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Sequence of the gene encoding the 16S rRNA of the beer spoilage organism *Megasphaera cerevisiae*

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The 16S ribosomal RNA gene from the beer-spoilage organism, *Megasphaera cerevisiae* was polymerase chain reaction (PCR)-amplified and sequenced. Analysis confirmed the phylogenetic position of *M. cerevisiae* as a sister taxon of *Megasphaera elsdenii*, within the obligately anaerobic, Gram-negative cocci. The sequence obtained should facilitate the development of DNA probes for early detection of this spoilage organism.

Keywords: *Megasphaera cerevisiae*; 16S rRNA; DNA probe; beer spoilage; phylogeny

Introduction

Megasphaera cerevisiae is a Gram-negative, strictly anaerobic coccus that causes spoilage of beer, particularly unpasteurized beer [6]. The bacterium causes turbidity in beer, and produces hydrogen sulfide and various short-chain fatty acids, including butyric, valeric and caproic acids, causing an unpleasant flavor in contaminated beer [3,7,16]. Both this organism and *Pectinatus* spp, which are Gram-negative, anaerobic rods producing propionic acid and organic sulfur compounds, have emerged over the last decade as important spoilers of packaged beer [6].

As a result of improved filling techniques in modern breweries, the dissolved oxygen content of beer and the volume of air in the headspace has been decreased considerably. Although this has led to an improvement in the chemical stability of beer, the conditions in bottled beers are now suitable for the growth of strictly anaerobic bacteria [1]. This phenomenon, coupled with the increase in consumption of draft beer, as opposed to pasteurized beer, in many countries, has made the monitoring of bottled beer for anaerobic spoilage bacteria of economic importance.

A central problem in monitoring anaerobic spoilers of beer is detection of *M. cerevisiae* in a reasonable length of time by conventional microbiological techniques. The organism grows very slowly and even with enrichment needs 3–4 weeks for detection [6]. However, polymerase chain reaction (PCR) technology can be applied to detect and identify beer spoilage organisms more rapidly [2,14,15]. When the species *M. cerevisiae* was described [3], beer isolates were attributed to the genus *Megasphaera* based primarily on phenotypic evaluation, although there were considerable differences at the DNA level (mol% G+C and DNA/DNA hybridization). We have determined the nucleotide sequence of the small subunit (16S) ribosomal RNA (rRNA) of the bacterium, thereby clarifying the phylogenetic position of *M. cerevisiae*. The potential development of a DNA probe to allow more rapid detection of this organism was also initiated.

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Materials and methods

Bacterial growth conditions

M. cerevisiae VTT E-85230 was obtained from the culture collection of the Technical Research Centre of Finland, Espoo, Finland. The strain was isolated from contaminated Finnish beer and its identity was confirmed by microscopic and biochemical characteristics [3,6]. Strain VTT E-85230 was grown on peptone-yeast extract broth containing 5% (w/v) fructose (PYF) as described by Engelmann and Weiss [3] at 30° C for 72 h under anaerobic conditions. Cultures were stored at –70° C in PYF medium supplemented with 40% (v/v) glycerol.

DNA extraction procedure

Bacteria were recovered by centrifugation (5000 × *g*, 4° C, 15 min) in a microcentrifuge and suspended in 0.2 ml of sterile water. Subsequently, 20 μl of 5 M NaCl, 3 μl of 10% sodium dodecyl sulfate (SDS) in water and 20 μl of lysozyme solution (10 mg ml^{-1}) were added to cell suspensions and incubated at 37° C for 20 min. After heating at 95° C for 10 min to complete lysis, 20 μl of 10 mg ml^{-1} proteinase K (Sigma, St Louis, MO, USA) was added, followed by incubation (55° C, 30 min), and digestion was stopped by denaturing the enzyme by heating (95° C, 10 min). A phenol–chloroform extraction mixture (120 μl Tris-equilibrated phenol and 120 μl chloroform-isoamyl alcohol [24 : 1]) was added to the suspension, followed by vortex mixing and centrifugation (14000 × *g*, 5 min). The aqueous layer was removed and reextracted with phenol–chloroform mixture. RNA in the aqueous extract was removed by treatment with 5 μl RNase solution (0.5 mg ml^{-1}) at 37° C for 45 min. DNA was precipitated by the addition of about 1 ml of cold absolute ethanol, storage at –70° C for 20 min and then centrifugation (14000 × *g*, 45 min). The supernatant fluid was discarded and the pellet of DNA was dried under vacuum and dissolved in about 200 μl of water. To determine the concentration of DNA present, a 10-μl sample of the DNA extract was electro-

phoresed on a 1% (w/v) agarose gel alongside known amounts of DNA and stained with 0.8 μg ml^{-1} of ethidium bromide (Boehringer-Mannheim, Mannheim, Germany). Subsequently, the DNA extract was diluted in water to a concentration of 20 μg ml^{-1} and stored at –20° C.

PCR amplification

PCR amplifications were carried out using a primer combination capable of amplifying nearly full-length 16S rRNA genes from most bacteria. The sequences of these oligonucleotides and their reference positions (according to *Escherichia coli* numbering) are: EB (bacteria specific), 5′-GAGTTTGATCCTGGCTCAG-3′, positions 8 to 27; and UN (universal), 5′-ACGGNWACCTTGTTACGAGTT-3′, positions 1492 to 1506 (IUPAC nomenclature applies to degenerate positions; N = A, T, G or C; W = A or T). During synthesis, the UN primer was phosphorylated at the 5′ terminus. This allows specific lambda exonuclease digestion of one strand of the PCR product [8]. The volume of the PCR amplification reaction was 50 μl and contained one-tenth volume of Taq (× 10) buffer, dNTPs at a final concentration of 200 mM, 200 pmol each of UN and EB primers, 1 unit of Taq DNA polymerase (all purchased from Promega, Madison, WI, USA) and 20 ng of template DNA. The reaction conditions were: denaturation, 95° C for 1 min; primer annealing, 52° C for 1 min; extension, 72° C for 1 min, for 30 cycles; and a final extension stage, 72° C for 5 min. The products of the PCR amplification procedure were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

Five PCR reactions were pooled and purified using the Magic PCR Preps Kit (Promega) according to the manufacturer's instructions. The amplified DNA was then subjected to digestion by lambda exonuclease, using 44 μl of DNA solution, 5 μl of lambda exonuclease (10 ×) buffer (Pharmacia, Uppsala, Sweden) and 1 μl (8 units) of cloned lambda exonuclease (Pharmacia), and incubated at 37° C for 30 min. After incubation, the DNA was purified further by using the Magic PCR Preps Kit, and dispensed in 250-ng amounts, each of which acted as template for sequencing. Single stranded sequencing reactions were performed using the T7 Sequencing Kit (Pharmacia) according to the manufacturer's instructions. Both strands were sequenced in a stepwise fashion using sequential primers designed from the sequence data obtained.

Results

Phylogenetic analyses

In total 1377 nucleotide positions were sequenced from the *M. cerevisiae* 16S rRNA gene (Figure 1). A comparative alignment of the most similar sequences to the *M. cerevisiae* sequence was retrieved from the Ribosomal Database Project [10] electronic mail server. This alignment contained 16 members of the sporomusa group and also sequences from *Heliobacter chlorum* and *Syntrophospora bryantii* as outgroups. The final alignment was 1409 nucleotide positions in length. Subsequently, the data were analyzed using the distance matrix/neighbor joining method (implemented by the PHYLIP computer package [5]) and a parsimony method (implemented by the PAUP computer

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   1 AGTGGCAAAC GGGTGAGTAA CGCGTAAACA ACCTGCCCTC CAGATGGGGA CAACAGCTGG
  61 AAACGGCTGC TAATACCGAA TACGATCTAA AGGCCGCATG ACCTTTAGAA GAAAAGATGG
 121 CCACTGAATA GTCTATCGCC GGAAGAGGGG TTTGCGTCTG ATTAGCTAGT TGGAGGGGTA
 181 ACGGCCCACC AAGGCAATGA TCAGTAGCCG GTCTGAGAGG ATGAACGGCC ACATTGGAAC
 241 TGAGACACGG TCCAGACTCC TACGGGAGGC AGCAGTGGGG AATCTTCCGC AATGGGCGAA
 301 AGCCTGACGG AGCAACGCCG CGTGAGTGAA GACGGCCTTC GGGTTGTAAA GCTCTGTTTT
 361 ATGGGACGAA CGGGYGTATG GTCAATACCC ATACGCCGTG ACGGTACCGT AAGAGAAAGC
 421 CACGGCTAAC TACGTGCCAG CAGCCGCGGT AATACGTAGG TGGCAAGCGT TGTCCGGAAT
 481 CATTGGGCGT AAAGGGCGCG CAGGCGGTTC GGTAAGTCGG TCTTAAAAGT GCGGGGCTTA
 541 ACCCCGTGAT GGGATTGAAA CTGCCGGACT GGAGTGTCGG AGAGGAAAGC AGAATTCCTA
 601 GTGTAGCGGT GAAATGCGTA GATATTAGGA GGAATACCAG TGGCGAAAGC GGCTTTCTGG
 661 ACGACAACTG ACGCTGAGGC GCGAAAGCCA GGGGAGCAAA CGGGATTAGA TACCCCGGTA
 721 GTCCTGGCCG TAAACGATGG ATACTAGGTG TAGGAGGTAT CGACCCCTTC TGTGCCGGAG
 781 TTAACGCAAT AAGTATCCCG CCTGGGGAGT ACGGCCGCAA GGTTGAAACT CAAAGGAATT
 841 GACGGGGGCC GCACAAGCGG TGGAGTATGT GGTTTAATTC GACGCAACGC GAAGAACCTT
 901 ACCAAGCCTT GACATTGAGT GCCAGGGATA GAGATATCCT GTTCTTCTTC GGAAGACGCG
 961 AAAACAGGTG GTGCNNNNNN NNNNNNNNNN NNTGTCGTGA GATGTTGGGT TAAGTCCCGC
1021 AACGAGCGCA ACCCCTATCT TCTGTTGCCA GCACGTAAAG GTGGGAACTC AGAAGAGACT
1081 GCCGCAGACA ATGCGGAGGA AGGCGGGGAT GACGTCAAGT CATCATGCCC CTTATGGCTT
1141 GGGCTACACA CGTACTACAA TGGCTCTTAA TAGAGGGAAG CGAAAGGAGC CGATCCTGGA
1201 GCAAACCCCA AAAACAGAGT CCCAGTTCGG ATTGCAGGCT GCAACTCGCC TGCATGAAGC
1261 AGGAATCGCT AGTAATCGCA GGTCAGCATA CTGCGGTGAA TACGTTCCCG GGCCTTGTAC
1321 ACACCGCCCG TCACACCACG AAAGTCATTC ACACCCGAAG CCGGTGAGGT AACCGGC
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Figure 1 Nucleotide sequence of the 16S rRNA gene of *M. cerevisiae*. The amplification primers, described in Materials and Methods, are not shown. IUPAC nomenclature is used: N = A, T, G or C. Two potential regions for subsequent construction of *M. cerevisiae*-specific DNA probes or PCR primers are underlined. The sequence has been deposited with GenBank under the accession number L37040

package [13]). Distances were calculated using a correction for superimposed substitutions [9] and phylogenetic hypotheses based upon these distances were created by the neighbor joining method [11]. Confidence limits were calculated by the bootstrapping method of sampling with replacement [4]. For parsimony analyses, gaps were treated as a fifth state and only parsimony informative sites, of which there were 516, were analyzed. Searches were performed using the 'Heuristic' search routine with the tree bisection-reconnection (TBR) branch swapping option in effect. Ten random sequence-addition replicates were performed for each search and one hundred bootstrap replicates were performed.

Figure 2 shows the phylogenetic tree obtained by applying the neighbor joining method and parsimony analyses. The sister group relationship between *M. cerevisiae* and *M. elsdenii* was confirmed since the two taxa were retrieved as a clade in 100% of the bootstrap resamplings of the 16S rRNA data. The *M. cerevisiae* and *M. elsdenii* 16S rRNA sequences show 92% similarity when both base differences and insertions/deletions are compared. *Selenomonas* spp and *Pectinatus* spp, which are members of clades closely related to that of *Megasphaera* spp, belong like *Megasphaera* spp to a group of bacterial species with Gram-negative cell walls within the phylum of Gram-positive bacteria [12]. This would suggest that these bacteria originated from a Gram-positive ancestor and subsequently developed a Gram-negative cell wall. In addition, *M. cerevisiae*, *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*, *Zymophilus paucivorans*, *Selenomonas lacticifex* are all obligately anaerobic, non-sporeforming beer spoilage bacteria.

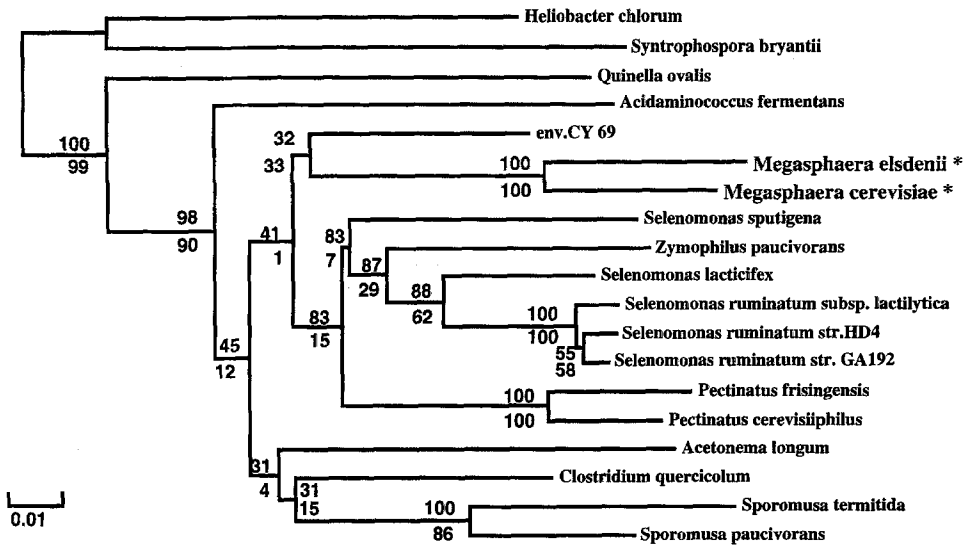

Figure 2 Phylogenetic tree showing the relationships between *M. cerevisiae* and organisms of the sporomusa group. The numbers to the left and right of the internal nodes of the tree are bootstrap proportions (ie the number of times the clade appeared in 100 bootstrap replicates). The upper numbers are the boostrap proportions obtained with the neighbor joining method, and the lower numbers are those obtained by parsimony analyses. Env. CY69 is a 16S rRNA gene sequence deposited in the database from an uncultured environmental organism

Comparative 16S rRNA sequence analysis amongst anaerobic beer spoilers

An alignment of four taxa (*M. cerevisiae*, *M. elsdenii*, *P. cerevisiiphilus*, and *P. frisingensis*) was performed to determine the potential for design of specific DNA probes or PCR primers that would only recognize *M. cerevisiae*, and discriminate between it and these other bacteria. Two potential regions for the design of suitable primers were found (Figure 3). The first region was found at positions 61 to 79 (AAAGGCCGCATGACCTTTA), the second at positions 355 to 374 (ATGGTCAATACCCATACGCC) of the 16S rRNA gene sequence. The number of differences in base pairs (bp) between the two identified regions of *M. cerevisiae* and the respective regions in the 16S rRNA gene sequences of *M. elsdenii* and *Pectinatus* spp varied between 11 to 12 bp over the 19-bp length of the first region, and 8 to 9 bp over the 20-bp length of the second region. This sequence variation is sufficient to design a PCR test for specific detection of *M. cerevisiae* and is presently under investigation.

16S rRNA base position

	positions 61-79:	positions 355-374:
Megasphaera cerevisiae	AAAGGCCGCATGACCTTTA	ATGGTCAATACCCATACGCC
Megasphaera elsdenii	TTTTGTCGCATGGCAGAGG	GCGGTCAATACCCGTTATGA
Pectinatus cerevisiae	TGCTGCTGCA---CAGTAT	ATAGCCAATAAGTATAGTGA
Pectinatus frisingensis	AGCGATTGCATAATGGCTT	AGTGTTAATACCACTATTGA
Heliobacter chlorum	CTGAAGGACATCCTTCAGG	------GTTT----------
Acidaminococcus fermentans	TTTTTCCGCATGGAGAGAG	ATCTATAATACATTTTGGTG
Acetonema longum	AGAGAMTGCAT-GTTTCTT	GTGGTAAATAATCACTCGTA
Clostridium quercicolum	TTTGAGCGCATGCTTGAAA	TATGTAAATAATGTAAGGCA
Sporomusa termitida	CTCGGTTGCATAACGGAGT	TATGTGAATAATRTAGACTA
Sporomusa paucivorans	CTTGGCTGCATGGCTGAGT	TGTGTGAATAATGCAGT-CA
Quinella ovalis	GAGAGTCGCATGACTTTGA	T----TAG------ATAGGC
Selenomonas sputigena	GGGGATGGCATCATCCCCG	CTTGAGAATATTGAGTTTGG
Zymophilus paucivorans	CTTTCCCACATGGGAAAGC	TATACGAATAATGTGCTGTA
Selenomonas lacticifex	ATTTTCCACATGGAAGATA	TATTTGAATAAAGTACTGCA
Selenomonas ruminatum subsp. lactilytica	GACTCCCGCATGGGAGCCT	GATGCGAATAGTTTCTTGCA
Selenomonas ruminatum HD4	GAYTCCCGCATGGGAGACT	GATGCGAATRGTTTCTAGCA
Selenomonas ruminatum 192	AGTTTCCGCATGGGAGCTT	AATGCGAATRGTTTTTCGCA
Syntrophospora bryantii	C---TGATTAATTG---GG	CGGAGTAATATACTGGAAGG

Figure 3 Nucleotide sequence alignment of the two most variable 16S rDNA regions between *M. cerevisiae* and most closely related bacterial species. A dash (–) indicates no homologous nucleotides at this position. These are the optimal regions for the design of *M. cerevisiae*-specific PCR primers

Discussion

When isolates of Gram-negative, obligately anaerobic, beer spoilage cocci (*M. cerevisiae*) were initially characterized by sugar fermentation tests, fermentation product analysis, peptidoglycan analysis and mol% G+C content, they were considered to differ from *M. elsdenii* because of significant differences in G+C content (*M. cerevisiae*: 42.4–44.8% G+C; *M. elsdenii*: 52.6% G+C) [16]. Further investigations of similar beer isolates attributed the isolates to the genus *Megasphaera* based primarily on phenotypic characteristics and since no genomic relatedness was observed to *M. elsdenii* (based on DNA/DNA hybridizations), a new species was proposed, *M. cerevisiae* [3]. The sister group relationship between *M. cerevisiae* and *M. elsdenii* was confirmed by the 16S rRNA data in the present study.

In conjunction, comparative sequence analysis of the 16S rRNA of *M. cerevisiae*, *M. elsdenii*, *P. cerevisiiphilus*, and *P. frisingensis* revealed sufficient differences to facilitate development of a DNA probe. Since PCR technology has been applied successfully to the detection of lactic acid bacteria in beer [2,14,15], the use of the primers described in the present study with PCR technology has potential for detection of *M. cerevisiae* in contaminated beer.

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References

1 Chelack BJ and WM Ingledew. 1987. Anaerobic Gram-negative bacteria in brewing—a review. J Amer Soc Brew Chem 45: 123–127.
2 DiMichele L and MJ Lewis. 1993. Rapid, species specific detection of lactic acid bacteria from beer using the polymerase chain reaction. J Amer Soc Brew Chem 51: 63–66.
3 Engelmann U and N Weiss. 1985. *Megasphaera cerevisiae* sp nov: a new Gram-negative obligately anaerobic coccus isolated from spoiled beer. Syst Appl Microbiol 6: 287–290.
4 Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.
5 Felsenstein J. 1993. PHYLIP version 3.5c: phylogeny inference package. Distributed by author, University of Washington, Seattle.
6 Haikara A. 1991. The genus *Pectinatus* and *Megasphaera*. In: The Prokaryotes, vol II, 2nd edn (Balows A, HG Trüper, M Dworkin, W Harder and K-H Schleifer, eds), pp 1993–2004, Springer-Verlag, New York.
7 Haikara A and K Lounatmaa. 1987. Characterization of *Megasphaera* sp, a new anaerobic beer spoilage coccus. Proc Congr Eur Brew Conv 23: 473–480.
8 Higuchi RG and H Ochman. 1989. Production of single-stranded DNA templates by exonuclease digestion following the polymerase chain reaction. Nucl Acids Res 17: 5865.
9 Jukes TH and CR Cantor. 1969. Evolution of protein molecules. In: Mammalian Protein Metabolism (Munro HN, ed), pp 21–132, Academic Press, New York.
10 Olsen GJ, N Larsen and CR Woese. 1991. The ribosomal RNA database project. Nucl Acids Res 19: 2017–2018.
11 Saitou N and M Nei. 1987. The neighbor-joining method: a new method for constructing phylogenetic trees. Mol Biol Evol 4: 406–425.
12 Schleifer KH, M Leuteritz, N Weiss, W Ludwig, G Kirchhof and H Seidel-Rufer. 1990. Taxonomic study of anaerobic, Gram-negative, rod-shaped bacteria from breweries: emended description of *Pectinatus cerevisiiphilus* and description of *Pectinatus frisingensis* sp nov, *Selenomonas lactifex* sp nov, *Zymophilus raffinosivorans* gen nov, sp nov, and *Zymophilus paucivorans* gen nov, sp nov. Int J Syst Bacteriol 40: 19–27.
13 Swofford DL. 1993. PAUP version 3.1.1: phylogenetic analysis using parsimony. Distributed by author, Smithsonian Institute, Washington, DC.
14 Tsuchiya Y, H Kaneda, Y Kano and S Koshino. 1992. Detection of beer spoilage organisms by polymerase chain reaction technology. J Amer Soc Brew Chem 50: 64–67.
15 Tsuchiya Y, Y Kano and S Koshino. 1993. Detection of *Lactobacillus brevis* in beer using polymerase chain reaction technology. J Amer Soc Brew Chem 51: 40–41.
16 Weiss N, H Seidel and W Back. 1979. Isolierung und systematische Zuordnung biershädlicher gramnegativer Bakterien. I: Gramnegative strikt anaerobe Kokken. Brauwissenschaft 32: 189–194.