ACTIVATION AND GERMINATION OF SPORES OF *BACILLUS THURINGIENSIS* VAR *ISRAELENSIS* BY ALKALINE PH AND LARVAL *(AEDES AEGYPTI)* GUT FLUID

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Abstract. Alkaline activation of the spores of crystalliferous (Cry+) and acrystalliferous (Cry-) strains of *Bacillus thuringiensis* var *israelensis*; wild type *B. cereus* and its transcipient crystalliferous derivatives and wild type *B. subtilis* was studied. Also the effect of larval (*Aedes aegypti*) gut fluid on the activation of spores of these strains was studied. Only the spores of the crystal forming strains were found to be activated by 0.1M K_2CO_3 (pH 10) and by the larval gut fluid. The process of alkaline activation was independent of whether crystals were present with the spores in the activation solution. This indicates that protoxin in the spore coat is responsible for the alkaline activation process and may have ecological implications for the organism.

INTRODUCTION

Bacillus thuringiensis, a gram-positive sporulating bacillus, produces proteinaceous parasporal crystal (delta-endotoxin) which is toxic against a number of lepidopterans and dipterans (Burges 1982). *B.thuringiensis* var *isralensis* produces crystal protein toxic against mosquito and blackfly larvae (de Barjack, 1978; Gonzalez *et al,* 1990). The spores exhibitsa number of unusual properties *eg*, high sensitivity to UV light (Benoit *et al,* 1990), presence of an outer layer of protoxin on the spore coat (Aronson *et al,* 1986) rapid disappearance fom the environment (Dulmage and Aizawa, 1982), activation of spores by treatment with alkaline solution prior to germination (Wilson and Benoit, 1993; Benoit *et al,* 1995).

The insecticidal crystal proteins are synthesized as protoxin which are solubilized by the alkaline pH in the gut of the susceptible insects and are activated by proteolysis by the mid gut proteases (Porter *et al,* 1993). Solubilization of proteins were also found *in vitro* (Aronson *et al,* 1991). The crystalliferous (Cry+) strains of *B.thuringiensis* possess an outer layer of protoxins on the spore coat but the acrystalliferous (Cry-) strains do not have protoxins on the outer sporecoat (Aronson *et al,* 1986).

Several species of *B.thuringiensis* spores found to be activated at the alkaline pH prior to germination (Wilson and Benoit 1990, 1993; Benoit *et al,* 1995). Earlier studies (Wilson and Benoit, 1990, 1993) showed that in addition to solubilization of protoxin, alkaline solutions which include 0.1M K_2CO_3 (pH 10) and native mid gut fluid from *Manduca sexta* larvae (pH 9.5) also activate *B. thuringiensis* var *kurstaki* spores to germinate *in vitro.* However, at lower pH (0.1M Tris HCl , pH 7.5) the spores were found not to be activated (Benoit *et al,* 1995). Even *Manduca sexta* midgut fluid after neutralization to pH 7.5 was found to loose its ability to activate the spores (Wilson and Benoit, 1993).

However, the situation with *B. thuringiensis* var *israelensis* was not studied so far. The effect of alkaline solution and mosquito (*Aedes aegypti*) larval gut fluid on the activation of spores of *B. thuringiensis* var *israelensis*, *B. cereus* and its transcipients harboring delta-endotoxin coding gene(s) and *B. subtilis* were studied. The activation phenomenon was found to be limited to sporogenous crystalliferous (Spo+ Cry+) strains only.

MATERIALS AND METHODS

Strains, spore and crystal production

The bacterial strains and their characteristics are listed in Table 1. Bacteria were grown in nutrient broth for 5 days. Spores and inclusions were harvested and resuspended in 0.1M NaCl with 0.1% (v/v) Triton x -100 and resuspended in distilled water as described previously (Wilson and Benoit, 1993). When required, crystals were purified by centrifugation at 35,000*g* for 40 minutes through 67% (v/v) renograffin as described previously (Benoit *et al,* 1990).

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Preparation of larval gut fluid

Gut fluid was obtained from *Aedes aegypti* fourth instar larvae. Approximately 300 larva were resuspended in 10 ml distilled water and kept frozen. After thaw, the suspension was homogenized in a homogenizer for 5 minutes. Particulate matter was removed by centrifugation at 10,000*g* for 30 minutes and the supernatant was passed through sterile 0.45 μm filter. The sterility of the supernatant was checked by streaking onto nutrient agar plate.

Activation and germination of spores

Spores were pretreated for 30 minutes with either heat (75°C), 0.1M Tris-HCl (pH 7.5) or 0.1M K_2CO_3 (pH 10) as described previously (Benoit *et al,* 1995) and were subsequently washed and resuspended in 0.1M Tris-HCl (pH 7.5) at an approximate density of 0.5 absorbance at 600 nm as described previously (Wilson and Benoit, 1993). Germination of these activated spores was measured as percentage decrease in initial absorbance at 600 nm, 20 minutes after addition of germinants L-alanine and adenosine (LAA) at a concentration of 4 mM each.

To study the effect of larval gut fluid, sterile gut fluid was substituted for germination buffer (0.1M Tris-HCl, pH 7.5) and LAA.

RESULTS AND DISCUSSION

Activation and germination of heat activated and without heat activated spores were determined in presence of water, 0.1M Tris-HCl (pH 7.5), 0.1M K_2CO_3 (pH 10) and germinants (LAA); and larval gut fluid. The bacterial strains used in this study are listed in Table 1 and the results are presented in Table 2.

Water induced no germination, 2 to 8% decrease of initial absorbance (Table 2) was noted irrespective of whether spores were heat activated or not. Activation of germination was also not observed when the spores were pretreated with 0.1M Tris-HCl (pH 7.5) (data not shown). Except the acrystalliferous (Cry-) and asporogenous (Spo-) strains and asporogenous crystalliferous (Spo- Cry+) strains all the spores could be activated to germinate by 0.1M K_2CO_3 (pH 10) and germinants (LAA), 53% to 63% decrease of initial absorbance was observed (Table 2). Heat activated spores showed slightly increased germination (data not shown) compared to spores without heat activation. This increased germination effect was observed irrespective of whether crystals were present in the spore suspension (unpurified spores, Table 2 A) or not (purified spores, Table 2 B). *B.thuringiensis* var *israelensis* Spo+ Cry+ strains were found to be activated almost equally well as the spores of *B.thuringiensis* var *israelensis* Spo+ Cry+ transcipients. It is interesting to note that *B.cereus* Spo- Cry+ strains, although expressed crystal toxins (Bhattacharya, 1994), their spores were found not to be activated by any agents (0.1M K_2CO_3, pH 10 and LAA or gut fluid) irrespective of blocked at different stages of sporulation. However, the spores of *B. cereus* transcipient (BC7) harboring crystal toxin plasmids could be activated by the different germination activating agents (Table 2A). The purified and unpurified spores of BC7 (*B. cereus* Spo+ Cry+ transcipient) did not exibit significant differences in activation of germination as observed by almost identical decrease in absorbance (Table 2A and 2B). Prior heat treatment of spores heat activation) of BC7 also did not exhibit significant differences in germination as compared to spores without prior heat activation (data not shown).

Larval gut fluid also found to activate the germination of *B. thuringiensis* var *israelensis* and BC7 spores. However, no activation was observed in case of *B. cereus* NCIB 9376 and *B. cereus* asporogenous mutant strains (BC2, BC4 and BC6) and *B. cereus* asporogenic crystalliferous (Spo-Cry+ strains, BC8, BC10 and BC12) and *B. subtilis* strains.

Almost identical activation of purified and unpurified spores by the germinants and gut fluid indicates that activation process is independent of whether crystal toxin is present in the activation solution or not. Moreover activation of germination of spores of Spo+ Cry+ strains only and not of the spores of Spo- Cry+ strains irrespective of blocked at different stages of sporulation or Spo+ strains, indicates that the activation phenomenon is restricted to sporogenous crystalliferous (Spo+ Cry+) strains only. These results are in agreement with earlier studies (Wilson and Benoit, 1993; Benoit *et al,* 1995) with other *B. thuringiensis* strains. These suggests that protoxin present in the spore coat gets dissolved at the alkaline pH and increases the porosity of the spore coats and thereby making the germinants more accessible to triger the activation of the germination process as has been suggested earlier (Wilson and Benoit, 1990, 1993; Benoit *et al,* 1995.) On the contrary, spores of the acrystalliferous (Cry-) strains, as they do not have any protoxin in the spore coats are not susceptible to alkaline treatment, remains intact maintaining their

Table 1
Description of bacterial strains.

Organism	Characteristics	Reference
1. *B. thuringiensis*		
a) var *israelensis*	Serotype H-14, Spo+Cry+	Bhattacharya, 1993
b) var *israelensis*	Serotype H-14, Spo+Cry+	Indigenous isolate
c) var *israelensis* BTSt R15	Streptomycin resistant Spot Cry- cured derivative of 1 (a)	Bhattacharya, 1993
d) var *israelensis* BTSt R36	Streptomycin resistant Spo+ Cry- cured derivative of 1 (a)	Bhattacharya, 1993
e) var *israelensis* BTSt R31	Streptomycin resistant Spo+ Cry+ transcipients derived from transconjugation of l(a) and l(c)	Bhattacharya, 1993
f) var *israelensis* BTSt R42	Streptomycin resistant Spo+ Cry- transcipients derived from transconjugation of l(a) and l(d)	Bhattacharya, 1993
2. a) *B. cereus* NCIB 9376	Spo+	Bhattacharya, 1994
b) BC1	Streptomycin resistant Spo+ derived from 2(a)	Bhattacharya, 1994
c) BC2	Streptomycin resistant Spo- at sporulation stage 0-II derived from 2(b)	Bhattacharya, 1994
d) BC4	Streptomycin resistant Spo- at sporulation stage IV derived from 2(b)	Bhattacharya, 1994
e) BC6	Streptomycin resistant Spo- at sporulation stage V-VI derived from 2(b)	Bhattacharya, 1994
f) BC7	Streptomycin resistant Spo+ Cry+ transcipient from transconjugation of l(a) and 2(b)	Bhattacharya, 1994
g) BC8	Streptomycin resistant Spo- Cry+ transcipient from transconjugation of l(a) and 2(c)	Bhattacharya, 1994
h) BC10	Streptomycin resistant Spo- Cry+ transcipient from transconjugation of l(a) and 2(d)	Bhattacharya, 1994
i) BC12	Streptomycin resistant Spo- Cry+ transcipient from transconjugation of l(a) and 2(e)	Bhattacharya, 1994
j) *B. cereus* var *mycoides* ATCC 11778	Spo+	Central Drugs Laboratory, Calcutta
3. *B. subtilis* ATCC 6633	Spo+	-do-

Table 2
Germination of spores, as measured by percentage decrease in absorbance when incubated with water, germinants (LAA, 20 minutes after treatment with 0.1M K_2CO_3); and larval gut fluid.

Strains[a]	Water	LAA	Gut fluid
A. Unpurified spores			
1 (a)	6,0(0.5)[b]	55(0.8)	57(0.28)
1 (b)	8.3(0.6)	56(0.8)	58(0.42)
1 (c)	5.0(0.7)	15(0.24)	14(0.21)
1 (d)	5.5(0.2)	17(0.21)	16(0.12)
1 (e)	6.8(0.3)	60(0.83)	62.5(0.8)
1 (f)	7.0(0.26)	63(1.4)	62(1.2)
2 (b)	2.0(0.2)	10(0.5)	20(0.3)
2 (c)	4.0(0.3)	12(1.0)	15(0.36)
2 (d)	6.8(0.8)	20(0.6)	17.6(0.26)
2 (e)	5.0(0.6)	13(0.7)	14.2(0.4)
2 (f)	7.4(0.42)	590.80)	62(1.2)
2 (g)	6.0(0.7)	18(0.1)	17(0.8)
2 (h)	2.0(0.5)	17(0.23)	21(0.8)
2 (i)	4.0(0.5)	16(0.54)	21.4(0.16)
2 (j)	4.6(0.7)	13(0.6)	18(0.72)
B. Purified spores			
1 (a)	7.8(0.26)	57(2.0)	60.4(0.83)
1 (b)	6.6(0.2)	58(0.47)	61(0.71)
1 (e)	5.0(0.5)	53(1.7)	60.5(0.8)
1 (f)	4.2(0.3)	55(0.24)	57.4(0.3)
2 (a)	6.5(0.6)	15.4(0.3)	17.4(0.42)
2 (f)	5.7(0.32)	61(1.8)	55(1.5)

a: For description of the strains vide Table 1.
b: Results are in averages of five independent experiments; in parenthesis is given the standard error of mean.

integrity and do not respond to activation process by the germinants. The fact that 0.1M Tris-HCl (pH 7.5) could not activate (data not shown) the germination of spores also supports this notion. The inability of the asporogenous crystalliferous (Spo-Cry+) strains (BC8, BC10 and BC12) to respond to the activation process indicates that the integrity of the intact spores is also essential for responding to the activation phenomenon, although the Spo-Cry+ strains were found to be toxic against mosquito larva (Bhattacharya, 1994). It is also interesting to note that not only the spores of *B. thuringiensis* var *israelensis* Spo+Cry+ strains but also the spores of *B. cereus* Spo+Cry+ strains responded to activation of germination. This indicates that presence of protoxin in the spore is an essential criterion for responding to activation phenomenon which has great ecological implication. As *B. thuringiensis* var *israelensis* is used as biolarvicides against mosquito larva and are exposed to a variety of pH under different environmental conditions in the field, they may be more effective in polluted water bodies, marshy lands and ditches rich in organic waste which are ideal habitats for mosquito breeding and at alkaline pH.

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