Specific Exosporium Reaction of *Bacillus megaterium*

BY J. TOMCSIK AND JOYCE B. BAUMANN-GRACE

Institute for Hygiene and Bacteriology, University of Basel, Switzerland

SUMMARY: The sporulation of 51 strains of *Bacillus megaterium* was examined in shaken cultures in potato extract and in Grelet's defined medium. Some abnormal strains are described in which the spores germinate within the sporangia or very soon after their liberation. The spores of *B. megaterium* could be completely freed from vegetative cell remnants by treatment with lysozyme followed by thorough washing. Rabbit anti-sera produced with these treated spores did not cross-react with the vegetative organisms. On the basis of their agglutination reactions the spores of 36 strains of *B. megaterium* were divided into 5 types.

A capsule-like exosporium was detected in wet Indian ink preparations on lysozyme-treated spores of more than half of the 36 strains. The exosporium was rendered visible in the phase-contrast microscope only after the addition of homologous spore antiserum. The exosporium of *Bacillus megaterium* consists of a capsule-like slimy substance which does not derive from the sporangium but which is probably produced during spore formation. The specific exosporium and spore coat reactions are described.

The exosporium was regarded by de Bary (1885) as a pale, weakly refractile, gelatinous structure, either capsule-like or appearing as a polar appendage which forms the outermost layer of the bacterial spore. This opinion was shared by Klein (1889), who observed that even in a dense spore suspension, contiguous spores cannot be seen when the exosporium is well developed. On this basis he rejected the idea that the exosporium is merely a refractile zone. More than half a century later, in his comprehensive review on the cytology of the bacterial endospore, Knaysi (1948) stated that the outer spore coat may be a slime layer. He sometimes designates this as exosporium but considers that its presence is debatable. In the remarkable electron microscopic study made by Knaysi & Hillier (1949), in which parallel beaded fibres were discovered in the spore coat of *Bacillus megaterium*, no trace of exosporium was found. A voluminous outer coat or giant capsule surrounding the main spore wall and corresponding to the exosporium of de Bary was first observed in the sections of spores of *B. cereus* in the electron microscope by Robinow (1951, 1953) and by Chapman (1956). No such structure was observed, however, in sections of spores of *B. megaterium*, although in this species the spore coat proper consists of two membranes lying close to one another, in contrast to the single layered wall of *B. cereus* (Robinow, 1951, 1953; Chapman, 1956; Mayall & Robinow, 1957). An exosporium was also observed by Dondero & Holbert (1957) in ultra-thin sections of *B. polymixa*.

This brief review of the literature on the exosporium is necessarily incomplete, but it shows that the observations have been contradictory even in a relatively small number of species. In recent years the examination of ultra-thin sections in the electron microscope has given considerable information on

the internal structure of the spore coat, but with this method it has not been possible to detect whether the spores of *Bacillus megaterium* are surrounded by a slime layer or whether the equivalent of an exosporium is completely lacking in this species (Chapman, 1956). No progress has been made in the last 70 years about the derivation of the exosporium which, as pointed out by Robinow (1953), has been considered by some workers as a condensation of the bacterial cytoplasm. Chapman (1956) regarded even the outer spore coat as a condensation of sporangial cytoplasm. Since Defalle (1902), Mellon & Anderson (1919), Howie & Cruickshank (1940) and Lamanna (1940*a, b*, 1942) clearly showed that the vegetative antigens are serologically distinct, we felt that it might be possible to solve the problem of the derivation of the exosporium by the use of immunocytological methods (Tomcsik, 1956*a, b*). *B. megaterium* was selected for systematic study of the 'specific exosporium reaction' for two reasons: (i) because the exosporium of this species cannot be detected in the electron microscope; (ii) because the vegetative organisms can be easily digested with lysozyme (Baumann-Grace & Tomcsik, 1957*a*) in contrast to most other species of *Bacillus*, thus providing an easy and reliable method for obtaining spores free from vegetative remnants.

METHODS

Organisms. Fifty-one *Bacillus megaterium* strains previously described (Baumann-Grace & Tomcsik, 1957*b*) were used.

Media. The potato media used by Behring (1889) for sporulation, and recommended on the basis of careful studies for the same purpose by Schreiber (1896), was more recently described by Robinow (1951). Following the recommendation of Powell & Strange (1956) 1/10 volume of Gladstone & Fildes broth (1940) was sometimes added.

A defined medium used by Grelet (1950, 1951) for studies on the sporulation of *Bacillus megaterium*, in which the sole sources of carbon and nitrogen are glucose and nitrate, respectively, was also used. The medium was prepared according to Grelet except that the glucose was decreased from 4.5 to 0.5% (w/v). The final pH value of the medium was 5.8. Some of our strains did not grow at this pH value and required adjustment to pH 6.8. Other strains required the addition of 0.5% (w/v) asparagine. Liquid cultures in potato and Grelet media were obtained by rocking in $\perp$ tubes (Kay & Fildes, 1950).

Lysozyme treatment. In order to free the spores from remnants of vegetative organism, the sporulating cultures were centrifuged and the deposit resuspended in the same volume of M/30 phosphate buffer (pH 7.0). An equal volume of buffer containing 1/10,000 dilution of crystalline lysozyme (Armour) was then added and the suspension incubated at 37° for 30 min. or until no vegetative remains were detectable microscopically. The spore suspensions were then chilled immediately and washed 5 times with distilled water in the cold. Previous observations had shown that, on the basis of the lysozyme sensitivity of young vegetative organisms, strains of *Bacillus megaterium* can be divided into two groups (Baumann-Grace & Tomcsik, 1957*a*). In the present

work lysozyme at 1/20,000 (w/v) was usually sufficient to remove the vegetative remnants from the sporulating organisms of both groups within 30 min.

'Vegetative' anti-sera were mostly those used previously (Baumann-Grace & Tomcsik, 1957*b*). They were prepared from 16 hr. cultures on Gladstone & Fildes agar, which is unfavourable for sporulation.

'Spore' anti-sera. Moderately thick suspensions of lysozyme-treated spores were washed 5 times with distilled water and freeze-dried in 2 ml. volumes. The spores were resuspended in 2 ml. saline for each injection. Rabbits were inoculated intravenously with 6-8 doses at 3- to 4-day intervals between each dose and were bled 7-8 days after the last injection. The resulting antisera agglutinated at a dilution of 1/640 or higher.

Spore agglutination. For this purpose the centrifugation method was used, the advantage being that the results are easier to read and incubation at 37°, which might lead to germination of the spores, is avoided. The tests were set up in the usual way with 0.2 ml. serum dilution and 0.2 ml. spore suspension and incubated at 4° for 30-60 min. The tubes were then centrifuged immediately for 4 min. and the results read by gently tapping the tube to shake up the deposit.

Specific sporangium reaction. This was carried out with 'vegetative' antisera as described previously (Tomcsik & Baumann-Grace, 1958).

Specific exosporium reaction. (a) One loopful of undiluted serum was applied to the edge of the coverslip of a wet mount of unstained spores and allowed to flow under the coverslip by capillary action. The preparations were examined by phase contrast. (b) Preparations obtained by mixing one loopful of spore suspension with antiserum on a slide and covering with a coverglass. This second method was used for the systematic examinations when each suspension was examined with 8 or more spore antisera. Readings taken immediately or after storing the preparations for several hours in a wet chamber were essentially the same, and were only complicated in a few instances by germination of the spores in serum mixture after a few hours at room temperature. Too high a concentration of spores in the suspension occasionally led to negative results; for instance strain Mg 29 gave a negative exosporium reaction when the optical density of the suspension was greater than BaSO₄ standard (McFarland) no. 4.

RESULTS

Production of spores and their purification from vegetative cell remnants

Since it was found that the growth and sporulation of several strains of *Bacillus megaterium* were as good in potato extract in rocked $\perp$ tubes at 37° or 28° whether or not 1/10 vol. Gladstone & Fildes (1940) broth was added, spore production was usually carried out in simple potato extract. Twelve out of 51 strains were asporogenous. This high proportion may be due to the fact that the strains have been subcultured monthly on Gladstone & Fildes

agar since we isolated or received them about three years ago. This medium was unfavourable for sporulation (Tomcsik & Baumann-Grace, 1958) and may therefore have favoured the production of asporogenous varieties. Of the remaining 39 strains, 29 sporulated rapidly in potato medium and showed 80–100 % spores which began to be liberated in 16–17 hr. at 28°. Compared with potato extract, maximum growth, spore production and liberation of the spores were obtained about 24 hr. later on Grelet's defined medium, even after re-incubation and addition of 0.5 asparagine. The most favourable time for harvesting the spores was at 23–24 hr. in Grelet's medium.

A few strains behaved unexpectedly in that abundant sporulation occurred only on potato agar and not in the shaken liquid medium. The 'M' strain (Tomcsik & Guex-Holzer, 1951) was most noticeable in this respect; only 1–2 % spores were formed after 3–4 days in shaken potato or Grelet's medium, but on potato agar almost 100 % sporulation was obtained in 2 days. The only further abnormality found in the sporulation of this strain was that no liberation of the spores took place. Even after incubation for 10 days there was no sign of disintegration of the long chains. Addition of glutamyl-polypeptide antibody or specific cell-wall antibody showed that the surface structures of the sporangia were still intact. The spores were completely liberated by lysozyme and remained refractile when stored at 4°.

Another phenomenon unknown to us was displayed by 8 of our strains (Mg 2, 11, 16, 21, 22, 27, 29, 40). Although typical refractile spores were found in the shaken potato cultures after 15–16 hr., they did not remain so but were rapidly converted, often within an hour or less, into large black ovoid bodies. Depending on the strain, 10–100 % of the organisms in the culture consisted of these elements. Seen in the phase-contrast microscope they resembled spores in the first stage of germination as described by Pulvertaft & Haynes (1951). Since these bodies were resistant to lysozyme they may represent incompletely germinated spores. Their production may be a hereditary property; for example, strain Mg 11 formed these black bodies regularly whether cultured in potato or in Grelet medium. An incomplete stage in spore production is shown in Pl. 2, fig. 5, in which most of the cellular elements consist of these ovoid structures. Contrary to their usual appearance, however, these structures are pale, suggesting some form of lysis. As can be seen from Pl. 2, fig. 7, these structures were resistant to lysozyme, whereas the vegetative organisms were completely dissolved. Addition of homologous vegetative antiserum to a lysozyme-treated washed suspension revealed no vegetative remnants.

*Immunologically specific reactions on the surface of
Bacillus megaterium spores*

On examination of lysozyme-treated washed spores in wet indian ink preparations, no trace of an exosporium was seen in more than half the strains; in the remainder the spores were surrounded by a distinct capsule-like zone into which the indian ink particles did not penetrate. With 9 of the strains the visibility of this structure was considered to be very good

(illustrated in Pl. 1, fig. 1). The preparations were made by inverting the cover-slip on to the mixture of indian ink and organisms and gently pressing it down with filter-paper. By increasing the pressure, the spores lost their refractility and increased in size, appearing as large black elements. These structures still retained their exosporium (see Pl. 1, fig. 2), although it was somewhat decreased in size. Despite the similarity in morphology between the black elements formed by crushing the spores and those produced by cultures of strain Mg 11 (Pl. 2, fig. 5), there is no relationship between the two.

An attempt was made to demonstrate the capsule-like exosporium in the phase-contrast microscope by using specific spore antiserum. Spore antisera against eight different strains of *Bacillus megaterium* were made; these agglutinated the homologous spores at a dilution of 1/64 and usually in 1/2560. The vegetative organisms were, however, not agglutinated in 1/20 dilution of the spore antisera and no specific cell-wall reaction could be elicited. After mixing one loopful of spore suspension, of a strain which showed a well-defined exosporium in indian ink, with one loopful of homologous spore antiserum, the exosporium became distinctly visible (Pl. 1, fig. 4). This reaction was specific; no trace of exosporium was seen without addition of antiserum or when homologous 'vegetative' or heterologous spore antiserum was substituted for homologous spore antiserum. For this reason the reaction was designated as the 'specific exosporium reaction', since it appeared to be similar in nature to the specific capsular reaction (Tomcsik, 1956*b*). This reaction was also carried out with a sporulating suspension of strain Mg11 before (Pl. 2, fig. 6) and after (Pl. 2, fig. 8) lysozyme treatment. Although addition of homologous 'vegetative' antiserum gave a strong vegetative cell-wall reaction with isolated cell walls of strain Mg11, its interaction with the intact bacilli was hardly visible (Pl. 2, fig. 5). No reaction was detected on the intact spores or on incompletely germinated spores, whether intact or partially empty (Pl. 2, figs. 5, 7). The last-mentioned elements, however, gave a specific spore-coat reaction on adding specific spore antiserum (Pl. 2, figs. 6, 8) and a weak exosporium reaction was observed on the refractile spores, although the latter can only be seen clearly with a magnifying glass (Pl. 2, fig. 8). Spore walls of other strains obtained by shaking intact spores in the Mickle disintegrator also showed a specific spore-coat reaction on adding homologous spore antiserum. This reaction is considered to be analogous to the specific cell-wall reaction (Tomcsik & Guex-Holzer, 1954).

Serological types of Bacillus megaterium spores

Spores of 4 strains selected at random (Mg 19, 45, 11, 17) were used to obtain our first spore antisera in rabbits. By cross-agglutination tests with the resulting antisera it was found that they represented 4 distinct spore types which showed no cross-reaction with one another. By agglutination tests between these antisera and spores of the remaining 35 strains, 24 were found to be agglutinated by one or other of the antisera. Of the remaining 11 strains which could not be typed, 4 (Mg 1, 6, 35 and 39) were again selected at random for rabbit immunization. However, contrary to the results with the first

4 antisera produced, the later antisera gave cross-reactions to some extent, but only 3 strains could not be agglutinated with one or more of the 8 spore antisera obtained. The specific exosporium reaction was always carried out before the agglutination test in order not to be influenced subjectively by knowing the results of the latter. Since it is difficult to evaluate this morphological reaction in strains which have a poorly developed exosporium, the initial readings were always carried out by one of us (J.T.).

Table 1. *Serological types of Bacillus megaterium spores established with agglutination and with specific exosporium reactions*

Spore type	Spores used for immunization	Agglutination*	Specific exosporium reaction	Exosporium visibility in indian ink		
				Negative or indistinct	Distinct	Very good
A	Mg 19	++	Distinct	.	2†	19, 25, 27, 30a†
A	Mg 19	++	Weak	9, 12, 13, 29	M	.
A	Mg 19	++	Negative	20, 41, 46, 51	.	.
A	Mg 19	+	Negative	48	.	.
B	Mg 45	++	Distinct	.	40, 45, 47	16, 21, 24, 31
B	Mg 45	++	Weak	37, 48	36	.
C	Mg 11	++	Distinct	.	11	—
D	Mg 17	++	Weak	17	.	—
D	Mg 17	++	Negative	53	.	.
E	Mg 1, 6, 35, 39	++	Distinct	.	6, 35	42
E	Mg 1, 6, 35, 39	++	Weak	39	22	.
E	Mg 1, 6, 35, 39	++	Negative	1, 3, 8		

* Agglutination: + positive in dilution 1/100; ++, positive in dilution 1/160 or more.

† No. of *B. megaterium* strain in our collection.

The results of the spore typing on the basis of the specific exosporium and agglutination reactions are summarized in Table 1. To facilitate the discussion, the first 4 spore types have been designated A, B, C, and D. Spores of 15 strains were grouped as A, 10 as B, 1 as C and 2 as D. The last spore type, E, contains several subtypes, some being related to group D; their complicated cross-relationship is summarized in Table 2. Table 1 also shows that the specific exosporium reaction can be used for serological typing when the exosporium is sufficiently well developed as judged by indian ink preparations. On the whole, it was found that the visibility of the exosporium in indian ink preparations was best with type B spores; the exosporium was correspondingly good with these strains.

The majority of the strains of the small-cell variety of *Bacillus megaterium* (Baumann-Grace & Tomcsik, 1957b) were asporogenous, but those which formed spores belonged entirely to type A. Some of the large-celled varieties, however, also belong to this group.

Table 2. *Selected tests comparing the strict specificity of Bacillus megaterium spore types A, B, C, with the irregular cross reactions of types D and E*

Strain no.	Exosporium visibility in	Spore serum types							
		A	B	C	D	E			
		19	45	11	17	1	6	85	89
Mg 19	indian ink	3, + + +	-	-	-	-	-	-	-
Mg 45	Very good	-	2, + + +	-	-	-	-	-	-
Mg 11	Distinct	-	-	2, + + +	-	-	-	-	-
Mg 17	Distinct	-	-	-	1, + + +	-	-	-	-
Mg 3	Indistinct	-	-	-	-	-	-	-	-
Mg 18	Negative	-	-	-	-	-	-	-	-
Mg 1	Negative	-	-	-	-	-	-	-	-
Mg 39	Indistinct	-	-	-	-	-	-	-	-
Mg 6	Indistinct	-	-	-	-	-	-	-	-
Mg 22	Distinct	-	-	-	1, +	-	2, + +	2, + +	1, + +
Mg 85	Distinct	-	-	-	2, + +	-	1, + +	1, + +	1, + +
Mg 42	Very good	-	-	-	-	-	-	2, + + +	-
	Very good	-	-	-	2, + +	-	2, + + +	1, + + +	2, + +

—, specific exosporium reaction, or agglutination negative; 1, specific exosporium reaction weak; 2, specific exosporium reaction distinct; 3, specific exosporium reaction strong; +, agglutination in 1/100 antiserum dilution; ++, agglutination in 1/160-1/320 antiserum dilution; + + +, agglutination in 1/640 antiserum dilution or more; 0, not examined.

DISCUSSION

In the present work the term exosporium has been given to the slimy, capsule-like outer layer which was observed on lysozyme-treated washed spores of more than half of our strains of *Bacillus megaterium* in wet indian ink preparations. A description of a similar structure is to be found in de Bary's book (1885), but he gave no name to it. The term exosporium was first used by Flügge (1886) and since then by many authors to designate widely different substances or membranes on the surface of the spores. We have, however, kept the term exosporium for *B. megaterium* spores, instead of inventing a new expression, in order to avoid increasing the confusion in the nomenclature of the various spore layers.

The capsule-like structure described here does not appear to correspond to either of the spore membranes found in ultra-thin sections of *Bacillus megaterium* spores. Robinow (1951, 1953) did not find an exosporium on spores of this species and Chapman (1956) stated even more categorically that *B. megaterium* lacks an exosporium. Our observations, however, suggest that although the exosporium of *B. megaterium* can be demonstrated by the very simple indian ink technique, it is most probably of a slimy nature and therefore not suitable for detection by electron microscopy.

According to Robinow (1953) most investigators believe that the exosporium is derived from the vegetative cell; Chapman (1956) considered even the outer spore coat to be a condensation of bacterial cytoplasm. We have been able to show with certainty that the capsule-like exosporium in *Bacillus megaterium* is a specific spore substance and is not derived from the vegetative cell.

Mellon & Anderson (1919) demonstrated that the spore and vegetative stages in *Bacillus subtilis* are serologically distinct, but also pointed out the difficulties of producing exclusively spore antisera unless the disorganized remnants of the vegetative cells are removed from the spore antigen used. Howie & Cruickshank (1940) and Lamanna (1940*a, b*) were apparently able to overcome these difficulties. With *B. megaterium* we found that this problem could be easily solved since the vegetative cells of this species are susceptible to lysozyme (Baumann-Grace & Tomcsik, 1957*a, b*), whereas the spores are completely resistant. Treatment of sporulating cultures with lysozyme and subsequent washing appears to remove all traces of 'vegetative' antigens since no 'vegetative' antibodies could be detected in the resulting spore antisera and the treated spores did not react with the specific 'vegetative' antisera. We found with the 'specific exosporium reaction' that the slimy, capsule-like exosporium of *B. megaterium* can be regarded as corresponding to the outermost layer of the spore and not to vegetative cell material. A similar reaction, which was designated the 'specific spore coat reaction' was observed on the empty spores. Like the specific cell-wall reaction, the specific spore coat reaction cannot be demonstrated on intact organisms. For this purpose the spore coats must be separated from the spore contents either by shaking in the Mickle disintegrator or by making use of empty, abnormally germinated

spores, before the reaction can be observed. A similar reaction which has been found on the spores of *B. cereus* will be described fully elsewhere.

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(Facing p. 674)

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EXPLANATION OF PLATES

Phase-contrast photomicrographs of *Bacillus megaterium* spores. Magnification *c.* $\times 2500$.

PLATE 1

- Fig. 1. Strain Mg 42; 24 hr. potato extract culture after dissolution of vegetative cell remnants with lysozyme. Wet indian ink preparation showing large, capsule-like exosporium on typical refractile spores.
- Fig. 2. Strain Mg 45; preparation as fig. 1. The coverslip has been firmly pressed down, crushing the spores which appear as black ovoid bodies surrounded by an exosporium.
- Fig. 3. Strain Mg 24; 38 hr. shaken culture from neutralized Grelet medium + 0.5 % asparagine after treatment with lysozyme. Wet mount in physiological saline showing refractile spores surrounded by a diffraction zone. No exosporium visible.
- Fig. 4. Same spore suspension as fig. 3 after addition of type B spore antiserum, showing a typical specific exosporium reaction.

PLATE 2

- Fig. 5. Strain Mg 11; 40 hr. shaken culture from Grelet medium (pH 5.8) + 0.5 % asparagine after the addition of specific 'vegetative' antiserum Mg 11, showing many intact vegetative organisms, 5 refractile spores and numerous empty black ovoid bodies which do not react with the antibody. Wet mount in saline.
- Fig. 6. A similar preparation as in fig. 5 except that specific spore antiserum has been added instead of 'vegetative' antiserum. A specific spore coat reaction can be seen on the black ovoid bodies.
- Fig. 7. The same suspension as in fig. 5 after total elimination of the vegetative organisms with lysozyme, and addition of 'vegetative' cell antiserum Mg 11.
- Fig. 8. The same suspension as in fig. 7 to which strain Mg 11 spore antiserum has been added in place of 'vegetative' antiserum. A specific spore coat reaction can be seen on the black ovoid bodies and a weak exosporium reaction on the refractile spores.

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