Characterization of plasmid pAW63, a second self-transmissible plasmid in Bacillus thuringiensis subsp. kurstaki HD73

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Bacillus thuringiensis subspecies kurstaki HD73, toxic for lepidopteran larvae, contains two large self-transmissible plasmids of approximately 75 kb, pHT73 and pAW63. The conjugative plasmid pHT73 has been studied extensively and has been shown to harbour the toxin gene cry1Ac, the transposon Tn4430 and several insertion sequences. In this study it was demonstrated that the minor plasmid pAW63 is also self-transmissible and about 10-30 times more efficient in mobilizing plasmid pBC16. To facilitate direct selection for pAW63 transfer, the plasmid was tagged with the tetracycline resistance transposon Tn5401 and in intraspecies matings it was found that after 2 h, all recipients had acquired a copy of the plasmid. Mating experiments demonstrated that pAW63 could be transferred to Bacillus thuringiensis subsp. israelensis, Bacillus cereus, Bacillus licheniformis, Bacillus subtilis and Bacillus sphaericus, and that the conjugative functions were expressed in these hosts. Hybridization studies showed that the replicons of pAW63 and pHT73 were distinct from one another. Sequences homologous to transposon Tn4430 and several insertion sequences were, however, shown to reside on both plasmids.

Keywords: Bacillus thuringiensis, HD73, self-transmissible plasmid, conjugation, mobilization

INTRODUCTION

Bacterial conjugation is a mechanism of genetic exchange that requires cell-to-cell contact and which is not susceptible to DNase present in the mating medium. Conjugation systems are encoded by large plasmids or by conjugative transposons (Clewell, 1993; Scott, 1993), which besides being capable of transferring themselves are able to co-transfer smaller mobilizable plasmids. Conjugation is a well-known process in Gram-negative bacteria with the F plasmid from Escherichia coli being particularly well studied. The cell-to-cell contact in Gram-negative bacteria is established by the sex pilus, which retracts and brings donor and recipient cells into close physical contact (Firth et al., 1996).

In Gram-positive bacteria, DNA transfer by conjugation has been found in an increasing number of species. In these bacteria cell-to-cell contact is not mediated by pili, but other, still scarcely characterized surface structures are involved. Recently, several Gram-positive systems capable of sustaining DNA transfer in liquid media have been discovered and characterized in some detail (for review, see Clewell, 1993). The best studied is the pheromone-induced conjugation system of Enterococcus faecalis (Dunny et al., 1978), which along with the conjugation systems of Lactococcus lactis (van der Lelie et al., 1991), Lactobacillus plantarum (Reniero et al., 1992) and the mosquito-toxic bacterium Bacillus thuringiensis (B.t.) subsp. israelensis (Andrup et al., 1993; Jensen et al., 1995), mediates plasmid transfer via the formation of large aggregates in liquid medium.

In 1982, González & Carlton reported the conjugative transfer of B.t. plasmids in mixed culture which led to the discovery that the insecticidal crystal protein genes were located on large conjugative or mobilizable plasmids in several subspecies (González et al., 1982). One of the subspecies analysed was B.t. kurstaki strain HD73. B.t. kurstaki flagella serotype 3ab comprises both strain HD1 (crystal serotype K-1) and HD73 (crystal serotype K-73) (Krywienczyk et al., 1978). Strain HD73 contains six plasmids ranging from 7.5 to 77 kb (González et al., 1981). The genetic basis of the insecticidal

Abbreviations: B.t., Bacillus thuringiensis; Cm, chloramphenicol; Em, erythromycin; Nal, nalidixic acid; Sm, streptomycin; Tc, tetracycline.
### Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference*</th>
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<tbody>
<tr>
<td><strong>B. t. kurstaki</strong></td>
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<tr>
<td>KT, HD73</td>
<td>4D4</td>
<td>de Barjac (1981)</td>
</tr>
<tr>
<td>AW05</td>
<td>HD73 cured of pAW63</td>
<td>BGSC</td>
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<td>AW06</td>
<td>HD73 cured of pHT73</td>
<td>This study</td>
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<tr>
<td>AW16</td>
<td>AW05, SmR</td>
<td>This study</td>
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<td>AW17</td>
<td>AW06, SmR</td>
<td>This study</td>
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<td>AW21</td>
<td>HD73 cured of both pAW63 and pHT73, SmR</td>
<td>This study</td>
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<tr>
<td>AW43</td>
<td>HD73 cured of both pAW63 and pHT73, NalR</td>
<td>This study</td>
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<tr>
<td>AW46</td>
<td>AW06 electroporated with pEG922, CmR, TcR</td>
<td>This study</td>
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<tr>
<td>AW48</td>
<td>AW43 mated with AW46, pAW63::Tn5401, NalR, TcR</td>
<td>This study</td>
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<td><strong>B. t. israelensis</strong></td>
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<tr>
<td>GBJ001</td>
<td>Plasmid-cured derivative, SmR</td>
<td>Jensen et al. (1995)</td>
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<tr>
<td>GBJ002</td>
<td>Plasmid-cured derivative, NalR</td>
<td>Jensen et al. (1996)</td>
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<td>AND940</td>
<td>GBJ002 containing pX016, NalR</td>
<td>Jensen et al. (1996)</td>
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<td><strong>Other Bacillus species</strong></td>
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<tr>
<td>B. cereus</td>
<td>AH183</td>
<td>A. B. Kolstø</td>
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<td>B. licheniformis</td>
<td>5A2</td>
<td>BGSC</td>
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<td>B. subtilis</td>
<td>SB202</td>
<td>L. Boe</td>
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<td>B. sphaericus</td>
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<td>BGSC</td>
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<td><strong>Plasmids</strong></td>
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<td>pBC16</td>
<td>Natural B. cereus plasmid, TcR</td>
<td>Bernhard et al. (1978)</td>
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<tr>
<td>pBC16ΔNdel</td>
<td>pBC16 with a 498 bp Ndel deletion in the mob gene</td>
<td>Andrup et al. (1996)</td>
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<tr>
<td>pEG588-8</td>
<td>Vector containing ori-44 from B.t. HD263</td>
<td>Baum et al. (1990)</td>
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<tr>
<td>pEG922</td>
<td>Tn5401 delivery vector</td>
<td>Baum (1994)</td>
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<tr>
<td>pHT232</td>
<td>Containing IS232A, corresponds to pHTZ232</td>
<td>Menou et al. (1990)</td>
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<tr>
<td>pHT44</td>
<td>Vector containing Tn4430</td>
<td>Lereclus et al. (1986)</td>
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<tr>
<td>pHTA2</td>
<td>Vector containing IS231C and cry1Ab</td>
<td>Sanchis et al. (1988)</td>
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*The Bacillus Genetic Stock Center, Columbus, OH, USA; A. B. Kolstø, University of Oslo, Norway; L. Boe, Technical University of Denmark.

Toxin has been located to a 77 kb plasmid in strain HD73 and it was suggested that there may be a second plasmid of similar size (González et al., 1981; Kronstad & Whiteley, 1984). Another *B. t. kurstaki* strain, KT, originating from Centre OILB (Institut Pasteur), has been shown to have a plasmid profile very similar to strain HD73 and the crystal toxin gene has been located to a 83 kb plasmid (Lereclus et al., 1985). We suggest that the two toxin-encoding plasmids are identical and should be named pHT73 (G. T. Vilas Boas, unpublished). It has been assumed that strain KT was identical to HD73 (Lereclus et al., 1985). However, as presented here, a plasmid analysis shows that strain KT only contains one large plasmid, the crystal toxin plasmid pHT73, whereas strain HD73 harbours a second plasmid, pAW63, somewhat smaller than pHT73. Plasmid pHT73 has been shown to contain a transposon, Tn4430 (Lereclus et al., 1986), and several insertion sequences (Menou et al., 1990; Mahillon et al., 1994) in addition to the toxin gene.

The objective of our study was to identify and analyse the conjugational activity of *B. t. kurstaki* strain HD73.

Evidence is presented that each of the two large plasmids of about 77 kb found in this strain, pHT73 and pAW63, is capable of bringing about its own transfer as well as that of mobilizable plasmids. Transconjugants of *B. t. israelensis, Bacillus cereus, Bacillus licheniformis, Bacillus subtilis* and *Bacillus sphaericus* that inherited plasmid pAW63 were, in turn, effective donors. It appears that plasmid pAW63, under the circumstances used here, was the more efficient of the two plasmids, both with regard to conjugative transfer and as a mobilizing agent.

### METHODS

**Strains, plasmids and media.** Bacterial strains and plasmids used in this study are listed in Table 1. Plasmids pEG922 and pEG588-8 were kindly provided by James A. Baum, Ecogen Inc., PA, USA. All cultures were grown in LB medium (Sambrook et al., 1989) containing antibiotics (Sigma), when appropriate, at the following concentrations (µg ml⁻¹): streptomycin (Sm), 100; nalidixic acid (Nal), 15; tetracycline (Tc), 4; chloramphenicol (Cm). 6. Restriction enzymes were used as recommended by the supplier (Gibco-BRL).
Plasmid transfer. Broth mating was conducted as described by Andrup et al. (1996). Equal amounts of exponentially growing cells (250 μl per OD_{600} unit) were combined in 7 ml prewarmed LB medium and incubated at 30 °C with moderate shaking (180 r.p.m.). After 3 h or as indicated, appropriate dilutions were plated on selective media and controls of donors and recipients, grown separately, were tested in parallel.

To exclude plasmid transfer by transformation, 50 μg DNase 1 ml^{-1} was added to mating mixtures. As donors B.t. kurstaki strains AW05 and AW06 containing pBC16 were used and matings to strain AW43 were performed. Transduction was excluded by adding supernatant from the donor cells filtered through a 0.2 μm filter to a potential recipient.

DNA isolation. Extraction of large plasmids from B.t. was conducted by the method of Jensen et al. (1995). Smaller plasmids were extracted by a modification of the alkaline-lysis method described by Andrup et al. (1993). DNA was analysed by horizontal gel electrophoresis (6-10 V cm^{-1}) in 0.5 or 0.8 % agarose (SeaKem GTG) with 1 x TBE buffer (Sambrook et al., 1989) for 2-3 h. After electrophoresis, the gel was stained in 1 μg ethidium bromide ml^{-1} for 5-10 min and destained in water. DNA fragments in agarose gels were purified using the QIAquick Gel Extraction Kit Protocol (Qiagen).

Electroporation. Electroporation was performed essentially as described by Bone & Ellar (1989). Exponentially growing culture (400 ml; OD_{600} ≈ 0.2) was chilled on ice and harvested in a precooled centrifuge. The cells were washed three times with cold water and resuspended in 500 μl cold 10 % (v/v) glycerol. The resuspended cells were mixed with 1 μg plasmid DNA and electroporated with a single pulse (1200 V, 100 Ω, 21 μF) using GeneZapper (International Biotechnologies). The cells were resuspended in 1 ml prewarm LB medium and incubated at 30 °C with moderate shaking for 3-16 h before plating on selective medium.

Transposon insertion in pAW63. Transposon insertion was performed as described by Jensen et al. (1996). Strain AW06 containing plasmid pAW63 was electroporated with pEG922 isolated from a B.t. israelensis strain (Jensen et al., 1996). Electrototransformants were selected on LB plates containing Cm and Tc. An exponentially growing culture of one of the electrototransformants, AW46, was diluted (1:100) into LB broth containing Tc at 42 °C and incubated overnight at 42 °C. A mating was conducted (at 30 °C) with this culture as donor and a plasmid-cured, Nal^R strain (AW43) as recipient. Cm^R, Tc^R and Nal^R transconjugants were isolated and their ability to transfer the Tc^R phenotype to a plasmid-cured Sm^R derivative of B.t. israelensis (GBJ001) was assessed. One of the transconjugants containing pAW63:: Tn5401 was designated AW48 and subsequently used in matings to determine the conjugative ability of each of the two large plasmids pHT73 and pAW63.

RESULTS

Characterization of the large plasmids in strain HD73

We isolated plasmid-cured derivatives of strain HD73 to determine the conjugal ability of each of the two large plasmids pHT73 and pAW63. Strain HD73 was cured of pAW63 (resulting in strain AW05, containing only pHT73) and pHT73 (resulting in strain AW06, containing only pAW63) by growth at 42 °C (Fig. 1, Table 1). To determine whether the two plasmids originated from a similar replicon, they were probed using the replication region ori-44 cloned in pEG588-8 (Baum et al., 1990). The ori-44 replicon is identical to that of pHT73 except for one nucleotide base change (T^301 → C) (Baum & Gilbert, 1991; Gamel & Pirot, 1992). Hybridization with plasmid pEG588-8, containing ori-44, gave a signal only with AW05 containing pHT73 and no hybridization to AW06 was observed (Fig. 1). This indicates that the two plasmids are basically different with regard to their replication functions. In Fig. 1 the plasmid profiles (large plasmids) of the donor strains containing the mobilizable plasmid pBC16 are also shown.

DNA homology between pHT73 and pAW63

As mentioned above there was no hybridization of the replicon of pHT73 to pAW63. To test whether pHT73 and pAW63 have sequences in common, hybridization with the crystal gene cry1Ab from B.t. aizawai 7.29 cloned into plasmid phTA2 (Sanchis et al., 1995) was performed. cry1Ab shares 86 % identity to the crystal gene from pHT73, cry1Ac (Hofte & Whiteley, 1989). As
probe we used a 1 kb internal HindIII fragment of cry1Ab. It was found that the cry1Ab probe only hybridized to fragments from pHT73 (Fig. 2b), so pAW63 contains no sequences with homology to the crystal gene. As seen in Fig. 2(b) the hybridization pattern of strains AW05, KT0 and HD73 were identical, verifying that the largest plasmid, pHT73, is present in these strains. To determine whether pAW63 harboured these plasmids. The restriction patterns of AW05, AW06 and KT0 in Fig. 2(a) showed that KT0 harbours plasmid pHT73, but no bands arising from plasmid pAW63 could be detected.

A plasmid preparation from strain AW06 was digested with HindIII and used as probe in hybridization studies with the following plasmids: pHT232 (containing an internal part of IS232A as a 1.7 kb Clal fragment) restricted with Clal; pHT44 (containing Tn4430 as a 4.2 kb KpnI fragment) restricted with KpnI; pHTA2 (containing cry1Ab and a part of IS231C as a 0.5 kb KpnI/PstI fragment) restricted with KpnI + PstI (Fig. 3). It was found that the plasmid preparation from AW06 hybridized to fragments containing Tn4430 and the insertion sequences IS232A and IS231C, while there was no hybridization to any fragment corresponding to cry1Ab. Hybridization to fragments of pHT73 (HindIII digest, Fig. 3) confirmed the partial homology between the two plasmids.

**Kinetics of transfer of plasmid pAW63**

By tagging pAW63 with the tetracycline resistance transposon Tn5401, resulting in the donor strain AW48 (see Methods), it was possible to follow the kinetics of transfer of pAW63::Tn5401. Broth matings to an HD73 strain resistant to streptomycin and cured of both large plasmids (strain AW21) were conducted. During mating appropriate dilutions of the mating mixture were plated on LB without antibiotics and after growth overnight, 100 colonies were picked on to selective media for the estimation of the number of donors, recipients and transconjugants. The results of such a mating experiment are shown in Fig. 4. It was found that after 2 h, practically all recipients had acquired a copy of plasmid pAW63::Tn5401 and were resistant to tetracycline. That the mechanism of transfer was by the process of conjugation, rather than by transformation, was demonstrated by performing matings in the presence of DNase. DNase had no significant effect on the frequency of transfer of pBC16. To exclude phage-mediated plasmid transfer, supernatant from a donor strain was added to a potential recipient strain. No transfer of pBC16 was detected.

**Mobilization of pBC16 by pHT73 or pAW63**

The mobilizable plasmid pBC16 from B. cereus was introduced by electroporation into strains HD73, AW05 and AW06. Matings were conducted to the following recipients: AW16 (SmR derivative of AW05), AW17 (SmR derivative of AW06) and AW43 (NalR and cured of both pHT73 and pAW63). Table 2 shows that both pHt73 and pAW63 were able to mobilize pBC16 to the recipients. The donor harbouring pAW63 (AW06) mobilized pBC16 at a frequency 10–30 times higher than the donor containing pHT73 (AW05) to recipients cured of pAW63 (AW16 and AW43). In contrast to this the two donors AW05 and AW06 mobilized pBC16 to the recipient harbouring pAW63 (AW17) with similar frequency. It was found that the frequency of mobilization drastically decreased when both donor and recipient harboured pAW63, indicating entry-exclusion. The HD73 donor, which contains both pAW63 and pHT73, had a reduced frequency of transfer to the three recipients compared to mobilization from strain AW06 alone.

Both strains AW05 and AW06 were able to mobilize pBC16 to strains of B. cereus (Table 2) and there were no significant differences in the mobilization capacity of pHT73 and pAW63. Neither did the presence of the aggregation-encoding plasmid pX016 (Jensen et al., 1995) in the recipient strain influence the frequency of transfer of pBC16. Analysis of the plasmid content of the transconjugants showed that besides pBC16 only transfer of pAW63 was detected. None of 28 B. cereus transconjugants examined received pHT73, while about 50% of the transconjugants from a mating with strain AW06 acquired plasmid pAW63. Subsequently, the B. cereus transconjugants harbouring pAW63 and pBC16 were able to mobilize pBC16 to B. cereus recipients (data not shown).

Recently Andrup et al. (1996) found that the aggregation-mediated conjugation system of B. sphaericus was demonstrated that pX016 encoded by pX016 is able to mobilize pBC16 deleted for the mobilization gene, mob, and for the origin of transfer oriT. To test whether a functional mob gene is necessary for mobilization by pHT73 and pAW63, the pBC16 derivative pBC16ΔNdel, which contains a deletion spanning the first half of the coding region of the mob gene, was introduced into strains AW05 and AW06. Using these strains as donors, no transfer of pBC16ΔNdel to the recipient strain AW21 (HD73 cured of both large plasmids) was found.

**Transfer of pAW63 to other species and subspecies**

To investigate the host range of conjugative plasmid pAW63, matings to various Bacillus strains were conducted. Strain AW48 was used as donor of pAW63 after 3 h broth-mating transfer of pAW63::Tn5401 to SmR recipient strains of B. cereus, B. subtilis and B. sphaericus was demonstrated (data not shown). Transconjugants from these matings were also capable of transferring pAW63::Tn5401 back
Characterization of pAW63

**Fig. 3.** (a) Agarose gel electrophoresis of: lane 1, pAW63 DNA digested with HindIII; lane 2, pHT232 digested with CiaI; lane 3, pHT44 digested with KpnI; lane 4, pHTA2 digested with KpnI and PstI; lane 5, pHTA2 digested with HindIII; lane 6, pHT73; lane 7, pHT73 digested with HindIII. Lane λ DNA digested with HindIII. (b) Autoradiograph of Southern blot using a 32P-labelled plasmid preparation of pAW63 digested with HindIII as probe.

**DISCUSSION**

This study demonstrates that *B.~. kurstaki* HD73 harbours two self-transmissible plasmids, pHT73 and pAW63. González *et al.* (1982) reported for the first time to a plasmid-cured HD73 (data not shown), indicating that pAW63 is also functional in these species.
Table 2. Mobilization of pBC16 by plasmids pH73 and pAW63

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Donors</th>
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<tbody>
<tr>
<td></td>
<td>HD73 (pHT73, pAW63, pBC16)</td>
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<tr>
<td>HD73</td>
<td>0.73±0.07</td>
</tr>
<tr>
<td>AW16 (pHT73)</td>
<td>0.084±0.017</td>
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<tr>
<td>AW17 (pAW63)</td>
<td>8.7±1.3</td>
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<td>AW43</td>
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<tr>
<td>B. t. israelensis</td>
<td>AND940 (pXO16)</td>
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<tr>
<td>GBJ002</td>
<td>ND</td>
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</table>

The data shown are from at least three experiments. Values shown are ± sd. The transfer frequency was 10⁻⁴ transconjugants per recipient. ND, Not determined.

The transfer of pHT73 from HD73 in a conjugation-like process in broth mating. They also reported the transfer of a smaller (66 kb) plasmid (González & Carlton, 1982), presumably pAW63, and they speculated whether the transfer of pHT73 was due to mobilization by the 66 kb plasmid. However, our work shows that both pH73 and pAW63 are independently able to mobilize pBC16. Furthermore, pAW63 is self-transmissible to B. t. israelensis, B. cereus, B. licheniformis, B. subtilis and B. sphaericus. Under the conditions used, pAW63 was more efficient than pH73 both regarding conjugative transfer and as a mobilizing agent. Strain KTₙ has been reported to be able to mobilize smaller non-conjugative plasmids (Lereclus et al., 1985) and was formerly assumed to be identical to HD73. However, this work showed that KTₙ only harbours pHT73 and not pAW63, hence mobilization must be caused by pHT73.

Mobilization of non-conjugative plasmids mediated by a co-resident conjugative plasmid by the process of donation (Clark & Warren, 1979) requires some functions on the non-conjugative plasmid: a trans-acting mob gene (encoding a mobility protein) and a cis-acting origin of transfer (oriT) (for a review, see Lanka & Wilkins, 1995). This has been shown also to apply to mobilization of pBC16, on which a mob gene and a site (RSₐ) suggested to function as origin of transfer (oriT) have been identified (Selinger et al., 1990), by the conjugative plasmid pLS20 in B. subtilis. However, recently Andrup et al. (1996) found that the aggregation-mediated conjugation system in B. t. israelensis is capable of mobilizing derivatives of pBC16 deleted for both these loci. In this study, we have demonstrated that both pAW63- and pHT73-mediated mobilization require the presence of the mob gene on pBC16, hence the mechanism of conjugation is different from the aggregation-mediated conjugation system encoded on pXO16 in B. t. israelensis.

Tagging plasmid pAW63 with the tetracycline resistance transposon Tn5401 enabled us to follow this plasmid in broth matings. We found very high transfer frequencies of pAW63 to a plasmid-cured derivative of HD73. After 2 h of mating more than 90% of the recipients were CₐR. This is comparable with the transfer frequency found for pXO16, the conjugative plasmid from B. t. israelensis (Jensen et al., 1996), but in contrast to this system neither pHT73- nor pAW63-mediated conjugation gene-
rated visible aggregates during broth mating. By microscopic examination the mating pairs were found to comprise 2–10 cells.

The transfer of pHT73 tagged with a gene encoding erythromycin resistance has been observed (G. T. Vilas Boas and others, unpublished). Matings were conducted both in broth culture, in soil and in infected larvae to a B. thuringiensis recipient. In broth mating pHT73-EmR transferred at frequencies between 2.4 × 10⁻⁴ and 1.6 × 10⁻³ c.f.u. per recipient c.f.u. This is comparable to the frequency we found for pAW63::Tn5401 transfer to recipients of B. israelensis.

To date, eight conjugative plasmids have been found in B.t. Two of these plasmids carry a gene for crystal production: pH73 (from HD73 and KT) and pXO12 (from thuringiensis) (Battisti et al., 1985). The other plasmids, i.e. pXO11 from B.t. thuringiensis (Battisti et al., 1985), pXO13 from B.t. morrisoni, pXO14 from B.t. tumanoffi and pXO15 from B.t. aesti (Reddy et al., 1987) have, apart from their conjugative ability, no known functions. Reddy et al. (1987) found DNA homology between pXO11, pXO12, pXO13 and pXO14 and showed that if donor and recipient contained the same conjugative plasmid, there was a reduced efficiency of pBC16 transfer, while transfer of pBC16 was enhanced if donor and recipient harboured different conjugative plasmids. Like Reddy et al. (1987) we observed entry-exclusion when both donor and recipient harboured pAW63; the transfer frequency, however, was reduced when donor and recipient each contained a different conjugative plasmid. This could indicate that pAW63 and pHT73 compete in the transfer process, perhaps by using the same system for mobilization and conjugation.

B.t. thuringiensis also harbours two conjugative plasmids: pXO12, containing the crystal toxin gene, and the cryptic plasmid pXO11 (Battisti et al., 1985). Both of these plasmids are capable of promoting the transfer of pBC16 to Bacillus anthracis and B. cereus, and the transconjugants harbouring either pXO12 or pXO11 are also efficient donors of pBC16. When B.t. thuringiensis is the donor, plasmid pXO12 is more efficient in transferring pBC16 than pXO11. This is in contrast to the situation in strain HD73 where the crystal plasmid, pH73, is less efficient than pAW63. The benefit for a cell to have two conjugative plasmids is unknown and it would be interesting to determine whether pAW63 harbours genes, other than cry genes, but expressing adaptive functions advantageous to B.t.

Work is in progress to map pAW63 and isolate relevant genes for conjugation and replication, hopefully thereby shedding some light on the structure and properties of pAW63 and conjugation in B.t. in general.

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