Plasmid Transfer between the *Bacillus thuringiensis* Subspecies *kurstaki* and *tenebrionis* in Laboratory Culture and Soil and in Lepidopteran and Coleopteran Larvae

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Plasmid transfer between *Bacillus thuringiensis* subsp. *kurstaki* HD1 and *B. thuringiensis* subsp. *tenebrionis* donor strains and a streptomycin-resistant *B. thuringiensis* subsp. *kurstaki* recipient was studied under environmentally relevant laboratory conditions in vitro, in soil, and in insects. Plasmid transfer was detected in vitro at temperatures of 5 to 37°C, at pH 5.9 to 9.0, and at water activities of 0.965 to 0.995, and the highest transfer ratios (up to 10⁻¹ transconjugant/donor) were detected within 4 h. In contrast, no plasmid transfer was detected in nonsterile soil, and rapid formation of spores by the introduced strains probably contributed most to the lack of plasmid transfer observed. When a *B. thuringiensis* subsp. *kurstaki* strain was used as the donor strain, plasmid transfer was detected in killed susceptible lepidopteran insect (*Lacanobia oleracea*) larvae but not in the nonsusceptible coleopteran insect *Phaedon choclariae*. When a *B. thuringiensis* subsp. *tenebrionis* strain was used as the donor strain, no plasmid transfer was detected in either of these insects even when they were killed. These results show that in larger susceptible lepidopteran insects there is a greater opportunity for growth of *B. thuringiensis* strains, and this finding, combined with decreased competition due to a low initial background bacterial population, can provide suitable conditions for efficient plasmid transfer in the environment.

Bacillus thuringiensis is a gram-positive, spore-forming bacterium that produces insecticidal crystal protein toxins during sporulation. *B. thuringiensis* was first found in diseased silkworms (*Bombyx mori*) in 1901 (11, 24) but has since been isolated from a range of environments, including insects, soil, dust from stored grain, and leaves from coniferous and deciduous trees (5, 15, 17, 20). *B. thuringiensis* has been shown to contain a range of toxins and virulence determinants that may enhance its pathogenicity. However, the insecticidal crystal protein toxins or δ -endotoxins are the primary determinants of pathogenicity. Generally, *B. thuringiensis* insecticidal protein toxin genes (*cry*) reside on large self-transmissible plasmids, and individual *B. thuringiensis* strains can harbor a diverse range of plasmids that can vary in number and in size from around 2 to 200 kb (4, 7–9, 16). Using plasmid curing, Gonzalez et al. (8, 9) demonstrated that the *cry* genes are present on large plasmids which are more than 50 kb long and can be self-transmissible between strains by a conjugation-like mechanism. The *cry* genes are not randomly distributed and tend to be confined to relatively few plasmids (2, 3). For example, *B. thuringiensis* subsp. *kurstaki* HD1 contains 12 plasmids, but four of its *cry* genes (*cryIAa*, *cryIAc*, *cry2A*, and *cry2B*) reside on a single 110-MDa plasmid (6); the remaining *cry* gene (*cryIAb*) occurs on an unstable 44-MDa plasmid (2, 3). Hence, plasmid behavior is of considerable significance for insecticidal activity in this bacterium.

The transfer frequency of some of the large self-transmissible plasmids has been shown to be almost one transconjugant per recipient, and thus movement of these plasmids can be detected simply by looking for the presence of crystals in *cry*-

negative recipients. However, in most cases the plasmid transfer frequency is low, and this approach is not feasible. Another method for studying the movement of large conjugative plasmids relies on the use of a mobilizable or *oriT*-bearing shuttle plasmid as a reporter of transfer frequency (2, 6). The cryptic conjugative plasmids pXO13, pXO14, pXO15, and pXO16 were originally discovered due to their ability to mobilize the small *Bacillus cereus* tetracycline resistance-encoding plasmid pBC16 (18).

B. thuringiensis strains have been used to study plasmid transfer in soil and insect species, as well as in laboratory broth (12, 25). The objective of this study was to obtain more detailed information concerning the transfer of plasmids between *B. thuringiensis* strains under environmentally relevant conditions. Experiments were carried out in vitro, in soil, and in larvae of lepidopteran and coleopteran insects. Each of the donor strains used was capable of killing one of the insects. By differentially labelling donor and recipient strains with antibiotic resistance markers, we were able to monitor the donor, recipient, transconjugant, and background microbial populations during the experiments.

MATERIALS AND METHODS

Bacteria and plasmids. The organisms used were *B. thuringiensis* subsp. *kurstaki* HD1, a streptomycin-resistant crystal toxin-negative mutant of this strain (*B. thuringiensis* subsp. *kurstaki* HD1 *cry* Sm^r) (12), and *B. thuringiensis* subsp. *tenebrionis* (14). *B. thuringiensis* subsp. *kurstaki* HD1 and *B. thuringiensis* subsp. *tenebrionis* were electroporated by using the method of Stephenson and Jarrett (22), plasmid pBC16, and a Bio-Rad gene pulser set at a field strength of 8.75 kV/cm (400 Ω , 25 μ F, 1.75 kV). To maintain the plasmid compositions of *B. thuringiensis* strains used frequently in this work (8, 9), cultures were stored as 5-ml aliquots in sterile bijoux bottles at -20°C in 20% (vol/vol) glycerol, and fresh cultures were used every month. All strains were routinely grown on nutrient agar (Oxoid) containing appropriate antibiotics when necessary at 30°C for 24 h.

Standard broth mating procedure. Plates containing a donor and plates containing a recipient were prepared by streaking loopfuls of cultures from stock plates onto nutrient agar containing 25 μ g of tetracycline ml⁻¹ for donor strains and 50 μ g of streptomycin ml⁻¹ for recipient strains. The plates were incubated

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at 30°C for 24 h. For each organism, a single colony was used to inoculate 50 ml of brain heart infusion (BHI) broth (Oxoid) containing the appropriate antibiotic, and the preparation was incubated at 30°C for 18 h with shaking (40 rpm). Each overnight culture was diluted to an optical density at 600 nm of 1.1 (approximately 10^8 CFU ml⁻¹) in 0.25× Ringer's solution. Then 0.5-ml aliquots of a donor suspension and 0.5-ml aliquots of a recipient cell suspension were added to three 250-ml conical flasks containing 50 ml of prewarmed BHI broth, and the preparations were incubated at 30°C for 6 h with shaking (40 rpm). Three broths preparations containing donor cells and three broth preparations containing recipient cells were treated in the same way and used as controls. After incubation, samples were serially diluted in 0.25× Ringer's solution and spread plated onto nutrient agar containing 25 µg of tetracycline ml⁻¹ to select for donor cells, onto nutrient agar containing 50 µg of streptomycin ml⁻¹ to select for recipient cells, and onto nutrient agar containing 25 µg of tetracycline ml⁻¹ and 50 µg of streptomycin ml⁻¹ to select for transconjugant cells. The plates were incubated at 30°C for 48 h, and the colonies were counted. Transfer ratios were calculated by dividing the number of transconjugants by the number of donors or recipients.

Effect of time, temperature, pH, and available water on the mobilization of pBC16. To study the effects of time and temperature on plasmid transfer, the standard broth mating procedure was used, and to study the effects of pH and available water, the following modifications were made. The effect of pH was investigated by adding 0.1 M Tris (Trizma; Sigma) to BHI broth preparations and adjusting the pH to 5.5 to 9.5 in 0.5-pH unit steps. Each batch of BHI broth was adjusted to the correct pH prior to autoclaving. BHI broth preparations supplemented with 0.5, 2, 3, 4, and 5% (wt/vol) NaCl were used to study the effect of water availability at water activities of 0.965 to 0.995.

Survival and gene transfer in soil. Soil samples (2 kg, wet weight) were collected from the top 25 cm of soil at two sites at Horticulture Research International, Wellesbourne, Warwickshire, United Kingdom. One of the soils (Covers) was a sandy loam (Wick series no. 1) that contained 0.9% organic carbon, 67.1% (vol/vol) sand, 16.6% (vol/vol) silt, and 16.3% (vol/vol) clay and had a pH of 6.8 (27). The second soil (Hunts Mill field) contained 1.91% organic matter, 75% (vol/vol) sand, 10% (vol/vol) silt, and 15% (vol/vol) clay and had a pH of 5.97 (26). The soil samples were sieved (mesh size, 2 mm), air dried at room temperature to a water content of 4% (wt/vol), and stored at 10°C until they were used.

Donor and recipient *B. thuringiensis* cells were grown in BHI broth at 30°C for 18 h with shaking (40 rpm). The cells were pelleted by centrifugation at 2,000 × g for 10 min, washed once in sterile distilled water, and resuspended in sterile distilled water to an optical density at 600 nm of 1.1 (approximately 10^8 CFU ml⁻¹). Suspensions of donor and recipient cells were added to a final density of 10^7 CFU g (dry weight) of soil⁻¹. The soil moisture content was adjusted so that the matric potential was -33 kPa. Subsamples (100 g, dry weight) of inoculated soil were placed in three 250-ml Duran bottles (Schott), compacted to a bulk density of 1.25 g cm⁻³, and incubated at 18, 25, and 30°C in the dark. Bottles containing soil inoculated with donor cells alone, bottles containing soil inoculated with recipient cells alone, and bottles containing only soil and water were included as controls and were treated in the same way. Each treatment was replicated three times. Samples (1 g, dry weight) of soil were taken at zero time and on days 1, 7, 21, and 28 and were placed in 9 ml of 0.25× Ringer's solution. The samples were vortexed for 20 s, diluted in 0.25× Ringer's solution, and plated onto nutrient agar supplemented with 12.5 µg of cycloheximide ml⁻¹ and the relevant antibiotics to select for donor, recipient, and transconjugant populations. In addition, to determine the number of spores in the soil after 7, 14, and 28 days, samples were heated at 70°C for 20 min and dilution plated onto media to select for donor and recipient cells as described above.

Insect rearing. Larvae of the tomato moth (*Lacanobia oleracea*) were obtained from the insect-rearing unit at Horticulture Research International, Wellesbourne, Warwickshire, United Kingdom. The larvae were maintained on tomato leaves (*Lycopersicon esculentum* cv. moneymaker). Larvae of the mustard beetle (*Phaedon cochleriae*) were reared on 10-week-old Chinese cabbage plants (*Brassica chinensis* var. *pekinensis* cv. kasumi).

Insect studies in which *L. oleracea* larvae were used. Donor and recipient spore and crystal mixtures were prepared by using nutrient agar plates that had been incubated at 30°C for 5 days. Spores and crystals were resuspended in sterile 0.25× Ringer's solution to an optical density at 600 nm of 1.1 (approximately 10^8 CFU ml⁻¹). The wetting agent Etalfix (Maag, Dielsdorf, Germany) was added to a concentration of 0.1% (vol/vol), and the suspension was spread over the surfaces of leaves by using a sterile 10-µl inoculation loop. Donor and recipient inocula were spread over three tomato leaves whose surface areas were approximately equal. To maintain leaf turgor, the petiole of each leaf was placed through a pierced lid on a small (50-ml) plastic pot filled with distilled water. Each sample was placed in a 400-ml plastic autoclavable beaker, and then seven third-instar *L. oleracea* larvae were added. Each beaker was placed in a perforated polyethylene bag and incubated at 25°C by using a cycle consisting of 18 h of light (24 W m⁻²) and 6 h of darkness. Three leaves inoculated with the donor alone, three leaves inoculated with the recipient alone, and three leaves inoculated with only water were included and treated in the same way. One larva was removed from each of the independent replicates after 1, 3, and 5 days. Each larva was crushed in 1 ml of 0.25× Ringer's solution in an Eppendorf tube by using a sterile pellet resuspender. The preparations were diluted in sterile 0.25×

Ringer's solution, and dilution plate counting was performed with nutrient agar and with nutrient agar containing the relevant antibiotics to select for transconjugant, donor, recipient, and background strains. In addition, samples were subjected to heat treatment at 70°C for 20 min to determine the number of heat-resistant donor and recipient spores present.

Insect studies in which *P. cochleriae* larvae were used. Gene transfer was studied by using second- and third-instar larvae of the mustard beetle (*P. cochleriae*). Leaf discs (diameter, 35 mm) were removed from a Chinese cabbage plant and placed abaxial surface down on 2% (wt/vol) water agar in a 90-mm-diameter petri dish. Fifty-microliter portions of donor and recipient inocula were spread over each leaf surface, and the preparations were air dried for 1 h. Thirty larvae were placed on each leaf disc by using an artist's brush. The petri dish was closed and incubated at 25°C by using a cycle consisting of 18 h of light and 6 h of darkness and a relative humidity of 65%. The experiment was performed in triplicate, and leaf discs inoculated with donor cells alone, leaf discs inoculated with recipient cells alone, and leaf discs inoculated with only water were used as controls. Samples consisting of three pooled insects from each plate were removed after 4, 24, 48, and 72 h and crushed in 1 ml of sterile 0.25× Ringer's solution, and the numbers CFUs per spore were determined.

Statistical analysis. For time course studies and quantitative-level treatments (e.g., temperature, pH, and available water treatments) a one-way model analysis of variance was used (21). Differences between times and levels were expressed by using least-significant-difference bars. In some instances a two-way analysis of variance was used.

RESULTS

Plasmid transfer in standard laboratory broth. Growth of *B. thuringiensis* subsp. *kurstaki* HD1(pBC16), *B. thuringiensis* subsp. *tenebrionis*(pBC16), and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r and mobilization of pBC16 between these strains under different laboratory conditions are shown in Fig. 1. When the *B. thuringiensis* subsp. *kurstaki* HD1(pBC16) donor strain was added to the broth at a concentration of approximately 10^6 CFU ml⁻¹, the concentration remained the same for approximately 4 h; then over the next 6 h the density increased to approximately 10^8 CFU ml⁻¹, and it remained at this level for the rest of the experiment (Fig. 1A). The growth of the recipient strain, *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r, was similar to the growth of the donor strain. Mobilization of pBC16 to *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r was detected after 4 h; however, neither the donor nor the recipient grew during this period. The concentration of transconjugants increased over the next 4 h to approximately 10^5 CFU ml⁻¹, and then the population level remained constant for the rest of the experiment. The initial ratio for transfer from the donor *B. thuringiensis* subsp. *kurstaki* HD1(pBC16) to the recipient *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r was 1.2×10^{-3} transconjugant per donor, and then the transfer ratio was 6.4×10^{-3} CFU ml⁻¹ for the rest of the experiment.

The concentrations of *B. thuringiensis* subsp. *tenebrionis*-(pBC16) and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r remained at the initial inoculum levels for 2 h and then increased to 1.52×10^7 and 1.46×10^7 CFU ml⁻¹, respectively, after 4 h (data not shown). Mobilization of pBC16 from *B. thuringiensis* subsp. *tenebrionis*(pBC16) to *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r was detected after 2 h; during this period no growth of the donor or the recipient was detected. The initial level of transconjugants was 6.36×10^2 CFU ml⁻¹; over the next 4 h the level of transconjugants increased to 7.40×10^4 CFU ml⁻¹. The transfer ratio for pBC16 was 2.5×10^{-3} .

Figure 1B shows the effect of temperature on the transfer of pBC16 from *B. thuringiensis* subsp. *kurstaki* HD1(pBC16) to *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r. The numbers of transconjugants obtained after incubation at 22.5 and 30°C were not significantly different. The highest numbers of transconjugants were produced in matings involving *B. thuringiensis* subsp. *kurstaki* HD1 when the preparations were incubated at 27.5°C. At higher temperatures, the numbers declined from 2.7×10^5 to 1.2×10^2 CFU ml⁻¹ at 37°C. The transfer ratio data were similar. The highest transfer ratio ($2.79 \times$

FIG. 1. Effects of various culture conditions on the growth of *B. thuringiensis* strains and transfer of plasmid pBC16. (A to D) Symbols: ●, *B. thuringiensis* subsp. *kurstaki* HD1(pBC16); ○, *B. thuringiensis* subsp. *kurstaki* HD1 *cry* Sm^r; ■, transconjugant. (E to H) Symbols: ■, *B. thuringiensis* subsp. *tenebrionis*(pBC16); □, *B. thuringiensis* subsp. *kurstaki* HD1 *cry* Sm^r; ●, transconjugant. The effects of time (A), temperature (B and E), pH at 25°C (C and G), pH at 30°C (F), and NaCl (D and H) were determined. The dashed lines indicate detection limits. The error bars indicate the least significant differences of the means ($n = 3$; $P > 0.05$).

10^{-2}) occurred at 22.5°C, and the ratio decreased sharply to 1.24×10^{-6} at 37°C. The effect of temperature on plasmid transfer between *B. thuringiensis* subsp. *tenebrionis*(pBC16) and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r is shown in Fig. 1E. Temperature did not have a significant effect on the growth of the donor and recipient bacteria. There was a significant difference between the number of transconjugants detected at 22.5°C and the number of transconjugants detected at 27.5°C. In addition, the number of transconjugants detected at 27.5°C was significantly different from the numbers of transconjugants detected at 30 and 37°C. The transfer ratio increased from 1.21×10^{-4} at 22.5°C to 6.48×10^{-3} at 30°C. There were not significant differences between transfer ratios obtained at temperatures ranging from 27.5 to 37°C. The segregational stability of pBC16 in the host, transconjugant, and donor strains was investigated, and pBC16 was found to be stably maintained (100%) at 25, 30, and 37°C in the donor, recipient, and transconjugant strains.

The effect of a range of pH values on mobilization of pBC16 from donor to recipient *B. thuringiensis* strains is shown in Fig. 1C, F, and G. Growth of the donor and recipient bacteria was not affected at pH values between 6.1 and 8.1, and growth of the recipient was not affected by a pH as high as 9.0. More variation between replicates was observed at higher pH values. The lowest pH at which transconjugants were detected was pH 5.9 (1.73×10^3 CFU ml⁻¹), and the highest pH at which transconjugants were detected was pH 9.0 (1.03×10^2 CFU ml⁻¹). The number of transconjugants at pH 6.1 and the number of transconjugants at pH 8.1 did not differ significantly (1.11×10^5 and 7.63×10^4 CFU ml⁻¹). There was a pH effect on the number of transconjugants at pH values greater than 8.1, but the transfer ratio did not decrease until the pH was 8.6. At higher pH values, the transfer ratio declined rapidly, and eventually at pH 9.3 no transconjugants were detected. The transfer ratio significantly decreased from 1.04×10^{-2} transconjugant per donor at pH 6.12 to 3.71×10^{-4} transconjugant per donor at pH 5.9. When mobilization of pBC16 between *B. thuringiensis* subsp. *tenebrionis*(pBC16) and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r was studied at a suboptimal temperature (25°C), the dual effects of temperature and pH resulted in a more clearly defined optimum pH between pH 7.1 and 7.6 (Fig. 1G).

In matings between both donor strains and the recipient *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r in the presence of various concentrations of NaCl, there were not significant differences in the growth of the donor and recipient strains at NaCl concentrations ranging from 0.5 to 3% (wt/vol) (Fig. 1D and H). Growth was significantly affected at NaCl concentrations greater than 3% (wt/vol), and the numbers of donor and recipient bacteria decreased under these conditions. Plasmid mobilization was detected at NaCl concentrations between 0.5 and 4% (wt/vol) but not at NaCl concentrations of 5 and 6% (wt/vol).

Plasmid transfer in soil. Figure 2 shows the results obtained when the two donor strains were inoculated individually along with the recipient strain into nonsterile soil. The experiments were performed at 18, 25, or 30°C. Each combination was inoculated into nonsterile soil at an initial level of 10^7 CFU g (dry weight)⁻¹, and the numbers of cells decreased by approximately 1 log unit when samples were obtained immediately after inoculation. After 7 days of incubation, the numbers of spores in the soil microcosms were determined after heat treatment, and the values obtained were not significantly different from the total numbers of donor and recipient cells. The numbers of spores were stable, approximately 10^4 CFU g (dry weight)⁻¹ of soil⁻¹, for at least 28 days. At no time during the

experiment was plasmid mobilization detected. After continued incubation, a stable population of donor and recipient cells was detected, which was probably due to the predominance of spores in the sample (data not shown). When inoculated separately (at both temperatures), the donor and recipient bacteria behaved in a similar fashion to when they were coinoculated into the same sample.

Plasmid transfer in *L. oleracea* larvae. When third-instar larvae of *L. oleracea* (tomato moth) were fed tomato leaves onto which spores and crystals of *B. thuringiensis* subsp. *kurstaki* HD1(pBC16) and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r had been spread, after 1 day the live larvae were found to contain 2.79×10^3 and 1.89×10^4 CFU of donor and recipient bacteria mg⁻¹, respectively (Fig. 3A). The larvae were observed throughout the experiment. On day 1 no dead *L. oleracea* larvae were detected, but on day 3 the larvae had died in those microcosms to which *B. thuringiensis* subsp. *kurstaki* HD1(pBC16) had been added. On day 3 one of the dead larvae was macerated, and the resulting preparation was examined by light microscopy. The contents of the insect were found to be predominantly *B. thuringiensis*. The total concentration of donor cells detected on day 3 was 3.28×10^5 CFU mg of larvae⁻¹, and the number of donor cells remained at this level for the remainder of the experiment. By day 3, the total concentration of recipient cells had increased to 2.02×10^5 CFU mg of larvae⁻¹; subsequently, the concentration of recipient cells decreased slightly to 6.62×10^4 CFU mg of larvae⁻¹ on day 5. There was not a significant difference between the total number of cells and the number of spores for the donor and recipient strains at any sampling time. Plasmid transfer was detected; the level of transconjugants was 3.76×10^3 CFU mg of larvae⁻¹ on day 3, but the level decreased to 1.24×10^2 CFU mg of larvae⁻¹ by day 5. The concentration of bacteria detected on nutrient agar increased from 4.60×10^4 CFU mg of larvae⁻¹ to 1.33×10^6 and 9.78×10^6 CFU mg of larvae⁻¹ on days 3 and 5, respectively. Plasmid mobilization was not detected in any of the control microcosms.

To explore the possibility that plasmid transfer between *B. thuringiensis* strains occurred in nonsusceptible species, we investigated mobilization of pBC16 between *B. thuringiensis* subsp. *tenebrionis* and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r in *L. oleracea* larvae. In this study, as expected, none of the larvae fed leaves spread with the donor and recipient bacteria died. The donor and recipient bacteria were found to be present at concentrations of 3.64×10^3 and 1.70×10^4 CFU mg of larvae⁻¹, respectively, after day 1 and at concentrations of 6.19×10^4 and 1.07×10^5 CFU mg of larvae⁻¹, respectively, after day 3 (data not shown). Subsequently, the concentrations of donor and recipient bacteria decreased to 3.54×10^5 and 5.98×10^4 CFU mg of larvae⁻¹, respectively, on day 5. The concentration of background bacteria increased from 8.49×10^4 CFU mg of larvae⁻¹ on day 1 to 5.22×10^6 CFU mg of larvae⁻¹ on day 5. Mobilization of pBC16 was not detected at any sampling time during the experiment.

Plasmid transfer in *P. cochleriae* larvae. We examined mobilization of pBC16 between *B. thuringiensis* subsp. *tenebrionis*(pBC16) and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r after spores and crystals were spread over leaves and ingested by *P. cochleriae* larvae. After 4 and 24 h all of the larvae were alive, and after 48 h the larvae in samples that contained *B. thuringiensis* subsp. *tenebrionis* were dead. Altogether, the concentration of donors initially decreased from 1.58×10^4 CFU mg of larvae⁻¹ after 4 h to 2.26×10^2 CFU mg of larvae⁻¹ after 24 h (Fig. 3B). Subsequently, the number of donors remained at comparable levels during the experiment. The concentration of recipients decreased from 5.24×10^4 CFU mg of

FIG. 2. Survival of *B. thuringiensis* strains and transfer of plasmid pBC16 in soil. *B. thuringiensis* subsp. *kurstaki* HD1(pBC16) was used as the donor strain in soil kept at 18°C (A) and 25°C (B), and *B. thuringiensis* subsp. *tenebrionis*(pBC16) was used as the donor strain in soil kept at 18°C (C) and 30°C (D). *B. thuringiensis* subsp. *kurstaki* HD1 *cry* Sm^r was used as the recipient strain, and no plasmid transfer was detected. Symbols: ●, donor cells; ○, donor spores; ■, recipient cells; □, recipient spores. The dashed lines indicate detection limits. The error bars indicate the least significant differences of the means ($n = 3$; $P > 0.05$).

larvae⁻¹ after 4 h to 1.28×10^3 CFU mg of larvae⁻¹ after 24 h of incubation. The concentration of background bacteria remained between 10^7 and 10^8 CFU mg of larvae⁻¹ throughout the experiment. Mobilization of pBC16 was not detected at any sampling time.

We also examined mobilization of pBC16 between *B. thuringiensis* subsp. *kurstaki* HD1 and *B. thuringiensis* subsp. *kurstaki* HD1 *cry* Sm^r in nonsusceptible *P. cochleriae* larvae. Samples of live larvae were obtained during the experiment. The levels of donor and recipient cells remained 10^1 and 10^3 CFU mg of larvae⁻¹, respectively, during the experiment (data not shown). The levels of donor and recipient spores detected were comparable to the total counts. The background concentration detected on nutrient agar increased significantly between 4 and 72 h from 1.29×10^4 to 2.11×10^6 CFU mg of larvae⁻¹. Plasmid mobilization was not detected in any of the samples.

DISCUSSION

The widespread occurrence of large self-transmissible plasmids in *B. thuringiensis* strains suggests that conjugation may be an important means of plasmid dissemination in *Bacillus* populations in nature. The experiments described in this paper were conducted in laboratory broth or by using nonselective conditions under which the donor, the recipient, and the

transconjugant can grow. This may have affected interpretation of the genuine transfer frequency, and hence the term transfer ratio was used. The results of the experiment in which we examined the effect of time on mobilization of pBC16 from *B. thuringiensis* subsp. *kurstaki* HD1(pBC16) indicate that after mobilization was detected initially, the transfer ratio did not change significantly, although the numbers of transconjugants and parental cells increased (Fig. 1A). This may imply that the transfer ratio and the transfer frequency are similar.

The strains which were constructed were used in sensitive studies to monitor the effects of a number of physical and chemical parameters on plasmid exchange between *B. thuringiensis* strains. Plasmid transfer was detected quickly, in some cases after as little as 2 h. However, the highest numbers of transconjugants were generally detected after 4 h, and longer incubation times did not seem to increase the numbers of transconjugants produced. Andrup et al. (1) detected plasmid transfer between *B. thuringiensis* strains after 1.5 h, and transfer ratios were maintained for 8 h. After 8 h the transfer rate decreased. When the temperature was varied, *B. thuringiensis* subsp. *kurstaki* HD1(pBC16) mobilized pBC16 better at lower temperatures ($\leq 30^\circ\text{C}$), whereas pBC16 was mobilized better from *B. thuringiensis* subsp. *tenebrionis* at higher temperatures (temperatures between 30 and 37°C). This was probably not an effect resulting from the use of plasmid pBC16, which was

FIG. 3. Growth of *B. thuringiensis* strains and transfer of pBC16 in insect larvae. (A) *B. thuringiensis* subsp. *kurstaki* HD1(pBC16) and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r in *L. oleracea* larvae (bar legend on the left-hand side). (B) *B. thuringiensis* subsp. *tenebrionis*(pBC16) and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r in *P. cochleriae* larvae (bar legend on the right-hand side). The error bars indicate the least significant differences of the means ($n = 3$; $P > 0.05$). B.t.k., *B. thuringiensis* subsp. *kurstaki*; B.t.t., *B. thuringiensis* subsp. *tenebrionis*.

originally isolated from *Bacillus cereus*, since it was the same for both strains. Rather, it was probably due to differences between the original strains, including differences in their plasmid compositions. The normal environmental temperature in which a plasmid predominates can play a role in the evolution of its transfer mechanism (13, 19). *B. thuringiensis* has been isolated from a range of different habitats, mainly as spores; hence, it is difficult to determine under which environmental conditions cells are most active and what evolutionary pressures may have influenced the development of the transfer mechanisms.

In contrast to the effect of temperature on plasmid transfer between the strains, no clear pH optimum was observed until a suboptimal temperature was used. This result is comparable to the results of Rochelle et al. (19), who found that in *Pseudomonas* species plasmid pQM1 exhibited a clear peak transfer ratio related to pH at 37°C but not at 25°C. More variation

between replicates was observed at pH values at the limits at which the donor and recipient strains were able to grow. Although our experiments were set up to allow for growth of donor and recipient cells together, some results, such as the results obtained at the pH extremes, showed that an increase in the size of the total population was not required for transfer to take place. However, at high salt levels both growth and plasmid transfer were inhibited. Growth of the donor and recipient cells together has been implicated in higher transfer ratios (12). In the present study, conditions that allowed optimal growth resulted in the greatest transfer ratios and the highest numbers of transconjugants.

B. thuringiensis is commonly isolated from soil at low levels, and spray application to crops can result in a large proportion of the inoculum entering the soil. The results which we obtained show that when *B. thuringiensis* entered soil environments, most of the cells did not acquire enough nutrients to be able to sustain growth and entered the sporulation phase. Formation of spores is a major factor which prevents plasmid exchange and explains the results which we observed. Conjugation-mediated gene exchange between *B. thuringiensis* strains has been detected in soil, but generally only after some kind of manipulation, such as addition of bentonite clay (23) or soil sterilization (25). Although Haack et al. (10) detected transfer in nonsterile soil, their data suggested that the sizes of the donor and recipient populations increased by as much as 2 log units. This is consistent with our data for mobilization of pBC16 in broth cultures rather than in soil, in which *B. thuringiensis* and most bacilli occur predominantly as spores or decline in number.

The main goal of the present study was to investigate plasmid transfer between *B. thuringiensis* strains that infect susceptible and nonsusceptible larvae under conditions resembling those found in nature. In susceptible *L. oleracea* larvae the levels of mobilization of pBC16 between *B. thuringiensis* subsp. *kurstaki* HD1(pBC16) and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r were high when spores and crystals were spread over tomato leaves. The high levels of mobilization observed in these experiments were similar to the levels observed in laboratory broth experiments. Our results were similar to those of Jarrett and Stephenson (12) and Vilas-Boas et al. (25); however, both of the latter groups of workers based their results on a single observation made after the larvae died. In the present study we investigated plasmid transfer during ingestion, larval death, and decomposition by destructively sampling a number of larvae at various times. This allowed us to estimate the initial level of a *B. thuringiensis* strain ingested and then monitor the increase or decrease in the number of cells during the experiments. Our data indicated that a low number of background bacteria were present in the insects; this allowed the donor and recipient strains to grow and aided plasmid transfer.

Plasmid transfer was also studied by using *B. thuringiensis* subsp. *tenebrionis*(pBC16) and coleopteran (*P. cochleriae*) susceptible larvae. Mobilization of pBC16 between *B. thuringiensis* subsp. *tenebrionis*(pBC16) and *B. thuringiensis* subsp. *kurstaki* HD1 cry Sm^r was not detected in *P. cochleriae* larvae. This may have been due to the low levels of donor and recipient strains detected in the presence of a high background level gut bacteria. This high background level was observed even in untreated larvae during control experiments. Hence, the *B. thuringiensis* strains may have been unable to compete effectively with the background organisms.

The rates of transfer of pBC16 between the *B. thuringiensis* strains used in the present study were higher in *L. oleracea* larvae than in *P. cochleriae*. This may have been because the *B. thuringiensis* strains grew better in the cadavers of *L. oleracea*

larvae than in the *P. cochleriae* larvae. In some cases, outgrowth of *B. thuringiensis* strains has been shown to be an important factor in *B. thuringiensis* insect pathology, but more importantly, in this study, we found that such outgrowth promotes plasmid transfer. The larger size of the *L. oleracea* larvae may have enabled the donor and recipient cells to grow together for a longer period of time in order to reach a critical population density that allowed plasmid transfer to occur. This may not be possible in small insects as cadavers can support only a finite number of cells; if the transfer frequency were below a certain level, gene exchange would be difficult to detect in a single cadaver, and many more insects would have to be sampled. In *P. cochleriae* larvae the resident microbial population appeared to utilize the insect cadaver, prevent establishment of a high density of *B. thuringiensis* cells, and prevent plasmid transfer.

It is apparent from this study that time, temperature, pH, and water activity (examples of environmental variables) alone do not hinder plasmid mobilization at the levels which are typically found in the environment. In any one situation the environmental variables studied combine to determine the ability of *B. thuringiensis* cells to interact. Although plasmid exchange between *B. thuringiensis* is less likely in soil, our results suggest that it can occur under conditions similar to those that might be encountered in the insect environment. However, our data probably represent the greatest potential for plasmid exchange since high numbers of donor and recipient cells were placed together and fed to insects. The levels of plasmid transfer predicted for infected larvae depend on the insect species, the larval food, the ability of the organisms to colonize the larvae, the *B. thuringiensis* strains used, and the dose received by the larvae.

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REFERENCES

- Andrup, L., J. Damgaard, and K. Wasserman. 1993. Mobilization of small plasmids in *Bacillus thuringiensis* subsp. *israelensis* is accompanied by specific aggregation. *J. Bacteriol.* **175**:6530–6536.
- Aronson, A. I. 1993. The two faces of *Bacillus thuringiensis*: insecticidal proteins and post-exponential survival. *Mol. Microbiol.* **7**:489–496.
- Aronson, A. I., and W. Beckman. 1987. Transfer of chromosomal genes and plasmids in *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **53**:1525–1530.
- Battisti, L., B. D. Green, and C. B. Thorne. 1985. Mating transfer of plasmids among *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*. *J. Bacteriol.* **162**:543–550.
- Bernhard, K., P. Jarrett, M. Meadows, J. Butt, D. J. Ellis, G. M. Roberts, S. Pauli, P. Rodgers, and D. Burgess. 1997. Natural isolates of *Bacillus thuringiensis*. Worldwide distribution, characterisation, and activity against insect pests. *J. Invertebr. Pathol.* **70**:59–68.
- Carlton, B. C., and J. M. Gonzalez. 1985. The genetics and molecular biology of *Bacillus thuringiensis*, p. 211–249. In D. A. Dubnau (ed.), *The molecular biology of the bacilli*, vol. 2. Academic Press Inc., New York, N.Y.
- Chapman, J. S., and B. C. Carlton. 1985. Conjugal transfer in *Bacillus thuringiensis*, p. 453–467. In D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), *Plasmids in bacteria*. Plenum Press, New York, N.Y.
- González, J. M. J., B. J. Brown, and B. C. Carlton. 1982. Transfer of *Bacillus thuringiensis* plasmids coding for delta-endotoxin among strains of *Bacillus thuringiensis* and *Bacillus cereus*. *Proc. Natl. Acad. Sci. USA* **79**:6951–6955.
- González, J. M. J., H. T. Dulmage, and B. C. Carlton. 1981. Correlation between specific plasmids and δ -endotoxin production in *Bacillus thuringiensis*. *Plasmid* **5**:351–365.
- Haack, B. J., R. E. Andrews, and T. E. Loynachan. 1996. Tn916-mediated genetic exchange in soil. *Soil Biol. Biochem.* **28**:765–771.
- Ishiwata, S. 1901. On a kind of severe flacherie (sotto) disease. *Dainihon Sanshi Kaiho*. **9**:1–5.
- Jarrett, P., and M. Stephenson. 1990. Plasmid transfer between strains of *Bacillus thuringiensis* infecting *Galleria mellonella* and *Spodoptera litoralis*. *Appl. Environ. Microbiol.* **56**:1608–1614.
- Kelly, W. J., and D. C. Reaney. 1984. Mercury resistance among soil bacteria: ecology and transferability of genes encoding resistance. *Soil Biol. Biochem.* **16**:1–8.
- Krieg, A., A. M. Huger, G. A. Lagenbruch, and W. Schnetter. 1983. *Bacillus thuringiensis* subsp. *tenebrionis*: a new pathotype effective against larvae of Coleoptera. *J. Appl. Entomol.* **96**:500–508.
- Martin, P. A. W., and R. S. Travers. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl. Environ. Microbiol.* **55**:2437–2442.
- Mazodier, P., and J. Davies. 1991. Gene transfer between distantly related bacteria. *Annu. Rev. Biochem.* **25**:147–171.
- Meadows, M. P., D. J. Ellis, J. Butt, P. J. Jarrett, and H. D. Burges. 1992. Distribution, frequency, and diversity of *Bacillus thuringiensis* in an animal feed mill. *Appl. Environ. Microbiol.* **58**:1344–1350.
- Reddy, A., L. Battisti, and C. B. Thorne. 1987. Identification of self-transmissible plasmids in four *Bacillus thuringiensis* subspecies. *J. Bacteriol.* **169**:5263–5270.
- Rochelle, P. A., J. C. Fry, and M. Day. 1989. Factors affecting conjugal transfer of plasmids encoding mercury resistance from pure cultures and mixed natural suspensions of epilithic bacteria. *J. Gen. Microbiol.* **135**:409–424.
- Smith, R. A., and G. A. Couche. 1991. The phylloplane as a source of *Bacillus thuringiensis* variants. *Appl. Environ. Microbiol.* **57**:311–315.
- Snedecor, G. W., and W. G. Cochran. 1989. One way and two way analysis of variance, p. 217–268. In *Statistical methods*, 8th ed. University of Iowa Press, Iowa City.
- Stephenson, M., and P. Jarrett. 1991. Transformation of *Bacillus subtilis* by electroporation. *Biotechnol. Tech.* **5**:9–12.
- Van Elsas, J. D., J. M. Govaert, and J. A. Van Veen. 1987. Transfer of plasmid pFT30 between bacilli in soil as influenced by bacterial population dynamics and soil conditions. *Soil Biol. Biochem.* **19**:639–647.
- Van Frankenhuyzen, K. 1993. The challenge of *Bacillus thuringiensis*, p. 1–35. In P. F. Entwistle, J. S. Cory, M. J. Bailey, and S. Higgs (ed.), *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. John Wiley and Sons, Chichester, United Kingdom.
- Vilas-Bóas, G. F. L. T., L. A. Vilas-Bóas, D. Lereclus, and O. M. N. Arantes. 1998. *Bacillus thuringiensis* conjugation under environmental conditions. *FEMS Microbiol. Ecol.* **25**:369–374.
- Walker, A., S. J. Welch, A. Melacini, and Y. H. Moon. 1996. Evaluation of three pesticide leaching models with experimental data for alachlor, atrazine and metribuzin. *Weed Res.* **36**:37–47.
- Whitfield, W. A. D. 1974. The soils of the National Vegetable Research Station, Wellesbourne, p. 21–30. In *Report for the National Vegetable Research Station 1973*. H.R.I., Wellesbourne, United Kingdom.