Cores, Microbial Organelles Possibly Specific to Group D Streptococci

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A long, thin, approximately cylindrical core spans the interior of cells of 24-hr cultures of all group D streptococci that were examined, five strains of *Streptococcus faecalis*, single strains of *S. faecalis* subsp. *zymogenes* and *S. durans*, and three strains of *Streptococcus* spp. In one strain of *S. faecalis*, serial section electron microscopy showed that most cells possess a core. The core is 0.10 to 0.16 μ m thick and consists of a matrix and an axial array of ribosomelike particles. It resembles one of two types of cores present in a stable protoplast form of one of the *S. faecalis* strains. Cores were not present in single strains of *S. pyogenes* (β -hemolytic group A), *S. agalactiae* (group B), *S. dysgalactiae* (group C), *S. equisimilis* (group C), and *S. mitis* (viridans group) that were examined; nor were cores observed in single strains of *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus megaterium*. Cores may be useful, therefore, in identification of group D streptococci. For preservation and rapid recognition of cores, a glutaraldehyde-osmium tetroxide sequence of fixation appears superior to the osmium tetroxide method often employed in processing bacteria for electron microscopy.

In comparison with most life forms, the ultrastructural organization of bacteria is deceptively simple. Attempts to analyze the complex metabolic and genetic fabric of bacterial life are based on very few identified visible cellular components. When bacterial (cell wall-bearing) forms are converted to viable protoplast (cytologically cell wall-free) forms, the structural pattern generally is even further simplified by retention of only the cell plasma membrane, ribosomes, fine fibrous material (nucleic acids, at least in part), and an apparently structureless "ground substance," seemingly a reduction to the minimum of the structural elements essential to cellular life. Obviously, an additional organelle, common to bacterial and protoplast forms of a microbe, could provide a key test structure for visible responses to treatments affecting microbial function.

Abrams et al. (1) discovered a tubular structure entrapped in ghosts of protoplasts derived from *Streptococcus faecalis* (ATCC 9790). We reported (7) tubular and nontubular types of long, thin, approximately cylindrical structures, termed cores, in a stable (nonreverting) protoplast form derived from the B₁₄ strain of *S. faecalis*. Both types of cores contain ribosomelike particles, which are irregularly arranged inside the electron

dense, cylindrical, thin wall of tubular cores but axially arranged in the matrix of nontubular cores. The cores are about 0.08 to 0.13 μ m in diameter, and they sometimes extend beyond the cell body without piercing the plasma membrane. Although more than one core per protoplast cell can occur, both types of cores have never been observed in the same cell.

This paper describes cores and their incidence in the parent bacterial form of *S. faecalis* strain B₁₄. Also reported is the presence of such cores in four other strains of *S. faecalis*, one strain of *S. faecalis* subsp. *zymogenes*, one strain of *S. durans*, and single strains of three other group D *Streptococcus* spp., as well as absence of cores in representatives of the A (β -hemolytic), B, C, and viridans groups of streptococci, and single strains of *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus megaterium*. The problem of detection of cores is considered in relation to two kinds of fixation for electron microscopy. The possible significance and taxonomic usefulness of cores are discussed.

MATERIALS AND METHODS

Strain B₁₄ of *S. faecalis*, originally isolated from urine of a pyelonephritic patient (17), was cultured in Difco Brain Heart Infusion broth (BHI) for 24 hr at 37 C.

In the early part of this study, the procedure for preparation of *S. faecalis* for electron microscopy, involving, in part, a glutaraldehyde-osmium tetroxide sequence of fixation and ethanolic uranyl acetate during dehydration for Epon embedment (25), was the same as we used for the derived protoplast form (7). Later, when it was apparent that no detectable advantage in ultrastructural preservation of the bacterial form resulted from inclusion of sucrose solution in the glutaraldehyde fixative and polyethylene glycol 200 in the glutaraldehyde and osmium tetroxide fixatives, as well as in the agar, used to embed the fixed bacteria, the procedure was modified by substitution of equivalent volumes of distilled water for the sucrose solution and polyethylene glycol. Regardless of the presence or absence of sucrose and polyethylene glycol in the fixatives, we use the term G-Os conditions to denote a glutaraldehyde-osmium tetroxide sequence of fixation.

A few samples were prepared by an osmium tetroxide fixation, termed R-K conditions (19), and then small blocks of buffered agar-embedded cells were stained with buffered uranyl acetate (35). After 5-min changes in 95% (v/v) and absolute ethyl alcohol, the blocks were dehydrated further for Epon embedment (25), starting with the first room temperature absolute ethyl alcohol step in the procedure used for protoplasts (7).

Sections, mounted on Formvar substrates, were stained with alkaline lead citrate (42) and then coated with carbon by vacuum evaporation. Electron photomicrographs were taken at original magnifications of 6,700 to 41,000 with an Hitachi HU-11A electron microscope operated at 75 kv, with a 150- μ m condenser and 50- μ m objective apertures.

Incidence of cells with cores was estimated by means of enlarged electron micrographs (16,700 to 22,000 $\times$ final magnification) of selected areas traced through sets of 11 serial sections (approximate average section thickness per set 42 to 54 nm). Each area was photographed first in the central section of the ribbon of serial sections; then that area was photographed in successive sections to both extremes of the ribbon. To be counted, a core had to lie within an ultrathin section in the oblique to longitudinal range of view, or, if in approximate cross-sectional view, had to appear, in successive serial sections, either in the same position in the cell or in successive positions linearly displaced across the cell. For each estimate of percentage of cells with cores, only those cells were considered which were present in every one of a set of 11 serial micrographs.

Less extensive comparative investigations, seeking to establish presence or absence of cores under comparable conditions of 24-hr culture and G-Os conditions of fixation, were performed with representatives of various serological groups of streptococci [β -hemolytic group A: *S. pyogenes* type 12, provided by A. I. Braude (3); group B: *S. agalactiae* (ATCC 13813); group C: *S. dysgalactiae* (ATCC 9926) and *S. equisimilis* (ATCC 9542); group D: four strains of *S. faecalis* (ATCC 882, 4079, 6057, 9790) and single strains of *S. faecalis* subsp. *zymogenes*, provided by W. B. Sarles (34), *S. durans* (ATCC 6056), and

Streptococcus spp. (ATCC 9854, 12958, 12959); viridans group: *S. mitis* (ATCC 6249)]. In addition, strain B₃₅ of *S. aureus* (isolated locally from a human infection and provided by S. Feinegold), *B. megaterium* (ATCC 13632), and strain B₂₅ of *E. coli* (originally an isolate from urine in a case of human pyelonephritis (16)) were studied. The B₁₄ strain of *S. faecalis* was always included as a control organism for demonstration of core preservation under G-Os conditions employed in each of the experiments with other strains and species. (The letter B followed by a numerical subscript is an identification symbol assigned by our laboratory. ATCC numerical instead of B numerical designations are given wherever possible.)

RESULTS

With 24-hr cultures of *S. faecalis* strain B₁₄ fixed under G-Os conditions, cells with a core are readily located during electron microscopic scanning of ultrathin sections (Fig. 1). Cores are approximately cylindrical and range between 0.10 and 0.16 μ m in thickness. From the appearance of these cores in various orientations with respect to the plane of sectioning and also from serial section reconstruction of core and cell geometrical relationships, it is apparent that the ends of the core are always close to the cell membrane. This is evident in single sections cut approximately parallel to the core axis (Fig. 3, 7, cf. Fig. 8). Electron dense ribosomelike particles, possibly in tight helical array (cf. Fig. 8), extend along the central axis and through the ends of the cylindrical core matrix. Surrounding electron-dense ribosomelike particles are responsible for the visible outline of the core.

The matrix of the core is either of two types. One kind appears of the same electron density as, and continuous with, protoplasmic "ground substance" (Fig. 2, 3). The other kind of matrix consists of three concentric zones, the electron density of the intermediate zone being greater than that of the inner and outer zones, and the electron density of the outer zone being of the same electron density as confluent protoplasmic "ground substance" (Fig. 4).

With R-K conditions, electron microscopy of the bacterial form of strain B₁₄ *S. faecalis* generally reveals the nuclear and cytoplasmic areas typical of this fixation technique (Fig. 5). Occasional cells with clearly visible core structure occur (Fig. 6), the electron density of the matrix being similar to that of the protoplasmic "ground substance." There is much less difference in electron density between ribosomelike particles and matrix of the core under R-K conditions than with G-Os conditions of fixation.

Serial sections reveal that most cells of *S. faecalis* in 24-hr culture are almost prolate

FIG. 1. Strain B₁₄ *S. faecalis*. Arrows indicate cores in various orientations (each core verified in serial sections, serial set D of Table 1). Fixation: G-Os conditions with sucrose and polyethylene glycol 200. All figures are from 24-hr cultures with embedment in Epon.

spheroidal in shape, except for shallow equatorial indentation toward the level of the developing cross wall. Generally, only one core per cell is present; it usually passes through the central gap in the developing cross wall. Rarely, there are two cores per cell, either full length and parallel to one another, or, when a developing cross wall is nearly complete, with a half-length core visible in each half of the cell. Occasional short cores appear in infrequent oblique (Fig. 7) to transverse (cf. Fig. 8) orientation, as though trapped in one half of a cell by the partially formed cross wall.

In two experiments, serial section estimates of core incidence in 24-hr cultures of *S. faecalis* strain B₁₄, fixed under G-Os conditions, indicated most, but not all, cells contain a core (Table 1). In experiment 1, with samples of the same culture, a single estimate of core incidence after R-K conditions of fixation was much lower than either of two estimates after G-Os conditions of fixation; this corroborated the impression of a significant difference in core incidence between the two kinds of preparations obtained simply by electron microscopic scanning of ultrathin sections.

Although each set of 11 serial sections was generally not of sufficient aggregate thickness to pass completely through a cell, this number of sections and 17 to 26 cells counted per electron microscopic field were sufficient for a good

estimate of core incidence in each set. These estimates were changed very little even when counts were limited to the central 8 or 9 sections of a serial set of 11. In one instance, with 17 serial sections and selection of those 21 cells in a field which could be traced in their entirety, a core incidence of 90% was obtained; yet, for the same field, with selection of those cells present in every one of the central 11 serial micrographs (serial set C of Table 1), an estimate of core incidence was only slightly lower.

In addition to their presence in the B₁₄ strain of *S. faecalis*, cores were apparent in cells of 24-hr cultures of all examined group D organisms (G-Os conditions): *S. faecalis* (four other strains), *S. faecalis* subsp. *zymogenes* (Fig. 9), *S. durans*, and three *Streptococcus* spp.

There may be some species and even strain variations in core morphology. As mentioned previously, the B₁₄ strain of *S. faecalis* exhibits cores with either a matrix of low electron density or a matrix with an intermediate zone of medium electron density. The core matrix of the cell of ATCC 12959 *Streptococcus* sp. (Fig. 8) is entirely of medium electron density and is clearly different from much of the cytoplasmic "ground substance." Although cores of *S. durans* were unmistakable in the single electron microscopic preparation studied, core incidence was relatively low, the periphery of the core was not as regular, and the contrast between core matrix and ribo-

FIG. 2. Cross-sectional and nearly longitudinal views of cores in strain B_{14} *S. faecalis*. The core matrix has the same electron density as that of the cytoplasmic "ground substance." Fixation: G-Os conditions with sucrose and polyethylene glycol 200.

FIG. 3. Strain B₁₄ *S. faecalis*. Core in nearly longitudinal section with matrix of electron density similar to that of the cytoplasmic "ground substance." The core is almost as long as the cell, which has just begun to form a cross wall. Fine fibrils (arrows), as thin as 0.3 nm, are scattered throughout the cytoplasm. The punctate zone above the lower arrow may represent a cluster of fibrils in cross section. Fixation: G-Os conditions with sucrose and polyethylene glycol 200.

FIG. 4. Oblique and cross-sectional (inset) views of cores in strain B_{14} *S. faecalis*. The matrix of these cores has an intermediate zone (arrows) of greater electron density than that of the cytoplasmic "ground substance." Fixation: G-Os conditions with sucrose and polyethylene glycol 200.

somelike particles was not as great as with the other core-bearing species under the same G-Os conditions.

In 24-hr cultures, the β -hemolytic group A *S. pyogenes* and the ATCC representatives selected for this study from the B, C, and viridans groups of streptococci exhibited no cores; nor

were cores present in *S. aureus*, *E. coli*, or *B. megaterium*.

DISCUSSION

A cylindrical organelle, termed core, which consists of a matrix and axially arranged ribosomal-like particles, occurs in the bacterial form

FIG. 5. Strain B₁₄ *S. faecalis*, showing central light fibrillar zone and fluffy material on the cell wall. Fixation: R-K conditions (19).

FIG. 6. Strain B₁₄ *S. faecalis*, showing core in longitudinal orientation. Fixation: R-K conditions. Core structure appears less discrete than it does with G-Os conditions of fixation.

FIG. 7. Serial sections through a core in strain B₁₄ *S. faecalis*. These sections were part of serial D (Table I) and the cell is also present in Fig. 1. Fixation: G-Os conditions with sucrose and polyethylene glycol 200.

of all nine strains of group D streptococci examined but not in examined representatives of the A (β -hemolytic), B, C, and viridans groups of streptococci, nor in *S. aureus*, *E. coli*, and *B. megaterium*. This kind of core, as well as a tubular type, also containing ribosomelike particles and originally described (1) in ghosts of protoplasts derived from *S. faecalis* (ATCC 9790), has been reported (7) for a cultured stable protoplast form of *S. faecalis*. But in reports of microbial ultrastructure, apparently none mentions cores of the kind described here. If these cores are specific to group D streptococci, the reason for lack of such reports for bacterial forms is simply an apparent absence of published examinations of ultrathin sections of group D streptococci.

There are, in the literature, descriptions of two other classes of linear bacterial organelles, helical ribosomal arrays in certain strains of *Mycoplasma gallisepticum* (12, 27, 28) and various ribosome-free tubular structures, some of which have been termed rhabidosomes (10, 21–24, 32, 45) or microtubules (18, 31), and which have been observed in a number of bacterial genera. The significance of the helical ribosomal arrays is unknown. The tubular structures constituting the other class have at times been considered to

bear some relationship to virus, bacteriophage, defective phage parts, or bacteriocins (4, 10, 13, 18, 33). Organelles of both classes are relatively small, and none combines in one structure the cylindrical matrix or tube and the contained ribosomelike particles which characterize the class of linear bacterial organelles which we term cores.

In contrast to the visibility of cores in the living protoplast form of *S. faecalis* (7), cells of the living parent bacterial form, grown in BHI, are opaque when viewed by phase contrast light microscopy. Thus, estimates of core incidence must, for the present, depend on the extremely laborious procedure of serial sectioning. The few serial section sets so far used in estimating core incidence prevent assessment of accuracy. At the apparent expense of a slight underestimate, however, a fairly small number of serial sections appear sufficient to establish that after 24-hr culture in BHI at 37 C and fixation under G-Os conditions, most cells of *S. faecalis* strain B₁₄ contain a core. Those relatively few cells lacking a core may arise when daughter cells fail to receive a core (Fig. 7 and 8).

For estimation of core incidence, fixation under G-Os conditions appears superior to fixation under R-K conditions. The latter tech-

FIG. 8. *Streptococcus* sp. (ATCC 12959). Longitudinal section of core in rare transverse orientation. Some of the ribosomelike particles are in zigzag array, indicating possible helical arrangement. Fibrils (arrows), as thin as 0.3 nm, are visible in the protoplasm. Small zones free of ribosomelike particles, at either end of the cell in this figure, are sometimes observed in other streptococci examined in this study; they are usually peripheral in location (cf. Fig. 3). Fixation: G-Os conditions without sucrose or polyethylene glycol 200.

nique seems to preserve only a small proportion of cores and leads to relatively little difference in contrast between cores and surrounding bacterial protoplasm. This probably accounts for our earlier, mistaken impression that cores were absent from the bacterial form of *S. faecalis* strain B₁₄.

This is not to suggest that one or the other method of fixation is best for the preservation of all structural features of the bacterial form of *S. faecalis*. The fluffy material, that is more likely to be present at the surface of and between cells after fixation under R-K conditions (compare Fig. 5, 6 with Fig. 2, 3, 4), could be a natu-

FIG. 9. *S. faecalis* subsp. *zymogenes*. Core in oblique section. The ultrastructure of this organism is similar to that of strain B₁₄ *S. faecalis*. Fixation: G-Os conditions without sucrose or polyethylene glycol 200.

TABLE 1. Incidence of cells with cores in 24-hr cultures of *S. faecalis* strain B₁₄

Expt no. ^a	Serial set	Fixation	Data based on 11 serial sections	
			No. of cells observed	Incidence of cells with cores (%)
1	A	R-K ^b	26	19
	B	G-Os ^c	17	94
	C ^d	G-Os	24	83
2	D	G-Os	25	88
	E	G-Os	26	59

^a Each experiment represents a single culture.

^b R-K conditions (19).

^c Glutaraldehyde-osmium tetroxide sequence.

^d Data for central 11 sections of a set of 17 serial sections. For the whole set of 17 serial micrographs, an estimate of core incidence, based on cells present in 11 or more of the micrographs, was 81% of 75 cells traced.

rally occurring cell secretion; it could be a precipitate of culture medium constituents or of cell components derived by a fixation-induced cell leakage. The finer cytoplasmic fibrils after G-Os

conditions of fixation could reflect a difference in response to electron staining, but the more general distribution of these fibrils through the cytoplasm, after this kind of fixation, implies a fixation artifact, either active dispersal of fibrils with G-Os conditions or phase separation of fibrillar from other cytoplasmic materials under R-K conditions. Mesosomes are evident, and the plasma membrane is much more easily visualized after R-K conditions. It appears, therefore, that the true nature of these features of *S. faecalis* cannot be established solely on the basis of these two kinds of fixation. It is noteworthy, however, that reports of osmium tetroxide as a source of artifacts in ultrastructural fixation (5, 11, 15, 20, 26, 29, 41, 43) are more frequent than reports of artifacts with either glutaraldehyde fixation (2) or fixation in a glutaraldehyde-osmium tetroxide sequence (29, 36).

The composition and significance of the core must remain, for the present, a matter of speculation. The nontubular or tubular core in a stable protoplast form, originally derived from *S. faecalis* strain B₁₄, is evidently a stiff structure and only becomes apparent in the late stages of growth in culture (7). Similarly, in the parent bacterial form, nontubular cores appear late in culture (*unpublished data*); that they, also, may

be stiff is suggested in Fig. 7, where, in successive serial sections, a part of the developing cross wall appears displaced slightly upward in a region close to the tip of a core. It is this apparent core stiffness which suggests a possible polymeric molecular structure of matrix or tube in non-tubular and tubular cores, respectively.

In the bacterial form, core matrix volume is equivalent to about 10 to 20% of cell wall volume but to an even greater percentage of that portion considered (6) to be new cell wall volume. Consequently, if core matrix were polymeric and consisted of any components (carbohydrate or amino acid or both) common to cell wall substance, a maximal rate of formation of cores or core matrix precursors at some time during exponential growth could place a severe competitive restriction on amount and integrity of new cell wall synthesis. In view of a current concept of cell wall growth (37, 39, 40) by autolysis of peptidoglycan glycosidic linkages (9, 39) and presumed insertion of disaccharide tetrapeptide subunits (14), such competition might explain the autolytic tendency reported [data of A. Stramp (37)] for ATCC 9790 *S. faecalis* in late exponential growth. In our examination of five strains of the bacterial form of *S. faecalis*, in 24-hr BHI culture, such competition might account for many incomplete new cross walls, a low incidence of cell wall fragments, and cytologically deranged protoplasts, some still with a core. [In osmotically stable medium (BHI plus sucrose), autolytically produced protoplasts (cf. 30) are in cytologically good condition and often possess a core (R. G. McCandless, et al., J. Ultrastruct. Res. 21:162-163, 1967).] If, on the other hand, the maximal rate of synthesis of core matrix or matrix precursors in the ATCC 9790 strain is a postexponential growth phenomenon, cores might be the result of discontinuance of a lysis and insertion type of cell wall growth, owing to rapid fall in cell wall autolysin activity after the log phase (9, 37, 39), consequent accumulation of cell wall precursors, and adaptive rerouting of these precursors toward core matrix synthesis.

If core matrix should represent, in polymerized form, components diverted from incorporation into cell wall, then determination of the presence or absence of a core in relation to the cell wall compositional patterns reported by Colman and Williams (8) for 197 streptococcal strains would help establish the extent to which the core may be useful as a taxonomic character. In some instances, these cell wall compositional patterns correlate with species and serological group differences. For example, patterns 9, 10, and 11

include all 15 group D strains, but none of Lancefield groups A, B, or C, and few viridans group strains studied by Colman and Williams. Since cores were absent from representatives of the A, B, C, and viridans groups examined by us, and since, in the study of Colman and Williams, pattern 11 was common to streptococcal species and strains in many Lancefield groups in addition to group D, the examination of representatives of pattern 11 for presence of a core is particularly crucial. This may permit a choice between the alternatives of a correlation of cores with certain cell wall compositional patterns or with the presence of the group D antigen. The latter alternative would suggest the use of the core as a taxonomic characteristic of group D streptococci.

The group D specific antigen is apparently an intracellular teichoic acid (38, 44), which, for the ATCC 9790 strain, is present in highest amount during stationary phase growth (38) and, for the NCIB 8191 strain, is associated, at least in part, with a centrifugally isolable ribosome fraction (44). This parallels our findings of intracellular cores in group D streptococci, highest incidence of cores in the later stages of growth (*unpublished data*) and presence of ribosomelike particles within the core. If further study should indicate a lack of correlation between cores and streptococcal cell wall compositional patterns, but, rather, a good correlation between cores and group D antigen, then the possibility that core matrix, or some part thereof, is group D antigen becomes a reasonable working hypothesis.

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