High Toxicity Asporogenous Mutants of *Bacillus sphaericus* 2362

Somchit Jiaviriyaboonya
Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Rangsit Campus, Pathumthani, 12121, THAILAND
e-mail: somchit@tu.ac.th

Abstract

By means of ethyl methane sulfonate (EMS) mutagenesis, 113 oligo-sporogenous mutants of *Bacillus sphaericus* 2362 were isolated. Meanwhile, 2 spontaneous asporogenous mutants were isolated from continuous culture. After a number of subculturings, 13 stable EMS-mutated asporogenous mutants (E-Spo$^-$) and 2 spontaneous asporogenous mutants (S-Spo$^-$) were selected for electron microscopic examination, SDS-PAGE and Western Blot analysis. No crystal toxin examined by either electron microscope or Western blot was observed from the 2 S-Spo$^-$. Five mutants of E-Spo$^-$ with atypical crystal and non-crystal toxins observed by electron microscopy and high toxin concentration detected by Western blot were further evaluated by bioassay using mosquito larvae of *Culex quinquefasciatus*. R24, with the LD_{50} of 3.32 x 10^2 cells/ml, was chosen for further study in batch fermentation. An enzyme linked immunosorbent assay (ELISA) was used for monitoring toxin production in batch culture. The maximum dry cell weight and cell count of R24 were lower than those of the parent strain. The toxin level, as measured by ELISA and bioactivity was comparable in both strains. This result for R24 showed a significant reduction in toxin activity compared to the level initially determined. The loss of bioactivity of R24 appeared to result from strain instability following extended subculturing over 240-300 generations as well as storing at –20°C in glycerol.

1. Introduction

Bacillus sphaericus (Bs) is a mesophilic, gram positive, rod-shaped, spore forming bacillus which is widespread in soil and aquatic environments. It is a strictly aerobic bacterium preferring organic acids to sugars as sources of carbon and energy [1]. As a significant role in biocontrol, it produces mosquito larvae toxin in the form of crystal during sporulation. The structure of sporulation associated crystal toxin from Bs 2362, one of the most toxicity strains of Bs, is characterized by a rectangular crystal lattice form or parallelepiped inclusion located alongside the spore (Figure 1). The spore and crystal toxin are enclosed by the exosporium, which perhaps serves as protection.

Among the most toxicity strains of Bs, i.e. Bs 2297, 1593 and 2362, there are two major proteins found in crystal toxin, 51.4 and 41.9 kDa, responsible for larvicidal activity. These two proteins, of course, are produced during sporulation [2]. In the low toxicity strain, Bs SSII-1, a 100-kDa mosquitocidal toxin (Mtx) was reported to be produced predominantly during the vegetative phase of growth. However, it was found to be unstable during sporualtion due to degradation by protease enzymes [3]. Unlike *Bacillus thuringiensis*, Bs possesses the ability to survive in medium to highly polluted water, and its toxicity appears to persist for a longer time. In addition, Bs spores can also recycle in *Culex* larvae. In the larval midgut, spores germinate and multiply, leading to production of new spores which are released into the aquatic environment as the larval cadaver disintegrates [4,5,6]. Due to its longer persistence in aquatic environments and related recycle ability, the World Health Organization is strongly encouraging further evaluation and development of Bs as a microbial larvicide [7].

Spore formation, which is mainly found in *Bacillus* and *Clostridium* spp., is a survival mechanism induced by some change in the

environment [8]. Depletion of carbon or nitrogen sources in batch culture or these nutrient limitations in continuous culture can lead to initiation of sporogenesis.

Development of spores in Bs has been reported to accompany crystal toxin formation [9], however various combinations of mutants, e.g. Spo^- Cry^- and Spo^- Cry^+ can occur. Asporogenous mutants of *Bacillus thuringiensis* producing either comparable or high toxin activity relative to the parent have been reported [10,11,12].

Since there are no reports of asporo-genous mutants of Bs 2362, the aim of this work was to study the morphology and insecticidal activity, both *in vitro* and *in vivo*, of Bs 2362 asporogenous mutants. Such mutants could have potential advantages such as enhanced toxicity or faster rates of toxin production. When the mutant with the highest yield of toxin has been evaluated in this manner, further fermentation studies for optimization of growth and toxin production will be carried out.

2. Materials and methods

Bacillus sphaericus 2362 was kindly provided by Professor S. Singer, Biology Department, Illinois University. For mutagenesis protocol, ethyl methane sulfonate (EMS) was employed at a concentration of 4% in NYSM medium containing 13 g/l nutrient broth, 0.5 g/l yeast extract, 1 ml $MnCl_2 \cdot 4H_2O$, 7 ml 0.1 M $CaCl_2 \cdot 2H_2O$ and 2 ml 0.5 M $MgCl_2 \cdot 6H_2O$.

Transmission electron microscope (TEM) and field emission scanning microscope (SEM) photos were taken using Hitachi H-7000 and Hitachi S900, respectively. Immunogold staining, a new technique using specific antibody to binary toxin labeled with gold particles to locate larvicidal toxin which can be examined by TEM, was described previously [13]. SDS-PAGE, Western blot, ELISA protocol and bioassay were described in a previous paper [14].

Batch fermentation was studied in a medium consisting of yeast extract 10 g/l, sodium acetate 15 g/l, $MnCl_2 \cdot 4H_2O$ 0.02 g/l, $CaCl_2 \cdot 2H_2O$ 0.2 g/l, $MgCl_2 \cdot 7H_2O$ 0.1 g/l and KH_2PO_4 0.1 g/l in a two-litre LH fermentor at 30° C and pH 7. Agitation was set at 700 rpm with air supply 1 vvm to maintain fully aerobic growth (DO greater than 20% air saturation). Samples were withdrawn at different stages of growth to determine cell number, spore number, dry cell weight and residual acetate concentration. The toxin was extracted from samples by alkaline extraction as previously described [14].

3. Results and discussion

3.1 Mutagenesis and selection

Since ethyl methane sulfonate (EMS) produces point mutations at high frequency, it was chosen for mutagenesis. Oligosporogenous mutants with translucent colonies, 113 in all, were isolated on NYSM plates. In continuous culture of Bs 2362 with carbon (acetate)-limitation [15], there was a spontaneous mutation, resulting in 2 asporogenous mutants.

3.2 Ultrastructure analysis by electron microscopy

From a number of subcultures on NYSM plates, 13 stable EMS-mutated asporogenous mutant (E-Spo^-) and 2 stable spontaneous asporogenous mutants (S-Spo^-) isolated from continuous culture, were chosen for electron microscopic study. The S-Spo^- mutants showed no crystal toxin at all. For E-Spo^- mutants, some contained crystal toxins but others did not. Crystal toxins were only found in mutants with incomplete spores (Figure 2,3).

Larvicidal crystal toxin production was concomitant with spore development. In *B. thuringiensis*, there were 7 stages of sporulation with successively developed crystal toxin formation [16]. Initiation of crystal formation in *B. thuringiensis*, as well as that in Bs, occurred in stage III of sporulation. For the Spo^- mutants blocked in the early stages of sporulation, neither spores nor crystalline inclusions were produced, while for the Spo^- mutants blocked at late stages of spore formation, incomplete spore and crystal-like inclusion formation were found to occur [17]. The results of the present study would indicate that many of the Bs asporogenous mutants were blocked in the later stages of sporulation. Since EMS causes deletion mutation randomly, only blockages at

late stages produce crystal toxins but incomplete spore. For spontaneous mutation in continuous fermentation, it perhaps caused early stages and permanent blocking of both sporulation and crystal toxin production.

Blocking the sporulation process in other *Bacilli* spp. such as *B. subtilis* has been shown to increase levels of carbon sensitive enzymes (e.g. glucose-6-phosphate dehydro-genase, glutamine synthetase, arbinose isomerase and histidase), possibly at the level of gene expression [18]. The cellular biosynthesis of crystal formation may have increased in the Spo$^-$ mutants in the present study resulting in higher toxicity. However, with the increased rate of synthesis, improperly folded crystals may have formed and resulted in various unusual shaped crystal inclusions found in some mutants as demonstrated in Figure 3. This event has been reported also in some recombinant *E. coli* [19]. In addition, in eliminating sporulation, suboptimal medium components have been found to affect crystal assembly [20].

Immunogold staining followed by transmitted electron microscope scanning demonstrated that crystal toxin and non-crystal toxin of the E-Spo$^-$ mutants was bound to immunogold inside the cells (Figure 4). Non-crystal form of toxin bound to immunogold was suggested to result from the non-ordered aggregated form of the binary toxin.

3.3 SDS-PAGE and Western blot analysis

To confirm toxin activity by immuno-detection, Bs 2362 and the mutants were subjected to alkaline extraction, followed by SDS-PAGE and Western blotting [14]. Two major protein bands corresponding to 53 and 41 kDa, were detected in the final Western blots. After scanning these bands, toxin activity was quantitatively estimated based on intensity relative to the parent strain. The S-Spo$^-$ strains appeared to have very low toxin band intensity while some of the E-Spo$^-$ strains showed high toxin band intensity, compared to that of the parent strain.

3.4 Bioassay

The mutants with crystal toxin observed by electron microscope and high toxin protein intensity detected by Western blot, namely R24, R29, R40, R63 and R74, were selected for bioassay. All mutants and the parent strain were grown in 250-ml flasks containing 100 ml NYSM for 2 days at 30°C with shaking (200 rpm). Cells were harvested for bioassay protocol which performed against third instar larvae of *Culex. quinque-fasciatus*. The method was based on that outlined by the World Health Organization, WHO [21]. The lethal dose to cause 50% mortality (LD_{50}) was calculated with a probit analysis program (PA-mod).

The results of LD_{50} of parent strain (Bs 2362) and the mutants, R24, R29, R40, R63 and R74 are shown in Table 1. All mutants, except R63, possessed higher bioactivity against *Culex quinquefasciatus* than the parent strain. As R74 showed some reversion of sporulation and R40 and R29 grew slower than others, R24 was chosen to study further in fermentation.

Table 1 LD_{50} of *Bacillus sphaericus* 2362 and the mutants

Strains	LD_{50} (cells/ml)	95% LMS*
Bs 2362	1.62×10^3	$1.36\text{-}1.90 \times 10^3$
R24	3.32×10^2	$2.23\text{-}4.94 \times 10^2$
R29	2.59×10^2	$1.43\text{-}4.72 \times 10^2$
R40	2.63×10^2	$1.58\text{-}4.39 \times 10^2$
R63	8.34×104	$4.6\times10^3\text{-}1.5\times10^5$
R74	1.27×10^3	$8.9\times10^2\text{-}1.7\times10^3$

* = 95% confidence limits

Typical sigmoidal curve was observed in lethality curves of the parent and the mutant strains. Zero (0%) mortality and 100% mortality were used to calculate LD_{50} by PA-mod. Unlike others, the R63 was not able to kill the larvae completely (100%) at a concentration of 10^5 cells/ml. Further increasing the cell concentration to 10^6 did not increase larval mortality to 100%, indicating the sublethal nature of the toxin produced. Besides the

delayed mortality, paralyzed and sick larvae were also observed as sublethal symptoms.

3.5 Fermentation study

Figure 5 shows growth and toxin profiles of Bs 2362 and R24, respectively. Free spores appeared when acetate was exhausted (17 h). Toxin measured by ELISA was detected from 10 h, 7 h before the appearance of free spores. Following its initial production, the toxin level profile paralleled the increase of sporulation.

Some parameters from batch fermentations of both strains are summarized in Table 2. The maximum specific growth rates (μ_{max}) of Bs 2362 and the mutant were 0.50 and 0.46 h^{-1}, respectively. Maximum DCW (dry cell weight) of the parent strain was also higher than that of the mutant, resulting in a slightly lower final yield of toxin produced per dry weight. However, the final toxin yield from both strains were similar following alkaline extraction (0.60 and 0.66 % DCW for Bs 2362 and R24, respectively). Overall, a comparative evaluation of the batch culture kinetics of both parent and mutant strains revealed very similar patterns of growth and toxin production.

Table 2 Some parameters from batch culture fermentations of Bs 2362 and the mutant

Parameter	Bs 2362	The mutant
μ_{max} (h^{-1})	0.50	0.46
DCW (g/l)	6.185	4.790
Cell count (x10^9/ml)	9.29±1.79	8.02±1.71
Spore count (x10^9/ml)	4.89 1.01	0
Toxin level (μg/ml)	15.12	13.92
Final toxin yield (%DCW)	0.60	0.66
LD_{50} (cells/ml)	1.04 x 10^3	2.38 x 10^3
	(6.43x10^2-1.69x10^3)*	(1.41-4.35 x 10^3)*

* = 95% confidence limits

Surprisingly, the LD_{50} value of the mutant produced under controlled culture conditions was higher than the LD_{50} of the parent strain (Table 2). In other words, activity *in vivo* or bioactivity of the mutant had decreased by at least 5 times compared to that initially measured (Table 1). This may have resulted from strain instability and reversion and/or secondary mutation occurring following a number of subculturings from plates.

To confirm whether or not reversion had occurred, an inoculum prepared directly from a glycerol stock of R24 stored for 6 months at -20°C was developed, followed by twice repeating batch fermentation in the same medium. The results for the two experiments had no significant difference (data not shown). However, these results revealed a loss of bioactivity of R24 after storage for 6 months at –20°C, as well as following successive plate subculturings and were evidence of significant instability in an asporogenous mutant of Bs 2362.

After 2 years storage at -20°C, the R24 was checked for morphology on NYSM plate. A mixed culture of normal and translucent colonies was found. This result showed that more reversion to sporulation has been continuing even when stored at -20°C as a glycerol stock.

To obtain stable asporogenous mutants, repeated subculturing was used in the present study. However, loss of bioactivity of some mutants (such as R24) has been demonstrated to relate to instability resulting from an increasing number of subculturings (~240-300 generations, from calculation), as well as storage for an extended period as a glycerol stock at -20°C. From the present results it was found with R24 that loss of bioactivity occurred from a relatively high level to the same level as the parent strain. It is possible that this was a necessary adaptation of the mutant to survive and to be stable. As mentioned previously, an imbalance in metabolism caused by EMS in generating asporogenous mutants, can result in changes in cell condition and cause instability. If the mutant can adapt to the new conditions, it may be more stable and survive with high toxicity. If not, the mutant will revert back to a more stable form (as the parent strain), or it will not survive.

The results however, indicate that an asporogenous mutant could be used for toxin production and this may have advantages in terms of faster overall rates of toxin production, as the additional time for sporulation is not required.

4. References

[1] Priest, F.G. (1992), Biological Control of Mosquitoes and Other Biting Flies by *Bacillus sphaericus* and *Bacillus thuringiensis*, J. Appl. Bacteriol., vol.72, pp.357-369.

[2] Baumann, P., Clark, M., Baumann, L. and Broadwell, A.H. (1991), *Bacillus sphaericus* as a Mosquito Pathogen: Properties of the Organism and Its Toxins, Microbiol. Rev., vol.55, pp.425-436.

[3] Thanabalu, T. and Porter, A.G. (1995), Efficient Expression of a 100-kilodalton Mosquitocidal Toxin in Protease-deficient Recombinant *Bacillus sphaericus*, Appl. Environ. Microbiol., vol.61, pp.4031-4036.

[4] Mulligan, F.S., Schaefer, C.H. and Wilder, W.H. (1980), Efficacy and Persistence of *Bacillus sphaericus* and *Bacillus thuringiensis* H14 against Mosquitoes under Laboratory and Field Conditions, J. Econ. Entomol., vol.73, pp.684-688.

[5] Rochers, D.B and Garcia, R. (1984) Evidence for Persistence and Recycling of *Bacillus sphaericus*, Mosq. News, vol.44, pp.160-165.

[6] Nicolas, L., Dossou-Yovo, J. and Hougard, J.-M. (1987), Persistence and Recycling of 2362 Spores in *Culex quinquefasciatus* Breeding Sites in West Africa, Appl. Microbiol. Biotechnol., vol.25, pp.341-345.

[7] Glazer, A.N. and Nikaido, H. (1995) (eds) Microbial Biotechnology: Fundamentals of Applied Microbiology, W.H. Freeman and Company, U.S.A.

[8] Murrell, W.G. (1961), Spore Formation and Germination as a Microbial Reaction to the Environment, **In**: The XI Symposium of the Society of General Microbiology, Megrell, G.G. and Gooder, H. (eds), Cambridge University Press, pp.100-150.

[9] Yousten, A.A. and Davidson, E.W. (1982), Ultrastructural Analysis of Spores and Parasporal Crystals Formed by *Bacillus sphaericus*, Appl. Environ. Microbiol., vol.44, pp. 1449-1455.

[10] Nishiitsutsuji-Uwo, J., Wakisaka, Y. and Eda, M. (1975), Sporeless Mutants of *Bacillus thuringiensis*, J. Invertebr. Pathol., vol.25, pp. 355-361.

[11] Johnson, D.E., Niezgodski, D.M. and Twaddle, G.M. (1980), Parasporal Crystals Produced by Oligosporogenous Mutants of *Bacillus thuringiensis* (Spo^- Cry^+), Can. J. Microbiol., vol.26, pp.486-491.

[12] Wakisaka, Y., Masaki, E., Koizumi, K., Nishimoto, Y., Endo, Y., Nishimura, M.S. and Nishiitsutsuji-Uwo, J. (1982), Asporogenous *Bacillus thuringiensis* Mutant Producing High Yields of δ-Endotoxin, Appl. Environ. Microbiol., vol.43, pp. 1498-1500.

[13] Jiaviriyaboonya, S., Marks, B.D.H., England, D.F. and Rogers, P.L. (1998) Mosquito Larvicidal Binary Toxin Localization in Recombinant Bacteria: Identifying Using an Immunoglobulin Technique, vol.12, no.7, pp. 533-538.

[14] Jiaviriyaboonya, S., Penfold, R.J. and Rogers, P.L. (1998) A sandwich enzyme-linked Immunosorbent Assay for the *Bacillus sphaericus* Binary Toxin, Curr. Microbiol., vol. 36, pp. 152-157.

[15] Idachaba, M.A. (1996) Batch and Continuous Culture Studies of the Mosquitocidal Pathogen *Bacillus sphaericus* 2362, Ph.D. Thesis, UNSW, Sydney, Australia.

[16] Bulla, L.A., Jr., Bechtel, D.B., Kramer, K.J., Shethna, Y.I., Aronson, A.I. and Fitz-James, P.C. (1980) Ultrastrcuture, Physiology and Biochemistry of *Bacillus thuringiensis*, CRC Crit. Rev. Microbiol., vol.6, pp.147-204.

[17] Charles, J.-F., Bourgouin, C. and de Barjac, H. (1988) *Bacillus sphaericus* Asporogenous Mutants: Morphology, Protein Pattern and Larvicidal Activity, Ann. Inst. Pasteur Microbiol, vol.139, pp. 243-259.

[18] Boylan, S.A., Chun, K.T., Edson, B.A. and Price, C.W. (1988), Early-blocked Sporulation Mutations Alter Expression of Enzyme under Carbon in *Bacillus subtilis*, Mol. Gen. Genet., vol.212, pp.271-280.

[19] Blum, P., Velligan, M., Lin, N. and Matin, A. (1992), DnaK-mediated Alterations I Human Growth Hormone Protein Inclusion Bodies, Bio/Technology, vol.10, pp.301-304.

[20] Sachidanandham, R. and Jayaraman, K. (1993), Formation of Spontaneous Asporogenic Variants of *Bacillus thuringiensis* subsp. *galleriae* in Continuous Cultures., Appl. Microbiol. Biotechnol., 40, pp.504-507.

[21] Dulmage, H.T. (1970) Production of the Spore-delta-endotoxin Complex by Variant of *Bacillus thuringiensis* in Two Culture Media, J. Invertebr. Pathol., vol.16, pp.385-389.

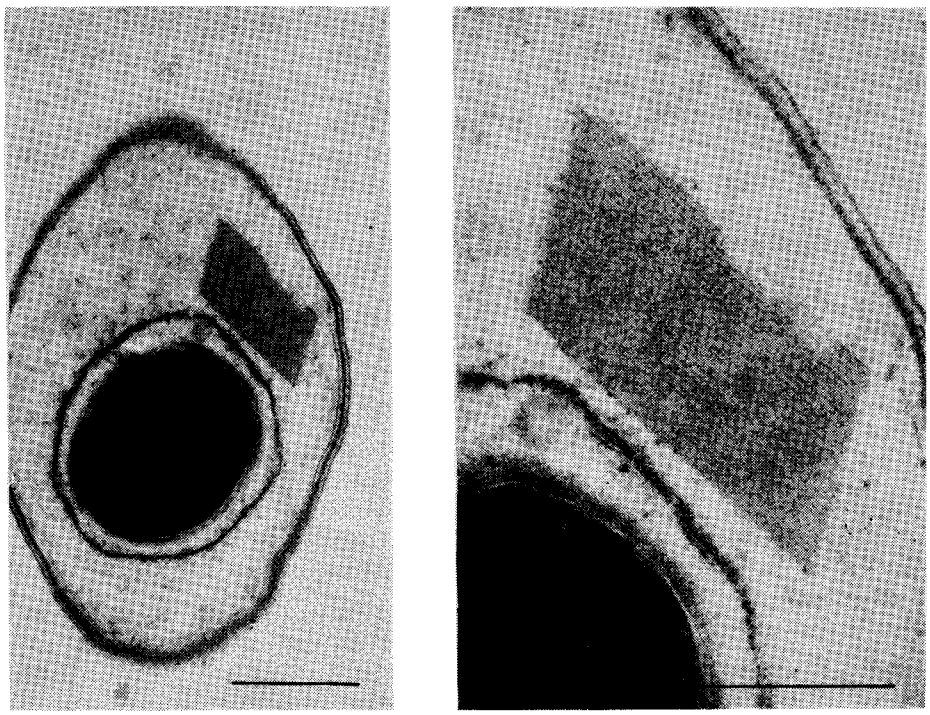

Figure 1 A spore containing toxin crystal from *Bacillus sphaericus* 2362, bar: 0.2 μm.

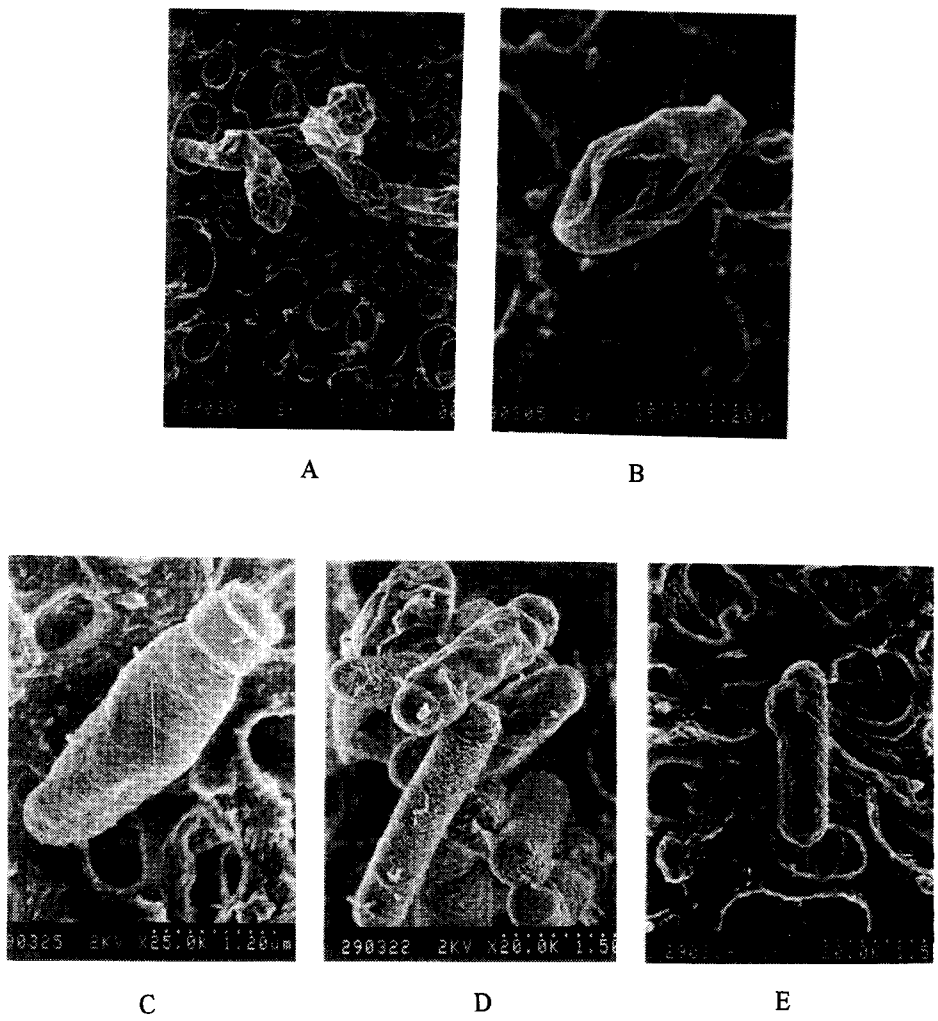

Figure 2 Scanning electron microscope (SEM) of *Bacillus sphaericus* 2362 spores
A, B: parent strain, C, D, E: E-Spo- mutants

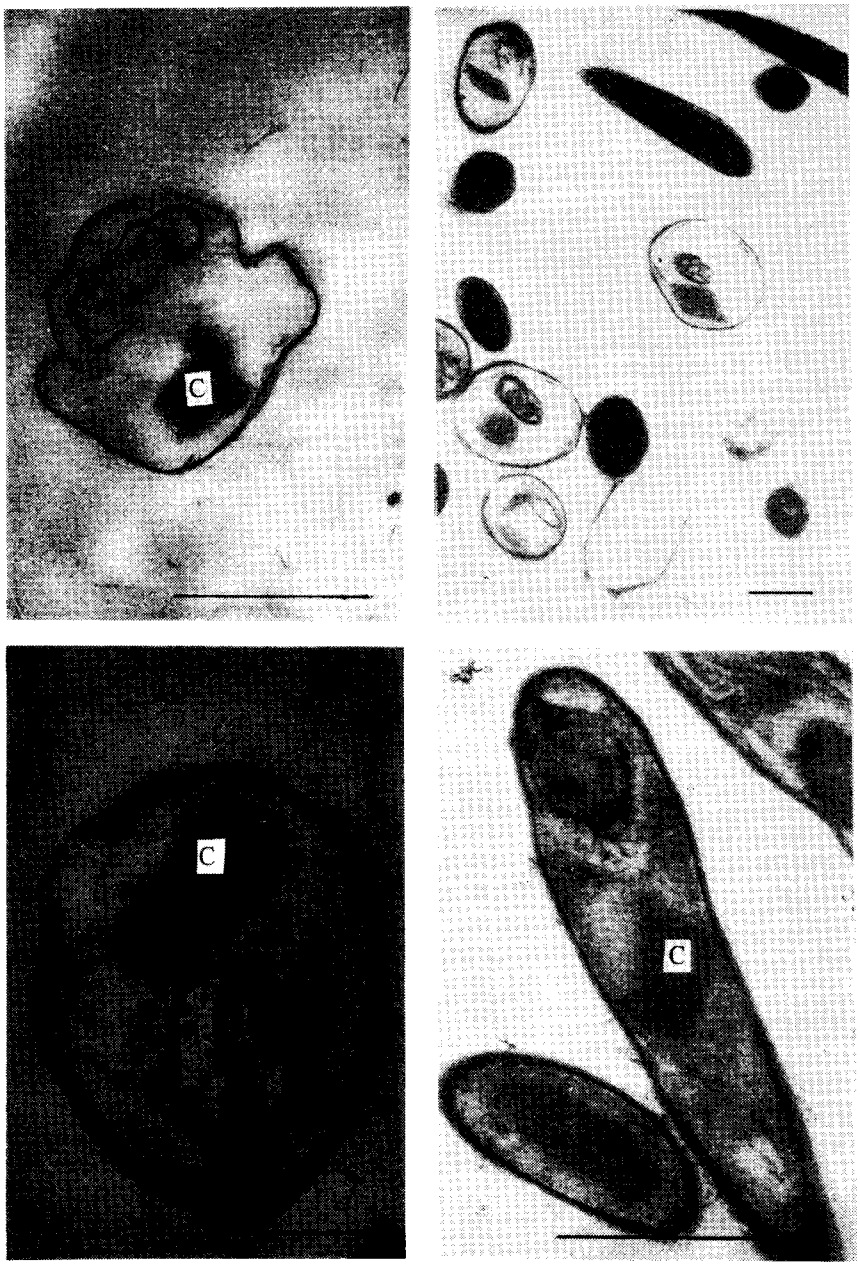

Figure 3 Atypical crystal toxins found in E-Spo⁻ mutants, C: crystal, bar: 0.5 μm.

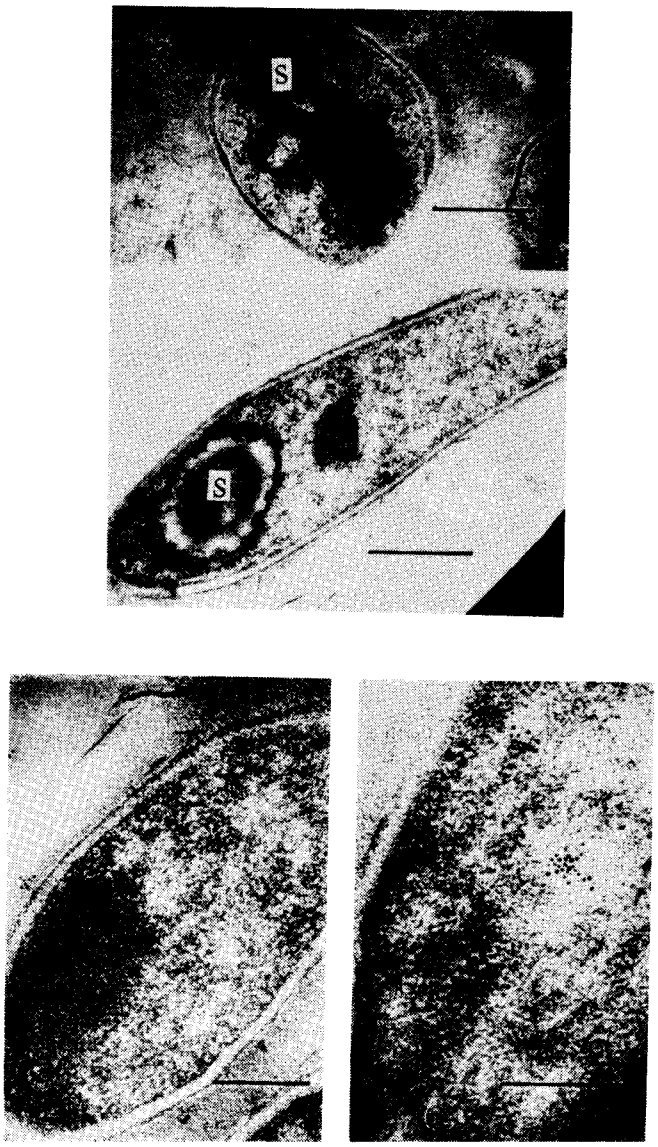

Figure 4 Immunogold labeling toxins in E-Spo$^-$ mutants, S: spore, C: crystal toxins, A: amorphous toxin, bar: 0.2 μm.

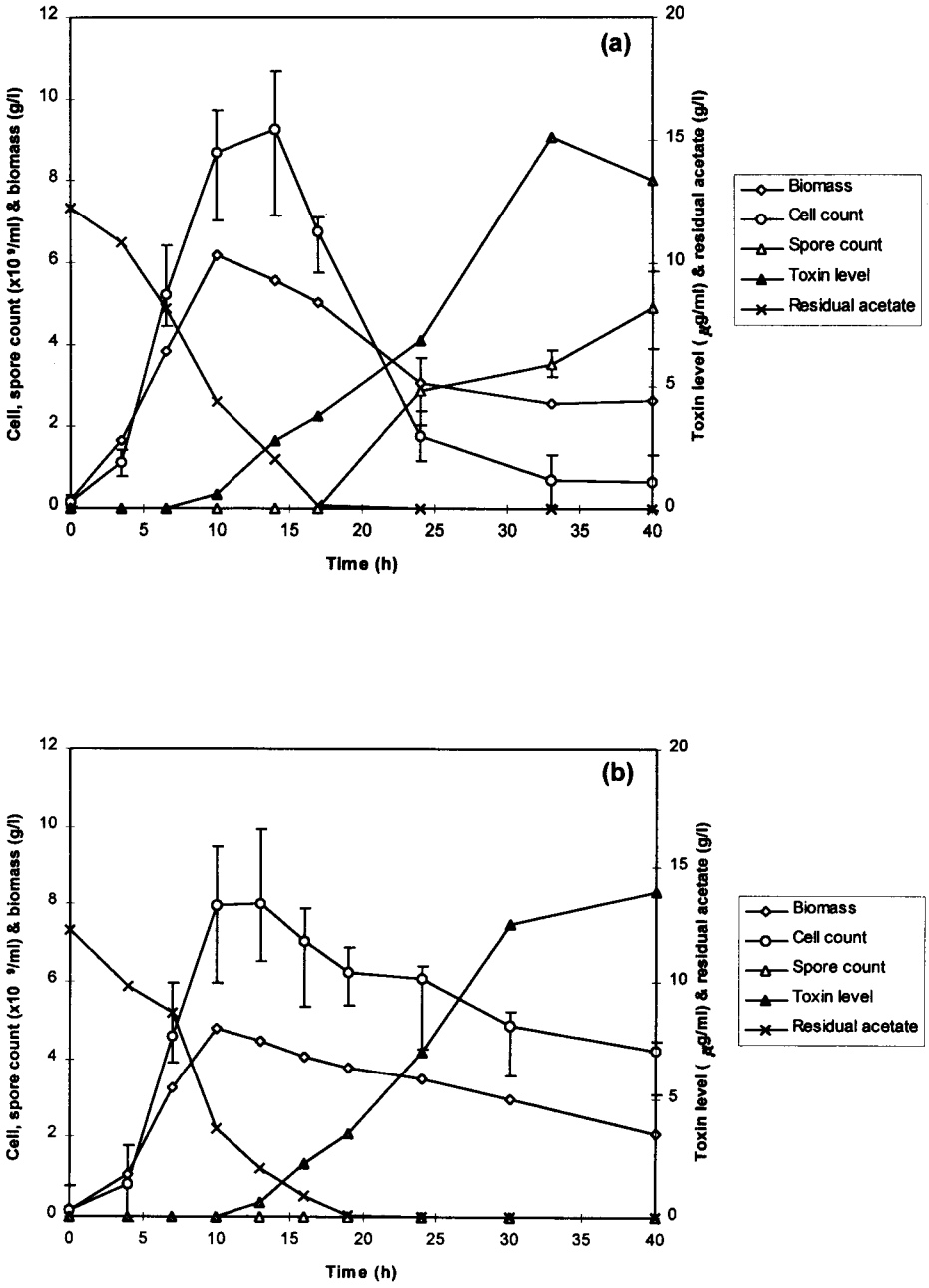

Figure 5 Growth, cell and spore counts and toxin level of *Bacillus sphaericus* 2362 (a) and the mutant (b).