Plasmid Patterns of *Bacillus thuringiensis* Type Strains

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Practically all *Bacillus thuringiensis* strains contain a set of self-replicating, extrachromosomal DNA molecules or plasmids, which vary in number and size in the different strains. The plasmid patterns obtained from gel electrophoresis have previously been used as a tool to characterize strains, but comparison of the plasmid patterns has been limited in the number and diversity of strains analyzed. In this report, we were able to compare the plasmid patterns of 83 type strains (out of 84) and 47 additional strains from six serotypes. The information obtained from this comparison showed the importance of this tool as a strain characterization procedure and indicates the complexity and uniqueness of this feature. For example, with one exception, all type strains showed a unique plasmid pattern. All were unique in such a way that none showed even a single comigrating plasmid in the agarose gels, and therefore, cluster analysis was impossible, indicating that plasmid patterns are qualitative rather than quantitative features. Furthermore, comparison between strains belonging to the same serotype showed a great difference in variability. Some serotypes (e.g., israelensis) showed the same basic pattern among all its strains, while other serotypes (e.g., morrisoni) showed a great diversity of patterns. These results indicate that plasmid patterns are valuable tools to discriminate strains below the serotype level.

*Bacillus thuringiensis* is a ubiquitous bacterium (25) isolated from a great diversity of habitats, which include soil, insects, stored crop products (31), phylloplane (32), and aquatic habitats (18). Its great biotechnological success resides in the production of highly specific insecticidal proteins (Cry proteins) simultaneously with the sporulation phase. These Cry proteins are coded by genes (cry genes) harbored in megaplasmids (10, 14, 21, 34), although it has also been suggested that they are present in the chromosome (21). Plasmids have also been associated with the production of a different toxin called β-exotoxin (23). The relevance of plasmids in *B. thuringiensis* strains is assumed by the regular presence of a set of plasmids, which can vary in number from 1 to 17 and in size from 2 to 80 MDa (2, 9). The set of plasmids harbored in a strain are normally visualized by agarose gel electrophoresis, where they form an electrophoretic pattern of bands, according to their differential migration in the gel. Although *B. thuringiensis* plasmids have been studied either to locate cry genes (9, 21) or to transfer them between different strains and species (3, 5, 10, 15, 30), plasmid patterns have frequently been used to characterize strains (2, 29, 33), especially compared to those of standard strains (16, 17, 27). A plasmid pattern seems to be related to each strain, serotype, or any other subgroup specific.

In a plasmid pattern, two different groups of plasmids can be recognized: those that are ≤30 MDa and those that are ≥30 MDa, called megaplasmids. For practical purposes, each group is divided by the so-called chromosomal band in the agarose gel. Small plasmids are below that band, and megaplasmids are above it. Although apparently all *B. thuringiensis* plasmids replicate following Gruss and Ehrlich’s model (13) for gram-positive bacteria, small plasmids generally use the rolling-circle replication mechanism, with single-stranded DNA intermediates, while megaplasmids normally use the “theta” replication mechanism (24). In addition, small plasmids are generally present in high copy numbers, while megaplasmids are present in low copy numbers. No specific function has been found for the small plasmids, which is the reason why they are called “cryptic” (24). As for the megaplasmids, their main recognized function is harboring cry genes, although the sequencing of some of these plasmids indicates the occurrence of other important genes (6, 8, 19).

In this report, we were able to determine the diversity of plasmid patterns from 130 type strains and also within six serotypes. The information obtained from this comparison showed the importance of this tool as a strain characterization procedure and indicates the complexity and uniqueness of this feature.

**MATERIALS AND METHODS**

**Bacterial strains.** All the bacterial strains included in this work were kindly provided by the International Entomopathogenic Bacillus Center (IEBC) at the Pasteur Institute, Paris, France (see Tables S1 and S2 in the supplemental material).

**Plasmid extraction.** Each strain was cultured in 50 ml Spizizen broth (0.2% NH₄SO₄, 1.4% K₂HPO₄, 0.6% KH₂PO₄, 0.1% sodium citrate, 0.02% MgSO₄·7H₂O) supplemented with 0.5% glucose, 0.1% Casamino Acids (Difco), and 0.01% yeast extract to an optical density at 600 nm of 0.9 to 1.1 at 30°C and 250 rpm shaking. Vegetative cells were pelleted at 20,200 × g for 15 min at 4°C. Each pellet was resuspended in 20 ml cold TES buffer (30 mM Tris base, 5 mM EDTA, 50 mM NaCl; pH 8.0 adjusted with 3 N HCl) and centrifuged in a 30 mM Tris base, 5 mM EDTA, 50 mM NaCl; pH 8.0 adjusted with 3 N HCl) and centrifuged at 20,000 × g for 20 min at 4°C. Each pellet was resuspended in 2 ml lysy buffer (TES buffer containing 20% sucrose, 2 mg/ml lysozyme, and 1 μl/ml of RNase from a 10-mg/ml stock solution) and incubated at 37°C for 90 min or until more than 90% spheroplast formation was achieved and monitored under the microscope (some strains required more than 3 hours). The spheroplast suspension was supplemented with 3 ml of 8% sodium dodecyl sulfate in TES buffer and incubated at 68°C for 10 min. Then 1.5 ml of 3 M sodium acetate (pH 4.8) was added, and the suspension was incubated at −20°C for 30 min. The suspension was centrifuged at 20,200 × g for 20 min at 4°C. The supernatant was centrifuged; if it was not, another centrifugation was done, and ultimately, if still required, it was filtered. Two volumes of cold absolute ethanol were added to the supernatant and incubated overnight at −20°C. Plasmid-enriched DNA was pelleted at...
20,200 g for 20 min at 4°C. Each pellet was dissolved in 100 µl Tris-EDTA (pH 8.0) (10 mM Tris-HCl, 1 mM EDTA) and stored at 20°C until further use.

Electrophoresis. In order to visualize the plasmid pattern from each strain, 10 µl of each plasmid-enriched DNA solution was loaded, along with the Supercoiled DNA ladder (Invitrogen), in 0.5% agarose gels (11 by 14 cm) and run in 1× Tris-borate-EDTA buffer (45 mM Tris-borate, 1 mM EDTA) at 2 V/cm for 15 to 20 h. Gel slabs were stained for 10 min in 0.4 g/ml ethidium bromide and washed in double-distilled H2O for 1 h. Gels were recorded in a Gel Doc 2000 gel system (Bio-Rad Corporation). All gels included the plasmid preparation from B. thuringiensis serovar kurstaki HD-1 strain as a reference.

RESULTS

Plasmid patterns of Bacillus thuringiensis type strains. The plasmid purification procedure described in this report is the outcome of numerous tests and modifications made to techniques reported earlier (2, 7, 9). After countless purifications made throughout this work, it finally proved to be simple, reliable, and reproducible.

Figures 1 to 3 show the plasmid patterns of all the B. thuringiensis type strains included in this report. All the strains contain at least one plasmid, and some strains have a maximum of 13 plasmids. In general, all plasmid patterns are unique to each strain. Efforts to arrange similar patterns in the same gel to identify comigrating bands and then try to develop a dendrogram were fruitless. Some strains, such as the type strains of serovars colmeri and iberica, showed very similar patterns (Fig. 1C), but more detailed analyses indicated that such similarity was not real, as bands showed a slight difference in their agarose gel migration speed. The same happened with the type strains of serovars leesis and kim (Fig. 2C). However, serovar israelensis and malaysiensis type strains (Fig. 1B) showed clear homology. Interestingly, serovars sharing the same antigenic determinants, such as sumiyoshi-
shiensis (serotype H3a,3d) and fukuokaensis (serotype H3a,3d,3e) (Fig. 2A), had different plasmid patterns.

Comparative resolution of megaplasmid bands (plasmids above the chromosomal DNA band) in the agarose gel was difficult and was a unreliable way to differentiate plasmid patterns. This is the reason why the comparison of plasmid patterns focused on the plasmids below the chromosomal band. However, this decision made it impossible to compare the plasmid patterns of those strains that contained only megaplasmids (Fig. 3B).

Plasmid patterns of strains within the same serotype. In order to find any diversity of plasmid patterns within a given serotype, a number of strains within six different serotypes were chosen and their plasmid patterns were analyzed. In contrast to the results obtained when plasmid patterns from different serotypes were compared, strains within the same serotype showed at least some degree of resemblance. Figure 4A shows the plasmid patterns of nine strains belonging to serovar kurstaki (H3a3b3c). Four strains show identical patterns, but most importantly, there were some obvious comigrating bands, indicating some degree of relationship among all of them. This relationship was even more evident among strains of serovar israelensis (H14) (Fig. 4B), where all plasmid patterns were almost identical, including that of the serovar malaysiensis type strain described above.

Also, some degree of relationship was observed when the plasmid patterns of nine strains from serovar thuringiensis (H1) were compared (Fig. 4C). All nine strains showed some comigrating bands, except for strains T01 110 and T01 194, whose plasmid patterns were identical but completely different from those of the rest of the strains analyzed. More diversity was observed when the plasmid patterns of nine strains belonging to serovar kenyae (H4a4b) were compared (Fig. 4D). Although some strains had the same plasmid pattern, most are unique to each strain. Likewise, high pattern diversity was observed in the group of 14 strains from serovars sotto and dendrolimus, which share the same antigenic determinants (H4a4b) (Fig. 5A and B). Only four strains had the same plasmid pattern, and five more contained only megaplasmids. In both cases, strains belonged to both serovars. Similarly diverse was the group constituted by nine strains from serovar morrisoni (H8a8b). Only two strains showed identical patterns, with the rest of the strains displaying unique patterns. These include the highly unique strains tenebrionis and PG-14.

**DISCUSSION**

Interest in *B. thuringiensis* plasmids started at the end of the 1970s when a correlation was established between the formation of crystals and the presence of certain plasmids (9, 10). Later on, attention was focused on the location of cry genes in plasmids and on the transfer of plasmids between different strains of *B. thuringiensis* and from *B. thuringiensis* to *Bacillus cereus* (3, 5, 10, 15, 30). However, little attention was paid to
the importance of plasmid patterns as a tool for characterization of strains. For example, Lereclus et al. (22) compared the plasmid patterns of 11 strains belonging to nine different serotypes, Ibarra and Federici (16) compared nine different isolates from serovar israelensis, and Aptsosoglou et al. (2) compared the plasmid patterns of 44 isolates, and there were other such reports. However, in spite of these and other reports, plasmid patterns were always underestimated, mostly due to the possibility that *B. thuringiensis* strains may spontaneously lose some of their plasmids and to the unreliability of the plasmid extraction techniques.

A major concern in this work was the reliability of the technique used to extract the plasmids from the strains. Because a great many plasmid purification techniques appear in the literature (3, 5, 19) and most of them are either time-consuming and/or unreliable, much time was spent on the development of a reliable and simple technique which could render reproducible results. After a series of tests and modifications to some reported techniques (2, 7, 9), we developed a procedure that proved to be reproducible and simple, corroborated by countless purifications made throughout the development of this work. Achieving efficient degradation of the typical thick cell wall of gram-positive bacteria and careful handling of megaplasmids were the major problems solved with this procedure.

In this report, we were able to compare the plasmid patterns of 83 type strains (only one was missing) and 47 additional strains from six serotypes. The information obtained from this comparison showed the importance of this tool as a strain characterization procedure and indicates the complexity and uniqueness of this feature. With the exception of serovars israelensis and malaysiensis, all type strains showed a unique plasmid pattern. Interestingly, all were unique in such a way that none showed even a single comigrating plasmid in the agarose gels. Such a distinctive feature made impossible to analyze the results by cluster analysis, indicating that this is a qualitative rather than a quantitative feature. Such uniqueness was suggested in previous reports (2, 9, 26) but was never corroborated by comparing a large number of strains.

Although special care was taken to obtain undegraded megaplasmids during the purification procedure and in spite of their importance in harboring the cry genes (2, 4, 11, 14), megaplasmids were finally overlooked during the plasmid pattern comparison. Their migration in the agarose gel during electrophoresis was very limited, and hence, comigrating bands were difficult to discriminate. Therefore, comparison focused only on those plasmids migrating below the chromosomal DNA band, and the megaplasmids (those above the chromosomal DNA band) were used as a secondary option to differentiate between patterns. Besides, megaplasmids are easily degraded during storage, losing information about each pattern. However, a major constraint of this decision was a lack of information to compare the plasmid patterns of all the type strains (eight in total) that harbored only megaplasmids (Fig. 3B). Interestingly, the serovar entomocidus and its biotype subtoxicus (both serotype H6) showed the same lack of small plasmids and an apparently comigrating megaplasmid. Similar results were observed in serovars finitimus (serotypes and IEBG code numbers are shown in parentheses) (serotype 2; IEBG T02001), tohchijensis (19; T19001), pondicheriensis (20a, 20c; T20A001), japonesis (23; T23001), balearica (48; T48001), navarrensis (50; T50001), pirenaica (57; T57001), and vazensis (67; T67001) (patterns not shown).

The uniqueness of each plasmid pattern was no longer observed when patterns from strains belonging to the same serotype were compared. Similar and sometimes identical plasmid patterns were observed between strains belonging to the same serotype. However, variation occurred on two levels, one that could be explained in terms of monophyletic strains, where comigrating plasmids occurred, and the other in terms of paraphyletic strains, where no comigrating plasmids occurred. That is, there were strains sharing the same basic plasmid patterns, albeit with variation in terms of one or several missing plasmids. These patterns always showed comigrating bands, and strains might have evolved from a single ancestor, in spite of the highly diverse geographical origin. On the other hand, there were strains which, regardless of belonging to the same serotype, showed a completely different pattern (i.e., no comigrating bands), indicating that they might...
have evolved from different ancestors. The degree of variation within a serotype differed among the different serotypes. While all the strains within serotype H14 (serovar israelensis) showed the same basic plasmid pattern, the strains in serotype H8a,8b (serovar morrisonii) showed almost one different pattern for each tested strain. The rest of the serovars tested (kurstaki, sotto, and kenyaec) showed different degrees of variations within each of them. The homogeneity shown between the strains of serovar israelensis has been reported earlier (1, 16); while the great diversity among the strains of serovar morrisonii has been demonstrated before (12, 20, 27, 28), as it contains strains toxic to lepidopteran larvae, strains toxic to mosquito larvae, and strains toxic to coleopteran larvae.

In conclusion, plasmid patterns are valuable tools to characterize B. thuringiensis strains. Rather than being quantitative features, where differences among patterns can be measured in terms of degrees of similarity, plasmid patterns are qualitative features, where differences among patterns can be measured in terms of degrees of similarity, plasmid patterns are qualitative features, represented by specific sets of plasmids. Strains can lose plasmids, but the basic set remains, visualized by comigrating bands in the agarose gel. Also, different sets of plasmids can be found in strains belonging to the same serotype. Due to this variation, plasmid patterns have limited use in discriminating between serovars, but they can be used at a lower level of discrimination. Because of this, plasmid patterns can be a useful tool to differentiate specific strains, something highly valuable in intellectual property claims.

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