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Influence of pH Extremes on Sporulation and Ultrastructure of *Sarcina ventriculi*

SUSAN E. LOWE,[1]* H. STUART PANKRATZ,[2] AND J. GREGORY ZEIKUS[1,2,3]

Department of Biochemistry[1] and Department of Microbiology and Public Health,[2] Michigan State University, East Lansing, Michigan 48824, and the Michigan Biotechnology Institute, Lansing, Michigan 48909[3]

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Distinct morphological changes in the ultrastructure of *Sarcina ventriculi* were observed when cells were grown in medium of constant composition at pH extremes of 3.0 and 8.0. Transmission electron microscopy revealed that at low pH (≤3.0) the cells formed regular packets and cell division was uniform. When the pH was increased (to ≥7.0), the cells became larger and cell division resulted in irregular cells that varied in shape and size. Sporulation occurred at high pH (i.e., ≥8.0). The sporulation cycle followed the conventional sequence of development for refractile endospores, with the appearance of a cortex and multiple wall layers. The spores were resistant to oxygen, lysozyme, or heating at 90°C for 15 min. Spores germinated within the pH range of 4.6 to 7.0.

Sarcina ventriculi was first observed in 1842 by Goodsir (13) in the contents of a human stomach. The organism has been cultivated from garden soil (2, 3) and stomach contents (4), and it has also been enriched and isolated from sand (26), river mud (8), and peat bog sediments (14).

The prevalence of this organism in sedimentary environments and acid or alkaline soils that have been stored for months to years (26) suggests the presence of resistant structures or spores. Examination of soil and sand by numerous workers has failed to reveal the presence of spores of *S. ventriculi*, even though the organism was readily cultivated from these samples. To date there has been only one, nondetailed report on the sporulation of *S. ventriculi* (20).

In addition, the ability of *S. ventriculi* to grow under conditions from pH 2.0 to 9.0 (15) has raised some interesting questions about physiological adaptations of this organism to extremes in pH. Goodwin and Zeikus (15) demonstrated that the internal pH of *S. ventriculi* shifted in relation to the external pH, with an internal pH of 7.1 during growth at an external pH of 7.0, versus an internal pH of 4.3 during growth at pH 3.0. In the present communication we report the ultrastructural and physiological changes which occur when this organism is grown at low versus high pHs. These findings represent the first detailed descriptions of the sporulation cycle of *S. ventriculi*.

MATERIALS AND METHODS

Chemicals and gases. All chemicals used were of reagent grade and were obtained from Sigma Chemical Co., St. Louis, Mo., or Mallinckrodt, Inc., St. Louis, Mo. The nitrogen gas was 99.9% pure and was passed over copper-filled Vycor furnaces (Sargent Welch Scientific Co., Skokie, Ill.) to remove oxygen.

Organism and culture conditions. *S. ventriculi* JK was cultivated as described previously (15). It was routinely grown at 37°C in 26-ml anaerobic bottles which contained 10 ml of glucose complex medium and a nitrogen headspace. The glucose complex medium contained the following (per liter of distilled water): glucose, 30 g; yeast extract, 5 g; tryptone, 5 g; titanium-nitrotriacetic acid solution, 2 ml (22). For growth under pH control, 4-liter Kimax jars (American Scientific Products, Romulus, Mich.) containing 3 liters of medium were used. The jars were equipped with a pH probe, and the contents were mixed by placing the jars on a magnetic stirrer. When the culture was grown at pH 3.0, the initial pH did not change during the fermentation; at pH 7.0 and above, the pH was controlled by the addition of 5 M NaOH.

Resistance of the spores. Approximately 10 g (wet weight) of sporulating cells was harvested and suspended in 50 ml of distilled water, and 10 ml of this suspension was placed into test tubes. Four treatments were used: incubation of the spores at room temperature to determine the effect of oxygen, incubation at 37°C with 0.2% (wt/vol) lysozyme, incubation at 80°C, and incubation 90°C. At various time intervals, 0.1-ml samples were removed and inoculated into anaerobic tubes containing 10 ml of glucose complex medium (pH 6.0). The tubes were incubated at 37°C, and the increase in A_{660} was monitored.

Preparation of cells for electron microscopy. Cells were harvested anaerobically from cultures in the mid- to late exponential phase of growth by being allowed to sediment. The culture vessel had a nozzle on its bottom that was fitted with a short piece of butyl tubing and a 1.0-ml syringe through which the cells were decanted into a 250-ml polycarbonate centrifuge bottle flushed with N_2. The cells were centrifuged at 8,000 × *g* for 15 min and washed once in anaerobic water containing 2 mM dithiothreitol.

Most cells were fixed overnight to 2 days in cold 2.5% glutaraldehyde in 0.1 M Na_2HPO_4–KH_2PO_4 (pH 7.2). The cells were embedded in agar and, after a buffer wash, postfixed for 1 h at room temperature in 1% OsO_4 in the same buffer as above. They were then dehydrated through an ethanol series, treated with propylene oxide, and embedded in either Poly/Bed 812 (Polysciences Inc., Warrington, Pa.) or VCD/HXSA (Ladd Research Industries, Inc., Burlington, Vt.) epoxy resin. Some cells were fixed in the

* Corresponding author.

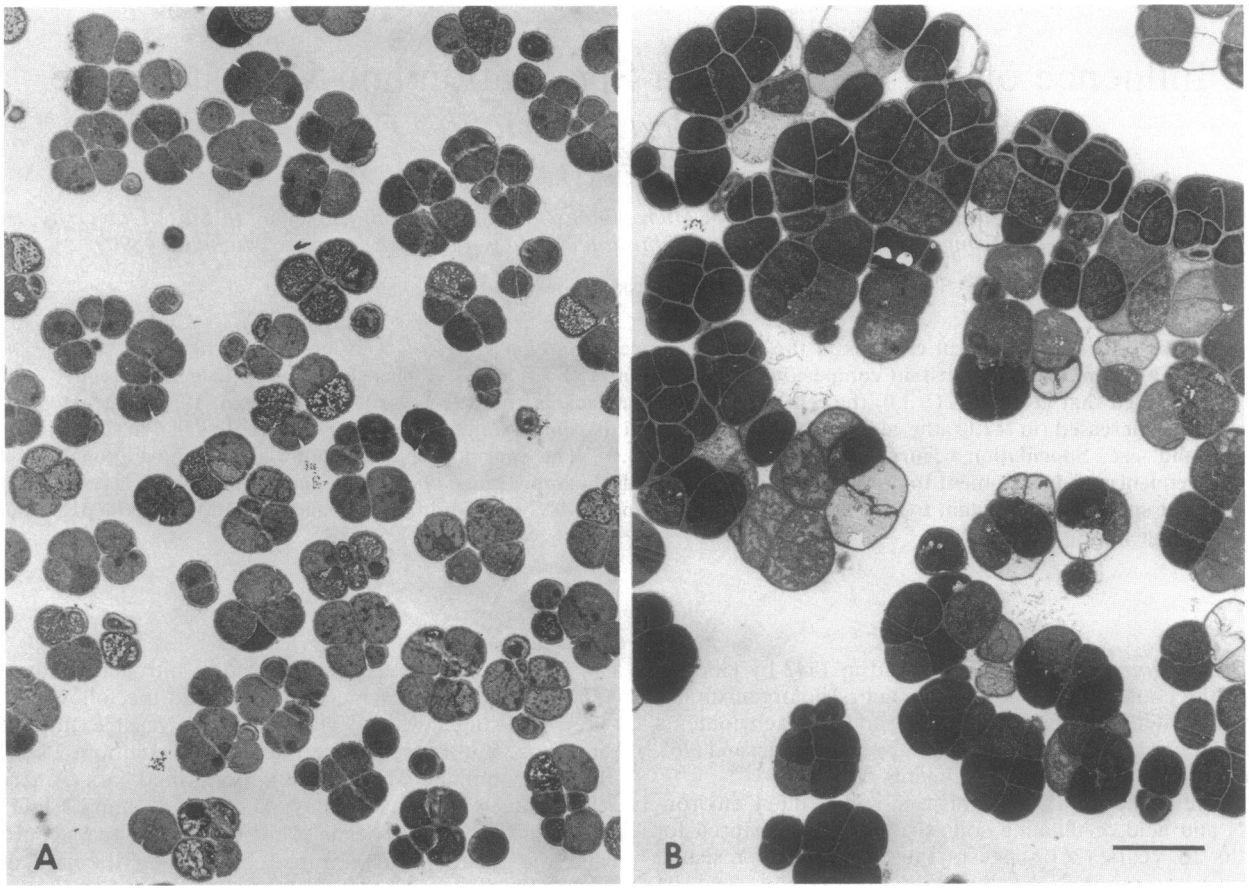

FIG. 1. Electron micrographs of grazing cells of *S. ventriculi* in thin section. (A) Cells from batch culture at a constant pH of 3. (B) Cells grown at a constant pH of 7. Scale bar, 5 μm.

presence of ruthenium red essentially as described by Pate and Ordal (23). Thin sections were cut with a diamond knife mounted on an Ultratome III (LKB Instruments, Inc., Rockville, Md.), stained with uranyl acetate and lead citrate, and examined with an electron microscope (CM-10; Philips Electronic Instruments, Co., Mahwah, N.J.).

RESULTS

Ultrastructure of cells grown at low and high pH. To study the effect of environmental pH on the ultrastructure of *S. ventriculi*, we grew the organism in batch culture with pH control. Thin sections of cells of *S. ventriculi* grown at pH 3.0 and 7.0 are shown in Fig. 1A and B, respectively. The most striking differences in ultrastructure are the increase in the number of cells per packet and the irregularity in cell division when the organism was grown at pH 7.0 compared with pH 3.0. The cells grown at low pH formed regular tetrads, with cell division being equal (resulting in cells of similar size) and only a small number of cells being present within each packet. In addition, not all the cells within each packet had a granular cytoplasm at pH 7.0, and autolyzed cells were observed.

A more detailed thin section of *S. ventriculi* cells grown at pH 3.0 and 7.0 is shown in Fig. 2A and B, respectively. At low pH the cell contents were densely granular and were enclosed by a cell membrane and thick cell wall. Cementing these cells together was a layer of material previously identified as cellulose (7). A developing transverse wall septum was observed to divide some cells into two nearly equal parts. In these dividing cells, and occasionally in other cells, membranous bodies resembling mesosomes were associated with the plasma membrane. Similar observations of mesosomes have been made with the cells of *S. maxima* (18). Large inclusion bodies of uniform appearance but unknown chemical composition were present (Fig. 2A). At high pH (Fig. 2B), in addition to irregular cell division with the formation of cells of variable size and shape, the cells did not round off, compared with the rounded cells of cultures grown at low pH. The cell wall was thinner than that of cells grown at low pH, and the cellulose layer between the cells was less densely stained (Fig. 2B).

Conditions for sporulation. Experiments were conducted by using batch culture to determine the physiological requirements for induction of sporulation in *S. ventriculi*. When *S. ventriculi* was grown in batch culture on 3.0% (wt/vol) glucose with the pH of the medium maintained at 8.0, sporulation occurred, with approximately 30% of the cells containing spores toward the end of growth.

Morphology and ultrastructure of sporulating cells. Light-microscopic photomicrographs of sporulating and vegetative cultures are shown in Fig. 3. Sporulating cultures contained cells of various refractilities, whereas in vegetative cultures the cells appeared uniformly phase dark. There was little synchrony in sporulating cultures, because not all of the cells within an aggregate contained spores. By and large, the

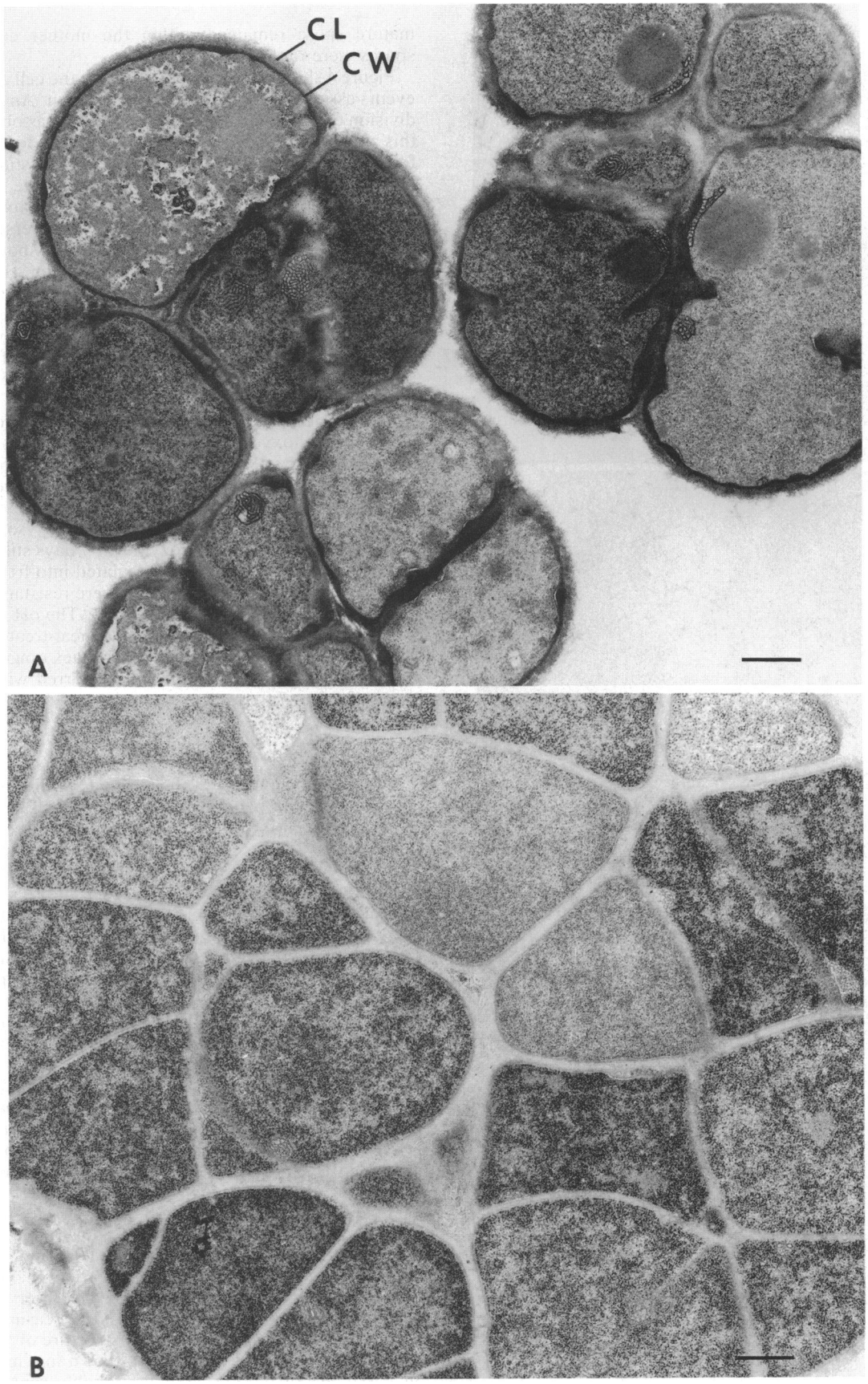

FIG. 2. Electron micrographs, at a higher magnification than in Fig. 1, of the ultrastructural differences between cells in thin section grown at pH 3 (A) and pH 7 (B). The cells are surrounded by a putative cellulose layer (CL) next to the cell wall (CW). Scale bar, 0.5 μm.

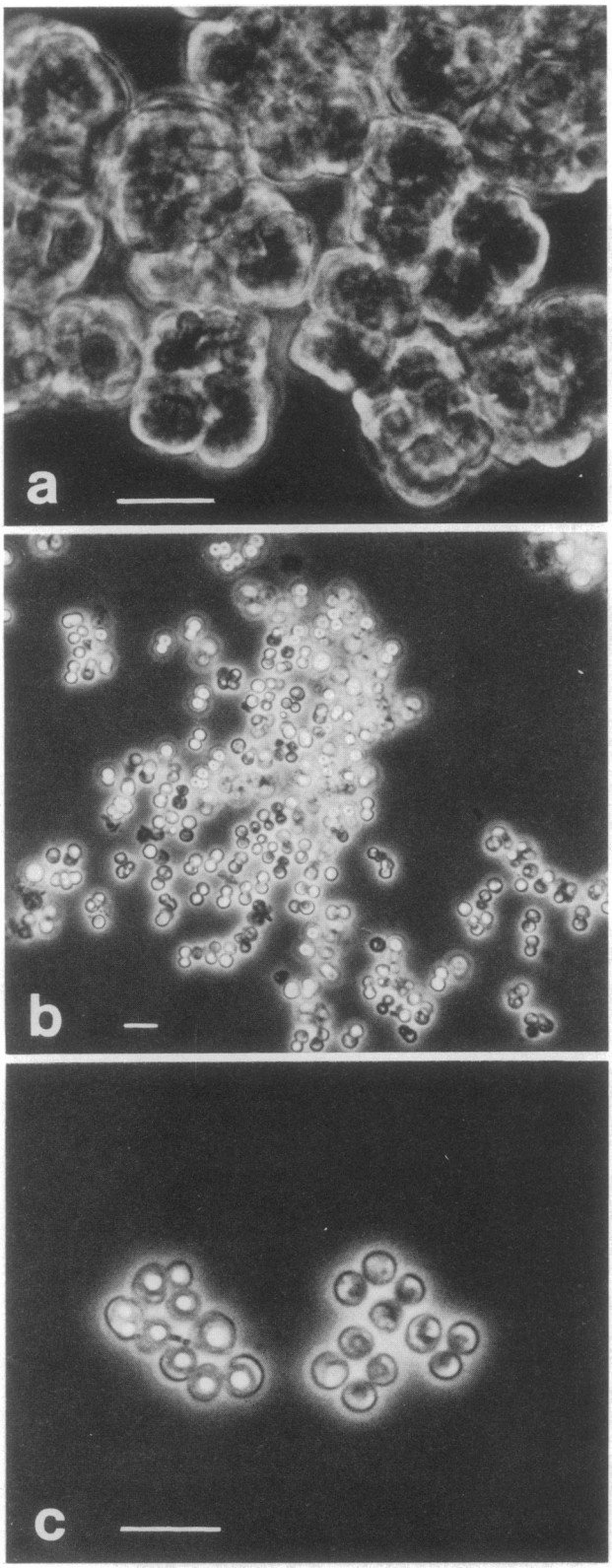

FIG. 3. Light-microscopic photomicrographs of sporulating and vegetative cells of *S. ventriculi*. Scale bar, 10 μm. (A) Vegetative cells under phase contrast (pH 8.0); (B) sporulating cells under phase contrast (pH 8.0); and, (C) nigrosin preparation of sporulating cells under bright field (pH 8.0).

mature spore remained within the mother cell and free spores were rarely observed.

Figure 4 shows thin sections in which the cell architectural events associated with sporulation are seen. An unequal cell division common to endospore formation was observed, and this was followed by the appearance of a forespore. The forespore was surrounded by a membrane distinct from the mother cell membrane and moved from the sporangium periphery to its center during development. Storage bodies, possibly poly-β-hydroxybutyrate, were dispersed throughout the cytoplasm. Biochemical analysis of sporulating cultures determined the presence of glycogen, a compound absent in cells grown at lower pH values under nonsporulating conditions. Subsequent spore development led to cortex formation and the appearance of clearly visible dark coat layers and an exosporium. The cytoplasm of the mature spore cell was dark, probably owing to ribosome concentration.

Physiological properties of mature spores. The resistance of the spores to oxygen, lysozyme, and heat was examined, together with the pH range which would permit germination. The spores germinated after exposure to oxygen for 3 h, demonstrating that they were not affected by aerobic conditions. *S. ventriculi* spores were resistant to lysozyme; incubation of the spores with lysozyme for 7 days still resulted in growth after the spores were inoculated into fresh medium. The spores were thermostable and were resistant to heating at 80°C for 35 min and 90°C for 15 min. The pH required for germination was examined by using a heat-treated sporulating culture. Medium with initial pH values ranging from 3.0 to 9.0 was inoculated, and growth occurred within the pH range of 4.6 to 7.0.

DISCUSSION

This study demonstrates that *S. ventriculi* forms spores as a consequence of pH-dependent alteration of growth physiology. The ability to form spores questions the taxonomic status of *S. ventriculi* and illustrates the importance of growing fermentative organisms under conditions of constant pH when investigating spore formation.

There have been relatively few reports of pH affecting cell division and morphology, owing to the limited pH range for growth of most bacteria. However, two organisms capable of growth over a broad pH range which undergo morphological changes are *Clostridium acetobutylicum* (19) and *Lactobacillus bulgaricus* (24). With *C. acetobutylicum*, the acidogenic phase of growth causes a decrease in the medium pH from 6.8 to around 5.0, and when the switch to the solventogenic phase occurs at low pH, the vegetative rods are converted to clostridial forms (19). When *L. bulgaricus* is grown at low pH (around pH 4.0), it exists as rods with a relatively small number of cells per chain; with increasing medium pH, filamentous cell growth occurs, with addition of cells to the chain, and when the pH reaches 8.0, the chains become folded into clumps (24). At high pH the enzymes responsible for dechaining activity in *L. bulgaricus* are inhibited. Although autolytic enzymes from *S. ventriculi* have not been studied, inhibition of such enzymes would seem to be a probable explanation of the observed increase in the number of cells per packet with increasing pH.

A detailed description of the fine structure of *S. ventriculi* was made when the organism was grown in a medium with an initial pH of 6.0 (18), but it probably represents cells grown in a final pH of about 4.2. The ultrastructure of the cells of *S. ventriculi* described previously (5, 18) closely

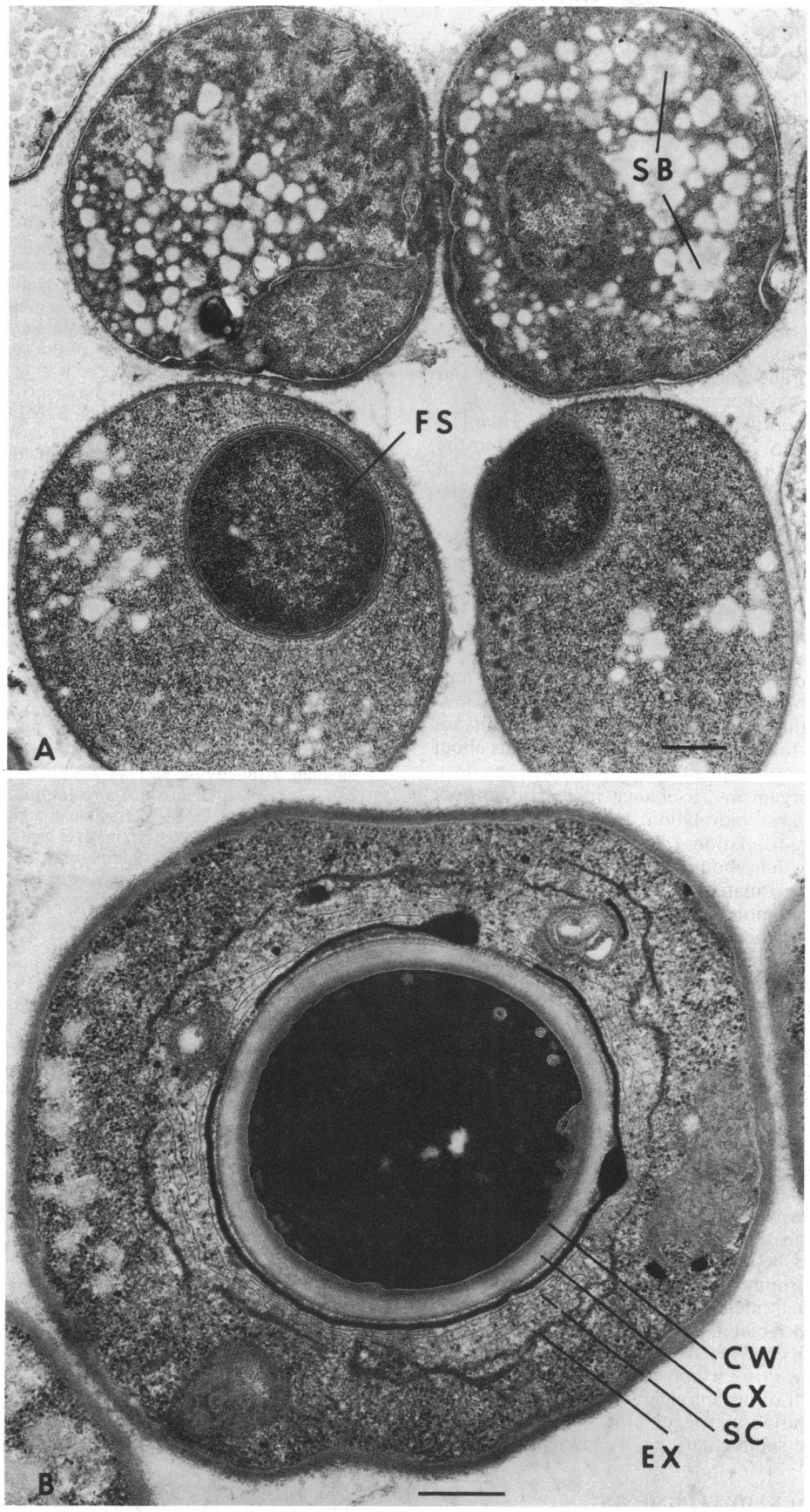

FIG. 4. (A) Cells in various stages of forespore (FS) development, with storage bodies (SB) present. (B) Mature spore within the sporangium. The exosporium (EX) surrounds the spore coat (SC), which encloses the cortex (CX) and the cell wall (CW). Scale bar, 0.5 μm.

resembles the fine structure of cells that we observed growing at pH 3.0. Although the cell yield and doubling time of *S. ventriculi* grown at pH 3.0 and 7.0 are comparable (15), from morphological observations of irregular cell division, cell lysis, and sporulation, conditions of high pH appear to be less favorable for growth. This supports the finding that cells gain a higher proton motive force at low than at high pH values (15).

The spore cell was enclosed within the inner membrane and was surrounded by the cortex, which was composed of two distinct layers, the small, dense inner cortical layer and the less-dense outer cortical layer, as described for the spores of *Bacillus cereus* (11). Surrounding the cortex was the spore coat and finally the exosporium, which was composed of a thin membrane which appeared to be lamellar and was similar to the exosporium present in spores of *C. sporogenes* (16), *C. botulinum* (27), and *B. megaterium* (1). The mature spores of *S. ventriculi* always remained in the mother cell and, in this respect, are similar to the spores of *Sporosarcina ureae* (25), *C. sporogenes* (16), *C. pasteurianum* (21), and *C. perfringens* (17).

The only endosporeforming cocci that have been described previously (19) are *Sporosarcina halophila* and *Sporosarcina ureae*; both organisms are aerobic and gram-positive and occur in packets of two, four, or eight cells. The ultrastructure of sporulation in *Sporosarcina ureae* has been described previously (26), and the process is similar to events leading to spore formation in *S. ventriculi*. Although *S. ventriculi* and *S. maxima* differ in physiology and G+C content from the other sporeforming cocci, the ability of these two species to form spores (20) raises questions about their taxonomic position among the nonsporeforming cocci.

In a number of organisms, a change in pH or nutrient concentration can cause sporulation. In *S. ventriculi*, high pH gives rise to spore formation. In *Sporosarcina ureae*, the optimum pH for growth is about 8.8, and a decrease in pH to 6.8 to 7.0 results in sporulation (12). In *B. subtilis*, sporulation occurs within the optimum pH range for growth (6.5 to 7.8) only when glucose becomes limiting (10).

The conditions required for the formation of spores and subsequent germination are interesting in terms of the survival of this organism in the environment. The normal habitat of *S. ventriculi* is considered by Smit (26) to be the diseased human stomach, although the ubiquitous presence of this organism in sediments and soils (26) suggests that the stomach is not the only natural habitat. The conditions in the stomach required for proliferation of *S. ventriculi* include a low pH (about 1.5) and a high concentration of carbohydrates (26), extreme conditions which prevent the growth of other organisms. *S. ventriculi* can also be isolated from the intestines of patients with cancer of the stomach (26). The role of sporulation in the life cycle of *S. ventriculi* is not known, but it could be postulated to occur upon passage from the acidic environment of the stomach to the alkaline conditions of the small intestine. In this regard, spores of *S. ventriculi* have been isolated from elephant feces (20) and human feces (6). Soil can vary from having acidic conditions due to the breakdown of carbohydrates to having alkaline conditions as a result of protein degradation; therefore, it is possible that pH variation triggers the sporulation of *S. ventriculi* in soil and sedimentary environments.

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LITERATURE CITED

1. **Beaman, T. C., H. S. Pankratz, and P. Gerhardt.** 1972. Ultrastructure of the exosporium and underlying inclusions in spores of *Bacillus megaterium* strains. J. Bacteriol. **109**:1198–1209.
2. **Beijerinck, M. W.** 1905. Een obligaat anaerobe gistingssarcine. Versl. Kon Akad. Wetensch. Amsterdam **13**:608–614.
3. **Beijerinck, M. W.** 1906. Une sarcine de fermentation anaerobie obligatoire. Arch. Neerl. Sci. Exact. Natur. Ser. 2 **11**:199–205.
4. **Beijerinck, M. W.** 1911. Een proefneming met maagsarcinae. Versl. Kon. Akad. Wetensch. Amsterdam **19**:1412–1415.
5. **Canale-Parola, E.** 1970. Biology of the sugar-fermenting sarcinae. Bacteriol. Rev. **34**:82–97.
6. **Canale-Parola, E.** 1986. Genus *Sarcina Goodsir* 1842, 434^{AL}, p. 1100–1103. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
7. **Canale-Parola, E., R. Borasky, and R. S. Wolfe.** 1961. Studies on *Sarcina ventriculi*. III. Localization of cellulose. J. Bacteriol. **81**:311–318.
8. **Canale-Parola, E., and R. S. Wolfe.** 1960. Studies on *Sarcina ventriculi*. I. Stock culture method. J. Bacteriol. **79**:857–859.
9. **Claus, D., and F. Fahmy.** 1986. Genus *Sporosarcina Kluyver* and van Niel 1936, 401^{AL}, p. 1202–1206. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
10. **Dawes, I. W., and J. Mandelstam.** 1970. Sporulation of *Bacillus subtilis* in continuous culture. J. Bacteriol. **103**:529–535.
11. **Ellar, D. J., and D. G. Lundgren.** 1966. Fine structure of sporulation in *Bacillus cereus* grown in chemically defined medium. J. Bacteriol. **92**:1748–1764.
12. **Gibson, T.** 1974. Genus V. *Sporosarcina* Kluyver and van Niel 1936, p. 573–574. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
13. **Goodsir, J.** 1842. History of a case in which a fluid, periodically ejected from the stomach, contained vegetable organisms of an undescribed form. With a chemical analysis of the fluid, by George Wilson. Edinb. Med. Surg. J. **57**:430–443.
14. **Goodwin, S., and J. G. Zeikus.** 1987. Ecophysiological adaptations of anaerobic bacteria to low pH: analysis of anaerobic digestion in acidic bog sediments. Appl. Environ. Microbiol. **53**:57–64.
15. **Goodwin, S., and J. G. Zeikus.** 1987. Physiological adaptations of anaerobic bacteria to low pH: metabolic control of proton motive force in *Sarcina ventriculi*. J. Bacteriol. **169**:2150–2157.
16. **Hashimoto, T., and H. B. Naylor.** 1958. Studies of the fine structure of microorganisms. II. Electron microscopic studies on sporulation of *Clostridium sporogenes*. J. Bacteriol. **75**: 647–653.
17. **Hoeniger, J. F. M., P. F. Stuart, and S. C. Holt.** 1968. Cytology of spore formation in *Clostridium perfringens*. J. Bacteriol. **96**:1818–1834.
18. **Holt, S. C., and E. Canale-Parola.** 1967. Fine structure of *Sarcina maxima* and *Sarcina ventriculi*. J. Bacteriol. **93**:399–410.
19. **Jones, D. T., A. van der Westhuizen, S. Long, E. R. Allocock, S. J. Reid, and D. R. Woods.** 1982. Solvent production and morphological changes in *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **43**:1434–1439.
20. **Knoll, H.** 1965. Zur biologie der garungssarcinen. Monatsber. Dtsch. Akad. Wiss. Berl. **7**:475–477.
21. **Mackey, B. M., and J. G. Morris.** 1971. Ultrastructural changes during sporulation of *Clostridium pasteurianum*. J. Gen. Microbiol. **66**:1–13.
22. **Moench, T. T., and J. G. Zeikus.** 1983. An improved preparation method for a titanium (III) media reductant. J. Microbiol. Methods **1**:199–202.

23. **Pate, J. L., and E. J. Ordal.** 1967. The fine structure of *Chundrococcus columnaris*. III. The surface layers of *Chundrococcus columnaris*. J. Cell Biol. **35**:37–51.
24. **Rhee, S. K., and M. Y. Pack.** 1980. Effect of environmental pH on chain length of *Lactobacillus bulgaricus*. J. Bacteriol. **144**: 865–868.
25. **Robinson, R. W., and C. R. Spotts.** 1983. The ultrastructure of sporulation in *Sporosarcina ureae*. Can. J. Microbiol. **29**:807–814.
26. **Smit, J.** 1933. The biology of the fermenting sarcinae. J. Pathol. Bacteriol. **36**:455–468.
27. **Stevenson, K. E., and R. H. Vaughn.** 1972. Exosporium formation in sporulating cells of *Clostridium botulinum* 78A. J. Bacteriol. **112**:618–621.