A Strain of *Bacillus circulans* Capable of Growing under Highly Alkaline Conditions

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(Received 28 July 1960)

SUMMARY

An organism capable of growing at pH values up to 11·0 appeared, presumably as a contaminant, in the course of 'training' experiments designed to produce alkali-resistant strains of *Bacillus cereus* Frankland & Frankland; this organism has now been characterized as *B. circulans* Jordan. Of 26 other strains of *B. circulans* studied, none grew at pH 10·7. The alkali-resistant *B. circulans* strain showed little loss of resistance after many transfers on neutral medium. When it grew in alkaline media it lowered the pH value of these media.

INTRODUCTION

It was reported previously (Kushner & Lisson, 1959) that strains of *Bacillus cereus* Frankland & Frankland, an organism that will not normally grow above pH 9·5, could be 'trained' by successive subcultures in media of gradually increasing pH value to grow at pH 10·8. The training process was a long one, requiring more than 50 transfers during more than a month. In four experiments it was not possible to make *B. cereus* grow at pH values higher than 10·8, even when it was transferred daily in media of this pH value for an additional month. In one experiment, however, the culture, which had been slowly trained to grow at pH 10·0 took on, in five transfers, the ability to grow at pH 11·0. Bacteria removed when the culture had acquired the ability to grow at pH 10·0 appeared, microscopically and in colonial form, like the alkali-resistant *B. cereus* described by Kushner & Lisson (1959). Those removed after the culture grew at pH 10·7 and subcultured on neutral or alkaline buffered agar, however, were quite distinct from *B. cereus* in that the rods were thinner than those of *B. cereus* and, on agar, formed small translucent colonies. This organism has now been kept for more than 2 years by transfer on neutral or alkaline buffered nutrient agar. It is not considered likely that it was derived from *B. cereus*, but it is thought to be a contaminant which entered during the training procedure. Because of its ability to grow under highly alkaline conditions, it was further investigated.

METHODS

The conditions of growth for the experiments reported in Fig. 1 were those described earlier (Kushner & Lisson, 1959). Phosphate-buffered nutrient broth (BNB) and phosphate-buffered nutrient agar (BNA), final pH 7·4, were prepared

by dissolving dried nutrient broth and dried nutrient agar (Difco) in 0.1 M phosphate buffer (pH 7.5) to give a medium of final pH 7.4. Alkaline media were prepared by the addition of sterile N-NaOH, according to previously determined titration curves. For examining this organism, the methods and media used were similar to those of Smith, Gordon & Clark (1952) and Knight & Proom (1950). Tests were made on BNA (pH 7.4 and 10.7); BNB (pH 7.4 and 10.7); in routine laboratory media (pH 7.0); in media made up in 0.1 M-phosphate buffer (pH 7.5). Incubation was at 37°, except where otherwise stated.

Spore sculpture. The surface configuration of spores was kindly examined for us by Mr D. E. Bradley by the electron microscope and the carbon replica method of Bradley & Williams (1957).

RESULTS

Morphological characters of the highly alkali-resistant organism

The organism conforms in all respects to the description of *Bacillus circulans* given by Smith *et al.* (1952). No difference was found in the morphology on BNA (pH 7.4) and nutrient agar (pH 7.0). On BNA (pH 10.7) at 24 hr. the rods were slightly thinner and longer than on BNA (pH 7.4). The swollen sporangia and oval spores were similar to those formed on BNA (pH 7.4).

Spore sculpture. The sculpture of the spore surface was found by Mr D. E. Bradley by using the electron microscope and carbon replica method of Bradley & Williams (1957) to be smooth.

Growth characters

No difference was obtained from the description of *Bacillus circulans* by Smith *et al.* (1952) apart from the inability to grow anaerobically. No growth was obtained on the surface of nutrient agar and BNA + glucose (1 %, w/v) slopes or streak plates in McIntosh and Fildes jar (aerobic controls positive), and surface growth only in BNA and nutrient agar + glucose (1 %, w/v) shake tubes. The organism appears therefore to be an obligate aerobe. Colonial form and amount of growth appeared similar on BNA (pH 7.4) and BNA (pH 10.7).

Physiological characters

The organism conforms to the description of *Bacillus circulans* by Smith *et al.* (1952) apart from the inability to produce acid from glucose. No growth was obtained on inorganic salts basal medium + sugar up to 7 days at 30° or 37°. Growth but no acid was obtained on nutrient agar slopes with bromcresol purple indicator + glucose, xylose, arabinose, sucrose, mannitol, maltose or raffinose after 7–14 days at 30° or 37°.

Nutrition. The nutritional requirements of this organism were satisfied by a medium containing salts, acid-hydrolysed casein (Knight & Proom, 1950) thiamine and biotin. An ammonia basal medium (Knight & Proom, 1950) did not replace the casein basal medium.

Examination of strains of Bacillus circulans for alkali resistance

The above evidence indicated that this organism could be classified as a strain of *Bacillus circulans* Jordan. A collection of 26 strains of *B. circulans* from different sources was therefore examined to assess the occurrence of alkali resistance in this

species. Fourteen strains from the Wellcome Research Laboratory collection (Knight & Proom, 1950) and 12 isolated from soil (by M.E.C.) were tested for ability to grow in alkaline media. The cultures were maintained routinely on nutrient agar (pH 7.0) and were subcultured several times in BNB (pH 7.4) at 30° and 37° before testing.

Cultures (24 hr.) in BNB (pH 7.4) at 30° and 37° were inoculated by adding two 4 mm. loopfuls to BNB at pH 7.4 and 10.7. Alkali resistance was indicated by growth in BNB at pH 10.7; results were recorded after growth for 24 hr. at 30° and 37°. None of the 26 strains tested grew in BNB at pH 10.7 in 24 hr.; with the exception of one strain (CN3878), all grew within 24 hr. in BNB at pH 7.4.

Fig. 1 *a, b*. The effect of pH value on growth of alkali-tolerant *Bacillus circulans*. *a*, Bacteria maintained for 1 year at pH 7.4; *b*, bacteria maintained for 1 year at pH 10.7. Bacteria were grown for 20 hr. on BNA of pH 7.4 (*a*) or 10.7 (*b*), resuspended in BNB (pH 7.4) and an inoculum equiv. 0.025 mg. dry wt. bacteria in 0.3–0.5 ml. broth added to tubes containing 10 ml. BNB of the pH values shown. Incubation, shaking, and measurement of optical density were as described by Kushner & Lisson (1959).

Growth at different pH values and the retention of alkali tolerance

The effect of the pH value of the medium on the growth of this organism in BNB is shown in Fig. 1. Comparison was made between bacteria which had been maintained for 1 year on BNA (pH 7.4) with transfers at 2-week intervals (Fig. 1*a*) and bacteria which had been similarly maintained at pH 10.7 (Fig. 1*b*). Both cultures grew after a slightly shorter lag period at pH 9.9 than at pH 7.4. In the highly alkaline media in which growth occurred, cultures reached a higher density than they did at pH 7.4. A 1-year long transfer on BNA of pH 7.4 had caused little loss of alkali resistance, the only difference between the two cultures appearing at pH 10.8, where the bacteria maintained at pH 7.4 grew after a lag period 11 hr. longer than did those maintained at pH 10.7. When first isolated, these bacteria grew in shaken cultures of pH 11.0. Two years later, at the time of the experiment shown in Fig. 1, they still grew on BNA at pH 11.0 but did not grow in shaken BNB of this pH value. In this, they resemble alkali-resistant *B. cereus* (Kushner & Lisson, 1959), which grew at a slightly higher pH value on agar than in liquid medium.

In the late stages of incubation with shaking, there was a decrease in the optical density of all cultures. No clumping of the bacteria was observed, and the culture became viscous, indicating that lysis was probably taking place. As with alkali-resistant *Bacillus cereus*, the growth of the present organism in alkaline media lowered the pH value. After incubation for 30 hr. the pH value of the culture which was originally at 7.4 had increased to 7.8, and the pH values of all other cultures had decreased to 8.8–9.0.

DISCUSSION

The swollen sporangia and oval spores produced by this alkali-resistant organism indicate that it is a *Bacillus* sp. of morphological group 2 (Smith *et al.* 1952). The results of the physiological tests classify the organism as a strain of *Bacillus circulans*. Inability to produce acid from glucose and to grow anaerobically is, however, not typical of *B. circulans*, although atypical reactions of this kind are not uncommon with strains of this species-complex. The smooth surface sculpture of the spores is similar to that found in some strains of *B. circulans*, although other strains of this species may show longitudinal parallel ribs or reticulation (Bradley & Franklin, 1958). The nutritional requirements of this strain are also consistent with those found by Knight & Proom (1950) for some strains of *B. circulans* and for *Bacillus* species in general. Since alkali resistance was not found in 25 other strains of *B. circulans* the high resistance shown by this particular strain is, thus far, unique.

The alkali-resistant *Bacillus circulans* strain has been sent to the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.

We are indebted to Mr D. E. Bradley (A.E.I. Research Laboratories, Aldermaston Court, Berkshire) for the examination of the spore sculpture by the carbon replica method, and to Professor B. C. J. G. Knight for advice during part of this investigation. Mr T. A. Lisson provided valuable technical assistance during the early stages of this work. This paper is contribution no. 658, Forest Biology Division, Research Branch, Department of Agriculture, Ottawa, Canada.

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