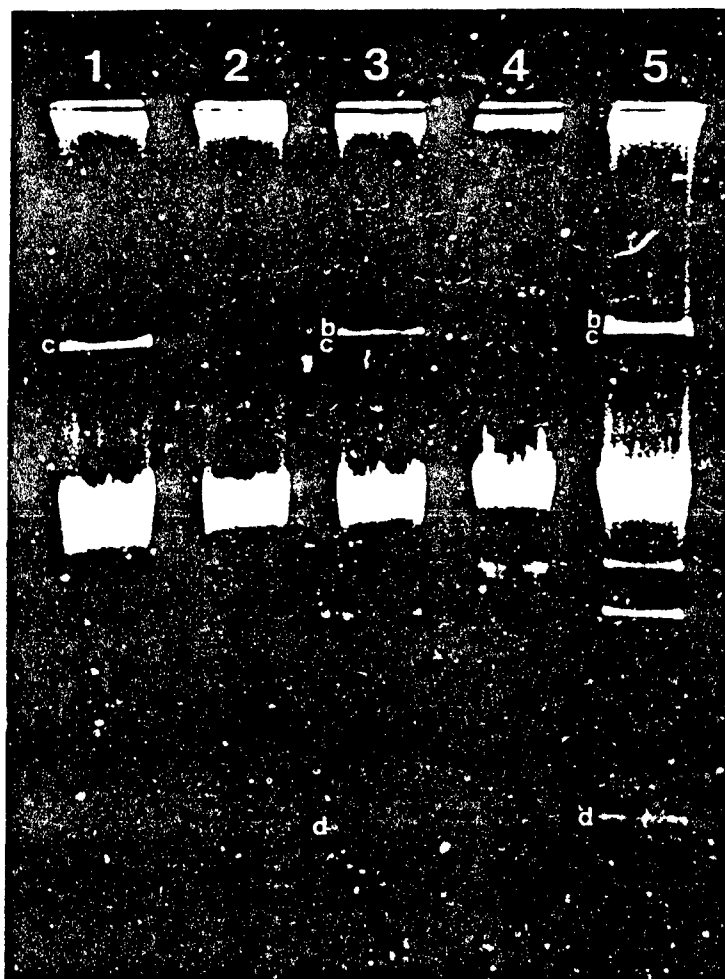


FIG. 4. Agarose gel electrophoresis of plasmid DNA from B. anthracis and B. cereus, demonstrating pX02 and its transfer to B. cereus. Lane 1, B. anthracis 6602 wild type; Lane 2, B. anthracis 6602 R1 (rough variant); Lane 3, B. anthracis 6602 tr172B-2, a transipient carrying pX02, pX012, and pBC16; Lane 4, B. cereus 569 UM20-1; Lane 5, B. cereus 569 UM20-1 tr49G-4, a transipient carrying pX02, pX012, and pBC16. Plasmids are labeled as follows: (b) pX012; (c) pX02; (d) pBC16.

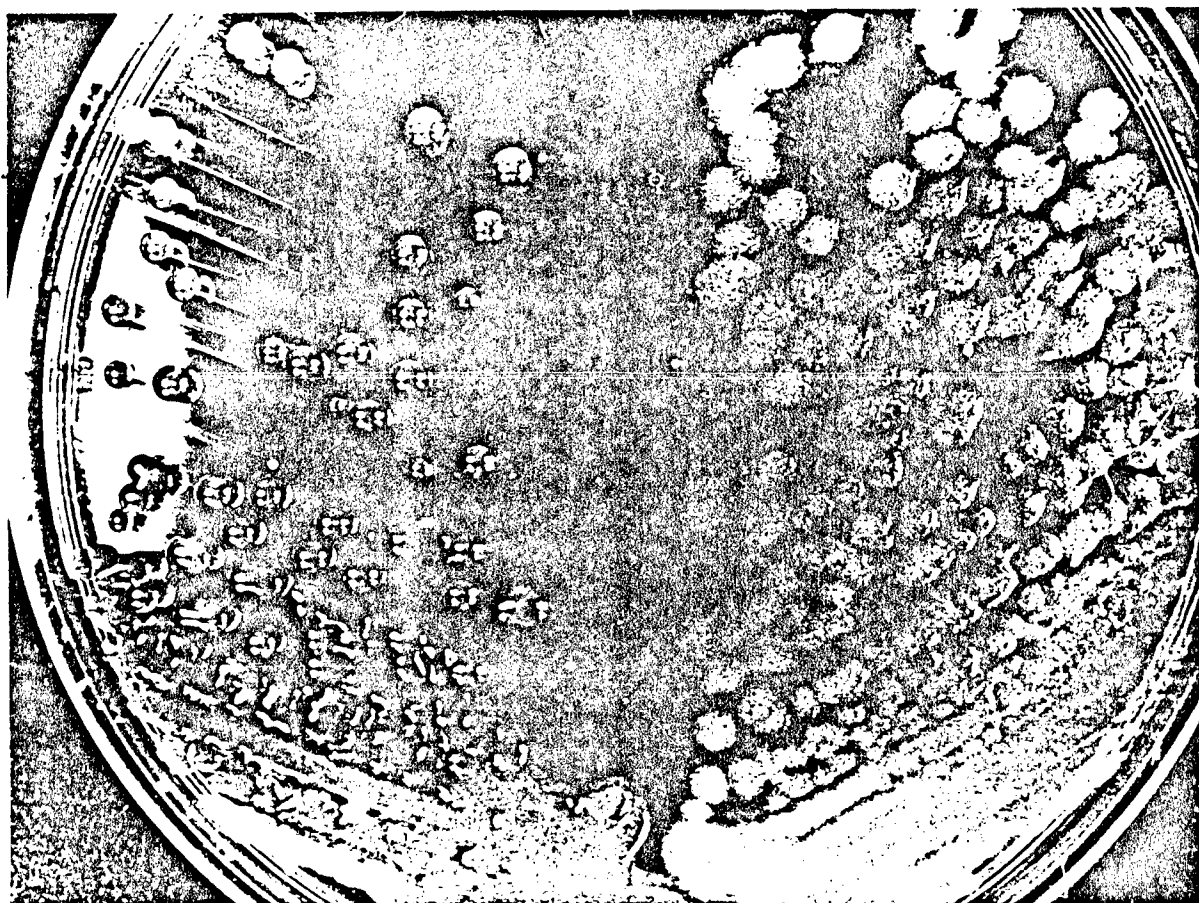


FIG. 5. Colonies of B. anthracis Pasteur strain grown on bicarbonate agar in 20% CO<sub>2</sub>. Left, strain 6602 wild type. Right, 6602 R1 (rough variant of 6602).



FIG. 6. Phase-contrast photomicrograph of cells of B. anthracis Pasteur strain grown on bicarbonate agar in 20% CO<sub>2</sub>. Left, strain 6602 wild type. Right, 6602 R1 (rough variant of 6602).

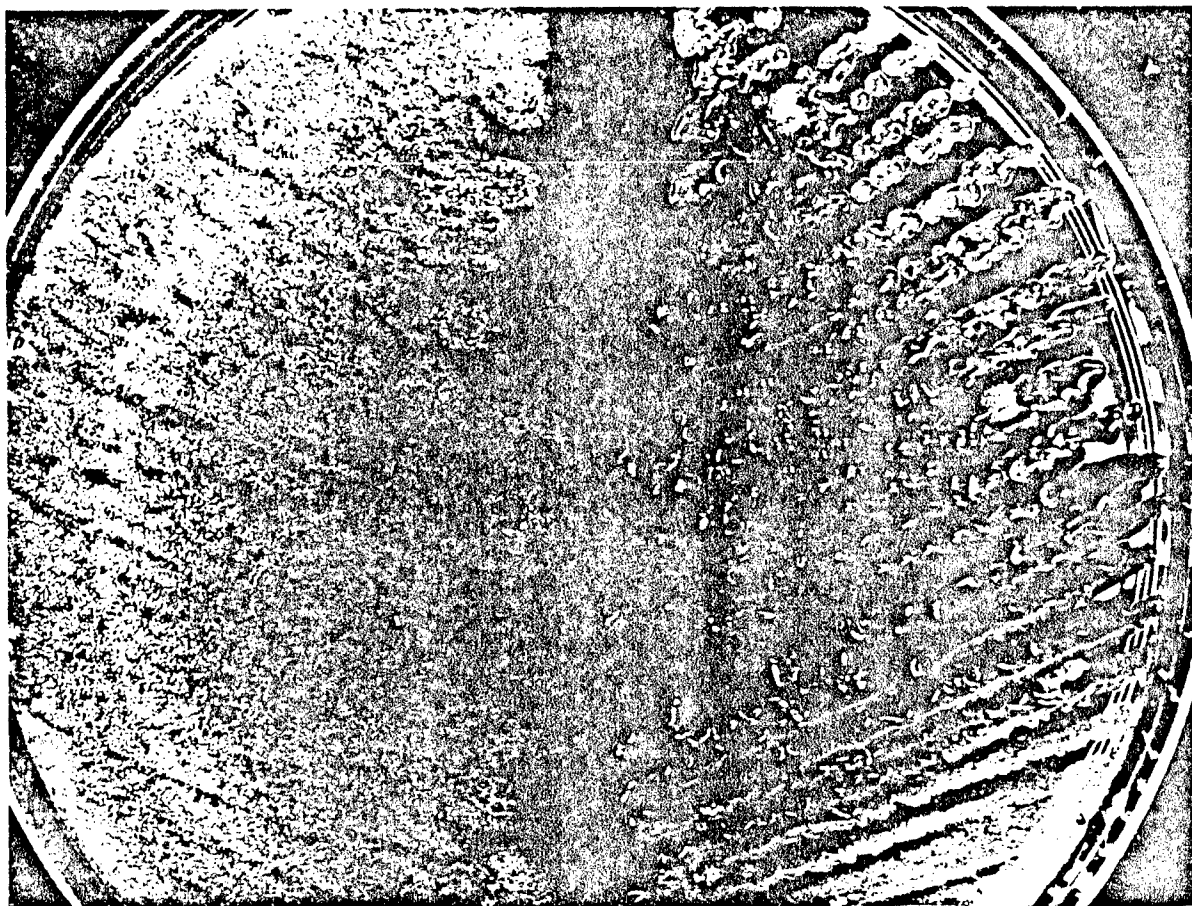


FIG. 7. Colonies of B. cereus grown on bicarbonate agar in 20% CO<sub>2</sub>. Left, B. cereus 569 UM20-1. Right, B. cereus 569 UM20-1 tr49G-4, a Cap<sup>+</sup> transcripient carrying pX02.



FIG. 8. Phase-contrast photomicrograph of cells of B. cereus grown on bicarbonate agar in 20% CO<sub>2</sub>. Left, B. cereus 569 UM20-1 Cap<sup>-</sup>. Right, B. cereus 569 UM20-1 tr49G-4, a Cap<sup>+</sup> transciptient carrying pX02.

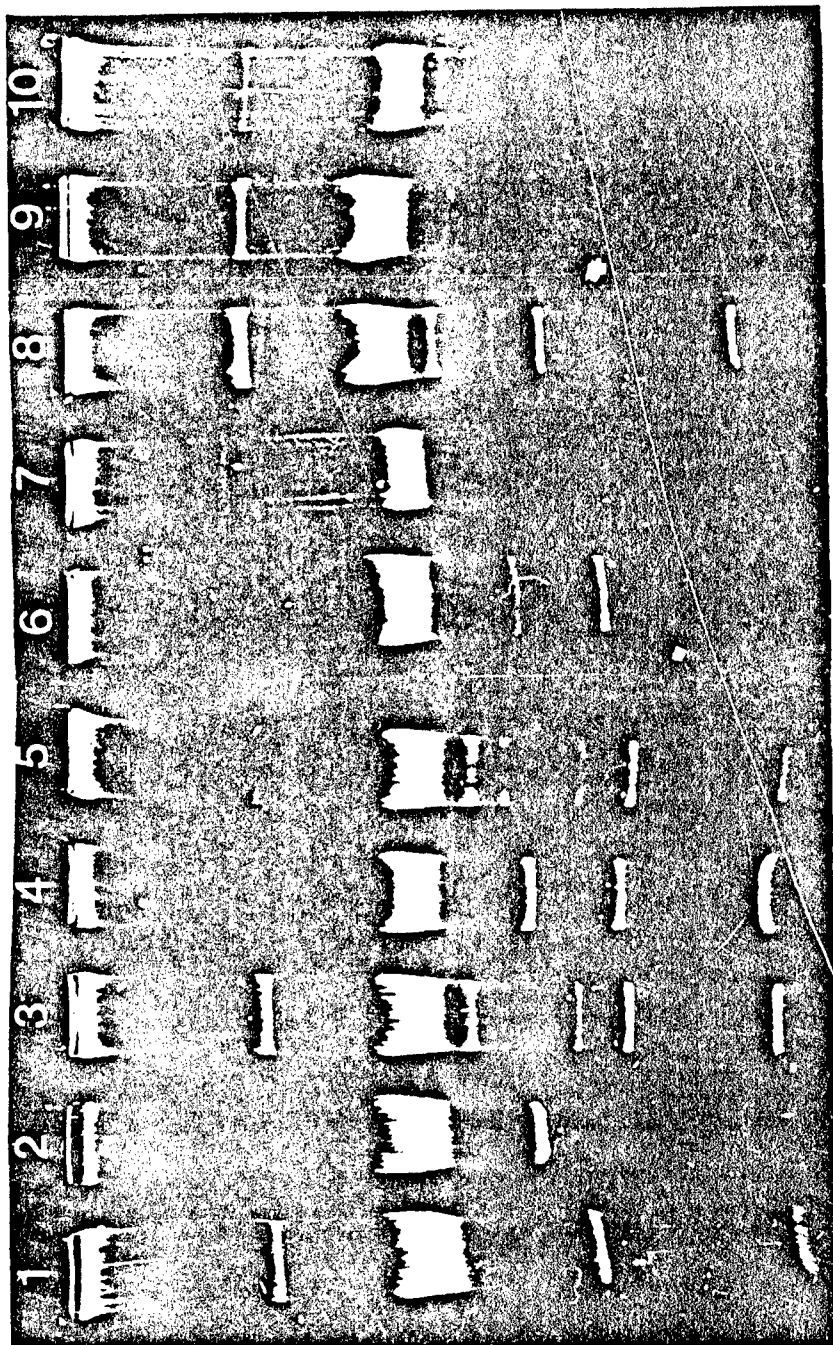


FIG. 9. Agarose gel electrophoresis of  $\text{Cap}^+$  and  $\text{Cap}^-$  donors, recipients, and transipients. Lane 1, B. anthracis 6602 tr172B-2  $\text{Cap}^+\text{Cry}^+\text{Tc}^{\text{r}}$ ; Lane 2, B. cereus 569 UM20-1; Lane 3, B. cereus 569 UM20-1 tr60G-6  $\text{Cap}^+\text{Cry}^+\text{Tc}^{\text{r}}$ ; Lane 4, B. cereus 569 UM20-1 tr60G-10  $\text{Cap}^+\text{Cry}^+\text{Tc}^{\text{r}}$ ; Lane 5, B. cereus 569 UM20-1 tr60G-11  $\text{Cap}^-\text{Cry}^+\text{Tc}^{\text{r}}$ ; Lane 6, B. cereus 569 UM20-1 tr60G-10  $\text{Cap}^+\text{Cry}^+\text{Tc}^{\text{r}}$  (same as lane 4); Lane 7, B. anthracis UM23C1-2 tr66G-1  $\text{Cap}^+\text{Cry}^+\text{Tc}^{\text{r}}$ ; Lane 8, B. cereus 569 UM20-1 tr49G-4  $\text{Cap}^+\text{Cry}^+\text{Tc}^{\text{r}}$ ; Lane 9, B. anthracis UM23C1-2 tr63G-10  $\text{Cap}^+\text{Cry}^+\text{Tc}^{\text{r}}$ ; Lane 10, B. anthracis UM23C1-2 tr63G-21  $\text{Cap}^-\text{Cry}^+\text{Tc}^{\text{r}}$ .

#### ABSTRACTS OF PAPERS TO BE PRESENTED

Two papers will be presented at the March 1985 meeting of the American Society for Microbiology. Abstracts of the two papers are included here.

##### Interspecies Plasmid Transfer during Bacillus Matings

Laurie Battisti, B. D. Green, and C. B. Thorne

A mating system has been developed which promotes plasmid transfer among the three Bacillus species, B. thuringiensis, B. anthracis, and B. cereus. Transfer of the selectable tetracycline resistance plasmid, pBC16, and other resident plasmids from B. thuringiensis donors to B. cereus and B. anthracis recipients occurred during mixed incubation in broth cultures. Plasmid transfer was resistant to DNase, and cell-free filtrates of donors were ineffective. Two plasmids, pX011 and pX012 from B. thuringiensis subsp. thuringiensis, were responsible for plasmid mobilization. Transcipients harboring either pX011 or pX012 were, in turn, effective donors. Phase microscopy revealed that transcipients inheriting pX012 had also acquired the ability to produce parasporal crystals (Cry<sup>+</sup>) resembling those produced by B. thuringiensis donors, while those inheriting pX011 were Cry<sup>-</sup>. This plasmid exchange system has been useful in the analysis of genetic determinants residing on the B. anthracis toxin plasmid, pX01 (112 megadaltons). B. anthracis transcipients inheriting pX012 were capable of transferring pX01 to genetically marked B. anthracis recipients that had been previously cured of pX01. Thus, a mating system has been developed which promotes interspecies transfer of numerous Bacillus plasmids by a conjugation-like process.

Demonstration of a Capsule Plasmid in Bacillus anthracis

Brian D. Green, L. Battisti, and C. B. Thorne

Virulence of Bacillus anthracis is attributed to two factors: a tripartite exotoxin and a capsular polypeptide. It has been demonstrated previously that a 112-Mdal plasmid, pX01, encodes the structural genes for toxin synthesis. We investigated whether a 60-Mdal plasmid, pX02, present in virulent and avirulent strains capable of producing capsules, was involved in the synthesis of the capsule. B. anthracis Pasteur vaccine strain (ATCC 6602), an avirulent strain cured of pX01 and unable to produce toxin, forms capsules when grown on agar containing bicarbonate and/or serum and incubated in 20% CO<sub>2</sub>. Analysis of the plasmid content of rough noncapsulated variants of this strain revealed two classes: those which were cured of pX02 and contained no detectable plasmid DNA, and those which still carried pX02. Reversion to Cap<sup>+</sup> was demonstrable only in rough variants which harbored pX02. By means of the Bacillus mating system in which plasmid transfer is mediated by the fertility plasmid, pX012, pX02 was transferred from B. anthracis 6602 to B. cereus 569R M20 Ant<sup>-</sup> str-2. A B. cereus transcient which acquired pX02 and retained the Ant<sup>-</sup> and Str<sup>r</sup> markers of the recipient produced capsules under the same conditions required for capsule formation by B. anthracis. This confirms that pX02 carries information required for the synthesis of capsules by B. anthracis.

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The following papers are in press or have been submitted for publication:

1. Thorne, C. B. Genetics of Bacillus anthracis. In Microbiology, 1985.  
American Society for Microbiology. In press.
2. Green, B. D., L. Battisti, C. B. Thorne, and B. E. Ivins. Demonstration of a capsule plasmid in Bacillus anthracis. Submitted for publication.
3. Battisti, L., B. D. Green, and C. B. Thorne. A mating system for transfer of plasmids among Bacillus anthracis, B. cereus, and B. thuringiensis.  
Submitted for publication.

The following Ph. D. dissertation was written on research carried out under this contract:

Robillard, Norman J. Changes associated with plasmid loss in Bacillus anthracis. Ph. D. Dissertation. University of Massachusetts. September 1984.

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