

SURVIVAL AND CONJUGAL TRANSFER BETWEEN *BACILLUS THURINGIENSIS* STRAINS IN AQUATIC ENVIRONMENT

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ABSTRACT

Field and laboratory studies were conducted to assess the survival of cells and spores and plasmid transfer between *Bacillus thuringiensis* strains in aquatic environment. Results indicated that cells and spores of *B. thuringiensis* can survive for 10 days in water, without altering their number. The sporulation process began after 12-15 hours of inoculation of water. *B. thuringiensis* was able to transfer conjugative plasmids in the aquatic environment.

Key words: *Bacillus thuringiensis*, conjugation, microbial ecology

INTRODUCTION

Bacillus thuringiensis δ -endotoxins are recognized safe biological insecticidal proteins. The insecticidal protein gene is usually found in large self-transmissible plasmids (7,26). The commercialized products are generally composed of spore-crystals, and large amounts of such product have been applied to aquatic environment in order to control disease vector flies.

The interest in the fate, survival and conjugation potential of bacteria introduced in field is increasing due to the risks to the environment in consequence of the unwanted release of biological control microorganisms. The ecological significance and importance of environmental gene transfer and survival of cells and spores of biological control microorganisms have been relatively poorly understood.

Many studies have been conducted on plasmid transfer in environmental conditions using *Pseudomonas* spp (3,23). However, few studies have examined the environmental transfer among *B. thuringiensis* strains. For *B. thuringiensis*, it has been shown that, after having grown in mixed cultures with other *Bacillus*, the large plasmids, encoding insecticidal toxins,

were transferred by a conjugation-like process (6,7). Mating between strains of *B. thuringiensis*, *B. cereus* and *B. anthracis* have been reported involving transference of small and large plasmids (4,13).

In soil microcosm the conjugation-like process has been poorly described (9,24) and no studies have been conducted in aquatic environment. Some information about persistence and survival of *B. thuringiensis* has been documented in soil. Saleh *et al.* (20) recovered *B. thuringiensis* from soil 14 to 40 days after the field application, and Pruett *et al.* (19) found spores after 6 months. Saleh *et al.* (20) showed germination of spores, when incubated in supplemented soil.

Studies conducted in non-supplemented soil indicated a decrease in the number of cells, and the maintenance in spore form (25). Laboratory studies indicate that spores of *B. thuringiensis* var *kurstaki* can survive for relatively long period in fresh water and marine environment (17).

The aim of this study was to determine the extent of conjugal transfer between *B. thuringiensis* strains in liquid broth, tap water, lake water, and sewage water in both laboratory and field conditions, and to describe the survival of cells and spores of this bacterium under the same conditions.

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MATERIALS AND METHODS

Bacterial strains

The strains of *B. thuringiensis* used in this study are listed in Table 1. *B. thuringiensis* var *kurstaki* KT0(pHT73-Em^R) harbors the 75kb pHT73-Em^R plasmid, which encodes the erythromycin resistance and the Cry1Ac toxin.

Strain 407-1 is an acrysaliferous brown pigment-producing derivative of strain 407 selected by streptomycin resistance. Acrysaliferous phenotype (407-1 and 4Q2-81R) was obtained by 42°C treatment.

Mating in liquid broth

KT0(pHT73Em^R) was used as the donor strain and either 407-1 or 4Q2.81R were used as the recipient strains. Donor and recipient cells were grown in Luria Bertani broth – LB (21) at 30°C, with aeration to logarithmic growth phase (10⁷cfu.ml⁻¹). Enumeration of donor and recipients strains was done by plating on LB agar with appropriate antibiotic. Equal amounts of donor and recipient cells (250 µl) were combined in 7 ml of prewarmed LB broth, and incubated at 30°C with shaking at 40 rpm (1). After 2 hours, 100 µl of the mixture was inoculated onto LB agar plates containing 10 µg.ml⁻¹ erythromycin and 200 µg.ml⁻¹ streptomycin. The frequencies of conjugation were calculated through the number of exconjugants per number of recipients.

Mating on nitrocellulose membrane filters

For mating on filters, donor and recipient cells (10⁷cfu.ml⁻¹) were mixed at the same ratio and filtered through a 0.45 µm-pore-size nitrocellulose membrane. Filters were placed, bacteria side up, on the surface of LB agar plates and incubated for 2 hours at 30°C. The cells were eluted from the filters and resuspended in saline solution, and inoculated on LB selective agar plates (16).

Mating in laboratory conditions

Matings were conducted in tap water, lake water and sewage water. Water samples from different sources were sterilized by autoclaving at 121°C for 1 hour. Donor and recipient cells were grown separately to logarithmic phase in 10 ml of LB broth,

centrifugated and resuspended to the volume of 10 ml of water (10⁸cfu.ml⁻¹) (18). The total volume of the mixture was incubated at 30°C for 24 hours with shaking at 40 rpm. Following the incubation, hourly samples were plated on LB agar plates containing erythromycin and streptomycin.

Mating in field conditions

Cells for mating in field conditions were prepared in the same manner as those for mating in laboratory conditions. The resuspended cells were placed in dialysis bags (Sigma n° 250-7U; 10 cm length, molecular weight cutoff > 12,000), each one containing 10 ml of the mixture of cells. The dialysis bags were placed in a sewage lake (22). After 2, 4, 6 and 24 hours, bacterial cells were recovered from each bag by removing 1 ml aliquots, following proper dilution and plating onto selective LB agar and incubated at 30°C.

Plasmid stability

To determine the stability of plasmid pHT73 in broth medium, culture of exconjugant originated from KT0(pHT73Em^R)/407-1 cells were kept exponentially growing by dilutions every 12 hours up to 48 hours, in prewarmed LB broth without antibiotics. Every 12 hours, serial dilutions in saline solution were spread on agar plates without antibiotics. After overnight incubation at 30°C, 100 individual colonies from exconjugant strain were transferred with a toothpick to agar plates containing erythromycin and streptomycin (15). The plasmid stability in water was calculated by adding exconjugant cells in water samples from different sources, and proceeding as described above.

Cell survival study

The experiments were conducted in the laboratory and in the field using the following types of water: sterile tap water, sterile lake water, sterile sewage and sewage water in field conditions. Strains 4Q2.81R and KT0(pHT73Em^R) were used.

The cell culture was grown in 50 ml LB broth at 30°C, with aeration, to logarithmic phase, centrifugated and washed twice in saline solution. Final concentrated cell suspensions (approximately 10⁸cfu.ml⁻¹) were inoculated into flasks with 50 ml

Table 1. Characteristics of *B. thuringiensis* strains used in mating and survival studies.

<i>B. thuringiensis</i> strain	Phenotype	Source
<i>kurstaki</i> KT0(pHT73Em ^R)	Cry ⁺ Em ^R	Vilas Bôas <i>et al.</i> (24)
<i>thuringiensis</i> 407-1	Cry ⁻ Sm ^R	Vilas Bôas <i>et al.</i> (24)
<i>israelensis</i> 4Q2-81R	Cry ⁻ Sm ^R	This study

Cry⁺ : crystaliferous; Cry⁻ : acrysaliferous; Em^R : erythromycin resistance; Sm^R : streptomycin resistance.

of water, and incubated at 30°C, with aeration. For cell enumeration, aliquots of 1 ml were withdrawn from each flask at intervals of 24 hours during 10 days, diluted and plated before and after heat treatment (70°C for 20 minutes) onto agar LB, and incubated at 30°C.

For the field study, dialysis bags containing 10 ml of resuspended vegetative cells (as described above) were placed in sewage water. At the time of 1, 2, 6, 8 and 10 days, a dialysis bag was removed and submitted to enumeration of bacteria using selective LB agar.

Spore survival study

The experiments were conducted in the laboratory using sterile tap, lake and sewage water.

The strains of *B. thuringiensis* were grown in 50 ml of Bacto Peptone broth – BP (14) for 3 days or up to total sporulation of the cells. The culture was heated for 20 min at 70°C. The spores were harvested by centrifugation, washed three times in saline solution and resuspended in sterile water to a final volume of 50 ml and incubated at 30°C with aeration. For enumeration of spores, water samples (1 ml) were taken every 24 hours up to 10 days, diluted and plated onto selective LB agar, before and after heat treatment. The plates were incubated at 30°C.

RESULTS

Conjugal transfer in broth

The conjugation potential transfer of the plasmid pHT73 in broth and filter was examined. The transference frequencies estimated after 2 hours of mating are listed in Table 2. After mating, exconjugant cells were selected on plates containing streptomycin and erythromycin followed by microscopic observation of crystal (donor strain phenotype). Exconjugant cells from KT0(pHT73Em^R) donor/407-1 recipient presented a brown pigment (recipient strain phenotype).

Concerning the KT0(pHT73Em^R)/4Q2-81R pair, the frequency of transfer of plasmid pHT73 in filter and in broth medium, after 2 hours, was similar. However, for the pair

KT0(pHT73Em^R)/407-1, the frequency was 10-fold higher in broth medium than in filter.

We also analysed if the exconjugant cells 4Q2.81RT that had gained pHT73 plasmid had become competent plasmid donors, when the recipient cell was the 407-1 strain, in mixed culture. The conjugation was detected but the conjugation frequency could not be determined because of the difficulty of selection.

Conjugal transfer in aquatic environment

For the experiments in water, in laboratory as well as in environmental conditions, the mating pair KT0(pHT73Em^R)/407-1 was used. Matings in tap water, lake water and sewage water conducted in the laboratory showed similar dynamics. The conjugation frequencies increased about 10 times in the three types of water in 6 hours, but in sewage water the conjugation frequencies were always higher (Fig. 1 and Table 3). A measure taken after 24 hour, indicated a similar frequency to that measured at time 6 hour. The pH of sewage water used in these studies varied between 9.0 and 10.0 before sterilization and between 7.0 and 8.0 after sterilization. The concentration

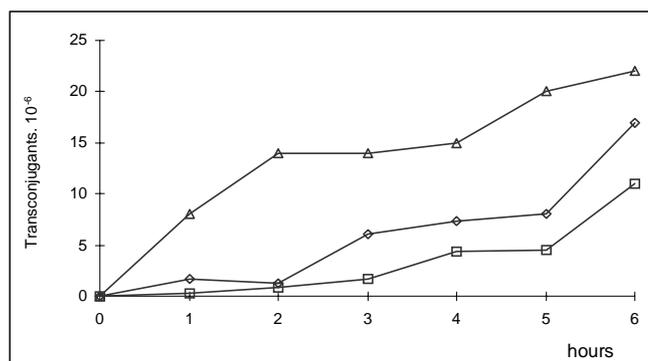


Figure 1. Transfer of plasmid pHT73 from KT0(pHT73-Em^R) donor to 407-1 recipient strains in tap water (◇), lake water (□) and sewage water (△) in laboratory conditions.

Table 2. Transfer frequencies between *B. thuringiensis* strains in broth culture and on nitrocellulose membrane.

Donor	Recipient	Transfer frequencies ¹	Transfer frequencies ¹
		(broth mating)	(membrane mating)
		2 hours	2 hours
KT0(pHT73Em ^R)	407-1	1.7x10 ⁻⁴	2.0x10 ⁻⁵
KT0(pHT73Em ^R)	4Q2-81R	6.0x10 ⁻⁸	3.0x10 ⁻⁸

¹ exconjugants per recipient. The values are average of three repetitions.

Table 3. Transfer frequencies between KT0(pHT73-Em^R) and 407-1 in different sources of water, in laboratory conditions.

	Transfer frequencies ¹		
	1 hour	3 hours	6 hours
tap water	1.6 x 10 ⁻⁶	6.0 x 10 ⁻⁶	1.7 x 10 ⁻⁵
lake water	1.2 x 10 ⁻⁶	2.5 x 10 ⁻⁶	1.0 x 10 ⁻⁵
sewage water	5.5 x 10 ⁻⁶	1.4 x 10 ⁻⁵	2.4 x 10 ⁻⁵

¹ exconjugants per recipient. The values, are average of three repetitions.

Table 4. Chemical characteristics of water samples, used in the conjugation experiments

mg.l ⁻¹	sample			
	1	2	3	4
Ca	28.72	13.77	14.40	15.70
Mg	3.84	4.48	4.67	3.91
P	<0.05	0.11	2.22	2.88

- 1 -sterile tap water
- 2 -sterile lake water
- 3 -sterile sewage water
- 4 - non sterilized sewage water

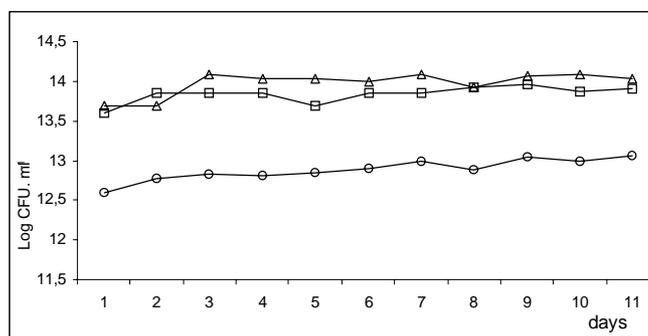
of some ions such as Ca, Mg, and P, in the three types of water remained the same after sterilization (Table 4).

In field conditions, the maximum and minimum concentration of O₂ dissolved in the water was 13.4 and 9.4, respectively. In the period of the experiment the temperature of sewage water varied between 32 to 35°C. In studies conducted in sewage water, in field conditions, the conjugation frequency detected after 6 hours of experiment was 1x10⁻⁶, and remained constant until 24 hours, which was the last measurement.

The experiments carried out in liquid medium and in water indicated the pHT73 plasmid maintained its stability when in non-selective conditions. The stability was estimated to be 100%.

Survival spore and cell study

In laboratory the survival of *B. thuringiensis* strains was monitored by counting of viable cells and spores. The sporulation dynamics of *B. thuringiensis* vegetative cells in the different types of water is shown in Fig. 2. In all trials, the numbers of viable cells remained inalterd after inoculation in water. After 24 hours of inoculation in water in laboratory,

**Figure 2.** Sporulation of *B. thuringiensis* strains in laboratory conditions. Tap water (O), lake water (Δ), sewage lake water (□). Each point corresponds to the average number of spores of strains 4Q2.81R and KT0(pHT73Em^R).

approximately 100% of the cells were sporulated, without decrease in this number. Endospores were observed after 8 hours of inoculation, and spores after 12 h in tap water and 15 in lake and sewage lake water. In the spores survival study, loss of spores or germination were not observed. The number of spores remained stable during the 10 days of experiment (Fig. 2).

In the studies conducted in the field a small decrease in the number of cells of strain 4Q2.81R was observed after 6 hours of inoculation, and this number did not change in the course of the experiment.

DISCUSSION

When grown in broth culture, *B. thuringiensis* strains are able to transfer plasmids in variable frequencies by a conjugation-like plasmid transfer process first described by Gonzalez *et al.* (7,8,10,15,24). In this study, experiments with mating broth using *B. thuringiensis* strains showed frequencies of 10⁻⁴ to 10⁻⁸ (Table 2). These results are in agreement with those observed by other authors. (2,10,24).

For mating between KT0(pHT73Em^R) and 4Q2.81R, the lower frequency observed can be attributed to the effect of either bacteriocins (1,11,12) or aggregation phenotype between strains (1,2). The method used for mating between this pair did not interfere in the frequency of transference (Table 2).

For the first time the results presented here indicate that plasmid transfer between *B. thuringiensis* strains can occur in aquatic environment both under laboratory and field conditions. It was observed that in the laboratory the conjugation frequency in sewage water was 10-fold higher than in field conditions. Parameters such as temperature, nutrients, and pH may interfere with gene transfer. Vilas Bôas (25) showed that the acid pH of soil does not alter the conjugation process. In our study, the pH value ranged from alkaline to neutral condition, and did not hinder the conjugation process.

Our results showed that survival and persistence of *B. thuringiensis* during 10 days in water in laboratory and in field conditions were similar. In field conditions, any contamination of water could be observed because cells and spores were suspended in a dialysis bag and not monitored at the bottom of sewage lake. However, these condition did not show the influence of the aquatic microbial environment. *B. thuringiensis* strains inoculated in water of different origins presented a constant kinetics in spore formation and persistence. In another study (17), a higher percentage of survival of *B. thuringiensis* in lake water than in tap and distilled water was shown. The authors observed a 40% decrease in viable cells in the three di treatments in the 10 days of experiment, and they remained relatively stable until the 70th day. In the present study, loss of viable cells was not observed. In field conditions, the basic pH did not alter the survival of *B. thuringiensis*.

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RESUMO

Sobrevivência e conjugação de *Bacillus thuringiensis* em ambiente aquático

O presente trabalho é um estudo sobre a sobrevivência e a conjugação de linhagens de *Bacillus thuringiensis* em água. Os experimentos conduzidos no laboratório mostram que as células e os esporos de *B. thuringiensis* podem persistir pelo menos 10 dias na água. A esporulação inicia-se 12-15 horas após a inoculação. O processo de conjugação foi demonstrado

em diferentes ambientes aquáticos, tanto em condições de laboratório quanto no meio ambiente.

Palavras-chave: *Bacillus thuringiensis*, conjugação, ecologia microbiana.

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