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High-Salt Stress Conditions Increase the pAW63 Transfer Frequency in *Bacillus thuringiensis*

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Conjugation experiments with *Bacillus thuringiensis* and transfer kinetics demonstrated that salt stress has a positive impact on plasmid transfer efficiency. Compared to standard osmotic conditions (0.5% NaCl), plasmid transfer occurred more rapidly, and at higher frequencies (>100-fold), when bacteria were exposed to a high-salt stress (5% NaCl) in liquid brain heart infusion (BHI). Under milder salt conditions (2.5% NaCl), only a 10-fold effect was observed in Luria-Bertani broth and no difference was detected in BHI. These observations are particularly relevant in the scope of potential gene exchanges among members of the *Bacillus cereus* group, which includes food-borne contaminants and pathogens.

Bacillus cereus sensu lato group members are Gram-positive, spore-forming bacteria that are widely distributed in the environment, in food products, and in processing lines (5, 7, 26). Although members of this group were historically classified as separate species on the basis of phenotypic criteria only, major evidences tend to consider them as forming a single taxon (8, 16, 22). Moreover, it is largely recognized that phenotypical features that were originally used to distinguish *B. cereus* sensu stricto, *Bacillus thuringiensis*, and *Bacillus anthracis* are directly related to the plasmid pool, making a cured strain of *B. thuringiensis* indistinguishable from that of *B. cereus* sensu stricto (14). This issue has led to the growing concern regarding the extended use of *B. thuringiensis* as a biopesticide that could end up in the food chain.

In light of the connections that exist between plasmids and virulence factors associated with *B. cereus* sensu lato, the study of plasmid transfer among these bacteria is of great interest. In this context, conjugation has been extensively studied in different niches, including insect gut (20), gnotobiotic rats (28), the rhizosphere of grass plant (18), soil (24), water (21), and, most recently, dairy products (15, 23). These habitats were found to variably affect the overall process of plasmid conjugation and mobilization, suggesting adaptative transfer mechanisms.

Among the numerous environmental conditions associated with *B. cereus*, salt stress is particularly relevant since it is largely used in the food industry as an additive and preservative. Moreover, the salt stress response of *B. cereus* has highlighted a negative effect of sodium chloride on bacterial growth and the important modulation of the *B. cereus* genetic expression profile (3). The upregulation of osmoprotectants, Na^+/H^+, and dipeptide and tripeptide transporters and the activation of an oxidative stress response are among the noticeable effects of the general salt stress transcriptome response that have been reported (4).

As suggested above, horizontal gene transfers are of particular interest in the case of the *B. cereus* group, which is known to harbor a plethora of plasmids and mobile elements (10, 17). In this study, the model plasmid pAW63, originating from *B. thuringiensis* subsp. *kurstaki* (27), was used to assess the conjugation dynamics among *B. thuringiensis* strains in various stress conditions.

The transfer behavior of pAW63, as well as its ability to mobilize pUB110 (through donation, which corresponds to mobilization by the pAW63 transfer machinery), originating from *Staphylococcus aureus*, in biparental matings were studied in Luria-Bertani (LB) and brain heart infusion (BHI) broths, under conditions of mild (2.5%) and high (5% [wt/vol] NaCl) salt stresses, and were compared to that displayed in standard liquid culture broth (0.5% NaCl). The strains used in the biparental mating systems are reported in Table 1. In order to select the transconjugants and the mating partners, appropriate combinations of antibiotics were added to the culture media at the following concentrations (μg ml^{-1}): nalidixic acid (Nal), 15; streptomycin (Sm), 100; tetracycline (Tet), 10; and kanamycin (Kan), 50. Mating experiments were performed by using inocula prepared from overnight bacterial cultures grown in the same media as the conjugation mixture. Equal amounts of donor and recipient cells were mixed in a glass bottle, under microaerophilic conditions (headspace, <10% of the total volume; no shaking), to obtain a mixture containing about 10^7 CFU ml^{-1}. Mating experiments were performed for 4 h at 30°C, without shaking. Appropriate dilutions of bacteria were plated on selective solid media, and transfer frequencies were estimated as the number of transconjugants per recipient cell (T/R). The presence of expected plasmids in transconjugants was confirmed by PCR using plasmid-specific primers (data not shown). All conjugation experiments were performed at least three times and kinetics at least twice. Differences between plasmid transfer frequencies under different salt stress conditions of mating experiments were compared using one-way analysis of variance (ANOVA) on arcsine-transformed data, followed by Student's *t* test performed using SAS (v9.2) to verify whether the differences were significant ($P < 0.05$) or not.

High-salt stress conditions greatly favor plasmid transfer between *B. thuringiensis* strains. *B. thuringiensis* conjugation was assessed using a donor strain containing the self-transfer plasmid pAW63 and the mobilizable plasmid pUB110 [named GBJ002(pAW63, pUB110)] and a cured isogenic recipient strain (named GBJ001). As shown in Fig. 1A, significant ($P < 0.05$) 5- to 10-fold decreases in the conjugation and mobilization frequencies were observed in mild salt stress conditions compared to those in

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Origin and/or features	Source or reference
B. thuringiensis subsp. *israelensis* strains		
4Q7	Derivative of wild-type isolate 4Q2, cured of all plasmids	BGSC[a]
GBJ001	Spontaneous Sm^r mutant of 4Q7, used as recipient	9
GBJ002	Spontaneous Nal^r mutant of 4Q7, used as donor	9
Plasmids		
pAW63::Tn*5401*	Derivative of natural conjugative *B. thuringiensis* subsp. *kurstaki* plasmid pAW63, Tet^r	27
pUB110	Natural mobilizable plasmid originating from *S. aureus*, Kan^r	6

[a] *Bacillus* Genetic Stock Center, Columbus, OH.

LB medium. Conversely, pAW63 and pUB110 transfer frequencies increased significantly ($P < 0.05$) in 5% salt stress conditions, with transfer frequencies reaching ca. 10^{-3} T/R for both conjugation and mobilization.

Unlike in LB medium, pAW63 conjugation and pUB110 mobilization frequencies in BHI were similar in both mild and standard salinity conditions (Fig. 1B), reaching frequencies of ca. 3×10^{-5} T/R. However, pAW63 conjugation and pUB110 mobilization frequencies showed a striking increase in BHI 5% NaCl ($P <$ 0.001), with transfer frequencies 100- to 500-fold higher in high-salt stress conditions than in standard salinity.

Transfer kinetics highlighted differential transfer parameters between high-salt stress and standard salinity conditions. To have a better understanding of the transfer behavior of pAW63 and pUB110 plasmids, kinetics were obtained under standard and high-salt stress conditions, in BHI medium, using *B. thuringiensis* subsp. *israelensis*. The mating mix was sampled every 10 min for 4 h for each condition. The kinetics were analyzed using several parameters, such as the frequencies of transfer, the detection time (t_S) at the onset of transfer, and the equilibrium time (t_E), which is the time from which the frequency remains constant (15).

In standard BHI conditions (0.5% NaCl) (Fig. 2A), pAW63 and pUB110 transfers were both detected after 90 min (t_S). pAW63 transfer frequency reached a maximum of 1×10^{-5} T/R and remained constant until the end of the mating period (4 h). The apparent mating period of pAW63 in standard conditions was estimated as 100 min (t_E = 190 min). Mobilization of pUB110 displayed the same transfer behavior.

In high-salt stress conditions (5% NaCl) (Fig. 2B), the onset of pAW63 conjugation and pUB110 mobilization took place after only 10 and 20 min (t_S), respectively, which is very fast compared to the 90 min observed in the plasmid transfers in standard BHI (Fig. 2A). Since, under these conditions, the stabilization of mating also occurred after 190 min (t_E), the resulting "mating time" was 180 min. Thus, compared to the standard BHI conditions, the higher transfer frequencies (reaching almost 1×10^{-2} T/R) resulted from both an earlier onset of transfer and a longer apparent mating period.

The sodium chloride concentrations used in this study mim-

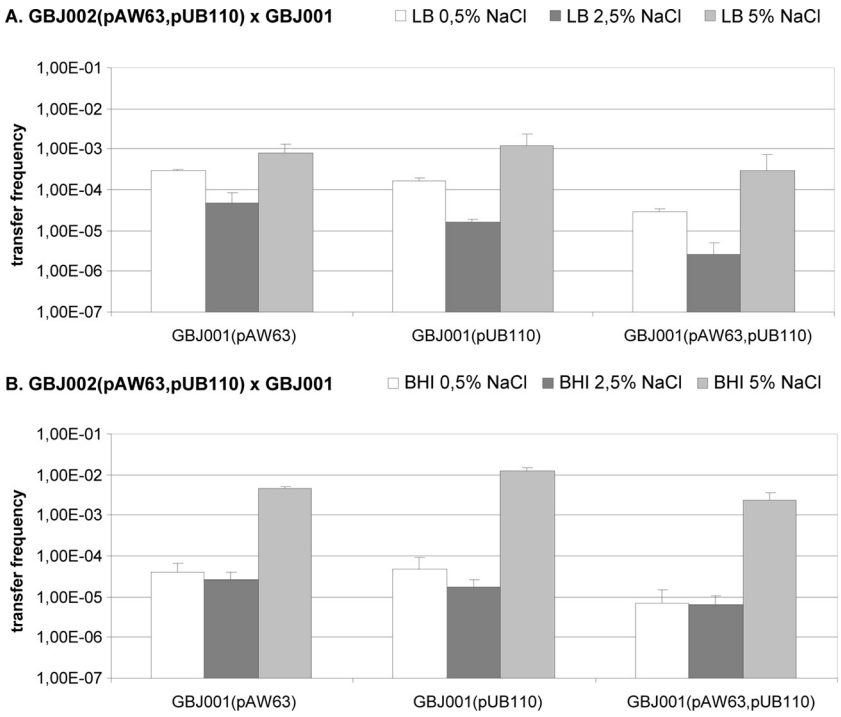

FIG 1 Conjugation among *B. thuringiensis* strains in salt stress conditions in LB (A) and BHI (B) media. The same number of donor and recipient *B. thuringiensis* strains was mixed and incubated for 4 h at 30°C. The following three different conditions were tested: standard (0.5%), mild (2.5%), and high-salt stress (5% [wt/vol] NaCl) conditions. pAW63 and pUB110 transfer frequencies into the GBJ001 recipient cells were assessed as the number of T/R. The interval bars indicate the standard deviations.

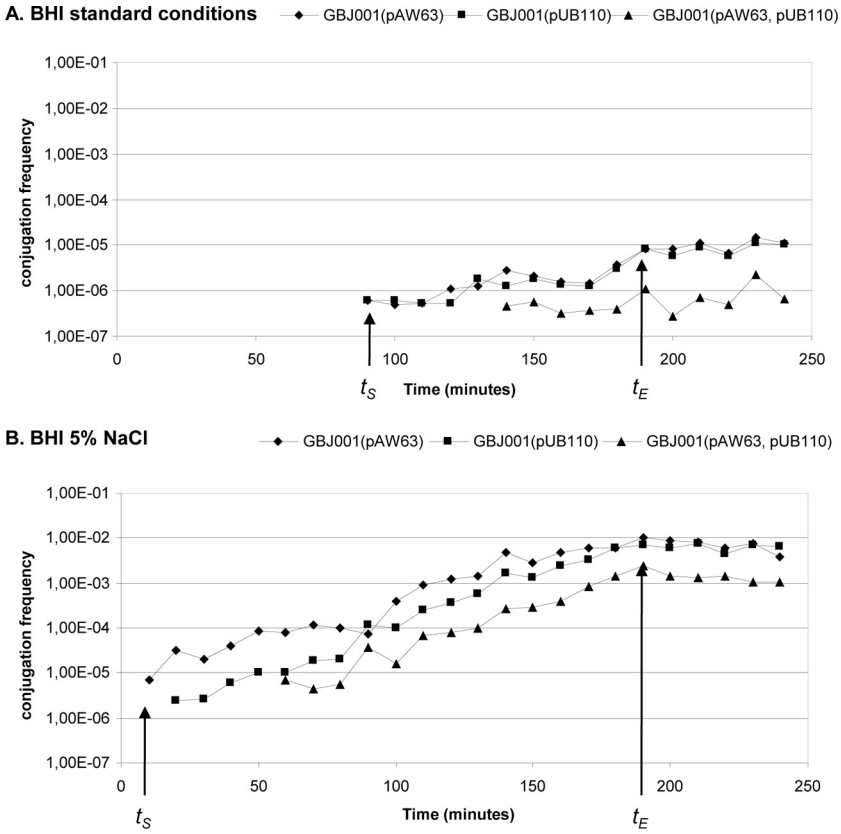

FIG 2 Kinetics of pAW63 and pUB110 transfer in a *B. thuringiensis* biparental mating. The experiments were performed in BHI standard conditions (0.5%) (A) and BHI high-salt stress conditions (5% [wt/vol] NaCl) (B). The following three types of transfer were detected every 10 min for 4 h: transfer of pAW63 (◆), mobilization of pUB110 (■), and transfer of both plasmids to the recipient cell (▲). The detection time (t_S) and the equilibrium time (t_E) for pAW63 transfer (15) are shown on each chart. pAW63 and pUB110 transfer frequencies were estimated as the number of T/R.

icked salt concentrations found in certain food products (e.g., 2% of NaCl in some cheese and up to 5% in sausages). Although *B. thuringiensis* can sustain these salt concentrations, we have observed the following phenotypic modifications of the bacterial cells: a decrease in motility, a reduced cell number, clumping of bacteria, and the formation of long bacterial filaments forming visible aggregates (Fig. 3). These phenotypic observations are consistent with previous studies, where it was demonstrated that high-salt stress has pleiotropic effects on the physiology of *Bacillus subtilis*, such as alteration in the composition of the cytoplasmic membrane (12, 13), adjustment of the cell wall properties (11), and impairment of the swarming capability of the cells (19). Similarly, a recent study (4) on the transcriptomic response of *B. cereus* exposed to a 5% NaCl stress has reported a downregulation of autolysin and murein hydrolase exporter genes potentially involved in hydrolyzing peptidoglycan when daughter cells separate.

These observations could obviously account for the reduced number of CFU/ml noticed in our experiments but could also possibly account for the increased frequencies in plasmid transfer. The formation of filaments observed when *B. thuringiensis* subsp. *israelensis* strains were exposed to a high-salt stress (Fig. 3B) is indeed reminiscent of the recent observations of a very efficient

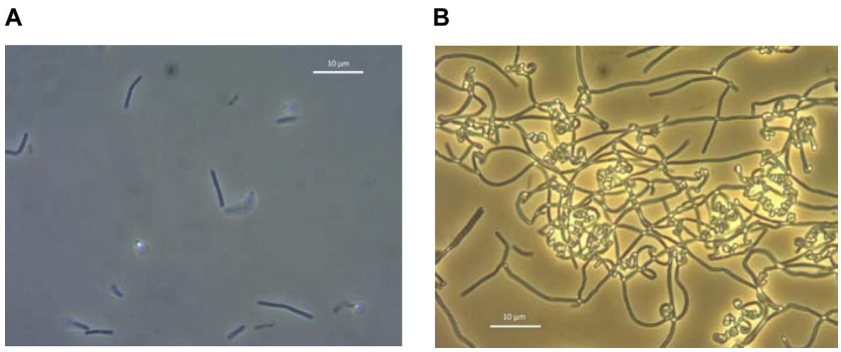

FIG 3 Phase-contrast micrograph of overnight precultures of *B. thuringiensis* subsp. *israelensis* in LB 0.5% NaCl (A) and in BHI 5% (wt/vol) NaCl (B).

transfer of the *B. subtilis* conjugative element ICE*Bs1* in bacterial cell chains (1). The high efficiency of conjugation seems to be likely due to an intimate and stable cell-cell contact when bacteria are present in a chain. Along the same lines, kinetics in BHI medium highlighted a faster transfer of plasmids in high-salt stress conditions, while the equilibrium time (t_E) remained the same. It is then tempting to speculate that the rapid formation of filaments and, subsequently, the quick propagation of plasmids between bacterial cell chains could explain the earlier onset of plasmid transfer and the higher frequencies observed. A similar mechanism could therefore explain the higher transfer frequencies of pAW63 and pUB110 in high-salt stress conditions. In addition, the subcellular localization of several conserved conjugation proteins encoded by pLS20, a plasmid found in *B. subtilis*, was recently investigated. This plasmid is largely membrane-associated, indicating that transfer takes place at the cell pole, which is a preferred site for the assembly of the active conjugation apparatus (2). This observation reinforces the assumption that the transfer of plasmids could occur more rapidly in bacterial cell chains, a characteristic also reminiscent of the plasmid transfer observed in *Streptomyces* species (25).

An alternative, but non-mutually exclusive hypothesis could stem from differential expression of plasmidic and/or chromosomic genetic determinants in stressed conditions. It has indeed been demonstrated that a high-salt stress induces a change in the expression pattern of *B. cereus* (4), where σ^B and many other members of the σ^B regulon are upregulated, while genes described to be involved in chemotaxis are downregulated. Further transcriptomic analysis of plasmid-borne and chromosomal gene expression, during bacterial conjugation and under salt stress conditions, will help better understand the regulatory pathways underlying plasmid transfer in the *B. cereus* group in response to variations of environmental conditions.

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