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The Genetic Basis of the Aggregation System in *Bacillus thuringiensis* subsp. *israelensis* Is Located on the Large Conjugative Plasmid pXO16

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The aggregation phenotypes Agr^+ and Agr^- of *Bacillus thuringiensis* subsp. *israelensis* are correlated with a conjugation-like plasmid transfer and characterized by the formation of aggregates when the bacteria are socialized during exponential growth. We present evidence for the association of the Agr^+ phenotype with the presence of the large (135-MDa) self-transmissible plasmid pXO16.

Bacillus thuringiensis subsp. *israelensis* is a gram-positive bacterium that is highly toxic to larvae of several dipteran aquatic insects (for a review, see reference 10). *B. thuringiensis* subsp. *israelensis* has been reported to contain up to 10 plasmids (5, 7). Two of the small plasmids, pTX14-1 and pTX14-3, have been cloned and characterized (2, 4, 9). A 75-MDa plasmid has been shown to encode the toxin genes (6, 7, 13, 16), and the plasmid transfer functions have been ascribed to a self-transmissible 135-MDa plasmid (7) identical to plasmid pXO16 reported by Reddy et al. (11, 15). A 68-MDa plasmid is associated with a phage-like particle and with satellite inclusion in *B. thuringiensis* subsp. *israelensis* (14).

Recently, we have found that mobilization of small plasmids between strains of *B. thuringiensis* subsp. *israelensis* is accompanied by non-pheromone-induced and protease-sensitive coaggregation between donor and recipient cells (1). Two aggregation phenotypes (Agr^+ and Agr^-) were identified. The aggregation phenotypes are characterized by the appearance of macroscopic aggregates when cells of exponentially growing strains of Agr^+ and Agr^- bacteria are mixed in broth.

The mobilization of small plasmids was found to be unidirectional, from Agr^+ cells to Agr^- cells (1). In addition, the Agr^+ phenotype was transferred at a high frequency (≈100%) to Agr^- cells during broth matings. The high transferability of the Agr^+ phenotype and the frequent appearance of Agr^- cells from Agr^+ strains suggest that loci involved in aggregate formation are located extrachromosomally. In this report, we present evidence for the localization of loci essential for the Agr^+ phenotype on plasmid pXO16 in *B. thuringiensis* subsp. *israelensis.*

Strains. The bacterial strains used in this study are listed in Table 1. Antibiotic-resistant mutants were isolated by using the following protocol. During exponential growth in Luria-Bertani broth (12) at 30°C, streptomycin was added to a final concentration of 20 μg/ml. After additional incubation for 2 days, dilutions were plated on solid media with streptomycin. After 16 h of incubation, single colonies were picked. Antibiotics (Sigma) in agar plates and broth medium were used at the following concentrations: streptomycin, 100 μg/ml; tetracycline, 10 μg/ml.

Aggregation, mating, and mobilization. To test the ability of a pair of strains to coaggregate, equal amounts of cells in the logarithmic growth phase (250 μl per unit of optical density at 600 nm) were combined in 7 ml of prewarmed Luria-Bertani medium and incubated at 30°C with moderate shaking (175 rpm). The aggregation mixture was regularly inspected for visible aggregates. Those strains which formed aggregates with a known Agr^+ strain and not with a known Agr^- strain were designated Agr^- and vice versa. Matings were carried out by using the aggregation assay, and after 2.5 to 3 h, a period after which maximum transfer of small plasmids is reached and aggregation ceased (1), appropriate dilutions were plated on selective medium. Controls of donors and recipients, grown separately, were tested in parallel.

Mobilization of plasmid pBC16 was accomplished by using strain AND508 electroporated with pBC16(Tet^r) as the donor and streptomycin-resistant Agr^- strains as recipients. Transconjugants were selected on agar plates containing both streptomycin and tetracycline.

Heat was used as a plasmid-curing agent (7) by inoculating prewarmed Luria-Bertani broth at 43°C with an exponentially growing Agr^+ strain and incubating it for 24 h. The culture was spread on agar plates, and single colonies were tested for their aggregation phenotype.

Plasmid isolation. Detection and isolation of large plasmids were done by using the methods of Kado and Liu (8) and Battisti et al. (3) with some alterations. The bacteria were grown in 7 ml of Luria-Bertani broth overnight (12 to 16 h) at 30°C to a final optical density at 600 nm of 11 to 15. A 2-ml volume of cells was pelleted and resuspended in 100 μl of E buffer (15% [wt/vol] sucrose, 40 mM Tris-hydroxide, 2 mM EDTA, pH 7.9) by being pipetted up and down. Cells were lysed by addition of 200 μl of lysing solution (3% [wt/vol] sodium dodecyl sulfate, 50 mM Tris, pH 12.5). The lysate was heated at 60°C for 30 min, and 5 U of proteinase K (Sigma) was added. The solution was inverted 20 times and incubated at 37°C for 90 min. About 1.0 ml of phenol-chloroform-isoamyl alcohol (Sigma) was added, and the solution was inverted 40 times. After centrifugation, the upper aqueous layer was subjected to electrophoresis. Cells harvested in the logarithmic growth phase (i.e., an optical density at 600 nm of 0.6 to 0.8)

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics	Source or reference
NB31	*B. thuringiensis* subsp. *israelensis*, Agr$^+$[a]	Novo[b]
4Q2	*B. thuringiensis* subsp. *israelensis*, Agr$^+$	BGSC[c]
W4Q30	Plasmid-cured derivative of 4Q2, Agr$^-$	BGSC
W4Q31	Plasmid-cured derivative of 4Q2, Agr$^+$	BGSC
W4Q50	Plasmid-cured derivative of 4Q2, Agr$^-$	BGSC
4Q7	Derivative of 4Q2 cured of all plasmids,[d] Agr$^-$	BGSC
AND406	NB31 cured of natural plasmid pTX14-1, Agr$^+$	1
AND508	AND406 cured of small plasmids, Agr$^+$	1
AND661	Spontaneous Str^r mutant of AND406, Agr$^-$	This study
AND699	Spontaneous Str^r mutant of 4Q7, Agr$^-$	This study
GBJ001	Spontaneous Str^r mutant of 4Q7, Agr$^-$	This study
AND668	Transconjugant of AND661 mated with AND508, Agr$^+$	This study
AND789	Transconjugant of AND699 mated with AND508, Agr$^+$	This study
AND801	Transconjugant of GBJ001 mated with AND508, Agr$^+$	This study
AND710	Isolate of heat-treated culture of AND508, Agr$^-$	This study
AND824	Isolate of heat-treated culture of AND801, Agr$^-$	This study
GBJ006	Isolate of heat-treated culture of AND508, Agr$^-$	This study
GBJ21	Isolate of heat-treated culture of AND508, Agr$^-$	This study
GBJ24	Isolate of heat-treated culture of AND668, Agr$^-$	This study
GBJ26	Isolate of heat-treated culture of AND789, Agr$^-$	This study

[a] Agr$^+$ or Agr$^-$ indicates the aggregation phenotype of the strain.
[b] Novo, Novo Nordisk A/S strain collection, Bagsvaerd, Denmark.
[c] BGSC, *Bacillus* Genetic Stock Center, Columbus, Ohio.
[d] Occasionally we saw a faint band migrating at the size of a 110-MDa plasmid in plasmid preparations from this strain and its derivatives (Fig. 2A).

rendered only small amounts of the largest plasmids (the 110-MDa plasmid and pXO16) relative to the amounts of the 68- and 75-MDa plasmids.

DNA was analyzed by horizontal agarose gel electrophoresis (8 to 10 V/cm) in 0.5% agarose (SeaKem GTG) with 1× TBE buffer (12) for 3 to 5 h. After electrophoresis, DNA was stained in 1 μg of ethidium bromide per ml for 15 min and destained in water for up to 7 days. Photographs of gels were taken by using UV light and exposing the film through a red filter.

Plasmid contents of different strains. As seen in Fig. 1, strain AND406 (lane a) displayed a plasmid pattern identical to that of strain 4Q2 (lane b). Both strains contained the 135-, 110-, 75-, and 68-MDa plasmids identified by González and Carlton (7), and they both exhibited the Agr$^+$ phenotype. The largest plasmid (135 MDa) is identical to pXO16 (11, 15) and will therefore be referred to as pXO16. Strain AND661 (lane e), a spontaneous Str^r and Agr$^-$ mutant of AND406, has lost three of the large plasmids (the 68- and 75-MDa plasmids and pXO16), and strain W4Q31 (lane c), a plasmid-cured derivative of 4Q2, harbored only the two largest plasmids (the 110-MDa plasmid and pXO16) but retained the Agr$^+$ phenotype. No smaller plasmids are involved in the aggregation phenotype as shown by the fact that strain AND508, a derivative of AND406 cured of all smaller plasmids, displayed the Agr$^+$ phenotype.

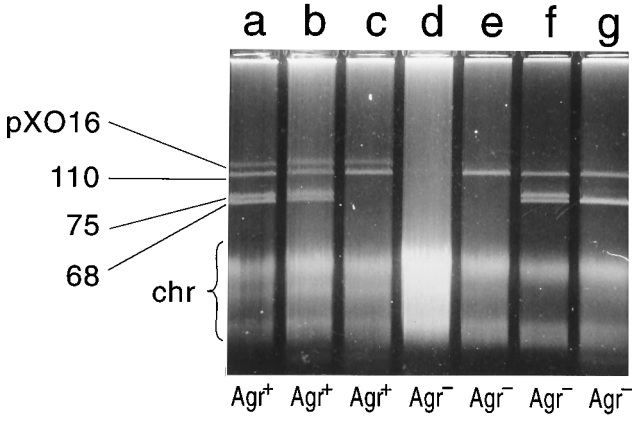

FIG. 1. Agarose gel electrophoresis of plasmid DNAs from various *B. thuringiensis* subsp. *israelensis* strains. The sizes of relevant plasmids are indicated in megadaltons. The designation chr indicates chromosomal and linear DNA fragments. The aggregation phenotype of each strain is marked at the bottom of each lane. The following strains were used: AND406 (lane a), 4Q2 (lane b), W4Q31 (lane c), 4Q7 (lane d), AND661 (lane e), W4Q30 (lane f), and W4Q50 (lane g).

Reference strains of *B. thuringiensis* subsp. *israelensis* (from the *Bacillus* Genetic Stock Center), reported to differ only in their plasmid contents, displayed the following pattern: the strains that were Agr$^+$ contained plasmid pXO16 (Fig. 1, lanes b and c), and those strains displaying the Agr$^-$ phenotype did not (Fig. 1, lanes d, f, and g).

To establish whether the Agr$^+$ phenotype, transferred to the recipients, was associated with the uptake of plasmid pXO16, three types of matings were performed. Strain AND508 (Agr$^+$) was mated with strains GBJ001 (Agr$^-$ Str^r) and AND699 (Agr$^-$ Str^r), respectively (Fig. 2A), and strain AND508 (Agr$^+$) electroporated with plasmid pBC16 (1) was mated with strain AND661 (Agr$^-$ Str^r). The acquisition of the Agr$^+$ phenotype of the transconjugants was verified. Figure 2A and B show the plasmid profiles of the donors (Fig. 2A and B, lane a), recipients (Fig. 2A, lanes b and e; Fig. 2B, lane b), and transconjugants (Fig. 2A, lanes c and f; Fig. 2B, lane c). It is apparent that in all cases the recipients acquired plasmid pXO16 along with the Agr$^+$ phenotype.

To verify that acquisition of the Agr$^+$ phenotype was reversibly correlated with the presence of plasmid pXO16, four Agr$^+$ strains, AND508, AND668, AND789, and AND801, were ex-

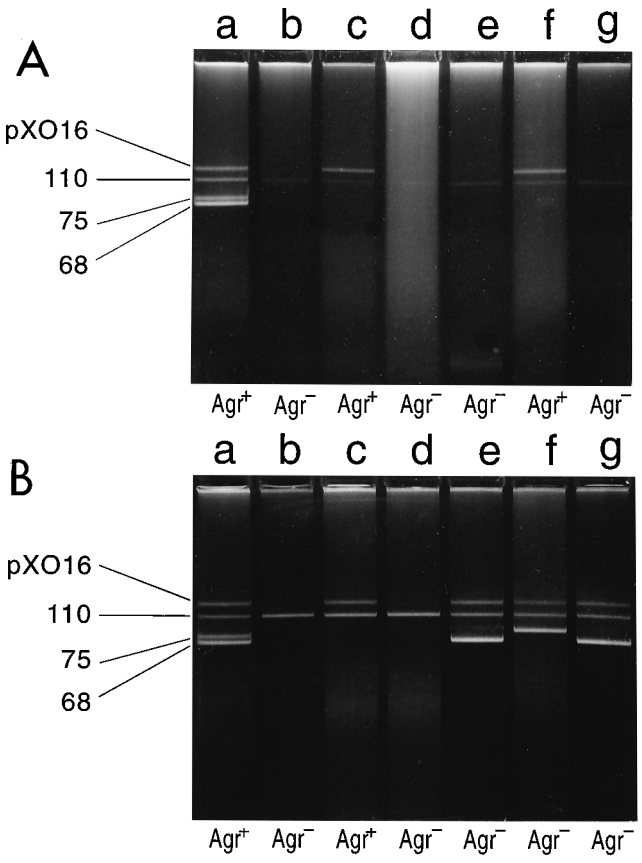

FIG. 2. (A) Agarose gel electrophoresis of plasmid DNAs from donors, recipients, transconjugants and heat-treated transconjugants of *B. thuringiensis* subsp. *israelensis* matings. The strains of two separate matings are shown: 1, donor AND508 (lane a), recipient AND699 (Str^r) (lane b), resulting transconjugant AND789 (Str^r) (lane c), strain GBJ26 (lane d) isolated after heat treatment of strain AND789; 2, recipient GBJ001 (Str^r) (lane e), resulting transconjugant AND801 (Str^r) (lane f), strain AND824 (lane g) isolated after heat treatment of strain AND801. (B) Agarose gel electrophoresis of plasmid DNAs from a donor, a recipient, and a transconjugant of a *B. thuringiensis* subsp. *israelensis* mating. The plasmid DNAs from heat-treated strains are also shown. The following strains are shown: donor, AND508 (lane a); recipient, AND661 (Str^r) (lane b); resulting transconjugant, AND668 (Str^r) (lane c); strain GBJ24 (lane d), isolated after heat treatment of strain AND668. Strains AND710 (lane e), GBJ006 (lane f), and GBJ21 (lane g) were isolated after heat treatment of strain AND508.

posed to heat treatment. When examined, the Agr$^-$ strains obtained after heat treatment of the Agr$^+$ strains exhibited plasmid contents that correlated plasmid pXO16 with the Agr$^+$ phenotype (Fig. 2A, lanes d and g; Fig. 2B, lane d).

Three mutants (AND710, GBJ006, and GBJ21) isolated after heat treatment of strain AND508 (Fig. 2B, lanes e, f, and g), however, all displayed the Agr$^-$ phenotype although they seemed to contain a plasmid of about 135 MDa (135* plasmid). The loss and appearance of plasmids in the range of 60 to 90 MDa were also recognized in these mutants. Recombinations of the 68- and 75-MDa plasmids giving rise to plasmids of 63 and 80 MDa have previously been reported (7). To examine whether the 135* plasmid is related to plasmid pXO16, the ability of strains AND710, GBJ006, and GBJ21 to function as recipients in matings with a donor harboring plasmid pXO16 and mobilizable Tet^r plasmid pBC16 (1) was investigated. Streptomycin-resistant mutants of strain AND710, GBJ006, and GBJ21 and strain GBJ001 were mated with strain AND508 electroporated with pBC16. As shown in Table 2, the

TABLE 2. Mobilization of pBC16

Donor	Recipient	Mean relative transfer frequency[a] ± SD
AND508(pBC16)	GBJ001	1.00
AND508(pBC16)	GBJ006[b]	0.464 ± 0.14
AND508(pBC16)	GBJ21[b]	0.448 ± 0.18
AND508(pBC16)	AND710[b]	0.653 ± 0.18

[a] The data shown are from five experiments, and the transfer frequency, calculated as the number of transconjugants per recipient, is relative to the number of transconjugants per GBJ001 recipient in each experiment.
[b] Streptomycin-resistant mutant of the strain was used.

transfer frequencies of pBC16 showed a significant difference between matings involving a recipient carrying the 135* plasmid and the control strain (GBJ001) harboring neither the 135* nor the pXO16 plasmid. This indicates that entry exclusion, incompatibility, or another means of conjugative inhibition was exerted by the 135* plasmid. This 135* plasmid could be a variant of plasmid pXO16 which contains some sort of aggregation mutation. Alternatively, the 135* plasmid may be the result of recombinogenic activity of the smaller plasmids present in strain AND508.

To examine the nature of the 135* plasmid, we conducted several experiments. First, we performed Southern blot analysis with DNA probes derived from the 68- and 75-MDa plasmids of *B. thuringiensis* subsp. *israelensis*. None of them hybridized with either the 135* plasmid or plasmid pXO16 (data not shown). This supports the first hypothesis, that the 135* plasmid is a mutated derivative of pXO16. An anonymous DNA probe hybridized to both the 135* plasmid and plasmid pXO16, which also supports this hypothesis. Second, we compared isogenic pairs of strains containing either the 135* plasmid or plasmid pXO16. We found that (i) the ones containing pXO16 grew more slowly (reduced growth rate of 4 to 7% [compared with the growth rates for strains with 135* plasmid]) (ii) the bands representing plasmid pXO16 on agarose gels were 30 to 40% more intense than the bands representing the 135* plasmid (scanning of negative photo), (iii) the strains containing plasmid pXO16 displayed the Agr$^+$ phenotype, and (iv) there were differences between the restriction patterns of the two plasmids (data not shown). These facts show that there is a difference between the 135* plasmid and plasmid pXO16.

From the above-described findings and from the fact that strains containing the 135* plasmid have a reduced ability to function as recipients, we conclude that the 135* plasmid is a derivative of pXO16 containing, at least, a mutation affecting the aggregation phenotype encoded by the plasmid. The mutation is not a large deletion (>5 kbp), since we detected no difference between the sizes of the plasmids. The nature of the mutation, however, remains to be unraveled. The 135* plasmid may prove convenient in locating the aggregation genes in future work.

Our results demonstrate that the genetic basis of the aggregation system is located on plasmid pXO16 in *B. thuringiensis* subsp. *israelensis*. We have analyzed the plasmid contents of various *B. thuringiensis* subsp. *israelensis* isolates, aggregation mutants, plasmid-cured derivatives, transconjugants, and recured transconjugants, and in all cases the Agr$^+$ phenotype was correlated with the presence of plasmid pXO16.

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