Evidence for a Close Phylogenetic Relationship between *Melissococcus pluton*, the Causative Agent of European Foulbrood Disease, and the Genus *Enterococcus*

J. CAI AND M. D. COLLINS*

Department of Microbiology, Institute of Food Research, Reading Laboratory, Reading RG6 2EF, United Kingdom

The 16S rRNA gene sequence of *Melissococcus pluton*, the causative agent of European foulbrood disease, was determined in order to investigate the phylogenetic relationships between this organism and other low-G+C-content gram-positive bacteria. A comparative sequence analysis revealed that *M. pluton* is a close phylogenetic relative of the genus *Enterococcus*.

"*Bacillus pluton*," the primary etiological agent of European foulbrood of the honey bee, *Apis mellifera*, was originally described by White (14). This bacterium was first cultured and characterized in detail by Bailey (1) and was reclassified as "*Streptococcus pluton*" by him. The taxonomic position of "*S. pluton*" has always been problematic, and the species was not included on the Approved Lists of Bacterial Names (12). Bailey and Collins (3) assigned "*S. pluton*" to a new genus, *Melissococcus*, on the basis of the results of phenotypic studies (2). Our knowledge concerning the genetic interrelationships of streptococci and related low-G+C-content gram-positive taxa has improved considerably in recent years with the application of nucleic acid hybridization and 16S rRNA sequencing techniques. As a result, the genus *Streptococcus* is now primarily restricted to pyogenic and oral species (10). The "lactic" (or Lancefield group N) and "fecal" (Lancefield group D) streptococci have been assigned to two new genera, *Lactococcus* and *Enterococcus*, respectively (9–11). More recent comparative 16S rRNA sequencing data have shown that the genus *Lactococcus* is phylogenetically more closely related to the genus *Streptococcus* than to the genus *Enterococcus* (4). The latter genus is phylogenetically closely related to the genera *Carnobacterium* and *Vagococcus* (4, 13, 15). Until now, there has been no information concerning the phylogenetic affinities of the genus *Melissococcus*. In this paper we describe the 16S rRNA gene sequence of *Melissococcus pluton* and the results of a comparative analysis performed with other catalase-negative, low-G+C-content, gram-positive bacteria.

M. pluton NCDO 2443^T (T = type strain) was grown as described by Bailey and Collins (2), and chromosomal DNA was prepared by using the method of Farrow et al. (6). The 16S rRNA gene of *M. pluton* NCDO 2443^T was amplified by a PCR and was sequenced directly by using a Sequenase version 2.0 sequencing kit (U.S. Biochemicals) as described previously (8). Sequences were aligned and similarity values were determined by using the Wisconsin Molecular Biology package (5). Phylogenetic trees were constructed by using neighbor-joining and unweighted pair group with mathematical average methods.

The 16S rRNA gene sequence of *M. pluton* NCDO 2443^T which we determined has been deposited in the GenBank data base under accession number X75751. This sequence consisted of 1,396 nucleotides, representing approximately 93% of the

total 16S rRNA primary structure. The derived 16S rRNA sequence of *M. pluton* was aligned with more than 150 other low-G+C-content gram-positive 16S rRNA sequences retrieved from the EMBL and GenBank data bases. For the initial homology analysis approximately 90 nucleotides proximal to the 5' end of the molecule were removed because of problems in aligning the hypervariable V1 region, which is different lengths in phylogenetically diverse taxa. After we established the closest relatives of *M. pluton*, however, this stretch of sequence could be aligned unambiguously and was

FIG. 1. Dendrogram showing the relationships of *M. pluton*, enterococci, and other low-G+C-content gram-positive organisms. Clustering was by the unweighted pair group method. T., *Tetragenococcus*; C., *Carnobacterium*; V., *Vagococcus*; E., *Enterococcus*.

* Corresponding author. Mailing address: AFRC Institute of Food Research, Reading Laboratory, Earley Gate, Whiteknights Road, Reading RG6 2EF, United Kingdom. Phone: 0734 357000. Fax: 0734 267917.

TABLE 1. Levels of sequence similarity for a 1,396-nucleotide region of the 16S rRNAs of *M. pluton* and closely related low-G+C-content gram-positive organisms

Strain	% Sequence similarity to:																				
	<i>Carnobacterium mobile</i> NCFB 2765 ^T	<i>Carnobacterium piscicola</i> NCDO 2762 ^T	<i>Enterococcus avium</i> NCDO 2369 ^T	<i>Enterococcus casseliflavus</i> NCDO 2376	<i>Enterococcus cecorum</i> NCDO 2674 ^T	<i>Enterococcus columbae</i> STR 345 ^T	<i>Enterococcus dispar</i> NCIMB 13000 ^T	<i>Enterococcus durans</i> NCDO 596 ^T	<i>Enterococcus faecalis</i> NCDO 581 ^T	<i>Enterococcus faecium</i> NCDO 942 ^T	<i>Enterococcus gallinarum</i> NCDO 2313 ^T	<i>Enterococcus hirae</i> NCDO 1258 ^T	<i>Enterococcus malodoratus</i> NCDO 846 ^T	<i>Enterococcus mundtii</i> NCDO 2375 ^T	<i>Enterococcus pseudoavium</i> NCDO 2138 ^T	<i>Enterococcus raffinosus</i> NCTC 1219 ^T	<i>Enterococcus saccharolyticus</i> NCDO 2594 ^T	<i>Enterococcus sulfureus</i> NCDO 2379 ^T	<i>Tetragenococcus halophilus</i> NCDO 1635 ^T	<i>Vagococcus fluvialis</i> NCDO 2497 ^T	<i>Vagococcus salmoninarum</i> NCFB 2777 ^T
<i>Melissococcus pluton</i> NCDO 2443 ^T	92.4	92.2	95.2	96.0	94.0	93.7	95.0	95.3	95.0	95.2	95.7	95.5	95.2	95.4	95.2	95.2	95.4	94.9	93.9	93.1	93.2
<i>Carnobacterium mobile</i> NCFB 2765 ^T		96.9	94.1	94.3	94.1	92.4	94.2	94.2	94.2	94.1	94.2	94.2	94.3	94.1	94.2	94.3	94.3	94.4	92.3	94.1	94.0
<i>Carnobacterium piscicola</i> NCDO 2762 ^T			94.0	93.8	93.1	92.8	93.8	93.8	92.9	93.6	93.7	93.9	94.4	93.7	94.1	94.1	93.8	93.6	91.4	92.6	94.2
<i>Enterococcus avium</i> NCDO 2369 ^T				98.7	96.1	96.1	97.4	98.8	96.2	98.8	98.7	99.0	99.6	98.8	99.4	99.5	96.8	96.5	93.7	94.5	93.9
<i>Enterococcus casseliflavus</i> NCDO 2376					96.5	96.3	98.1	98.3	96.5	98.1	99.8	98.2	98.9	97.9	98.8	98.7	97.8	97.3	94.5	95.0	94.3
<i>Enterococcus cecorum</i> NCDO 2674 ^T						97.4	95.6	95.9	94.5	95.7	96.5	95.7	96.3	95.4	96.1	95.9	95.7	95.4	92.9	93.9	92.8
<i>Enterococcus columbae</i> STR 345 ^T							96.0	95.6	93.8	95.5	96.3	95.3	96.2	95.1	95.9	95.8	95.5	94.4	92.2	93.2	92.3
<i>Enterococcus dispar</i> NCIMB 13000 ^T								97.7	95.6	97.6	98.1	97.7	97.8	97.4	97.6	97.2	97.4	95.9	93.9	94.4	93.7
<i>Enterococcus durans</i> NCDO 596 ^T									96.2	99.7	98.3	99.7	98.8	99.1	98.5	98.4	97.0	95.9	93.6	94.5	94.0
<i>Enterococcus faecalis</i> NCDO 581 ^T										96.2	96.5	96.4	96.2	96.3	96.0	95.6	95.4	94.8	93.0	93.8	93.9
<i>Enterococcus faecium</i> NCDO 942 ^T											98.2	99.7	98.8	99.0	98.5	98.5	97.1	96.0	93.3	94.0	93.9
<i>Enterococcus gallinarum</i> NCDO 2313 ^T												98.3	99.2	98.0	98.9	98.8	97.8	97.2	94.4	94.7	94.2
<i>Enterococcus hirae</i> NCDO 1258 ^T													98.8	99.3	98.5	98.7	97.1	96.1	93.2	94.0	93.8
<i>Enterococcus malodoratus</i> NCDO 846 ^T														98.5	99.5	99.5	97.3	96.7	93.5	94.6	94.1
<i>Enterococcus mundtii</i> NCDO 2375 ^T															98.3	98.3	96.6	96.2	93.7	94.3	94.1
<i>Enterococcus pseudoavium</i> NCDO 2138 ^T																99.2	96.7	96.5	93.6	94.7	93.8
<i>Enterococcus raffinosus</i> NCTC 1219 ^T																	97.0	96.3	93.5	94.7	94.0
<i>Enterococcus saccharolyticus</i> NCDO 2594 ^T																		96.9	94.2	93.8	93.8
<i>Enterococcus sulfureus</i> NCDO 2379 ^T																			93.7	94.0	93.7
<i>Tetragenococcus halophilus</i> NCDO 1635 ^T																				91.0	91.5
<i>Vagococcus fluvialis</i> NCDO 2497 ^T																					94.7
<i>Vagococcus salmoninarum</i> NCFB 2777 ^T																					

therefore reintroduced to calculate percentages of similarity. Representative sequence similarity values for *M. pluton* and its closest relatives are shown in Table 1, and a phylogenetic tree is shown in Fig. 1.

The 16S rRNA sequence of *M. pluton* exhibited very low levels of similarity with the sequences of streptococci (approx-

imately 87 to 89%), and thus *M. pluton* is phylogenetically only remotely related to the genus *Streptococcus* (data not shown). *M. pluton* exhibited the highest levels of sequence similarity with enterococci (range, approximately 94 to 96%), carnobacteria (92%), and vagococci (93%) (Table 1). The results of the treeing programs were consistent with the findings described

above, and *M. pluton* clustered at the periphery of the genus *Enterococcus* (15). Close, albeit more distant, relationships to the genera *Carnobacterium*, *Vagococcus*, and *Tetragenococcus* (formerly *Pediococcus halophilus*) were also observed (Fig. 1).

We found that on the basis of 16S rRNA sequence data *M. pluton* is phylogenetically only remotely related to streptococci. Both evolutionary distance calculations and treeing programs revealed that *M. pluton* and enterococci are closely related. Additional evidence for a close affinity between these taxa comes from the report that Lancefield group D antigen occurs in *M. pluton* (7). Because of the close relationship between *M. pluton* and enterococci, it could be argued that these organisms should be placed in a single genus. However, we consider such a change in nomenclature to be unwise, as the monospecific genus *Melissococcus* (Bailey and Collins 1983) has nomenclatural priority over the genus *Enterococcus* (Schleifer and Kilpper-Bälz 1984). The genus *Enterococcus* currently contains 18 species, and this genus name is widely used and accepted by the scientific community. A change of name would in our opinion serve no useful purpose and would create unnecessary nomenclatural confusion. Hence, we believe that the fastidious cultural requirements and low DNA G+C content of *M. pluton* (2), together with the branch point of *M. pluton* at the periphery of the *Enterococcus* cluster (Fig. 1), are sufficient to justify retention of *Melissococcus* as a separate genus.

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