Involvement of Tn4430 in Transfer of Bacillus anthracis Plasmids Mediated by Bacillus thuringiensis Plasmid pXO12

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The self-transmissible plasmid pXO12 (112.5 kilobases [kb]), originally isolated from strain 4042A of Bacillus thuringiensis subsp. thuringiensis, codes for production of the insecticidal crystal protein (Cry'). The mechanism of pXO12-mediated plasmid transfer was investigated by monitoring the cotransfer of the tetracycline resistance plasmid pBC16 (4.2 kb) and the Bacillus anthracis toxin and capsule plasmids, pXO1 (168 kb) and pXO2 (85.6 kb), respectively. In matings of B. anthracis donors with B. anthracis and Bacillus cereus recipients, the number of Tc' transipients ranged from 4.8 × 10^4 to 3.9 × 10^6/ml (frequencies ranged from 1.6 × 10^-4 to 7.3 × 10^-2), and 0.3 to 0.4% of them simultaneously inherited pXO1 or pXO2. Physical analysis of the transferred plasmids suggested that pBC16 was transferred by the process of donation and that the large B. anthracis plasmids were transferred by the process of conduction. The transfer of pXO1 and pXO2 involved the transposition of Tn4430 from pXO1 onto these plasmids. DNA-DNA hybridization experiments demonstrated that Tn4430 was located on a 16.0-kb AvaI fragment of pXO12. Examination of Tra' and Cry' derivatives of pXO12 showed that this fragment also harbored information involved in crystal formation and was adjacent to a restriction fragment containing DNA sequences carrying information required for conjugal transfer.

Recent reports from our laboratory have concerned the identification and characterization of six self-transmissible plasmids from five subspecies of Bacillus thuringiensis (1, 19). One of these plasmids, designated pXO12 (112.5 kilobases [kb]), was isolated from strain 4042A of Bacillus thuringiensis subsp. thuringiensis. Plasmid pXO12 is capable of mediating its own transfer as well as that of a large range of Bacillus plasmids among strains of Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis. In addition to conjugal transfer functions, pXO12 also encodes production of the insecticidal toxin known as the endotoxin or the parasporal crystal (Cry').

The widespread occurrence of large self-transmissible plasmids among B. thuringiensis strains suggests that conjugation may be an important means of plasmid dissemination in naturally occurring Bacillus populations (4). In the laboratory, this mating system has provided us with an efficient method of shuttling a wide range of plasmids among B. thuringiensis, B. cereus and B. anthracis. For example, the utility of this conjugation system in the genetic analysis of the B. anthracis toxin and capsule plasmids, pXO1 and pXO2, respectively, has been well documented (1, 6, 19, 24).

Prior to the development of this mating system, the most reliable system available for transferring plasmids among these three Bacillus species was transduction (21). Therefore, the importance of this system as an additional means of genetic exchange among these organisms warrants further genetic and physical analysis of these conjugative plasmids. We have been interested in determining the mechanism of pXO12-mediated transfer of both large and small plasmids. To this end, we have monitored the transfer of pBC16 (4.2 kb) and the B. anthracis toxin and capsule plasmids, pXO1 (168 kb) and pXO2 (85.6 kb), respectively.

Physical analysis of the transferred plasmids suggested that pBC16 was transferred by the process of donation, whereas the large B. anthracis plasmids were transferred by the process of conduction. Recent reports (12, 15) of the occurrence of the 4,149-base-pair transposon Tn4430 in several strains of B. thuringiensis, including B. thuringiensis subsp. thuringiensis, prompted us to investigate whether it was involved in pXO12-mediated conduction of large plasmids. Consequently, we have found that transfer of pXO1 and pXO2 does involve transposition of Tn4430 from pXO12 onto these plasmids. DNA-DNA hybridization experiments demonstrated that Tn4430 was located on a 16.0-kb AvaI fragment of pXO12. Examination of Tra' and Cry' derivatives of pXO12 showed that this fragment also harbored information involved in crystal formation and was close to DNA sequences involved in conjugal transfer ability. Thus, it appears that the aforementioned genetic elements are located close together on pXO12.

MATERIALS AND METHODS

Bacterial strains. The strains of B. anthracis and B. cereus used in this study are listed in Table 1. Escherichia coli JM83(pHT44), provided by M.-M. Lecadet, was used as a source of Tn4430 DNA. Plasmid pHT44 contained one copy of Tn4430 with 2 base pairs deleted at each end and was constructed by inserting the internal 4.2-kb KpnI fragment of Tn4430 into the KpnI site of vector plasmid pUC18 (12). Bacillus subtilis PSL1 (strain AS10 of the Bacillus Genetic Stock Center, Ohio State University) was transformed with the transposition selection vector pTV1 (27), provided by P. Youngman, and the transformant was used as a source of Tn917 (25).

Media. L broth, brain heart infusion broth, NBY medium, and the minimal medium Min IC agar were prepared as described previously (1, 6). Casamino Acids agarose me-
### Table 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics of the strain or plasmid | Origin or reference |
|-------------------|-----------------------------------------------|----------------------|
| **B. anthracis**  |                                               |                      |
| Weybridge         | Avirulent, Tox+ Cap* pXO1                     | 24                   |
| Weybridge UM44-1  | Ind- Tox+ Str+, pXO1                          |                      |
| Weybridge UM44-2  | Ind- Tox+ Str- pXO1                           |                      |
| Weybridge UM44-4  | Ind- Tox+ Str+ Cry+ Tra+ Te+, pXO1, pXO12, pBC16 |                      |
| Weybridge UM44-5  | Ind- Tox+ Str+ Cry+ Tra+, pXO12-4, pXO1, pBC16 |                      |
| Weybridge UM44-6  | Ind- Tox+ Str+ Cry+ Te+, pXO1-3, pXO12, pBC16  |                      |
| Weybridge UM44-7  | Ind- Tox+ Str+ Cry- pXO1-2, pXO12, pBC16       |                      |
| Weybridge UM44-8  | Ind- Tox+ Str- Cry+ Te+, pXO1-4, pXO12, pBC16  |                      |
| Weybridge UM44-9  | Ind- Tox+ Str+ Cry+ Te+, pXO11, pXO12, pBC16   |                      |
| Weybridge UM44-10 | Ind- Tox+ Str+ Cap+ MLS+ Te+, pXO2-8, pXO12, pBC16 |                      |
| Weybridge A       | Colony variant of Weybridge, Tox+ pXO1        | 24                   |
| Weybridge A UM2   | Trp-, Tox+, pXO1                              |                      |
| Weybridge A UM2-3 | Trp- Tox+ Cry+ Te-, pXO1-1, pXO12, pBC16       |                      |
| Weybridge A UM2-4 | Trp- Tox+ Cry-, pXO1-1, pXO12, pBC16           |                      |
| Weybridge A UM7   | Ade- Tox+, pXO1                               |                      |
| Weybridge A UM17  | Ade- Tox- Cry+ Te-, pXO1, pXO12, pBC16         |                      |
| Weybridge A UM17-3| Ade- Tox- Cry- Te-, pXO1, pXO12, pBC16         |                      |
| Weybridge A UM18-5| pyrA1 Tox- Str-, pXO1                         |                      |
| Weybridge A UM18-6| pyrA1 Tox- Str+ Cry- Tra+ Te-, pXO1, pXO12-3, pBC16 |                      |
| Weybridge A UM18-7| pyrA1 Tox- Str+ Cry- Tra-, pXO12-3            |                      |
| Weybridge A UM23-3| Ura- Tox+ Str-, pXO1                           |                      |
| Weybridge A UM23-4| Ura- Tox- Rif+ pXO1                           |                      |
| Weybridge A UM23-5| Ura- Tox+ Rif+ MLS- Tra-, pXO503               |                      |
| Weybridge A UM23-6| Ura- Tox- Cap+ Str+, pXO2-1                   |                      |
| Weybridge A UM23-7| Ura- Tox- Cap+ Str-, pXO2-2                   |                      |
| Weybridge A UM23-8| Ura- Tox- Rif+ Cry+ Te-, pXO12, pBC16         |                      |
| Weybridge A UM23-9| Ura- Tox+ Rif+ Te-, pXO1-3, pBC16             |                      |
| Weybridge A UM23-10| Ura- Tox+ Rif-, pXO1-3, pBC16                 |                      |
| Weybridge A UM23-11| Ura- Tox+ Rif+ Cry+ Te-, pXO1-5, pXO12, pBC16 |                      |
| Weybridge A UM23-12| Ura- Tox+ Rif+ Cry- Te-, pXO11-3, pXO12, pBC16 |                      |
| Weybridge A UM23-13| Ura- Tox- Rif+ Cry- Te-, pXO1-6, pXO12, pBC16  |                      |
| Weybridge A UM23-14| Ura- Tox- Rif+ Cry+ Te-, pXO1-7, pXO12, pBC16  |                      |
| Weybridge A UM23-15| Ura- Tox+ Rif+ Te-, pXO1, pXO12, pBC16         |                      |
| Weybridge A UM23-16| Ura- Tox- Rif+ Cry+ Te MLS-, pXO12, pXO503, pBC16 |                      |
| Weybridge A UM23-18| Ura- Tox- Rif+ Cry- Tra-, MLS-, pXO12-1        |                      |
| Weybridge A UM23-20| Ura- Tox+ Cry- Tra+ Te-, pXO12, pBC16         |                      |
| Weybridge A UM23-21| Ura- Tox- Cry+ Tra+ Te-, pXO12, pBC16         |                      |
| 4229 (Pasteur)   | Cap+ Tox-, pXO2                               | 6                    |
| 4229 UM12        | Cap+ Nal+, pXO2                               | UV of 4229           |
| 4229 UM12-1      | Cap+ Nal- Cry+ Te-, pXO2, pXO12, pBC16         | 4229 UM12 tr299-3 (6) |
| 4229 UM12-2      | Cap+ Nal- Cry- Te-, pXO2, pXO12, pBC16         | B. cereus 569 UM20-5  × 4229 UM12 |
| 6602 (Pasteur)   | Cap+ Tox-, pXO2                               | 6                    |
| 6602 UM4         | Cap+ Mls+, Cry+ Te-, pXO2, pXO12, pBC16       | A UM23-21 × 6602     |
| 6602 UM5         | Cap+ Mls+, Cry- Tra+ Te-, pXO2, pXO12-2        | B. cereus 569 UM20-13 × 6602 |

### B. cereus

| Strain or plasmid | Relevant characteristics of the strain or plasmid | Origin or reference |
|-------------------|-----------------------------------------------|----------------------|
| 569 UM20-1        | Ant+ Str+                                      | 1                    |
| 569 UM20-2        | Ant+ Str+ Cap+ Mls- Cry+ Tra+, pXO2-2, pXO12, pBC16 |                      |
| 569 UM20-3        | Ant+ Str+ Cap+ Mls- Cry+ Tra+, pXO2-3, pXO12, pBC16 |                      |
| 569 UM20-4        | Ant+ Str+ Cap+ Mls- Cry+ Tra+, pXO2-4, pXO12, pBC16 |                      |
| 569 UM20-5        | Ant+ Str+ Cap+ Mls- Cry+ Tra+, pXO2-5, pXO12, pBC16 |                      |
| 569 UM20-6        | Ant+ Str+ Cap+ Mls- Cry+ Tra+, pXO2-6, pXO12, pBC16 |                      |
| 569 UM20-7        | Ant+ Str+ Cap+ Mls- Cry+ Tra+, pXO2-7, pXO12, pBC16 |                      |
| 569 UM20-8        | Ant+ Str+ Cap+ Mls- Cry+ Tra+, pXO2-8, pXO12, pBC16 |                      |
| 569 UM20-9        | Ant+ Str+ Cap+ Mls- Cry+ Tra+, pXO2-9, pXO12, pBC16 |                      |
| 569 UM20-10       | Ant+ Str+ Cap+ Mls- Cry+ Tra+, pXO2-10, pXO12, pBC16 |                      |
| 569 UM20-11       | Ant+ Str+ Cap+ Mls- Cry+ Tra+, pXO2-11, pXO12, pBC16 |                      |
| 569 UM20-12       | Ant+ Str+ Mls+ Cry- Tra+, pXO2-12, pBC16        |                      |
| 569 UM20-13       | Ant+ Str+ Mls+ Cry- Tra+, pXO2-13, pBC16        |                      |

### Plasmids

| Plasmid | Relevant characteristics | Origin or reference |
|---------|--------------------------|----------------------|
| pXO1    | Encodes synthesis of B. anthracis toxin, Tox+ | 17, 24               |
| pXO1-1  | pXO1 with 2.6-kb insert from pXO12, Tox+     | This study           |
| pXO1-2 to pXO1-6 | pXO1-1::Tn4430, Tox+    | This study           |
TABLE 1—Continued

| Strain or plasmid | Relevant characteristicsa and plasmids | Origin or referenceb |
|-------------------|----------------------------------------|----------------------|
| pXO1-7            | pXO1::Tn4430, Tox*                     | This study           |
| pXO2             | Encodes synthesis of B. anthracis capsule, Cap* | 6                    |
| pXO2-1 to pXO2-7 |                                        | This study           |
| pXO2-8            | pXO2::Tn917, Cap*                      | This study           |
| pXO12             | Tra"Cry" from B. thuringiensis         | 1                    |
| pXO12-1 and pXO12-2 | pXO2::Tn917, Tra"Cry"       | This study           |
| pXO12-3 and pXO12-4 | Deletion derivatives of pXO12, Tra"Cry" | 8                    |
| pXO503           | pLS20::Tn917, Tra"             | This study           |
| pBC16           | Te"                                    | 2                    |

a Abbreviations: Ade, adenine; Ant, anthranilic acid; Cry, synthesis of parasporal crystal; Ind, indole; Trp, tryptophan; Ura, uracil; MLS*, Te917-encoded macrolide-lincosamide-streptogramin B resistance; Nal', nalidixic acid resistance; Rif', rifampin resistance; Str', streptomycin resistance; Te', pBC16-encoded tetracycline resistance; Cap, synthesis of polyglutamate capsule; Tox, synthesis of protective antigen component of the anthrax toxin; Tra, mediation of plasmid transfer by mating.

b A presumed cointegrate of pXO1-1 and pXO12.

c pXO12 in 6602 UM4 is an uncharacterized deletion derivative.

d A presumed cointegrate of pXO2 and pXO12.

dium was prepared as described by Reddy et al. (19). For solid medium, 15 g of Difco agar (Difco Laboratories, Detroit, Mich.) was added per liter. Streptomycin and rifampin were used at final concentrations of 200 and 10 μg/ml, respectively. For selection of macrolide--lincosamide--streptogramin B-resistant (MLS') cells, both erythromycin (1 μg/ml) and lincomycin (20 μg/ml) were used. Tetracycline was used at a final concentration of 5 or 25 μg/ml as described previously (1).

**Mating procedure.** Matings were carried out in broth as described by Battisti et al. (1).

**Transduction.** Methods used for the transfer of plasmids by bacteriophage CP-51-mediated transduction were those described previously (6, 21).

**Extraction and restriction analyses of plasmid DNA.** For routine screening, plasmid DNA was extracted by a modification (19) of the procedure described by Kado and Liu (7). Extracts were analyzed by agarose gel electrophoresis as described previously (1).

Restriction endonucleases were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) or International Biotechnologies, Inc. (New Haven, Conn.) and were used according to the instructions of the manufacturer. Digests were examined on 0.7% agarose (SeaKem GTG Agarose; FMC BioProducts, Rockland, Maine) gels. Molecular weights of DNA fragments were determined by comparing their mobilities to those of a kilobase ladder obtained from Bethesda Research Laboratories.

**Electrophoresis of restriction fragments from agarose gels.** Electroelution of DNA restriction fragments was carried out with an Elutrap electroseparation chamber according to the instructions of the manufacturer (Schleicher & Schuell, Inc., Keene, N.H.).

**Hybridization procedure.** The DNA probes used for hybridization experiments were prepared by the following procedures. pHHT44 DNA (pUC18::Tn4430) was extracted from E. coli by the alkaline lysis method of Birnboim and Doly (3), as described by Maniatis et al. (16), and purified by isopycnic centrifugation in CsCl. The plasmid DNA was cut with KpnI, and the 4.2-kb restriction fragment (Tn4430) was electrophoretically distributed above. Tn917-containing plasmid pTV1 (27) was extracted from B. subtilis by the procedure of Koller and Thorne (8) and purified by isopycnic centrifugation in CsCl. The three AvrI restriction fragments (2.2, 1.8, and 1.2 kb) representing Tn917 DNA were electrophoretically separated from agarose gel slices. The DNA preparations were radio-

labeled by nick translation (20) with [α-32P]dGTP purchased from Amersham Corp., Arlington Heights, Ill., and a kit obtained from Bethesda Research Laboratories. The specific activity of the resulting labeled DNA ranged from 1 × 10^7 to 3 × 10^7 cpm/μg. Plasmid DNA restriction fragments were separated on 0.7% agarose gels and transferred to GeneScreen Plus nylon membranes (Du Pont, NEN Research Products) by a modification (19) of the Southern blotting technique (22). DNA-DNA hybridizations were performed as described previously (19).

**Detection of pXO1.** B. anthracis strains containing pXO1 were detected by screening (19) for the production of the protective antigen component of the anthrax toxin (10).

**Capsule production.** Strains containing pXO2 were detected by their ability to produce capsules when grown on NB agar containing bicarbonate and serum as described previously (6).

**Plasmid-curing procedures.** Strains were cured of pBc16 by growing colonies on L agar at 42°C for 24 h, and they were cured of pXO12 by cultivation in the presence of novobiocin (6) or by serial passage at 42°C. The last procedure was also used to cure strains of pXO1 (17, 24).

**Generation of pXO12::Tn917 derivatives.** To construct pXO12::Tn917 derivatives, a Tn917-carrying derivative of the B. subtilis (natto) conjugative plasmid pLS20, designated pXO503 (8), was used as a transposition vector. A mating was performed between strains Weybridge UM44-4(pXO1, pXO12, pBc16) and Weybridge A UM23-5(pXO503), and they MSL' RPS' transconjugants were selected. A transposant designated Weybridge A UM23-16 and containing pXO12, pXO503, and pBc16 was isolated.

To induce transposition of Tn917 from pXO503 onto pXO12, Weybridge A UM23-16(pXO12, pXO503, pBc16) was grown in the presence of inducing concentrations of erythromycin (20 ng/ml) and mitomycin C (10 ng/ml) (26). Cells from the induced culture were used to prepare broth mating mixtures, with B. cereus 569 UM20-1 as recipient. Previous work in our laboratory has shown that, in contrast to pXO12, pLS20 mediates transfer of itself and other plasmids more readily on solid medium than in broth (T. M. Koehler, Ph.D. thesis, University of Massachusetts, Amherst, 1987). Therefore, these matings would favor the transfer of pLS20 over that of the pLS20 derivative pXO503. Representative MSL' Str' transconjugants from the mating mentioned above were streaked for single-colony
isolation and then examined for plasmid content to identify isolates which contained pXO12::Tn917 derivatives.

Generation of pXO2::Tn917-containing strains. To construct a pXO2::Tn917 derivative, a mating was performed between B. cereus 569 UM20-1(pXO12-2) and B. anthracis 6602(pXO2), and prototrophic MLS' transcipients were selected on Min IC agar containing erythromycin. From this mating, an MLS' Cap' transipient designated 6602 UM5 and containing plasmids pXO2 and pXO12-2 was isolated. To effect the transposition of Tn917 from pXO12 to pXO2, a mating was carried out between strains 6602 UM5(pXO2, pXO12-2) and Weybridge UM44-2. MLS' Str' transipients were selected, and these were screened for capsule production. An MLS' Str' Cap' transipient designated Weybridge UM44-10 and harboring only pXO2-8, a pXO2::Tn917 derivative, was isolated.

To allow us to determine the frequency of pXO2-mediated transfer of the pXO2::Tn917 derivative, we introduced pXO2 into Weybridge UM44-10 (pXO2-8) by mating it with the donor, Weybridge A UM23-8(pXO12, pBC16). A transipient designated Weybridge UM44-11 and containing pXO2-8, pXO12, and pBC16 was isolated.

RESULTS

Transfer of B. anthracis resident plasmids by pXO2. We previously reported transfer of the tetracycline resistance plasmid pBC16 and the B. anthracis plasmids pXO1 and pXO2 from strains harboring the B. thuringiensis fertility plasmid pXO12 (1, 6, 24). More recent experiments suggest that the frequency of transfer of the small plasmid pBC16 is considerably higher than that of the large plasmids pXO1 and pXO2. The number of Tc' transipients ranged from 10^3 to 10^5/ml, whereas the number of Tc' transipients that simultaneously inherited pXO1 was only 0.3 to 0.4% of the total (Table 2, experiment A). Similar results were obtained in pXO1-mediated transfer of pXO2 (6).

To compare more accurately the frequency of pXO2 transfer with that of pBC16, Weybridge UM44-11, which contained pXO2-8, pXO12, and pBC16, was mated with Weybridge A UM23-4. This allowed direct selection of transipients which acquired pXO2-8 or pBC16 by plating on agar containing rifampin and either erythromycin and lincomycin or tetracycline, respectively. The results of two independent matings are shown in Table 2, experiment B. The number of Tc' transipients obtained was approximately 2 x 10^5/ml, and the number of MLS' transipients ranged from 4.0 x 10^2 to 2.7 x 10^3. Interestingly, only 45 to 46% of the MLS' transipients were Cap'. Thus, the number of transipients that inherited pXO2-8 was 200-1000-fold lower than the number that inherited pBC16. Examination of the plasmid profile of representative MLS' Cap' transipients showed that they contained a pXO12 plasmid which carried a copy of Tn917. Transipients which were MLS' Cap' were found to have inherited pXO12 and pXO2::Tn917 plasmids which conigrated with the respective plasmids of the donor strain.

Plasmids pXO1 and pXO2 contain Tn4430 after pXO2-mediated mobilization. Plasmid analyses were performed on representative Tox' or Cap' transipients to confirm the acquisition of pXO1 or pXO2 in addition to pBC16. These analyses showed that there were a variety of plasmid profiles represented among the transipients (Fig. 1A and 2A). The majority of Tox' or Cap' transipients examined harbored pXO12 (Cry') and either pXO1 or pXO2, which on agarose gels appeared to be indistinguishable from the respective plasmids present in the donor strains. However, other transients which were Cry' and either Tox' or Cap' contained a single large plasmid migrating above the chromosomal DNA in agarose gels. This suggested the formation of cointegrate plasmids (Fig. 1A, lane 6; Fig. 2A, lane 5). In contrast to the variability of Tox' and Cap' transients, all Tc' transients inherited plasmid DNA which conjugated with pBC16 from the donor strains.

The high frequency of pBC16 transfer suggests that transfer of this plasmid occurs by donation. In contrast, the lower frequency of transients which acquired pXO1 or pXO2 suggests that transfer of these large plasmids may be conducted, a process which requires physical contact between the conjugative and nonconjugative plasmids. Since in other conjugation systems physical contact between the fertility plasmid and other mobilizable plasmids is often mediated by transposable elements (5), we examined pXO1 and pXO2 before and after mobilization for hybridization to Tn4430. Plasmid DNA from the agarose gels shown in Fig. 1A and 2A was transferred to nitro membranes and probed with 32P-labeled Tn4430. The results of these hybridizations are shown in Fig. 1B and 2B. Tn4430 hybridized to pXO1 from six of seven Tox' transients tested and to pXO2 from seven of eight Cap' transients tested. There was no detectable hybridization with pXO1 or pXO2 before mobilization (Fig. 1B, lanes 1 and 8; Fig. 2B, lanes 1, 6, and 10). As expected, Tn4430 also hybridized to the presumed cointegrate plasmids (Fig. 1B, lane 6; Fig. 2B, lane 5).

To determine the sites of insertion of Tn4430, we examined several pXO1 and pXO2 plasmids by restriction endonuclease digestion. To facilitate isolation of pXO1 from various Tc' transients generated from matings described in Table 2, we cured them of pXO12 and pBC16 sequentially by cultivation in the presence of novobiocin followed by serial passage at 42°C. In this way, strains which harbored pXO1 alone were obtained. pXO1 DNA was isolated, digested with EcoRI or KpnI, and electrophoresed in 0.7% agarose gels. These two enzymes were chosen because Tn4430 has no EcoRI sites and because it has two KpnI sites,
FIG. 1. Demonstration of Tn4430 transposition from pX012 to pX01 plasmids after their transfer by pX012-mediated conjugation. Weybridge A UM2-3 was the donor for transciipients in lanes 2 through 7, and Weybridge A UM17-4 was the donor for those in lanes 9 and 10. The recipients (not shown), Weybridge UM44-2 and Weybridge A UM23-4, contained no plasmids before mating. (A) Agarose gel electrophoresis of plasmid DNA from donors and Tox* transcipients. Bands: a, pX01::pX012; b, pX01 and pX01 derivatives; c, chromosomal DNA; d, pX012; e, pBC16. The light band above pX01 in lanes 1 through 5 and 7 through 10 is an alternate form of pX01. It migrated at a slightly slower rate than the cointegrate plasmid pX01::pX012 in lane 6. Lanes: 1, Weybridge A UM2-3(pX01-1, pX012, pBC16); 2, Weybridge UM44-6(pX01-2, pX012, pBC16); 3, Weybridge UM44-8(pX01-4, pX012, pBC16); 4, Weybridge A UM23-9(pX01-3, pBC16); 5, Weybridge A UM23-11(pX01-5, pX012, pBC16); 6, Weybridge A UM23-12(pX01-1, pX012, pBC16); 7, Weybridge A UM23-13(pX01-6, pX012, pBC16); 8, Weybridge A UM17-4(pX01, pX012, pBC16); 9, Weybridge A UM23-14(pX01-7, pX012, pBC16); 10, Weybridge A UM23-15(pX01, pX012, pBC16). (B) Autoradiograph of 32P-labeled Tn4430 DNA hybridized to the plasmid DNAs shown in panel A. Lanes 1 through 10 correspond to those shown in panel A.

each located 2 base pairs from an end of the transposon. The restriction patterns of the pX01 derivatives were compared with those of similarly digested pX01 from Weybridge A UM2, which was taken as wild type. There were noticeable differences in the restriction patterns of wild-type pX01 and the toxin plasmid from the transciipients. For example, the 7.95-kb EcoRI fragment present in wild-type pX01 was missing from the toxin plasmid of donor strain A UM2-3, and two new fragments, 4.15 and 6.45 kb, were present (Fig. 3A, lanes 7 and 8). This was the result of the acquisition of an uncharacterized 2.6-kb element from pX012. This altered pX01 plasmid of Weybridge A UM2-3 was designated pX01-1. There were further alterations in pX01-1 after it was mobilized by pX012. For example, in pX01-2 from Weybridge UM44-6, the 6.45-kb EcoRI fragment was replaced with a 10.6-kb fragment (Fig. 3A, lane 9). In pX01-3

FIG. 2. Demonstration of Tn4430 transposition from pX012 to pX02 plasmids after their transfer from B. anthracis to B. cereus 569 UM20-1 by pX012-mediated conjugation. (A) Agarose gel electrophoresis of plasmid LNA from donors, recipient, and Cap* transciipients. Bands: a, pX02::pX012; b, pX012; c, chromosomal DNA; d, pX02; e, unnamed nonconjugative plasmid inherited from B. thuringiensis (1); f, pBC16. Lanes: 1, B. anthracis 6602 UM4(pX02, pX012 [see below], pBC16), donor for transciipients in lanes 3 through 5; 2, B. cereus 569 UM20-1, recipient; 3, B. cereus 569 UM20-2(pX02-2, pX012, pBC16); 4, B. cereus 569 UM20-3(pX02-3, pX012, pBC16); 5, B. cereus 569 UM20-4(pX02::pX012, pBC16); 6, B. anthracis 4229 UM12-1(pX02, pX012, pBC16), donor for transciipients in lanes 7 through 9; 7, B. cereus 569 UM20-6(pX02-1, pX012, pBC16); 8, B. cereus 569 UM20-8(pX02, pX012, pBC16); 9, B. cereus 569 UM20-7(pX02-4, pX012, pBC16); 10, B. anthracis 4229 UM12-2(pX02, pX012, pBC16), donor for transciipients in lanes 11 through 13; 11, B. cereus 569 UM20-9(pX02-5, pX012, pBC16); 12, B. cereus 569 UM20-10(pX02-6, pX012, pBC16); 13, B. cereus 569 UM20-11(pX02-7, pX012, pBC16). (B) Autoradiograph of 32P-labeled Tn4430 DNA hybridized to the plasmid DNAs shown in panel A. Lanes 1 through 13 correspond to those shown in panel A. Plasmid pX012 in 6602 UM4 and transciipients derived from 6602 UM4 is an uncharacterized deletion derivative. It is about the same size as pX02, and the two plasmids did not separate under the electrophoretic conditions used here.
from Weybridge A UM23-9, the 5.6-kb EcoRI fragment was replaced with a 9.8-kb fragment (Fig. 3A, lane 10). Comparisons of KpnI digests of pXO1, pXO1-1, pXO1-2, and pXO1-3 showed that the last two plasmids had acquired a 4.2-kb fragment which comigrated with the KpnI fragment that we have shown to be Tn4430 (Fig. 3A, lanes 5 and 6). When the DNA fragments from the gel shown in Fig. 3A were transferred to nylon membranes and probed with \(^{32}\)P-labeled Tn4430, only those fragments which were unique to pXO1-2 and pXO1-3 showed homology to the transposon (Fig. 3B).

To determine the sites of insertion of Tn4430 in pXO2 after mobilization, we examined pXO2 DNA before and after transfer by pXO12. To facilitate isolation of pXO2 DNA from transcipients which also carried pXO12 and pBC16, we transferred pXO2 by transduction with phage CP-51 to B. anthracis, which had been cured of all plasmids. Plasmid DNA from representative Cap\(^{+}\) transductants was isolated and digested with restriction endonuclease KpnI or EcoRI. Figure 4A compares KpnI and EcoRI digestion patterns of the pXO2 plasmids, designated pXO2-1 and pXO2-2, originally found in the Cap\(^{+}\) transcipients B. cereus 569 UM20-6 and 569 UM20-2, respectively, with similarly digested pXO2 from B. anthracis 6602. Digestion of pXO2-1 and pXO2-2 with KpnI showed that these plasmids had acquired a 4.2-kb fragment which comigrated with the 4.2-kb KpnI fragment of pXO12 (Fig. 4A, lanes 4 and 5). EcoRI-digested pXO2-1 showed the loss of the 6.1-kb fragment present in digests of wild-type pXO2 and the presence of a new 10.3-kb fragment (Fig. 4A, lane 7). Examination of EcoRI-digested pXO2-2 showed that it had lost the 6.1- and 9.4-kb fragments and gained two new fragments of 5.2 and 10.3 kb (Fig. 4A, lane 8). The pXO2 DNA fragments of Fig. 4A were transferred to nylon membranes and probed with \(^{32}\)P-labeled Tn4430. The fragments that showed homology to Tn4430 were unique to pXO2-1 and pXO2-2 (Fig. 4B).

Plasmid pBC16 is unaltered after transfer. As mentioned earlier, the high frequency of pBC16 transfer suggests that this plasmid is transferred by donation. Since transfer of nonconjugative plasmids by donation does not require physical contact with a fertility plasmid, there should not be any alteration in pBC16 after it has been transferred. To confirm this, we examined pBC16 before and after mobilization by pXO12. pBC16 from each of two independently derived B. anthracis transcipients was moved into plasmid-free Weybridge A UM23-3 by transduction with CP-51, and plasmid DNA was isolated from transductants. As a control, we used pBC16 which had been transduced into B. anthracis but had not been exposed to pXO12 in our laboratory. Restriction analysis of the various pBC16 plasmids confirmed that there were no alterations after transfer, and tests for hybridization with \(^{32}\)P-labeled pXO12 showed that there was no homology.
FIG. 4. Agarose gel electrophoresis and corresponding autoradiograph demonstrating insertion of Tn4430 in pXO2-1 and pXO2-2. (A) Agarose gel electrophoresis of plasmid DNAs digested with KpnI and EcoRI. Fragment sizes are given in kilobases. Arrows designate fragments which show homology to probe DNA. Lanes: 1, BRL 1-kb ladder; 2 through 5, pXO12, pXO2, pXO2-1, and pXO2-2 DNAs, respectively, cut with KpnI; 6 through 8, pXO2, pXO2-1, and pXO2-2 DNAs, respectively, cut with EcoRI. (B) Autoradiograph of 32P-labeled Tn4430 DNA hybridized to the restriction digests shown in panel A. Lanes 2 through 8 correspond to those shown in panel A.

The results discussed above suggest that Tn4430 has a role in pXO2-mediated transfer of the large B. anthracis plasmids. Experiments have shown that there is a copy of Tn4430 on pXO2 as well as on three other self-transmissible B. thuringiensis plasmids currently under investigation in our laboratory (19). Therefore, it would be interesting to determine whether Tn4430 is near DNA sequences carrying information required for conjugal transfer ability. To determine the location of Tn4430 on pXO2, we probed a blot of the AvaI restriction digest of pXO2 for hybridization with 32P-labeled Tn4430.

This experiment showed that there was strong homology between Tn4430 and the 16.0-kb AvaI fragment of pXO2 (L. A. Battisti, Ph.D. thesis, University of Massachusetts, Amherst, 1988). As discussed below, this fragment was found to be adjacent to sequences commonly lost in Tn deletion derivatives of pXO2.

Restriction analysis of transfer-deficient deletion derivatives of pXO2. We isolated two Tra− Cry+ deletion derivatives of pXO2: one designated pXO2::pXO12 and the other designated pXO2::pXO12. Both strains were obtained from conjugations of B. anthracis and B. cereus (19). The presence of Tn4430 in pXO12 was confirmed by hybridization with 32P-labeled Tn4430 DNA.

| Expt | Donor | B. anthracis Webridge recipient | Tc' transciptantsa | No./ml | Frequencyb | % Tc' Cry+ | % Cap' Cry+c |
|------|-------|---------------------------------|-------------------|--------|------------|------------|------------|
| A    | B. anthracis Weybridge UM44-9 (pXO1::pXO12, pBC16) | A UM23-4 | 4.0 × 10^5 | 1.4 × 10^-2 | 57 (495/874) | NA |
|      | B. anthracis Weybridge A UM23-12 (pXO1::pXO12, pBC16) | UM44-2 | 6.8 × 10^5 | 2.1 × 10^-4 | 49 (83/170) | NA |
| B    | B. cereus 569 UM20-4 (pXO2::pXO12, pBC16) | A UM23-4 | 3.5 × 10^4 | ND     | NA | 24 |
|      | B. cereus 569 UM20-4 (pXO2::pXO12, pBC16) | A UM23-4 | 1.4 × 10^4 | 1.7 × 10^-4 | NA | 17 |

a Numbers in parentheses are ratios of number of Tc' Cry+ transciptants/number of Tc' transciptants tested. NA, Not applicable. ND, Not determined.
b Number of Tc' transciptants per donor.
c Transciptants were selected on agar plates containing tetracycline and NaHCO3 and incubated in 20% CO2, allowing direct scoring of Tc' Cap' colonies. All of 50 or more Tc' Cap' transciptants tested were Cry+.

between the two plasmids (B. D. Green, Ph.D. thesis, University of Massachusetts, Amherst, 1988).

Generation of cointegrate plasmids of pXO12 and B. anthracis resident plasmids. In mating mixtures that were screened for cotransfer of the Tc' and Cry+ phenotypes from donors carrying pXO1 and pXO2, some of the transciptants that inherited both phenotypes did not contain pXO1 or pXO2. Instead, they contained a plasmid which was higher in molecular weight than either of the two expected plasmids, suggesting the formation of cointegrate plasmids. To test the ability of these strains to transfer the large plasmid and pBC16, matings were performed with two independent B. anthracis transciptants, Weybridge UM44-9 and Weybridge A UM23-12, as donors to cured strains of B. anthracis. The results of these matings are shown in Table 3 (experiment A). The donor strains harboring the putative pXO1::pXO12 cointegrate transferred pBC16 at frequencies comparable to those of pXO12-mediated transfer of pBC16 (10^-2 to 10^-5). However, the proportion of Tc' transciptants which were also Tc'_, i.e., approximately 50%, was much larger than had been observed previously. In addition, all of the Tc' transciptants were also Cry+. Plasmid analysis revealed that the Tc' Cry+ transciptants inherited a large plasmid that comigrated in electrophoretic gels with the suspected cointegrate present in the donors. None of several Tc' Cry+ transciptants examined carried a similar large plasmid.

Results analogous to those with pXO1 were also obtained with pXO2. A Cap' Cry+ transciptant designated B. cereus 569 UM20-4 harboring pBC16 and an apparent cointegrate of pXO2 and pXO12 (Fig. 2A, lane 5) was obtained. When this strain was used as the donor in matings with Weybridge A UM23-4, pBC16 was transferred at a frequency comparable to that of donor strains containing wild-type pXO12 (Table 3, experiment B). The proportion of Tc' transciptants which simultaneously acquired the ability to make capsules was much larger than had been observed previously with donor strains that contained wild-type pXO2 and pXO12. Plasmid analysis of Tc' Cap' Cry+ transciptants revealed that these strains had inherited, in addition to pBC16, a single large plasmid which migrated at the same rate as the presumed cointegrate plasmid in the donor strain. The large plasmid was not found in several Tc' Cap' Cry+ transciptants examined.

Localization of Tn4430 on pXO12. The results discussed above suggest that Tn4430 has a role in pXO12-mediated transfer of the large B. anthracis plasmids. Experiments have shown that there is a copy of Tn4430 on pXO2 as well as on three other self-transmissible B. thuringiensis plasmids currently under investigation in our laboratory (19). Therefore, it would be interesting to determine whether Tn4430 is near DNA sequences carrying information required for conjugal transfer ability. To determine the location of Tn4430 on pXO12, we probed a blot of the AvaI restriction digest of pXO12 for hybridization with 32P-labeled Tn4430.

This experiment showed that there was strong homology between Tn4430 and the 16.0-kb AvaI fragment of pXO12 (L. A. Battisti, Ph.D. thesis, University of Massachusetts, Amherst, 1988). As discussed below, this fragment was found to be adjacent to sequences commonly lost in Tn deletion derivatives of pXO12.

Restriction analysis of transfer-deficient deletion derivatives of pXO12. We isolated two Tra− Cry+ deletion derivatives of pXO12. The first, designated pXO12::pXO12, contains the entire plasmid pXO12, while the second, designated pXO12::pXO12, contains only the Tc' region of pXO12. The results of these experiments are shown in Table 3. The data indicate that Tn4430 is located between the two plasmids, as predicted by the results of the localization experiments.
pXO12, designated pXO12-3 and pXO12-4, after growth of pXO12-containing *B. anthracis* strains at 42°C to cure them of pBC16 and pXO1. Restriction analysis of the two derivatives revealed that both had lost the 48.5-kb *Ava*I restriction fragment (Fig. 5, lanes 3 and 4). These results suggest that the 48.5-kb *Ava*I fragment of pXO12 carries information required for conjugal transfer ability. The simultaneous loss of the 48.5- and the 16.0-kb *Ava*I fragments to produce pXO12-4 (Fig. 5, lane 4) suggests that these fragments are adjacent on pXO12.

**Physical and genetic analyses of pXO12::Tn917 derivatives.** Two Tn917-carrying derivatives of pXO12, designated pXO12-1 and pXO12-2, were isolated from the MLS' Str' transciptiens Weybridge A UM23-18 and *B. cereus* 569 UM20-13, respectively. Restriction analysis with *Ava*I confirmed the insertion of Tn917 DNA into the two plasmids. Tn917 was found to contain four *Ava*I cleavage sites when propagated in *B. subtilis* PSL1(pTV1) and in *B. anthracis* Weybridge A UM23-5(pXO503). As reported earlier (18), two of the four *Ava*I sites in Tn917 are symmetrically located 7 base pairs from each end of its terminal inverted repeats. Thus, digestion of Tn917 with *Ava*I produced three distinct fragments approximately 2.2, 1.8, and 1.2 kb in size (Fig. 6A, lanes 1 and 2). Perkins and Youngman (18) reported a similar cleavage pattern for *Ava*I digested Tn917 DNA that had been subcloned in an *E. coli* host. Digestion of pXO12-1 and pXO12-2 with *Ava*I generated three new restriction frag-

ments that comigrated with the 2.2-, 1.8-, and 1.2-kb fragments of Tn917 (Fig. 6A, lanes 4 and 5). DNA-DNA hybridization experiments corroborated these results; 32P-labeled Tn917 DNA isolated from pTV1 hybridized to the 2.2-, 1.8-, and 1.2-kb fragments of the pXO12::Tn917 derivatives (Fig. 6B, lanes 4 and 5).

Mating experiments showed that *B. anthracis* Weybridge A UM23-18 and *B. cereus* 569 UM20-13 could both act as donors of the pXO12::Tn917 derivatives (Table 4). In addition, phase microscopy revealed that both donors were Cry'-. Restriction analysis of pXO12-1 and pXO12-2 showed that the Tra' Cry' plasmids had both lost the 16.0-kb *Ava*I fragment of pXO12 (Fig. 6A, lanes 4 and 5), suggesting that this fragment carries information required for the synthesis of parasporal crystals. Absence of the 16.0-kb *Ava*I fragment from pXO12-2 suggests that insertion of Tn917 occurred in that fragment and that the insertion generated two new *Ava*I fragments of approximately 8.3 and 4.2 kb (Fig. 6A, lane 5).

Because of the number of DNA alterations exhibited by pXO12-1 (Fig. 6A, lane 4), the site of Tn917 insertion in that plasmid could not be determined by this analysis.

**Transfer frequency of pXO12::Tn917 derivatives.** The Tn917-carrying derivatives of pXO12 were used to determine the transfer frequency of pXO12 by monitoring the

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**FIG. 5.** Agarose gel electrophoresis of *Ava*I-digested pXO12 DNA and of DNA from two Tra' Cry' pXO12 deletion derivatives, pXO12-3 and pXO12-4. Fragment sizes are given in kilobases. Lanes: 1, BRL 1-kb ladder; 2, pXO12 DNA; 3, pXO12-3 DNA; 4, pXO12-4 DNA.

**FIG. 6.** Agarose gel electrophoresis and corresponding autoradiograph confirming the presence of Tn917 in two Tra' Cry' derivatives of pXO12, pXO12-1, and pXO12-2. Fragment sizes are given in kilobases. (A) Agarose gel electrophoresis of plasmid DNAs digested with *Ava*I. Lanes: 1, pTV1 DNA; 2, pXO503 DNA; 3, pXO12 DNA; 4, pXO12-1 DNA; 5, pXO12-2 DNA. (B) Autoradiograph of 32P-labeled Tn917 DNA hybridized to the restriction digests shown in panel A. Lanes 1 through 5 correspond to those shown in panel A.
TABLE 4. Frequency of pXO12-mediated transfer of pBC16 compared with transfer frequency of pXO12::Tn917 derivatives

| Donor strain | Transcipients with recipient* | B. anthracis | B. cereus |
|--------------|-------------------------------|-------------|-----------|
| B. anthracis Weybridge A UM23-20(pXO12, pBC16) | 9.0 x 10^5 | 1.9 x 10^-2 | 3.9 x 10^6 | 7.1 x 10^-2 |
| B. cereus 569 UM20-5 (pXO12, pBC16) | 4.8 x 10^4 | 1.6 x 10^-4 | ND | ND |
| B. anthracis Weybridge A UM23-18(pXO12-1) | 2.3 x 10^5 | 1.3 x 10^-2 | 4.5 x 10^5 | 2.6 x 10^-2 |
| B. cereus 569 UM20-13(pXO12-2) | 2.2 x 10^4 | 9.2 x 10^-5 | ND | ND |

* Tc' transcipients were selected when the donor strain contained pXO12 and pBC16. MLS' transcipients were selected when the donor strain contained pXO12::Tn917, i.e., pXO12-1 and pXO12-2. Counterselection of donors was facilitated by the use of recipient strains that were resistant to streptomycin or rifampin. Frequencies are expressed as the number of transcipients per donor. ND, Not determined.

DISCUSSION

The results presented here suggest that pXO12-mediated transfer of the high-molecular-weight B. anthracis plasmids pXO1 and pXO2 occurs by conduction. The evidence for this is twofold. (i) The transfer frequency of pXO1 or pXO2 was very low relative to that of pXO12::Tn917 or pBC16. (ii) pXO1 and pXO2, in a majority of transcipients which acquired either plasmid, contained a copy of Tn4430. The frequencies of pXO12-mediated transfer of pBC16 ranged from 1.6 x 10^-7 to 7.1 x 10^-2, but only 0.3 to 0.4% of the Tc' transcipients acquired pXO1 or pXO2 in addition to pBC16. The implied lower frequency of pXO12-mediated transfer of the B. anthracis plasmids, compared with that of pBC16, was confirmed by matings using a donor strain which contained pXO12, pBC16, and a pXO2 plasmid which had been tagged with Tn917. The results of such matings showed that the number of transcipients which inherited the pXO2 derivative was 200- to 1,000-fold lower than the number which inherited pBC16.

Transcipients that had acquired pXO1 or pXO2 by mating were examined for the presence of Tn4430 on the transferred plasmids. In six of seven pXO1' transcipients and in seven of eight pXO2' transcipients that were examined, each of the transferred plasmids contained a copy of the transposon. Several transcipients obtained from independent matings contained presumed cointegrate plasmids of pXO12 and the B. anthracis plasmid pXO1 or pXO2. When strains containing these plasmids were used as donors in matings with cured strains of B. anthracis, the proportion of Tc' transcipients which were also Tox' was higher than normal. For example, Weybridge Bridge UMM4-9(pXO1::pXO12, pBC16) and Weybridge Bridge UMM2-12(pXO1::pXO12, pBC16) transferred pBC16 at frequencies between 10^-2 and 10^-4. Approximately 50% of the Tc' transcipients tested were also Tox'. All of the Tox' transcipients examined were also Cry' and harbored a plasmid indistinguishable from that found in the donor strain. Similarly, B. cereus 569 UM20-4, a strain carrying a putative pXO2::pXO12 cointegrate, transferred pBC16 at a frequency of 1.7 x 10^-4, and approximately 20% of the Tc' transcipients were Cap'. All of the Cap' transcipients were Cry' and harbored a plasmid which was indistinguishable from the large plasmid in the donor strain.

These studies suggest that the function of Tn4430 in the mobilization of pXO1 and pXO2 is to mediate the formation of cointegrate molecules between the fertility plasmid pXO12 and the nonconjugative B. anthracis plasmids. The cointegrate plasmid is then transferred to recipient cells, where it usually resolves into pXO12 and the respective B. anthracis plasmid. However, our results have shown that in some instances these cointegrate plasmids are stably maintained in transconjugant cells. We have also found that Tn4430 is not unique in its function of mediating the formation of cointegrate plasmids with pXO12. In experiments not described here, we used a pXO12::Tn917 plasmid to mobilize pXO2. Several Cap' transcipients obtained from these matings inherited a pXO2 plasmid, in which a copy of Tn917, but not Tn4430, was inserted.

The results presented here demonstrate that Tn4430 is located in the same vicinity on pXO12 as the crystal gene and information involved in conjugal transfer ability. Lereclus et al. (14) reported a model for the structural organization of Tn4430 (4.2 kb), a crystal gene (ca. 3.7 kb), and two sets of inverted repeats, IR1 (1.7 kb) and IR2 (2.1 kb), on plasmid pBT42, indigenous to B. thuringiensis subsp. berliner. They found that these genetic elements were arranged to form a composite transposon-like element on the plasmid. Tn4430 and the crystal gene were separated by one copy of IR1, and these three elements were flanked on each side by another copy of IR1 (14). Similarly, Krost and Whiteley (9) have found that two sets of inverted repeat sequences, IR2150 and IR1750, flank the crystal toxin gene on the 75-kb plasmid of B. thuringiensis subsp. kurstaki HD73. Because of the molecular relatedness of crystal-encoding plasmids in general (4, 11), it is likely that the crystal gene and Tn4430 are similarly arranged on pXO12. Further studies are needed to map the exact location of the crystal gene on pXO12 and to determine whether there are also copies of inverted repeat elements present on this region of pXO12.

Studies by Lereclus et al. (12, 13) have shown that Tn4430 promotes deletions in DNA sequences adjacent to its endpoints. It is possible then that Tn4430 or insertion elements related to IR1 are responsible for the spontaneous loss of DNA sequences on this region of pXO12. Therefore, Tn4430 may be directly responsible for generating deletion derivatives of pXO12 that are Tra'..

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