CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

THE ULTRASTRUCTURE OF SPORULATION
IN
SPOROSARCINA UREAE

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requirements for the degree of Master of Science in

Biology

by

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DEDICATION AND ACKNOWLEDGMENTS

I would like to dedicate this thesis to my parents. Without their help I would not have had the chance.

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ABSTRACT

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The morphology and ultrastructure of two strains of Sporosarcina ureae at various stages of growth and sporulation were examined using electron microscopy.

Sporulating cells were embedded, sectioned, then examined in the transmission electron microscope. Sporulation begins with non-polar cross wall growth as in vegetative division. Normal wall extension stops before complete septation, but membrane extension together with narrower than normal extension of the wall continues to completely bisect the cell. One of the cells so formed then engulfs the other by extension of the membrane. Wall-like material is formed between the cells during engulfment, unlike the process in most sporeforming bacilli. Cortex material is then laid down and the engulfed cell develops into a mature endospore in much the same way as in Bacillus species. After maturation in broth cultures the spore remains in the mother cell. Lysis has not been observed over a period of several months. There are significant similarities between the phenotypic and ultrastructural characteristics of S. ureae and certain members of Bacillus to suggest a close phylogenetic relationship.

INTRODUCTION

Sporosarcina ureae is a Gram-positive, catalase-positive coccus that occurs in packets of two, four, or eight cells and is motile by means of peritrichous flagella. It is chemoheterotrophic and strictly aerobic and inhabits soils that generally contain high concentrations of urea. It is the only coccus possessing the ability to form refractile heat-resistant endospores. Other known endospore-formers are rod-shaped and belong to the genera Sporolactobacillus, Clostridium, Desulfotomaculum, and Bacillus. All endospore-formers, including Sporosarcina, are currently classified in the family Bacillaceae (Buchanan, Gibbons, 1974).

Although it is unusual for cocci to be motile and form endospores, motile endospore-forming cocci have been described since before the turn of the century (cf. Gibson, 1935; Chester, 1901). Beijerinck (1901) named a motile sporeforming coccus, capable of hydrolyzing urea, Planosarcina ureae Beijerinck 1901. Beijerinck preserved the type strain which today is available from the American Type Culture Collection (ATCC 6473) and was used in this study. In the seventh edition of Bergey's manual (1957), this motile sporeforming coccus was placed with the family Micrococcaceae and referred to as Sarcina ureae (Beijerinck) Lohnis 1911. In edition eight of Bergey's Manual (1974), it

was placed in the family Bacillaceae (Part 15. Endospore-forming Rods and Cocci), and referred to as Sporosarcina ureae (Beijerinck) Kluver and van Niel 1936. Studies on the mature spore, nucleic acid (G+C mole %, DNA-DNA homology, DNA-RNA homology), and allosteric enzymes of Sporosarcina ureae show a closer relationship of S. ureae with the sporeformers rather than the micrococci (Janssen, Lund, Anderson, 1958; Thompson, Leadbetter, 1962, 1963; Kocur, Martinec, 1963, 1965; Mazanac, Kocur, Martinec, 1965; Auletta, Kennedy, 1966; Ellar, Lundgren, 1966; Venner, 1967; Bohacek, Kocur, Martinec, 1968; Herndon, Bott, 1969; Jensen, Stenmark, 1970; Kocur, Bergan, Mortensen, 1971, Silva et al., 1973; Stackebrandt, Woese, 1979).

Pechman et al. (1976) and Fox et al. (1977) compared S. ureae with several other aerobic endospore-formers by means of 16S ribosomal RNA (rRNA) cataloging. They found the most closely related species to be Bacillus pasteurii which has many phenotypic characteristics in common with S. ureae. They recommended, but did not make a formal nomenclatural proposal, to place S. ureae and B. pasteurii into one genus. However, their study did not include Bacillus sphaericus which also shares many phenotypic characteristics with S. ureae (Table 1).

The DNA homology of 62 strains of Bacillus sphaericus was studied by Krych, Johnson, and Yousten (1980). B. sphaericus is of particular interest because the ultra-

TABLE 1

Phenotypic characteristics of S. ureae, B. sphaericus,
B. pasteurii.

	<u>B. pasteurii</u> ^a	<u>S. ureae</u> ^b	<u>B. sphaericus</u> ^a
strain	ATCC (11859)	ATCC (6473)	NCTC (9602)
Gram Rx	+	+	+
catalase	+	+	+
cell shape	rods	cocci	rods
cell size (μm)	0.5-1.1 X 1.3-4.0	0.8-1.3	0.6-1.0 X 1.5-5.0
arrangement	chains	tetrads	chains
motile	+	+	+
aerobic	+	+	+
glucose-acid	-	-	-
-gas	-	-	-
-acetoin	-	-	-
G+C mole %	42	43	43
T _{max} °C	33-42	37 ^(d)	30-45
T _{opt} °C	N/A	30	N/A
T _{min} °C	N/A	N/A	5-15
spore-spherical	+	+	+
-size μm	0.8-1.3	1.0-1.1 ^(d)	0.8-1.1 ^(C)
-location	terminal	central	terminal
sporangium distended	+	-	+

a- Gibson, Gordon, 1974

b- Gibson, 1974

c- Holt, Gauthier, Tipper, 1975

d- Pregerson, 1973

structure of its sporulation has also been described (Holt, Gauthier, Tipper, 1975). To better understand the relationships of these organisms, their ultrastructural characteristics should be compared. The present study examined the ultrastructure of sporulation in Sporosarcina ureae.

An endospore is one of the most complicated structures manufactured by bacteria. The mature spore is the ultimate stage of sporulation which represents a cryptobiotic state with a minimum or nonexistent metabolic activity. The spore is resistant to much higher temperatures than vegetative cells and can withstand drying, ultraviolet radiation, and chemical and physical abuse.

In general, spores (Figure 1) are composed of a cytoplasmic core or protoplast containing a complete genome. The core is surrounded by protective layers beginning with a membrane, a germ cell wall, and a rather thick cortex. An outer spore membrane is next, followed by one of two types of protein coats, lamellar or dense. Some species (e.g. Bacillus cereus, Clostridium perfringens) possess an outer envelope, the exosporium.

The process of sporulation has been analyzed biochemically and morphologically on several members of the Bacillaceae but not Sporosarcina. Using light and electron microscopy, the process has been found to show the same general pattern in all organisms which have been examined.

Detailed descriptions can be found to show the same general pattern in all organisms which have been examined. Detailed descriptions can be found in several books and articles (Gould, Hurst, 1969; Dawes, Hansen, 1972; Freeze, 1972; Holt Gauthier, Tipper, 1975; Aronson, Fitz-James, 1976; Bechtel, Bulla, 1976; Fuller, Lovelock, 1976; Piggot, Coote, 1976; Doi, 1977; Sonenshein, Campbell, 1978; Bulla, et al., 1980).

Sporulation initiates a period of dormancy in the bacterial life cycle that it is believed can be induced by at least two external factors. One is the depletion of a necessary nutrient or substance that inhibits sporulation or upsets the carbon/nitrogen ratio in the vegetative cell. The other factor is the accumulation of catabolites in the cells' environment (Grelet, 1957; Murrell, 1967; Vinter, 1969; Sonenshien, Campbell, 1978).

The morphological changes during sporulation were first elucidated with thin section electron microscopy by Robinow (1953). This work was followed by Chapman (1956) on the sporulation of Bacillus megaterium and Bacillus subtilis, and by Young and Fitz-James (1959) for that of Bacillus cereus. Schaeffer et al. (1965) studied B. subtilis and described seven morphological stages that have been used as a general model in subsequent investigations. These seven stages represent the sequential course of sporulation and generally apply to all bacterial sporulating

FIGURE 1

Mature Spore

systems studied thus far. These are shown diagrammatically in Figure 2 as: axial filament formation; spore septum formation; engulfment, formation of the forespore, germ cell wall, cortex, and coats; maturation and sporangial lysis. The times given are for Bacillus subtilis and indicate the approximate duration of each stage (Dawes, Kay, Mandelstam, 1968).

Stage one represents the transition from vegetative growth to sporulation (Fig 2a). The nuclear material consisting of two or more copies of the chromosome condenses from a loose fibrous network into an "axial filament". A sporal mesosome appears and is associated with the part of the filament later to be enclosed within the spore. Mesosomes found in exponentially growing cells are reported to be lamellar in structure while those examined just prior to and during sporulation are vesicular and more dense (Highton, 1969). The existence of an axial filament is not unique to the sporulation process since it is also found in cells where growth conditions are changed abruptly (Young, Mandelstam, 1979). The chromosomes subsequently separate allowing the onset of septum formation.

Septum formation (stage two, Fig. 2b) begins with membrane invagination from a point near one pole of the cell. This annular membrane fuses and forms two layers of double unit membrane comprising the spore septum. There are now two membrane-bound cytoplasmic areas within the cell. One

FIGURE 2
Sporulation in Bacillus

polar, known as the incipient forespore, and the other, known as the mother cell. This new membrane septum differs from a division septum in that no wall materials are synthesized and the cellular location is polar rather than central.

Stage three (Fig. 2c,d) is a unique process called engulfment. The spore septum proliferates at the original site of invagination and the junction of the septum with the plasma membrane migrates toward the cell pole until the entire incipient spore cytoplasm and nucleoid is "engulfed" and is surrounded by two double unit membranes. This internal structure is now referred to as the forespore. Upon separation of the forespore membrane from the plasma membrane the cell is committed to sporulation. Before this time the addition of fresh media to the culture will result in the resumption of vegetative growth. But when the incipient spore cytoplasm is enclosed with its own semipermeable membrane, the sporulation process continues to completion (Fitz-James, 1964). At the completion of engulfment, there is the unique situation of one cell existing within another.

Cytological changes in stages four through six are not readily discernable as being separate. Each stage is identified by the production of a particular spore structure, but these events overlap considerably.

Cortex formation (stage four, Fig. 2d) is associated

with the deposition of an electron dense cortical material between the two forespore membranes. This material is composed mainly of mucopeptide polymers, and is incorporated to make up the immature cortex of the spore.

Coat formation occurs in stage five (Fig. 2f) with the assembly of coat protein. This forms a lamellar coat that incorporates some of the mother cell cytoplasm and is sometimes further surrounded by another homogenous layer of protein. The protein has a very high cysteine content, four to five times to that found in vegetative cells, and comprises 80% of the total spore protein (Tipper, Gauthier, 1972). During stage four and five the spore becomes dark gray when viewed with Normarski interference optics (Green, Holt, Leadbetter, Slepecky, 1971).

Full refractility of the spore is obtained during final maturation (stage six, Fig. 2g) with the uptake of Ca^{++} ions. These ions associate with the dipicolinic acid (DPA), conferring heat resistance to the spore. Dehydration also takes place at this time. Stage six is readily observed with dark phase contrast microscopy, with the endospore appearing brightly refractile.

At stage seven (Fig. 2h) the spore enters a dormant state; and it is normally released by lysis of the mother cell.

The spore remains in a dormant state until environmental conditions are favorable for the onset of germination

and subsequent vegetative growth. There does not appear to be a time limit on the viability of spores, since it is said those found in Egyptian tombs are still capable of germination.

The sporulation process has been well standardized and documented by the numerous studies on the endospore-forming rods found in the family Bacillaceae. However, this process has not been examined with the unique morphological member of this group, Sporosarcina. It is the purpose of this investigation to elucidate the fine structure of Sporosarcina ureae with special emphasis placed on sporulation. Also, similarities and discrepancies in these events with other sporeformers, specifically B. sphaericus and B. subtilis, will be discussed in relation to the taxonomic classification of Sporosarcina ureae.

MATERIALS AND METHODS

I. CULTURES

Two strains of Sporosarcina ureae were examined. Strain C₁ is the type strain of S. ureae (ATCC 6473), and strain P₁₅ is from local garden soil (Pregerson 1973). Strain P₁₅ was a typical representation of the 61 strains studied by Pregerson (1973). Stock cultures were maintained on tryptic-soy-yeast extract (TSY) slants under refrigeration; they were not transferred with any regularity since cultures several years old were readily viable.

II. MEDIA

Several media were used in attempts to synchronize sporulation and obtain the highest percentage of spores in a culture. The strains were cultured in TSY or brain heart infusion broth (BHI) of full strength and 1/2, 1/4, 1/6, 1/8, and 1/10 dilutions as well as on full strength and TSY and BHI agar plates. Also used was a bi-phasic medium system (Tyrell, MacDonald, Gerhardt, 1958; MacDonald, MacDonald, 1962) of modified MacDonald sporulating broth over a layer of the same medium solidified with agar. Since MacDonald and MacDonald (1962) noted increased sporulation of S. ureae in the presence of 1% (w/v) CaCO₃, cultures were also grown with and without 1% (w/v) CaCO₃, in one or both phases of this medium system. Single phase media of either modified MacDonald broth or agar were also tested

with and without CaCO_3 . The composition of the media are as follows:

Modified MacDonald medium:

Difco yeast extract	2.0 g
Difco peptone	4.0 g
Difco malt extract	3.0 g
K_2HPO_4	1.0 g
$(\text{NH}_4)_2\text{SO}_4$	4.0 g
CaCl_2	0.1 g
MgSO_3 (when used)	0.8 g
Bacto agar (solid medium)	30.0 g
CaCO_3 (when used)	10.0 g
trace elements solution	1.0 ml
distilled water	1000 ml

Trace elements solution:

$\text{NaB}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$	880 mg
$\text{CoSO}_4 \cdot 7 \text{H}_2\text{O}$	240 mg
$\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$	440 mg
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	16.5 mg
$\text{CuCl}_2 \cdot \text{H}_2\text{O}$	13.5 mg
Versonol iron solution	110 ml
distilled water to make one liter	

Tryptic-soy-yeast extract (TSY) and brain heart infusion (BHI) media were purchased from Difco Company and prepared according to the directions.

Inoculations were made from stationary phase cultures grown in 1% (w/v) tryptone broth (Difco) which does not support sporulation. The formation of spores in the various media was monitored with a phase contrast microscope. None of the media supported synchronized sporulation, but

the bi-phasic system gave the highest yield of spores. The bi-phasic medium presented problems, however, with loose agar and CaCO_3 (when used) separating out with the cells when harvested. The medium used to obtain the final results was single phase MacDonald broth without CaCO_3 .

In an attempt to grow spores in a minimal salts defined medium, the method devised by Pregerson (1973) for vegetative growth was modified by adding spore wall components as suggested by Powell and Strang (1957). This medium had the following composition:

Minimal salts medium:

Tris-HCl (Sigma)	12.11 g
K_2HPO_4	0.2 g
$\text{MgSO}_4 \cdot \text{H}_2\text{O}$	0.2 g
NH_4Cl	1.6 g
CaCl_2	0.02 g
trace elements solution	1.0 ml
distilled water	1000 ml

Sterile 1N HCl was added after autoclaving to give a pH of 8.5.

The following filter sterilized solutions were also added aseptically after autoclaving:

Carbon and energy source	Final concentration
sodium acetate (20% w/v)	5.0 mg/ml
Vitamins	
biotin (2.0 $\mu\text{g}/\text{ml}$)	0.002 $\mu\text{g}/\text{ml}$
niacin (2.0 mg/ml)	1.0 $\mu\text{g}/\text{ml}$
thiamine (2.0 mg/ml)	1.0 $\mu\text{g}/\text{ml}$

Spore wall components

DL-alanine (200 mg/ml)	200	µg/ml
L-aspartic acid (200 mg/ml)	200	µg/ml
DL-diaminopimelic acid (200 mg/ml)	200	µg/ml
D-glucosaminic acid (200 mg/ml)	200	µg/ml

III. CULTURE CONDITIONS AND GROWTH MEASUREMENT

Growth was measured turbidimetrically in sidearm flasks with a Bausch and Lomb Spectronic 20 colorimeter at 660 nm. The percentage sporulated cells was determined in wet mounts by counting the number of spores in a total of 200 cells with a Zeiss phase contrast microscope. The germination process was observed with phase contrast microscopy using the method by Kuhn and Starr (1972) using thin agar MacDonald medium.

All cultures were incubated in a New Brunswick incubator shaker at 22 or 30°C and 200 rpm for broth cultures.

IV. HARVEST OF CELLS

To obtain cells at the onset of spore formation, four cultures of 25 ml of modified MacDonald broth in 250 ml flasks were inoculated with varying amounts of tryptone-grown stationary phase cultures. When the flask receiving the largest inoculum showed the first refractile forespores under phase contrast microscopy, the other three cultures were harvest and washed with 1% tryptone broth supplemented with 0.5% NaCl (Silva *et al.*, 1973).

Free spores were obtained by treating 25 ml cultures,

that had completed sporulation, with 12.5 mg of lysozyme (Final conc. 0.5 mg/ml) overnight on a rotary shaker at 4°C. Two ml of 10% (w/v) sodium dodecyl sulfate (SDS) was added at room temperature and after twenty minutes the free spores were washed by centrifugation five times with 0.1M KCl and stored in the refrigerator.

Sporangia containing two-week old mature spores were fixed for electron microscopy in the growth medium to prevent the activation of the spores during the washing process. After the primary fixation the cells were harvested and washed in 1% tryptone broth supplemented with 0.5% NaCl.

V. TRANSMISSION ELECTRON MICROSCOPY (TEM)

A. Preparation of Thin Sections

Primary fixation of cells was performed with buffered 4% glutaraldehyde for 10 minutes. The buffers were either cacodylate, Veronal-acetate, or S-collidine. Since S. ureae occurs normally in an alkaline environment, a pH of 8.5 was tried along with 7.4 and 6.8. Veronal-acetate buffer with a pH of 6.8 is the standard buffer when using the almost universal method of Kellenburger et al. (1958) for fixing and embedding bacteria.

After the primary fixation, the samples were washed and suspended in the respective buffer for one hour to remove unreacted glutaraldehyde. After washing, the main fixation was performed in buffered 1% osmium tetroxide for

14 hours. During this time, the samples were agitated in test tubes on a rotary shaker at 4°C.

To facilitate handling for subsequent treatment, cells were embedded in agar blocks. Cells, washed three times after main fixation were placed in a 47°C water bath, mixed with 3% agar (Difco, Noble Agar) in a 1:1 ratio. The mixture was blended in a vortex mixer and poured onto microscope slides where they hardened into a thin film. Blocks measuring 1mm X 1mm were cut with a clean razor blade while the agar was kept moist with buffer. Forty to fifty blocks of each sample were placed in vials and dehydrated for ten minutes at each step of an aqueous acetone series of 10, 20, 30, 50, 70, 90%, and twice in 100% acetone. Post fixation was carried out for 1.5 hours with saturated uranyl acetate at the 50% acetone step. The dehydrated blocks were embedded in either Epon 812, Epon/Araldite, or Spurr low viscosity embedding medium in one hour steps of plastic/acetone ratios of 1:2, 1:1, 2:1, and three changes of 100% plastic.

Due to the slow polymerization of the Spurr embedding medium at room temperature, its hardener was added at the outset of embedding with the last 100% plastic change allowed to penetrate overnight. Because both Epon and Epon/Araldite harden quite rapidly, the hardener was added only in the 100% plastic step. Six to ten agar blocks of each sample were placed in individual BEEM cap-

sules and polymerized at 60°C for 48 hours.

Thin sections were cut on a Porter-Blum MT-2 ultra microtome (Ivan Sorvall, Inc., Norwalk, CT.) with glass knives which had been made on an LKB knife maker (LKB Instruments, Inc., Tustin, CA.) grids and were stained for contrast with 0.35% lead citrate (Reynolds, 1963) for 15 minutes followed by 2% uranyl acetate for 20 minutes. Grids were observed in a Zeiss EM-9S-2 electron microscope operating at 60 Kev accelerating voltage with a 30 μ m objective aperture. Electron micrographs were recorded on Kodak electron micrograph film (#4463), developed with Dektol developer, and printed on Kodak Polycontrast paper with an F-2 filter in a Durst-Laborator enlarger.

BUFFERS

<u>S-collidine</u>	0.2M
S-collidine (Pelco)	5.34 ml
1N HCl added to adjust pH	(7.4)
distilled water to 200 ml	
 <u>Veronal-acetate</u>	 0.28 M
sodium acetate	1.94 g
sodium veronal	2.94 g
NaCl	3.4 g
CaCl (1.0 <u>N</u>)	5.0 ml
HCl (1.0 <u>N</u>)	added to adjust pH (7.4, 6.8, 8.5)
distilled water	360 ml

<u>Cacodylate</u>	0.2 M
sodium cacodylate	42.8 g
HCl (1.0 N)	added to adjust pH (7.4, 6.8)
distilled water	1000 ml

PLASTICS

Epon 812

solution A

Epon 812	100	ml
dodecenyl succinic anhydride (DDSA)	117	ml

solution B

Epon 812	100	ml
nadic methyl anhydride (NMA)	80	ml

Solutions A and B were mixed in various ratios to vary the final hardness. The hardener was DMP-30 at a 3% final concentration.

Epon/Araldite (Mollenhauer, 1964)

solution A

Epon 812	31	ml
Araldite	40.5	ml
dibutyl phthalate	3.5	ml

solution B

DDSA	75	ml
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Solutions A and B were mixed 1:1 just prior to use with the hardener, DMP-30 added to a 2% final concentration.

Spurr (Spurr, 1969)

vinylcyclohexene dioxide (VCD)	10	g
diglycidyl ether of propypropy- leneglycol (D.E.R. 736)	6	g
nonenyl succinic anhydride (NSA)	26	g
dimethylaminoethanol (DMAE)	0.4	g

B. Preparation of Negative Stains

Single drops of log phase cultures were placed on 200 mesh carbon coated grids. The cells were allowed to settle and adhere to the carbon film for two minutes before the grids were washed with distilled water. A drop of 2% phosphotungstic acid brought to a pH of 7.4 with 0.1 N NaOH, was added for 30 seconds then removed with filter paper. Specimens were observed in the transmission electron microscope.

VI. SCANNING ELECTRON MICROSCOPY (SEM)

Cells of log phase broth cultures were allowed to settle and adhere to cover slips that had been covered with 0.1% polylysine. Fourteen-hour colonies, grown on thin layers of agar, were removed with the agar and also placed on cover slips. The slips were flooded with Karnovsky's fixative for 12 hours at 4°C, then dehydrated in a graded aqueous acetone series of 10, 20, 50, 70, 90%, and twice in 100% acetone for five minutes at each step. Samples were dried in a critical point drying apparatus (Pelco, Inc., Tustin, CA.) using liquid CO₂, mounted on SEM specimen holders, and coated with a 15 nm layer of gold in a Hummer-II coating device. The specimens were observed with an ISI Super MINI SEM II (International Scientific Instruments) scanning electron microscope and photomicrographs recorded with Polaroid type 55 Land film.

Karnovsky's fixative (Graham, Karnovsky, 1966)

glutaraldehyde (50%)	10	ml
formaldehyde (50%)	8	ml
cacodylate buffer (0.1 M), pH 7.4	82	ml

VII. LIGHT MICROSCOPY

Vegetative and sporulating cells were fixed with Karnovsky's fixative and observed on thin agar (Kuhn, Starr, 1972) with a Zeiss light microscope equipped with phase optics. Photomicrographs were recorded on 35 mm Kodak Panatomic X film, developed, then printed on Polycontrast paper.

RESULTS

Vegetative cells from log phase cultures of Sporosarcina ureae P₁₅ viewed with phase contrast light microscopy were 1.25 μ m in diameter with those of S. ureae C₁ 1.4 μ m in diameter. The cocci occurred singly, in pairs, tetrads, and packets of eight with the tetrad arrangement being most common (Plate 1). Scanning electron microscopy of Log phase cells of strain P₁₅ grown on agar showed individual cells being 1.1 μ m in diameter (Plate 2). These cells did not show tetrad arrangements as clearly as broth-grown cultures and seem to be manufacturing an extracellular material (Plate 2-B). When grown on agar, young colony morphology can be seen in Plate 3-B.

Negatively stained cells revealed the flagellar organization of the tetrads. There were 7 to 8 flagella per tetrad with 2 to 5 flagella per cell (Plates 5, 6). The number of flagella was observed to vary depending on the growth conditions and media. The flagella were 21 nm thick (Plate 3-A) and varied in length up to 17 μ m (Plate 4).

When examined with the transmission electron microscope, thin sections of log phase cells appeared similar to stationary phase vegetative cells found in sporulating cultures. Cells of strain P₁₅ measured 1.2 μ m, while strain C₁ were 1.4 μ m in diameter. Except for cell size, there was no morphological difference between the two strains.

Cells possessed a cytoplasmic envelope consisting of a plasma membrane 7.0 nm thick, a Gram-positive wall of 11.0 nm, and an outer wall layer 18.0 nm thick. The periplasmic space and the space separating the two wall layers both measured 11.0 nm. The outer wall was not continuous around each cell but surrounded the entire packet of cells (Plates 7-12). Cross walls were continuous with the Gram-positive wall but were about twice as thick (25 nm).

Neither flagella nor baseplates were observed in thin sections. Depending on the plane of sectioning, it was sometimes possible to observe a midline cut through a tetrad with four cells at their maximum diameter. In sporulating cultures, there was little synchrony of sporulation within a tetrad, indeed, one cell could contain a mature spore, while the adjacent three cells were in the vegetative state.

Thin sections of S. ureae also revealed the sporulation process (Fig. 3). With the exception of axial filament formation (stage 1) which was not observed, the process followed the conventional sequence of steps. Starting with stage two, the forespore septum was formed (Fig. 3 a & b) followed by engulfment (Fig. 3c - e), spore development (Fig. 3f & g), and final maturation (Fig. 3h). Neither parasporal crystals, sporal pili, nor an exosporium was observed. Upon maturation of the endospore, the sporangium did not lyse as with many other bacilli.

FIGURE 3
Sporulation in Sporosarcina ureae

a

e

b

f

c

g

d

h

The forespore was initiated by invagination of the plasma membrane and wall at a point central to the cell (Plate 7). Initially, this process was indistinguishable from vegetative cell division with the cross wall being twice as thick as that of the Gram-positive wall surrounding the cells. The innermost portion of the septum which was synthesized last, however, had the thickness of only a single layer of Gram-positive wall. This central portion of the septum was presumed to be the germ cell wall of the spore. It was not possible to determine which portion of the cytoplasm was destined to become forespore until engulfment began.

Forespore formation commenced by the septum bulging into the mother cell cytoplasm. This process was followed by movement of the junction of the forespore septum and the plasma membrane toward one end of the mother cell. The septal wall material detached from the cross wall that initiated sporulation and remained detached from the Gram-positive wall surrounding the mother cell. The separation usually began on one side of the mother cell resulting in the eventual fusion occurring near the end of the cell (Plates 8-11). When the septum-plasma membrane junction had fused, the forespore had been engulfed within the mother cell.

After engulfment, the forespore membranes had a ragged wavelike appearance (Plate 12). By the time the fore-

spore had become centrally located within the cell, the membrane was regular and smooth. Cortical material then appeared between the wall and the outer membrane of the forespore. While the cortex thickened, protein coats became visible in the mother cell cytoplasm. A homogenous layer of cytoplasm was trapped just outside the outer membrane but within the lamellar coats (Plate 13). The lamellar coats eventually formed a five layered polygon around the forespore. These layers developed one layer at a time and the sides of the polygon formed individually. During final spore maturation, the cytoplasm became more homogenous and more electron dense than the mother cell cytoplasm (Plates 13-15). Plates 14 and 15 illustrate a nearly mature endospore.

Examinations with the light microscope revealed that lysis of the mother cell did not occur over a period of several months, but electron microscopic observations of two-week old cultures showed extensive degradation of the mother cell cytoplasm with the plasma membrane separated from the wall (Plate 16). The spore protein stained very heavily and the nucleoid was fibrous. A mature spore measured $0.87\text{ }\mu\text{m}$ in diameter with the cortex 56 nm in thickness. The space between the cortex and coats that contained the incorporated mother cell cytoplasm measured 28 to 42 nm while the outer lamellar coats were 25 nm thick. The inner spore membrane measured 7.0 nm . The outer spore

membrane was not visible.

PLATE 1

- A- Phase contrast photomicrograph of S. ureae
P₁₅ cells grown in tryptone broth.

3,600 X

- B- Phase contrast photomicrograph of S. ureae P₁₅
cells grown in MacDonald broth. Note mature
refractile endospores (S).

3,600 X

A

B

PLATE 2

Scanning electron micrographs of early colony morphology on agar. Cells are S. ureae P₁₅. Note extracellular material (E) in Plate 2B.

A- 3,000 X

B- 9,000 X

A

5.0 μ m

B

10 μ m

PLATE 3

A- S. ureae P₁₅ flagella negatively stained
in 2% phosphotungstic acid.

184,500 X

B- Scanning electron micrograph of fourteen-
hour colony of S. ureae P₁₅ on MacDonald agar.

2,400 X

A

50 nm

B

50 μ m

PLATE 4

Electron micrograph of negatively stained
S. ureae P₁₅ showing flagellar length and arrange-
ment. Cells were treated with 2% phosphotungstic
acid.

8,500 X

PLATE 5

Negative stain of S. ureae P₁₅ showing
flagellar arrangement.

36,000 X

PLATE 6

Higher magnification electron micrograph of
cells in Plate 4 showing flagellar arrangement.

84,000 X

0.1 μ m

PLATE 7

Initiation of Sporulation (see Fig. 3a)

Thin section electron micrograph of Sporosarcina ureae C₁ fixed in Veronal acetate buffer pH 7.4 and embedded in Epon 812.

OW - outer wall

W - Gram-positive wall

pM - plasma membrane

C - cytoplasm

CW - cross wall

A - invaginating wall

B - invaginating membrane

112,000 X

0.1μm

PLATE 8

Mid-Phase Engulfment (Fig. 3c)

Sporosarcina ureae P₁₅ fixed in veronal acetate buffer pH 6.8 and embedded in Spurr low viscosity embedding medium.

C - mother cell cytoplasm

CW - cross wall

sm - septal membranes

sw - septal wall

J - septum-plasma membrane junction

P - forespore cytoplasm

A - invaginated cell wall

Q - adjoining cell

112,000 X

PLATE 9

Mid-Phase Engulfment (Fig. 3b)

Sporosarcina ureae P₁₅ fixed in Veronal acetate buffer pH 6.8 and embedded in Spurr.

C - mother cell cytoplasm

pM - plasma membrane

S - sporol septum

J - septum-plasma membrane
junction

P - forespore cytoplasm

112,000 X

PLATE 10

Late-Phase Engulfment (Fig. 3d)

Sporosarcina ureae P₁₅ fixed in Veronal acetate buffer pH 6.8 and embedded in Spurr.

- C - mother cell cytoplasm
- J - septum-plasma membrane junction
- P - forespore cytoplasm
- S - sporal septum
- Q - adjoining cell

112,000 X

PLATE 11

Near Completion of Engulfment (Fig. 3d)

Sporosarcina ureae P₁₅ fixed in Veronal acetate buffer pH 6.8 and embedded in Spurr.

- OW - outer wall
- W - Gram-positive wall
- pM - plasma membrane
- C - mother cell cytoplasm
- CW - cross wall
- sm - engulfment membranes
- sw - engulfment wall
- J - septal-plasma
membrane junction
- P - forespore cytoplasm
- Q - adjoining cell

246,000 X

PLATE 12

Completed Forespore Formation (Fig. 3e)

Sporosarcina ureae P₁₅ fixed in Veronal acetate buffer pH 6.8 and embedded in Spurr.

C - mother cell cytoplasm

P - forespore cytoplasm

S - forespore membranes

Q - adjoining cell

112,000 X

PLATE 13

Maturing forespore (Fig. 3f, g)

Sporosarcina ureae fixed in Veronal acetate
buffer pH 6.8 and embedded in Spurr.

- OW - outer wall
- W - Gram-positive wall
- pM - plasma membrane
- C - mother cell cytoplasm
- P - forespore cytoplasm
- im - inner membrane
- om - outer membrane
- cX - immature cortex
- Ic - incorporated mother
cell cytoplasm
- L - lamellar coats

112,000 X

PLATE 14

Nearly Mature Endospore (approaching Fig. 3h)

Sporosarcina ureae P₁₅ fixed in Veronal acetate buffer pH 6.8 and embedded in Spurr.

C - mother cell cytoplasm

P - spore cytoplasm or core

im - inner membrane

Gw - germ cell wall

cX - cortex

Ic - incorporated mother cell
cytoplasm

L - lamellar coats

A - point of sporal
septum formation

ri - ribosomes

112,000 X

PLATE 15

Higher Magnification Electron Micrograph of
Endospore in Plate 8

C - mother cell cytoplasm
P - spore cytoplasm or core
im - inner membrane
Gw - germ cell wall
cX - cortex
Ic - incorporated mother
cell cytoplasm
L - lamellar coats
ri - ribosomes

246,000 X

PLATE 16

Two-week-old Endospore (Fig. 3h)

Sporosarcina ureae P₁₅ fixed in cacodylate buffer
pH 7.4 and embedded in Spurr.

- OW - outer wall
- W - Gram-positive wall
- pM - plasma membrane
- C - mother cell cytoplasm
- CW - cross wall
- P - spore cytoplasm or core
- im - inner membrane
- cX - cortex
- L - lamellar coat
- N - nucleoid
- Q - adjoining cell

DISCUSSION

The formation of an endospore represents one of the most complex morphological changes that bacteria undergo in their life cycle. Any life form with the ability to enter a dormant state and resist harsh environments has a selective value for survival and represents an evolutionary advancement in differentiation. The geneticist attempts to determine how the complex controls function in developmental differentiation. First, however, the morphological events must be determined. Indeed, in bacteria that have been extensively studied (e.g., Bacillus subtilis) much progress has been made in understanding this process.

The present fine structural details of S. ureae is in agreement with the limited amount of previous electron microscopic work on this organism (Mazenec, Kocur, Martinec, 1965; Silva, et al., 1973; Beveridge, 1979a,b; Stewart, Beveridge, 1980). In the first fine structural analysis, Mazenec and others (1965) used methacrylate for embedding, which is inferior to modern plastic resins.

Of the three embedding media utilized in this study, Spurr resin gives the best results. Epon/Araldite proves unsatisfactory due to slow penetration into spores. Epon alone works well for vegetative and early sporulating cells, but again, slow penetration prevents proper embedding of spores. Spurr resin has a very low viscosity and much slower polymerization times at room temperature, and there-

fore allows the embedding procedure to take place over a longer period. The method devised by Chiovetti (1978) for embedding fungal spores with Spurr resin allows for thorough penetration over a period of four days. This illustrates the problems of embedding spores which are much more difficult to work with than vegetative cells, but the development of low viscosity resins has been a great improvement.

The type of buffer and pH greatly affect the quality of fixation of S. ureae. S-collidine has been reported to enhance contrast of membranes (Ellar, Lundgren, 1966) but, unfortunately, poor results were obtained whenever this buffer was used. Veronal acetate which is generally used with bacteria for examination by electron microscopy, proves satisfactory (Plates 7 - 15). Cacodylate buffer, however, allows for the best preservation of spore structure with increased contrast (Plate 16).

Since S. ureae is found in highly alkaline environments, it was thought that fixation at a high pH might give better preservation of the fine structure. However, fixation at pH 8.5 caused the cells to lyse releasing cytoplasm. Fixatives, especially glutaraldehyde, do not readily cross link at high pH. The best results are obtained when the pH is lowered to 6.8 or 7.4.

Recently, Beveridge (1979a, b) published excellent micrographs of S. ureae showing the outer wall layer. This

structure is not unique to Sporosarcina; many Gram-positive and Gram-negative bacteria possess regular outer walls, but the structure of these walls is not consistent and varies considerably among species (Beveridge, Murray, 1974; Sleytr, Glauert, 1975; Stewart, Murray, Beveridge, 1980).

The outer layer in Sporosarcina has been analyzed and found to be composed of a regularly structured protein of 150,000 daltons (Stewart, Beveridge, 1980). It is believed this protein layer is of significant ecological importance by protecting the cell from alkaline environments (Beveridge, 1979a). In addition, this layer possibly prevents infection by bacteriophages, since it has been noted that viruses have not been isolated from this organism. An attempt was made to induce Sporosarcina not to manufacture this outer wall by growing it in successive cultures of minimal salts media, but the outer wall persisted throughout fifteen successive transfers. It would be worthwhile to search for a mutant strain that lacks this layer and examine its bacteriophage susceptibility and environmental tolerances.

The outer protein layer may also be responsible for the retention of the spore by the mother cell and could protect the spore during germination. Since this layer cannot be identified in mature spores, germinating spores would lack its protection until a new layer was manufac-

tured. The benefits of this layer would be available to the newly outgrown cell, however, if it were still surrounded by the wall and protein layer from the spore mother cell. Upon the first cell division, the old walls would then be ruptured and shed. Since this process cannot be observed clearly with the light microscope, a further electron microscopic investigation is necessary.

The fine structure of the sporulation process described here is similar to other sporeformers studied thus far (Green, Holt, Leadbetter, 1971; Freeze, 1972; Holt, Gauthier, Tipper, 1975; Bechtel, Bulla, 1976; Young, Mandelstam, 1979; Bulla et al., 1980). However, S. ureae differs from the general models of sporulation. The first stage of sporulation, consisting of the formation of an axial filament from the nuclear material (Fig. 2a), is not observed in S. ureae. It is possible this structure is altered during fixation or, more likely, that filament formation is not required in sporulation. This hypothesis has recently been proposed by several researchers who noted that axial filaments are not formed during microcycle sporulation, where the germinated outgrown spore is induced to sporulate rather than proceed through the first cell division (Hanson, Peterson, Yousten, 1970; Young, Mandelstam, 1979). It is possible that axial filaments are merely a response to a change in growth conditions and are not involved with sporulation. The lack of this structure in

S. ureae supports this hypothesis.

Sporulation of S. ureae is initiated by the formation of the sporal septum by invagination of membrane and wall rather than membrane alone, as is the case in most other sporeformers. In S. ureae, wall synthesis can be extensive (Plate 8) and does not completely stop. Instead, it is reduced presumably to become the germ cell wall of the spore. Material interpreted as wall extension can be seen during late-phase engulfment (Plates 10, 11). In other systems the germ cell wall is manufactured after engulfment is complete. Also, in other sporeformers, the septum is polar with the terminal cytoplasm later incorporated into the spore. With S. ureae the septum is central to the cell with no distinction found between mother and spore cytoplasm.

Stage seven, consisting of mother cell lysis, does not appear to occur in S. ureae. Other sporeformers release the endospore soon after maturation. Lysis is induced by the release of lytic enzymes that kill the mother cell. With S. ureae death of the vegetative mother cell occurs within two weeks with considerable cytoplasmic degradation (Plate 16), but the outer protein layer remains, preventing release of the spore.

Sporulation in Sporosarcina ureae is therefore similar to Bacillus sp. during stages two through six, but stages one and seven are absent. Also, the sporal septum is cen-

tral rather than polar and is accompanied with considerable wall involvement, including the synthesis of the apparent germ cell wall during the engulfment stage.

The present study indicates that sporulation in Sporosarcina resembles that of other members of the Bacillaceae, particularly Bacillus sphaericus, and strongly supports the present classification of Sporosarcina in the family Bacillaceae. Sporulation is a process that requires a complex genome with developmental controls. Bacteria able to undergo sporulation presumably have a strong phylogenetic relationship.

The ultrastructure of sporulation in B. sphaericus has been described (Holt, Gauthier, Tipper, 1975). Both S. ureae and B. sphaericus share unique qualities with respect to the ultrastructure of the vegetative cell and the ultrastructure of the sporulation process. Vegetative cells of B. sphaericus possess an outer protein wall layer that appears similar to the outer protein layer found around S. ureae. B. sphaericus forms the sporal septum with considerable cell wall involvement at the outset of formation, and completes the septum with reduced wall involvement in the same manner as S. ureae. However, with B. sphaericus, the septum is polar, as with other bacilli, and the incorporated wall material is degraded at the onset of engulfment. In S. ureae, the wall material remains, presumably to become the germ cell wall of the spore. B.

sphaericus releases the mature spore which possesses an exosporium, while S. ureae retains the spore and does not manufacture an exosporium.

B. sphaericus also shares many phenotypic characteristics with S. ureae (Table 1) which, along with the ultrastructural similarities, indicate a close relationship.

Bacillus pasteurii also has phenotypic characteristics in common with both B. sphaericus and S. ureae (Table 1), however, the ultrastructure of B. pasteurii has not been examined. The suggestion by Pechman et al. (1976) and Fox et al. (1977) to place S. ureae and B. pasteurii together in the same genus on the sole basis of rRNA nucleotide sequencing represents a departure from the traditional use of cell morphology and mode of division in classifying sporeformers.

S. ureae, B. sphaericus, and B. pasteurii have certain characteristics that place them closer to one another than to any other members of the family Bacillaceae. However, due to the sparcity of data showing a close relationship, further studies should be conducted before they be considered as a taxonomically distinct group.

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