Analysis of the β' Subunit of DNA-Dependent RNA Polymerase Does Not Support the Hypothesis Inferred from 16S rRNA Analysis that *Oenococcus oeni* (Formerly *Leuconostoc oenos*) Is a Tachytelic (Fast-Evolving) Bacterium

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rRNA sequencing has shown that leuconostocs comprise three distinct phylogenetic lineages which have been designated separate genera (viz., the genera *Leuconostoc sensu stricto*, *Oenococcus*, and *Weissella*). In addition, the 16S rRNA line formed by *Oenococcus oeni* (formerly *Leuconostoc oenos*) is exceptionally long; this fact, together with variations in the compositions of conserved positions in the 16S rRNA, has led to the hypothesis (D. Yang and C. R. Woese, *Syst. Appl. Microbiol.* 12:145–149, 1989) that this organism is a fast-evolving bacterium. Previous evidence that the leuconostocs should be divided into three genera and that *O. oeni* is an example of tachytelic evolution has come solely from rRNA analyses. In this study we sequenced the *rpoC* gene encoding the β' subunit of DNA-dependent RNA polymerase of leuconostocs and performed a comparative phylogenetic analysis. The subdivision of the leuconostocs into three distinct lineages was confirmed by the *rpoC* gene data, but no evidence that indicated that *O. oeni* is evolving at an extraordinary rate was found. If *O. oeni* is truly tachytelic, then fast-evolving phenomena would be expected to occur throughout the whole genome, including this independent molecular chronometer.

Recently, the gram-positive coccus genus *Leuconostoc* has undergone extensive taxonomic reorganization. 16S rRNA (14, 25) and, more recently, 23S rRNA (15) studies have shown that the leuconostocs form three distinct lines of descent (viz., the genus *Leuconostoc sensu stricto*, the paramesenteroides group of species, and *Leuconostoc oenos*). As a result of these phylogenetic studies, the genus *Leuconostoc* is now restricted to *Leuconostoc mesenteroides* (the type species of the genus) and its close relatives, whereas *Leuconostoc paramesenteroides* and some atypical lactobacilli have been placed in the new genus *Weissella* (2). *L. oenos*, which forms a third and independent line, has been reclassified as *Oenococcus oeni* (5). The latter organism is unusual in that it exhibits an exceptionally long 16S rRNA branch and normally conserved positions in this molecule are altered (25). It has been hypothesized by Yang and Woese (25) that these characteristics, together with the purportedly “unusual” phenotypic properties of *O. oeni*, indicate that this bacterium is a rapidly evolving organism (an organism which exhibits so-called tempo-mode evolution [23]).

Previously, the only evidence that *O. oeni* is an example of tachytelic evolution at the bacterial level has come from rRNA sequence analysis data. Since rRNA is a neutral indicator of evolutionary change, the “fast-evolving” phenomenon should be evident throughout the whole genome (i.e., evident from other chronometers). DNA-dependent RNA polymerase (RNAP) is a multisubunit enzyme consisting of two α subunits (encoded by the *rpoA* gene), one β subunit (encoded by *rpoB*), and one β' subunit (encoded by *rpoC*). The *rpoB* and *rpoC* genes are adjacent to each other on maps (Fig. 1) in a transcriptional organization that is found in most bacteria (1, 10, 21). The large sizes of the β and β' subunits (151 and 156 kDa, respectively, in *Escherichia coli*), together with their ancient origin in evolutionary terms, indicate that these molecules could be

immensely powerful molecular chronometers for phylogenetic analysis (11, 16, 17, 19). The key role of RNAP in transcription of DNA makes this molecule independent of the translational apparatus (i.e., rRNA). In this study, we sequenced almost the entire gene (*rpoC*) encoding the largest subunit of RNAP of leuconostocs and a selection of gram-positive bacteria to (i) determine the phylogenetic validity of subdividing the leuconostocs into three genera, and (ii) test the inference (25) from 16S rRNA analysis data that *O. oeni* is an example of tachytelic evolution.

MATERIALS AND METHODS

Bacterial strains and DNA preparation. The bacterial strains used in this study are listed in Table 1. Strains were cultured in appropriate media and at temperatures recommended in the relevant culture collection catalog. Chromosomal DNA was extracted as described previously (9). The authenticity of each strain examined was confirmed by performing a partial 16S rRNA gene sequence analysis (data not shown).

PCR amplification. To obtain almost complete copies of the *rpoC* gene, PCR products were generated (Fig. 1) by using oligonucleotide primers 208F and 3768R (Table 2), which are complementary to DNA sequences that code for conserved amino acid sequences in the *rpoC* genes of *E. coli* and *Staphylococcus aureus* (1). Two overlapping PCR products were generated for *Leuconostoc carnosum* (Fig. 1) by using primers 208F and 1391R and primers 1064F and 3937R (Table 2). PCR were performed in 1× PCR buffer (Perkin-Elmer, Warrington, United Kingdom) containing deoxynucleotide triphosphates at a concentration of 10 mM and 4 ng of each oligonucleotide primer per μ l in a final volume of 50 μ l under a layer of PCR grade mineral oil (Sigma, Poole, United Kingdom) by using a PCR thermal cycler (Biometa, Maidstone, United Kingdom). After an initial denaturation step consisting of 94°C for 5 min, AmpliTaq polymerase (Perkin-Elmer) was added to a final concentration of 0.02 U/ μ l and 25 cycles consisting of 92°C for 1 min, 48°C for 1 min, and 65°C for 3 min were performed; in addition, there was a final extension step consisting of 65°C for 10 min.

Cloning, transformation, and sequencing. PCR products were purified from primers, nucleotides, and enzyme by using a QIAquick PCR purification kit (Qiagen, Dorking, United Kingdom); then the products were ligated into a pCR II vector (TA cloning kit; Invitrogen, Abingdon, United Kingdom) and transformed into *E. coli* INV α cells as recommended by the manufacturer. DNA sequencing was performed by using the dideoxynucleotide chain termination method for both positive and negative strands of each cloned PCR product. Two independent PCR products were sequenced for each *rpoC* gene to check AmpliTaq polymerase fidelity, and a third PCR product was generated to resolve

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FIG. 1. Map showing the organization of the *rpoB* (only the carboxy terminus is shown) and *rpoC* genes found in most bacteria, together with the approximate positions of oligonucleotide primers used in PCR to generate a partial fragment of the *rpoC* gene for DNA sequencing. The arrows indicate the binding sites and directions of primers.

any nucleotide base discrepancies. On average, one nucleotide base error was detected for every 3 kb of sequence.

Sequence analysis. Sequences were aligned by using the PILEUP program of the Wisconsin Molecular Biology software package (3), and the multiple alignments obtained were edited manually. Distance matrices for the DNA and amino acid alignments were calculated by using the DNADIST and PROTDIST programs, respectively, in the PHYLIP software package (7). Unrooted distance matrix trees were generated by using the Fitch-Margoliash method and were then statistically tested by performing a bootstrap analysis (7). Bootstrap values were calculated from 200 replicates. Maximum-parsimony trees were also generated by using the PROTPARS and DNAPARS programs in the PHYLIP software package (7).

Nucleotide sequence accession numbers. The EMBL accession numbers of the *rpoC* gene nucleotide sequences which we determined are shown in Table 1.

RESULTS AND DISCUSSION

The PCR amplification strategy used to generate and clone an approximately 3-kb central portion of the *rpoC* gene from three *Leuconostoc* species, two *Weissella* species and *O. oeni* is shown in Fig. 1. The region that was sequenced consisted of 2,967 to 3,216 nucleotides and was equivalent to approximately 85% of the *rpoC* gene of *E. coli* (18). The fidelity of the sequence data generated from the PCR analysis was vigorously checked, and discrepancies (on average, 1 base per 1,500 to 2,000 bases) were resolved by sequencing additional products obtained from independent PCR. A multiple alignment of the amino acid sequence deduced from the partial gene sequences together with the equivalent regions of some other low-G+C-content gram-positive bacteria is shown in Fig. 2. The *E. coli* β' subunit consists of 1,407 amino acid residues, and our alignment was equivalent to residues 77 through 1249 of this subunit.

Obtaining a good sequence alignment is prerequisite for a molecular systematic analysis because each aligned position has to include only homologous residues of the different molecules. As shown in Fig. 2 and previously (16), the numerous regions which exhibit high levels of primary structural conservation that are interspersed throughout the β' molecule greatly facilitate alignment of less well-conserved regions. However, several insertion and deletion differences (indels), particularly differences between gram-positive and gram-negative species, were evident from the multiple-alignment analysis data. In order to minimize alignment ambiguities due to these differences and alleviate possible errors in phylogenetic reconstruction due to incorrect weighting of the differences between species, major indels specific to gram-negative bacteria (e.g., residues 942 to 1129 in *E. coli*) or gram-positive bacteria (e.g., residues 579 to 613 in *Staphylococcus aureus*) were omitted. The reliability of phylogenetic inferences based on the results of comparative analyses of 16S rRNAs of leuconostocs and closely related organisms was assessed by constructing distance matrix trees from the β' subunit data. Trees were generated by using both amino acid similarities and the equivalent DNA sequences at the second codon position. Studies in which workers used the B subunit of RNAP have shown that the nucleotide at the second codon position is unbiased by G+C content and is more reliable than the nucleotides at positions 1 and 3 for phylogenetic reconstructions (11). Since differences in species sampling influence tree generation (12), a 16S rRNA tree

TABLE 1. Bacterial strains used and their *rpoC* gene sequence accession numbers

Species	Strain ^a	<i>rpoC</i> gene EMBL accession no.
<i>Leuconostoc carnosum</i>	NCFB2776 ^{Tb}	X95811
<i>Leuconostoc mesenteroides</i>	NCDO523 ^T	X95810
<i>Leuconostoc pseudomesenteroides</i>	NCDO768 ^T	X95812
<i>Oenococcus oeni</i>	NCDO1674 ^T	X96384
<i>Weissella hellenica</i>	NCFB2973 ^T	X96470
<i>Weissella paramesenteroides</i>	NCDO803 ^T	X95813
<i>Streptococcus pyogenes</i>	NCDO923	X96385

^a NCDO, National Collection of Dairy Organisms, Reading, United Kingdom; NCFB, National Collection of Food Bacteria, Reading, United Kingdom.

^b T = type strain.

was constructed by using the same set of species to assess congruence between the two chronometers. The Fitch-Margoliash trees derived from the β' subunit and 16S rRNA analyses are compared in Fig. 3. The topologies of the two β' subunit trees, which were based on amino acid similarities and the second codon position of *rpoC*, are very similar. All statistically significant groups (bootstrap values, ≥ 90) were also confirmed by performing a maximum-parsimony analysis (data not shown). The overall topology of the 16S rRNA tree is also in good agreement with the topologies of the trees constructed with the β' chronometer data, with the leuconostocs forming three distinct sublines (viz., the genera *Leuconostoc*, *Oenococcus*, and *Weissella*). There are only minor topological differences between the 16S rRNA and β' subunit trees, and these differences invariably involve reference species (e.g., *Brochothrix thermosphacta*) for which bootstrap values are low (data not shown) and, therefore, the branching order is uncertain. Therefore, little significance can be attributed to these differences. The topological pattern in the leuconostoc branch is identical in all three trees. The three *Leuconostoc* species and two *Weissella* species form two well-separated and statistically significant groups (bootstrap values, 100), and *O. oeni* forms the deepest line on the leuconostoc branch. However, a noticeable difference between the trees derived from the two chronometers concerns the length of the *O. oeni* line. Consistent with previous studies (14, 25) *O. oeni* exhibits a particularly long 16S rRNA line, whereas with the β' subunit a pronounced length is not apparent (Fig. 3). To ascertain whether there was discordance in the observed lengths of the *O. oeni* branch when the two chronometers were used, we compared β' subunit amino acid sequence distances with the 16S rRNA distances for each pair of strains belonging to the low-G+C-content gram-positive group (Fig. 4). Figure 4 shows that for pairwise comparisons involving *O. oeni*, values were consistently biased toward higher 16S rRNA distances (i.e., to the right of the diagonal line). No other single species consistently occurred off the diagonal line. If the β' and 16S rRNA distances were in agreement, then the *O. oeni* values should have

TABLE 2. Oligonucleotides used to generate PCR products

Primer	Sequence ^a	Gene	Position ^a
208F	TG ^C TC ^A TG ^C GG ^A AA ^A TA ^C AA	<i>rpoC</i>	208–227
3768R	CAT ^A AC ^T TC ^T AC ^A TG ^T TT ^A TC	<i>rpoC</i>	3768–3748
1064F	TCGATGTTGGTCCCTTTCTTGAA	<i>rpoC</i>	1064–1085
3937R	AAGC ^T TT ^C GT ^A ATNCC ^T AG ^A CA	<i>rpoC</i>	3937–3920
1391R	TCNCC ^G TC ^A GAA ^A TCNGC ^G ATT	<i>rpoC</i>	1391–1375

^a All sequences and positions are 5'-3', and the positions are *E. coli* *rpoC* gene positions.

W.h.	R	I	R	Y	K	G	I	V	C	D	R	C	G	V	E	V	T	S	A	K	V	R	R	E	R	M	G	H	I	E	L	A	A	P	V	S	H	I	W	Y	F	K	G	I	P	S	R	M	G	L	V	L	D	M	S	P	R	S	L	E	60	
W.p.	R	I	R	Y	K	G	I	V	C	D	R	C	G	V	E	V	T	S	A	K	V	R	R	E	R	M	G	H	I	E	L	A	A	P	V	S	H	I	W	Y	F	K	G	I	P	S	R	M	G	L	V	L	D	M	S	P	R	S	L	E	60	
L.m.	R	I	R	Y	K	G	I	V	C	D	R	C	G	V	E	V	T	S	S	K	V	R	R	E	R	M	G	H	I	E	L	A	A	P	V	T	H	I	W	Y	F	K	G	I	P	S	R	M	G	L	V	L	D	M	S	P	R	S	L	E	60	
L.p.	R	I	R	Y	K	G	I	V	C	D	R	C	G	V	E	V	T	S	S	K	V	R	R	E	R	M	G	H	I	E	L	A	A	P	V	T	H	I	W	Y	F	K	G	I	P	S	R	M	G	L	V	L	D	M	S	P	R	S	L	E	60	
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W.h.	G	G	R	F	A	T	S	D	L	N	D	L	Y	R	R	V	I	N	R	N	N	R	L	K	R	L	L	E	L	N	A	P	G	I	I	V	Q	N	E	K	R	M	L	Q	E	A	A	D	A	L	V	D	N	G	R	R	G	R	P	V	239	
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L.m.	G	G	R	F	A	T	S	D	L	N	D	L	Y	R	R	V	I	N	R	N	N	R	L	K	R	L	L	E	L	N	A	P	G	I	I	V	Q	N	E	K	R	M	L	Q	E	A	V	D	A	L	I	D	N	G	R	R	G	R	P	V	239	
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W.h.	T	G	P	G	N	R	P	L	K	S	L	S	H	M	L	K	G	K	Q	G	R	F	R	Q	N	L	L	G	K	R	V	D	Y	S	G	R	S	V	I	D	V	G	P	F	L	K	M	N	Q	M	G	L	P	R	P	M	A	I	E	L	299	
W.p.	T	G	P	G	N	R	P	L	K	S	L	S	H	M	L	K	G	K	Q	G	R	F	R	Q	N	L	L	G	K	R	V	D	Y	S	G	R	S	V	I	D	V	G	P	F	L	K	M	N	Q	M	G	L	P	R	P	M	A	I	E	L	299	
L.m.	A	G	P	G	N	R	P	L	K	S	L	S	H	M	L	K	G	K	Q	G	R	F	R	Q	N	L	L	G	K	R	V	D	Y	S	G	R	S	V	I	D	V	G	P	F	L	K	M	N	Q	M	G	L	P	R	P	M	A	I	E	L	299	
L.p.	A	G	P	G	N	R	P	L	K	S	L	S	H	M	L	K	G	K	Q	G	R	F	R	Q	N	L	L	G	K	R	V	D	Y	S	G	R	S	V	I	D	V	G	P	F	L	K	M	N	Q	M	G	L	P	R	P	M	A	I	E	L	299	
L.c.	A	G	P	G	N	R	P	L	K	S	L	S	H	M	L	K	G	K	Q	G	R	F	R	Q	N	L	L	G	K	R	V	D	Y	S	G	R	S	V	I	D	V	G	P	F	L	K	M	N	Q	M	G	L	P	R	P	M	A	I	E	L	299	
O.o.	S	G	P	G	N	R	P	L	K	S	L	S	H	M	L	K	G	K	Q	G	R	F	R	Q	N	L	L	G	K	R	V	D	Y	S	G	R	S	V	I	D	V	G	P	F	L	K	M	N	Q	M	G	L	P	R	P	M	A	I	E	L	300	
P.a.	A	G	P	G	N	R	P	L	K	S	L	S	H	M	L	K	G	K																																												

W.h.	D	I	K	A	R	Y	A	E	T	P	I	L	P	P	F	F	K	K	G	Y	L	S	D	I	I	A	E	V	Y	K	I	Y	K	V	T	E	T	S	L	L	D	R	M	K	D	L	G	Y	D	E	S	T	K	S	G	L	T	V	G	V	594	
W.p.	D	I	K	A	H	Y	A	E	T	P	I	L	P	P	F	F	K	K	G	Y	L	S	D	I	I	A	E	V	Y	K	I	Y	K	V	T	E	T	S	L	L	D	R	M	K	D	L	G	Y	D	E	S	T	K	S	G	L	T	V	G	V	594	
L.m.	N	I	H	D	Y	L	A	D	T	S	I	V	N	P	F	F	K	K	G	F	L	S	D	I	I	A	E	V	Y	K	R	Y	K	V	T	E	T	S	L	L	D	R	M	K	D	L	G	Y	D	E	S	T	K	S	G	L	T	V	S	M	594	
L.p.	D	I	H	D	Y	L	A	E	T	P	I	I	G	A	F	F	K	K	G	F	L	S	D	V	I	A	E	V	Y	K	R	Y	K	V	T	E	T	S	L	L	D	R	M	K	D	L	G	Y	D	E	S	T	K	S	G	L	T	V	S	M	594	
L.c.	D	I	N	E	F	L	S	D	A	P	I	I	G	A	F	F	K	K	G	F	L	S	D	V	I	A	E	V	Y	K	R	Y	K	V	T	E	T	S	L	L	D	R	M	K	D	L	G	Y	D	E	S	T	K	S	G	L	T	V	S	M	594	
O.o.	D	I	V	G	W	F	K	N	E	S	L	N	G	P	F	F	K	S	G	F	L	S	D	I	A	O	I	Y	A	R	Y	Q	V	T	R	T	S	V	L	L	D	D	M	K	D	L	G	Y	N	I	S	T	R	S	G	L	T	V	R	M	599	
P.a.	D	I	H	E	Q	L	N	Q	R	P	L	L	S	P	F	F	K	S	G	F	L	S	D	V	I	A	E	V	Y	K	Q	Y	K	V	T	E	T	S	L	L	D	R	M	K	D	L	G	F	Y	R	S	T	L	S	G	L	T	V	G	I	594	
S.p.	D	I	Q	E	V	I	D	G	L	D	I	N	V	P	F	F	K	K	K	N	L	G	N	I	A	E	T	F	K	R	F	R	T	E	T	S	A	F	L	D	R	L	K	D	L	G	Y	Y	H	S	T	L	A	G	L	T	V	G	I	595		
W.h.	A	D	V	T	D	L	K	E	K	P	E	I	I	E	D	A	H	K	Q	V	A	T	V	T	K	Q	F	R	R	G	L	I	T	D	S	E	R	Y	E	R	V	I	A	I	W	N	K	A	K	D	I	T	E	K	L	I	E	H	F	654		
W.p.	S	D	V	T	D	L	K	E	K	P	E	I	I	E	D	A	H	K	Q	V	A	T	V	T	K	Q	F	R	R	G	L	I	T	D	S	E	R	Y	E	R	V	I	A	I	W	N	K	A	K	D	I	I	Q	D	K	L	I	E	S	F	654	
L.m.	T	D	V	T	D	L	K	E	K	P	A	I	I	E	D	A	H	S	Q	V	A	T	V	T	K	Q	F	R	R	G	L	I	T	D	S	E	R	Y	Q	R	V	T	E	I	W	T	K	A	K	D	I	I	Q	D	K	L	I	E	S	F	654	
L.p.	T	D	V	T	D	L	K	E	K	P	A	I	I	E	D	A	H	S	Q	V	A	T	V	T	K	Q	F	R	R	G	L	I	T	D	S	E	R	Y	Q	R	V	T	E	I	W	T	K	A	K	D	I	I	Q	D	K	L	I	E	S	F	654	
L.c.	N	D	V	T	E	L	T	E	K	P	A	I	I	E	D	A	H	K	Q	V	A	T	V	T	K	Q	F	R	R	G	L	I	T	D	S	E	R	Y	Q	R	V	T	E	I	W	T	K	A	K	D	I	I	Q	D	K	L	I	E	S	F	654	
O.o.	S	D	V	T	E	L	P	E	K	G	P	A	I	I	E	D	A	H	K	Q	V	A	T	V	T	K	Q	F	R	R	G	L	I	T	D	S	E	R	Y	I	Q	V	T	Q	T	W	S	D	A	Q	D	K	I	K	S	M	L	I	A	S	F	659
P.a.	A	D	I	T	N	L	P	K	P	A	I	I	A	I	E	D	A	H	K	Q	V	A	T	V	T	K	Q	F	R	R	G	L	I	T	D	S	E	R	Y	E	R	V	I	G	I	W	N	D	A	K	D	E	I	S	Q	R	L	M	D	T	F	654
S.p.	A	D	I	P	V	I	D	N	K	A	E	I	I	E	D	A	H	H	R	V	E	E	I	N	K	A	F	R	R	G	L	M	T	D	D	D	R	Y	V	A	V	T	T	W	R	B	A	K	E	A	L	E	K	R	L	I	E	T	Q	655		
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L.c.	E	P	T	N	P	I	F	M	M	Q	D	S	G	A	R	G	N	I	S	N	F	V	Q	L	A	G	M	R	G	L	M	A	G	P	G	G	K	I	I	E	L	P	V	T	A	N	F	R	E	G	L	T	V	M	E	M	F	I	S	T	714	
O.o.	D	S	K	N	P	I	F	M	M	S	D	S	G	A	R	G	N	I	S	N	F	F	Q	L	A	G	M	R	G	L	M	A	A	P	N	G	K	V	I	E	L	P	V	T	A	N	F	R	E	G	L	C	V	L	E	M	F	I	S	T	719	
P.a.	D	P	Q	N	P	I	F	M	M	S	D	S	G	A	R	G	N	I	S	N	F	S	Q	L	A	G	M	R	G	L	M	A	A	P	N	G	K	I	M	E	L	P	I	L	S	N	F	R	E	G	L	S	V	L	E	M	F	I	S	T	714	
S.p.	D	P	K	N	P	I	F	M	M	S	D	S	G	A	R	G	N	I	S	N	F	S	Q	L	A	G	M	R	G	L	M	A	A	P	N	G	R	I	M	E	L	P	I	L	S	N	F	R	E	G	L	S	V	L	E	M	F	I	S	T	715	
W.h.	H	G	A	R	K	G	M	T	D	T	A	L	K	T	A	D	S	G	Y	M	T	R	R	L	V	D	V	A	Q	D	V	I	I	R	E	D	D	C	G	S	D	R	G	L	D	V	S	A	I	M	N	G	N	E	V	I	E	S	L	Y	774	
W.p.	H	G	A	R	K	G	M	T	D	T	A	L	K	T	A	D	S	G	Y	M	T	R	R	L	V	D	V	A	Q	D	V	I	I	R	E	D	D	C	H	S	D	R	G	L	D	V	T	A	I	M	D	G	N	E	V	I	E	P	L	Y	774	
L.m.	H	G	A	R	K	G	M	S	D	T	A	L	K	T	A	N	S	G	Y	L	T	R	R	L	V	D	V	A	Q	D	V	I	V	R	E	F	D	N	D	S	D	R	G	V	A	V	K	A	I	M	D	G	T	S	V	E	P	L	Y	774		
L.p.	H	G	A	R	K	G	M	S	D	T	A	L	K	T	A	N	S	G	Y	L	T	R	R	L	V	D	V	A	Q	D	V	I	V	R	E	F	D	N	D	S	D	R	G	V	A	V	K	A	I	M	D	G	T	S	V	E	P	L	Y	774		
L.c.	H	G	A	R	K	G	M	S	D	T	A	L	K	T	A	N	S	G	Y	L	T	R	R	L	V	D	V	A	Q	D	V	I	V	R	E	F	D	N	D	S	D	R	G	V	P	V	Q	A	I	M	D	G	T	S	V	E	P	L	Y	774		
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P.a.	H	G	A	R	K	G	M	T	D	T	A	L	K	T	A	N	S	G	Y	L	T	R	R	L	V	D	V	A	Q	D	V	I	V	R	E	K	D	C	G	T	D	R	G	L	L	I	T	A	I	A	E	G	N	E	I	E	P	L	Y	774		
S.p.	H	S	A	R	K	G	M	T	D	T	A	L	K	T	A	D	S	G	Y	L	T	R	R	L	V	D	V	A	Q	D	V	I	I	R	E	D	D	C	G	T	D	R	G	L	L	I	T	A	I	T	D	G	K	E	V	T	E	T	L	E	775	
W.h.	E	R	I	L	G	R	Y	A	Q	K	S	V	F	E	P	Q	T	G	D	K	L	V	G	H	N	E	M	I	T	E	D	I	A	K	R	I	I	E	A	G	I	T	T	V	T	I	R	S	A	F	T	C	N	T	E	H	G	V	C	V	834	
W.p.	E	R	I	L	G	R	Y	A	Q	K	S	V	F	D	P	E	T	G	E	T	L	V	N	H	N	E	M	I	T	E	D	I	A	K	R	I	I	E	A	G	I	T	T	V	T	I	R	S	A	F	T	C	N	T	E	H	G	V	C	V	834	
L.m.	V	R	I	L	G	R	Y	A	M	K	S	V	F	D	P	E	T	G	E	K	I	C	S	R	N	E	M	I	D	E	D	V	A	K	K	I	V	N	A	G	I	K	E	V	T	I	R	S	V	F	T	S	T	T	E	H	G	V	S	V	834	
L.p.	D	R	I	L	G	R	Y	A	M	K	S	V	F	D	P	E	T	G	E	K	I	V	S	R	N	E	M	I	D	E	D	V	A	K	A	I	V	N	A	G	I	E	E	V	T	I	R	S	V	F	T	S	T	T	E	H	G	V	S	V	834	
L.c.	D	R	I	L	G	R	Y	A	M	K	S	V	F	D	P	E	T	G	E	K	I	V	S	R	N	E	M	I	D	E	D	V	A	K	I	V	N	A	G	I	E	K	V	T	I	R	S	V	F	T	S	T	T	E	H	G	V	S	V	834		
O.o.	D	R	I	L	G	R	Y	A	M	K	P	V	I	D	P	K	T	G	E	V	I	A	K	K	N	O	M	I	D	E	H	V	A	D	Q	I	I	D	A	G	I	Q	T	V	T	I	R	S	I	F	T	C	R	T	E	H	G	V	C

FIG. 3. Comparison of phylogenetic trees for leuconostocs inferred from the amino acid sequence of the β' subunit of RNAP, the second codon position of *rpoC*, and 16S rRNA. The numbers are bootstrap values. Abbreviations: B., *Bacillus*; Broc., *Brochothrix*; E., *Escherichia*; H., *Haemophilus*; L., *Leuconostoc*; List., *Listeria*; M., *Mycobacterium*; O., *Oenococcus*; P., *Pediococcus*; Porph., *Porphyromonas*; Pseud., *Pseudomonas*; Stap., *Staphylococcus*; S., *Streptococcus*; W., *Weissella*.

in other tachtelic bacteria (e.g., mycoplasmas [23, 24]). Utilizing the somewhat limited sequence data set available for the β' subunit, we examined whether *O. oeni* shows a greater-than-normal tendency to have different compositions at positions that are generally conserved. The results of this analysis are summarized in Table 3, and these results failed to provide evidence of enhanced variations in the mutation rate at these sites in *O. oeni* compared with other leuconostocs and closely related or-

FIG. 4. Scatter plot of 16S rRNA distances versus β' subunit amino acid distances. Symbols: ●, distances between pairs of low-G+C-content gram-positive strains excluding *O. oeni*; △, distances between *O. oeni* and other low-G+C-content gram-positive strains.

ganisms. This finding with the β' subunit is in marked contrast to the findings obtained with the 16S rRNA chronometer (25).

Since the pioneering work of Woese in the 1970s (for a review see reference 23) 16S rRNA has increasingly occupied the central stage in microbial systematics and has revolutionized our understanding of prokaryote evolution and diversity. Until recently, nearly all phylogenetic reconstructions for bacteria were based on this single molecular chronometer. Over the past decade, however, systematics researchers have begun to compare and contrast phylogenetic relationships inferred from data for other molecules (e.g., RecA [6], GroEL [22], EF-TU [13], B subunit RNA polymerase [11]) with phylogenetic relationships inferred from 16S rRNA data. These studies have in the main demonstrated that 16S rRNA is a truly excellent molecule for reconstructing phylogenetic relationships. In this study, using the β' subunit of RNAP, we found that the subdivision of leuconostocs into three distinct sublines inferred from 16S rRNA data (14, 25) is phylogenetically valid. Our findings provide strong support for the recent taxonomic reorganization of these organisms into the three phylogenetically separate genera (the genera *Leuconostoc* sensu stricto, *Oenococcus*, and *Weissella*) (2, 5). The results of our analysis of

TABLE 3. Levels of variation at relatively conserved sites in the β' subunits of some leuconostocs and related lactic acid bacteria

Species	% Variation in bacterial consensus sequence ^a	% Variation in low-G+C-content consensus sequence ^a
<i>Leuconostoc carnosum</i>	3.3	2.4
<i>Leuconostoc mesenteroides</i>	3.3	1.9
<i>Leuconostoc pseudomesenteroides</i>	3.5	2.1
<i>Oenococcus oeni</i>	2.2	2.4
<i>Pediococcus acidilactici</i>	2.6	2.1
<i>Streptococcus pyogenes</i>	2.8	4.5
<i>Weissella hellenica</i>	1.4	1.2
<i>Weissella paramesenteroides</i>	1.9	1.2

^a The consensus sequences were generated by using a selection of gram-positive and gram-negative bacteria (amino acids that are approximately 90% conserved) or low-G+C-content gram-positive bacteria (amino acids that are 90% conserved) and included three representatives of the leuconostoc branch (*Leuconostoc mesenteroides*, *Weissella hellenica*, and *Oenococcus oeni*).

the β' subunit do not, however, support the hypothesis (25) inferred from 16S rRNA data that *O. oeni* is a fast-evolving organism. Woese stated that (23) rapidly evolving organisms have extraordinarily long 16S rRNA branches and a pronounced tendency to have different compositions at otherwise conserved positions. Furthermore, the phenotype of such organisms is "expected to be highly unusual or bizarre" (23). In other words, what appears to be an increased evolutionary tempo on the genotypic (i.e., 16S rRNA sequence) level translates into a variety of bizarre traits on the phenotypic level (the so-called "tempo-mode relationship" [23–25]). Since 16S rRNA appears to be a neutral indicator of evolutionary change, this suggests that the fast-evolving phenomenon should be evident throughout the whole genome (i.e., that the entire genome is evolving rapidly). If this is true, evidence of such fast-evolving behavior should be found in β' subunit studies. In this study *O. oeni* did not form a long β' branch, nor was there any evidence of exceptional variation in the composition of conserved sites of the β' subunit. It is important to note that *O. oeni* was "purported" by Yang and Woese (25) to have an "atypical or unusual phenotype." Although *O. oeni* differs from other leuconostocs in its ability to grow at low pH values (pH 4.2 to 4.8) and in the presence of 10% ethanol, it has a large number of biochemical, physiological, and chemical features in common with these organisms. Data in *Bergey's Manual of Systematic Bacteriology* (8) reveal that *O. oeni* has much in common with other members of the leuconostoc branch and is not bizarre phenotypically. Yang and Woese (25) suggested that the numerical taxonomic analysis of leuconostocs performed by Priest and Barbour (20) identified *O. oeni* as the deepest branch of the leuconostoc lineages, thereby supporting the phenotypic distinctiveness of this taxon. However, an examination of the numerical phenetic data of Priest and Barbour (20) shows that this is not the case. Further evidence that *O. oeni* is not phenotypically bizarre enough to warrant the tempo-mode hypothesis comes from a numerical analysis of the total soluble cell proteins (4). Although the analyses mentioned above demonstrated that *O. oeni* is a distinct species, they did not in any way indicate that this organism is particularly different phenotypically from other leuconostocs, nor did this organism form the deepest cluster in the numerical analysis (4). Thus, there is no convincing phenotypic evidence that supports the tempo-mode hypothesis for *O. oeni*. This, together with the absence of fast-evolving behavior in the β' subunit of RNAP, suggests that *O. oeni* is not a true tachytelic bacterium. It is clear that caution is required when long rRNA stalk lengths are interpreted. Without supporting evidence from other independent chronometers, this rRNA behavior should not be presumed to reflect the behavior of the whole genome. Why 16S rRNA exhibits such aberrant behavior in organisms like *O. oeni* will require further investigation. It is important to emphasize that although the β' subunit analysis results do not support the hypothesis that *O. oeni* is a fast-evolving organism, they do not preclude the possibility that other taxa that have long rRNA lines are tachytelic. Clearly this phenomenon needs to be examined not only with a range of different organisms but also with taxa that exhibit quite different phylogenetic depths.

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