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Conjugal transfer between *Bacillus thuringiensis* and *Bacillus cereus* strains is not directly correlated with growth of recipient strains

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ABSTRACT

Bacillus thuringiensis and *Bacillus cereus* belong to the *B. cereus* species group. The two species share substantial chromosomal similarity and differ mostly in their plasmid content. The phylogenetic relationship between these species remains a matter of debate. There is genetic exchange both within and between these species, and current evidence indicates that insects are a particularly suitable environment for the growth of and genetic exchange between these species. We investigated the conjugation efficiency of *B. thuringiensis* var. *kurstaki* KT0 (pHT73-Em^R) as a donor and a *B. thuringiensis* and several *B. cereus* strains as recipients; we used one-recipient and two-recipient conjugal transfer systems *in vitro* (broth and filter) and in *Bombyx mori* larvae, and assessed multiplication following conjugation between *Bacillus* strains. The *B. thuringiensis* KT0 strain did not show preference for genetic exchange with the *B. thuringiensis* recipient strain over that with the *B. cereus* recipient strains. However, *B. thuringiensis* strains germinated and multiplied more efficiently than *B. cereus* strains in insect larvae and only *B. thuringiensis* maintained complete spore germination for at least 24 h in *B. mori* larvae. These findings show that there is no positive association between bacterial multiplication efficiency and conjugation ability in infected insects for the used strains.

1. Introduction

Bacillus thuringiensis is an important bacterial species for biological control of agricultural insect pests and disease vectors. Its entomopathogenic properties are due to the action of crystal (Cry) proteins (Schnepf et al., 1998), often encoded by genes on large, self-transmissible plasmids. The presence of these plasmids provides *B. thuringiensis* strains with the potential to colonize a range of insect species.

Bacillus cereus is an opportunistic human pathogen. It is responsible for gastrointestinal intoxications associated with food poisoning and causes diarrhea and emetic symptoms; it is also associated with other local and systemic diseases. The opportunistic nature of *B. cereus* as a pathogen is related to its capacity to produce a variety of non-specific virulence factors, including cereulide, phospholipases and enterotoxins (Vilas-Bôas et al., 2007; Arnesen et al., 2008).

Both *B. thuringiensis* and *B. cereus* belong to the *B.* cereus taxonomic group, which also includes *Bacillus anthracis, Bacillus mycoides, Bacillus pseudomycoides,* and *Bacillus weihenstephanensis.* The two species share substantial chromosomal similarity and are phenotypically nearly identical. *B. cereus, B. thuringiensis,* and *B. anthracis,* the best-studied species, display differences in plasmid characteristics associated with their pathogenicity (Rasko et al., 2007).

Although *B. thuringiensis* is readily isolated from soil, it is incapable of multiplying effectively in either bulk soil or water (Ohana et al., 1987; Thomas et al., 2000, 2001; Vilas-Bôas et al., 2000; Raymond et al., 2010b). There is evidence that insects are the optimal sites both for multiplication of *B. thuringiensis* and for it exchanging of genetic material (Aly et al., 1985; Jarrett and Stephenson, 1990; Vilas-Bôas et al., 1998; Takatsuka and Kunimi, 2000; Thomas et al., 2000, 2001, 2002; Suzuki et al., 2004; Raymond et al., 2010a).

Conjugation between *B. thuringiensis* strains resulting in the transfer of plasmids harboring a *cry* gene was first reported by González et al. (1982). Since then, considerable efforts have been made to understand the significance of this behavior to the evolution of this group of bacteria. This phenomenon has now been described in various conditions (Jarrett and Stephenson, 1990; Andrup et al., 1993, 1998; Wilcks et al., 1998; Jensen et al., 1995; Vilas-Bôas et al., 1998, 2000; Thomas et al., 2000, 2001, 2002). Plasmids may be transferred from *B. thuringiensis* to *B. cereus*, and from *B. thuringiensis* to other species and genera (González et al., 1982; Lereclus et al., 1983; Battisti et al., 1985; Reddy et al., 1987; Hu

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et al., 2004, 2005; Van der Auwera et al., 2007; Yuan et al., 2007). However, little is known about the preference of donor *B. thuringiensis* strains for genetic exchange with *B. thuringiensis* or *B. cereus* strains, or about the relationship between bacterial multiplication efficiency and the mating process. Therefore, we investigated the efficiency of conjugation between *B. thuringiensis* and *B. cereus* strains in one-recipient (a donor and a recipient strain) and two-recipient (a donor and two-recipient strains) systems. We also tested for any correlation between growth and conjugal transfer. We compared conjugative efficiency and the multiplication of vegetative cells of *B. thuringiensis* and several *B. cereus* strains following conjugation both *in vitro* and in *Bombyx mori* larvae of a *B. thuringiensis* plasmid bearing a gene encoding a Cry protein.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *B. cereus* group strains used are listed in Table 1. *B. thuringiensis* var. *kurstaki* KT0 (pHT73-Em^R) harbors the 75 kb resident plasmid pHT73-Em^R which carries the *cry*1Ac gene tagged with an *ermC* gene conferring erythromycin resistance (Vilas-Bôas et al., 1998).

B. thuringiensis and *B. cereus* strains were cultured in Luria–Bertani (LB, Difco) broth with shaking at 30 °C for the analysis of vegetative growth. Sporulated cultures were prepared by cultivating strains in Bacto-Peptone medium (BP, Lecadet et al., 1980), with shaking at 30 °C for 72 h and were observed by phase-contrast microscopy. The cultures were then harvested by centrifugation, and the pellets were washed twice and resuspended in sterile saline solution. CFU were counted for each suspension before assays with *B. mori* larvae, by plating serial dilutions on LB agar.

Streptomycin-resistant mutants and rifampicin-resistant mutants of *B. cereus* were isolated by plating bacteria on BP agar plates containing 200 μg mL^{-1} of streptomycin or 100 μg mL^{-1} of rifampicin. Single colonies were selected after 2 days of incubation at 30 °C. *B. thuringiensis* var. *kurstaki* KT0 (pHT73-Em^R) was cultured in culture media containing 100 μg mL^{-1} of erythromycin.

Plasmid stability in the exconjugants during vegetative growth was assessed in brain–heart infusion (BHI, Difco).

2.2. Mating procedures

B. thuringiensis var. *kurstaki* KT0 (pHT73-Em^R) was used as donor strain in all experiments; recipient strains are described in Table 1. The recipient and donor controls, cultured separately, were tested in parallel. All experiments were performed in triplicate. Conjugation frequencies were calculated as the ratio of exconjugants to recipient cells (EC/R).

Table 1
Properties of *B. thuringiensis* and *B. cereus* strains used in this study.

Strains	Characteristics	Source
B. thuringiensis var. *kurstaki* KT0 (pHT73-Em^R)	Donor, Cry$^+$, Em^R	[a]
B. thuringiensis var. *thuringiensis* 407-1	Recipient, Pig$^+$, Cry$^-$, Sm^R	[b]
B. cereus D1 4430	Recipient, Sm^R	[c]
B. cereus 569	Recipient, Sm^R	[c]
B. cereus 388	Recipient, Rif^R	[c]
B. cereus 433	Recipient, Rif^R	[c]
B. cereus ATCC 14579	Recipient, Sm^R	[d]
B. cereus ATCC 10987	Recipient, Sm^R	[d]
B. cereus MADM 1279R	Recipient, Rif^R	[e]

Cry$^+$: produces an insecticidal crystal; Cry$^-$: does not produce an insecticidal crystal; Pig$^+$: produces a brown pigment; Em^R: erythromycin resistant; Sm^R: streptomycin resistant; Rif^R: rifampicin resistant.

[a] Vilas-Bôas et al. (1998).
[b] This study.
[c] INRA-Génétique Microbienne et Environnement, Guyancourt/France.
[d] American Type Culture Collection, Rockville, MD, USA.
[e] National Environmental Research Institute, Roskilde/Denmark.

2.2.1. Plasmid transfer in broth

The procedure was as described by Vilas-Bôas et al. (1998). Equal volumes (250 μL) of donor and recipient strain cultures were grown to an OD_{600} of 1.0, and then added to 7 mL of freshly, pre-warmed LB broth without antibiotics and incubated at 30 °C for 2 h. Appropriate dilutions were plated onto LB agar containing either erythromycin and streptomycin or erythromycin and rifampicin. LB agar containing the appropriate antibiotic was used for counting recipient and donor cells. Matings that did not yield exconjugants were repeated and the entire contents of the mating mix (7 mL) were concentrated and plated.

Exconjugants derived from the recipient strain of *B. thuringiensis* var. *thuringiensis* 407-1 were tested for resistance to erythromycin and streptomycin, the presence of a crystal (by phase-contrast microscopy), and production of brown pigment; those derived from streptomycin-resistant strains of *B. cereus* were tested for resistance to erythromycin and streptomycin, and the presence of a crystal; and those from rifampicin-resistant strains of *B. cereus* were tested for resistance to erythromycin and rifampicin and the presence of a crystal.

Only recipient strains that successfully received pHT73-Em^R in one-recipient matings in LB broth were used for two-recipient mating experiments. This system was composed of the donor and two-recipient strains, *B. thuringiensis* var. *thuringiensis* 407-1 and a rifampicin resistant *B. cereus* strain. Aliquots of 250 μL of each of the three strains grown to an OD_{600} of 1.0 were mixed with 7 mL of fresh, pre-warmed LB broth. Appropriate dilutions were plated onto LB agar containing either erythromycin/streptomycin or erythromycin/rifampicin. Exconjugants were assessed as described for the one-recipient mating experiments.

2.2.2. Plasmid transfer on nitrocellulose filters

The procedure was as previously described by Lereclus et al. (1983). Broth cultures of the donor and recipient strains at an OD_{600} of 1.0 were mixed (0.5 mL of each) on membrane filters (Millipore 0.22 μm pore size). The membranes were dried and transferred onto LB agar without antibiotics and incubated for 24 h at 30 °C. The mating mixture was resuspended in 1 mL of LB broth and spread on selective medium containing the appropriate antibiotics. Dilutions plated separately onto LB agar containing either streptomycin or rifampicin were used to calculate the numbers of CFU of the recipient strains.

2.2.3. Bacterial growth and plasmid transfer in B. mori

Plasmid transfer and spore germination capacity, vegetative cell multiplication and sporulation were studied in third-instar larvae of the silkworm, *B. mori*. Larvae were obtained from the insect rearing unit at Fujimura do Brasil S/A, Arapongas/PR, Brazil. Experiments were carried out by free ingestion on mulberry leaves (*Morus nigra*, Moraceae) in acrylic boxes covered with lids that allowed aeration at 25 °C and with a 16/8 h light/dark photoperiod.

Fourteen larvae were maintained in individual boxes and each larva was fed on a mulberry leaf disk inoculated with 10 μL of a sporulated culture without heat treatment containing 10^9 CFU of the recipient strain. After 12 h at 25 °C, the larvae were transferred to leaves treated with 10^9 CFU of the sporulated culture without heat treatment of donor strain and incubation was continued at 25 °C until death. The experiments were performed in triplicate. In parallel control experiments, fourteen larvae were similarly fed with mulberry leaves without either donor or recipients. Bacteria were recovered from seven larvae immediately after death (t_0)