Sporulation of *Bacillus sphaericus* 2297: an Electron Microscope Study of Crystal-like Inclusion Biogenesis and Toxicity to Mosquito Larvae

By A. KALFON, J.-F. CHARLES,* C. BOURGOUIN AND H. DE BARJAC

Unité de Lutte Biologique II, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France

(Received 29 June 1983; revised 18 September 1983)

Sporulation of *Bacillus sphaericus* strain 2297 in a synchronous liquid culture was studied by electron microscopy. The t₀ of sporulation occurred 7 h after the beginning of the lag phase. Crystal-like inclusions first appeared at t₂ and reached their final size between t₅ and t₆. The release of the spore/inclusion complex occurred at about t₁₅ (22 h after inoculation).

Toxicity against *Culex pipiens* larvae was related to sporulation and appeared during the early stages of sporulation. The LC₅₀ (24 h) decreased about 10⁵-fold between t₀₋₂ and t₅, in correlation with the formation of crystalline inclusions. Heat resistance of spores appeared later than toxicity.

**INTRODUCTION**

Use of bacteria for biological control of mosquitoes, vectors of tropical diseases, is a promising alternative to chemical insecticides with their problems of pollution and insect resistance. Discovery of the highly toxic *Bacillus thuringiensis* var. *israelescens*, serotype H-14, was a promising breakthrough in this direction (Goldberg & Margalit, 1977; de Barjac, 1978). Some strains of *Bacillus sphaericus* also develop a toxic activity against mosquito larvae (Kellen et al., 1965; Singer, 1980), essentially *Culex* and *Anopheles* species. Most studies have been done on one of the more potent strains, 1593 (Singer, 1980), the toxicity of which increases during sporulation (Myers et al., 1979). Recently the isolation and characterization of toxic components from spores have been reported (Tinelli et al., 1980; Tinelli & Bourgouin, 1982; Davidson, 1983). A new *B. sphaericus* strain, 2297 (or MR4), highly toxic for *Culex* and *Anopheles* larvae, was recently isolated in Sri Lanka (Wickremensinghe & Mendis, 1980); it is characterized by large crystal-like inclusions (Wickremensinghe & Mendis, 1980; Yousten & Davidson, 1982; de Barjac & Charles, 1983). Unlike strain 1593, *B. sphaericus* 2297 is not toxic for *Aedes* larvae. Alkaline treatment of strain 2297 spores dissolves their inclusions, giving an alkaline extract which is toxic for mosquito larvae (de Barjac & Charles, 1983).

The relation between sporulation and toxicity in *B. sphaericus* strains has not been clearly established, perhaps due to a lack of appropriate sporulation media for these bacteria, which sporulate poorly in common media. We have recently defined a sporulation medium, MBS, which gives a high yield of spores and great larvicidal activity (Kalfon et al., 1983). When *B. sphaericus* was grown in this medium, the toxicity increased markedly as the spore yield increased. Using this medium, we found that strain 2297 was significantly more active than strain 1593 against *Culex pipiens* larvae. This property, together with the production of crystal-like inclusions, led us to study in detail the relation between sporulation, larvicidal activity and the biogenesis of inclusions in synchronous sporulating cultures of strain 2297.

**METHODS**

*Bacterium. Bacillus sphaericus* strain 2297 was sent to our laboratory (which is a World Health Organization Reference Center for entomopathogenic *Bacillus*) by the WHO.

*Media and culture conditions.* All studies were done in MBS liquid medium (Kalfon et al., 1983), which contains (g L⁻¹): MgSO₄, 7H₂O, 0.3; MnSO₄, 0.02; Fe₂(SO₄)₃, 0.02; ZnSO₄, 7H₂O, 0.02; CaCl₂, 0.2; tryptose (Difco), 10; yeast extract (Difco), 2; the pH was adjusted to 7.4. For solid medium Difco Bacto-agar (20 g L⁻¹) was added.
Cultures were incubated at 30°C with vigorous shaking, in 1-litre Erlenmeyer flasks containing 100 ml culture medium. We used as inoculum 5 ml of a sporulated culture that had been heat shocked (78°C, 10 min) in order to synchronize the bacterial growth. Optical density was measured in a Zeiss spectrophotometer at 650 nm. Samples of 1-4 ml were taken every hour, and kept at -70°C with 18% (v/v, final concentration) glycerol. Freezing and thawing has no effect on the morphology or toxicity of the bacterial cells, even during the early sporulation stages (M. Lecadet, personal communication). Portions of the same samples were used for bioassays, electron microscopy and studies on resistance to heat and to chloroform.

Bio-assay procedures. Ten fourth-instar larvae of Culex pipiens subsp. pipiens were put in Petri dishes containing 30 ml demineralized water. Assays were done at 25°C in duplicate, using fivefold dilutions of the bacterial cultures. Mortalities were determined at 24 h; the LC50 and LC90 (expressed as dilution of samples of culture at different ages) were calculated by probit analysis and graphed on log-probit paper; the confidence limits of LC50 were obtained by the method of Litchfield & Wilcoxon (1949).

Electron microscopy. Samples were fixed, post-fixed and embedded in resin as described previously (Charles & de Barjac, 1982) and ultrathin sections were observed at 80 kV with an Elmiskop 101 Siemens electron microscope.

Spores counts and resistance. Counts of c.f.u. were done at different culture times, by plating on solid MBS medium. Heat resistance was determined by comparing c.f.u. before and after treatment at 80°C for 10 min. Resistance to chloroform was determined by shaking the samples for 15 min with 1/3 volume chloroform in a Vortex mixer and plating the aqueous phase.

RESULTS

In MBS liquid medium, under the conditions previously described, B. sphaericus 2297 sporulates quickly: complete spore formation, with about 70% sporulation, occurred within 24 h of inoculation of the culture medium. With heat-shocked spores as inoculum, a high degree of synchronization was achieved: 83-90% of the population were in the same morphological stage, as observed by electron microscopy.

Sporulation \( t_0 \) was determined graphically from the growth curve (Fig. 1). Mean generation time during the exponential phase was about 60 min. Exponential-phase cells are shown in Fig. 2. Initiation of the forespore septum began at \( t_1 \) (Fig. 3). Septum formation was complete before \( t_2 \) (stage II of sporulation according to the nomenclature established by Ryter, 1965). The inclusions first appeared immediately afterwards (Fig. 4) at \( t_2 \). A regular striation of the inclusions was observed from this time on. Envelopment of the forespore occurred during \( t_3 \) (Fig. 5). The end of stage III, at \( t_4 \) (Fig. 6), was characterized by an irregular oval prespore and a crystal-like inclusion, 0.5 µm in width which already had the final typical shape and structure. Synthesis of exosporium and spore coat began at \( t_5 \) (Fig. 7). The two layers of the cortex, \( c_1 \) and \( c_2 \), were present at \( t_6 \) (Fig. 8). By this time the lamellar structure of the medial layer of the spore

![Fig. 1. Growth curve of B. sphaericus strain 2297 in MBS medium culture, with graphic determination of \( t_0 \) of sporulation.](image-url)
B. sphaericus 2297 sporulation and toxicity

Figs 2-4: C, crystalline inclusions; CW, cell wall; LI, lipid inclusion; M, mesosome; PM, plasma membrane; S, completed sporulation septum. The bar markers represent 0.5 μm.

Fig. 2. Vegetative cells just before division.

Fig. 3. Cells at t₁: formation of the sporulation septum (arrows).

Fig. 4. (a) First appearance of the crystalline inclusion; (b) enlargement of the inclusion, showing the regular striation.

ccoat was clearly visible and completely enveloped the forespore. At t₈ (Fig. 9), cortex and spore coats were entirely formed, and an electron-transparent zone appeared around the outer layer of the spore coat. The exosporium was completely formed by t₉ (Fig. 10). At t₁₀ (Fig. 11) the
Fig. 5. Cells at $t_3$: engulfment of the forespore.

Fig. 6. Cells at $t_4$: engulfed forespore and first appearance of the inclusion envelope.

Fig. 7. Cells at $t_5$: beginning of spore coat and exosporium synthesis.

sporangium separated and the beginning of lysis was observed (Fig. 12). Sporangia were completely lysed and spores containing inclusions were released into the medium (stage VII) by $t_15$.

Larvicidal activity of cultures of different ages against fourth-instar larvae of *Culex pipiens* was determined (Fig. 13). The $LC_{50}$ at 24 h reached $1.09 \times 10^{-7}$ (Fig. 13a, b). Toxicity of vegetative cells ($t_{0-1}$) was about 10$^5$-fold lower ($LC_{50}$ 8.4 $\times$ 10$^{-5}$). The principal decrease of $LC_{50}$ and $LC_{90}$ during sporulation occurred by $t_4$ (Fig. 13b).
Figs 8–10: C, crystalline inclusion; CX, cortex (c₁, internal dark layer; c₂, external light layer); E, exosporium; IC, inner coat; LDM, low-density material; LMC, lamellar midcoat; OC, outer coat. The bar markers represent 0.5 µm.
Fig. 8. Cells at t₈: synthesis of cortex.
Fig. 9. Cells at t₉: end of cortex and spore coat synthesis. The crystalline inclusion has reached its final size.
Fig. 10. Cells at t₁₀: end of exosporium synthesis.

Heat resistance of sporulated cells appeared later than toxicity (Table 1): at t₇, 30% of the cells were resistant, and 50% at t₁₁. Chloroform resistance was complete by t₇, but the count of chloroform-resistant cells at t₁₁ was 10% of the untreated control. This result is almost certainly an underestimate, perhaps due to the presence of resistant spores in the organic phase after chloroform treatment.

**DISCUSSION**

In this study, we found a strict correlation in synchronous liquid cultures between sporulation stages, development of spore inclusions and toxicity for mosquito larvae. This confirms the relation between sporulation and toxin genesis which was found also by using two different
Table 1. Chloroform and heat resistance of spores at different stages of sporulation

Each c.f.u. value is the average of two replicates, and is significantly different from the control. The heat and chloroform treatments are described in Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$t_{0-2}$</th>
<th>$t_{7}$</th>
<th>$t_{11}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$1.30 \pm 0.05 \times 10^9$</td>
<td>$5.05 \pm 1.65 \times 10^8$</td>
<td>$2.02 \pm 0.38 \times 10^9$</td>
</tr>
<tr>
<td>Heat</td>
<td>$3.90 \pm 0.70 \times 10^8$</td>
<td>$6.05 \pm 1.80 \times 10^7$</td>
<td>$9.70 \pm 1.00 \times 10^8$</td>
</tr>
<tr>
<td>Chloroform</td>
<td>$8.00 \pm 2.00 \times 10^6$</td>
<td>$2.69 \pm 1.10 \times 10^9$</td>
<td>$2.35 \pm 0.35 \times 10^9$</td>
</tr>
</tbody>
</table>

Figs 11 and 12: C, crystalline inclusion; CE, crystal envelope; CW, cell wall; E, exosporium; Ia, Ib, non-crystalline inclusions; MS, mature spore. The bar markers represent 0.5 μm.

Fig. 11. Cells at $t_{10}$, before lysis, showing two non-crystalline inclusions. (Ia and Ib).

Fig. 12. Cells at $t_{11}$; liberation of the spore/inclusion complex.

culture media (Kalfon et al., 1983). Industrial development of sporulation media for *B. sphaericus* may lead to an increase in toxicity, making this bacillus a very potent larvicidal agent.

The relation between the development of crystal-like inclusions and that of toxicity points to their participation in the toxicity. This was also shown by purification of these inclusion bodies (Davidson, 1983; C. Bourgouin & J.-F. Charles, unpublished data) and by alkaline extraction from spores (de Barjac & Charles, 1983). Not only are inclusion bodies toxic, but they appear to account for a major part of the toxicity.

The sporulation and morphological stages of *B. sphaericus* strain 2297 are similar to those of strain 9602 described by Holt *et al.* (1975) even though the time-scale of sporulation is shorter. This might be due to differences in medium composition.

The genesis of crystal-like inclusions in strain 2297 seems to follow the same pattern as that of *B. thuringiensis*, but the inclusions seemed to appear earlier, morphologically, during the
sporulation process in *B. sphaericus* 2297 (Ribier & Lecadet, 1973; Charles & de Barjac, 1982). The presence of inclusions in *B. sphaericus* strain 2297 might be responsible for its higher larvicidal activity in comparison with less toxic strains like 1593, which does not contain such large inclusions (Kalfon *et al.*, 1983). An hypothesis to explain this could be that biogenesis of inclusions might occur only after saturation of the toxic compound within the spores, which is thus present in larger quantities.

Our results are in agreement with the recent study of Yousten & Davidson (1982), although they worked on solid medium or in liquid medium with low sporulation level. This emphasizes the importance of sporulation medium and synchronous culture in the study of kinetics of sporulation dependent processes.

This study will be followed by one on the protein pattern during sporulation and on various asporogenous mutants, blocked at different stages. Synchronous genesis of the toxin should enable us later to study the mRNAs for toxin synthesis during sporulation and, by cloning the toxin gene, to understand the mechanism of high expression of this gene.

This investigation received support from the Vector Biology and Control Component of the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases. We thank Dr I. Larget-Thiéry for advice concerning bio-assay procedures, S. Hamon and G. Viviani for technical assistance, and the Electron Microscopy Centre of the Pasteur Institute.

**REFERENCES**


