

Plasmid Transfer between *Bacillus thuringiensis* subsp. *israelensis* Strains in Laboratory Culture, River Water, and Dipteran Larvae

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Plasmid transfer between strains of *Bacillus thuringiensis* subsp. *israelensis* was studied under a range of environmentally relevant laboratory conditions in vitro, in river water, and in mosquito larvae. Mobilization of pBC16 was detected in vitro at a range of temperatures, pH values, and available water conditions, and the maximum transfer ratio was 10^{-3} transconjugant per recipient under optimal conditions. Transfer of conjugative plasmid pXO16::Tn5401 was also detected under this range of conditions. However, a maximum transfer ratio of 1.0 transconjugant per recipient was attained, and every recipient became a transconjugant. In river water, transfer of pBC16 was not detected, probably as a result of the low transfer frequency for this plasmid and the formation of spores by the introduced donor and recipient strains. In contrast, transfer of plasmid pXO16::Tn5401 was detected in water, but at a lower transfer ratio (ca. 10^{-2} transconjugant per donor). The number of transconjugants increased over the first 7 days, probably as a result of new transfer events between cells, since growth of both donor and recipient cells in water was not detected. Mobilization of pBC16 was not detected in killed mosquito larvae, but transfer of plasmid pXO16::Tn5401 was evident, with a maximum rate of 10^{-3} transconjugant per donor. The reduced transfer rate in insects compared to broth cultures may be accounted for by competition from the background bacterial population present in the mosquito gut and diet or by the maintenance of a large population of *B. thuringiensis* spores in the insects.

Bacillus thuringiensis is a gram-positive bacterium that produces insecticidal crystal protein toxins during sporulation. *B. thuringiensis* was first discovered in diseased silkworms in 1901 (19, 31) but has since been isolated from a range of environments, including insects, soil, dust from stored grain, and leaves of coniferous and deciduous trees (7, 24, 25, 28). In 1977, *B. thuringiensis* subsp. *israelensis* was isolated from a mosquito-breeding pond in the Negev Desert of Israel and was found to be highly active towards dipteran larvae (16). A number of insecticidal protein toxins that are encoded on a single 72-MDa plasmid have been identified in *B. thuringiensis* subsp. *israelensis*. Movement of plasmids within *B. thuringiensis* strains has been proposed to be the main mechanism for generating diversity in toxin genes. In addition to plasmids carrying insecticidal toxin genes, many other plasmids, such as pXO11, pXO13, pXO14, pXO15, pXO16, and pAW63, have been detected in *B. thuringiensis*, and these plasmids have no known function apart from their conjugative ability (6, 26, 32). Strains of *B. thuringiensis* can have as few as one plasmid to more than six plasmids.

Movement of plasmids between *B. thuringiensis* strains has been of interest in the construction of strains with novel toxin combinations, the study of the mechanisms of plasmid transfer in bacteria, and the study of the evolution of toxin combinations. Furthermore, since *B. thuringiensis* is closely related to

the human pathogens *Bacillus anthracis* and *Bacillus cereus*, interest has been focused on the genetic exchange systems of these bacteria in relation to biosafety (22). Plasmid movement between *B. thuringiensis* strains can be monitored directly by plasmid profiling analysis of cells. This is possible when transfer frequencies are high and all recipients in a population become transconjugants. When the transfer frequency is lower, a shuttle plasmid with an antibiotic resistance gene can be used to monitor the movement of conjugative plasmids (3). The shuttle plasmids are either mobilizable or oriT bearing, such as pBC16. Differences in the abilities of conjugative plasmids to move the reporter plasmids have been demonstrated. Green et al. (17) transposon tagged conjugative plasmid pXO12 and demonstrated that it transferred itself and pBC16 at similar rates (10^{-2} transconjugant per recipient). In contrast, transposon tagging of plasmid pXO16 showed that it was transferred to every recipient cell but mobilized pBC16 only at a frequency of approximately 10^{-3} to 10^{-4} transconjugant per recipient (1).

Very few studies have examined plasmid transfer between *B. thuringiensis* strains and, in particular, between *B. thuringiensis* subsp. *israelensis* strains in the environment. Haack et al. (18) reported transfer of the broad-host-range conjugative transposon Tn916 between *Bacillus subtilis* and *B. thuringiensis* subsp. *israelensis* in nonsterile sandy soil. Significantly, although *B. thuringiensis* is applied to waterways to control pests such as mosquitoes and blackflies, no studies have examined plasmid transfer between *B. thuringiensis* strains in water yet. In nature *B. thuringiensis* strains may find themselves in the midgut environment of killed susceptible larvae. This environment has

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TABLE 1. Bacterial strains and plasmids

Strain	Plasmid	Relevant genotype or characteristics	Source or reference
<i>B. thuringiensis</i> subsp. <i>kurstaki</i> HD1 cry Sm ^r		Cry ^r Sm ^r	20
<i>B. thuringiensis</i> subsp. <i>israelensis</i> IPS82		cry4A cry4B	16
<i>B. thuringiensis</i> subsp. <i>israelensis</i> IPS82(pBC16)	pBC16	cry11A cry1A Tc ^r	This study
<i>B. thuringiensis</i> subsp. <i>israelensis</i> IPS78(pXO16)	pXO16::Tn5401	Tc ^r	This study
<i>B. thuringiensis</i> subsp. <i>israelensis</i> 658 cry Sm ^r		Cry ^r Sm ^r	P. Jarrett
<i>B. thuringiensis</i> subsp. <i>israelensis</i> AND931(pXO16::Tn5401)	pXO16::Tn5401	Tc ^r Sm ^r	2
<i>B. thuringiensis</i> subsp. <i>israelensis</i> GBJ002/IPS70		Nal ^r	21

proven to be conducive to gene exchange. Studies have found that plasmids can move between donor and recipient strains of *B. thuringiensis* in larvae of the lepidopteran insects *Galleria mellonella*, *Spodoptera littoralis*, and *Lacanobia oleracea* at levels similar to that found in broth culture (20, 30) but not in the midgut environment of the coleopteran insect *Phaedon cochleariae*. So far, there have been no studies of transfer between *B. thuringiensis* strains in dipteran insects.

The primary objective of this study was to obtain information regarding mobilization and transfer of plasmids between *B. thuringiensis* subsp. *israelensis* strains under environmentally realistic conditions. Insecticidal strains of *B. thuringiensis* subsp. *israelensis* were constructed with either a mobilizable plasmid (pBC16) or a transposon-tagged conjugative plasmid (pXO16::Tn5401) so that gene transfer in laboratory media, water, and insects could be studied.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this study are listed in Table 1. All strains were kept at -80°C in storage medium (Protect; Technical Consultants Limited). To maintain the plasmid compositions of *B. thuringiensis* strains used frequently in this work, cultures were stored as 5-ml aliquots in sterile bijoux bottles at -20°C in 20% (vol/vol) glycerol. Every month, a bijoux bottle was removed from the freezer and the contents were thawed, streaked onto nutrient agar (Oxoid, Basingstoke, Hampshire, United Kingdom), and grown at 30°C for 2 days. The plate was kept as a source of inoculum at 5°C for up to 1 month, after which a new inoculum plate was prepared from glycerol stocks.

Construction of *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) and IPS78 (pXO16::Tn5401). Plasmid pBC16 was introduced into *B. thuringiensis* subsp. *israelensis* IPS82 by electroporation by using a field strength of 8.75 kV/cm (400 Ω , 25 μF , 1.75 kV) and a Bio-Rad gene pulser (30). *B. thuringiensis* subsp. *israelensis* IPS78(pXO16::Tn5401) was constructed by plasmid mating. *B. thuringiensis* subsp. *israelensis* AND931(pXO16::Tn5401) contains a selectable tetracycline resistance gene marker on transposon Tn5401. This strain is also resistant to streptomycin (chromosomal mutation), but it does not contain the insecticidal proteins present in the original strain. To create a marked insecticidal strain, *B. thuringiensis* subsp. *israelensis* AND931(pXO16::Tn5401) was mated with *B. thuringiensis* subsp. *israelensis* IPS78. The cell mixture was plated on nutrient agar containing 5 μg of tetracycline ml^{-1} , which selected for strains harboring pXO16::Tn5401, and included both the donor and transconjugant strains. Since pXO16::Tn5401 transfers at a high frequency, all recipients should have become transconjugants, and these transconjugants could have accounted for up to 50% of the population obtained. One hundred colonies were examined by light microscopy for the presence of ovoid insecticidal protein toxin crystals, which were present only in the recipient strain. All colonies with ovoid inclusions were then retested for the ability to grow on nutrient agar containing 50 μg of streptomycin ml^{-1} . Any colonies unable to grow on media containing streptomycin were confirmed to be *B. thuringiensis* subsp. *israelensis* IPS78(pXO16::Tn5401) colonies.

Standard broth mating procedure. The standard broth mating procedure used was that described by Thomas et al. (30). Briefly, a single colony was used to inoculate separately 50 ml of brain heart infusion (BHI) (Oxoid) broth containing the appropriate antibiotic and incubated at 30°C for 18 h with shaking (40

rpm). The overnight culture was diluted to an optical density at 600 nm of 1.1 (approximately 10^8 CFU ml^{-1}) in 0.25 \times Ringer's solution (BDH). Aliquots (0.5 ml) of both donor and recipient cell suspensions were added to three 250-ml conical flasks containing 50 ml of pre-warmed BHI broth and incubated at 30°C for 6 h with shaking (40 rpm). Three broth preparations with donor cells and three preparations with recipient cells were treated in the same fashion and used as controls. After the incubation period, samples were serially diluted in 0.25 \times Ringer's solution and spread plated onto nutrient agar containing the appropriate antibiotics to select for donor, recipient, and transconjugant CFU. In experiments in which mobilization of pBC16 was studied, the bacteria were enumerated on nutrient agar containing 25 μg of tetracycline ml^{-1} (donor), 50 μg of streptomycin ml^{-1} (recipient), or both 25 μg of tetracycline ml^{-1} and 50 μg of streptomycin ml^{-1} (transconjugant). When the transfer of pXO16::Tn5401 was measured, the bacteria were enumerated on nutrient agar containing 5 μg of tetracycline ml^{-1} (donor), 25 μg of nalidixic acid ml^{-1} (recipient), or both 5 μg of tetracycline ml^{-1} and 25 μg of nalidixic acid ml^{-1} (transconjugant). The effects of time, temperature, pH, and salt concentration on plasmid transfer were measured by using the standard broth mating procedure with appropriate modifications (30).

Survival and gene transfer in water. Donor and recipient *B. thuringiensis* strains were used to inoculate individual nutrient agar plates containing the appropriate antibiotics. After incubation at 30°C for 24 h, a single colony was used to inoculate 50 ml of BHI broth, and the inoculated flask was incubated at 30°C for 18 h with shaking (40 rpm). The overnight culture was pelleted by centrifugation at 2,000 $\times g$ for 5 min and resuspended and diluted to an optical density at 600 nm of 1.1 (approximately 10^8 CFU ml^{-1}) in filtered (pore size, 0.22 μm ; Whatman) river water. The water was collected from the River Avon at Tiddington, Stratford-upon-Avon, United Kingdom, in August 1997 and October 1999 for experiments to measure the movement of pBC16 and pXO16::Tn5401, respectively. To three 90-ml replicates of fresh nonsterile River Avon water in 250-ml conical flasks, 10 ml of a bacterial suspension containing donor and recipient cells (ratio, 1:1) suspended in filtered river water was added, and the preparations were incubated at 10 and 25°C . Flasks inoculated with only donor and recipient cells and flasks containing only filtered river water were included and treated in the same fashion. Samples were taken, diluted in 0.25 \times Ringer's solution, and spread plated onto nutrient agar alone and nutrient agar containing the relevant antibiotics to determine the levels of background, donor, recipient, and transconjugant populations. In addition, samples were heated at 70°C for 20 min and dilution plate counted on the same media described above to determine the numbers of donor and recipient spores.

Insect rearing. Mosquito eggs (*Aedes aegypti*, rock strain) on filter paper were obtained from the School of Biological Sciences, Manchester University, Manchester, United Kingdom. Eggs were hatched by placing the filter paper on which they were laid in a Buchner flask containing 100 ml of distilled water under a vacuum (-0.65×10^5 Pa) for 1 h to break open the egg casings. The larvae were transferred to a 5-liter polyethylene bowl containing hamster feed pellets and incubated at 25°C for up to 1 week.

Studies with *A. aegypti* larvae. Spore and crystal mixtures of donor and recipient strains were prepared from nutrient agar plates that had been incubated at 30°C for 5 days. Spores and crystals were resuspended in sterile 0.25 \times Ringer's solution to an optical density at 600 nm of 1.1 (approximately 1×10^8 CFU ml^{-1}). Gene transfer was studied in second- or third-instar larvae of the mosquito (*A. aegypti*). Aliquots (250 ml) of donor and recipient cell suspension were added to 25 ml of distilled water containing 12 mosquito larvae in a plastic autoclavable beaker. Water microcosms were incubated at 25°C with a light cycle consisting of 18 h of light (24 W m^{-2}) and 6 h of darkness and 65% relative humidity. Microcosms containing only mosquito larvae and donor inocula, only mosquito larvae and recipient inocula, and only mosquito larvae and water were

included as controls and treated in the same fashion. Samples of three pooled mosquito larvae from each of the three replicate beakers were taken after 4, 24, and 48 h. The three mosquito larvae were washed in 1 liter of fresh sterile distilled water and placed in 1 ml of sterile distilled water in a 1.5-ml Eppendorf tube. A sterile pellet resuspender was used to crush the larvae, and dilutions were made in sterile 0.25× Ringer's solution and plated onto nutrient agar alone and nutrient agar containing the relevant antibiotics to select for transconjugant, donor, recipient, and background populations. In addition, samples were heat treated at 70°C for 20 min and dilution plate counted to determine the numbers of donor and recipient spores in samples.

Statistical analysis. For time course studies and quantitative-level treatments (e.g., temperature, pH, and available water) a one-way model of analysis of variance was used (29). Differences between times and levels were expressed by using a least significant difference bar. In some instances a two-way analysis of variance was used when two factors were compared.

RESULTS

Strain construction. *B. thuringiensis* subsp. *israelensis* IPS82 (pBC16) was shown to contain plasmid pBC16 and was insecticidal to susceptible *A. aegypti* larvae. In the standard mating broth *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) was unable to mobilize pBC16 to a *B. thuringiensis* subsp. *kurstaki* HD1 *cry* Sm^r recipient. However, plasmid pBC16 was mobilized efficiently from this strain to the recipient *B. thuringiensis* subsp. *israelensis* HD658 *cry* Sm^r. The transconjugant strain *B. thuringiensis* subsp. *israelensis* IPS78(pXO16::Tn5401) was also shown to be insecticidal and capable of transferring plasmid pXO16::Tn5401 into *B. thuringiensis* subsp. *israelensis* GBJ002 (IPS70).

Transfer of pBC16 and pXO16::Tn5401 in liquid culture. Mobilization of pBC16 from *B. thuringiensis* subsp. *israelensis* IPS82 to *B. thuringiensis* subsp. *israelensis* 658 *cry* Sm^r was studied over a 24-h period (data not shown). The donor and recipient strains had antagonistic effects on each other. There was a slight, nonsignificant increase in the number of donors from 7.1×10^5 CFU ml⁻¹ at the time of inoculation to 1.7×10^6 CFU ml⁻¹ after 2 h. This was followed by a decrease in the number of donors to 4.6×10^5 CFU ml⁻¹ after 6 h of incubation. Subsequently, the number of donors increased to 7.8×10^7 CFU ml⁻¹ after 10 h and remained at this level for the rest of the experiment, whereas there was an initial rise in the number of recipients from 3.9×10^5 CFU ml⁻¹ at the time of inoculation to 1.4×10^7 CFU ml⁻¹ after 6 h of incubation. The highest density of recipients was detected after 6 h, and thereafter the density decreased to 4.1×10^4 CFU ml⁻¹ after 24 h. The number of transconjugants detected initially after 2 h was 5.3×10^1 CFU ml⁻¹, and the number increased to 1.1×10^4 after 10 h of incubation, after which there was a decline in the size of the population to 2.5×10^2 CFU ml⁻¹ after 24 h. The increase in the number of donor cells resulted in an accompanying decrease in the number of recipient cells, which was reflected in the number of transconjugants. The curve for the number of transconjugants produced reflected the curve for the number of recipients. When transfer of pXO16::Tn5401 between *B. thuringiensis* subsp. *israelensis* IPS78 (pXO16::Tn5401) and *B. thuringiensis* subsp. *israelensis* GBJ002 after as little as 4 h was studied, the transfer ratio was found to be 1.0; in effect, all of the recipients gained a copy of the plasmid (data not shown).

The effect of temperature on transfer of pBC16 between *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) and *B. thuringiensis* subsp. *israelensis* 658 *cry* Sm^r is shown in Fig. 1A. There was

not a significant difference between the final population sizes of the donor after incubation at different temperatures, and this was also true for the recipient. The largest number of transconjugants was detected at 25°C (1.2×10^4 CFU ml⁻¹). The lowest transfer ratio was recorded at 22.5°C. The transfer ratio increased to 8.9×10^{-3} transconjugant per donor at 25°C. No significant decrease in the transfer ratio was observed between 25 and 37°C although the ratio varied by almost 1 order of magnitude. Over this temperature range the average transfer ratio was 8×10^{-2} transconjugant per donor. The highest transfer ratio per recipient was observed at 37°C (3.4×10^{-4} transconjugant per recipient) due to the decrease in the number of recipient cells. When the effect of temperature on the transfer of pXO16::Tn5401 from *B. thuringiensis* subsp. *israelensis* IPS78 (pXO16::Tn5401) to *B. thuringiensis* subsp. *israelensis* GBJ002 was studied, the results were comparable to the results obtained for transfer of pBC16 (Fig. 1B). The transfer ratio was approximately 1 transconjugant per donor or recipient between 22.5 and 30°C and then decreased to 4.5×10^{-2} transconjugant per donor and 5×10^{-2} transconjugant per recipient at 37°C.

The effects of pH on mobilization of pBC16 from *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) to *B. thuringiensis* subsp. *israelensis* 658 *cry* Sm^r and transfer are shown in Fig. 1C. The pH of the broth did not have a significant effect on growth of the donor strain at pH values between 5.8 and 8.8. The largest numbers of transconjugants were seen between pH 7.1 and 8.1 (1.0×10^4 to 1.3×10^4 CFU ml⁻¹). No reduction in the number of donor cells was observed at pH 8.4. Consequently, due to the decrease in the number of transconjugants at this pH, the transfer ratio expressed on a per-donor basis decreased significantly from 7.8×10^{-3} at pH 7.9 to 3.7×10^{-5} at pH 8.4.

Similar results were obtained when the transfer of pXO16::Tn5401 between *B. thuringiensis* subsp. *israelensis* IPS78(pXO16::Tn5401) and *B. thuringiensis* subsp. *israelensis* GBJ002 was studied over a range of pH values (Fig. 1D). The transfer ratio remained approximately 1 at pH values between 6.0 and 8.0. Growth of the recipient bacterium decreased significantly at pH values above pH 7.5. Consequently, the number of transconjugants and the transfer ratio decreased to 2×10^{-1} CFU ml⁻¹ and 1.2×10^{-1} , respectively, at pH 8.4 and 8.8. At pH 9.1 the sizes of both the donor and recipient populations decreased significantly, and no transconjugants were detected.

The effects of water activity on mobilization of pBC16 and growth of *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) and *B. thuringiensis* subsp. *israelensis* 658 *cry* Sm^r are shown in Fig. 1E. The numbers of donor and recipient bacteria fell gradually from 4.9×10^7 and 1.9×10^7 CFU ml⁻¹, respectively, at 0.5% (wt/vol) NaCl to 6.3×10^4 and 1.1×10^5 CFU ml⁻¹, respectively, at 5% (wt/vol) NaCl. Mobilization of pBC16 from *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) to *B. thuringiensis* subsp. *israelensis* 658 *cry* Sm^r was found to occur at NaCl concentrations between 0.5 and 3% (wt/vol) (Fig. 1E). The decreases in the numbers of donor and recipient cells in this mating experiment at NaCl concentrations of 0.5 to 3% (wt/vol) were mirrored by concomitant decreases in the number of transconjugants. The transfer ratio decreased from 3.6×10^{-4} at 0.5% (wt/vol) NaCl to 6.7×10^{-5} at 3% (wt/vol) NaCl. Plasmid mobilization was not detected at NaCl concentrations

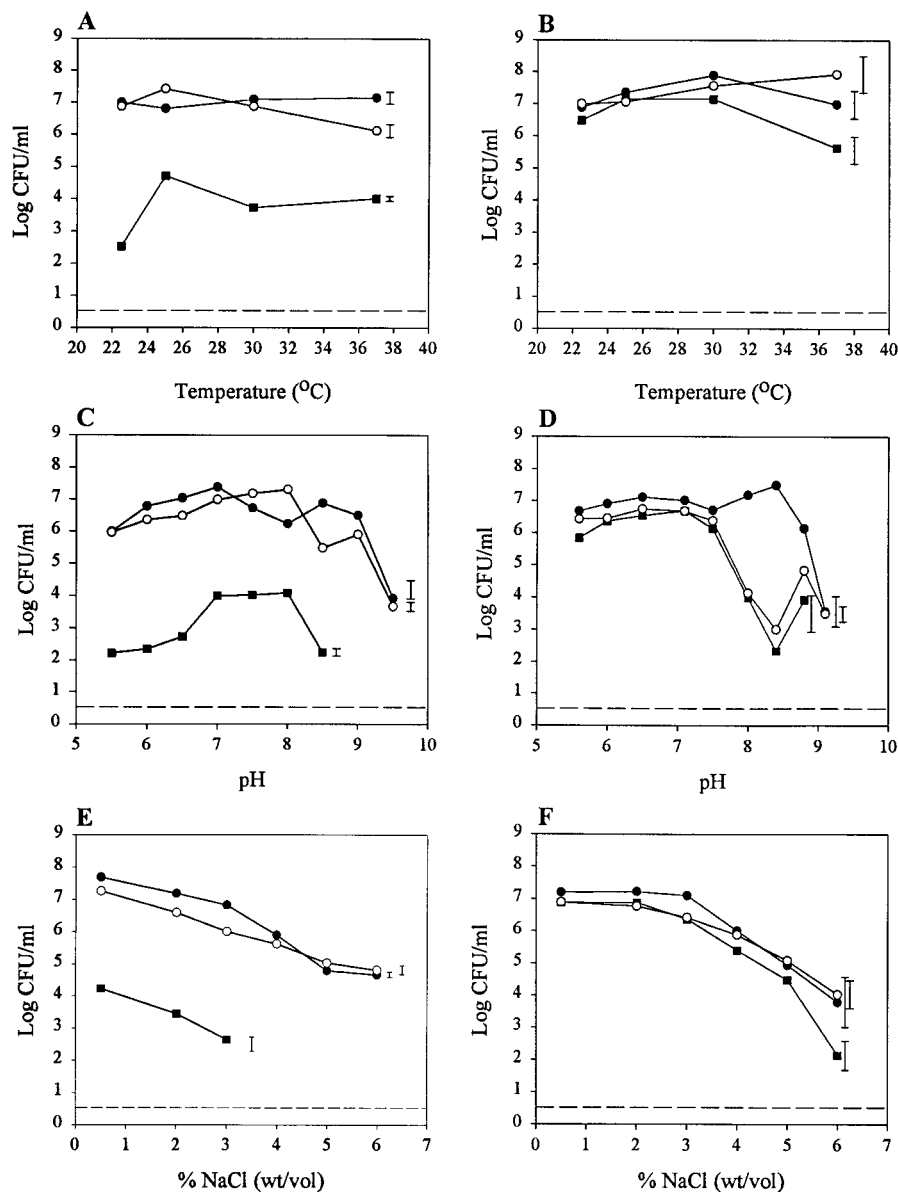


FIG. 1. Effects of various culture conditions on growth of strains and transfer of plasmids pBC16 and pXO16::Tn5401 in *B. thuringiensis* subsp. *israelensis*. (A, C, and E) Studies with *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) and *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r. Symbols: ●, *B. thuringiensis* subsp. *israelensis* IPS82(pBC16); ○, *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r; ■, transconjugant. (B, D, and F) Studies with *B. thuringiensis* subsp. *israelensis* IPS78(pXO16::Tn5401) and *B. thuringiensis* subsp. *israelensis* GBJ002. Symbols: ●, *B. thuringiensis* subsp. *israelensis* IPS78(pXO16::Tn5401); ○, *B. thuringiensis* subsp. *israelensis* GBJ002; ■, transconjugant. The effects of temperature (A and B), pH (C and D), and NaCl concentration (E and F) were examined. The dashed lines indicate the detection limit. The error bars indicate the least significant differences of the means ($n = 3$; $P < 0.05$).

greater than 3% (wt/vol). Again, a similar pattern was seen when transfer of pXO16::Tn5401 between *B. thuringiensis* subsp. *israelensis* IPS78 (pXO16::Tn5401) and *B. thuringiensis* subsp. *israelensis* GBJ002 was studied. There were decreases in the numbers of donor and recipient bacteria at NaCl concentrations between 3 and 6% (wt/vol) (Fig. 1F). The transfer ratios also decreased from 1 transconjugant per donor or recipient at 2% (wt/vol) NaCl to 2.4×10^{-2} transconjugant per donor and 1.4×10^{-2} transconjugant per recipient at 6% (wt/vol) NaCl, but notable numbers of transconjugants could still be detected at the highest NaCl concentration studied.

Survival of *B. thuringiensis* and mobilization of pBC16 in river water. Mobilization experiments were performed with nonsterile river water which was incubated in the dark without shaking at 10°C, a typical temperature encountered in a river in a temperate region, and at 25°C, the temperature at which mobilization for this strain was found to take place most efficiently in laboratory media (Fig. 1A). *B. thuringiensis* subsp. *israelensis*(pBC16) and *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r were coinoculated into river water at levels of 6.3×10^6 and 4.8×10^6 CFU ml⁻¹, respectively, and incubated at 10°C (Fig. 2A). The number of *B. thuringiensis* subsp. *israelensis*

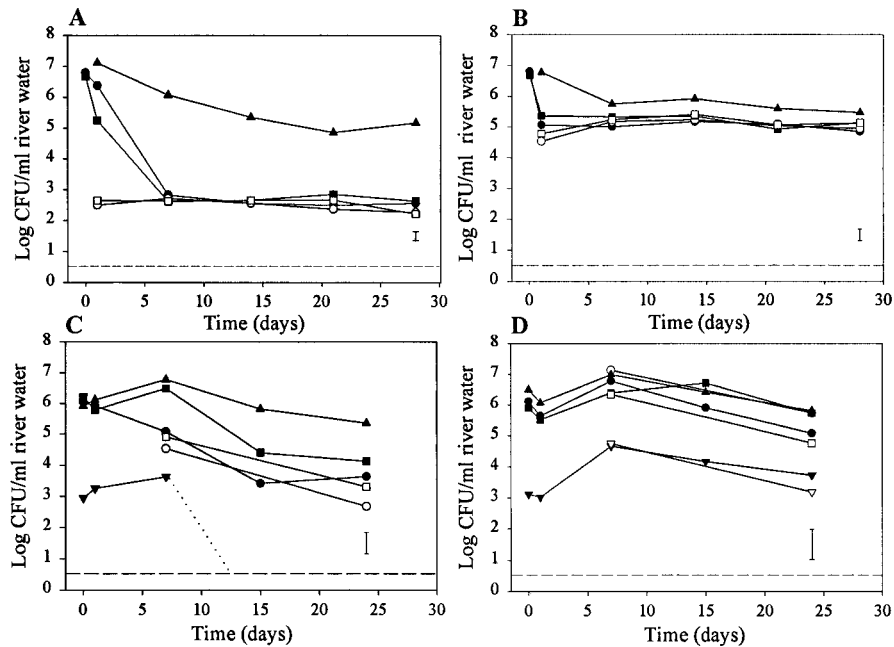


FIG. 2. Survival of *B. thuringiensis* strains and transfer of plasmids pBC16 and pXO16::Tn5401 in river water at 10°C (A and C) and 25°C (B and D). (A and B) *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) was used as the donor strain, and *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r was used as the recipient. (C and D) *B. thuringiensis* subsp. *israelensis* IPS78 (pXO16::Tn5401) was used as the donor, and *B. thuringiensis* subsp. *israelensis* GBJ002 was used as the recipient strain. Symbols: ▼, transconjugant cells; ▽, transconjugant spores; ●, donor cells; ○, donor spores; ■, recipient cells; □, recipient spores; ▲, CFU on nutrient agar. The dashed lines indicate the detection limit; the dotted line illustrates a decline to the detection limit. The error bars indicate the least significant differences of the means ($n = 3$; $P < 0.05$).

(pBC16) cells fell slightly to 2.4×10^6 CFU ml⁻¹ after 1 day of incubation, whereas the number of *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r cells declined to 2.3×10^5 CFU ml⁻¹ after 1 day of incubation. Subsequently, the total sizes of the donor and recipient populations declined sharply to 6.9×10^2 and 4.2×10^2 CFU ml⁻¹, respectively, after 7 days of incubation. The proportion of the population present as spores increased from 0.0064% of the CFU detected at inoculation to nearly 88% of the total *B. thuringiensis* CFU detected after 7 days of incubation, due to a rapid decline in the size of the population of vegetative cells. The donor and recipient bacteria were then maintained in the river water at levels around 4×10^2 CFU ml⁻¹, predominantly as spores. During this experiment, the size of the background culturable population was monitored and was found to increase from an ambient level of 8.7×10^3 to 1.3×10^7 CFU ml⁻¹ 1 day after inoculation of the donor and recipient populations. However, once this initial high level was attained, the population size declined gradually during the experiment and was 1.5×10^5 CFU ml⁻¹ after 28 days. No transconjugants were detected. The background culturable population during the experiment was predominantly composed of bacteria that did not display the colony morphology of *B. cereus* and *B. thuringiensis*. When *B. thuringiensis* subsp. *israelensis*(pBC16) and *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r were inoculated individually into river water, a similar pattern of survival was observed when the results were compared to the results of the coinoculation experiments (data not shown).

In water kept at 25°C the sizes of the *B. thuringiensis* subsp. *israelensis*(pBC16) and *B. thuringiensis* subsp. *israelensis* 658 cry

Sm^r populations declined from 6.3×10^6 and 4.8×10^6 CFU ml⁻¹, respectively, immediately after inoculation to 1.2×10^5 and 2.2×10^5 CFU ml⁻¹, respectively, after 24 h of incubation in river water (Fig. 2B). The numbers of spores of *B. thuringiensis* subsp. *israelensis* (pBC16) and *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r increased slightly from 3.4×10^4 and 6.1×10^4 CFU ml of river water⁻¹, respectively, after 1 day to 1.5×10^5 and 1.8×10^5 CFU ml of river water⁻¹, respectively, after 7 days of incubation. Plasmid mobilization was not detected when the donor and recipient strains were incubated at 25°C. The sizes of the populations of donor and recipient bacteria remained approximately 1×10^5 CFU ml⁻¹ during the experiment (28 days). The size of the culturable bacterial population present in the river water increased from 8.6×10^3 to 6.0×10^6 CFU ml⁻¹ after 24 h following inoculation of the donor and recipient strains, and the organisms in this and subsequent samples collected during the experiment were found to predominantly display the morphology of *B. cereus* and *B. thuringiensis* strains. Subsequently, the numbers of culturable bacteria present in the river water declined to approximately 5×10^5 CFU ml⁻¹ during the experiment. When *B. thuringiensis* subsp. *israelensis*(pBC16) and *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r were inoculated individually into river water, the patterns of survival were similar to that observed when the donor and recipient strains were coinoculated (data not shown).

Survival of *B. thuringiensis* and transfer of pXO16::Tn5401 in river water. *B. thuringiensis* subsp. *israelensis* IPS78(pXO16::Tn5401) and *B. thuringiensis* subsp. *israelensis* GBJ002 were coinoculated into river water at levels of 6.3×10^6 and $4.8 \times$

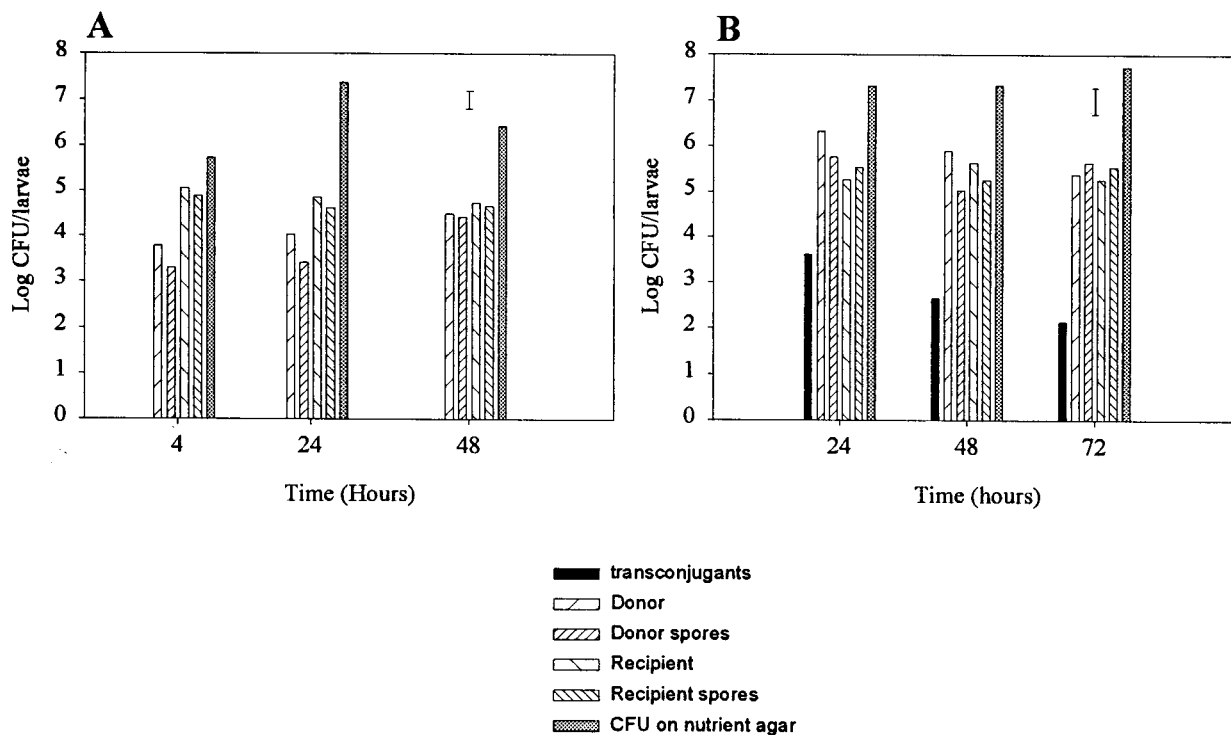


FIG. 3. Growth of *B. thuringiensis* strains and transfer of pBC16 and pXO16::Tn5401 in *A. aegypti* larvae. (A) *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) donor and *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r recipient. (B) *B. thuringiensis* subsp. *israelensis* IPS78(pXO16::Tn5401) donor and *B. thuringiensis* subsp. *israelensis* GBJ002 recipient. The error bars indicate the least significant differences of the means ($n = 3$; $P < 0.05$).

10^6 CFU ml⁻¹, respectively, and incubated at 10 and 25°C. Figures 2C and D show the survival of the donor and recipient strains. At both temperatures, the sizes of the populations of both donor and recipient bacteria exhibited slight, nonsignificant decreases after 1 day and slight, nonsignificant increases after 7 days. Subsequently, the sizes of all the bacterial populations monitored slowly decreased in the river water, and after 30 days the bacteria were detected predominantly as spores. Transfer of pXO16::Tn5401 between the donor and recipient strains was detected at both temperatures initially after entry into water and at later sampling times. At 10°C the transfer ratio was initially 7.7×10^{-4} transconjugant per donor and rose to 3.5×10^{-2} transconjugant per donor on day 7. However, the number of transconjugants declined to undetectable levels by 15 days after inoculation (Fig. 2C). At 25°C, initially the size of the transconjugant population was 1.3×10^3 CFU ml⁻¹, and the size of this population increased significantly to 3.0×10^4 CFU ml⁻¹ after 7 days. After this the transconjugant population was found to be composed predominantly of spores. The transfer ratio increased during the experiment from 9.7×10^{-4} to 4.4×10^{-2} transconjugant per donor.

Mobilization of pBC16 in *A. aegypti* larvae. Third- or fourth-instar larvae of *A. aegypti* were fed the donor *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) along with the recipient *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r. After 4 h all the *A. aegypti* larvae were dead. Altogether, the total number of donor bacteria per larva increased from 6.1×10^3 CFU larva⁻¹ after 4 h to 3.2×10^5 CFU larva⁻¹ after 48 h of incubation (Fig. 3A). The number of recipient bacteria remained approx-

imately 8×10^4 CFU larva⁻¹ throughout the experiment. There was not a significant difference in the number of donor or recipient spores at any time point. Mobilization of pBC16 between *B. thuringiensis* subsp. *israelensis* IPS82(pBC16) and *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r was not detected. Microscopic examination of the dead larvae indicated that bacteria other than *B. thuringiensis* dominated the cadavers. This was reflected in the higher number of background bacteria detected, which increased from 5.6×10^5 CFU larva⁻¹ after 4 h to 2.5×10^7 CFU larva⁻¹ after 48 h.

The total number of bacteria in the donor control increased from 1.8×10^3 CFU larva⁻¹ after 24 h to 3.8×10^4 CFU larva⁻¹ after 48 h (data not shown). The levels of background bacteria were much higher in all samples, reaching a maximum of 3.4×10^7 CFU larva⁻¹ after 48 h. In the recipient control, live mosquitoes were sampled during the entire experiment, and the number of recipient cells and spores remained approximately 3×10^5 CFU larva⁻¹ during the experiment. The level of background bacteria in the recipient control increased during the experiment from 3.8×10^5 CFU larva⁻¹ after 4 h to 4.6×10^7 CFU larva⁻¹ after 48 h. The level of background bacteria increased in the water-only control to a maximum of 3.4×10^5 CFU larva⁻¹ after 48 h of incubation.

Transfer of pXO16::Tn5401 in *A. aegypti* larvae. *A. aegypti* larvae were fed a lethal dose of the donor *B. thuringiensis* subsp. *israelensis* IPS78(pXO16::Tn5401) along with an equal dose of the recipient *B. thuringiensis* subsp. *israelensis* GBJ002. After 4 h all larvae were dead in microcosms that contained the active *B. thuringiensis* subsp. *israelensis* IPS78 (pXO16::

Tn5401) strain. The total number of donor and recipient bacteria remained between 5×10^5 and 5×10^6 CFU larva⁻¹ between 24 and 72 h (Fig. 3B). There was not a significant difference in the number of donor or recipient spores at any sampling point. The level of background bacteria remained approximately 3×10^7 CFU larva⁻¹. Transconjugants were detected after 24 h at a level of 4.8×10^3 CFU larva⁻¹; after this the level of transconjugants decreased to 1.6×10^3 and 1.6×10^2 CFU larva⁻¹ at 48 and 72 h, respectively. No plasmid transfer between donor and recipient in water initially inoculated with spores was detected during the 3-day study.

The donor-only control behaved in a similar fashion, with both the donor and background bacteria present at high levels (data not shown). In the recipient control experiment, in which live mosquitoes were sampled during the entire experiment, the number of recipient cells and spores remained approximately 7×10^4 CFU larva⁻¹. The level of background bacteria in the recipient control remained approximately 1×10^5 CFU larva⁻¹ throughout the experiment. The number of background bacteria in the water-only control remained approximately 5×10^4 CFU larva⁻¹ (data not shown).

DISCUSSION

The widespread occurrence of different toxin genes and plasmids in *B. thuringiensis* strains suggests that conjugation may be an important means for dissemination of these genes and plasmids in *Bacillus* populations in nature. Constructed strains were used in sensitive studies to monitor the effects of a range of physical and chemical parameters on plasmid exchange between *B. thuringiensis* strains (Fig. 1). Initial experiments indicated that plasmid pBC16 could not be transferred from *B. thuringiensis* subsp. *israelensis* IPS82 to *B. thuringiensis* subsp. *kurstaki* cry Sm^r, a strain which had previously been successfully used in studies to monitor mobilization of pBC16 under environmental conditions (30). However, these experiments did indicate that pBC16 could be mobilized to another strain, *B. thuringiensis* subsp. *israelensis* 658 cry Sm^r, with an average transfer ratio of 8×10^{-2} . For transfer of pXO16::Tn5401, the recipient *B. thuringiensis* subsp. *israelensis* GBJ002 has previously been shown to act as an efficient recipient with a transfer ratio of 1.0, as found here (2, 21). On occasion, the donor and recipient strains were found to have a slight antagonistic effect on each other even when the donor and recipient strains were essentially the same except for their plasmid compositions (IPS70, IPS78, IPS82). Previous studies of plasmid transfer between *B. thuringiensis* strains in laboratory broth have shown that *B. thuringiensis* subsp. *israelensis* donors can be antagonistic to recipients (21). This can have a significant effect on the transfer rate detected. In one study the presence of bacteriocins was shown to be responsible for antagonism (26). Since the genes for production of and resistance to antagonistic molecules could be plasmid or phage encoded, production of plasmid-free strains that can act as recipients may still provide strains that are sensitive to the donor strain. This could explain the results seen in our experiments. However, the antagonistic interaction observed between *B. thuringiensis* strains is also likely to occur in the environment and to hinder gene exchange.

Plasmid transfer was detected quickly, in some cases after as

little as 2 h. When different temperatures were used, pBC16 and pXO16::Tn5401 were found to transfer better at lower temperatures ($\leq 30^\circ\text{C}$). This behavior is similar to that of *B. thuringiensis* subsp. *kurstaki*, whereas *B. thuringiensis* subsp. *tenebrionis* mobilized pBC16 better at higher temperatures (between 30 and 37°C) (30). The experiments described in this paper were conducted in laboratory broth or under nonselective conditions under which growth of the donor, recipient, and transconjugant could take place. This may have affected interpretation of the genuine transfer frequency, and hence the term transfer ratio was used instead (30).

No clear pH optimum was observed for plasmid transfer. This result is comparable to the results of Rochelle et al. (27), who found that in *Pseudomonas* species, plasmid pQM1 exhibited a clear peak transfer ratio related to pH only at a suboptimal temperature, 37°C . Greater variation between replicates was observed at pH values at the limits at which the donor and recipient strains were able to grow. Although the present experiments were designed to allow for growth of donor and recipient cells together, some results, such as those obtained at the pH extremes, showed that an increase in the total population size was not required for transfer to take place. In the case of transfer of pXO16::Tn5401, even decreases in the numbers of donor and recipients could be tolerated. However, at high salt levels both growth and plasmid transfer decreased. Growth of donor and recipient cells together has been implicated in a higher transfer ratio (20). In the present study, conditions that allowed optimal growth produced the greatest transfer ratios and transconjugant numbers. However, even under relatively severe pH and water activity conditions that did not allow growth of the donor or recipient bacteria, pXO16::Tn5401 was shown to transfer at relatively high levels.

The results obtained for plasmid transfer between strains of *B. thuringiensis* subsp. *israelensis* in river water demonstrated that at lower temperatures, the number of cells able to form spores was significantly decreased and, hence, the surviving population was smaller than that found at a higher temperature. This was clearly the case when mobilization of pBC16 was studied. This was unexpected, as other authors have reported that in the presence of a natural microbiota (as in the present study), survival is reduced at higher temperatures due to increased predation by protozoans (4). In the soil environment, in contrast to their effect on *Pseudomonas* species, protozoans do not affect *B. thuringiensis* spore populations, possibly due to toxicity of the spores or crystals (8). However, in the aquatic environment *B. thuringiensis* subsp. *israelensis* has been shown to actively grow in the food vacuoles of the protozoan *Tetrahymena pyriformis* (23). *B. thuringiensis* subsp. *israelensis* has been shown to form spores efficiently after a nutritional and osmotic downshift at 32°C under sterile conditions (12). In the present study the best spore survival was seen at 25°C in the presence of an indigenous population. Although no transfer of pBC16 was detected in water, transfer of pXO16::Tn5401 was detected at both temperatures examined. Since transfer was detected on day 0, transfer in freshly cultured cells that were in an active state could have been the only transfer that took place. This transfer would still have had to occur in water, as both strains were kept separate until they were mixed in the final water sample. However, the transfer ratio at this point was only 9.7×10^{-4} transconjugant per donor, compared to

the transfer ratio of 1.0 transconjugant per donor that was commonly obtained for pXO16::Tn5401 in laboratory broth. Certainly, Andrup et al. (2) found that pXO16::Tn5401 was able to transfer in as little as 3.5 to 4 min in laboratory broth. However, the increase in the number of transconjugants over the first 7 days was significant, and no significant increase in the size of the donor or recipient population was detected. This indicates that plasmid transfer between cells occurred over a longer period in water or (less likely) that the transconjugant population grew under conditions under which the parental strains did not grow. The increase in the transfer ratio on day 7 coincided with the start of sporulation for the donor and recipient strains and may indicate that stationary-phase cells or cells in the early stages of sporulation are better able to transfer plasmids.

In *A. aegypti* larvae, as *B. thuringiensis* subsp. *israelensis* utilized the cadavers the sizes of the populations increased. However, the size of the background population increased more dramatically, and this population dominated the resources available. Mobilization of plasmid pBC16 between *B. thuringiensis* strains in killed insects was not detected in the present study. Although movement of pXO16::Tn5401 was detected in *A. aegypti* larvae, the transfer ratio was significantly lower (10^{-3} transconjugant per donor) than that observed in broth cultures. Transfer between donors and recipients in water was not detected in these experiments; this was probably due to the fact that spores were added and did not germinate until they were within the killed larvae. The lower transfer ratio of the normally highly potent plasmid and the lack of transfer of pBC16 may have been due to the poor environmental or microbiological conditions present in the larval gut. However, it may have been within the insect larvae that only a fraction of the engulfed spores were able to germinate and that the plasmid did indeed transfer to all of the available recipient cells, as it may do in broth matings. It was not possible to determine the numbers of donor and recipient cells in the samples without also counting spores. In killed larvae, the levels of background bacteria present in the larval cadavers may have affected the plasmid transfer rate. It is likely that a high level of background bacteria decreased the plasmid transfer rate, probably due to competition for resources. However, if the transfer rate is lower than the total number of donors and recipients that can be supported by an individual larva, transfer may not be detected unless a large sample of insects is studied. In the case of the mosquito *A. aegypti*, the larvae usually congregate at the surface of water when they are killed, before the cadavers eventually disintegrate and fall to the bottom and produce, as a mass, a unit larger than an individual insect. Therefore, during this process, a larger number of cells may come into contact with each other near the sediment surface, and this may be an area where increased gene transfer occurs.

Due to the high rates of transfer seen in this work, it is not surprising that gene transfer between *B. thuringiensis* and non-*B. thuringiensis* species seems to have taken place during evolution. The high numbers of background bacteria found in some experiments indicate that such transfer may be more likely than transfer to *B. thuringiensis* strains. It was only comparatively recently that strains of the anaerobic bacterium *Clostridium bifermentans* subsp. *malaysia* that contain *cry* genes were discovered, indicating that gene exchange between two

very different species has taken place (5). Coincidentally, both *B. thuringiensis* and *C. bifermentans* spores are activated by the alkaline pH values (15, 33) which are normally encountered in the dipteran midgut environment (14).

In a similar study with *Bacillus sphaericus*, no transfer of plasmid pAM β 1 between strains infecting *Culex quinquefasciatus* was detected (11). The lack of plasmid transfer is surprising since it was shown that *B. sphaericus* spores were able to germinate and cells were able to grow vegetatively in the presence of large numbers of bacteria indigenous to the larvae (10). Since plasmid transfer between *B. sphaericus* strains takes place better on membranes than in broth, the lack of transfer in insects was attributed to conditions in the decomposing larvae that resembled the conditions in a broth culture. In this respect *B. thuringiensis* strains may have an advantage as they are in general thought to transfer plasmids better in broth rather than on surfaces, such as membranes (9). In our experiments, donor and recipient *B. thuringiensis* strains were introduced together, and both colonized killed insects. A key question is whether two *B. thuringiensis* strains are found together in the insect environment. Damgaard et al. (13) have found that strains displaying different serotypes and activities can be isolated from the same location. Therefore, dual infection is a likely event, particularly in environments that select for high populations of *B. thuringiensis*, such as stored grain (25).

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