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Transfer of plasmids and chromosomal genes amongst subspecies of *Bacillus thuringiensis*

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SUMMARY

The plasmids pBC16 and pC194 from *Bacillus thuringiensis* subsp. *israelensis* strains A084-16-194 were transferred to 25 subspecies of *B. thuringiensis* by a conjugation-like process using broth mating technique. The frequencies of transfer varied considerably between different mating pairs, ranging from 1.1×10^{-9} to 9.8×10^{-5}. Additionally, chromosomal transfer could also be demonstrated in ten *B. thuringiensis* subspecies with very low frequencies (4.3×10^{-9} to 3.7×10^{-7}). The intersubspecies matings within a group of eight subspecies strains gave higher frequencies of transfer than the matings between the subspecies. Furthermore, the results indicated that the capability to transfer plasmids among these various subspecies did not depend on the presence of large plasmid.

INTRODUCTION

Bacillus thuringiensis is one of the most effective bacterial insecticides and it is being used widely to control populations of insect pests and vectors [1,3,16]. In contrast with chemical insecticides, the strains of this bacterium produce toxins with highly selective modes of action [1,3] such that the toxic activity of a particular isolate may be limited to very specific target insects and not be toxic to non-target insects or to other organisms including man [2]. Also in contrast with many chemical insecticides, these biopesticides will not persist for an extended period in nature and, are thus, unlikely to destroy the environmental equilibrium. For these reasons, many strains of *B. thuringiensis* have become widely used as bioinsecticides for agriculture and for certain vectors of human diseases [2,5].

More than 20 subspecies of *B. thuringiensis* produce different insecticidal toxins which can be categorized into three major groups; δ-endotoxins, α-endotoxins and β-exotoxins [7]. These toxins are encoded by various toxin genes located either on plasmids or on chromosomal DNA [3,6]. Furthermore, each subspecies of *B. thuringiensis* has been found to harbor many cryptic plasmids [6,10].

In 1982, Gonzalez et al. [9] discovered an effective plasmid transfer system among strains of *B. thuringiensis* and *B. cereus* via cell mating. This mode of gene transfer has been named a conjugation-like process. Subsequently, there were many reports [4,12,13,15,18] on the use of this conjugation-like process for transferring specific plasmids in *B. thuringiensis*. Klier et al. [12] transferred the toxin gene of subsp. *berliner* via the conjugation-like process to an acrystalliferous *B. thuringiensis* mutant of subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis*. Later, Battisti et al. [4] and Reddy et al. [18] demonstrated the transfer of plasmids by mating from *B. thuringiensis* subspecies to *B. anthracis* and *B. cereus*. In 1986, Loprasert et al. [15] showed that the plasmid pC194 from *B. thuringiensis* strain 0016 and pBC16 from *B. cereus* could be transferred to *B. thuringiensis* subsp. *israelensis* by using the conjugation-like process. In 1987, Koehler and Thorne [13] also demonstrated the transfer of the plasmid pBC16 from *B. subtilis* to *B. anthracis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. subtilis* and *B. thuringiensis* using the conjugation-like process.

Despite these numerous examples of the conjugation-like transfer of plasmids and genes in *B. thuringiensis* and related bacteria, there has as yet, been no report on the mechanism of this gene transfer. This study was initiated

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to investigate the nature of the conjugation-like transfer of plasmids and chromosomal markers from *B. thuringiensis* subsp. *israelensis* to various subspecies of *B. thuringiensis*.

MATERIALS AND METHODS

Bacterial strains. The strains of various subspecies of *B. thuringiensis* used in this study are listed in Table 1. Spontaneous rifampicin resistant (Rif^r) mutants were isolated on nutrient agar plates containing rifampicin at 50 μg/ml. A streptomycin resistant strain of *B. thuringiensis* subsp. *israelensis* A084-16-194 [15], harboring plasmids pBC16 of *B. cereus* GP7 and pC194 of *B. subtilis* HVS62 (which conferred tetracycline and chloramphenicol resistance, respectively) was used as the donor strain unless indicated otherwise. All cultures were maintained on nutrient agar slants and grown in L-broth medium for the mating procedure.

Media and chemicals. All bacteriological media were obtained from Difco Laboratories, Detroit, MI. Antibiotics and chemicals were purchased from Sigma Chemical Company, St. Louis, MO.

The conjugation-like condition. Donor and recipient cells

TABLE 1

Bacillus thuringiensis subspecies and their relevant phenotypes

Strain	Strain number[a]	Flagella serotype	Phenotype	Remark
B.t.i. A084-16-194	T014001	14	Cam^r (pC194) Tet^r (pBC16) Str^r	Ref. 15
B.t. thuringiensis	T01001	1	Pen^r Rif^r	This study[b]
B.t. finitimus	T02001	2	Pen^r Rif^r	This study
B.t. finitimus	T02001	2	Pen^r Str^r	This study
B.t. kurstaki	T03A001	3a3b	Pen^r Rif^r	This study
B.t. dendrolimus	T04A001	4a4b	Pen^r Rif^r	This study
B.t. sotto	T04001	4a4b	Pen^r Rif^r	This study
B.t. sotto	T04001	4a4b	Pen^r Str$^{r'}$	This study
B.t. kenyae	T04B001	4a4c	Pen^r Rif^r	This study
B.t. galleriae	T05001	5a5b	Pen^r Rif^r	This study
B.t. entomocidus	T06001	6	Pen^r Rif^r	This study
B.t. subtoxicus	T06A001	6	Pen^r Rif^r	This study
B.t. ostrinae	T08A001	8a8c	Pen^r Rif^r	This study
B.t. ostrinae	T08A001	8a8c	Pen^r Str$^{r'}$	This study
B.t. morrisoni	T08001	8a8c	Pen^r Rif^r	This study
B.t. tolworthi	T09001	9	Pen^r Rif^r	This study
B.t. caucasicus	T10007	10a	Pen^r Rif^r	This study
B.t. toumanoffi	T11001	11	Pen^r Rif^r	This study
B.t. toumanoffi	T11001	11	Pen^r Str$^{r'}$	This study
B.t. kyushuensis	T11A001	11a11c	Pen^r Rif^r	This study
B.t. thompsoni	T12001	12	Pen^r Rif^r	This study
B.t. dakota	T15001	15	Pen^r Rif^r	This study
B.t. indiana	T16001	16	Pen^r Rif^r	This study
B.t. tohokuensis	T17001	17	Pen^r Rif^r	This study
B.t. kumantoensis	T18001	18	Pen^r Rif^r	This study
B.t. tochigiensis	T19001	19	Pen^r Rif^r	This study
B.t. darmstadiensis	T10001	10	Pen^r Kan^r Rif^r	This study
B.t. pakistani	T13001	13	Pen^r Rif^r	This study
B.t. subage yannansuis	T20001	20a20b	Pen^r Rif^r	This study
B.t. wuhenensis	TX1001	–	Pen^r Rif^r	This study

[a] Original strain number (IEBC No.) provided by WHO center through Prof. H. de Barjac.

[b] All strains were originally obtained from WHO and subsequently subjected to selection for Rif^r or Str^r.

were grown separately in 1.5 × 15 mm test tubes containing 4 ml of L-broth. They were incubated at 37 °C with shaking at 200 rpm for 14 h. Then, each culture was separately transferred (1% inoculum) to a fresh batch of the same medium and further incubated under the same conditions for an additionnal 3 to 4 h, to assure exponential growth. Mating mixtures were prepared by mixing 2 ml of donor cells with 2 ml of recipient cells in 1.5 × 15 mm test tubes containing 4 ml of L-broth medium. Control tubes contained 4 ml of L-broth and 2 ml of either donor or recipient cells.

For mating experiments, mixtures were incubated at 37 °C with slow shaking for 8 h. Samples were removed and plated on appropriate selective media for determining the number of donors, recipients and transconjugants. Dilutions were made in 0.05 M phosphate buffer pH 7.0. Plates were incubated at 37 °C, and colonies were scored after 24 to 48 h. The frequency of transfer was calculated by dividing the number of transconjugants by the lesser number of the mating pair. All figures reported were the average of three independent experiments.

Extraction of plasmid DNA. Plasmid DNA was extracted by a modification of the procedure described by Kado and Liu [11].

Cells were grown in 1.5 × 15 mm test tubes containing 4 ml of L-broth supplemented with appropriate antibiotics. Cultures were incubated for 12–14 h at 37 °C on a rotary shaker. Cells in 1.5 ml of culture broth were collected by centrifugation at 7000 rpm in a microcentrifuge for 1 min at room temperature and suspended in 100 μl E buffer (0.04 M Tris-hydroxide, 0.002 M EDTA tetra-sodium salt, 15% sucrose, pH 7.9) by gentle vortexing. Cells were lysed by adding 200 μl of lysis solution (prepared by adding 3 g of sodium dodecyl sulfate and 5 ml of 3 N NaOH to 100 ml of 15%, wt/vol., sucrose in 0.05 M Tris-hydroxide). The eppendorf tubes were rapidly inverted 20 times to mix the cells and lysis solution and were then held in a 60 °C water bath for 30 min. The lysate was precipitated with 150 μl 3 M sodium acetate pH 5.0 by inverting and placing the tubes in an ice box for 40 min. The plasmid DNA was obtained by centrifugation at 10 000 rpm for 10 min. The supernatant was removed to a new eppendorf tube, and the DNA preparations were concentrated by adding 2.5 volume of 95% ethanol followed by treatment at −20 °C for 1 h.

The plasmid DNA was separated by centrifugation at 12 000 rpm for 10 min. The precipitate was dissolved in 10 μl Tris-EDTA pH 8.0 and mixed with 3 μl BJII solution (50% sucrose, 50 mM EDTA, 0.05% Bromophenol blue). The mixture was applied to horizontal 0.7% agarose gels

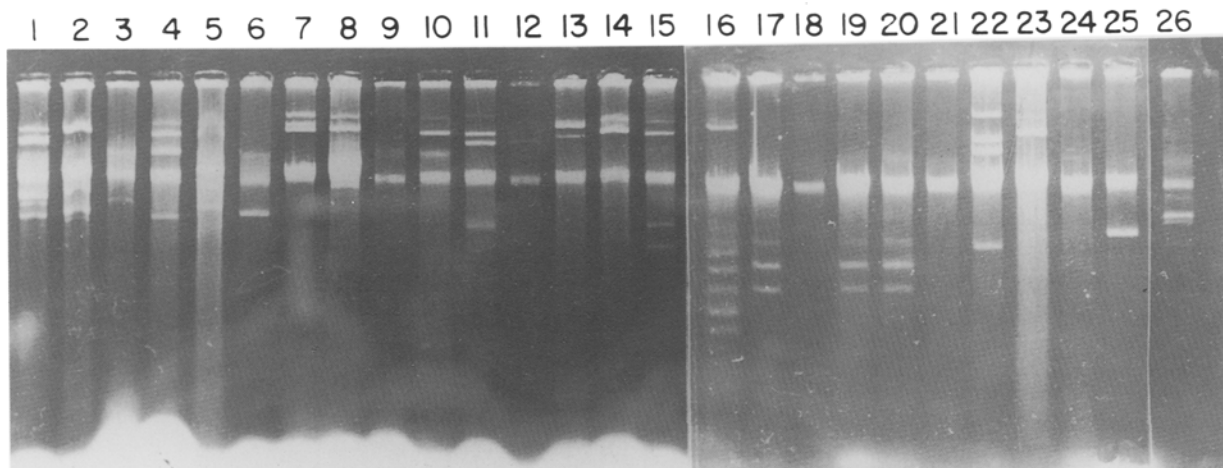

Fig. 1. Agarose gel electrophoresis of plasmid DNA extracts from various subspecies of *Bacillus thuringiensis*. All samples were extracted from 1.5 ml of overnight cultures with A_{600} = 1.2. Conditions used for extraction and for electrophoresis are described in the Materials and Methods. Lane 1, *B.t. thuringiensis*; lane 2, *B.t. kurstaki*; lane 3, *B.t. dendrolimus*; lane 4, *B.t. sotto*; lane 5, *B.t. kenyae*; lane 6, *B.t. galleriae*; lane 7, *B.t. ostrinae*; lane 8, *B.t. tolworthi*; lane 9, *B.t. kyushuensis*; lane 10, *B.t. thompsoni*; lane 11, *B.t. dakota*; lane 12, *B.t. tohokuensis*; lane 13, *B.t. darmstadiensis*; lane 14, *B.t. pakistani*; lane 15, *B.t. subtoxicus*; lane 16, *B.t.i.* A084-16-194; lane 17, *B.t. finitimus*; lane 18, *B.t. entomocidus*; lane 19, *B.t. subage yannansuis*; lane 20, *B.t. morrisoni*; lane 21, *B.t. caucasicus*; lane 22, *B.t. indiana*; lane 23, *B.t. kumantoensis*; lane 24, *B.t. tochigiensis*; lane 25, *B.t. wuhenensis*; lane 26, *B.t. tolworthi*.

prepared and run in Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 100 V at room temperature for 1 h. Gels were stained with ethidium bromide (1 μg/ml) for 30 min and destained in distilled water for 30–60 min.

RESULTS

Plasmid patterns of B. thuringiensis subspecies

The donor strain, *B. thuringiensis* subsp. *israelensis* A084-16-194 harbored its own plasmids in addition to the 4.25 kb pBC16 plasmid and the 2.91 kb pC194 plasmid which conferred tetracycline and chloramphenicol resistances, respectively. The plasmid pattern of all 25 *B. thuringiensis* subspecies which were used as recipients were also examined (Fig. 1).

All subspecies except one contained plasmid DNA ranging from 1 to 7 plasmids. Only one subspecies, namely subspecies *entomocidus*, did not harbor any plasmids. Four subspecies, i.e., *finitimus*, *subage yannansuis*, *tolworthi* and a non-motile *wuhenensis* carried only small plasmids which migrated faster than chromosomal DNA. Other subspecies contained ranges of both small and large plasmids.

Transfer of plasmids pBC16 and pC194

B. thuringiensis subsp. *israelensis* A084-16-194 (Str^r Tet^r Cam^r) was tested for the ability to transfer pBC16 and/or pC194 plasmids into various subspecies of *B. thuringiensis* by the broth mating technique. As a selective pressure, a rifampicin resistant marker was used in each of the recipient strains. The transconjugants which acquired plasmids pBC16 were scored on nutrient agar plates containing 20 μg/ml tetracycline and 50 μg/ml rifampicin, whereas the transconjugants with pC194 were selected on nutrient agar plates containing 15 μg/ml chloramphenicol and 50 μg/ml rifampicin. Similarly, the transconjugants which acquired the chromosomal marker were selected on nutrient agar plates containing 40 μg/ml streptomycin and 50 μg/ml rifampicin.

Tables 2 and 3 show the frequencies of plasmids transfer (average number of three independent experiments) into recipient subspecies by the conjugation-like process. The frequencies of plasmid transfer were found to differ

TABLE 2

Frequencies of transfer of plasmid pBC16 in various subspecies of *Bacillus thuringiensis*

Frequency of transfer[a]						
High[b]		Moderate[c]		Low[d]		No transfer[e]
finitimus	(9.8×10^{-5})	*thuringiensis*	(1.1×10^{-7})	*kenyae*	(6.5×10^{-9})	*tochigiensis*
ostrinae	(5.5×10^{-5})	*kurstaki*	(1.3×10^{-6})	*kyushuensis*	(2.5×10^{-8})	*caucasicus*
entomocidus	(1.3×10^{-5})	*dendrolimus*	(1.2×10^{-7})	*dakota*	(6.0×10^{-8})	
		sotto	(1.7×10^{-7})	*tohokuensis*	(2.4×10^{-8})	
		morrisoni	(7.7×10^{-6})	*kumantoensis*	(7.8×10^{-8})	
		toumanoffi	(2.3×10^{-7})	*pakistani*	(2.1×10^{-9})	
		thompsoni	(4.8×10^{-6})	*galleriae*	(1.1×10^{-8})	
		subtoxicus	(2.5×10^{-6})	*tolworthi*	(7.7×10^{-8})	
		darmstadiensis	(1.1×10^{-7})	*indiana*	(2.5×10^{-8})	
		subage yannansuis	(2.2×10^{-7})			
		wuhenensis	(2.2×10^{-7})			

[a] The frequencies of transfer are listed in parentheses after the name of the subspecies. Each number was the averaged from 3 independent experiments.

[b] Frequencies of transfer greater than 1×10^{-5}.

[c] Frequencies of transfer between 1×10^{-6} to 1×10^{-7}.

[d] Frequencies of transfer less than 1×10^{-8}.

[e] No transconjugant was detected when 1×10^{9} cells of donors and recipients were plated on NA plates containing tetracycline and rifampicin.

TABLE 3

Frequencies of transfer of plasmid pC194 in various subspecies of *Bacillus thuringiensis*

Frequency of transfer[a]				
Moderate		Low		No transfer
thuringiensis	(1.6×10^{-6})	*sotto*	(9.5×10^{-8})	*pakistani*
finitimus	(6.8×10^{-7})	*morrisoni*	(4.6×10^{-8})	
tochigiensis	(1.3×10^{-7})	*toumanoffi*	(1.8×10^{-8})	
kurstaki	(2.3×10^{-7})	*subtoxicus*	(6.2×10^{-9})	
dendrolimus	(2.5×10^{-7})	*dakota*	(4.3×10^{-8})	
kenyae	(1.1×10^{-7})	*tohokuensis*	(1.7×10^{-8})	
ostrinae	(2.6×10^{-7})	*darmstadiensis*	(1.0×10^{-8})	
caucasicus	(4.3×10^{-7})	*indiana*	(4.8×10^{-9})	
kyushuensis	(1.5×10^{-6})			
thompsoni	(6.3×10^{-7})			
entomocidus	(6.0×10^{-7})			
kumantoensis	(3.8×10^{-7})			
subage yannansuis	(1.9×10^{-7})			
wuhenensis	(9.0×10^{-7})			
galleriae	(4.0×10^{-7})			
tolworthi	(1.1×10^{-7})			

[a] See details in Table 2.

depending on the recipient subspecies. There appeared to be three different categories of recipients based on their ability to acquire plasmids pBC16 and pC194. In group I, subsp. *pakistani* acquired only pBC16 from the donor strain, and there were no transconjugants which contained the plasmid pC194. In group II, subsp. *tochigiensis* and *caucasicus* acquired only pC194 from the donor strain, and there were no transconjugants which contained plasmid pBC16. In group III, there were 22 subspecies which were capable of acquiring both plasmids (i.e., pBC16 and pC194). The frequencies of transfer for pBC16 ranged from 2.1×10^{-9} for transfer into subspecies *pakistani* to 9.8×10^{-5} for transfer into subspecies *finitimus*. The frequency of transfer for pC194 ranged from 4.8×10^{-9} in subsp. *indiana* to 1.6×10^{-6} in subsp. *thuringiensis*. The members of group III were subsp. *thuringiensis*, *finitimus*, *kurstaki*, *dendrolimus*, *sotto*, *kenyae*, *ostrinae*, *morrisoni*, *caucasicus*, *toumanoffi*, *kyushuensis*, *thompsoni*, *entomocidus*, *subtoxicus*, *dakota*, *tohokuensis*, *kumantoensis*, *darmstadiensis*, *subage yannansuis*, *wuhenensis*, *galleriae*, *tolworthi* and *indiana*.

The ability to transfer the relevant plasmids was confirmed by analysis of the plasmid patterns of the various transconjugants. Fig. 2 shows the plasmid profiles of the

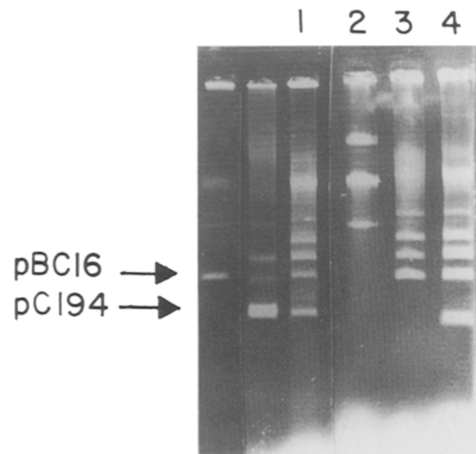

Fig. 2. Agarose gel electrophoresis of plasmid DNA extracts from a donor, *B.t.i.* A084-16-194 (lane 1); a recipient, *B.t. sotto*, (lane 2); a transconjugant that received pBC16 (lane 3), and a transconjugant that received pC194 (lane 4). Bands for pBC16 and pC194 are marked with arrows. The position of pBC16 and pC194 were obtained from extracts of *B. cereus* GP7 and *B. subtilis* HVS62, respectively.

representative mating pairs of donor strains, recipients, and transconjugants. Plasmid patterns from the randomly selected transconjugants revealed clearly that the plasmids pBC16, or pC194 or both had been transferred to the various recipient subspecies. However, except for subsp. *finitimus* and *ostrinae*, the rates of transfer of plasmids in all the subspecies were relatively low. The frequency of transfer for plasmids between strains within subspecies, i.e., between *B.t.i.* and *B.t.i.*, were found to be 1.6×10^{-4} and 1.5×10^{-5} for pBC16 and pC194, respectively. This was comparable to the transfer between the subsp. *israelensis* and *ostrinae*. Interestingly, the plasmids pBC16 and pC194 were either independently transferred or co-transferred into different subspecies, and the ability to transfer did not depend upon the plasmid pattern of the subspecies or of the recipients. The large plasmids of recipients did not seem to play an important role in acquisition of these two drug resistant plasmids because recipients harboring either small or large plasmids acquired plasmids pBC16 and/or pC194. Furthermore, *B. thuringiensis* subsp. *entomocidus*, which lacked plasmids, could acquire both pBC16 and pC194 at transfer frequencies of 1.3×10^{-5} and 6.0×10^{-7}, respectively.

In addition, the flagella type of the recipient did not play any significant role in the rate of plasmid transfer in this conjugation-like process. *B. thuringiensis* subsp. *wuhenensis*, which did not possess any flagella, acquired plasmids pBC16 and pC194 by the broth mating technique with frequencies of 2.2×10^{-7} and 9.0×10^{-7}, respectively.

On the basis of plasmid aquisition frequency, the strain studied could also be categorized into three groups; namely, those with a high frequency of transfer (greater than 1×10^{-5}), those with a moderate frequency of transfer (between 10^{-6} to 10^{-7}) and those with a low frequency of transfer (less than 1×10^{-8}). Using this classification for acquisition pBC16, the subspecies which fell in the high frequency group included subsp. *finitimus*, *ostrinae*, and *entomocidus*. Those in the moderate frequency group were subsp. *thuringiensis*, *kurstaki*, *dendrolimus sotto*, *morrisoni*, *toumanoffi*, *thompsoni*, *subtoxicus*, *darmstadiensis*, *subage yannansuis* and *wuhenensis*. Those in the low frequency group were subspecies *kenyae*, *kyushuensis*, *dakota*, *tohokuensis*, *kumatoensis*, *pakistani*, *galleriae*, *tolworthi* and *indiana*.

The frequency of acquisition plasmid pBC16 was found to be higher than that of pC194 plasmid. There were sixteen subspecies (i.e., *thuringiensis*, *finitimus*, *tochigiensis*, *kurstaki*, *dendrolimus*, *kenyae*, *ostrinae*, *caucasicus*, *kyushuensis*, *thompsoni*, *entomocidus*, *kumantoensis*, *subage*

TABLE 4

Transfer of chromosomal marker (streptomycin or rifampicin resistant) in *Bacillus thuringiensis*

Frequency of transfer[a]				
Moderate		Low		No transfer
thuringiensis	(1.0×10^{-7})	*finitimus*	(9.3×10^{-9})	*tochigiensis*
kurstaki	(3.7×10^{-7})	*sotto*	(1.7×10^{-8})	*dendrolimus*
subage yanansuis	(2.7×10^{-7})	*toumanoffi*	(4.7×10^{-9})	*kenyae*
		thompsoni	(4.0×10^{-8})	*ostrinae*
		kumantoensis	(5.5×10^{-8})	*morrisoni*
		darmstadiensis	(1.0×10^{-8})	*caucasicus*
		wuhenensis	(4.3×10^{-9})	*kyushuensis*
				entomocidus
				subtoxicus
				dakota
				tohokuensis
				pakistani
				galleriae
				tolworthi
				indiana

[a] See details in Table 2.

yannansuis, *wuhenensis*, *galleriae* and *tolworthi*) which acquired plasmid pC194 at a moderate frequency of transfer, while there were only eight subspecies (*sotto*, *morrisoni*, *toumanoffi*, *subtoxicus*, *dakota*, *tohokunensis*, *darmstadiensis* and *indiana*) which acquired pC194 at low frequency of transfer.

Transfer of chromosomal DNA

When attempts were made to detect the transfer of chromosomal markers, it was found that transconjugants acquiring the chromosomal marker Str^r from the donor, *B. thuringiensis* subsp. *israelensis* A084-16-194 [15] could be selected on nutrient agar plates containing streptomycin at 40 μg/ml and rifampicin at 50 μg/ml. Spontaneous mutation was ruled out by the inability to detect similar colonies after plating only donor or recipient cells on the same media.

Table 4 shows that ten *B. thuringiensis* subspecies acquired chromosomal DNA from the donor strain. The frequency of transfer was found to be very low and ranged from 4.3×10^{-9} in subsp. *wuhenensis* to 3.7×10^{-7} in subsp. *kurstaki*. There was no mating pair which was found to transfer solely the chromosomal marker. In all cases where the chromosomal marker was transferred, both plasmids pBC16 and pC194 were also transferred.

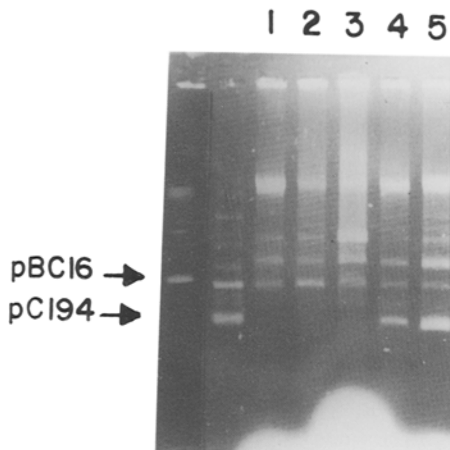

Fig. 3. Agarose gel electrophoresis of plasmid DNA extracts from, a recipient, *B.t. finitimus* (lane 1); a donor with pBC16, *B.t. finitimus*-pBC16 (lane 2); a transconjugant that received pBC16 (lane 3); a donor with pC194 (lane 4); a transconjugant that received pC194 (lane 5). The positions of pBC16 and pC194 are marked with arrows.

TABLE 5

Frequency of transfer of plasmids pBC16 and pC194, among the intrasubspecific mating pairs

Mating pair	Frequency of transfer	
	pBC16	pC194
B.t. fin – pBC16 × *B.t. fin*	2.2×10^{-7}	–
B.t. fin – pC194 × *B.t. fin*	–	4.0×10^{-6}
B.t. sot – pBC16 × *B.t. sot*	1.8×10^{-5}	–
B.t. sot – pC194 × *B.t. sot*	–	1.6×10^{-6}
B.t. ost – pBC16 × *B.t. ost*	8.7×10^{-6}	–
B.t. ost – pC194 × *B.t. ost*	–	2.1×10^{-5}
B.t. tou – pBC16 × *B.t. tou*	9.2×10^{-6}	–
B.t. tou – pC194 × *B.t. tou*	–	4.0×10^{-6}
B.t.i. A084-16-19 × *B.t.i.*	1.6×10^{-4}	1.5×10^{-5}

Transfer of plasmids pBC16 and pC194 between and within subspecies

Eight transconjugants which resulted from mating between *B. thuringiensis* subsp. *israelensis* A084-16-194 and subspecies *finitimus*, *sotto*, *ostrinae*, and *toumanoffi* were selected as representative donor strains. These harbored the plasmids pBC16 or pC194 (Fig. 3), and they were used to investigate the frequency of plasmid transfer between subspecies and within subspecies. In all mating pairs, streptomycin-resistant mutants of the corresponding subspecies strains were selected and employed as recipient cells.

The frequency of transfer within subspecies for these five different subspecies ranged from 2.2×10^{-7} to 1.6×10^{-4} (Table 5). Comparison with the transfer rate between subspecies (Tables 3–5) indicated that mating within subspecies gave a higher frequency of transfer than did mating between subspecies. However, there were two exceptions. The frequency of transfer of pBC16 within the subspecies *ostrinae* and *finitimus* was lower than the frequency of transfer between them.

DISCUSSION

Our study of the ability of *B.t.i.* strain A084-16-194 to transfer its plasmids and chromosomal marker genes to

various subspecies recipients required an initial examination of the plasmid pattern of the various subspecies used. As has been reported by numerous investigators [3,5,6], the different subspecies possessed different plasmid patterns. In full agreement with Carlton and Gonzalez [6], we showed that the subspecies *ostrinae* possessed 3 plasmids. The plasmids of *israelensis*, *kurstaki*, *sotto*, and *subtoxicus* also showed very similar patterns to those reported by Lereclus et al. [14].

When transfer of plasmids pBC16 and pC194 from *B.t.i.* A084-16-194 was attempted with various subspecies of *B. thuringiensis* using the conjugation-like mating process, it was interesting that not all subspecies were able to successfully receive both plasmids. For example, all subspecies except *pakistani* were capable of acquiring and maintaining the pBC16 plasmid as demonstrated by tetracycline resistance and the presence of the plasmid on agarose gels. Likewise, all subspecies except *tochigiensis* and *caucasicus* were capable of acquiring and maintaining the pC194 plasmid. It is not known whether the lack of success depended upon an inability to transfer or an inability to retain the relevant plasmid after transfer or both. However, since a large number of the subspecies did acquire both pBC16 and pC194, it is unlikely that inability to transfer the plasmids was the reason for lack of success in only a few subspecies. Rather, it is likely that the inability to acquire certain plasmids depended upon the ability of a particular strain to retain a particular plasmid. This contention is further supported by the fact that all successful transfers of drug resistance plasmids in recipient strain, resulted in the co-transfer and maintenance of other *B.t.i.* plasmids to the recipients. Again, this demonstrated that plasmids could be transferred from one subspecies of *B. thuringiensis* to another freely via the conjugation-like process.

In this study, there was no correlation between the plasmid patterns of the recipient *B. thuringiensis* subspecies and their ability to maintain the plasmids pBC16 and pC194. The subspecies *thuringiensis*, *dakota*, *indiana*, *sotto*, *toumanoffi*, *thompsoni*, and *subtoxicus*, which contained large plasmids, readily accepted plasmids pBC16 and pC194 at the same rate as subspecies *galleriae*, *finitimus*, and *wuhenensis*, which contained only small plasmids, and at the same rate as subspecies *entomocidus*, which did not possess any detectable plasmid at all. Strain *B.t.i.* A084-16-194, used as the donor in most of this study, contained large plasmids, but subspecies *finitimus*, which acted as the donor in an intrasubspecific transfer test, did not possess any large plasmids (Figs. 1 and 3). Thus, there was also no correlation between plasmid pattern and ability to donate plasmids via the conjugation-like process. This finding contrasts to that of Battisti et al. [4]. The discrepancy may arise from differences in the strains employed, in the method of conjugation employed or in the level of detection of transcojugants.

The frequency of plasmid transfer from *B.t.i.* A084-16-194 to various recipients varied greatly depending upon the subspecies recipient. The frequencies varied from 2.1×10^{-9} in subspecies *pakistani* to 9.8×10^{-5} in subspecies *finitimus*. These rates of transfer were similar to those reported by Fisher et al. [8]. Even so, the rate of gene transfer via the conjugation-like process varies greatly from one report to another [4,8,9,12,15,18]. Nonetheless, the transfer frequency obtained in this study is well within the range of figures being reported elsewhere [8,9,15].

Using the same plasmids, and the same conjugation-like conditions, the frequency of transfer within subspecies was much higher than the frequency between subspecies. The result was to be as expected, since the restriction/modification of foreign plasmids may play a significant role in successful plasmid transfer. Furthermore, the transfer of genes within a subspecies may be supported by better conditions for "pairing" between two conjugants than those found with transfer between subspecies. A specific study on the nature of "pairing" between two cells may shed more light onto the mechanism of this poorly understood conjugation-like process.

With very limited data, we were able to demonstrate chromosomal transfer from subsp. *israelensis* to subspecies *thuringiensis*, *finitimus*, *kurstaki*, and *sotto* using streptomycin and rifampicin resistant markers. Although, chromosomal transfer of these resistance phenotypes was attempted in all our conjugation experiments involving 25 subspecies, only 10 successful mating pairs could be demonstrated. However, it is possible that chromosomal transfer occurs at such a low rate that the methods used in this study were not sensitive enough to allow for its detection. If the conjugation-like gene transfer process were to be performed using the membrane filter technique [15], perhaps more chromosomal transfer could be detected [17]. Nonetheless, demonstration of chromosomal transfer opens the way for further optimization of the process. Perhaps it could be used to obtain a better understanding of the genetic organization of this microorganism and to obtain genetically improved strains of *B. thuringiensis*.

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