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Genetic Basis for Alkaline Activation of Germination in *Bacillus thuringiensis* subsp. *israelensis*▽

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Differences in activation between spores from strains of *Bacillus thuringiensis* subsp. *israelensis* with and without the toxin-encoding plasmid pBtoxis are demonstrated. Following alkaline activation, the strain bearing pBtoxis shows a significantly greater germination rate. Expression of just three genes constituting a previously identified, putative *ger* operon from this plasmid is sufficient to produce the same phenotype and characterizes this operon as a genetic determinant of alkaline activation.

Bacillus thuringiensis is a member of the *Bacillus cereus* sensu lato complex of bacilli and is distinguished by its ability to produce parasporal crystalline inclusions that may be toxic to invertebrates, usually insects (13). Spores and crystals are ingested by susceptible larvae, and the constituent Cry and/or Cyt crystal proteins are solubilized and proteolytically processed in the gut to release the active toxins (7). *B. thuringiensis* spores are able to germinate in the intoxicated larva and exploit the rich nutrient resources of the insect cadaver. Germination is triggered in response to germinants, usually nutrients that signal favorable conditions for growth (15). Like other *B. cereus* group bacteria, *B. thuringiensis* will germinate in response to nonspecific germinants, such as nutrient broth, and to specific germinants including L-alanine or ribonucleosides, such as inosine, and the process can be visualized by a loss of phase brightness (2).

In the laboratory, spores can be "activated" (induced to relatively synchronous germination) by conditions such as brief sublethal heat treatment or preincubation in an alkaline environment. The latter treatment is used most often in laboratories studying *B. thuringiensis* since such a treatment is more physiologically relevant than heat activation because most insects have alkaline guts in which the pH stimulates germination (18). Despite this interesting and relevant phenomenon, the genetic basis for alkaline activation has not been elucidated, although an association between alkaline activation and crystal production in strains of *Bacillus thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* has been noted (2, 4).

The genes encoding the crystal toxins are usually extrachromosomally borne on high-molecular-weight, stringent plasmids (14), and to date the sequence of only one such plasmid, pBtoxis from *B. thuringiensis* subsp. *israelensis*, has been reported (3). In addition to genes encoding four Cry proteins and three Cyt proteins, pBtoxis appears to encode a range of other proteins, including factors that may be involved in spore germination. The pBtoxis genes pBt084, pBt085, and pBt086 show similarities to previously reported *ger* genes (encoding proteins similar to the *Bacillus subtilis* germination protein GerAC and *B. cereus* proteins GerIB and GerIA, respectively). In addition, the plasmid bears two apparent pseudogenes, pBt060 and pBt063, that are similar to pBt086 and pBt085, respectively (3). Transcriptional analysis showed no mRNA production from the apparent pseudogenes but confirmed transcription from pBt084, pBt085, and pBt086 as a single operon (17). Related operons appear to exist in the genomes of *B. cereus* G9842 and *Bacillus weihenstephanensis* KBAB4 (EMBL accession numbers CP001186 and CP000903, respectively), but only distantly related genes appear in the completed genomes of *B. thuringiensis* strains. A close homolog (>70% identity at the level of the three proteins) has, however, recently been reported from *B. thuringiensis* subsp. *kurstaki* HD73 (accession number EF618567), but it is not clear whether this is a genomic or plasmid-borne operon in this strain.

In this study, we demonstrate that *B. thuringiensis* subsp. *israelensis* strains with and without the pBtoxis plasmid show different germination responses. In particular, different rates of germination are seen following an alkaline activation step, and the pBt084-pBt085-pBt086 operon alone is responsible for this phenomenon.

Bacterial strains, plasmids, and growth conditions. The plasmid pHT304 (1) was kindly provided by D. Lereclus of the Institut Pasteur, Paris, France. *Bacillus thuringiensis* subsp. *israelensis* strain 4Q5, cured of all plasmids except pBtoxis and an approximately 15-kb linear DNA element, and strain 4Q7, which is cured of all plasmids, including pBtoxis (5), were obtained from the *Bacillus* Genetic Stock Center, Ohio State University. All strains were grown at 30°C in Embrapa medium (16) for 4 to 7 days until sporulation, as assessed by phase-contrast microscopy, was in excess of 95%. Sporulated cultures (1 ml) were centrifuged in a microcentrifuge at 1,300 rpm and washed six times in a concentration of 1/4 strength Ringer's solution (Oxoid) by repeated centrifugation before resuspension in 50 μl of the same solution.

Activation of spores. Spores were activated by two alternative methods. Incubation of spore resuspensions in a water bath at 70°C for 20 min was used to achieve heat activation without a loss of spore viability (12). To induce alkaline activation, spores were harvested and washed twice by centrifugation with sterile water. Spore pellets were then resuspended (to

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an approximate attenuance of 0.5 at 600 nm) in 0.1 M sodium carbonate (pH 10) and incubated at room temperature for 30 min with gentle rocking using a shaking platform. The spores were then centrifuged and washed three times in 1/4-strength Ringer's solution before use in germination assays.

Germination. The following three germinants were used in this study: (i) the complex germinant nutrient broth (Sigma); (ii) the single germinant inosine (5 mM); and (iii) the single germinant L-alanine (10 mM), to which 13 mM D-cycloserine (Sigma) was added to prevent the activity of alanine racemase, which converts L-alanine to D-alanine, an inhibitor of germination (6).

Germinant solutions (5 μl) were added to equal volumes of the spore suspensions in PCR tubes and mixed thoroughly. Then, 5 μl of the mixture was pipetted onto a microscope slide, which had been incubated at 35°C over a water bath, and a coverslip was added to allow examination of the sample. Germination was measured using a Leitz Diaplan phase-contrast microscope at a magnification of ×1,250. Prior to germination, all spores appear phase bright, but when the germination process begins they rapidly become phase dark. On this basis, germination can be measured by counting the proportion of phase-dark spores out of a sample of 300 spores in the field of view at different time points. The experiment was repeated at least three times with each strain for each germinant. We used general linear models to explain the variance in the response, using strain, replicate, and time as candidate predictor variables. We considered models including the main effects and each of the possible two-way interaction terms. Analyses were carried out using the SPSS v16 program (SPSS Inc., Chicago, IL).

Cloning of pBtoxis *ger* operon. The germination genes of the pBtoxis plasmid (nucleotides 69,060 to 73,121 [3]) were amplified from vegetative cells of *B. thuringiensis* subsp. *israelensis* strain 4Q5 by using the following primers: 5′-CATTGAAAC GAGCAAATGTCA-3′ and 5′-CCTTTGCACCAAATCCTT TT-3′. The PCR analysis using *Taq* polymerase (Promega) was carried out with an annealing temperature of 49°C and an extension time of 4 min at 72°C for the first 10 cycles, followed by a further 20 cycles with a 1-min extension period. The approximately 4-kb amplicon was cloned using the TOPO TA cloning kit (Invitrogen). The region was then excised using EcoRI, and the fragment was recloned into pHT304 (1) and transformed into *Escherichia coli* strain DH5α. The resultant pHT304-ger plasmid DNA was isolated using the Qiagen Midi kit following the manufacturer's recommendations, and the inserted DNA was sequenced prior to its introduction into strain 4Q7 by electroporation as previously described (11) to produce the strain 4Q7-ger.

Germination of strains. In the absence of any prior activation step, *B. thuringiensis* strain 4Q5 containing pBtoxis or strain 4Q7 cured of this plasmid was incubated with inosine as the germinant, and the percent germination was followed over 20 min. Germination for strain 4Q5 reached approximately 28%, a level that was significantly higher ($P = 0.002$) than that for 4Q7 and 4Q7-ger (approximately 23%) (data not shown).

Following heat activation, germination rates were higher than those without heat treatment, with no significant differences between strains when either nutrient broth or inosine was used as a germinant (not shown). In response to L-alanine, strain 4Q5 showed a slightly higher germination rate ($P = 0.004$) at 20 min than 4Q7 (Fig. 1), but strain 4Q7-ger was indistinguishable from 4Q7 ($P = 0.862$), indicating that the *ger* genes play no role in the heat activation response to L-alanine. Both strains 4Q5 and 4Q7 were produced from the same parental *B. thuringiensis* strain 4Q2 (5), so the difference in germination phenotype between 4Q5 and 4Q7 could be due to factors present on elements found in the former strain but cured from the latter, including the pBtoxis plasmid and the linear DNA element first described in *B. thuringiensis* subsp. *israelensis* by González and Carlton (9). Although no other transcriptionally active *ger*-like genes were identified on pBtoxis (3, 17), there are numerous coding sequences of unknown function and many putative regulator genes that could, in principle, help to produce these responses by their action on other plasmid or chromosomal genes.

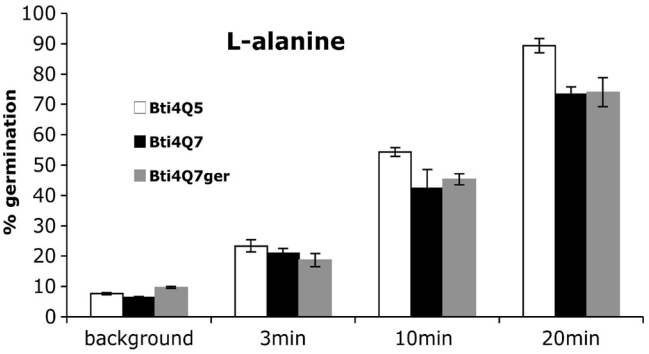

FIG. 1. Germination responses of *Bacillus thuringiensis* subsp. *israelensis* (Bti) strains after heat activation with 10 mM L-alanine. Experiments were repeated three times. Error bars are shown as standard errors of the means after calculation of standard deviation.

Following alkaline treatment, strain 4Q5 germinated at a high rate in response to all germinants, while strain 4Q7 showed a much lower germination rate (a P value of 0.043 for the inosine germinant) (Fig. 2), indicating a role for the pBtoxis plasmid in enhancing germination following alkaline activation. Strain 4Q7-ger germinated very strongly in a manner statistically indistinguishable from strain 4Q5 (a P value of 0.973 for the inosine germinant) (Fig. 2). To analyze this effect further, the germination responses were followed over a time course of 40 min. Again, strains containing the pBtoxis *ger* genes (4Q5 and 4Q7-ger) germinate more rapidly than strain 4Q7 in response to both inosine and alanine after alkaline activation, with differences that are noticeable at 10 min and very clear from 15 min onwards (Fig. 3).

Alkaline activation and different pH. We then investigated the effect of the pH of preincubation on the ability to promote alkaline activation. Spores of strains 4Q5, 4Q7, and 4Q7-ger were incubated in either Tris-HCl buffer (pH 8 and pH 8.5) or sodium carbonate buffer (pH 9 and pH 10) to attempt spore activation. At pH values of 8 and 8.5, germination rates were comparable to those of unactivated spores. At pH values of 9 and 10, a weak alkaline induction of germination in response to inosine was seen with strain 4Q7; however, the presence of the *ger* genes (on pBtoxis or pHT304-ger) leads to a marked increase in responsiveness to alkaline activation at these pH values (Fig. 4A to C).

Following alkaline activation, significant differences were

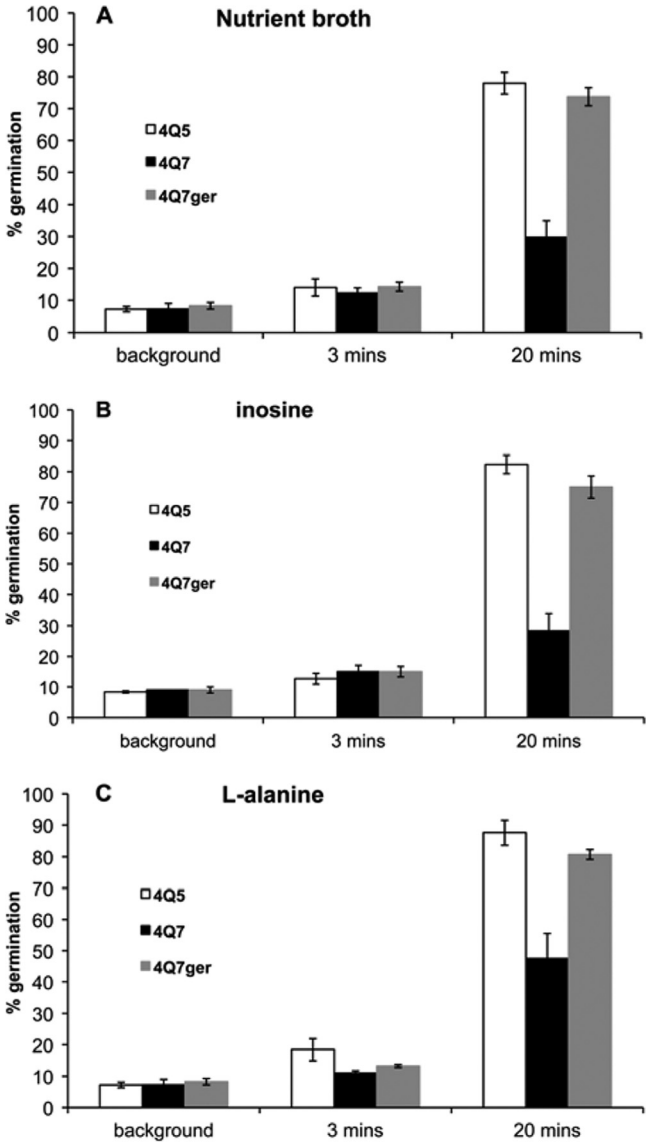

FIG. 2. Germination responses of *B. thuringiensis* subsp. *israelensis* strains after alkaline activation at pH 10. Shown are results for nutrient broth (A), 5 mM inosine (B), or 10 mM L-alanine (containing 13 mM D-cycloserine) (C). Experiments were repeated three times. Error bars represent the standard errors of the means after calculation of the standard deviation.

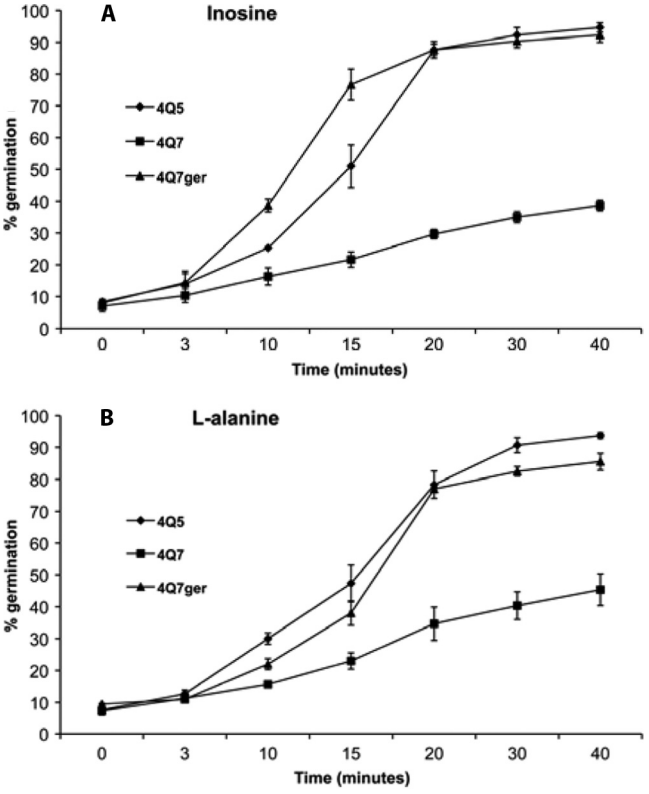

FIG. 3. Time course of germination responses of *B. thuringiensis* subsp. *israelensis* (Bti) strains after alkaline activation. Germinants used were 5 mM inosine (A) and 10 mM L-alanine (containing 13 mM D-cycloserine) (B). Experiments were repeated three times. Error bars represent the standard errors of the means after calculation of the standard deviation.

observed in the germination rates with all the germinants between strains 4Q5 and 4Q7. In contrast to the unactivated and heat-activated responses to L-alanine (as described above), the loss of the alkaline activation response in strain 4Q7 could be reversed by complementation with the pBt084, pBt085, and pBt086 genes in strain 4Q7-ger. This clearly indicates a role for these genes in the alkaline activation of spores.

The nature of the alkaline activation response is unknown and little studied. In contrast to heat activation, alkaline activation is irreversible and sensitive to ethanol (8), perhaps suggesting some permanent change as a result of alkaline treatment. We might speculate that the alkaline conditions may be causing an effect such as a change in permeability of the spore, structural changes in spore proteins, or stripping of the spore coat. Whatever the mechanism, the pBtoxis *ger* genes appear to mediate this effect in some way to offer a specific alkaline activation response that is not available to strains lacking these genes. The midguts of many insects, including mosquitoes, are alkaline and it is possible that a selective advantage may be gained by spores able to germinate at the appropriate time in the gut (10).

Our findings significantly extend those of several other authors (2, 4, 8) who have shown in vitro and using insect gut extracts that alkaline activation is a feature of crystal-forming strains that is lacking from acrystalliferous strains. These previous results have been interpreted to suggest a role for the protoxin in the coat of the spore in the alkaline activation phenotype. However, since the *ger* genes described in our study colocalize with the crystal toxin genes on the pBtoxis plasmid, a similar situation may prevail in the *B. thuringiensis* strains used in previous studies, and the loss of crystal production may reflect curing of the toxin-coding plasmid along with *ger* genes of the type found on pBtoxis. The complementation of the alkaline activation response by pHT304-ger indicates that the *ger* genes pBt084, pBt085, and pBt086 can produce this effect in the absence of any toxin production in strain 4Q7-ger.

The combination on the pBtoxis plasmid of toxin genes with

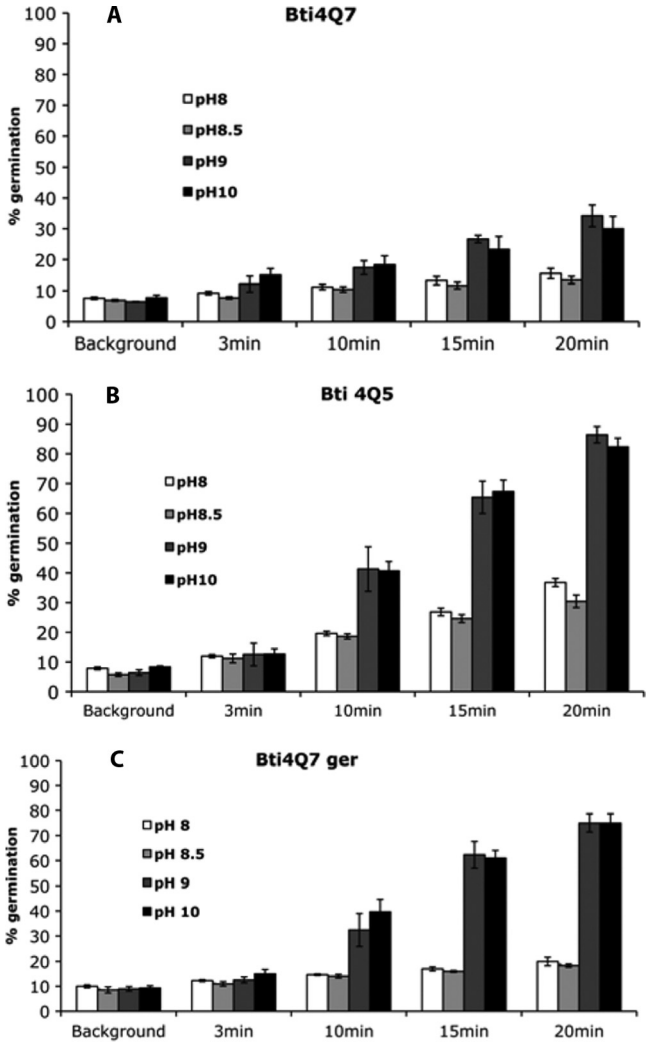

FIG. 4. Germination responses of *B. thuringiensis* subsp. *israelensis* (Bti) strains after alkaline activation at a range of pH with inosine germinant. Shown are the results for strains 4Q7 (A), 4Q5 (B), and 4Q7-ger (C). The experiments were repeated three times. Error bars represent the standard errors of the means after calculation of the standard deviation.

ger genes may exert a multifactorial influence on the phenotype of the host bacterium to coordinate germination under the alkaline conditions of the gut with toxin damage to the gut and death of the host insect. The potential significance of the effect of germination rate on the virulence of *B. thuringiensis* subsp. *israelensis* is currently under investigation.

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