Germination Under Alkaline Conditions and Transmission of Alkali Resistance by Endospores of Certain Strains of *Bacillus cereus* and *Bacillus circulans*

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SUMMARY

Endospores of *Bacillus cereus* Frankland and Frankland strain R (which is able to grow under highly alkaline conditions) germinated at a higher pH value than spores of an alkali-sensitive strain, Mu-3055. Spores of *B. circulans* Jordan strain Ru 38 (able to grow at pH 11.0) germinated at pH 11.0. Spore germination at any pH value was generally followed by outgrowth to the vegetative rod. Outgrowth of germinated spores of *B. cereus* R at pH 10.0 and of those of *B. circulans* Ru 38 at pH 11.0 took place at 30° but not at 37°. Growth from a vegetative inoculum of *B. cereus* R at pH 10.0 or 10.3 took place more readily at 30° than at 37°. The ability of the vegetative forms of both bacterial species to grow under alkaline conditions was transmitted through the endospore.

INTRODUCTION

Two strains of alkali-resistant *Bacillus* species have recently been described: a strain of *B. cereus* Frankland and Frankland able to grow in media of pH 10.3 (Kushner & Lisson, 1959), and a strain of *B. circulans* Jordan able to grow at pH 11.0 (Chislett & Kushner, 1961). Previous work was largely concerned with the effect of alkaline media on the vegetative growth of these organisms, but gave no information about the effect of such alkaline media on endospore germination. We have now examined the effect of alkaline conditions on the development of the vegetative form from the endospore and have examined how the ability of the vegetative form to grow under alkaline conditions is transmitted through the endospore.

METHODS

Organisms. *Bacillus cereus* strain Mu-3055 (non-adapted: sensitive to alkali) and *B. cereus* strain R (adapted from Mu-3055 and resistant to alkali); both described by Kushner & Lisson (1959); *B. circulans* strain Ru 38 (maintained routinely on buffered nutrient agar (pH 7.4) but capable of growth at pH 11.0); described by Chislett & Kushner (1961).

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Culture media. The media used were: phosphate-buffered nutrient broth (BNB) and phosphate-buffered nutrient agar (BNA) of final pH values 7.4 and higher, prepared as before (Kushner & Lisson, 1959; Chislett & Kushner, 1961).

Preparation of spore suspensions. The three strains were subcultured several times in BNB (pH 7.4), with incubation at 30°, before finally spreading 1 ml. of 6 hr. culture on the surface of 50 ml. BNA (pH 7.4) in a 20 oz. bottle; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ had been added to the BNA medium to a final concentration of 5 p.p.m. Mn^{++} , and additional agar (Davis, New Zealand) was added to give a final concentration of 3% (w/v) agar in the medium. The *Bacillus cereus* strains were incubated at 30° for 7 days and the *B. circulans* strain for 14 days. Spores were harvested by washing off the growth with sterile distilled water and glass beads. The resulting suspension was washed six times in sterile distilled water at 5° and the deposit, consisting almost entirely of spores, resuspended in 0.01 M-phosphate buffer (pH 7.4). After making viable spore counts on nutrient agar plates, the spore suspensions were diluted with 0.01 M-phosphate buffer (pH 7.4) to contain 10^9 viable spores/ml. and held at 5° until required. Before making viable counts and before use in the experiments described in Table 1, the portion of each spore suspension used was heated at 80° for 15 min., then cooled rapidly in an ice bath and held at 5° until required. In the experiments shown in Table 2 and in those with shaken cultures of *B. circulans*, the spores used had been prepared in a similar manner, with the following variations in technique: BNA medium (pH 7.4) with 5 p.p.m. Mn^{++} added was used; incubation was for 7 days at 37° instead of at 30°. At the end of this time there was a high degree of sporulation and comparatively few vegetative forms remained. The washed suspensions of spores + bacteria were heated for 1 hr. at 60°, adjusted to a concentration of 3×10^8 viable spores/ml. and stored in distilled water at 5°.

Germination of spores at pH 7.4 and at alkaline pH values. Spore suspensions were added to 10 ml. BNB medium in 6 in. $\times$ $\frac{5}{8}$ in. tubes at pH values from 7.4 to 11.0, to give a final concentration of 10^7 viable spores/ml. Incubation was at 30° and 37°, and examinations were made at 2, 4, 6, 8, 10, 12 and 24 hr. Spore germination was detected by examining the suspension in a haemocytometer, by using a 4 mm. phase contrast objective (see Berger & Marr, 1960). Under phase contrast, the ungerminated spores appeared refringent, thus allowing a direct spore count. The staining method of Conklin (1934) with malachite green and mercurochrome, whereby ungerminated spores stained green and germinated spores and vegetative forms red, gave a good correlation with the results obtained by the haemocytometer and phase contrast method. For quantitative determinations of the number of germinated and ungerminated spores/ml., and to estimate percentage germination, 0.01 ml. spore suspension was delivered by a dropping pipette on to a marked area of 1 cm.² on a slide and the suspension stained by Conklin's method. As soon as microscopic observation showed that the spores were germinating, the pH values of the cultures and of control tubes of uninoculated media were measured. The results have been recorded as the relative extent of germination at the earliest time this process was observed (see Table 1). Once germination of even a small proportion of spores had taken place, subsequent vegetative cell growth lowered the pH value of the medium (Kushner & Lisson, 1959; Chislett & Kushner, 1961). For this reason, information about the time-course of germination of a spore population at a given pH value could not readily be obtained in these experiments.

Outgrowth. The successive changes leading to the formation of a vegetative rod from a germinated spore have been termed 'outgrowth' (Campbell, 1957). This process has been recorded here qualitatively simply by noting the first appearance of vegetative rods in the culture medium.

Transmission of alkali resistance. Growing cultures in BNB medium (pH 7.4) resulting from spore germination at this pH value were inoculated into duplicate tubes of BNB medium at different pH values between 7.4 and 11.0. The inocula were two 4 mm. loopfuls of an 18–21 hr. culture at 37°. The tubes were incubated at 30° or 37° and examined at intervals. As soon as growth was visible as turbidity the cultures were examined microscopically, and pH measurements were made of the cultures and of control tubes of uninoculated media.

RESULTS

Spore germination and outgrowth

At pH 7.4, both alkali-sensitive and alkali-resistant strains of *Bacillus cereus* showed considerable germination and outgrowth after 2 hr. at 30° or 37° (Table 1). At pH 9.5, spore germination and outgrowth took place much more rapidly and extensively with the alkali-resistant strain than in the alkali-sensitive strain. Both processes were seen at pH 10.0 with *B. cereus* R, but not with *B. cereus* Mu-3055. Germination did not take place at pH values higher than 10.0 with either strain. At the highest pH values at which germination was possible for each strain, this process and/or outgrowth took place more slowly, if at all, at 37° than at 30°.

Table 1. *Germination and outgrowth of spores of alkali-sensitive and alkali-resistant Bacillus cereus and of alkali-resistant B. circulans*

Organism	pH value	Temperature	Spore germination*	Outgrowth†
<i>B. cereus</i> Mu 3055 (alkali-sensitive)	7.4	30°	+++ at 2 hr.	+ at 2 hr.
		37°	+++ at 2 hr.	+ at 2 hr.
	9.5	30°	(+) at 4 hr.	+ at 4 hr.
		37°	(+) at 24 hr.	+ at 24 hr.
	10.0, 10.3	30°	— at 24 hr.	— at 24 hr.
		37°	— at 24 hr.	— at 24 hr.
<i>B. cereus</i> R (alkali-resistant)	7.4	30°	+++ at 2 hr.	+ at 2 hr.
		37°	+++ at 2 hr.	+ at 2 hr.
	9.5	30°	+++ at 2 hr.	+ at 4 hr.
		37°	++ at 2 hr.	+ at 4 hr.
	10.0	30°	(+) at 12 hr.	+ at 24 hr.
		37°	(+) at 12 hr.	— at 24 hr.
	10.3, 10.7	30°	— at 24 hr.	— at 24 hr.
		37°	— at 24 hr.	— at 24 hr.
<i>B. circulans</i> Ru 38 (alkali-resistant)	7.4	30°	+ at 2 hr.	+ at 4 hr.
		37°	+ at 2 hr.	+ at 4 hr.
	9.5, 10.0,	30°	+++ at 2 hr.	+ at 2 hr.
		37°	+++ at 2 hr.	+ at 2 hr.
	10.3	30°	+ at 2 hr.	+ at 4 hr.
		37°	+ at 2 hr.	+ at 4 hr.
	10.7	30°	+ at 2 hr.	+ at 4 hr.
		37°	+ at 4 hr.	— at 24 hr.

* % spores germinated reported as follows: (+) 5%, + 5–20%, ++ 20–50%, +++ > 50%.

† Outgrowth = appearance of rods at time indicated.

The results obtained with spores of the alkali-resistant *Bacillus circulans* strain Ru 38 differed markedly from those obtained with the *B. cereus* strains in that germination was more extensive after 2 hr. at pH values from 9.5 to 10.3 than at pH 7.4. Outgrowth was observed within 2 hr. with the pH range 9.5–10.3, but was not observed until 4 hr. at pH 7.4. Results at pH 10.7 or 11.0 were similar to those found at pH 7.4, except that at pH 11.0, outgrowth took place at 30° but not at 37°.

Transmission of alkali resistance

Following spore germination at pH 7.4, the vegetative form of *Bacillus cereus* strain Mu-3055 in standing tubes showed growth after 4 hr. at pH 7.4 and pH 9.5, at 30° and at 37°; no growth occurred at pH 10.0 or higher. *B. cereus* strain R showed growth after 4 hr. at pH 7.4 and pH 9.5, at 30° and at 37°. At pH 10.0 and pH 10.3, growth was observed within 12 hr. at 30°, but not until 24 hr. at 37°. *B. circulans* strain Ru 38 showed growth after 4 hr. at 37° in the pH range 7.4 to 11.0, but at 30° growth was not observed in this pH range until after 6 hr. incubation.

Table 2. *Effect of pH value on the growth of alkali-sensitive and alkali-resistant Bacillus cereus following spore germination at pH 7.4*

In Expts. 2 and 3 one drop of spore suspension of strain Mu-3055 or strain R was added to 10 ml. BNB (pH 7.4) medium and incubated 4 hr. with shaking at 37°, to an optical density of 0.7–0.8. Three drops of each culture were added to tubes of BNB medium of different pH values. Shaking and measurements of optical density were as described by Kushner & Lisson (1959).

Data for Expt. 1 from Table 3 of Kushner & Lisson (1959).

Expt.	Inoculum	pH value	Sensitive (strain Mu-3055)	Resistant (strain R)
			$T_{0.4}$ (hr.)*	
1	Bacteria maintained on plates (mixture of spores and vegetative forms)	9.5	16	7.2
		9.8	> 25	11.0
		10.0	> 25	24.8
		10.1	> 25	> 25
2	Bacteria obtained by spore germination, 2 weeks after preparation of spores	9.4	9	5
		9.8	> 24	10
		9.9	> 24	20
3	Bacteria obtained by spore germination, 16 weeks after preparation of spores	9.4	10	4
		9.8	> 24	21
		9.9	> 24	> 24

* Time at which cultures reached a (net) optical density of 0.4.

An examination of the effects of pH value on the vegetative growth of *Bacillus cereus* derived from spores was also made in shaken cultures at 37° (Table 2). Once growth had begun there was, as before noted (Kushner & Lisson, 1959), little effect of the initial pH value on the growth rate; and the growth rates observed were similar to those reported by Kushner & Lisson (1959). Thus vegetative forms derived from spores 2 weeks after sporulation had retained full alkali resistance. After an additional 14 weeks storage of the spores in distilled water at 5°, however, there was some loss of alkali resistance; the vegetative forms from germinated spores were no longer able to grow at pH 9.9 in 24 hr.

In an experiment with spores of the alkali-resistant *Bacillus circulans*, an inoculum of the vegetative form was prepared by incubating these spores overnight on a BNA medium plate (pH 7.4), and a series of tubes containing BNB medium of different pH values was inoculated and shaken as before (Chislett & Kushner, 1961). The growth curves obtained were virtually identical with those given by the alkali-resistant *B. circulans* which had been maintained at pH 7.4 and transferred as a mixture of spores and vegetative forms (Chislett & Kushner, 1961, Fig. 1a).

These results indicated that growth of the alkali-resistant bacteria took place at a slightly higher pH value in standing than in shaken cultures. However, in standing cultures at 30° and 37° *Bacillus cereus* R showed growth at pH 10.3 (as above) but not at pH 10.7; and *B. circulans* Ru 38 showed growth at pH 11.0 (as above) but not at pH 12.0. The highest pH value at which growth was observed for *B. circulans* Ru 38 was pH 11.4 (pH value of standing culture) after 18 hr. at 37°.

DISCUSSION

In obtaining *Bacillus cereus* strain R the culture method used was designed to select vegetative forms with ability to grow at higher pH values than could the original culture, rather than to select spores that could germinate at higher pH values (Kushner & Lisson, 1959). It was conceivable that such alkali-resistant vegetative forms might produce spores whose germination was as sensitive to highly alkaline conditions as were spores of the original alkali-sensitive cultures. It has now been found that spores of this selected alkali-resistant strain can germinate and develop into vegetative rods at higher pH values than spores of the original strain Mu-3055. The upper pH limit for spore germination of *B. cereus* strain R spores is slightly lower than the upper limit for growth.

Spores of the alkali-resistant *Bacillus circulans* Ru 38 can also germinate in highly alkaline media; when incubation is at 30°, they can germinate in a medium of the highest pH value at which growth is possible. Germination and outgrowth took place more rapidly at pH 9.5–10.3 than at pH 7.4; and it should be noted that vegetative growth of *B. circulans* Ru 38 began after a shorter lag period at pH 9.9 than it did at pH 7.4 (Chislett & Kushner, 1961). The fact that spores of the bacteria studied had the ability to develop into vegetative forms and these to multiply at high pH values suggests that the alkali resistance of these processes may have a common physiological basis.

In so far as it has been studied, the effect of temperature on the processes of growth and spore germination at different pH values seems complex. Although a temperature of 37° could cause growth to take place more rapidly than a temperature of 30°, at the highest pH values at which germination and growth were possible, germination, outgrowth and vegetative growth of *Bacillus cereus* R and outgrowth of *B. circulans* Ru 38 took place more rapidly, or only at 30°. Thus, despite its ability to accelerate growth, the higher temperature also increased the toxic effects of highly alkaline media.

The alkali-resistant *Bacillus circulans* strain Ru 38 has been deposited with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, and allotted the number NCIB 9218.

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