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Phylogenetic Analysis of *Metabacterium polyspora*: Clues to the Evolutionary Origin of Daughter Cell Production in *Epulopiscium* Species, the Largest Bacteria

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It is rare that there are molecular clues to the evolutionary origin of developmental traits. We have encountered an evolutionary juxtaposition that may explain the origin of the unique replicative morphology of *Epulopiscium* spp., the largest known bacteria, which reproduce by the internal production of multiple live offspring. We report here a 16S rRNA-based phylogenetic analysis of *Metabacterium polyspora*, a multiple-endospore-forming, uncultivated inhabitant of guinea pig cecum. Cells of *M. polyspora* were harvested from cecum contents by sedimentation in a Ficoll gradient and lysed. The bacterial 16S rRNA genes of this lysate were amplified by PCR. Sequence analysis of the cloned PCR products revealed two dominant, closely related 16S rRNA types. In situ hybridization of cecum contents with fluorescently labeled oligonucleotides, diagnostic of these two sequences, showed that they represent distinct strains of *M. polyspora*. Phylogenetic analyses of the sequences showed that *M. polyspora* is closely related to *Epulopiscium* spp. On the basis of this result and other correlations, we propose that the process of sporulation was modified in a predecessor of *Epulopiscium* spp. to produce live offspring instead of quiescent endospores.

Twenty-four years prior to articulating the concept of the prokaryote-eukaryote dichotomy, Édouard Chatton and his colleague, Charles Pérard, described several unusual microbes found in the ceca of guinea pigs (6, 7). One notable component of this community is a large bacterium, capable of producing multiple endospores, which they named *Metabacterium polyspora* (7). This bacterium is common among the complex microbiota of the guinea pig cecum (7, 23, 32, 36), and similar symbionts have been found in the intestinal tracts of other rodents (23). *M. polyspora* bears a remarkable physical resemblance to the surgeonfish intestinal symbiont *Epulopiscium* spp. A distinct characteristic of both *Metabacterium* spp. and *Epulopiscium* spp. is the production of multiple, large inclusions (23) (Fig. 1). For *M. polyspora* these inclusions are the products of sporulation, while in *Epulopiscium* spp. they are daughter cells.

Epulopiscium spp. reproduce by the internal production of multiple offspring referred to as daughter cells (8, 16, 27). Daughter cells are initiated in the tips of an *Epulopiscium* cell and grow until they completely fill the parental cell (16, 27). The mature offspring are subsequently released from a slit in the parental cell cortex. The parental cell cortex is discarded as an empty husk (27).

The phylogenetic analysis of *M. polyspora* presented here was used to determine if the similarities of morphology and habitat between these organisms reflect a close evolutionary relationship. Together with other morphological comparisons, this phylogenetic analysis provides insight into the possible origin of the unique form of reproduction exhibited by *Epulopiscium* spp.

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MATERIALS AND METHODS

***M. polyspora* enrichment.** The cecum removed from a guinea pig, *Cavia porcellus*, was wrapped in moist towels and aluminum foil and stored frozen at −20°C until processed. A pea-sized portion of cecum contents was sliced out of a frozen cecum with a fresh razor blade and placed in sterile ST buffer (145 mM NaCl, 50 mM Tris-HCl [pH 8.3]). Formaldehyde was added to a final concentration of 0.5%. The suspension was placed on ice for approximately 1 h to fix and thaw cecum contents. To remove large pieces of vegetal matter, the suspension was filtered through glass wool that was loosely packed in the bottom of a large syringe.

A Ficoll step gradient was prepared in a 15-ml disposable centrifuge tube. Steps, 3 ml in volume, were layered into the tube in the following order from the bottom: 25, 20, 15, and 10% Ficoll. Ficoll stock solution was added to the filtered cecum sample to a final concentration of 5% Ficoll, and 1.5 ml of this sample was layered on top of the gradient. The tube was spun in a clinical centrifuge at setting 2 for 10 min. Fractions containing *M. polyspora* cells and little debris or other bacteria were pooled and diluted with an equal volume of ST buffer. The cells in this suspension were pelleted, washed twice with ST buffer, transferred to a microcentrifuge tube, and washed twice with 10 mM Tris-HCl, pH 8.3.

Cell disruption, amplification of genes coding for rRNA (rDNA), and PCR product cloning. The cell pellet was disrupted by using an alkaline lysis procedure as previously described (2) with the addition of five rounds of rapid freezing and thawing, performed by transferring the tube between a dry ice-ethanol bath and a 70°C heating block. Primers 8FPL (GCGGATCCGCGGCCGCTGCA GAGTTTGATCCTGGCTCAG) and 1492RPL (GGCTCGAGCGGCCGCCC GGGTTACCTTGTTACGACTT) were used in a PCR to amplify bacterial 16S rRNA genes from the lysate. Lysate (1 μl of a 1:10 dilution) was added to an amplification reaction mixture which contained 30 mM Tris-HCl (pH 8.3); 30 mM KCl; 1.5 mM $MgCl_2$; 0.05% Nonidet P-40; 0.2 μg of each primer; 0.2 mM (each) dATP, dCTP, dGTP, dTTP; and 1 U of *Thermus aquaticus* DNA polymerase in a 100-μl reaction mixture. The PCR mixture was overlaid with mineral oil and incubated in a Thermal Cycler (Perkin-Elmer Cetus). The cycler regimen was as follows: an initial heating step of 94°C for 4 min, followed by 30 rounds of 1.5 min at 92°C, 1.5 min at 37°C, and 2 min at 72°C. PCR products were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated from 0.3 M sodium acetate with ethanol. By using standard procedures, the products were restricted with *Not*I and *Pst*I, gel purified, and cloned into pBluescript KS^- (Stratagene) that had also been digested with *Not*I and *Pst*I (33).

Analysis of the rDNA clone library. A PCR assay with *Epulopiscium* group-specific primers was used to identify likely *Metabacterium* rDNA clones. Each of the 89 rDNA-containing clones was assessed with three PCRs containing the universal primer 515F with either an *Epulopiscium* group-specific primer, 822R

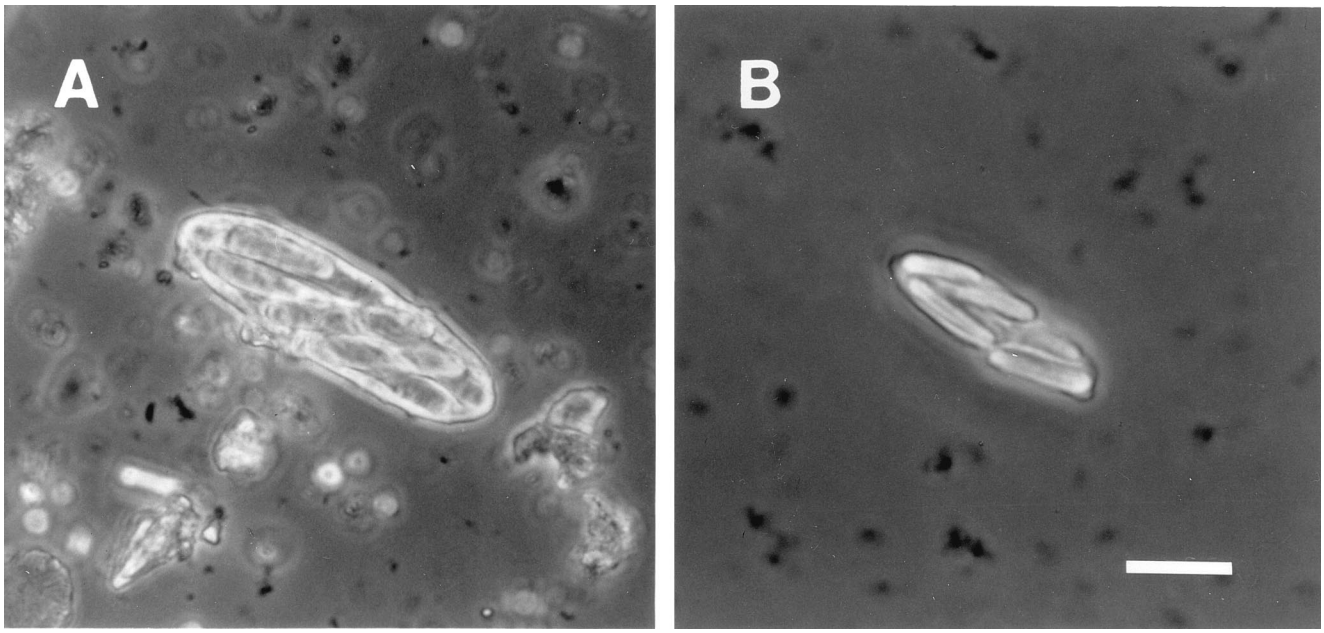

FIG. 1. Phase-contrast micrographs of a type F *Epulopiscium*-like intestinal symbiont from the surgeonfish *Acanthurus olivaceus* and of *M. polyspora* from the cecum contents of a guinea pig. (A) Type F surgeonfish symbiont containing seven daughter cells. The F morphotype is defined as ranging in length from 15 to 60 μm with the ability to produce as many as seven daughter cells (8). Daughter cell-producing *Epulopiscium*-like surgeonfish symbionts are closely related on the basis of in situ hybridization analyses (unpublished results). (B) *M. polyspora* with seven highly refractile endospores. A single *M. polyspora* cell can produce as many as nine endospores (32). While bacteria with the ability to produce two endospores are well documented (4, 10, 11, 25), the ability of *Metabacterium* spp. to produce more than two endospores is unique among known bacteria. *M. polyspora* cells range in length from 10 to 25 μm and are similar to *Epulopiscium* spp. in morphology and habitat. These photographs are at the same magnification. Bar, 10 μm.

or 1423R, or the universal primer 1492R (serving as a positive control). Amplifications were performed as described above except that the annealing temperature was increased to 50°C. Approximately 1 ng of plasmid DNA or 10 ng of genomic DNA was used in each reaction.

Plasmid DNA was sequenced by a dideoxy chain termination method (34).

In situ hybridization analyses. All in situ hybridizations were performed as previously described (12). Oligonucleotide probes that were used in the initial in situ hybridization analysis were the *Epulopiscium* group-specific probes 822R (CCCGTAAGSCCCGACACCTAGTATT) and 1423R (TTGCGGTTAGGT CACTGACGTTGGGCCCCCT). Two *M. polyspora* strain-specific oligonucleotides, M 3.29 (CATGCGATTTTGACAGT) and M 1.6 (CATGCAATACTC CATAGT), were designed and used to confirm the source of the rDNA clones. The strain-specific probes anneal to an extended helical element just downstream of *Escherichia coli* 16S rRNA position number 183.

Hybridization of oligonucleotide probes to cells that contain mature endo spores was enhanced by treatment with a strong acid, a modification of a previously described method (31). Slides coated with cecum contents were first treated with 1 N HCl in a 60°C water bath for 3 min. The slides were removed from the acid, rinsed with distilled water, and immersed in 100 mM Tris-HCl, pH 8.0, for 2 min, rinsed again, and air dried. These slides were hybridized as previously described (12). The acid treatment caused the extrusion of endospore contents which enhanced probe hybridization (data not shown).

Phylogenetic analyses. Clone sequences were manually aligned with bacterial sequences available through the Ribosomal Database Project (26). Alignments were based on conserved sequences, and a secondary structure model of the cloned rDNA gene product was based on the established 16S rRNA secondary structure (3, 20, 21, 39, 42). Several different algorithms were used to compare phylogenetic results, including distance matrix (29), the DeSoete method (13), and fastDNAml (14, 30).

Nucleotide sequence accession numbers. GenBank accession numbers for the 16S rRNA sequences for *M. polyspora* strains M 3.29 and M 1.6 are U22331 and U22332, respectively.

RESULTS

In situ hybridization analysis with *Epulopiscium* group-specific probes. We were intrigued by the gross morphological resemblance of *M. polyspora* and *Epulopiscium* spp. and, therefore, undertook to determine their phylogenetic relatedness. Initially, two fluorescently labeled *Epulopiscium* group-specific rRNA probes (2) were applied to guinea pig cecum contents. Both probes specifically hybridized to *M. polyspora* (data not shown). These results indicated a close phylogenetic affiliation between these organisms. A 16S rDNA sequence-based phylogenetic analysis was performed to define this relationship further.

Identification of *M. polyspora* rDNA clones. *M. polyspora* is not available in pure culture; consequently, cells for these analyses were collected directly from guinea pig cecum contents. As described in Materials and Methods, an enrichment of *M. polyspora* cells was obtained by centrifugation of cecum contents through a Ficoll step gradient. Cells from this enrichment were disrupted by treatment with alkali, and the lysate was used directly as the source of template for PCR amplification of bacterial 16S rRNA genes. The amplification products were cloned into a plasmid vector.

Since the *Epulopiscium* group-specific oligonucleotides exclusively hybridized to *M. polyspora* cells in the initial in situ analysis, they were used as primers in a PCR-based assay to identify likely *Metabacterium* sequences in the rDNA clone library. The assay involved subjecting cloned rDNAs to a series of three PCRs with the following combinations of primers: 515F (universally conserved) with 822R (*Epulopiscium* group specific), 515F with 1423R (*Epulopiscium* group specific), and 515F with 1492R (also universal, serving as a positive control). The PCR assay was first tested with a selection of bacterial genomic and plasmid DNAs. The three combinations of primers gave PCR products of the predicted size only for cloned *Epulopiscium* rDNAs. Corresponding products with all sets of primers were not generated with DNA templates from other gram-positive bacteria tested: *Heliobacterium chlorum*, *Bacillus subtilis*, *Bacillus megaterium*, *Eubacterium thermomarinus*, *Streptomyces coelicolor*, and the proteobacterium *Xenorhabdus*.

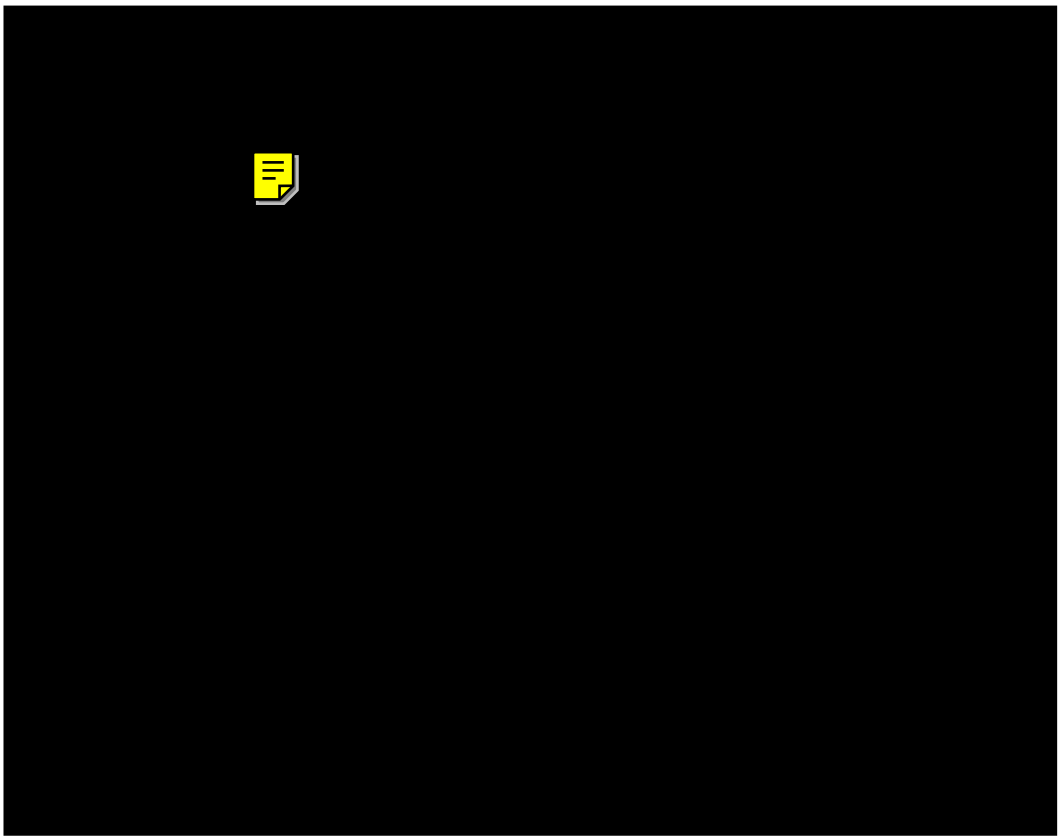

FIG. 2. In situ hybridization of guinea pig cecum contents with a probe designed to specifically identify putative *M. polyspora* rRNA. The oligonucleotide probe to identify M 3.29 was labeled with fluorescein and hybridized as previously described (12). Hybridization results of the M 1.6-specific probe that was labeled with Texas Red are not shown. These probes were designed to anneal to a hypervariable, extended helical element in the 183 (*E. coli* numbering) region of the 16S rRNA (see Materials and Methods). Panels show pairs of micrographs of fields hybridized with the M 3.29 probe, phase contrast on the left and epifluorescence on the right. (A and B) A vegetative *M. polyspora* cell. (C and D) The rare occurrence of fluorescent probe halos that presumably result from hybridization of the probe to rRNAs in cytoplasm but exclusion of the probe from the impermeable, mature endospores. The cecum contains material with varying degrees of autofluorescence. Despite treatment with sodium borohydride, residual autofluorescence can be seen in these samples but can easily be distinguished from fluorescence of bound probes by differences in color and by viewing the fields with several different fluorescent filter sets. All photographs were taken at the same magnification. Bar, 10 μm.

Abundant amplification products were obtained with all sets of primers for 25 of the 89 assayed clones of the cecum-derived bacterial rDNA clone library.

To ascertain the diversity of these putative *Metabacterium* rDNA clones, the sequence of approximately 200 nucleotides of the 3′ end of each of these 25 rDNA fragments was determined. Two categories of clones were identified. Within a category, sequences varied from one clone to another by less than 0.7%. This level of variation is consistent with one theme emerging from microbial community analyses, that a particular type of organism in the environment is not represented by a single type of rRNA but is represented by clusters of very similar rRNAs (18). These variants may represent intergenic differences within the same organism or subtle variations between closely related lineages.

For each category of sequences, one representative clone, exhibiting the consensus over the 200-nucleotide span, was selected for further analysis. Both strands of the representatives, M 3.29 and M 1.6, were sequenced in their entirety. These two rDNAs are 97.6% similar over the length of 1,316 positions used in the phylogenetic analyses. The inferred secondary structures of the two types of rRNA showed no idiosyncrasies in expected structural elements or conserved features. Therefore, the observed sequence variation is thought to represent rRNA strain diversity within the *M. polyspora* population and not PCR artifacts.

In situ hybridization analysis of *M. polyspora*. Further in situ hybridization analyses were used to confirm the sources of the rDNA clones. Two oligonucleotide probes, complementary to unique sequences in each of the putative *Metabacterium* rRNAs, were labeled with different fluorescent markers, which allowed for simultaneous hybridization of the probes to cecum contents. The probe for M 3.29 hybridized to the majority of *M. polyspora* vegetative cells (Fig. 2). Hybridization of the M 1.6 probe to *M. polyspora* cells was rare (data not shown), indicating that this strain was a minor component of the community. No cross-hybridization was observed, indicating that these rRNA sequences are derived from discrete strains of *M. polyspora*. Each probe hybridized to both vegetative and spore-containing cells, which rules out the possibility that M 3.29 and M 1.6 represent stage-specific rRNAs, a situation previously observed in the eukaryote *Plasmodium bergheii* (19). Individual *Metabacterium* cells have not been monitored from the vegetative state through spore formation. Therefore, the observation that both probes hybridize to both vegetative and endospore-containing cells is the first direct evidence establishing these morphologically distinct cells as stages in the *M. polyspora* life cycle.

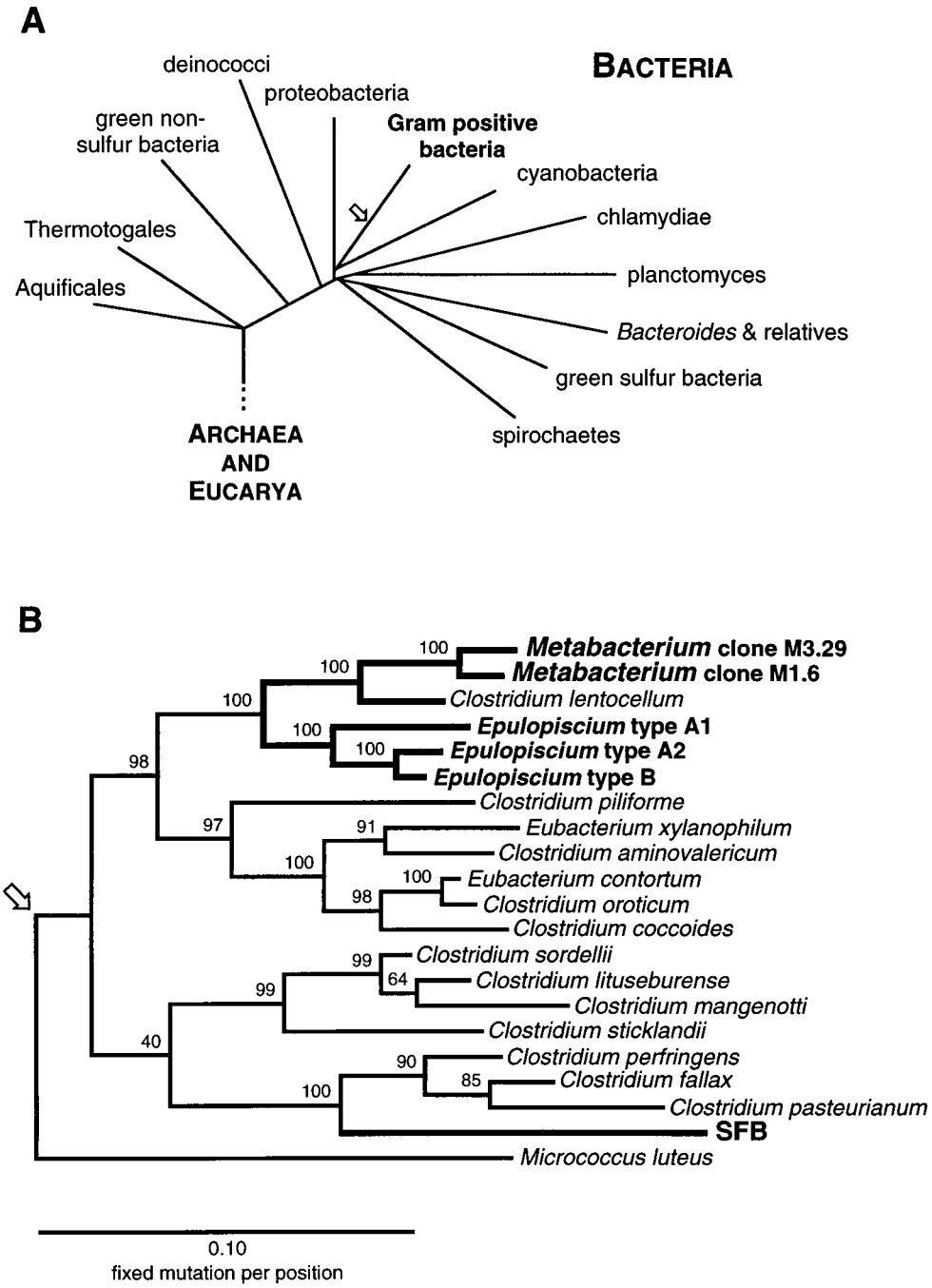

FIG. 3. Phylogenetic trees relating *M. polyspora* to other bacteria. (A) Diagrammatic representation of the bacterial phylogenetic domain (38, 40). *M. polyspora* belongs to the group of low-G+C gram-positive bacteria (41). The arrow represents the approximate point of divergence of the low-G+C gram-positive bacteria shown in panel B. (B) Phylogenetic comparison of the two identified *M. polyspora* sequences with other bacterial 16S rRNA sequences using the fastDNAml program. The high-G+C gram-positive bacterium *M. luteus* served as the outgroup for the low-G+C gram-positive bacteria used in this analysis. The tree shows *Metabacterium* spp. with some of their closest known clostridial cluster XIV relatives (9). Also included are the only other phylogenetically characterized daughter cell-producing bacteria, SFB, with some of their group I (22)-cluster I (9) clostridia relatives: *C. pasteurianum*, *C. fallax*, and *C. perfringens*. *C. sordellii*, *C. lituseburense*, *C. mangenotti*, and *C. sticklandii* are members of the clostridial cluster XIV (9). Numbers at nodes represent the proportion of 100 bootstrap resamplings that support the shown topology (15).

Phylogenetic analysis of the *Metabacterium* rDNA clones. The phylogenetic analyses of M 3.29 and M 1.6 revealed that these clones were derived from low-G+C gram-positive bacteria, most closely associated with *Clostridium lentocellum* and *Epulopiscium* spp. Figure 3 shows the results of a maximum likelihood tree of the *M. polyspora* rDNAs with other gram-positive bacteria. Distance matrix, Desoete, and maximum likelihood analyses all resulted in the same topology for the clade containing *M. polyspora*, *C. lentocellum*, and *Epulopiscium* spp. These organisms constitute a coherent relatedness group to the exclusion of 774 gram-positive rRNA sequences that were available through the ribosomal database project at

the time of this analysis (26). These results confirm the close affiliation of *M. polyspora* and *Epulopiscium* spp. indicated by the initial in situ hybridization analysis. The cellulolytic bacterium *C. lentocellum* is presently the only cultivated representative of this clade (28). The culture conditions used for *C. lentocellum* may aid in the development of culture conditions for *Metabacterium* or *Epulopiscium* species. Since *Epulopiscium* spp. and *Metabacterium* spp. both inhabit the intestinal tracts of herbivores, it is possible that they, too, are cellulolytic.

DISCUSSION

The results of these analyses prove *M. polyspora* to be one of the closest known relatives of *Epulopiscium* spp., the largest known bacterium. This result may lend insight into the evolutionary origin of daughter cell production in *Epulopiscium* spp. Aside from *Epulopiscium* spp., few known bacteria produce viviparous offspring intracellularly. A morphologically distinct form of reproduction occurs in the pleurocapsalean cyanobacteria. In these organisms, nucleoid divisions followed by septation result in the production of large numbers of tiny baeocytes. Lysis of the parental cell then releases the baeocytes, which are not resistant spores but independent, often motile, offspring (37).

Another group of microbes that produce offspring internally are the uncultivated and unnamed segmented filamentous bacteria (SFB) that live anchored, by means of a holdfast, to the internal ileal walls of rats and mice (5, 17). Microscopic studies have found that as these SFB enter a reproductive mode, the distal segments, followed sequentially by more proximal segments, initiate the internal production of two holdfast-bearing cells. These offspring then follow one of two fates. Live daughter cells can be released upon the deterioration of the mother cell, to settle into the ileal wall and produce another filament. Alternatively, the internal offspring can be encased in a thick spore coat, which would facilitate dispersal outside the host. A phylogenetic analysis of this murine symbiont proved it, too, to be a member of the low-G+C gram-positive bacteria but not a specific relative of *Epulopiscium* spp. (35) (Fig. 3). More genetic and morphological data are needed to determine if daughter cell formation in these SFB is a process related to daughter cell formation in *Epulopiscium* spp. Since all identified *Epulopiscium*-like symbionts are closely related (based on in situ hybridizations) (1, 2) and are phylogenetically isolated from other organisms that produce daughter cells, it appears that daughter cell production has arisen independently, in this group of bacteria. (It is of course possible, but we think unlikely, that the internal production of offspring arose prior to endospore production and was lost in all other known lineages.)

The close phylogenetic relationship of *M. polyspora* and *Epulopiscium* spp. suggests an evolutionary pathway for the novel form of reproduction exhibited by *Epulopiscium* spp. Since its closest relatives, *Metabacterium* spp. and *Clostridium* spp., are endospore-forming organisms, we hypothesize that the bacterial differentiation process of sporulation was modified in a predecessor of *Epulopiscium* spp., to produce multiple viviparous offspring instead of dormant endospores. This notion is supported by the morphological similarities of *Epulopiscium* spp. and *Metabacterium* spp. and similarities between sporulation and daughter cell production. Where endospore production is best characterized, in *B. subtilis*, a forespore is initiated in the tip of a differentiating cell. The first morphological change associated with sporulation is asymmetric septation. Next, the membrane of the larger compartment, the mother cell, migrates around the smaller forespore, engulfing it and eventually pinching it off as a free protoplast within the mother cell. This is followed by the elaboration of a spore coat and, eventually, the programmed lysis of the mother cell (for a review, see reference 24). We suggest that the earliest stages of sporulation, septation followed by engulfment, were coopted for the production of live offspring in a predecessor of *Epulopiscium* spp. Consistent with this notion is the observation that in *Epulopiscium* spp. which produce two daughter cells, the newly formed daughters are seen closely associated with the tips of the mother cell (16, 27). The earliest morphological stages of sporulation in *M. polyspora* and daughter cell development in *Epulopiscium* spp., particularly for those that produce more than two offspring, have yet to be defined. The apparent ability of the distantly related gram-positive SFB to produce daughter cells while retaining sporulation capability is also consistent with this hypothesis.

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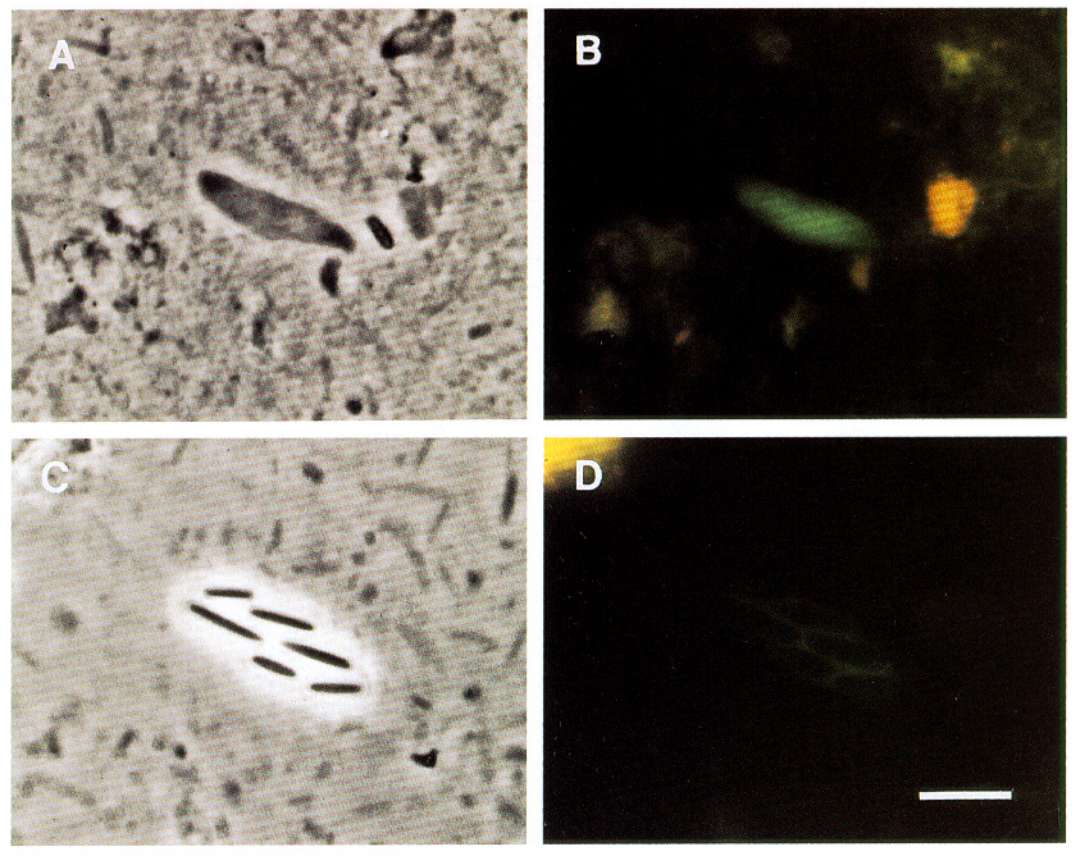
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