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POSSIBLE ORIGIN AND FUNCTION OF THE PARASPORAL CRYSTALS IN BACILLUS THURINGIENSIS

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Summary: Acrystalliferous strains of Bacillus thuringiensis subsp. kurstaki were isolated at a high frequency following heat treatment of spores. Spores of these strains lacked a 130,000 dalton glycoprotein, the major component common to both parasporal crystals and coats and were nontoxic to tobacco hornworm larvae. Moreover, the deficiency of this glycoprotein resulted in lysozyme sensitivity of the spores of some mutants and the presence of new spore coat proteins in others. All nontoxic acrystalliferous mutants lacked the complete array of at least six plasmids present in the wild type, implying a relationship between presence of plasmid(s) and toxicity. The unique capacity of this species to alter the surface coating of spores which appears to be related to crystal formation may provide flexibility for germination and growth in diverse soil environments.

A variety of strains of Bacillus thuringiensis form a crystalline inclusion adjacent to the spore during post-exponential cellular development (1-4). In B. thuringiensis subsp. kurstaki, the inclusion is a glycoprotein that represents 20-30 percent of the cell dry weight and is comprised of a single repeating subunit whose molecular weight is approximately 1.3×10^5 (5,6). When ingested by lepidopteran larvae, the crystal dissolves and the protoxic subunit is converted to one or more toxic components (7).

An intriguing relationship exists between the spore and crystalline inclusion because the spore coat is comprised primarily of the same glycoprotein subunit found in the crystal (8). This component, when extracted from the spore surface, exhibits the same lethality as soluble crystal fed to tobacco hornworm larvae (9). To further study the origin of the crystalline glycoprotein and its presumed dual function as a protoxin and protective spore coating, we have isolated a variety of acrystalliferous mutants and analyzed

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for (i) presence of the protoxic glycoprotein, (ii) toxicity to tobacco hornworm larvae, (iii) extrachromosomal DNA, and (iv) spore germination properties.

MATERIALS AND METHODS

For production of spores and isolation of mutants, B. thuringiensis subsp. kurstaki was grown in liquid yeast extract-glucose medium (G Tris, 10) at 30 C on a rotary shaker (300 rpm). Mutants resistant to cycloserine were selected by first incubating exponentially growing cells in the presence of cycloserine (50 µg/ml) for 3-4 hr and then spreading 10^7-10^8 cells on G Tris agar containing cycloserine (50 µg/ml). The cycloserine-resistant strain was checked for crystalline production by phase-contrast microscopy and SDS polyacrylamide gel electrophoresis(11) and toxicity to tobacco hornworm larvae (9, 12). Resistance to 20 µg/ml tetracycline was then introduced into the cycloserine-resistant strain in the same manner as described above.

Most of the acrystalliferous mutants were isolated from spores that had been heated at 80 C for 40 min or at 80 C for two successive 20-min intervals with a 20 min cooling interval. The heat-shocked spores were spread on G Tris agar and incubated at 30 C for 48 hr until colonies consisted almost exclusively of spores. Lysozyme-sensitive spore mutants (S_1 in Table 1) were detected by pipetting 1.5 ml of a solution containing 100 µg of lysozyme (EC 3.2.1.17) per ml of 0.03M tris (hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.8) onto the G Tris agar plates and incubating them at 27 C for one hr. Colonies containing lysozyme-sensitive spores turned dark (13). Some acrystalliferous mutants (cry-B in Table 1) were selected at random. Occasionally, a slight difference in colony morphology was exploited but usually these mutants occurred with sufficient frequency (1 per 500-1000 colonies), after heat shocking the spores, to permit screening by phase-contrast microscopy. The presence or absence of protoxic glycoprotein was determined by electrophoresis of spore coat extracts in polyacrylamide containing 0.1% SDS and by insect bioassay (9,12).

A third class of mutants, R_6, was derived from the lysozyme-sensitive strain, S_1. It had been noted earlier that the parental strain germinated very slowly (3-4 times slower than germination of B. cereus spores) in response to germinants that are optimal for B. cereus (14). Because B. thuringiensis is thought to be closely related to B. cereus on the basis of cell and spore morphology as well as limited nutritional data (15), response to germinants similar to that of B. cereus was anticipated. Although a rather wide variety of germinants was tested, none produced as rapid a germination response as in B. cereus. The two acrystalliferous mutants, S_1 and cry B, also germinated as slowly as the parental strain. Slow germination by S_1 was expected because all lysozyme-sensitive mutants (coat defective) of B. cereus germinate slowly (16).

We selected rapid germinators of B. thuringiensis S_1 by heat shocking spores, treating them with 10mM L-alanine plus 1mM inosine for 5 min and then layering the spores on 30-60% Renografin (from 76% stock solution consisting of 66% meglumine diatrizoate and 10% sodium diatrizoate) gradients (17) and centrifuging them in an SW41 rotor at 23,000 rpm for 30 min. The region of the gradient that contained phase-dark, germinated spores was removed and inoculated into liquid G Tris medium containing cycloserine (50 µg/ml). Spores produced in this medium again were heat shocked, plated on G Tris agar, and rapidly appearing colonies were selected. Germination studies of spores and electrophoresis of crystal or spore extracts in SDS polyacrylamide gels were done as previously described (5, 14).

Table 1. Properties of acrystalliferous strains of B. thuringiensis subsp. kurstaki.

Strain(1)	Phenotype(2)	Germination time (min)(3)	Toxicity (% mortality)(4)	Major extractable spore coat polypeptides(5)	Number of plasmids by size (6)
parental	$cyclo^R$, lys^R, cry^+	14-16	100	ca. 130,000 daltons	6
S_1	$cyclo^R$, lys^S, cry^-	14-16	0	none	0
cry^-B	$cyclo^R$, lys^R, cry^-	14-16	0	none	0
R_6	$cyclo^R$, lys^R, cry^-	6-8	0	13,000 and 26,000 daltons	0

(1) Either cycloserine-resistant or cycloserine plus tetracycline resistant strains were used. These antibiotic resistant strains produced crystals and were as toxic as the parental strain.

(2) $Cyclo^R$: resistance to cycloserine; lys^R or lys^S: resistance or sensitivity of spores to lysozyme (E.C. 3.2.1.17). Lysozyme treated spores (see Materials and Methods) were examined by phase-contrast microscopy. Less than 10% of the resistant spore population turned phase-dark whereas >60% of the sensitive spores became phase-dark. cry^+ or cry^-: presence or absence of parasporal crystal based on visual examination with phase-contrast microscopy, presence or absence of crystal band in Renografin gradients (24), and presence or absence of 130,000-dalton protein in SDS polyacrylamide gels.

(3) Measured as previously described (14) following heat activation and addition of 10mM L-alanine plus 1mM inosine. Values are times of decrease of A_{650}nm values to 50% of final plateau. These values correlate well with the fraction of spores that have turned dark in the phase microscope. Value for B. cereus is 5-6 min.

(4) Toxicity was based on survival of neonate tobacco hornworm larvae exposed to diet surface treated with either crystal or spore extract (9, 12). A mortality count was made 1-7 days after exposure of the larvae to the treated surface. Dead larvae were characterized according to instar or stage of growth. Mean 50% lethal dose value (LD_{50}) for spores against the neonate larvae was 7.4×10^{-8} g/cm^2.

(5) Spores were extracted twice with 8M urea-0.07M dithioerythritol-1% (w/v)SDS-0.005M cyclohexylaminoethane (Ches), pH 9.6, at 37 C for 90 min. Extracts were electrophoresed in SDS polyacrylamide gels as previously described (5, 14).

(6) Plasmids were isolated, fractionated, and examined as previously described (18-20).

Plasmids were isolated in cesium chloride-ethidium bromide gradients (18) and examined in the electron microscope as previously described (19). The plasmids were additionally fractionated by electrophoresis in 0.8% agarose gels containing Tris-sodium borate buffer, pH 8.2 (20).

RESULTS

Three different classes of acrystalliferous mutants were analyzed (Table 1). They all retained the antibiotic resistance properties of the parental strain (similar mutants have been isolated from the cycloserine plus tetracycline-resistant strains). Mutant S_1 produced lysozyme-sensitive spores, lacked a discrete crystal, was devoid of the 130,000-dalton polypeptide subunit (5,6) as determined by polyacrylamide gel electrophoresis, and was not toxic to tobacco hornworm larvae. There was very little extractable protein on these spores (10-20% of that found in the parental strain) and there were no prominent bands seen after electrophoresis of extracts in polyacrylamide gels.

The phenotype of cry$^-$B mutants was similar except that its spores were resistant to lysozyme and contained only 25% as much extractable protein as the parental strain. Again, there were no major protein bands seen in polyacrylamide gels.

The third class, represented by R_6 in Table 1, was selected from S_1 on the basis of rapid germination of spores. This mutant still lacked a crystal, was nontoxic, but produced lysozyme-resistant spores. There were two prominent proteins present in spore extracts with molecular weights of approximately 13,000 and 26,000 daltons. The spore coat protein profile in polyacrylamide gels was virtually identical to that of *B.cereus* (16). Several such revertants have been independently isolated and they all exhibit the resistance to cycloserine or to cycloserine plus tetracycline as in the parental strains. Furthermore, these strains grow well in a minimal medium designed for *B. thuringiensis* (21) but poorly in one formulated for *B. cereus* (22). The major difference in the two media is the lower concentration of glutamic acid in the latter.

Similar differences were found in two strains of B. thuringiensis subsp. alesti, one a crystal former and the other an acrystalliferous derivative (kindly provided by Dr. P. C. Fitz-James, University of Western Ontario, Ontario, Canada). The latter strain germinated rapidly and contained spore coat proteins similar to B. cereus whereas the coat protein profile of the crystal forming parental strain was the same as that of B. thuringiensis subsp. kurstaki.

The very high frequency at which acrystalliferous mutants appeared suggested involvement of an unstable genetic element such as a plasmid. No revertants to crystal formation were obtained either by selecting for fast germinators (R_6 in Table 1) or for lysozyme-resistant revertants of strain S_1. The latter were obtained by killing lysozyme-sensitive spores with heat (80 C for 20 min). After two cycles of lysozyme and heat treatment, no crystal producers were found ($< 1/10^6$).

There were at least six plasmids in the parental strain distinguishable by contour analysis (Table 2). Obviously, some of the size classes may represent multimers. Any highly twisted molecules were not measured, causing the analysis to be biased for smaller molecules. Consequently, the relative number of molecules in each size class is an approximation. The three acrystalliferous mutants listed in Table 1 lacked all six plasmids. In one series of experiments, the cells were steady-state labeled with [^{3}H] thymidine, DNA was extracted and fractionated by centrifugation in cesium chloride-ethidium bromide gradients. Radioactivity in the region of plasmid DNA was negligible i.e. <0.5% of the wild type value. When wild type and mutant cells were mixed prior to lysis, there was no dimunition of plasmid bands as evidenced by agarose gel electrophoresis, i.e. mutant extracts did not cause aggregation or degradation of plasmids.

DISCUSSION

The high frequency of non-crystal forming strains and the correlation with absence of plasmids implies an involvement of the latter in crystal

Table 2. Frequency distribution by number and weight of the extrachromosomal DNA molecules in B. thuringiensis subsp. kurstaki.[1]

Size class	No. of molecules	Average contour length ± S.D.(μm)	% S.D.	Mol. wt (daltons)	% of total molecules (by number)	% of total circular DNA (by weight)
I	190	0.66± 0.03	4.7	1.32×10^6	35.7	8.0
II	84	2.43± 0.10[2]	4.0	4.90×10^6	15.8	13.2
III	200	2.70± 0.10[2]	3.7	5.45×10^6	37.6	34.9
IV	24	4.77± 0.18	3.7	9.64×10^6	4.5	7.4
V	27	14.90± 0.76	5.1	30.10×10^6	5.1	26.0
VI	7	23.33± 1.19	5.1	47.13×10^6	1.3	10.5
Total	532			Total	100	Total 100

(1) Isolated and characterized as previously described (18-20).

(2) Overlapping contour lengths but separable by agarose gel electrophoresis.

production. One or more of these plasmids, or even a portion of one, could code for or regulate the production of the protoxic glycoprotein. It is puzzling that all plasmids are always lost in the mutants. This phenomenon could be related to the methods used for enriching for mutants and may result in heat inactivation of a specific protein needed for replication of all the plasmids. Alternatively, all of the plasmids may be closely related in base sequence and, therefore, may have a common origin or a common replication site that was affected by heat.

The capacity of some of the acrystalliferous strains to germinate rapidly and produce spore coats similar to B. cereus is intriguing. Possibly, crystal production affects spore coat formation. Loss of crystal synthesis generally results in spores that are deficient in the major coat proteins and that germinate slowly (S_1 and cry$^-$B of Table 1). A second mutation may be required to obtain fast germinators such as R6, since a well defined B. cereus type coat may be required in order to respond to germinants. It may be possible, however, to select fast germinators directly from the parental strain and obtain B. cereus-like spores in a single step, perhaps as in the case of B. thuringiensis subsp. alesti.

The capacity of B. thuringiensis to produce either rapidly or slowly germinating spores in the absence or presence of a crystal may have adaptive significance. The protoxin provides the organism a capacity to invade insect larval hemolymph, a rather unique environment suitable for spore germination and vegetative growth (23). In such a niche, rapid germination would not be essential because there is little competition from other species. Without a crystal, however, survival outside an insect in a more competitive soil environment may be dependent on the ability of B. thuringiensis spores to respond more rapidly to germinants.

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