Proposal of *Quinella ovalis* gen. nov., sp. nov., Based on Phylogenetic Analysis

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Quin's oval is a relatively large bacterium often seen in the rumens of sheep fed diets containing some readily fermented carbohydrates. It has not been obtained in axenic cultures, but a number of its features have been determined by various methods, such as studying cell suspensions purified from rumen fluid by differential centrifugation. We obtained similarly purified suspensions from a sheep fed a diet containing a large amount of molasses. Nearly complete 16S rRNA sequence analysis of these cells as well as cells of *Selenomonas ruminantium* subsp. *ruminantium* GA192 (ATCC 12561; type strain) and *S. ruminantium* subsp. *lactilytica* HD₄ (ATCC 27209) was done. These sequences were compared with those of other bacteria. Evolutionary distance estimates indicated that Quin's oval was most closely related to the *Selenomonas-Megasphaera-Sporomusa* group in the gram-positive phylum but that it belongs in a new genus. We propose the name *Quinella ovalis* gen. nov., sp. nov., with its description based on previously known features.

Quin's oval (QO) was briefly described by Woodcock and Lapage in 1913 (27) and separately discovered by Quin (16) in 1943. Quin found it in large numbers in the rumens of sheep fed diets of sugar-rich fresh alfalfa either alone or supplemented with molasses, sucrose, or glucose. Although he did not isolate it, he named it "*Schizosaccharomyces ovis*" because of its yeast-like (gassy) rapid fermentation of sugar, its relatively large (4-by-8 μ m) oval to oblong-oval shape, and its reproduction by binary fission. He also found that it produces glycogen intracellular reserve material from part of the sugar used.

Further studies on QO have been hampered by the inability of researchers to obtain pure cultures; however, a considerable number of its features have been determined by various techniques. For example, Howard and coworkers (2, 6, 23) obtained highly enriched cells by differential centrifugation of rumen fluid from sheep fed diets that promoted the growth of large numbers of QO. Using these suspensions, they obtained information on the carbohydrate fermented and the products produced and definitive information on glycogen storage. They also determined that since the cell walls of QO were shown to contain 2,4-diaminopimelic and muramic acids, it is a bacterium. Other studies, such as those of Orpin (13), which used very slow and meticulous culture techniques, have confirmed and extended the information on carbohydrate fermentation, motility, gram-negative staining, and several other features.

QO is considered an important part of the microbiota of the rumens of sheep, and it occurs in numbers from 1.9×10^5 to 3.0×10^8 /ml (13). It has been found in numbers as high as 10^{11} /ml (21) in sheep fed mainly molasses.

While no taxonomic information has been published on QO since the definitive studies showing that it is a bacterium (23), one of us (M.P.B.) has long surmised that it is at least

somewhat related to *Selenomonas ruminantium* subspecies (4, 15). Its fermentation products, propionate and acetate, its glycogen reserve material and unusual flagellation, and its fermentation of mannitol (2, 13, 15), which is specific to few rumen microbes, are similar (6, 14, 15).

The information from these previous studies provides the essence of a phenotypic description for QO; thus, a phylogenetic assessment of the organism was done by 16S rRNA sequence analysis of cells purified from ruminal fluid by differential centrifugation.

Also, while 16S rRNA oligonucleotide cataloging indicated that *Selenomonas*, *Sporomusa*, and *Megasphaera* form a cluster in the gram-positive phylum (18), no sequencing of 16S rRNA of *Selenomonas* species has been done. Thus, we sequenced the RNA of *S. ruminantium* subsp. *ruminantium* and that of *S. ruminantium* subsp. *lactilytica* for comparison with that of QO in this study.

MATERIALS AND METHODS

Source of strains and preparation of cells. A sheep with a cannulated rumen was fed a diet consisting of pellets (0.5 kg; 80% alfalfa hay, 20% dry molasses) and a liquid molasses mixture (2.5 kg) twice daily (21). After the sheep had been on the diet for a 3-week period, QO dominated the rumen microbial population, and rumen contents were removed and strained through cheesecloth. Cells of QO were enriched from 500 ml of rumen contents by differential centrifugation as previously described (23). The resulting cell pellet contained greater than 90% QO cells (cell count) as determined by phase-contrast microscopy, with the other morphotypes being small bacteria. These cells were frozen at -20°C until needed for sequencing.

S. ruminantium subsp. *ruminantium* GA192 (ATCC 12561^T) and *S. ruminantium* subsp. *lactilytica* HD₄ (ATCC 27209) were from the collection of M. P. Bryant. The anaerobic techniques used for cultivation were those of Hungate (8) as modified by Bryant (3) and Balch and Wolfe (1). The medium was composed of 30% (vol/vol) clarified rumen fluid and 0.2% (wt/vol) glucose, 0.1% cellobiose, 0.1% starch, 0.0002% resazurin, 0.05% cysteine-HCl, 0.05% $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.4% CO_2 -equilibrated Na_2CO_3 , and minerals

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[0.045% K_2HPO_4 , 0.045% KH_2PO_4 , 0.045% $(NH_4)_2SO_4$, 0.09% NaCl, 0.019% $MgSO_4 \cdot 7H_2O$, 0.012% $CaCl_2 \cdot 2H_2O$].

Ribosomal purification and RNA sequencing. Cells were fractured by mechanical disruption with glass beads (19). Ribosomes were isolated on a sucrose step gradient, and the rRNA was isolated by phenol, phenol-chloroform (4:1), and chloroform extractions followed by ethanol precipitation (10, 19). Dideoxy nucleotide sequencing was carried out with reverse transcriptase and ^{35}S -dATP as described by Lane et al. (10) with three universal oligonucleotide primers (10). The remaining sequence was determined with four additional primers. Their sequences and complementary priming sites (*Escherichia coli* 16S rRNA numbering) are CTACGGG (G/A)(G/C)GCAGCAG (residues 342 to 357), TCTACGCAT TTCACC (residues 690 to 704), AGGGTTGCGCTCGTTG (residues 1100 to 1115), and GGGTACCTTGTTACG (residues 1496 to 1510).

Phylogenetic analysis. Sequences were aligned by using conserved features of the primary and secondary structures of 16S rRNA genes (26). Sequence similarities were calculated by comparing only those positions at which bases could be unambiguously aligned (approximately 1,300 bases). Evolutionary distances were calculated by the method of Jukes and Cantor (9).

A phylogeny was determined from the evolutionary distance by the algorithm of De Soete (5). This inferred phylogeny was consistent with that derived by a second method in which the optimal branching order was deduced by an iterative program that tested alternative branching orders that minimized tree error (12). Error was defined as the sum of the squares of the differences between the pairwise evolutionary distance estimates and the corresponding tree representation of the distance. Each difference was weighted by the corresponding statistical uncertainty of the distance estimate (12).

Nucleotide sequence accession numbers. GenBank accession numbers for the sequences for the various strains and species (Table 1 and Fig. 1) include M23928 (*Lactobacillus casei*), M23732 (*Clostridium innocuum*), M23731 (*Clostridium ramosum*), M23927 (*Clostridium barkeri*), M23930 (*Clostridium pasteurianum*), M23929 (*Clostridium aminovalericum*), M26494 (*Clostridium sticklandii*), M62701 (QO [*Quinella ovalis*]), M26493 (*Megasphaera elsdenii*), M62702 (*S. ruminantium* subsp. *ruminantium*), M62703 (*S. ruminantium* subsp. *lactilytica*), and M11212 (*Heliobacterium chlorum*).

RESULTS AND DISCUSSION

Analysis of the 16S rRNA sequence data (Table 1) shows the values for estimated evolutionary distances between QO 16S rRNA and those of other more or less related bacteria. The 16S rRNA sequence of QO is related to those of the gram-positive group, even though QO has a gram-negative ultrastructure (23). Similar relatedness within the gram-positive groups has been found in the sequences of other gram-negative bacteria, such as those in the genera *Selenomonas*, *Megasphaera*, and *Sporomusa*, to which the sequence of QO is most closely related. On the basis of the 16S rRNA oligonucleotide-cataloging technique, these three genera were previously demonstrated to form a distinct evolutionary group (18). QO has now been shown to belong to this group.

A phylogenetic tree, inferred from an evolutionary distance matrix (Table 1), illustrates the evolutionary distances among selected phylogenetically gram-positive *Eubacteria*

TABLE 1. Evolutionary distance matrix^a

Bacterium	Evolutionary distance from:									
	<i>B. subtilis</i>	<i>L. casei</i>	<i>C. innocuum</i>	<i>C. ramosum</i>	<i>C. pasteurianum</i>	<i>C. aminovalericum</i>	<i>C. sticklandii</i>	<i>C. barkeri</i>	<i>H. chlorum</i>	<i>M. elsdenii</i> <i>S. ruminantium</i>
<i>Bacillus subtilis</i>										
<i>Lactobacillus casei</i>	12.2									
<i>Clostridium innocuum</i>	17.9	17.5								
<i>Clostridium ramosum</i>	17.1	18.0	16.6							
<i>Clostridium pasteurianum</i>	16.5	17.1	22.9	21.3						
<i>Clostridium aminovalericum</i>	18.8	18.7	21.5	19.2	17.1					
<i>Clostridium sticklandii</i>	18.3	18.9	23.2	22.7	17.1	17.2				
<i>Clostridium barkeri</i>	16.9	19.1	22.1	22.3	17.2	18.9	17.2			
<i>Heliobacterium chlorum</i>	18.3	19.9	24.1	21.5	18.9	21.2	22.0	21.5		
Quin's oval	21.7	21.9	23.4	25.2	20.4	23.1	23.0	21.9	19.4	
<i>Megasphaera elsdenii</i>	21.5	20.9	21.4	21.1	22.2	21.3	21.8	23.0	20.8	18.6
<i>Selenomonas ruminantium</i>	18.9	17.8	20.8	21.5	17.9	19.7	21.1	19.5	18.1	14.2
										12.3

^a The evolutionary distances are based on aligned 16S rRNA sequences as described in Materials and Methods. The sources of sequences not determined in this study were *B. subtilis* (7), *L. casei*, *C. pasteurianum*, *C. aminovalericum*, *C. sticklandii*, *C. barkeri*, *M. elsdenii* (28), *H. chlorum* (25), *C. innocuum*, and *C. ramosum* (22). The root of the tree was inferred by use of the cyanobacterium *Anacystis nidulans* 16S rRNA sequence (20) as an outgroup. The sequence of *S. ruminantium* subsp. *ruminantium* GA192 was used in the analysis and differs in 17 positions from approximately 1,200 nucleotides of sequence determined for *S. ruminantium* subsp. *lactilytica* HD₄ (positions 98 to 1466 *E. coli* numbering).

FIG. 1. Phylogenetic relationship of QO to other selected bacteria. The phylogenetic tree was inferred from the evolutionary distance matrix (Table 1) by the algorithm of De Soete (5).

genera and their relationship to QO (Fig. 1). However, QO is not closely related to either *M. elsdenii* or *S. ruminantium*. Neither is it specifically related to *Sporomusa paucivorans*. The evolutionary distances separating *S. paucivorans* from QO, *M. elsdenii*, and *S. ruminantium* GA192 are 18.5, 14.8, and 13.2 (24). Thus, QO is shown to be a distinct member of this assemblage of organisms that share a gram-negative ultrastructure but are affiliated with gram-positive bacteria. Absolute boundaries, based on 16S rRNA sequence similarities, have not been proposed for defining a genus, species, or higher taxons, nor may it be appropriate to do so, since a working taxonomy must consider both the phenotype and the phylogenetic position of the organism. However, on the basis of the evolutionary distances separating QO and its known relatives in relationship to distances separating other described genera, we propose that QO be placed in a separate genus and species.

Description of the new genus and species. *Quinella* gen. nov. *Quinella* (Quin.el'la. N.L. fem. n.) is named for the pioneering ruminologist, J. I. Quin (16), who described it in some detail. It includes nonsporing, heterotrophic, mesophilic, anaerobic ovals with tumbling motilities and linear tufts of flagella on one side of their cells (14). The genus is gram negative, and its cell wall contains a distinct outer membrane and muramic and *m*-diaminopimelic acids (23). It reproduces by binary fission, and it usually occurs as singles and pairs. It ferments a small number of carbohydrates, and it produces lactate, acetate, propionate, and CO₂ as the main fermentation products (2, 13) and glycogen-like reserve material (6).

The type species is *Q. ovalis*.

Q. ovalis sp. nov. The description of *Q. ovalis* sp. nov. (o.val'is. L. fem. adj., egg-shaped) is that of the genus plus the following. Cells are oval. They are about 3 to 4 by 5 to 8

µm in size but can be considerably smaller in the rumen if crowding occurs (21). Photomicrographs that represent the morphology have been published (11, 16, 21, 23).

The species has not been axenically cultivated, and therefore, this description is the type of the species (rule 18 in the *International Code of Nomenclature of Bacteria*). Highly enriched and partially purified cell suspensions ferment glucose, fructose, sucrose, and mannitol and slowly metabolize maltose. Polysaccharides, amino acids, proteins, lactate, melibiose, mannose, glucosamine, galactose, rhamnose, cellobiose, lactose, glucuronic acid, xylose, arabinose, and soluble starch are not fermented or are fermented at extremely low rates (2, 13).

Rapid growth occurs at 37 to 39°C, and slow growth occurs at 44°C. No growth occurs at 25 or 50°C (13). It grows in the rumen at pH values at least somewhat lower than 6.0. The higher pH limit is not known. It produces mainly lactic acid as a fermentation product when the sugar energy source is not limiting growth, but produces mainly acetate, propionate, and CO₂ when the energy source is limiting and growth is slow (21), as does *S. ruminantium* (17). When it is by far the main organism in the rumens of sheep fed mainly molasses (21), acetate and propionate are the main organic acids produced and no lactate is detectable at any time during the 24-h feeding cycle.

Q. ovalis is observed in mesophilic, anaerobic, and rumen environments such as the rumens of sheep, but it is probably also widely distributed in other ruminants such as llamas (13). The number observed in the rumen is strongly influenced by the amount of fermentable sugars, such as glucose or sucrose, in the animal's diet (2, 13, 16, 21).

Relationships of *Q. ovalis*. Its closest relatives, on the basis of 16S rRNA sequence analyses, are *S. ruminantium* subsp. *ruminantium* and *S. ruminantium* subsp. *lactilytica*, which

as subspecies share approximately 98.8% 16S rRNA sequence similarity (Table 1). These two subspecies are also similar to *Q. ovalis* in their fermentation products, but they differ from *Q. ovalis* in their abilities to ferment many more sugars, glycosides, and amino acids and in their curved-to-helical shape (4). The large selenomonad "*S. ruminantium* subsp. *bryanti*" differs from *Q. ovalis* as do the other two subspecies, except that it ferments fewer energy sources than the other two subspecies (4, 15). However, unlike *Q. ovalis*, it ferments both cellobiose and mannose. Cultures of *S. ruminantium* subsp. *bryanti* are not extant, so it would be quite difficult to obtain cells for 16S rRNA sequencing for comparison with the sequence of *Q. ovalis*.

Another rumen oval bacterium, "*Magnooovum eadui*," that produces glycogen reserves and the fermentation products lactate, acetate, propionate, and CO₂ is similar to *Q. ovalis* in the above respects but differs greatly in that it is much larger, it has a gliding motility with a very large number of peritrichously arranged flagella, and, while it has a similarly gram-negative ultrastructure, it contains no ribose but does contain galactosamine in the cell wall (14).

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