CHITINASE PRODUCING BT STRAINS

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Screening of 402 strains of more than 18 varieties of *Bacillus thuringiensis* showed chitinase to be inducible in virtually every serovar tested. Though the chitinase titre varied among strains, there was a strong correlation between enhanced lethality to spruce budworm, *Choristoneura fumiferana* (Clemens), and an increase in chitinase titre within the serovar. Strains grown in chitinase inducing medium gave a more rapid and higher kill than those grown in sporulation medium, and a faster kill than the currently used commercial strain.

Introduction

The sustained and growing assault on vast areas of spruce, balsam and Douglas fir forests by *Choristoneura fumiferana* (Clemens), the Spruce Budworm (SBW), has occasioned a variety of treatment regimens. Among these, and still representing an area of controversy, is the application of chitinase as an adjuvant in *Bacillus thuringiensis (B.t.)* treatments (Smirnoff 1974, 1977; Morris 1976). Indeed, little clear-cut evidence is available, at this time, on which to base a definitive opinion. Work in our laboratory (Dubois 1977; Daoust 1978) has demonstrated that the presence of microorganisms originally isolated from their insect host, induced in growth medium for chitinase production, and subsequently applied to leaf tissue, is associated with lesions produced in the peritrophic membrane of larvae which have ingested these chitinase active microorganisms. Ultimately, death occurs as a result of the generalized septicemia which follows spore and cell invasion through these perforations. Accordingly, it was decided to search among the various strains of *B.t.* for chitinase active cultivars. The underlying hypothesis was that such chitinase active organisms could, in hydrolyzing gut membranes, mediate spore entry and activity and, augmented by the activity of the crystal toxin on the epithelial cells of the gut, prove more lethal than non-chitinase active strains.

In the report which follows, we survey the incidence of chitinase activity in a wide range of *B.t.* crystal and serotypes, and indicate the relationship between chitinase activity, crystal type and lethal effectiveness to SBW.

Methods and Materials

Bacillus thuringiensis

Cultures of *Bacillus thuringiensis*, used in this study, were obtained from Dr. N. R. Dubois of the Center for Biological Control of the Northeastern Forest Experiment Station, U.S. Forest Service, Hamden, CT. These were maintained on nutrient agar slants and stored at 4^0C until needed. The various *B.t.* cultures were tested for chitinolytic activity by streaking on petri plates containing a mineral salts medium as described by Dubois (1977) in which 0.1% purified chitin served as the sole source of carbon. Replicate plates containing 0.2% casamino acids as well as chitin, were streaked with *B.t.* to assess the effects of this enrichment on the growth of the organism. Chitinolytic activity was estimated from the degree of clearing around each colony at 4, 6 and 12 days after inoculation.

Strains of *B.t.* were tested quantitatively for chitinase production colorimetrically. Chitinase titers were measured by the addition of 0.5 ml of 0.1M citrate buffer (pH 5.0) and 0.5 ml purified colloidal chitin (25 mg/ml) to 0.5 ml of supernatant from each test culture. The supernatant was obtained by centrifuging the whole culture for 15 min at 10,000 rpm in an RC-2 Sorvall refrigerated centrifuge. Chitin, one batch used throughout, was purified as described by Daoust (1978); broth culture techniques are described below.

The assay mixtures were incubated for one hour at 50^0C in a water bath. This temperature favors the action of the enzyme complex which attacks the chitin polymer, as opposed to those chitinase components which hydrolyze oligomer or dimer units (Weir 1978). Controls contained 0.5 ml distilled water instead of culture supernatant. Colorimetric determination of N-Acetyl D-glucosamine (NAG) levels, as a measure of chitinase titers, were made using a B & L Spectronic 21 colorimeter at 585 nm according to the method of Reissig *et al* (1955).

Cultures showing strong chitinolytic activity were selected for further testing in bioassays of SBW mortality. They were cultured in quantity using two media, one medium designed to amplify production of spores and crystals, and the other selected to enhance chitinase production while still permitting sporulation and crystal formation.

When maximal spore and crystal production was desired, flasks were prepared containing 100 ml of *B.t.* sporulation medium as described by Dubois (1968). Inocula were obtained from 24 hour cultures grown in trypticase soy broth (TSB), transferred again to TSB for 4-6 hours to obtain log phase growth and added as 1% of the volume of the medium in the sporulation growth flasks. These flasks were incubated at 27^0C for 3 to 5 days on a shaker table. Cultures were then examined microscopically for the presence of spores and crystals and only cultures which contained both were used in bioassays. Acceptable levels were considered to be 90% spores and crystals as compared to vegetative cells, and a 1:1 ratio of spores to crystals was virtually always found.

B.t. cultures were also grown in a chitin based liquid medium designed to increase the chitinolytic activity of the bacteria. It contained the mineral salts trace elements present in the solid medium used in the agar plates, and 3% crude chitin as the sole source of carbon.

The chitin was obtained from Pfaltz and Bauer, Stamford, CT. A single batch of crude chitin from this source sufficed for all experiments described in this report. The crude chitin was boiled 10 min in distilled water, followed by 5-6 distilled water rinses. After each rinse, the chitin was allowed to settle and the water was decanted by suction. The cleaned chitin was dried in an oven at 30°C, and further ground for approximately 20 minutes (2-3 min pulses) in a blender.

TSB tubes (5 ml) were inoculated from slant cultures and incubated 20-24 hours at 25°C, after which 1 ml from the tubes was inoculated into 99 ml of mineral salts, 3.0% chitin broth in 250 ml flasks. The flasks were incubated at 27°C in the dark on a rotary shaker (115 oscillation/min).

Spruce Budworm

Cultures of *B.t.* were subsequently tested for virulence on SBW larvae which were reared as follows: hibernacula of SBW were obtained from the USDA Forest Service in Hamden, CT. and kept refrigerated. As needed, squares (approx. 1 cm^2) of cheesecloth containing the hibernacula were cut and placed on the underside of the lids of 1 oz plastic cups. The cups were filled approximately 1 cm high with "Bio-Serv" SBW diet medium containing aureomycin (Bio-Serv, Inc., Frenchtown, N. J.). The medium, its water and agar components sterilized to reduce contamination, was poured at about 120°F, and allowed to solidify within the cups.

Cups were incubated at 25°C (approx. 70% relative humidity) in 24 hours of light a day for approximately 3 weeks, or until larvae had attained fourth instar. Cups were incubated in an inverted position to take advantage of the larvae's photopositive behavior and to reduce mold infection in the cups. Larvae were transferred to new cups if they became overcrowded, if the diet medium was depleted or dried out, or if mold began to grow on the medium. Larvae were discarded if they showed any signs of infection.

The eclosure of SBW larvae is not synchronous, and in fact may span a week. Consequently, the growth of the larger larvae in a group occasionally was slowed down by sequestering them in a 12°C incubator, permitting smaller larvae to catch up in size. Larvae in the three penultimate instars were used in bioassays. These larger larvae were separated into empty cups and starved for 24 hours at 12°C prior to each bioassay. No more than 5 larvae were placed in a cup to reduce cannibalism.

Larvae were then sorted for size and instar, and transferred to replicate groups of 7 comparable individuals into prepared bioassay dishes. The bioassay dish consisted of an 8.5 cm plastic petri dish, floored with a piece of filter paper.

A small sprig of balsam fir was placed in each dish. Each sprig contained about 30 needles, and had a branch stem approximately 4 cm in length. In most experiments, the treatment to which the SBW larvae were subjected was applied on the surface of this balsam sprig.

For each treatment, balsam fir sprigs were washed in tap water, then dried at room temperature. Sprigs were next dipped into an aliquot of whole culture in beakers (usually 6 ml in a 25-50 ml beaker) followed by air drying for approximately one half hour. Triton X-100, a surfactant, was added at 0.1% to all treatments to enhance leaf wetting and adhesion by active agents.

Seven SBW larvae were added to each bioassay dish after a suitably prepared fir sprig was in place. They were allowed to feed (or not) on the sprig for 48 hours after which an untreated sprig replaced the treated one as a food source. Consumed sprigs were replaced as needed. Five to ten replicates of each dish were used, and controls dipped in sporulation medium or chitin broth were maintained. For most experiments, the number of viable bacteria (cells and spores), in the cultures employed was determined by the "drop plate technique" of Reed and Reed (1948). Five replicates of 0.01 ml from dilutions of cultures (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}) were delivered with a 0.1 ml Mohr pipet onto the surface of TSA plates. Plates were allowed to dry and were incubated in an inverted position at 27°C for 20-24 hours. Bacterial counts were made using a Bausch and Lomb dissection microscope, and replicates were averaged.

For each experiment, specific details of numbers, replicates and variations in technique are presented in the results together with the data deriving from that experiment.

Results

As will be noted from Table 1, chitinase production is a characteristic widely distributed among the varieties of *B. thuringiensis* tested. Indeed few varieties were without a chitinolytic strain, and their absence in varieties of *B.t. sotto* and *subtoxicus* may simply reflect the minimal number of strains available for testing. Although the chitinolytic propensity seemed to cluster within specific crystal or serovar types (e.g. *kurstaki, entomocidus* or *darmstadiensis*) it was not by itself a predicator of lethal potential.

On the other hand, there was a clear correlation between chitinase titre and lethality (Figs. 1,2). In Fig. 1 are shown results obtained when 8 cultivars of the *kurstaki* variety (H3a3b) were tested for effectiveness against spruce budworm. Mortality corresponded very clearly with concentration of chitinase generated. In Fig. 2 are shown results achieved with repeated trials of the cultivar HD-110 (*B.t.* var. *entomocidus*) harvested at different periods under the same conditions and yielding different amounts of chitinase. In this case too, there is an unequivocal relationship between the amount of chitinase present and mortality.

Table 1. The production of chitinase by selected taxonomic serovars of *Bacillus thuringiensis*[1]

Varietal epithet	Serovar	Degree of Plate Clearing ++	+	+/-	0	Totals
kurstaki	H3a,3b	5	44	8	14	71
thuringiensis	H1	3	25	4	39	71
finitimus	H2	4	0	1	3	8
alesti	H3a	1	16	3	4	24
kenyae	H4a,4c	0	5	4	8	17
sotto	H4a,4b	0	0	0	1	1
dendrolimus	H4a,4b	6	1	2	1	10
galleriae	H5a,5b	1	43	12	19	65
entomocidus	H6	2	0	0	3	5
subtoxicus	H6	0	0	0	2	2
aizawai	H7	1	37	6	2	47
morrisoni	H8a,8b	0	5	3	6	14
tolworthi	H9	1	4	0	1	6
canadensis	H5a,5c	1	2	0	1	4
darmstadiensis	H10	6	4	0	3	13
toumanoffi	H11a,11b	0	1	1	0	2
israeliensis	H14	1	5	0	3	9
other	-	2	2	1	2	7
unidentified	-	3	8	0	6	17

[1] The degree of chitin-agar plate clearing was defined as follows: 0, no clear-zone visible in the agar surrounding colonies; +/-, a thin cleared ring around colonies just visible to the naked eye; +, a cleared ring around colonies about 1 mm wide; and ++, a cleared ring of 2 mm or more visible around the colonies on plate.

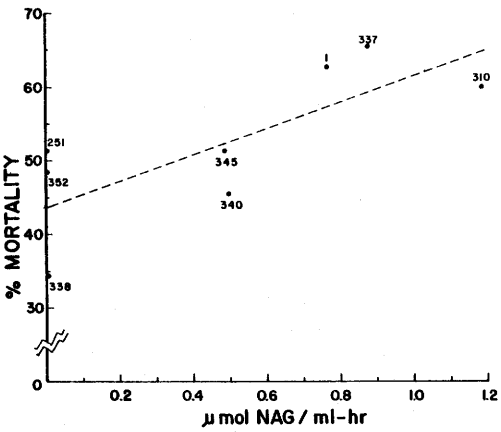

Fig. 1. Spruce Budworm mortality produced by 8 strains of *B.t.* var. *kurstaki* of varying chitinase titre. The strains were selected from one variety (serovar and crystal types, H3a,b and k-1, respectively) to minimize the effects of variables other than chitinase titre. All cultures were grown for 16 days in chitin broth medium. The initial number of larvae tested for each group was 35. Control mortality (not shown) was 5.7%. The correlation coefficient obtained for these points was r = 0.75; with 6 df, this gave 95% confidence limits by t-test. The number by each point is its HD number: HD-1 is the standard commercial strain.

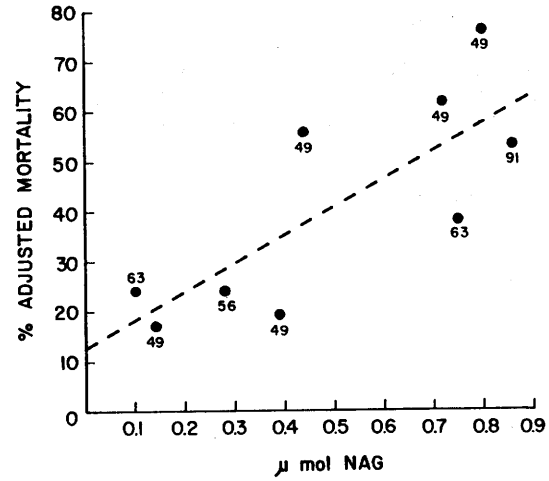

Fig. 2. The relationship of chitinase production in cultures of *B.t.* HD-110 (var. *entomocidus*) to mortality in spruce budworm.

The data points were calculated by means of Abbott's formula (1925) on mortality figures in each experiment to yield the "adjusted mortality" shown. The number by each data point gives the n for each experiment, the initial number of larvae in each treatment (and control) group. For these data, r = 0.785, significant at the 97% level of confidence by t-test with df = 7.

If one compares the overall efficacy of chitinase producing cultivars to that of the HD-1 strain currently in commercial use, the significant trend which emerges is the greater immediacy of kill achieved even though after 5 days the ultimate kill may be equivalent. It is clear (Fig. 3) that the presence of chitinase accelerates the general lethal action of *B.t.*

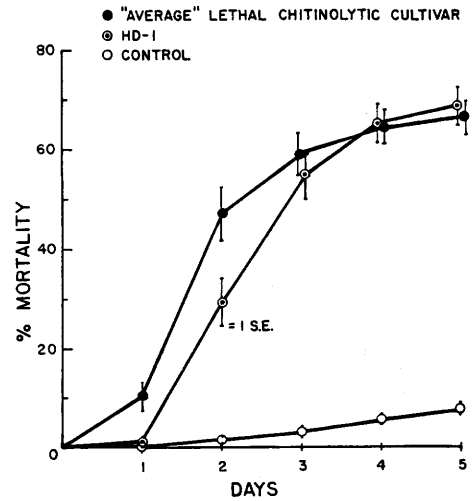

Fig. 3. Rate of kill of chitin grown *B.t.* strains compared with that of the commercial strain HD-1.

A composite of 9 separate experiments. The chitinolytic cultivars tested were HD's 7, 37, 48, 124a, 234, 287, 319, 340 and 539. Initial numbers per treatment group in each experiment ranged from 49 to 119, but each experiment was weighted equally to produce the average points

shown in the graph. The points for HD-1 and the controls are obtained from the same 9 experiments, and had the same initial n per group as their respective chitinolytic treatment groups.

The "chitinolytic cultivars" group results tested against that of HD-1 by ANOVA proved significantly different at the 0.05 level ($F = 4.92$, $df_1/df_2 = 1/80$). As the HD-1 actually produced higher ultimate mortality on day 5, this difference is plainly due to the distinctly higher mortality which the chitinolytic cultivars produced at the onset of the treatment exposure period.

That these effects are conditioned by the induction of chitinase is confirmed by the data in Table 2 and Fig. 4. The mortality achieved by cultivars grown in sporulation medium, i.e. in the absence of chitinase, is consistently and significantly lower than that achieved by the same cultures grown in a chitin medium for the induction of chitinase. As might be anticipated this, of course, did not hold true where the culture lacked the potential for inducible chitinases in significant amounts, the threshold level of available chitinase appearing at or above 0.6 µmol of NAG per ml-hour (see Table 2).

Table 2. A comparison of *B.t.* the lethality to spruce budworm of *B.t.* cultures grown in sporulation and chitin broth media, respectively.

Varietal	% mortality on 5th day: Sporulation medium	Chitin medium	Chitinase titre
HD-1	80.0	43.0	0.53[1]
HD-37	28.5	51.8	0.93
HD-48	44.8	44.8	2.04[2]
HD-110	32.6	36.7	1.15[2]
HD-124a	20.6	23.8	1.5
HD-198	63.2	36.7	0.33[2]
HD-198	45.6	47.3	0.4[3]
HD-224	21.4	42.8	0.68
HD-226	37.4	58.7	0.96[3]
HD-232	40.2	42.8	5.5[4]
HD-234	46.4	66.0	1.27
HD-287	41.0	71.4	1.4
HD-323	58.4	61.4	0.75[3]
HD-498	44.1	62.7	1.62[3]
HD-500	22.0	19.0	0.1
HD-539	51.7	46.4	0.6[5]

[1] = µMol NAG/hr/ml. Usually HD-1 grown in chitin medium killed over 60% by day 5 (see Fig 1).

[2] = in this experiment, control mortality = 22.8% on day 5 (over 2x normal).

[3] = sporulation culture tested alone. Chitin results are mean data from other experiments.

[4] = reading on chitinase probably aberrant.

[5] = in 4 other experiments, chitin cultures of HD-539 averaged 71.9% mortality and averaged over 1 in chitinase titre.

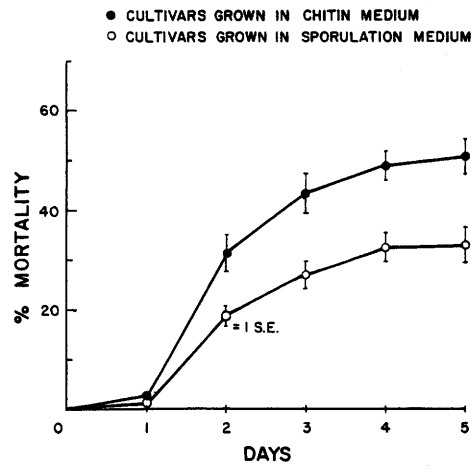

Fig. 4. Differential mortality to SBW achieved by cultures grown in chitin medium and sporulation medium.

This graph is a composite drawn from the data in Table 2. Cultivars HD-1, 198 and 500 have been omitted because of their low chitinase production, below 0.6 µmol NAG. The differences seen in HD-1 on Table 2 are extreme: see figs 1 and 3 for more usual values.

A further confirmation of the potential of chitinase active strains is shown in Fig. 5. In this instance, *B.t.* var. *galleriae* HD-287 (crystal type k-1) is consistently more lethal when induced for chitinase than commercial strain HD-1 or when grown in conventional sporulation medium. Again, as shown in Fig. 4, the significance of these results lies not only in the ultimate percentage kill achieved, but in the more rapid inception of kill and the minimizing of foliar damage this implies.

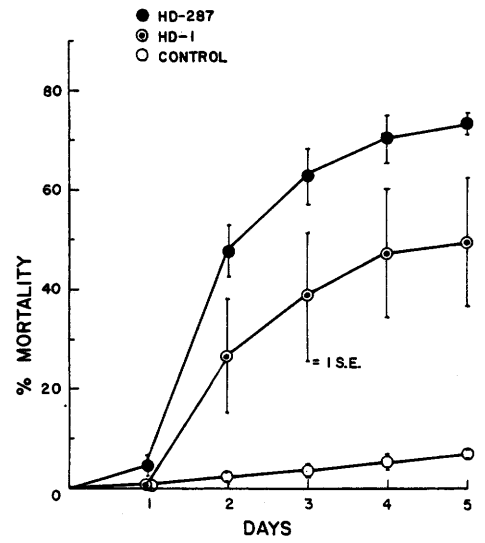

Fig. 5. Mortality to spruce budworm induced by *B.t.* HD-287, var. *galleriae* (crystal type k-1), and strain HD-1.

Composite data for 9 experiments. Although n, the initial number of larvae in each experiment

treatment group, varied from experiment to experiment (range = 42 to 70) data from separate experiments were averaged equally; it was felt that variation in mortality was due to larval batch tested and not random independent factors.

Data for HD-1 was drawn from only 4 experiments. In other cases, final mortality fell below 20% and was discarded as unrepresentative (see Fig. 3 for other HD-1 data).

This extreme variability in HD-1 lethality, as reflected in the S.E. shown here, is a common problem with this strain (N. Dubois, pers. comm.).

For 3 experiments (run as part of those above) *B.t.* HD-287 was tested as grown in sporulation medium. On day 5, mortality was $\bar{x}$ = 50.6% $\pm$ 6.84 (= S.E.).

Discussion

In contrast to the findings of Smirnoff and Valero (1977), the capacity for chitinase production would appear to be widespread and not confined to a varietal type. Within each varietal type there is, to be sure, a wide range of chitinolytic activity. For the *kurstaki* variety on which we report, the degree of enhanced lethality appears directly related to the titre of active chitinase. Certainly these results invite investigation of the relationship between chitinase and lethality within other varietal groups.

Although the phenomenon of chitinase enhanced lethality by *B.t.* to SBW seems unequivocal from the foregoing, further work is clearly required to elucidate the precise, operative mechanism. On the basis of the work reported by Dubois (1977), one may infer that there is an erosion of the peritrophic membrane. This facilitates entrance of spores as well as adventitious microflora into the larval haemocoel resulting in a broader and more diffuse disruption of various physiological and metabolic processes. Death occurs more rapidly in a population rendered more susceptible by these plural effects. A number of issues, however, remain speculative at this time. There is a discomfiting variability in the chitinase yield generated from the same culture grown under presumably identical conditions. This might suggest the presence of a catabolite repressor analagous to the cellulase system and a feedback mechanism that may inhibit the sustained production of chitinase. Additionally, it should be mentioned that the production of chitinase and the dissolution of the parasporal crystal and subsequent release of the toxic fraction represent antithetic pH demands: the pH optimum for chitinase lies at 5.5 to 5.8; for crystal dissolution above about 8. It is therefore remarkable that sites are present in the insect gut where both these agents may express themselves. However, there does appear to be a marked potential for chitinase activity at the peritrophic membrane of the Douglas fir tussock moth, *Orgyia pseudotsugata*. Studies by Brandt *et al* (1978) report that both chitinase and protease degraded the peritrophic membrane, releasing products of hydrolysis and effecting structural changes in the membrane. Although work in our laboratory showed that many strains of *B.t.* such as HD-287 do produce proteases, no clear-cut relationship between protease titres and mortality could be demonstrated.

A final question around which there has been much speculation has been our sustained inability to achieve any consistent enhancement of *B.t.* virulence with commercial chitinase. It might have been presumed that the lytic effect generated by this enzyme could have provided an additive measure of lethality in the same way provided by the chitinase induced in the chitinase active *B.t.* strains. Two explanations offer themselves for this failure: 1. the chitinase must be of a specific character capable of catalyzing the depolymerization of the chitin in the SBW gut; or 2. that the enzyme is operative only when membrane bound and responds to the presence of an additional factor provided by this linkage. The role of vegetative cells, always present in small numbers in treatment aliquots is also unclear. Though the event of increased lethality as a consequence of the presence of microbial chitinase seems certain enough, the precise details of its nature await further clarification.

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