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Survival of diverse *Bacillus thuringiensis* strains in gypsy moth (Lepidoptera: Lymantriidae) is correlated with urease production

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ABSTRACT

Bacillus thuringiensis is an entomopathogenic bacterium that can kill a variety of pests, but seldom causes epizootics because it replicates poorly in insects. We have tested lepidopteran-toxic *B. thuringiensis* strains with diverse substrate utilization profiles for the ability to survive repeated passages through larvae of the gypsy moth, *Lymantria dispar*, without intervening growth on artificial media. These experiments have revealed a remarkable correlation between the production of urease by the bacteria and its ability to survive repeated passages through larvae. Of 26 urease-positive strains tested, 23 were capable of surviving five passages through gypsy moth larvae. In contrast, none of the 24 urease-negative strains tested survived to the 4th passage, with only three strains surviving to the 3rd passage. Selection of *B. thuringiensis* strains with phenotypic traits favoring replication in the environment, such as urease production, may improve their efficacy as biological control agents.

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1. Introduction

Bacillus thuringiensis Berliner (Bt) is a spore-forming Gram-positive bacterium that produces insecticidal crystalline proteins $(\delta$ -endotoxins) when sporulating. Although Bt is an insect pathogen, it is generally used as a bio-insecticide. The δ -endotoxins are sufficient to kill an insect, while the bacterium itself generally achieves only limited replication within the host (Aly, 1985; Prasertphon et al., 1973; Raymond et al., 2008). Although a few apparent epizootics have been described [reviewed by Dangaard (2000)], Bt does not typically persist in a purely pathogenic fashion. It has even been questioned whether Bt replication in the host is actually involved in insect toxicity (Broderick et al., 2006). Thus, the selection of strains for use as control agents has been based largely on their compliment of δ -endotoxins, and the quantity of these proteins that are produced upon sporulation. Although the δ -endotoxin compliment of a strain is clearly a critical consideration, we wished to investigate other traits that might significantly impact the utility of a strain for a particular application. Different Bt strains may differ in their ability to persist in certain soils or on certain plants, and they may differ in their ability to replicate in the target insect. At high pest densities, it is possible that better replication in the host could lead to improved availability of Bt over time. With a fuller understanding of the ecological niches occupied by various Bt biotypes, it may be possible to find situations where Bt actually func-

* Corresponding author. *E-mail address:* phyllis.martin@ars.usda.gov (P.A.W. Martin). tions as a recycling biological control agent, as is the case for *Paenibacillus popilliae* (Dutky) Pettersson, which has successfully controlled Japanese beetles, *Popillia japonica* Newman, for extended periods when applied as an inoculum (Faust and Bulla, 1982).

Our laboratory possesses a large collection of Bt strains (Martin and Travers, 1989), which includes approximately 3500 Bt strains that have been characterized by crystal morphology, toxicity and biochemical phenotype. We tested strains that were toxic to gypsy moth larvae, *Lymantria dispar* (L.), for the ability to kill, replicate, and form spores and toxic crystals in gypsy moth cadavers for at least five passes without growth on laboratory media. Although all the strains tested appeared to have similar toxicity to gypsy moth larvae, only those strains that produced urease had the ability to survive repeated passages through gypsy moth larvae.

2. Materials and methods

2.1. Bt strains

Fifty isolates of Bt were tested for their ability to survive repeated passage through gypsy moth larvae. These strains were chosen such that ca. half (26) exhibited urease activity while the remainder (24) did not. All of the strains chosen produced bi-pyramidal crystals and had previously been shown to be toxic to a lepidopteran insect, *Trichoplusia ni* (Hübner). The strains and their phenotypic profiles, based on substrate utilization, are presented in Table 1. In all, 20 phenotypes were represented. With the



Table 1

<i>Bacillus thuringlensis</i> strains used and their phenoi	types.	
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IBL No.	Avg. number of passes survived	Positive phenotype ^a
950, 1496	5	ur
110, 120, 357, 420, 447, 563, 635, 860, 866, 888, 3087	4.8	ur le
402	2	ur su
1405	5	ur le es
714, 743, 1115, 1380	4.3	ur le sa
556	5	ur le su
116, 1161	5	ur le ma
1140	5	ur le sa ma
661, 749	5	ur le es sa
658	5	ur le sa su
363, 1402	2.5	le
1376, 1378, 1387, 1392, 1399	2	le sa
443	4	le sa su
547, 554, 572	1.7	le su ma
299, 300	1.5	le es sa su
111	2	le es sa su ma
118, 119	2	es su
285	1	sa su
279, 280, 281, 1444, 3084, 3085	2.1	su
1310	3	su ma

^a Phenotypes: ur, production of urease; le, production of lecithinase (phospholipase C); es, utilization of esculin; sa, acid production from salicin; su, acid production from sucrose; ma, acid production from mannose.

exception of IBL 743, all bacteria tested were isolated by acetate selection (Travers et al., 1987) from soil samples originating in Iceland, Jamaica, Mexico, Nepal, Norway, Sweden, and the USA. Strain IBL 743 was isolated from the gut of an *Aedes aeygpti* (L.) mosquito larva by T. Clark (Beltsville, MD).

2.2. Phenotypic tests and enumeration

Biochemical tests used in this study included production of urease and lecithinase, utilization of esculin, and acid production from salicin, sucrose, and mannose. Substrate utilization testing was performed on agar dots as described by Martin et al. (1985), which was a modification of standard biochemical tests using 2% agar (Parry et al., 1983). Acid production from sugars used a 0.1% tryptose with 1% sugar and bromcresol purple as an acid indicator. Urease medium was also peptone based with 2% urea and phenol red as a basic indicator. For lecithinase (phospholipase C) tests, yolks of two eggs less than 24-h-old were aseptically added to 500 ml of selective agar for Bacillus cereus Frankland and Frankland (Oxoid, Basingstoke, UK) at 55 °C. For each battery of tests on a particular strain, each test was replicated on 30 dots. Strains IBL 455 (isolated from a 1980 preparation of Dipel[®], Abbott Laboratories Chicago, IL) and IBL 1410 (isolated from a 1994 preparation of Novodor[®], Mycogen, San Diego, CA) were used as controls for substrate utilization; together they provide a complimentary range of positive and negative responses to the substrate utilization tests. IBL 455 displays positive tests for lecithinase, urease, utilization of esculin and acid production from salicin, while IBL 1410 is positive for the production of acid from sucrose and mannose. Bacteria were enumerated on half-strength Luria-agar (Atlas, 2004) without glucose.

2.3. Survival of strains in gypsy moth larvae

Gypsy moth egg masses were received from USDA/APHIS, Otis Air National Guard Base (MA). Eggs were hatched and larvae reared to 2nd instar on a wheat germ-based diet (Bell et al., 1981) at 25 °C and a 16:8 h L:D cycle without humidity control.

Experiments were conducted to determine if Bt strains could survive up to five passages through gypsy moth larvae without intervening growth on artificial media. To initiate the process, gypsy moth larvae were fed Bt strains grown on artificial media. The dead larvae resulting from this initial feeding, and all subsequent passages, served as the source of Bt spores and crystals for the following round of feeding. For each Bt strain, this cycle was repeated for five iterations or until no insects died. In order to demonstrate that the correlation between urease production and survival of strains through repeated passages is a general phenomenon that is not restricted to certain strains, we chose to test each strain as a single replicate, allowing us to maximize the number of strains included in the experiment. In all, 26 urease-positive and 24 urease-negative strains were assayed.

Initial spore-crystal mixtures for the first round of feeding were obtained by growing strains on 100 mm plates of T3 agar at 30 °C for 2-5 days until spores and crystals had formed (Travers et al., 1987). Plates were then scraped into 10 ml sterile water and vortexed to form a suspension that was used to feed the larvae. For the initial passage, $300 \mu l$ of the spore-crystal suspension for each strain was applied to each of two freeze-dried pellets of gypsy moth diet (Martin, 2004). Two 2nd instar larvae were then placed onto each of these treated diet pellets, for a total of four insects per Bt strain. Dead insects were removed on days 3 and 6, placed in sterile 1.5 ml microfuge tubes (cadavers from each strain were pooled), and kept at 25 °C until day 7. On day 7, dead larvae were homogenized in 300 µl sterile water per insect. Diet pellets for the next passage were each treated with $300 \,\mu$ l of the resulting homogenate. Control pellets, treated with 300 µl sterile water only, were included with each passage. Chi square analyses were performed to determine if the frequencies of the various phenotypes were the same among all strains tested and those strains that could survive for five passages in larvae.

To ensure that the initial suspensions used in the first passage contained similar numbers of spores, selected suspensions containing either urease-positive (IBL 110, IBL 116, IBL 120, IBL 357, IBL 402, IBL 743, IBL 749 and IBL 950) or urease-negative strains (IBL 111, IBL 118, IBL 119, IBL 285, IBL 443, IBL 1310 and IBL 1444) were enumerated. For some strains that survived repeated passages (IBL 110, IBL 116, IBL 357, IBL 743, IBL 749 and IBL 950). Bt was recovered and enumerated from dead larvae at each passage. In most cases, phenotypic tests were performed on these strains as well, with testing of 30 individual colonies isolated for each strain at each passage. Analysis of variance (PROC Mixed; SAS, 2008) was used to determine if there were significant differences among spore titers of urease-positive and urease-negative suspensions used for initial feedings. Analysis of variance was also used to compare Bt counts recovered from killed larvae by both strain and passage number. The effect of passage on titers of individual strains was also analyzed by regression.

To determine if Bt germinated and grew on the diet pellets, fresh pellets were treated as above with T3-grown spores of IBL 120, IBL 743, IBL 749, IBL 402 or sterile water (as a control). Treated diet pellets were collected immediately following application of the suspensions and at 24 h intervals for 3 days. Collected pellets were suspended in 5 ml sterile water in a sterile plastic bag and ground in a stomacher blender (Techmar, Cincinnati, OH) for 60 s on high. Cells were then serially diluted and enumerated on half-strength L-agar without glucose. Growth of bacteria was determined by comparing plate counts from pellets collected at 24, 48 and 72 h post-inoculation.

2.4. Urease assay

To determine if urease was produced in the absence of urea, selected strains were grown for 24 h in half-strength Luria-broth without glucose at 25 °C and 200 rpm in an orbital shaker. Cells were removed by centrifugation, and the resulting supernatant was filtered through a 0.22 μ m cellulose acetate filter (Costar, Corning NY USA). A 500 μ l aliquot of this sterile supernatant was added to filter-sterilized urea broth (Parry et al., 1983) and the change in pH was recorded after 24 h incubation at 30 °C (ammonia liberated from urea raises the pH). Protein concentration of the sterile supernatant was measured by the Bradford assay (Sigma-Aldrich, St. Louis MO, USA). Urease activity was expressed as the change in pH units/mg protein concentration. Strains assayed included the urease-positive strains IBL 110, IBL 116, IBL 120, IBL 357, IBL 402, IBL 420, IBL 563, IBL 635, IBL 661, IBL 714, IBL 743, IBL 749, IBL 888, IBL 950, IBL 1115, IBL 1140, IBL 1380, IBL 1405 and IBL 3087, and the urease-negative strains IPS-82 [Bt *israelensis*; urease-negative according to deBarjac (1981)], IBL 118 and IBL 119 as negative controls.

3. Results

3.1. Survival of strains in gypsy moth larvae

Of the 50 Bt strains that we tested, 23 of 26 urease-positive strains were capable of completing five passages in gypsy moth larvae. None of the 24 urease-negative strains persisted for five passages; only four urease-negative strains survived three passages, with a single strain surviving to the fourth passage. Comparison of survival of urease-positive and urease-negative strains by Chi square analysis showed highly significant differences between the two phenotypes ($\chi^2 = 39.3$; df = 1; *P* < 0.0001). There was no mortality on control (water only) pellets. The average number of passes survived by each of the phenotypes represented in the experiment is summarized in Table 1. Overall, the mean number of passages survived by the urease-negative strains was 2.12 ± 0.83 (*n* = 24).

From our results it also appears that the ability to make acid from sucrose is negatively correlated with the ability of an isolate to persist through multiple passages. However, this result is due to the highly skewed distribution of sucrose-positive strains between urease-positive and urease-negative strains (3 vs. 17, respectively). Of the three strains positive for both traits, two survived five passes. Of the eight strains negative for both traits, none survived five passes (with a mean of 2.0 passes). These results are summarized in Table 2. The remaining phenotypic traits (lecithinase, utilization of esculin, and production of acid from salicin and mannose) were more evenly distributed between urease-positive and urease-negative strains, and the frequency of those traits in the survivors was not different from the frequency in the total population tested using χ^2 test at the 0.01 level (Table 3).

For selected urease-positive isolates, we followed replication through all five passages by cell count (Table 4). Nearly all bacterial colonies recovered from dead larvae that had been fed urease-positive strains had *Bacillus* morphology. The number of bacteria recovered was not significantly affected by strain (F = 1.42; df = 5, 18; P = 0.27) or by pass (F = 0.18; df = 4, 18; P = 0.95). Bacteria isolated from insects killed by two urease-negative strains (IBL 118 and IBL 119) at the stage they failed to propagate, revealed numer-

Table 3

Differences of individual traits among surviving Bacillus thuringiensis strains.

Positive phenotypic trait ^a	Frequency in total strains tested	Frequency in survivors	χ2 df = 1	Probability
ur	0.52	1.0	24.0	<0.01*
su	0.36	0.09	7.96	<0.01*
le	0.70	0.91	3.54	0.035
es	0.14	0.17	0.35	0.43
sa	0.32	0.35	0.10	0.75
ma	0.12	0.13	0.04	0.86

^a Phenotypes: ur, production of urease; le, production of lecithinase (phospholipase C); es, utilization of esculin; sa, acid production from salicin; su, acid production from sucrose; ma, acid production from mannose.

Different at the 0.01 level.

Table 4

Bacillus thuringiensis recovered from gypsy moth larvae by pass^a.

Strain IBL No.	Positive phenotype ^b	Pass 1	Pass 2	Pass 3	Pass 4	Pass 5	Slope	R ^{b,**}
950	ur	7.62	6.64	7.62	7.69	7.61	0.11	0.13
357	ur le	*	7.23	6.78	7.20	7.47	0.11	0.26
110	ur le	7.27	7.23	7.70	7.10	7.11	-0.05	0.08
743	ur le sa	7.43	7.37	7.47	6.15	6.66	-0.28	0.55
749	ur le es sa	7.60	7.70	7.60	7.12	6.88	-0.20	0.79
116	ur le ma	7.37	7.86	7.69	7.81	7.60	0.04	0.11

^a Titers are expressed log₁₀ colony-forming units (cfu) recovered per insect.

^b Phenotypes: ur, production of urease; le, production of lecithinase (phospholipase C); es, utilization of esculin; sa, acid production from salicin; su, acid production from sucrose; ma, acid production from mannose.

 * All bacteria recovered were used to feed the next larvae, so no titer was performed.

* All regressions were non-significant (P > 0.05).

ous bacteria that did not appear to be Bt. For the urease-positive strains shown in Table 4, we were generally able to perform phenotypic determinations on bacteria recovered from killed insects at each passage. The predominant phenotype exhibited by these recovered bacteria remained the same as that of the strain initially fed to the insects. Urease production was a particularly stable trait, with 94 \pm 1.8% of isolated bacteria testing urease-positive across all five passages (930 recovered colonies tested).

Enumeration of selected isolate preparations fed to larvae in the first passage revealed no systematic bias in the inocula used to treat the diet pellets that could explain the results obtained. The average titers of urease-positive strains $[5.04 \pm 0.86 \times 10^7 \text{ colony-forming units (cfu) per diet pellet, } n = 8]$, and those of urease-negative strains $(5.15 \pm 0.92 \times 10^7 \text{ cfu per diet pellet, } n = 7)$ were not significantly different (*F* = 0.01; df = 1, 13; *P* = 0.94).

It is unlikely that growth of urease-positive Bt strains on the diet pellets contributes to their ability to survive multiple passages in our experimental system; there was no indication that strains grew on diet pellets. The number of bacteria recovered from pellets after 3 days never exceeded the initial number recovered from similar pellets 0 days after inoculation.

Table 2

Phenotypes of surviving Bacillus thuringiensis strains versus non survivors.

••	-		
Phenotype	Surviving strains	Non-surviving strains	χ2 (df = 1); P
Urease + sucrose-	110, 116, 357, 420, 447, 563, 635, 661, 743, 749, 860, 866, 888, 950, 1115, 1140, 1161, 1380, 1405, 1496, 3087	120, 714	16.7; <0.01
Urease- sucrose+		111, 118, 119, 279, 280, 281, 285, 299, 300, 443, 547, 554, 572, 1310, 1444, 3084, 3085	13.6; <0.01
Urease— sucrose—		363, 1376, 1378, 1387, 1392, 1399, 1402	6.8; <0.01
Urease+ sucrose+	658, 556	402	0.52; 0.48

3.2. Urease production

At 24 h, the three urease-negative strains produced a change in pH/mg protein of 39.1 ± 16.1 while the positive strains, excluding IBL 402 and IBL 714, produced a change in pH/mg protein of 617.8 ± 427.9 (a range of 211.2-975.1). IBL 402 and IBL 714, two of the three strains that gave positive responses for urease in the agar-dot assay but failed to survive five passages in gypsy moth, produced changes of 58.8 and 60.0 pH units/mg of protein, respectively. IBL 120, the third urease-positive strain that failed to survive multiple passages through larvae, produced a change in pH/mg protein of 607.2, which is essentially the average of the urease-positive group.

4. Discussion

At the present time, Bt is used as a biological insecticide. To be a true biological control agent, the bacterium would have to achieve a sustainable presence in the environment. One necessary component of sustainability is the capacity of the bacterium to replicate and sporulate effectively in the environment, preferably where the pest species will come in contact with it. Here we have shown that among 50 Bt isolates having 20 different phenotypes, only those producing urease activity had the ability to pass repeatedly from one gypsy moth larva to the next. Of the 26 strains positive for urease by the agar-dot assay, only three failed to survive five passages; two of these strains were subsequently shown to produce markedly less urease activity than other urease-positive strains. Although considerably more work would be required to establish that production of urease itself is required for Bt to complete their life cycle in lepidopteran larvae, the degree to which the two phenomena are correlated strongly suggests this.

The ability to produce acid from sucrose appears to be negatively correlated with the capacity to survive repeated passages in larvae; however, this trend is due to a skewed distribution of phenotypes in this study. This skewed distribution is present in our collection and is probably reflective of Bt populations in nature. Very few urease-positive isolates produce acid from sucrose. Among the three isolates we found that were toxic to Lepidoptera, produced bi-pyramidal crystals, and were positive for both urease and the production of acid from sucrose, two survived five passes in larvae. Eight isolates that were negative for both traits were tested; among these, none could pass repeatedly from one larva to the next. At the least, this suggests that absence of acid production from sucrose is not a sufficient condition for Bt to complete its life cycle in gypsy moth larvae.

Interestingly, plant ureases have been shown to have insecticidal properties and do share sequence similarities with bacterial ureases (Carlini and Polacco, 2008). However, the toxic effects of plant ureases appear restricted to insects utilizing cathepsins as their major gut proteases. They are not toxic to insect groups with alkaline guts, such as the Lepidoptera, where trypsin-like proteases predominate (Carlini et al., 1997). Likewise, the fact that the endotoxins of many urease-negative Bt strains are entirely adequate for killing gypsy moth larvae would seem to diminish the probability that urease toxicity plays any role the long-term passage of strains in insects.

A more likely role for urease in Bt is that of biodegradation once the host has been killed. Urease activity is common among soil microbes and is required for the breakdown of proteins and nucleic acids. Heermann and Fuchs (2008) note that the insect-associated bacteria *Photorhabdus luminescens* (Thomas and Poinar) Boemare and *Yersinia enterocolitica* (Schleifstein and Coleman) Fredriksen produce both insecticidal toxins and urease, and speculate that urease has adaptive value for proliferation in hemolymph. In *Y*. *enterocolitica*, also a pathogen of humans, expression of both the urease and the insecticidal toxin complex operons is induced by low-temperatures that may be associated with an insect-associated life stage (Bresolin et al., 2006; Heermann and Fuchs, 2008).

In our study, the urease-positive strains that survived five passages through gypsy moth larvae were by far the predominant species of bacteria isolated from dead larvae. While this may simply reflect success by these strains in utilizing the host insect, it is entirely possible that urease-positive Bt strains possess some advantage in competing with the enteric bacteria of the host insect. In recent work dealing with the interaction of Bt with the gut flora of the gypsy moth, Broderick et al. (2006) reported that Bt var. kurstaki (urease-positive) did not cause mortality when fed to larvae "cured" of their normal gut flora by antibiotic treatment. Their experiments suggest that Bt itself cannot cause a fatal septicemia, but permeabilizes the gut, allowing the insect's normal gut bacteria entry into the hemocoel. The gut bacteria then replicate and cause the septicemia. Superficially, this seems at odds with our results, but the two studies cannot really be compared. Broderick et al. (2006) focused on events leading up to death, while our study simply shows that Bt (at least the urease-positive strains) predominates in the larval cadaver 4-6 days after death. This area certainly deserves further investigation.

Genomic analyses performed on *Bacilli* related to Bt have shown that the occurrence of genes required for urease is variable (Hu et al., 2008). Urease genes are not present in Bacillus anthracis Cohn (Ames strain), B. thuringiensis konkukian (strain 97-27), or B. cereus ATCC 14579 but are present in B. cereus ATCC 10987 and the more distantly related Bacillus sphaericus Meyer and Neide (strain B3-41). Interestingly, B. sphaericus has been documented to germinate and grow in mosquito cadavers (Correa and Yousten, 1995). For B. cereus ATCC 10987, Mols and Abee (2008) have shown that urease production does not seem to confer better fitness to low pH environments, as Lee et al. (1993) demonstrated for Helicobacter pylori (Marshall) Goodwin, and speculated that the enzyme provides nitrogen to the bacteria in nitrogen-limited environments. The presence or absence of urease among Bt strains probably reflects adaptations to unrecognized environmental niches. According to deBarjac (1981), urease-positive Bt subspecies include the most commercially exploited biotypes, kurstaki and aizawai, as well as kenyae, thompsoni, galleriae and toumanoffi. Urease-negative subspecies include thuringiensis, finitimus, israelensis, sotto, dendrolimus, darmstadiensis and others. It should be noted that although B. thuringiensis subsp. israelensis does not produce urease activity (deBarjac, 1981) it has been shown to replicate in mosquitoes (Ali et al., 1989; Suzuki et al., 2004), although only short-term growth has been monitored.

Although we remain cautious about extrapolating our results to other species of insects, our observation that only urease producing strains are capable of sustaining levels of replication in gypsy moth may have significant ramifications for the development of more effective control agents. Urease production is a relatively uncommon trait among Bt strains. Of ca. 3500 well characterized isolates in our collection, 650 produce bi-pyramidal crystals and are toxic to *T. ni*; of these, only 129 are urease positive. Thus, the overall frequency of Lepidoptera-toxic isolates that might be expected to maintain sustainable replication levels in larvae is 3–4% of the characterized collection. Interestingly, of 216 urease-positive isolates tested, 59% were toxic to *T. ni*, compared with only 19% of tested isolates in the entire collection.

Bt has been used effectively as a biopesticide for decades. As Bt typically does not persist in the environment at levels adequate to control pests, repeated applications are often necessary. There may be systems, however, where repeated applications are not practical or economical. In such systems, a Bt strain that grows and sporulates in its host could provide longer term control with fewer applications. In order to improve Bt as a biological control agent, more emphasis should be placed on traits such as the presence of urease, that will allow the bacterium to persist in the environment.

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References

- Ali, A., Weaver, M., Costenmoyer, E., 1989. Effectiveness of *Bacillus thuringiensis* serovar israelensis (Vectobac 12 AS) and *Bacillus sphaericus* 2362 (ABG-6232) against *Culex* spp. Mosquitoes in a dairy lagoon in central Florida. Florida Entomologist 72, 585–591.
- Aly, C., 1985. Germination of Bacillus thuringiensis var. israelensis spores in the gut of Aedes larvae (Diptera: Culicidae). Journal of Invertebrate Pathology 45, 1–8.
- Atlas, R.M., 2004. Handbook of Microbiological Media. CRC Press, Boca Raton, FL. p. 887.
- Bell, R.A., Owens, C.D., Shapiro, M., Tardif, J.G.R., 1981. Development of mass rearing technology. In: Doane, C.C., McManus, M.L. (Eds.), The Gypsy Moth: Research Toward Integrated Pest Management. U.S. Department of Agriculture Technical Bulletin 1584, Washington, DC, pp. 599–633.
- Bresolin, G., Neuhaus, K., Scherer, S., Fuchs, T.M., 2006. Transcriptional analysis of long-term adaptation of *Yersinia enterocolitica* to low-temperature growth. Journal of Bacteriology 188, 2945–2958.
- Broderick, N.A., Raffa, K.F., Handelsman, J., 2006. Midgut bacteria required for Bacillus thuringiensis insecticidal activity. Proceedings of the National Academy of Sciences USA 103, 15196–15199.
- Carlini, C.R., Polacco, J.C., 2008. Toxic properties of urease. Crop Science 48, 1665– 1672.
- Carlini, C.R., Oliveira, A.E., Azambuja, P., Xavier-Filho, J., Wells, M.A., 1997. Biological effects of canatoxin in different insect models: evidence for a proteolytic activation of the toxin by insect cathepsin-like enzymes. Journal of Economic Entomology 90, 340–348.
- Correa, M., Yousten, A.A., 1995. Bacillus sphaericus spore germination and recycling in mosquito cadavers. Journal of Invertebrate Pathology 66, 76–81.

- Dangaard, P.H., 2000. Natural occurrence and dispersal of *Bacillus thuringiensis* in the environment. In: Charles, J.F., Delécluse, A., Nielsen-Le Roux, C. (Eds.). Kluwer Academic Publishers, Dorchecht, The Netherlands, pp. 23–40.
- deBarjac, H., 1981. Identification of H-serotypes of *Bacillus thuringiensis*. In: Burges, H.D. (Ed.), Microbial Control of Pests and Plant Diseases 1970–1980. Academic Press Inc., London, pp. 35–45.
- Faust, R.M., Bulla, L.A., 1982. Bacteria and their toxins as insecticides. In: Kurstak, E. (Ed.), Microbial and Viral Pesticides. Marcel Dekker Inc., New York, pp. 75–208.
- Heermann, R., Fuchs, T.M., 2008. Comparative analysis of the *Photorhabdus luminescens* and the *Yersinia enterocolitica* genomes: uncovering candidate genes involved in insect pathogenicity. BMC Genomics 9, 40.
- Hu, X., Fan, W., Han, B., Liu, H., Zheng, D., Li, Q., Dong, W., 2008. Bacterium Bacillus sphaericus C3-41 and comparison with those of closely related Bacillus species. Journal of Bacteriology 190, 2892–2902.
- Lee, A., Fox, J., Hazel, S., 1993. Pathogenicity of *Helicobacter pylori*: a perspective. Infection and Immunity 61, 1601–1610.
- Martin, P.A.W., 2004. A freeze-dried diet to test pathogens of Colorado potato beetle. Biological Control 29, 109–114.
- Martin, P.A.W., Haransky, E.B., Travers, R.S., Reichelderfer, C.F., 1985. Rapid biochemical testing of large numbers of *Bacillus thuringiensis* isolates using agar dots. BioTechniques 3, 386–392.
- Martin, P.A.W., Travers, R.S., 1989. Worldwide abundance and distribution of Bacillus thuringiensis isolates. Applied and Environmental Microbiology 55, 2437–2442.
- Mols, M., Abee, T., 2008. Role of ureolytic activity of *Bacillus cereus* in nitrogen metabolism and acid survival. Applied and Environmental Microbiology 47, 2370–2378.
- Parry, J.M., Turnball, P.C.B., Gibson, J.R., 1983. A Colour Atlas of *Bacillus* Species. Wolfe Medical Publications Ltd., London.
- Prasertphon, S., Areekul, P., Tanada, Y., 1973. Sporulation of *Bacillus thuringiensis* in host cadavers. Journal of Invertebrate Pathology 21, 205–207.
- Raymond, B., Elliot, S.L., Ellis, R.J., 2008. Quantifying the reproduction of *Bacillus thuringiensis* HD1 in cadavers and live larvae of *Plutella xylostella*. Journal of Invertebrate Pathology 98, 307–313.
- SAS Institute Inc., 2008. SAS OnlineDoc. version 9. SAS Institute Inc., Cary, NC..
- Suzuki, M.T., Lereclus, D., Arantes, O.M.N., 2004. Fate of *Bacillus thuringiensis* strains in different insect larvae. Canadian Journal of Microbiology 50, 973– 975.
- Travers, R.S., Martin, P.A.W., Reichelderfer, C.F., 1987. Selective process for efficient isolation of soil *Bacillus* spp.. Applied and Environmental Microbiology 53, 1263–1266.