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Conjugative Transfer of Insecticidal Plasmid pHT73 from *Bacillus thuringiensis* to *B. anthracis* and Compatibility of This Plasmid with pXO1 and pXO2[▽]

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***Bacillus anthracis*, the etiologic agent of anthrax, is genetically close to and commonly shares a giant gene pool with *B. cereus* and *B. thuringiensis*. In view of the human pathogenicity and the long persistence in the environment of *B. anthracis*, there is growing concern about the effects of genetic exchange with *B. anthracis* on public health. In this work, we demonstrate that an insecticidal plasmid, pHT73, from *B. thuringiensis* strain KT0 could be efficiently transferred into two attenuated *B. anthracis* strains, Ba63002R (pXO1$^+$ pXO2$^-$) and Ba63605R (pXO1$^-$ pXO2$^+$), by conjugation in liquid medium in the laboratory, with transfer rates of 2.3×10^{-4} and 1.6×10^{-4} CFU/donor, respectively. The *B. anthracis* transconjugants containing both pHT73 and pXO1 or pXO2 could produce crystal protein Cry1Ac encoded by plasmid pHT73 and had high toxicity to *Helicoverpa armigera* larvae. Furthermore, the compatibility and stability of pHT73 with pXO1/pXO2 were demonstrated. The data are informative for further investigation of the safety of *B. thuringiensis* and closely related strains in food and in the environment.**

In the *Bacillus cereus sensu lato* family, the classification of three species, *B. thuringiensis*, *B. cereus*, and *B. anthracis*, is based mainly on the presence of different functional plasmids in the clusters (26). *B. thuringiensis* is an insect pathogen, harboring plasmids encoding insecticidal proteins with toxicity against insects of the orders Diptera, Lepidoptera, and Coleoptera and nematodes (30); *B. cereus* is a ubiquitous food spoilage bacterium associated with two forms of human food poisoning (diarrheal and emetic syndromes), and the cereulide toxin (which causes the emetic syndrome) is encoded on a large virulence plasmid, pCERE01 (also named pCER270; ≈270 kb) (15); *B. anthracis* is the active agent of anthrax, and its virulence is attributed mainly to the presence of plasmids pXO1 (≈182 kbp) and pXO2 (≈95 kbp) since curing any of the plasmids attenuates the strain (5).

Recent studies demonstrated that the genetic backbones of both the pXO1 and pXO2 plasmids are not restricted only to *B. anthracis* but rather can be found in isolates of the related species *B. cereus* and *B. thuringiensis* as well (14, 24, 33). It was reported that *B. cereus* strain G9241, isolated from a patient with life-threatening pneumonia, carried a specific protective antigen gene (*pagA*) on plasmid pBCXO1 and a gene encoding a capsule made mainly of putative polysaccharide on plasmid pBC218 (14, 18). In further studies, the specific pXO1 fragments and pXO2 *cap* genes were detected in *B. cereus* strains 03BB102 and 03BB108 (13) and a backbone similar to the common backbone of pXO2 was found in a *B. thuringiensis* subsp. *konkukian* strain (serotype H34) isolated from a wounded soldier (12). These findings indicate that *B. anthracis* and related species may share a large gene pool and that genetic exchange among these species, followed by diversification, may be a major cause for their genomic plasticity and evolution.

The vegetative cells of the *B. cereus sensu lato* family strains can grow under nutrient-rich conditions as ubiquitous inhabitants, and they provide a large gene pool for horizontal gene exchange and coevolution, especially by conjugation. Some reports have confirmed that strains of *B. thuringiensis* and *B. cereus* exhibit low degrees of clonality and that exchange of genetic material between them occurs frequently in river water (31), soil (35), food preparation environments (33), and even the guts of insects (31, 37). Previous reports revealed that the toxin genes or whole toxin plasmids of *B. anthracis* can transfer to other *Bacillus* sp. strains in broth culture (28) and in the rhizosphere of grass plants (29) and that, conversely, exotic genes or plasmids can also be transferred into *B. anthracis* through conjugation. The frequent genetic change among *B. cereus* group strains raises concerns that *B. anthracis* and related species cannot be well distinguished by traditional species definitions and that the possible impact of *B. cereus* group strains on food and environments needs to be elucidated.

Our work focused on the conjugational transfer of the insecticidal plasmid pHT73 from *B. thuringiensis* into *B. anthracis* in liquid medium and in "ready-to-drink" milk. Our findings demonstrated that *B. anthracis* strains could receive the conjugative insecticidal plasmid pHT73 during food preparation and that pHT73 was stably coexistent with pXO1 or pXO2. Furthermore, the *B. anthracis* transconjugants could produce parasporal crystals with high toxicity to *Helicoverpa armigera*. The data are informative for further investigations into the safety of *B. thuringiensis* in food and the environment.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Strains and plasmids used in this study are listed in Table 1. The erythromycin-resistant strain *B. thuringiensis* subsp. *kurstaki* KT0(pHT73-Erm^R) (34) was used as the conjugational

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TABLE 1. Strains and plasmid used in this study

Strain or plasmid	Relevant characteristics[a]	Origin or reference
B. thuringiensis strains		
KT0	Contains plasmid pHT73-Erm^R	34
GBJ001	Mutant of 4Q7 cured of plasmids; Str^r	17
B. anthracis strains		
CMCC(B) 63002	pXO1$^+$ pXO2$^-$	CMCC[b]
CMCC(B) 63605	pXO1$^-$ pXO2$^+$	CMCC
Ba63605R	Mutant of CMCC(B) 63605; Rif^r; filament-like vegetative strain	This study
Ba63002R	Mutant of CMCC(B) 63002; Rif^r	This study
Ba16	pXO1$^+$ pXO2$^+$	This study
B. cereus strains		
F3502/73S	Emetic strain; Str^r	7
MADM1279S	Isolated from sugar candy; Str^r	11
AND1309S	Isolated from curry powder; Str^r	11
Plasmid pHT73	Conjugative 75-kb plasmid; *cry1AC* Erm^r	34

[a] Str^r, streptomycin resistant; Rif^r, rifampin resistant; Erm^r, erythromycin resistant.
[b] CMCC, National Center for Medical Culture Collections, China.

donor. The attenuated *B. anthracis* strains CMCC(B) 63002 and CMCC(B) 63605, carrying only plasmids pXO1 and pXO2, respectively, were used as recipients, and the recipient strains were designated Ba63002R and Ba63605R. Three strains of *B. cereus* were originally isolated from foodstuffs. Spontaneous mutants resistant to rifampin or streptomycin were selected in Luria-Bertani (LB) medium. pHT73-Erm^R is a conjugative plasmid that carries a *cry1Ac* gene.

LB liquid medium and agar plates were used for strain culture and CFU counting (1). Ultrahigh-temperature (UHT)-sterilized whole milk was a commercial product (Wuhan, China) with a pH of 6.7. The sterility of the milk was tested by plating 100-μl samples onto LB agar plates and evaluating CFU after 48 h of incubation at 30°C. Antibiotics in agar plates and broth media were used at the following concentrations: erythromycin, 100 μg/ml; rifampin, 50 μg/ml; and streptomycin, 100 μg/ml.

Conjugation experiments. The mating experiments were performed according to a modified procedure (2). The overnight precultures of donor and recipient strains were incubated separately with shaking (120 rpm) in either LB medium or milk (depending on the mating medium) without antibiotics for 10 h at 30°C. Equal amounts (500 μl) of donor and recipient strains were combined in 5 ml prewarmed mating medium and incubated at 30°C without shaking. After 5 h, 100-μl samples of the mating mixtures were subjected to a vortex for 10 s. Appropriate dilutions of mixtures were plated onto selective media for counting of donor, recipient, and transconjugant bacteria. In parallel, donor and recipient strains were grown separately under the same conditions as a control.

PCR and RAPD analyses. Five primer pairs were designed for detection of the specific genes in the donor and recipient strains and the resulting transconjugants. Primer pairs bcerm426S-bcerm929A and lep2A-lep2B were used for detecting the erythromycin resistance gene (16) and the *cry1Ac* gene (4), respectively, carried by pHT73; primer pair PA-1 (5′-TCCTAACACTAACGAAGTC G-3′) and PA-2 (5′-GAGGTAGAAGGATATACGGT-3′) was used for the *pag* gene carried by plasmid pXO1; primer pair Cap-1 (5′-ACTCGTTTTTAATCA GCCCG-3′) and Cap-2 (5′-GGTAACCCTTGTCTTTGAAT-3′) was used for pXO2 (the capsule gene *cap*); and primer pair Ba813-1 (5′-TTAATTCACTTG CAACTGATGGG-3′) and Ba813-2 (5′-AACGATAGCTCCTACATTTGGA G-3′) was used for a chromosomal marker, Ba813 (27). The random amplified polymorphic DNA (RAPD) analysis was performed with random primers OPA2 and OPA9 (Operon Technologies, Inc., Alameda, CA) to distinguish the donor from recipients and transconjugants (10).

Plasmid detection and stability test. Plasmid DNA was extracted from the donor, recipients, and transconjugants by the procedure described by Kado and Liu and was analyzed on 0.6% Tris-borate-EDTA agarose gel (19). The stability of the exterior plasmid in *B. anthracis* was investigated by a method described previously (16).

Protein analysis. The donor, recipients, and transconjugants were incubated in LB medium until sporulation. Then the cultures were collected, washed with distilled water, and used for SDS-PAGE (20) and electron microscopy studies with a Hitachi H-7000FA microscope.

Bioassay of toxicity. The toxicities of donor, recipient, and transconjugant strains against *H. armigera* were evaluated by bioassays (21). The target insects were a stable susceptible *H. armigera* colony maintained in the Experimental Insect Center, Wuhan Institute of Virology, Chinese Academy of Sciences. The bioassays of individual and combined sporulated cultures were performed with larvae confined singly to compartments of a 24-well tissue culture plate. The larvae were fed a semiartificial diet composed of wheat flour, soybean flour, yeast powder, and a mixture of vitamins and agar. Diluted recombinant *B. thuringiensis* sporulated cultures alone and in combination were well mixed with the melted feed at about 55°C, giving final concentrations ranging from 6.25 to 500 mg culture per g feed, and the mixtures were then transferred into separate cubes. One neonate larva was placed into each cell, and a plastic cover was used to confine the larvae. The plates were incubated at 26 ± 1°C, with humidity at 85% and a photoperiod of 12:12 h (light-dark). Duplicate sets of 24 larvae for each dose and five doses for each dose-response experiment were used. The bioassay was performed two or three times on different days, and the mortalities were recorded after 72 h. A 50% lethal concentration (LC_{50}) with 95% fiducial limits was determined using probit analysis with a probit program (E. Frachon, Institut Pasteur), and the LC_{50} and LC_{90} were expressed in milligrams of culture per milliliter of artificial feed.

RESULTS

Plasmid transfer from *B. thuringiensis* to *B. anthracis*. The mating experiment results indicated that attenuated *B. anthracis* strains Ba63002R and Ba63605R could receive the insecticidal plasmid pHT73 from the donor strain KT0 in LB broth medium and UHT-sterilized milk, with transfer ratios ranging from 6.9×10^{-4} to 1.9×10^{-7} transconjugants/donor (Table 2). It was evident that the conjugational transfer of the plasmid from *B. thuringiensis* to *B. anthracis* occurred with significantly higher frequency in UHT-sterilized milk (2.3×10^{-4} and 1.6×10^{-4} transconjugants/donor) than in LB medium (3.8×10^{-6} and 2.6×10^{-6} transconjugants/donor). All tested transconjugants retained the same phenotypes as the original recipient *B. anthracis* strains, being sensitive to penicillin G and unable to ferment salicin and having no hemolytic activity on blood agar (data not shown). The level of stability of plasmid pHT73 in the transconjugants was above 52% after culturing of the transconjugants for about 200 generations (Table 2). Two transconjugants, named KBa63002R and KBa63605R, were selected for further study.

Plasmid analysis and detection of marker genes. Plasmid profiles indicated that the donor harbored a pHT73 plasmid of

TABLE 2. Transfer rates and stability of pHT73 in *B. anthracis*

Transconjugant	Transfer rate (CFU/donor) (95% confidence interval) in:		Plasmid stability (200 generations)
	Milk	LB medium	
KBa63002R	2.3×10^{-4} (9.1×10^{-5}–6.9×10^{-4})	3.8×10^{-6} (1.9×10^{-7}–8.1×10^{-6})	63%
KBa63605R	1.6×10^{-4} (8.2×10^{-5}–5.4×10^{-4})	2.6×10^{-6} (9.2×10^{-7}–5.3×10^{-6})	52%

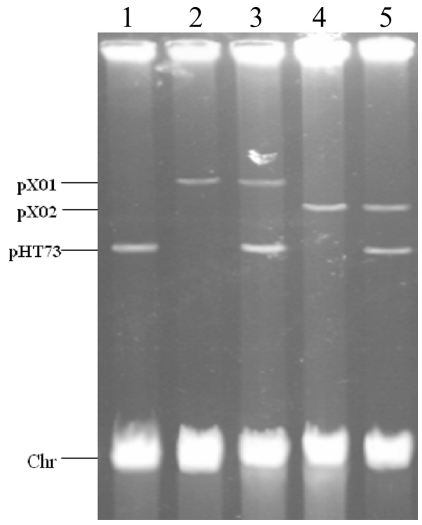

FIG. 1. Plasmid profiles of donor, recipient, and transconjugant strains. Lanes: 1, KT0; 2, Ba63002R; 3, KBa63002R; 4, Ba63605R; and 5, KBa63605R. Chr, chromosomal products.

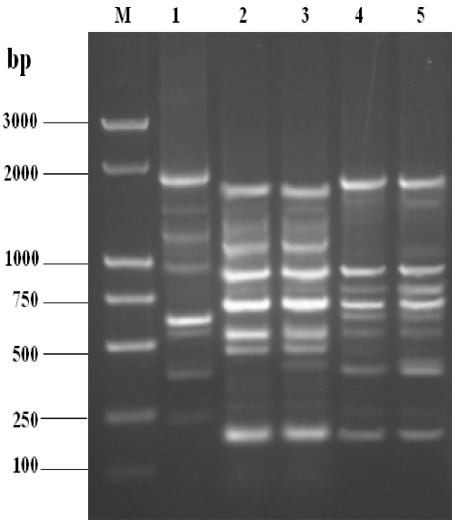

FIG. 3. RAPD patterns of donor, recipient, and transconjugant strains. Lanes: M, marker; 1, KT0; 2, Ba63002R; 3, KBa63002R; and 4, Ba63605R.

75 kbp, that the recipients Ba63002R and Ba63605R contained a 185-kbp pXO1 plasmid and a 94-kbp pXO2 plasmid, respectively, and that the transconjugants harbored both their own anthrax plasmid and pHT73 (Fig. 1). The PCR analysis of specific marker genes confirmed that donor KT0 and transconjugants KBa63002R and KBa63605R contained the erythromycin resistance gene and the *cry1Aa* gene on plasmid pHT73, while the recipient strains Ba63002R and Ba63605R and transconjugants KBa63002R and KBa63605R contained the specific genes *cap* and *pag* and the marker Ba813 on plasmids pXO1 and pXO2, respectively (Fig. 2). Furthermore, the donor could be clearly distinguished from the recipients and transconjugants by RAPD analysis, all recipients and transconjugants exhibiting similar RAPD patterns (Fig. 3).

Protein analysis. The donor and all transconjugants produced parasporal crystals during sporulation (Fig. 4B, D, E, and F), while no parasporal crystals could be observed in the two recipient strains (Fig. 4A and C). SDS-PAGE showed that strains that produced parasporal crystals gave a 130-kDa band of Cry1Ac (data not shown). Bioassay results indicated that the transconjugants derived from recipient *B. anthracis* strains had toxicity against larvae of *H. armigera*, with LC_{50}s of 47.4 mg/ml

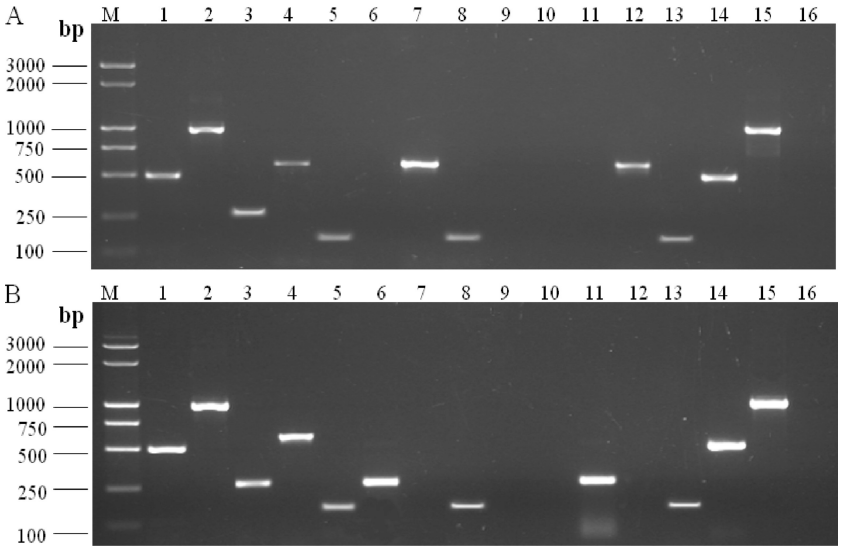

FIG. 2. Results from PCR amplification to detect the relative gene markers in donor KT0 and *B. anthracis* recipient and transconjugant strains. (A and B) Lanes: M, DL3000; 1, KT0 (*erm*); 2, KT0 (*cry1Ac*); 3, Ba16 (*cap*); 4, Ba16 (*pag*); and 5, Ba16 (Ba813). (A) Lanes: 6, Ba63002R (*cap*); 7, Ba63002R (*pag*); 8, Ba63002R (Ba813); 9, Ba63002R (*erm*); 10, Ba63002R (*cry1Ac*); 11, KBa63002R (*cap*); 12, KBa63002R (*pag*); 13, KBa63002R (Ba813); 14, KBa63002R (*erm*); 15, KBa63002R (*cry1Ac*); and 16, control. (B) Lanes: 6, Ba63605R (*cap*); 7, Ba63605R (*pag*); 8, Ba63605R (Ba813); 9, Ba63605R (*erm*); 10, Ba63605R (*cry1Ac*); 11, KBa63605R (*cap*); 12, KBa63605R (*pag*); 13, KBa63605R (Ba813); 14, KBa63605R (*erm*); 15, KBa63605R (*cry1Ac*); and 16, control.

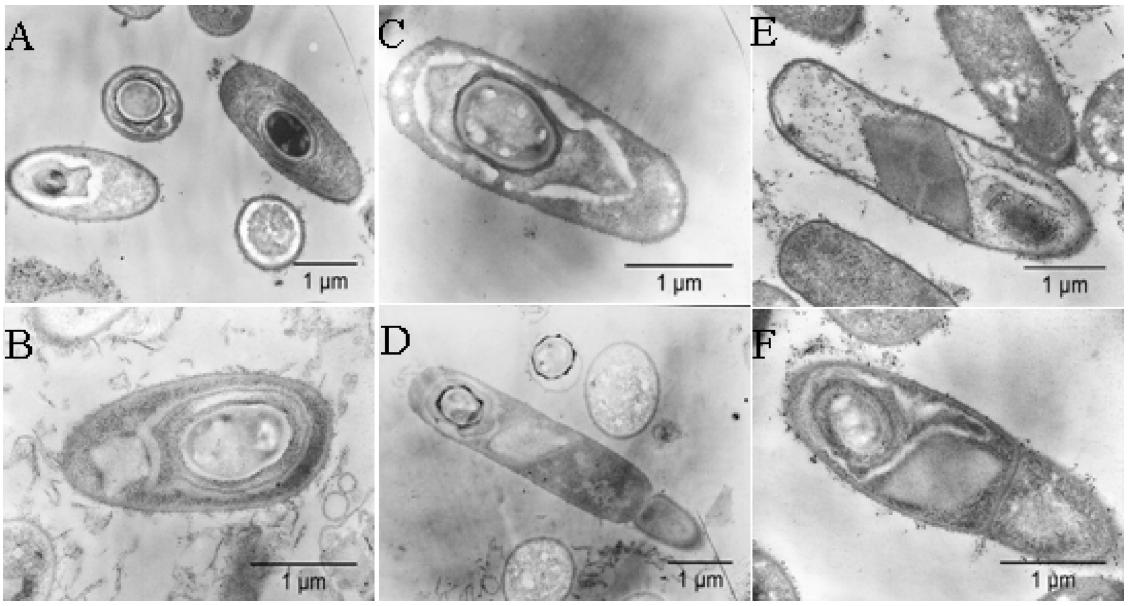

FIG. 4. Ultrathin electron micrographs of crystals produced in the following donor and transconjugant strains: Ba62002R (A); KBa62002R (B); Ba63605R (C); KBa63605R (D); and KT0 (E and F).

(for KBa63605R) and 54.4 mg/ml (for KBa63002R), comparable with that of donor strain KT0 (Table 3). Contrarily, recipient *B. anthracis* strains exhibited no toxicity to the target insects at treatment concentrations as high as 500 mg culture per g feed.

Plasmid transfer from *B. anthracis* to other *B. cereus* strains. Mating experiments demonstrated that plasmid pHT73 could be transferred from two transconjugants, KBa63002R and KBa63605R, to other *B. cereus* group strains and that all recipient stains (*B. thuringiensis* strain GBJ001 and *B. cereus* strains F3502/73S, MADM1279S, and AND1309S) could effectively receive the conjugative plasmid pHT73 with frequencies ranging from 9.4×10^{-4} to 9.1×10^{-6} transconjugants/donor (Table 4).

DISCUSSION

In this study, the conjugational transfer of an insecticidal plasmid, pHT73, from *B. thuringiensis* strain KT0 into attenuated *B. anthracis* strains in liquid media was evaluated. Our results demonstrated that the *B. anthracis* transconjugants harboring an insecticidal plasmid produced parasporal crystals during their sporulation and exhibited high toxicity against *H. amergira* insect larvae. This finding highlights the importance of gene exchanges among strains within the *B. cereus* group and the ecological impact of commercialized *B. thuringiensis* in the environment.

The results also showed that pHT73 can stably coexist with pXO1 or pXO2 in transconjugants. Previous studies revealed that pXO2 is a member of the pAMβ1 family of theta-replicating plasmids, which includes pXO2-like plasmids, pAW63 (originating from *B. thuringiensis* subsp. *kurstaki*), and pBT9727 (originating from *B. thuringiensis* subsp. *konkukian*) (23, 24, 32), and that the replicon of pHT73 belongs to the non-pAMβ1 family of theta-replicating plasmids (9, 36). Likewise, the replication gene *repX* and the quite recently recognized minimal replicon of pXO1 have no significant similarity to the replicon of pHT73 (23, 25). Thus, the three plasmids may utilize different replication systems, and there is no competition limiting their coexistence in a single cell.

There is no significant difference in the strain growth curve for bacteria cultured in LB medium and milk, while higher conjugational transfer frequencies were observed in the latter,

TABLE 3. Toxicity of donor, recipient, and transconjugant strains to *H. armigera*

Strain type	Strain designation	Toxicity[a] to susceptible *H. armigera*	
		LC_{50} (95% confidence interval)	LC_{90} (95% confidence interval)
Donor	KT0	16.9 (11.0–24.0)	58.1 (37.5–141)
Recipient	Ba63605R	>500	>500
	Ba63002R	>500	>500
Transconjugant	KBa63605R	47.4 (40.6–55.7)	202 (157–281)
	KBa63002R	54.4 (46.6–64.1)	244 (186–347)

[a] LC_{50}s and LC_{90}s are expressed as milliliters of fermented culture per milligram of artificial feed ($P \approx 1.2$ g/ml).

TABLE 4. Plasmid transfer from *B. anthracis* to *B. thuringiensis* and *B. cereus* strains

Donor	Recipient strain	Transfer rate (CFU/donor) in milk (95% confidence interval)
KBa63002R	*B. thuringiensis* GBJ001	3.8×10^{-4} (1.2×10^{-5}–6.8×10^{-4})
	B. cereus F3502/73S	2.3×10^{-4} (9.5×10^{-5}–7.1×10^{-4})
	B. cereus MADM1279S	4.9×10^{-5} (2.0×10^{-5}–6.9×10^{-5})
	B. cereus AND1309S	3.2×10^{-5} (9.1×10^{-6}–4.6×10^{-5})
KBa63605R	*B. thuringiensis* GBJ001	6.5×10^{-4} (8.3×10^{-5}–9.4×10^{-4})
	B. cereus F3502/73S	3.1×10^{-4} (9.5×10^{-5}–6.2×10^{-4})
	B. cereus MADM1279S	5.6×10^{-5} (1.2×10^{-5}–8.6×10^{-5})
	B. cereus AND1309S	4.7×10^{-5} (0.7×10^{-5}–6.8×10^{-5})

which was consistent with the results of Modrie and colleagues (22). They observed earlier onset of conjugation of pAW63 in food matrices (e.g., milk and soya milk) than in LB medium, suggesting that the higher frequencies were caused by a higher transfer rate and/or longer mating period.

Although the insecticidal plasmid could be transferred into *B. anthracis* in liquid medium in the laboratory, no naturally occurring insecticidal *B. anthracis* strain has been reported until now. In natural environments, conjugational gene transfer from *B. thuringiensis* to *B. anthracis* rarely happens because *B. anthracis* exists mainly as dormant spores and the vegetative cells of *B. anthracis* survive poorly outside a host or complex artificial medium (6). However, plasmid transfer from other *B. cereus* group strains to *B. anthracis*, and vice versa, may occur under some specific conditions. It is well documented that the spores of insecticidal *B. thuringiensis* can germinate and then multiply in the midguts of dead target insects, and plasmid transfer at different frequencies under these conditions have been recorded previously (31, 34). The large-scale application of commercial *B. thuringiensis* for agricultural pest control may facilitate gene exchange among *B. cereus* group strains in target insects and then endow the strains with insecticidal properties and wider host ranges. However, the role of applied *B. thuringiensis* in horizontal gene transfer among *B. cereus* group strains needs to be further evaluated.

It is already known that members of the *B. cereus* group display high levels of chromosomal similarity and are phenotypically similar, and their species classification is based mainly on the presence of different functional plasmids (3). However, it has been noted previously that plasmids in *B. thuringiensis* and *B. anthracis* can be cured after continuous multiplication outside the target hosts (8), and also that the bacteria can acquire extra exotic plasmids through conjugational transfer in different environments. Our present results confirmed that the *B. anthracis* transconjugants harbored both native plasmid pXO1 or pXO2 and exotic pHT73, which have been reported to have different replication mechanisms (9). This finding makes the classification of separate species in this group more ambiguous. Therefore, further investigation of virulence genes related to anthrax in *B. thuringiensis* will be of importance for assessing its potential impact in food and in the environment.

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