Systematic and Applied Microbiology 33 (2010) 204–208

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Distribution of phenotypes among *Bacillus thuringiensis* strains

Phyllis A.W. Martin*, Dawn E. Gundersen-Rindal, Michael B. Blackburn

USDA/ARS Invasive Insect Biocontrol and Behavior Laboratory, Beltsville, MD 20705, USA

ARTICLE INFO

Article history:
Received 15 October 2009

Keywords:
Bacillus cereus group
Bacillus
Parasporal crystal
Insect pathogen
Biocontrol

ABSTRACT

An extensive collection of *Bacillus thuringiensis* isolates from around the world were phenotypically profiled using standard biochemical tests. Six phenotypic traits occurred in 20–86% of the isolates and were useful in distinguishing isolates: production of urease (U; 20.5% of isolates), hydrolysis of esculin (E; 32.3% of isolates), acid production from salicin (A; 37.4% of isolates), acid production from sucrose (S; 34.0% of isolates), production of phospholipase C or lecithinase (L; 79.7% of isolates), and hydrolysis of starch (T; 85.8% of isolates). With the exception of acid production from salicin and hydrolysis of esculin, which were associated, the traits assorted independently. Of the 64 possible combinations of these six phenotypic characteristics, 15 combinations accounted for ca. 80% of all isolates, with the most common phenotype being TL (23.6% of isolates). Surprisingly, while the biochemical traits generally assorted independently, certain phenotypic traits associated with the parasporal crystal were correlated with certain combinations of biochemical traits. Crystals that remained attached to spores (which tended to be non-toxic to insects) were highly correlated with the phenotypes that included both L and S. Among the 15 most abundant phenotypes characterizing *B. thuringiensis* strains, amorphous crystals were associated with TLE, TL, T, and Ø (the absence of positive tested biochemical traits). Amorphous crystal types displayed a distinct bias toward toxicity to dipteran insects. Although all common phenotypes included *B. thuringiensis* isolates producing bipyramidal crystals toxic to lepidopteran insects, those with the highest abundance of these toxic crystals displayed phenotypes TLU, TLUA, TLUAE, and TLAE.

Published by Elsevier GmbH.

Introduction

Historically, many methods have been used to distinguish among strains of *Bacillus thuringiensis* (*Bt*), including substrate utilization tests, serotyping of flagellar antigens, plasmid profiles, and comparison of genetic sequences. Early attempts to classify varieties of *Bt* typically relied on substrate utilization tests and serotyping (de Barjac 1981; Heimpel 1967), while more recent approaches have been based on multilocus enzyme electrophoresis (Helgason et al. 2000), amplified fragment length polymorphism (Hill et al. 2004), or multilocus sequence typing (Helgason et al. 2004; Priest et al. 2004; Sorokin et al. 2006).

Prior to beginning our own phylogenetic analysis of *Bt* by multilocus sequence typing, we wished to systematically approach how to ensure adequate genetic diversity among isolates chosen for typing. We chose to utilize some of the earliest methods used for distinguishing *Bt* isolates, performing substrate utilization profiles on an extensive collection of *Bt* isolates from around the world. Some of these biochemical traits may reflect adaptations for survival under specific growth conditions. For instance, we recently found a strong correlation between urease production and the ability of *Bt* strains to replicate successfully in gypsy moth larvae (Martin et al. 2009). Results of the analysis presented here provide a unique glimpse into the relative frequencies of biochemical phenotypes originally ascribed to specific *Bt* serotypes by de Barjac (1981), and expand greatly upon the phenotypic analysis of Martin and Travers (1989). Surprisingly, although the biochemical traits we monitored generally appear to be randomly assorted among strains, we found that certain crystal traits were highly correlated with specific biochemical phenotypic profiles.

Materials and methods

The 3639 *Bt* strains analyzed for this study were isolated from approximately 350 soil samples originating from 34 countries around the world, with 42% of isolates from samples obtained within the United States. The vast majority (3429 isolates) of these were isolated by acetate selection as described by Travers et al. (1987). Following acetate selection, isolates that tested positive for protease and the production of acid from glucose, and negative for acid production from arabinose, mannitol, and xylose were examined for the presence of parasporal crystals. Isolates with all these attributes were considered to be *Bt* (Martin and Travers 1989).

* Corresponding author at: 10300 Baltimore Ave., Bldg 011 HH 17 BARC-West, Beltsville, MD 20705, USA. Tel.: +1 301 504 6331.
E-mail address: Phyllis.martin@ars.usda.gov (P.A.W. Martin).

0723-2020/$ – see front matter. Published by Elsevier GmbH.
doi:10.1016/j.syapm.2010.04.002

Those isolates not isolated by acetate selection (210 isolates) were generally isolated by pasteurization of aqueous soil suspensions, with growth on T3 (Travers et al. 1987) and microscopic examination of resulting colonies for parasporal crystals.

Phenotypic tests were conducted as described by Martin et al. (1985) on plates or agar dots. Media for the various phenotypic tests were as described by Parry et al. (1983) except that tests for production of acid from sugars were conducted on media with a tryptose base. Fifteen traits were initially tested. Acid production from arabinose, cellobiose, glucose, mannitol, mannose, salicin, sucrose, and xylose was determined in using 1% sugar in a 0.5% tryptose base with bromcresol purple as indicator. Utilization of citrate was conducted in Simmons citrate agar (Difco, Detroit, MI). Lysis of sheep red blood cells was tested for on Columbia blood agar with 5% sheep blood (Difco, Detroit, MI). Hydrolysis of esculin was monitored using 0.1% esculin in a 1% peptone base. Production of protease was tested on skim milk agar. Phospholipase production (lecithinase) was monitored on *Bacillus cereus* selective agar (Oxoid, Cambridge, UK). Amylase activity was detected by clearing zones on nutrient agar containing 1% corn starch, and urease activity monitored by ammonia formation on 2% urea in a glucose–phosphate–peptone base with phenol red as an indicator. To distinguish among *Bt* isolates, we selected traits that occurred in our collection in greater than 10% and less than 90% of the isolates. Phenotypes were designated by the positive characters they possessed. *Bt* strains IBL 455 isolated from a 1980 preparation of Dipel® (Abbot Laboratories, Chicago, IL) and IBL 1410 isolated from a 1994 preparation of Novodur® (Mycogen, San Diego, CA) were used as controls for phenotypic tests. IBL 455 was positive for production of amylase, lecithinase, protease, and urease; acid production from glucose and salicin, hydrolysis of esculin, and lysis of sheep red blood cells. IBL 1410 was positive for production of amylase, protease, and acid production from sucrose, mannose, and glucose. Thus, IBL 455 and IBL 1410 provided complementary positive and negative controls for five of the six tests that we found most useful for distinguishing among *Bt* isolates: production of urease and lecithinase, acid production from salicin and sucrose, and hydrolysis of esculin.

To allow observation of crystal morphology, strains were grown 48–72 h at 30 °C on T3. Crystals were characterized using phase contrast microscopy as forming bipyramidal (typical *kurstaki* crystal), amorphous or irregular (*israelensis* type crystal), attached (*finitimus* type crystal, where the crystal is not released from the spore), or "other" (such as irregularly pointed, cubic, or the flat crystals of *tenebrionis*).

Toxicity was determined against several insect species as described in Martin and Travers (1989) in single-dose tests. Toxicity assays were performed either as spread on the surface of artificial diet or incorporated into freeze-dried diets (Martin 2004) for Lepidoptera.

Un-rooted phenograms were generated using PAUP 4.03 software (Sinauer Associates, Sunderland, MA) after binary coding of strain phenotypic character states. Frequency distribution of crystal types was analyzed by chi-square (SAS Institute Inc. 2008). Circles with areas proportional to frequencies of phenotypes or crystal morphologies within phenotypes were superimposed upon the resulting phenogram using Photoshop.

Results

Some phenotypic traits were so nearly invariant that they were of little use in differentiating *Bt* isolates; these included acid production from glucose (96%), mannitol (3.2%), arabinose (0.1%), and xylose (2.7%); haemolysis of sheep blood (98.4%), protease production (91.2%); and citrate utilization (2.7%). The frequencies of these traits were consistent with a previous study (Martin and Travers 1989). Acid production from mannose occurred in 10.5% of all isolates, but most of these were in uncommon phenotypes. In the most common phenotypes, acid production from mannose occurred at a frequency of 3.2% and thus this trait was not found to be useful for differentiation. Six traits occurred in 20–86% of the isolates and were more useful for differentiation; hydrolysis of starch (T, 85.8%), production of phospholipase C or lecithinase (L, 79.7%), production of urease (U, 20.5%), acid production from sucrose (S, 34.0%), acid production from salicin (A, 37.4%), and hydrolysis of esculin (E, 32.3%).

Together, these six traits allowed a total of 64 possible phenotypes. In this sample only 60 occurred. The combinations of UA, UAE, USA and LUSE did not occur. Four traits (T, L, U, and S) were found to assort in random fashion. However, A and E tended to occur together almost twice as often as one would predict from their frequencies in the population (794 AE observed, 440 expected).

All observed phenotypes, the number of countries from which they were isolated, and their frequencies in the collection as a whole are listed in Table S1. Cumulatively, 80% of all isolates belonged to one of the 15 most abundant phenotypes. The most abundant phenotype encountered was TL, producing amylase and a lecithinase. In all, 23.6% of all isolates belonged to phenotype TL, with another 22% of isolates belonging to four phenotypes differing by the addition of a single trait from TL (TLS, TLU, TLA, and TLE). Analysis of the relative occurrence of the 15 most common phenotypes yielded an un-rooted phenogram with TL at its center, shown in Fig. 1A. The significance of this particular arrangement of phenotypes should become clear from the results which follow.

For the characterized collection as a whole, the distribution of crystal types was 43.9% bipyramidal, 27.0% amorphous, 12.3% attached and 16.8% other. The distribution of crystal types observed among the 15 most abundant phenotypes is shown in Fig. 1B–D, and Table S2. It is immediately obvious that the distribution of crystal types within phenotypes was generally different than that of the collection as a whole. Only TLS and TLA had distributions of crystals that were not statistically different from the overall distribution by chi-square analysis.

While all phenotypes produced bipyramidal crystals, their occurrence within phenotypes ranged from 85.4% of crystals for TLAE to 21.7% for T alone. Fig. 1B illustrates the relative frequencies of bipyramidal crystals among phenotypes superimposed upon the phenogram of Fig. 1A.

While bipyramidal crystals were much more prevalent in the collection as a whole, amorphous crystals were generally restricted to strains displaying phenotypes TL, TLE, Ø (negative for all traits), and T. In fact, 71.2% of amorphous crystals observed in the 15 most abundant phenotypes occurred in these four types, with the TL phenotype alone responsible for 55.0% of all amorphous crystals observed (Fig. 1C).

Strains displaying phenotypes that were positive for both lecithinase (phospholipase C) and acid production from sucrose (LS) had the highest proportion of attached crystal types (Fig. 1D). Strains displaying phenotypes TLS, TLSA, TLSAE, TLSE, and LSAE averaged 47.0% attached crystals, while phenotypes that were not *both* L+ and S+ averaged only 2.5% attached crystals. At the extremes, LSAE had 83.7% attached crystals, while TLUAE had none.

Not surprisingly, the frequency of lepidopteran toxicity in a particular phenotype was related to the proportion of bipyramidal crystals produced. Overall, 52% of strains producing bipyramidal crystals were toxic to lepidopteran larvae (Table 1). However, in the case of phenotypes TLUAE, TLAE, TLU, and TLUA 100%, 96%, 91% and 76% (respectively) of the isolates producing bipyramidal crystals were lepidopteran toxic.

On the other hand, dipteran toxicity was correlated with amorphous crystals. Fifty-eight percent of amorphous crystals were mosquito toxic (78% in TL).