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Original Research Article

pXO16, the large conjugative plasmid from *Bacillus thuringiensis* serovar *israelensis* displays an extended host spectrum

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ABSTRACT

pXO16, the large conjugative plasmid from *Bacillus thuringiensis* serovar *israelensis* is able to efficient self-transfer, to mobilize and retro-mobilize non-conjugative plasmids, including "non-mobilizable" plasmids, and to transfer chromosomal loci. It also displays a remarkable aggregation phenotype associated with conjugation under liquid conditions. However, it was recently shown that aggregation boosts pXO16 transfer but is not mandatory. In this paper, we have further explored pXO16 transfers under various mating conditions and with different members of the *Bacillus cereus* group. The results indicated that colony or filter mating largely compensate the transfer deficit observed when using a pXO16 aggregation-minus mutant. Using filter mating, pXO16 transfer efficiency and host range were both improved. For instance, pXO16 was shown to transfer itself, and to mobilize the small pUB110 plasmid, from *B. thuringiensis* serovar *israelensis* to the thermotolerant *Bacillus cytotoxicus* at frequencies of 3.3×10^{-3} and 5.2×10^{-4} transconjugants per donor (T/D), respectively. All together, these results indicate that pXO16 can potentially "circulate" among members of the *Bacillus cereus* group. Yet, this is contrasting with pXO16's known natural distribution, which is apparently limited to the *israelensis* serovar of *B. thuringiensis*.

1. Introduction

pXO16 is a large conjugative plasmid associated with the entomopathogen *Bacillus thuringiensis* serovar *israelensis*. It is used as biopesticide to control insect larvae of mosquitoes and blackflies, the vectors of animal and human diseases. This entomopathogenic activity results from the production, during sporulation, of delta-endotoxins (Cry) and cytolysins (Cyt), which form crystals in the bacterial sporanges (Lacey, 2007; Palma et al., 2014). *B. thuringiensis* belongs to *Bacillus cereus sensu lato (s.l.)*, a group of closely related bacteria whose members display differential virulence (Jensen et al., 2003; Okinaka and Keim, 2016). *Bacillus anthracis* is a mammal and human pathogen, while some strains of *B. cereus sensu stricto* are known as foodborne pathogens, provoking emesis or diarrhoea with a potentially lethal outcome. Interestingly, most of these bacteria harbour a plethora of plasmids, including conjugative and mobilizable ones. Moreover, some of the extrachromosomal molecules contain the genetic determinants for the virulent genes, as is the case for the virulent plasmids pXO1 and pXO2 of *B. anthracis*, the causative agent of anthrax (Hu et al., 2009).

One intriguing element of this extrachromosomal pool is pXO16. This 350-kb conjugative plasmid transfers itself in a fast and efficient way (100% within < 5 min) (Andrup et al., 1998). Another peculiar feature of pXO16 is its macroscopic aggregation phenotype displayed during conjugation (Andrup et al., 1993; Jensen et al., 1995). Typically, within minutes of contact, donor and recipient cells aggregate and form visible clumps that last for several minutes until pXO16 transfer is achieved. Recently, a 25-kb "aggregation" locus was identified in pXO16 sequence. Deletion of this region hampered the appearance of aggregation. However, although the transfer efficiency was reduced in the aggregation-minus mutant, pXO16 could be transferred at frequencies of *ca.* 10^{-4} transconjugants per donor cell (T/D). Similar transfer reductions were observed for mobilization of small plasmids (Makart et al., 2018).

In this paper, we report the transfer of pXO16 aggregation-minus mutant in colony and filter matings. We have also further investigated pXO16 conjugation and mobilization properties among other members of the *B. cereus* group using filter mating. In these conditions, and contrary to what was previously thought, the host range of this *B. thuringiensis* serovar *israelensis* plasmid appears to cover various members of the group, including its most distantly related kin, the thermotolerant *Bacillus cytotoxicus*.

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Table 1
Bacterial strains and plasmids used in this work.

Strains/plasmids	Main features	Source or references
Bacterial strains		
B. thuringiensis sv. *israelensis* GBJ001	Sm^R spontaneous mutant of reference strain 4Q7 cured of its plasmids	(Jensen et al., 1995)
B. thuringiensis sv. *israelensis* GBJ002	Nd^R spontaneous mutant of reference strain 4Q7 cured of its plasmids, except pBtic235	(Jensen et al., 1996; Gillis et al., 2017)
B. thuringiensis sv. *israelensis* GBJ002(pXO16)	GBJ002-derivative containing pXO16::Tn*5401* (Tc^R)	(Timmery et al., 2009)
B. thuringiensis sv. *israelensis* GBJ002(pXO16Δ*agr*)	GBJ002-derivative containing pXO16Δ*agr* (Cm^R)	(Makart et al., 2018)
B. thuringiensis sv. *israelensis* GBJ002(pXO16, pUB110)	GBJ002-derivative containing pXO16::Tn*5401* (Tc^R) and pUB110 (Km^R)	(Timmery et al., 2009)
B. thuringiensis sv. *entomocidus* HD9	Wild-type isolate, BGSC-ID: 4I4	BGSC[a]
B. thuringiensis sv. *entomocidus* HD9.1	Sm^R spontaneous mutant of strain HD9	(Makart et al., 2018)
B. thuringiensis sv. *aizawai* HD11	Wild-type isolate, BGSC-ID: 4J4	BGSC[a]
B. thuringiensis sv. *aizawai* HD11.1	Sm^R spontaneous mutant of strain HD11	(Makart et al., 2018)
B. weihenstephanensis WSBC10204	Wild-type isolate	(Lechner et al., 1998)
B. weihenstephanensis WSBC10204.1	Sm^R spontaneous mutant of strain WSBC10204	(Makart et al., 2018)
B. cereus sensu stricto (*s.s.*) H3081.97	Wild-type emetic isolate	(Hoffmaster et al., 2008)
B. cereus s.s. H3081.97.1	Sm^R spontaneous mutant of strain H308197	(Makart et al., 2018)
B. cereus s.s. IS075	Wild-type emetic isolate	(Hoton et al., 2009)
B. cereus s.s. IS075.1	Sm^R spontaneous mutant of strain IS075	(Makart et al., 2018)
B. cereus s.s. VD021	Wild-type isolate	(Van der Auwera et al., 2013)
B. cereus s.s. VD021.1	Sm^R spontaneous mutant of strain VD021	This work
B. cytotoxicus E28.3	Wild-type isolate from potato flakes	(Kone et al., submitted)
B. cytotoxicus E28.3.1	Sm^R spontaneous mutant of strain E28.3	This work
B. cytotoxicus E28.3.1(pXO16)	E28.3.1-derivative containing pXO16::Tn*5401* (Tc^R)	This work
B. cytotoxicus E28.3.2	Rf^R spontaneous mutant of strain E28.3	This work
Plasmids		
pXO16::Tn*5401*	Derivative of pXO16, conjugative plasmid naturally occurring in strain 4Q2 of *B. thuringiensis* sv. *israelensis*, Tc^R	(Jensen et al., 1996)
pXO16Δ*agr*	Aggregation-deficient pXO16-derivative due to the deletion of its *agr* region, Cm^R	(Makart et al., 2018)
pUB110	Mobilizable plasmid, Km^R	(Keggins et al., 1978

[a] BGSC: *Bacillus* genetic Stock Center, Ohio State University, USA.

2. Materials and methods

2.1. Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are reported in Table 1. Bacteria were grown in Lysogeny Broth (LB) medium containing NaCl (5 g L^{-1}), yeast extract (5 g L^{-1}) and Tryptone (10 g L^{-1}). LB was solidified with 1.4% (*w/v*) agar for agar plates. When appropriate, antibiotics (Sigma) were added at the following concentrations (μg mL^{-1}): Sm, streptomycin (100); Nd, nalidixic acid (15); Tc, tetracycline (10); Cm, chloramphenicol (10); Rf, rifampicin (50); Km, kanamycin (50).

2.2. Spontaneous antibiotic-resistant mutants

Spontaneous antibiotic-resistant mutants were obtained by plating the equivalent of at least 15 mL of an overnight culture (*ca.* 10^8 CFU mL^{-1}) on LB plates with the appropriate antibiotics from which growing colonies were retrieved and confirmed on new antibiotic-containing LB plates.

2.3. pXO16 mating assays

2.3.1. Matings in liquid media

Plasmid transfers in liquid media were performed as previously described in Timmery et al. (2009) with some modifications. Briefly, the mating partners (donor and recipient cells) were streaked separately on LB plates containing the appropriate antibiotic(s) for strain and plasmid selection and incubated at 30 °C overnight. Separate pre-cultures from initial plates were grown in 10 mL of LB medium without antibiotics and incubated overnight at 30 °C without shaking. Equal amounts of mating partner cells from pre-cultures (maximum 1 mL per pre-culture) were mixed in 10 mL of LB medium. The mixture was incubated at 30 °C, without shaking, during the mating time (4 h). After mating, appropriate dilutions were plated on selective media for parental and transconjugant strains and incubated overnight at 30 °C. Each mating experiment included a plating control of donor and recipient cell pre-cultures on selective media to exclude the presence of potential spontaneous mutants. The transfer frequencies were calculated as the ratio of transconjugants per donor cells (T/D) at the end of the conjugation time. All mating experiments were repeated at least three times, independently.

2.3.2. Filter matings

Filter mating experiments were performed three times during 4 h, as described hereinabove. The distinction with liquid matings is the establishment of contact between conjugative cells. Equal amounts of donor and recipient cells were successively passed through a 0,20 μm filter, which was then collected, placed on a LB plate without antibiotic and incubated at 30 °C during the defined mating time. After mating, cells were collected from the filter by resuspension in LB medium, before plating out the appropriate dilutions on selective media.

2.3.3. Drop-on-drop or colony mating

Drop-on-drop mating experiments were performed three times, as described above, during 4 h. Five μL of overnight pre-cultures of donor and recipient cells were placed on the same spot of a LB plate without antibiotic and incubated at 30 °C. After mating, the resulting colony was collected from the plate with a sterile loop and resuspended in LB medium, before plating out the appropriate dilutions on selective media.

2.3.4. Mobilization experiments

Mobilization of the small Km^R plasmid pUB110 (Table 1) by pXO16 was performed as previously described (Timmery et al., 2009), except that the matings were done on filter, as detailed above for pXO16.

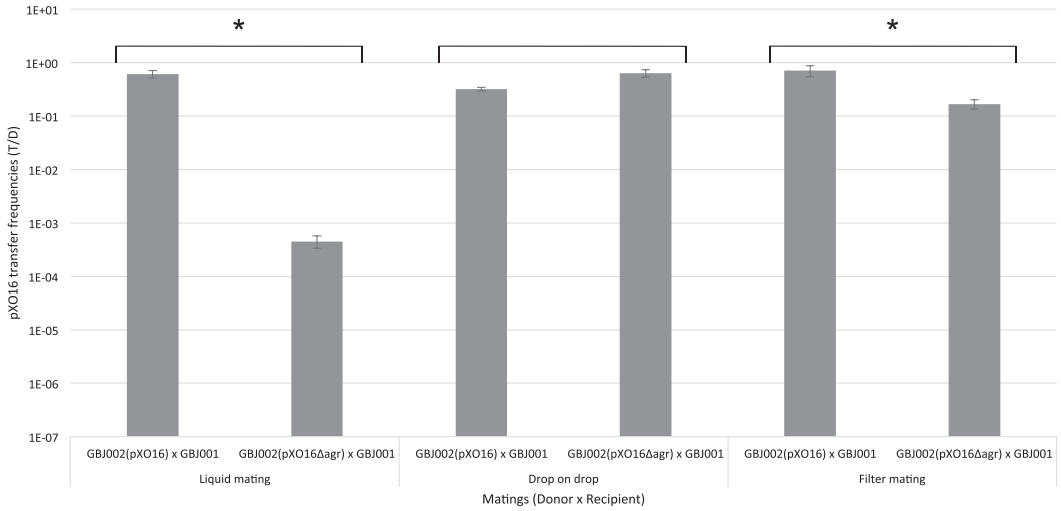

Fig. 1. Conjugative transfer frequencies of pXO16 and pXO16Δ*agr* under different mating conditions. Matings are always noted as "Donor x Recipient". Transfer frequencies are expressed as transconjugants per donor cells (T/D). Each frequency corresponds to the mean ± SEM out of three replicates. A star indicates that the differences between frequencies (under the same bracket) were significant, following a non-parametric Wilcoxon-Mann-Whitney test ($p < .05$). Otherwise, the differences were not significant.

3. Results

3.1. pXO16 aggregation is dispensable in filter and colony matings

One of the predictions of the study on pXO16 aggregation-minus mutant (Makart et al., 2018) was that the transfer deficit, in liquid medium, might be compensated by providing alternative physical contact between donor and recipient cells. In order to test this hypothesis, two complementary experiments were performed: the mating partners were either mixed together as drops on the surface of LB plates (drop-on-drop or colony mating) or were first deposited on a single filter and then incubated on LB plates (filter mating). As shown in Fig. 1, transfer of wild type pXO16 between *B. thuringiensis* sv. *israelensis* strain GBJ002 and GBJ001 was equally efficient, irrespective of the conditions, with average transfer frequencies of 6.1×10^{-1}, 3.2×10^{-1} and 7.1×10^{-1} T/D for liquid, drop-on-drop and filter matings, respectively. As shown before, the pXO16Δ*agr* mutant displayed a reduction of *ca.* 10^3 log in transfer efficiency in liquid conditions (Makart et al., 2018). However, when the transfer of pXO16Δ*agr* was performed with either colony or filter mating, the transfer efficiency observed of the type wild-type pXO16 was restored, although with a slight decrease in the case of filter mating (1.7×10^{-1} *versus* 7.1×10^{-1} T/D) (Fig. 1).

3.2. Filter mating allows pXO16 to move to various members of the B. cereus group

Based on the observation that filter mating provided appropriate conditions for pXO16 transfer among *B. thuringiensis* sv. *israelensis*, we further explored this mating set-up for various members of the *B. cereus* group and compared the transfer efficiencies between liquid and filter matings (Fig. 2). Three distinct situations could be observed. In the case of pXO16 transfer between *B. thuringiensis* sv. *israelensis* strain GBJ002 and the emetic strain ISO75.1 of *B. cereus*, no significant differences were noticed between liquid and filter matings with frequencies of *ca.* 1.6×10^{-2} T/D. In two other cases, where the recipient strains were either *B. thuringiensis* sv. *entomocidus* HD9.1 or the psychrotolerant *B. weihenstephanensis* strain WSBC10204.1, nine and ninety-five fold increase, respectively, were observed when the matings took place in filter rather than liquid conditions. Yet, the more striking observation was made for the matings with strains *B. thuringiensis* sv. *aizawai* HD11.1, *B. cereus* VD021.1 and the thermotolerant *B. cytotoxicus* E28.3.1. In each case, while no transfer could be detected under liquid condition, efficiencies of 2.5×10^{-2}, 3.7×10^{-3} and 3.2×10^{-3} T/D, respectively, were recorded when filter mating was used (Fig. 2).

These results demonstrate that, provided that the donor and recipient cells are in close contact with each other, pXO16 can "circulate" among a much broader spectrum of bacteria inside the *B. cereus* group, with frequencies of transfer reaching 8.6×10^{-2} T/D. However, in order to further investigate the influence of aggregation in plasmid transfer to these strains, a wild type and an aggregation-minus mutant of pXO16 were tested under condition of liquid matings. As shown in Fig. 3, while in the case of the emetic strain ISO75.1 of *B. cereus* no noticeable difference in transfer was observed in the absence of aggregation, less efficient pXO16 transfers were noted between *B. thuringiensis* sv. *israelensis* strain GBJ002 and either *B. weihenstephanensis* WSBC10204.1 (9-fold drop) or H3081.97.1 (19-fold drop), another emetic strain of *B. cereus*. These observations confirmed the importance of the aggregation phenomenon, but also indicate the influence of the partner cells in the extent of its contribution.

3.3. Plasmid mobilization by pXO16 is also efficient in filter mating

Under liquid conditions, pXO16 was shown to mobilize non-conjugative plasmids among strains of *B. thuringiensis* sv. *israelensis* (Timmery et al., 2009). Using filter-mating experiments, the Km^R plasmid pUB110 was transferred from *B. thuringiensis* sv. *israelensis* GBJ002(pXO16, pUB110) to *B. cytotoxicus* E28.3.1. The transfer of pUB110 was observed with an average frequency of 5.2×10^{-4} T/D, tenfold less than for pXO16 itself.

3.4. pXO16 can circulate among members of the B. cereus group

As indicated above, in the conditions of filter mating (Fig. 2), pXO16 can transfer itself to the thermotolerant *B. cytotoxicus*, certainly the most "divergent" member within the *B. cereus* group (Guinebretière et al., 2013). It was therefore interesting to see whether this pXO16 move could be followed by further migration across members the *B. cereus* group. First, a pXO16 "back-transfer" from *B. cytotoxicus* E28.3.1 to *B. thuringiensis* sv. *israelensis* GBJ002 was tested using a combination of appropriate antibiotic-resistant mutants. As shown in Fig. 4, this transfer was at least 100-fold more efficient than the original transfer (4.9×10^{-1} *versus* 3.3×10^{-3} T/D). Second, the transfer between two antibiotic-resistant mutants of *B. cytotoxicus* (E28.3.1 and E28.3.2) was

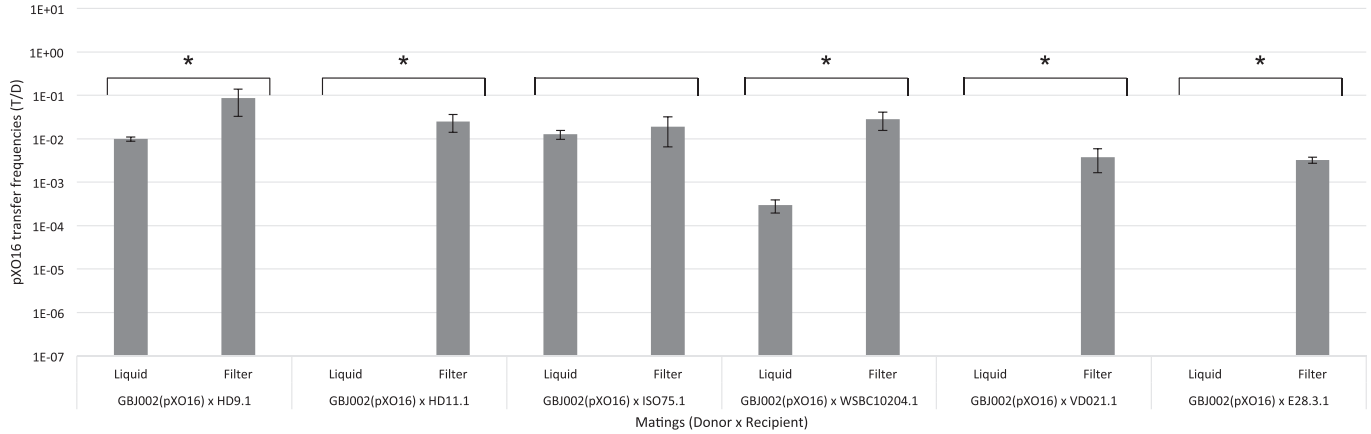

Fig. 2. Comparison of conjugative transfer frequencies of pXO16 to *B. cereus s.l.* strains under conditions of liquid and filter matings. The recipient strains are *B. thuringiensis* sv. *entomocidus* HD9.1, *B. thuringiensis* sv. *aizawai* HD11.1, emetic *B. cereus* ISO75.1, *B. weihenstephanensis* WSBC10204.1, *B. cereus* VD021.1 and B. cytotoxicus E28.3.1. A star indicates that the differences between frequencies (under the same bracket) were significant, following a non-parametric Wilcoxon-Mann-Whitney test ($p < .05$). Otherwise, the differences were not significant.

also rather efficient, with an average frequency of 1.7×10^{-1} T/D (Fig. 4). Again, these results indicate that, under the condition of filter mating, pXO16 can travel at relatively high frequencies across the different species of *B. cereus s.l.*

4. Discussion

Discovered in 1979, in a mosquito pond in Israel, *B. thuringiensis* sv. *israelensis* rapidly became an important biopesticide widely used to control mosquitoes and blackflies, in particular in countries where these insects contribute to the spread of human and animal diseases (Ben-Dov, 2014). Also named serotype H14, these bacteria contain a large pool of extrachromosomal molecules, including small, large and very large circular plasmids, as well as a linear prophage (Bolotin et al., 2017; Gillis et al., 2017; Gillis et al., 2018). pXO16 is one of the two largest plasmids and was found to display an efficient aggregation-associated conjugation (Andrup et al., 1998) that allows the transfer of "non-mobilizable" plasmids (Andrup et al., 1998) and even chromosomal loci (Makart et al., 2017). Until recently, the transfer of pXO16 was thought to be dependent of its aggregation, and only mating pairs forming clumps were considered and studied. However this is not the case. pXO16 aggregation is not mandatory for its DNA transfer. Rather, it helps donor and recipient cells to get into close contact and increases the transfer efficiency by 1000 to 10,000 folds (Makart et al., 2018). In the present paper, the extent of the aggregation contribution was further investigated, together with alternative transfer conditions (*i.e.* filter and colony matings).

The first and foremost observation is the fact that pXO16 aggregative phenotype is designed for DNA exchanges in "liquid" conditions, with "select" partners. In these conditions, transfers of almost 100% can be easily reached. However, the specific features displayed by the select recipient cells recognised in pXO16 aggregation remain so far unknown. The recent development of an exquisite assay using AFM (Feuillie et al., 2018) should bring more insights into this relevant question of cognitive interactions between the mating partners.

The second output of this work is the discovery of alternative routes for pXO16 DNA transfer, either inside colonies of mixed mating pairs or on artificially compacted cell partners (filter mating). These set-ups not only elude the necessity for aggregation, but also often increase pXO16 transfer frequencies and broaden its host spectrum. Using filter mating, the plasmid was indeed shown to move from *B. thuringiensis* sv. *israelensis* to various members of the *B. cereus* group, including strains of the psychrotolerant *B. weihenstephanensis* and the thermotolerant *B. cytotoxicus*, at average frequencies from *ca.* 3.3×10^{-3} to 8.6×10^{-2} (T/D) (Fig. 2). This extends pXO16 host range, which was so far limited to a few strains of *B. thuringiensis*, *B. cereus* (Jensen et al., 1996) or *B. anthracis* (Reddy et al., 1987). It should be noted that attempts to transfer the plasmid outside the *B. cereus* group (*e.g. Bacillus pumilus*) by filter mating have so far failed (data not shown) as reported before in liquid conditions (Jensen et al., 1996).

This paper also indicates that pXO16 is able to move among different members of the *B. cereus* group, as illustrated in the case of *B. cytotoxicus* (Fig. 4). One should however mention that in these transfers, the presence of large and potentially conjugative plasmids in *B. cytotoxicus* might have contributed to the transfer of pXO16. Nevertheless,

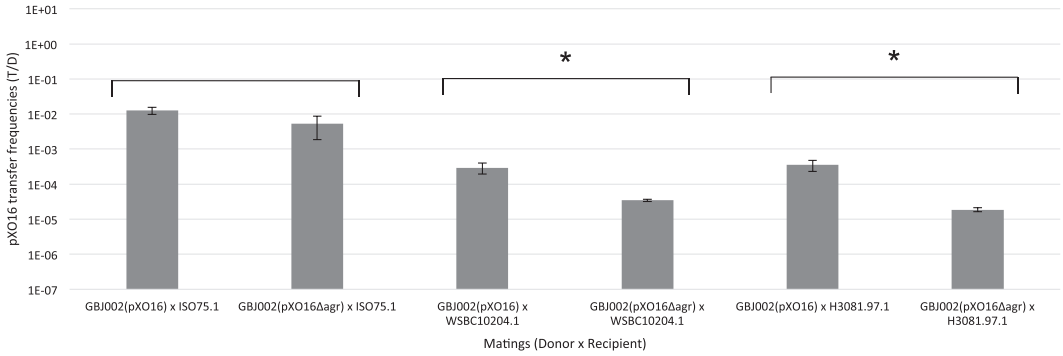

Fig. 3. Comparison of conjugative transfer frequencies of pXO16 and pXO16Δ*agr* to *B. cereus s.l.* strains under conditions of liquid mating. Detailed legend as for Fig. 1.

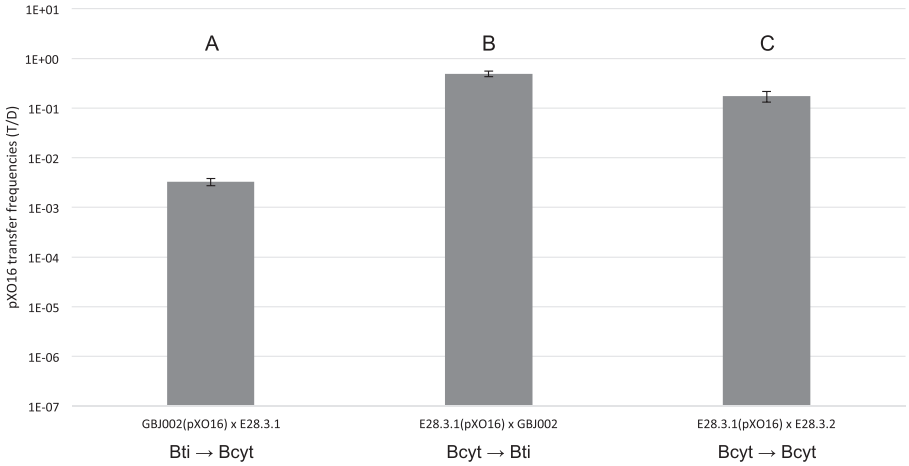

Fig. 4. Conjugative transfer frequencies of pXO16 between *B. thuringiensis* serovar *israelensis* and *B. cytotoxicus*. Different letters on frequencies indicate that the differences between the differently labeled frequencies were significant, following a non-parametric Wilcoxon-Mann-Whitney test ($p < .05$).

the fact that pXO16 is able to migrate, at rather high frequencies, and to mobilize other non-conjugative plasmids (*e.g.* pUB110, the Km^R plasmid) along its migration, is potentially relevant in the scope of gene exchanges between members of *B. cereus s.l.*

This brings us to the following paradox: why then is pXO16 only observed in strains of the *israelensis* serovar (Gillis et al., 2018)? One possible explanation is its potential instability in the new host. This was indeed shown in the case of *B. cytotoxicus* where pXO16 is highly unstable, albeit at high temperature (50 °C, data not shown). Another possible reason for pXO16's restricted natural host range is to be found in its *raison d'être*: indeed, *B. thurigiensis* sv. *israelensis* peculiar habitat, mosquito larvae, may act as positive selection for the presence of pXO16 in the bacterium. This hypothesis remains however to be tested.

Finally, since biofilms are a natural and common lifestyle of bacteria, and members of the *B. cereus* are no exception (Majed et al., 2016; Candela et al., 2018), it would be particularly interesting to further explore the transfer behavior of pXO16 in biofilms, including under flow cell conditions which may be closer to the actual lifestyle of its bacterial host.

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