Cellular fatty acyl and alkenyl residues in *Megasphaera* and *Pectinatus* species: contrasting profiles and detection of beer spoilage

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The strictly anaerobic Gram-negative beer spoilage bacteria *Megasphaera cerevisiae*, *Pectinatus cerevisiiphilus* and *P. frisingensis* were subjected to cellular fatty acid analysis, employing acid- and base-catalysed cleavage, gas chromatography and mass spectrometry. *M. cerevisiae* contained 12:0, 16:0, 16:1, 18:1, 17:cyc, 19:cyc, 12:0(3OH), 14:0(3OH) as the main fatty acids, and alk-1-enyl chains instead of acyl chains were detected to a considerable extent (14% of total fatty acids), indicating the presence of plasmalogens. The fatty acid pattern of *M. cerevisiae* was almost identical to that of *M. elsdenii*, the only species previously assigned to this genus. *P. cerevisiiphilus* and *P. frisingensis* yielded fatty acids that were heavily dominated by odd-numbered chains; 11:0, 15:0, 17:1, 18:cyc and 13:0(3OH) were the main fatty acids detected in both species. Alk-1-enyl chains with similar chain lengths were also found. Both *Pectinatus* species contained six different 3-hydroxy fatty acids with chain lengths between 11 and 15 carbons, 13:0(3OH) being dominant and the others accounting for generally less than 1% of total fatty acids. Among the minor components, an unsaturated 3-hydroxy fatty acid was detected which was shown to be 13:1(3OH). In addition, fatty acid analysis was shown to be applicable to detection of bacterial contamination of beer.

Keywords: *Megasphaera cerevisiae*, *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*, fatty acids

INTRODUCTION

Since the 1970s, strictly anaerobic Gram-negative bacteria assigned to the genera *Pectinatus* and *Megasphaera* have been recognized as spoilage organisms in packaged unpasteurized beer (Haikara, 1991). Their emergence has been associated with the advances in filling techniques resulting in decreased volumes of air in the head space of beer bottles and cans. The genus *Pectinatus* currently contains two species, *P. cerevisiiphilus* (Lee *et al.*, 1978) and *P. frisingensis* (Schleifer *et al.*, 1990), whereas the beer isolates of the genus *Megasphaera* have been named *M. cerevisiae* (Engelmann & Weiss, 1985) to distinguish them from the only species previously assigned to this genus, the rumen bacterium *M. elsdenii*.

Cellular fatty acid analysis is a useful tool for investigating taxonomic relatedness of bacteria. Furthermore, fatty acid analysis could possibly serve in identifying spoilage organisms in beer. In this study we have analysed cellular fatty acids of *M. cerevisiae*, about which there is no previous information. Both species of the genus *Pectinatus* were also analysed, since there are contradictory data concerning the fatty acid composition; a recent report of the cellular fatty acids of *Pectinatus* (Moore *et al.*, 1994) does not mention, for instance, the major 3-hydroxy fatty acid that we have described as a component of the lipopolysaccharide (LPS) of *P. cerevisiiphilus* and *P. frisingensis* (Helander *et al.*, 1992, 1994).

METHODS

Bacterial strains and growth conditions. *M. cerevisiae* DSM 20462 (Engelmann & Weiss, 1985) and VTT E-84195 (Haikara, 1991) and *M. elsdenii* ATCC 25940 were cultivated for 2 d at 30 °C in PYF broth (10 g peptone, 10 g yeast extract, and 20 g

Abbreviations: BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; CD₃OD, tetrauteriomethanol; DMA, 1,1-dimethylacetals; FAMES, fatty acid methyl esters; FID, flame ionization detector; LPS, lipopolysaccharide; TMS, 3-*O*-trimethylsilyl.

fructose, per litre) anaerobically, with stirring (250 r.p.m.) under nitrogen atmosphere. *P. cerevisiophilus* ATCC 29359^T and *P. frisingensis* ATCC 33332^T were cultivated as above, but the medium was PYG broth (as PYF but fructose replaced by 20 g glucose l⁻¹). The bacteria were collected by centrifugation and freeze-dried.

Liberation and derivatization of fatty acids and fatty alk-1-enyl chains. For each assay, 5–10 mg freeze-dried cells were used. Methanolysis (2 M HCl in methanol, 1 ml) was carried out in sealed tubes for 48 h at 100 °C. The lysate was filtered through glass wool, concentrated under N₂ flow, and dissolved in n-hexane for gas chromatographic analysis. Tetra-deuteriomethanol (CD₃OD, Merck) was alternatively used in methanolysis. Conditions of acid hydrolysis were 4 M HCl (1 ml), 4 h at 100 °C, followed by extraction with n-hexane and methylation by ethereal diazomethane. Saponification was performed essentially as described by Lambert & Moss (1983). Briefly, the cells were first suspended in 1 ml 15% (w/v) NaOH in 50% (v/v) aqueous methanol, and heated at 100 °C for 30 min. After cooling, 1.2 ml HCl/methanol reagent (25 ml of 37% HCl mixed with 75 ml methanol) was added, and the vial was further heated at 80 °C for 10 min. The mixture was extracted with diethyl ether/hexane (1:2, v/v) and treated with ethereal diazomethane. The internal standard in the *Megasphaera* samples was n-heptadecanoic acid (100 µg, Sigma). Trimethylsilylation of fatty acid methyl esters (FAMES) was done with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Fluka) by treating dried aliquots of the methanolysates with 25 µl BSTFA for 2 h at 65 °C.

Gas chromatography and mass spectrometry. Gas chromatography (GLC) was carried out with a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID). Quantification of FAMES and 1,1-dimethylacetals (DMAs) was performed with a 25 m × 0.32 mm phenyl methyl silicone fused silica capillary column (HP-5, Hewlett-Packard). The carrier gas was helium. The temperature programme was 170 °C isothermally for 3 min, then by 5 °C min⁻¹ to 260 °C. Temperatures of the injector and the detector were 260 °C and 280 °C, respectively. For identification of isomeric unsaturated FAMES, samples were also analysed using a polar column NB-9C (25 m × 0.32 mm, HNU-Nordion, Helsinki) which was operated from 140 °C (isothermally for 3 min) to 260 °C, increasing 5 °C min⁻¹. Standard mixtures (C₁₂–C₁₈) containing known amounts of saturated, unsaturated, and hydroxy FAMES were analysed before and after each set of analyses, to evaluate chromatographic performance and to obtain response factors for quantification of different fatty acid classes. Typically, the FID responses for saturated and unsaturated FAMES were directly proportional to the amount of analyte (response factor = 1.00), whereas for 3-hydroxy acids the response was lower (response factor = 1.11). For DMAs a response factor of 1.00 was used.

All mixtures were analysed by combined gas chromatography and mass spectrometry (GLC-MS), using a 5890 gas chromatograph equipped with a 25 m × 0.2 mm HP-5 column connected to a HP5971A mass-selective detector (Hewlett-Packard). The temperature programme was 170 °C isothermally for 3 min, then increasing by 5 °C min⁻¹ to 280 °C. Temperatures of the injector and the interface were 260 °C and 280 °C, respectively. Electron impact mass spectra were recorded at 70 eV. Saturated, unsaturated and cyclopropane FAMES normally yielded ions corresponding to M⁺, and diagnostic fragment ions were screened for the identification of different fatty acid classes as described elsewhere (Asselineau & Asselineau, 1984; Wollenweber & Rietschel, 1990). DMAs yielded, besides ions M⁺ and M–31⁺, the ion at m/z 75 as the

base peak. Chemical ionization mass spectra were acquired with ammonia as the reactant gas.

Beer samples. A bottle of commercial Finnish beer was inoculated with a drop of an actively growing culture of *P. frisingensis* ATCC 33332 and incubated at 30 °C until turbidity developed. Aliquots (1 ml) from the beer were freeze-dried, as were similar samples from uncontaminated beer. The dried materials were subjected to saponification as described above.

RESULTS

Identification of fatty acids and alk-1-enyl chains: technical considerations

Fatty acids (including alk-1-enyl chains) were analysed from *M. cerevisiae* strains DSM 20462 and VTT E-84195 and, for comparison, from *M. elsdenii*, which is the only species previously assigned to this genus. In addition, type strains of *P. cerevisiophilus* and *P. frisingensis* were analysed. For each of these strains, cellular fatty acid analysis by methanolysis and saponification, respectively, yielded markedly different chromatograms, as exemplified in Figs 1 and 2 for *P. cerevisiophilus*. Analysis of the methanolysate revealed a pattern of saturated, unsatu-

Fig. 1. Gas chromatogram of cellular fatty acids and alk-1-enyl chains of *P. cerevisiophilus* prepared by methanolysis. The analysis was carried out with flame ionization detection on a HP-5 column with a temperature programme from 170 °C (isothermally for 3 min) to 260 °C, increasing at 5 °C min⁻¹. Peaks arise from FAMES and DMAs. The notation includes the number of carbon atoms, double bonds, and hydroxylation or O-methylation, respectively.

Fig. 2. Gas chromatogram of cellular fatty acids of *P. cerevisiophilus* prepared by saponification. Chromatographic details were as in Fig. 1.

rated, and 3-hydroxylated FAMES, DMAs and several methoxy FAMES (Fig. 1). Since 3-hydroxy fatty acids are known to produce significant amounts of the corresponding 3-methoxy and α , β -unsaturated fatty acids as artefacts in various analytical procedures (Wollenweber & Rietschel, 1990), the presence of small amounts of 13:1 and 13:0(3OMe) derived from the major 3-hydroxy acid, 13:0(3OH) was expected in the chromatograms of *P. cerevisiophilus*, and they were added to 13:0(3OH) in the subsequent quantification. However, the methanolysate also contained several other peaks which were identified by GLC-MS as methoxy FAMES and DMAs; notably, 18:0(9OMe) was detected as the major component among these substances. Analysis of *P. cerevisiophilus* cells by methanolysis in CD_3OD yielded 18:0(9OMe), in which not only the carboxymethyl group but also the 9-methoxy group contained three deuterium atoms instead of hydrogen atoms, as indicated by the base peak (carboxy-terminal fragment cleaved between C9 and C10) at m/z 207. In the nondeuterium methanolysate this fragment was observed at m/z 201. It was thus concluded that the 9-methoxy group was artificially created during methanolysis, most likely from a cyclopropane acid as described by Vulliet *et al.* (1974). This was verified by analysis of the saponified sample (Fig. 2), in which no 9-methoxy acids were found; instead, two major peaks were present which were absent from the methanolysate. These were analysed by GLC-MS, and were identified as

methylene 9,10-heptadecanoic acid FAME (18:cyc) and the corresponding DMA. By similar techniques cyclopropane fatty acids and alk-1-enyl chains were identified also in *P. frisingensis* and in each *Megasphaera* strain.

In addition to the formation of artefacts, chromatograms obtained from methanolysed versus saponified samples differed in that the proportions of 3-hydroxy FAMES were low in the saponified samples. Typically, the latter yielded only 65% of the amount of the major 3-hydroxy FAME found in methanolysis. In Gram-negative bacteria, 3-hydroxy fatty acids are components of the lipid A part of the LPS of the outer membrane, and some of the 3-hydroxy fatty acids in each lipid A molecule are always amide-linked (Zähringer *et al.*, 1994). The amide-linked fatty acids require strong acid-catalysed conditions for liberation, and are not properly released by saponification. Therefore, it is generally agreed that to obtain reliable fatty acid patterns from bacteria with good coverage of the relatively alkali-stable amide-linked (hydroxy) fatty acids and acid-labile cyclopropane fatty acids, two methods in combination are needed (Lambert & Moss, 1983; Wollenweber & Rietschel, 1990; Mayberry & Lane, 1993). Accordingly, we combined the proportions of cellular fatty acids and alk-1-enyl chains as shown in Table 1, counting the proportions of fatty acids and alk-1-enyl groups other than those with cyclopropane chains from the methanolysis data. The proportion of cyclopropane fatty acids and cyclopropane alk-1-enyl chains were counted from data obtained by saponification.

The genus *Megasphaera*

It is evident from Table 1 that the fatty acid and alk-1-enyl chain composition of *M. cerevisiae* and *M. elsdenii* differed only slightly. The two strains of *M. cerevisiae* yielded practically identical patterns (data not shown for strain VTT E-84195). The pattern of saturated fatty acids differed in that 10:0 was exclusively present in *M. cerevisiae*, and the proportion of 16:0 was larger in *M. elsdenii*. The unsaturated fatty acids were dominated by 18:1 ω 9 cis (oleic acid) in both species, and they also contained 17:cyc and 19:cyc fatty acids. Three different 3-hydroxy fatty acids were detected in *M. cerevisiae*, the dominant one being 14:0(3OH). Several alk-1-enyl compounds with saturated, unsaturated, and cyclopropyl chains were detected; they generally displayed chain lengths and types which were also present in fatty acids. Alk-1-enyl chains accounted for 14% of the analysed material in both *Megasphaera* species.

The genus *Pectinatus*

P. cerevisiophilus and *P. frisingensis* displayed a striking predominance of odd-numbered fatty acids and alk-1-enyl chains. Among the saturated FAMES, 11:0 and 15:0 were the most abundant ones, and the rare 17:1 and its alk-1-enyl analogue dominated among the unsaturated chains. Altogether six different 3-hydroxy fatty acids were detected in *P. cerevisiophilus* and *P. frisingensis*, their proportions being similar in the two species. Their identity was additionally confirmed by GLC-MS analysis

Table 1. Fatty acid composition of *M. cerevisiae*, *M. elsdenii*, *P. cerevisiophilus* and *P. frisingensis*

Compositions are expressed as percentages of total fatty residues, combined from analyses by methanolysis and saponification. Results are means of three determinations that did not vary by more than 5% from the mean.

Component	<i>M. cerevisiae</i> DSM 20462	<i>M. elsdenii</i> ATCC 25940	<i>P. cerevisiophilus</i> ATCC 29359	<i>P. frisingensis</i> ATCC 33332
Saturated fatty acids				
10:0	2.9	ND	ND	ND
11:0	ND	ND	10.1	9.7
12:0	4.7	9.8	0.3	0.5
13:0	ND	ND	3.0	4.1
14:0	2.0	2.1	1.1	0.6
15:0	0.3	0.1	16.8	11.0
16:0	4.9	12.4	1.1	0.8
17:0	ND	ND	2.9	2.4
18:0	0.7	4.3	0.6	1.0
Unsaturated fatty acids				
15:1*	ND	ND	3.5	2.6
16:1 ω 7 cis	4.5	6.9	ND	ND
16:1 ω 7 $trans$	ND	ND	0.3	0.5
16:1*	2.7	3.0	ND	ND
17:1*	ND	ND	6.8	4.0
18:1 ω 9 cis	9.7	11.8	2.6	0.8
18:1 ω 9 $trans$	1.8	1.6	0.7	0.4
Cyclopropane fatty acids				
17:cyc	5.6	5.4	ND	ND
18:cyc	ND	ND	8.1	10.5
19:cyc	13.8	7.6	ND	ND
Hydroxy fatty acid				
11:0(3OH)	ND	ND	0.8	0.8
12:0(3OH)	4.6	3.9	0.4	0.5
13:0(3OH)	2.5	TR	24.9	26.1
13:1(3OH)	ND	ND	1.1	0.5
14:0(3OH)	22.1	17.4	0.3	0.4
15:0(3OH)	ND	ND	0.1	0.3
Alk-1-enyl chains				
15:0	ND	ND	0.6	1.2
16:0	2.0	3.5	ND	ND
16:1*	1.4	2.6	ND	ND
17:0	ND	ND	2.0	4.2
17:1*	ND	ND	6.2	4.7
18:0	0.7	0.7	ND	ND
18:1*	1.1	3.0	ND	0.8
17:cyc	4.3	2.4	ND	ND
18:cyc	ND	ND	5.7	11.6
19:cyc	4.6	1.5	ND	ND

ND, None detected; TR, < 0.1%, detectable by selective ion monitoring.

* Isomeric form not determined.

of 3-O-trimethylsilyl (TMS) ether methyl ester derivatives, which yielded prominent (usually base peak) fragments at m/z $M-15$. Among the hydroxy fatty acids the most abundant was 13:0(3OH), which was also the major cellular fatty acid. A minor FAME eluted slightly before 13:0(3OH) from the HP-5 column, in both methanolysed and saponified samples (Figs 1 and 2). This compound yielded a mass spectrum reminiscent of a 3-hydroxy

fatty acid, as indicated by the prominent fragment ion at m/z 103. Fragment ions at m/z 224 and 150, assigned to ions $M-18^+$ and $M-18-74^+$, indicated a molecular mass of 242. GLC-MS of the TMS ether methyl ester-derivatives confirmed that this fatty acid had a molecular mass that was two mass units lower than that of the 13:0(3OH), as indicated by the $M-15$ fragments at m/z 299 and 301, respectively. This fatty acid was thus

Fig. 3. Gas chromatogram (total ion chromatogram from GLC-MS) of a saponified 1 ml sample of beer in which *P. frisingensis* was growing. The amount of internal standard (17:0) was 100 μ g, and 1 μ l out of 100 μ l was injected.

identified as 13:1(3OH). Alk-1-enyl chains accounted for 15% of analysed material in *P. cerevisiophilus* and 23% in *P. frisingensis*. The latter was particularly rich in the alk-1-enyl group with the 18:cyc chain.

Analysis of contaminated beer

To assess whether fatty acid analysis could be applied to detection of bacterial contamination in beer, we analysed by saponification a 1 ml aliquot of beer in which *P. frisingensis* was growing. In parallel, a similar sample of uncontaminated beer was analysed. The analysis (Fig. 3) of the contaminated beer sample revealed FAMES including 18:cyc, 13:0(3OH), and other odd-numbered fatty acids, which are characteristic for *Pectinatus*. The uncontaminated beer analysed by this technique yielded only a minor peak of 16:0, accounting for less than 1 μ g fatty acid per ml beer.

DISCUSSION

The cellular fatty acid analysis revealed, both in *Megasphaera* and in *Pectinatus*, considerable proportions of alk-1-enyl chains in addition to acyl chains, indicating the presence of plasmalogens in these bacteria. Among the prokaryotes, plasmalogens are typically found in strictly anaerobic bacteria, including both Gram-positive and Gram-negative species (for a review see Goldfine, 1982); they have been well characterized, for instance, in *M. elsdenii* (Van Golde *et al.*, 1973; Johnston & Goldfine,

1982). *M. cerevisiae* and *Pectinatus* species thus represent no exception.

The fatty acyl and alk-1-enyl composition of two isolates of *M. cerevisiae* were nearly identical. Furthermore, the composition of *M. cerevisiae* closely resembled that of *M. elsdenii*, differing only in the quantitative proportions of some fatty acids. Our results for *M. elsdenii* agree well with the fatty acyl and alk-1-enyl composition reported for phospholipids of *M. elsdenii* ATCC 17752 (Johnston & Goldfine, 1982). There is no information concerning the LPS composition in the genus *Megasphaera*, but it is likely that the 3-hydroxy fatty acids detected are LPS components.

The striking predominance of odd-numbered fatty acyl and alk-1-enyl chains of both *P. cerevisiophilus* and *P. frisingensis* is a remarkable property seldom encountered among bacteria. In this respect *Pectinatus* species closely resemble *Veillonella alcalescens* and *V. parvula*, parasitic anaerobic cocci, in which the acyl and alk-1-enyl chains of phospholipids (Hewett *et al.*, 1971; Johnston & Goldfine, 1982), and also the LPS (Hewett *et al.*, 1971), exhibit the same major chain lengths as *Pectinatus*. The major 3-hydroxy fatty acid of *Pectinatus*, 13:0(3OH), was previously shown to be the main fatty acid substituting the lipid A backbone in both *Pectinatus* species (Helander *et al.*, 1994). This fatty acid is rarely found in LPS, but has been reported in *Legionella* species (Sonesson *et al.*, 1989, 1994), *Pseudomonas diminuta* (Wilkinson *et al.*, 1973; Kasai

et al., 1987) and *Selenomonas ruminantium* (Kamio *et al.*, 1971). Among the minor fatty acids of *P. cerevisiophilus* and *P. frisingensis*, the unsaturated 3-hydroxy acid 13:1(3OH) is exceptional as, to our knowledge, this fatty acid has not been previously reported in bacteria or in LPS (Wilkinson, 1988). Other unsaturated 3-hydroxy fatty acids have been sporadically found in bacteria (Wilkinson, 1988), notably in some species of *Rhizobium* [18:1(3-OH)] (Russa *et al.*, 1985; Urbanik-Sypniewska *et al.*, 1989). In the recent description of *Pectinatus* lipid A (Helander *et al.*, 1994), this fatty acid was not detected. However, micro-heterogeneity is a common feature in the fatty acids of lipid A (Zähringer *et al.*, 1994), and 13:1(3OH) may well be a component of this lipid, since minor fatty acids contribute little to the main structure which usually is chemically analysed for structure.

Pectinatus was included in a recent report comparing the cellular fatty acid patterns of Gram-negative anaerobes (Moore *et al.*, 1994). Our results disagree in several major fatty acids: Moore and co-workers reported no 13:0(3OH), which in our present study was the most abundant fatty acid (25 % of total), and which we have on several occasions reported as the main fatty acid of *Pectinatus* LPS (Helander *et al.*, 1983, 1992, 1994), nor did they detect any cyclopropane fatty acids. Instead, they report 18 % of 14:0 DMA, which was not present in our *Pectinatus* strains, and a large proportion of 18:1 DMA which was a minor component (< 1 % of total) in *P. frisingensis* and not detectable in *P. cerevisiophilus*. We believe that Moore and co-workers have erroneously identified these FAMES and DMAs by relying on GLC retention times only.

Finally, the fatty acid analysis was shown to be applicable for the detection of bacterial contamination in beer. As was also found in our experiment, fatty acids with carbon chains between C₁₂ and C₁₈ have been reported to exist in beer in submicrogram amounts per ml (Wackerbauer & Bender, 1983). The low amount of saponifiable fatty acids in uncontaminated beer will make it possible to detect small amounts of bacterial fatty acids in beer samples, possibly also allowing the determination of the type of contaminant on the basis of the types of fatty acids detected.

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