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n-Alkane degradation by *Marinobacter hydrocarbonoclasticus* strain SP 17: long chain β-hydroxy acids as indicators of bacterial activity

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Abstract

The lipids of the Gram-negative marine bacterium *Marinobacter hydrocarbonoclasticus*, cultivated in synthetic seawater on a single carbon source, acetate or *n*-icosane, were isolated, purified and their structures determined. Three different pools of lipids were isolated according to the sequential procedure used: "unbound" lipids extractable by solvents, lipids released under basic conditions ("ester bound") and lipids released by acid hydrolysis ("amide bound"). Even-carbon-numbered, *n*-fatty acids were identified in the "unbound" lipids of both the acetate and *n*-icosane cultures. In addition to these compounds, *n*-icosane induced the formation of *n*-icosan-1-ol and *n*-icos-11-en-1-ol, and also of a series of β-hydroxy acids ranging from C_{12} to C_{20}. In the "ester bound" lipids of the two cultures, short and long chain fatty acids were identified together with the β-hydroxy $C_{12:0}$ acid. This hydroxy acid was, by far, the major compound identified in the "amide bound" lipids of the two cultures. Comparison of the analytical data for the two cultures, and the differences in composition thus observed for the "unbound" pool, suggest the following metabolic pathway for *n*-icosane: hydroxylation to the C_{20} primary alcohol, transformation into the C_{20} β-hydroxy acid and subsequent degradation into lower homologues. In sharp contrast, lipids from the "ester bound" and "amide bound" pools were quite unaffected by the change of nutrient. Lipids from *Escherichia coli* were also examined in the same manner. The results are discussed in terms of geochemical implications, relative to the presence of "unbound" β-hydroxy acids in particulate matter and sediments.

Keywords: *n*-Alkanes; Biodegradation; Fatty alcohols; Fatty acids; Hydroxy fatty acids; *Marinobacter hydrocarbonoclasticus*; *Escherichia coli*

1. Introduction

Numerous bacteria have adapted, naturally, to degrade hydrocarbons, and bacterial strains exhibiting such properties have been isolated from, for example, polluted areas (Ratledge, 1978; Leahy and Colwell, 1990). Among these bacteria *Marinobacter hydrocarbonoclasticus,* isolated from sediments collected in the Gulf of Fos (French Mediterranean coast) at the mouth of a petroleum refinery and formerly considered as an *Alteromonas* species (Al-Mallah et al., 1990), was able to use various hydrocarbons as its sole carbon source (Gauthier et al., 1992). Moreover, this Gram-negative,

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facultative anaerobe, is slightly halophilic and extremely halotolerant (from 0.08 M and up to 3.5 M in NaCl, respectively; Fernandez-Linares et al., 1996). Subsequently, *Marinobacter* sp. have been found to be ubiquitous in seawater (Spröer et al., 1998; Huu et al., 1999).

The ability of *M. hydrocarbonoclasticus* to grow under various conditions, and its ability to actively degrade hydrocarbons, produced some specific lipids and/or variations in its lipid composition. The aim of the present work was, therefore, to examine these two points. To this end, lipid analyses were performed on bacterial cells cultured in synthetic seawater supplemented with ammonium acetate or *n*-icosane as the sole carbon and energy source. The cells were harvested at the stationary growth phase. Fatty acid-containing lipids, extracted from living organisms, are commonly separated into neutral, glyco- and phospholipids. With a view to focusing our study on the fatty moieties of *M. hydrocarbonoclasticus*, separation of the lipids into these three classes was not carried out; however, sequential extraction was performed. It is well known that the recovery of total lipids from micro-organisms, and sediments, requires classical solvent extraction coupled with more drastic basic and acid treatment (Mendoza et al., 1987a,b; Goossens et al., 1986, 1989a,b; Fukushima et al., 1992a,b; Zegouagh et al., 1996; Wakeham, 1999; Stefanova and Disnar, 2000). However, only a limited number of microbial species have been examined for their lipid composition using such successive treatments (Goossens et al., 1986, 1989a). In the present study, basic treatment of pre-extracted *M. hydrocarbonoclasticus* released fatty acids bound to macromolecular structures by ester bonds. Acid treatment of the residual material liberated fatty acids bound by amide functions which occur, in particular, in the membrane lipopolysaccharides (LPS) of Gram-negative bacteria. These two types of "bound" fatty acids were analysed together with the "unbound" fatty acids from the extracts. The hydrocarbon fractions of the extracts were also examined. We have also investigated the fatty acids of *Escherichia coli*, a facultative anaerobic Gram-negative bacterium, by the same procedure for comparison and to test the sequential procedure used.

2. Materials and methods

2.1. Materials

Silica gel for column chromatography (CC) (Silica gel 60, 70–230 mesh) was from Merck. Standards of fatty acid methyl esters were prepared from the acids, obtained from Aldrich, by addition of an etheral solution of diazomethane, generated from Diazald® (Aldrich).

2.2. Microorganisms and culture conditions

Marinobacter hydrocarbonoclasticus strain SP17, ATCC 49840 (Rockville, Maryland, USA), was used. Cells were grown in 500 ml inverted T-shaped flasks, containing 175 ml of synthetic seawater supplemented with ammonium acetate (2 g/l) or *n*-icosane (1 g/l). Synthetic seawater was composed of (g/l, in distilled water): Tris(hydroxymethyl amino methane), 5; KCl, 0.75; NH_4Cl, 1; $MgSO_4{\cdot}7H_2O$, 3.91; $MgCl_2{\cdot}6H_2O$, 5.08; $CaCl_2$, 1.5; NaCl, 23. The pH was adjusted to 7.8 with 10 N HCl; 2 ml and 4 ml of autoclaved (120 °C, 20 min) solutions of $FeSO_4$ (1 g/l) and dipotassium phosphate (18.6 g/l), respectively, were added to 1 l of synthetic seawater immediately before use. The organism was grown under aerobic conditions (aeration was provided by agitation on a reciprocal shaker, 96 rpm) at 20 °C. The biomass was harvested at the stationary growth phase by centrifugation (13,000 rpm, 15 min), washed twice with 300 ml of synthetic seawater and freeze-dried. Lyophilised cells of *E. coli*, strain K-12, were obtained from Sigma-Aldrich (cultured on a rich medium containing peptone and glucose).

2.3. Extraction and fractionation of lipids

The dry biomass of *M. hydrocarbonoclasticus* (1.07 g) was extracted using chloroform/methanol (2:1, vol/vol; 150 ml) with stirring at room temperature, 2×18 h. The mixture was filtered (0.5 μm FH type membrane; Millipore) and the retained solid was rinsed with methanol. The combined filtrates and washings were concentrated under reduced pressure, and fractionated by column chromatography on silica gel in the proportion of 12 g silica to 180 mg lipids. A hydrocarbon fraction was eluted with 45 ml heptane and the other lipids were recovered by elution with, respectively, 1220 ml acetone and 305 mL methanol. The mixed polar fractions were transesterified in 30 ml of methanol–KOH (0.1 N), at 0 °C for 2 h. The reaction mixture was acidified to pH 1 with aqueous HCl 5% (v/v) and extracted with diethyl ether, giving the "unbound" lipids. The solvent-extracted biomass was then reacted with 30 ml of methanol–KOH (1 N), under reflux for 2 h, to cleave ester bonds. The reaction mixture was filtered and the residue washed with methanol, as above. The combined filtrates were acidified with aqueous HCl (5%), diluted with water and the lipids extracted with diethyl ether. The concentrated extract constituted the "ester bound" lipids. The residual biomass was finally hydrolysed in 20 ml of aqueous HCl (4 N), for 6 h under reflux, to cleave amide bonds. After cooling, the reaction mixture was filtered, the residue washed with methanol and the combined filtrates were extracted with diethyl ether. The concentrated extract constituted the "amide bound" lipids. The two latter concentrated extracts were esterified

at room temperature, using diazomethane in diethyl ether (2 ml, 0.12 N). The reaction mixtures were concentrated under reduced pressure. Lyophilised cells of *E. coli* (1 g) were similarly treated, except that the hydrocarbon fraction was not separated.

2.4. Trimethylsilyl ether derivatives

Samples of the three esterified lipid pools isolated from each culture (2 mg), in pyridine solution (0.5 ml), were treated with 0.1 ml of 1,1,1,3,3,3-hexamethyldisilazane and 0.05 ml of chlorotrimethylsilane. After stirring at room temperature (1 h), the reaction mixture was extracted with diethyl ether and washed with water. Concentration of the organic phase under reduced pressure furnished the crude trimethylsilyl products which were analysed by combined gas chromatography–mass spectrometry (GC–MS).

2.5. Dimethyl disulfide (DMDS) adduction

DMDS adducts were prepared as previously described (Scribe et al., 1990). A portion of each lipid pool as methyl esters, trimethylsilylated or not depending on the presence of hydroxyl functions (ca 1 mg), was treated in 100 μl of *n*-heptane with 100 μl of DMDS and 1.2 mg of iodine in 20 μl of diethyl ether. The reaction was carried out in a 10 ml closed tube, for 48 h at 50 °C. Thereafter, 200 μl of *n*-heptane was added to the reaction mixture, and the excess of iodine was removed using 200 μl of an aqueous solution of 5% Na_2SO_3. After recovery of the organic phase, the aqueous phase was extracted a second time with 200 μl *n*-heptane. The combined extracts were concentrated under a stream of nitrogen and immediately analysed by GC–MS.

2.6. GC–MS

Hydrocarbon and esterified lipid fractions were analysed by GC–MS using an HP 5890 chromatograph coupled to an HP 5989 mass spectrometer. The chromatograph was equipped with a CPSil-5CB fused-silica capillary column (25 m×0.25 mm) coated with polydimethylsiloxane (film thickness 0.25 μm). The temperature program was from 140 to 300 °C (4 °C min^{-1}) for the hydrocarbon fraction, from 100 to 300 °C (4 °C min^{-1}) for fatty acid methyl esters and trimethylsilylether derivatives and from 240 to 300 °C at 4 °C min^{-1} for the DMDS adducts.

3. Results and discussion

Quantitative data (Table 1) shows that the contents in the three lipid pools, obtained by sequential extraction, are of the same order of magnitude in *E. coli* and *M. hydrocarbonoclasticus* grown on ammonium acetate and *n*-icosane.

The acetate culture of *M. hydrocarbonoclasticus* afforded hydrocarbons accounting for 0.014% of the dry biomass. GC–MS analysis showed the presence of *n*-alkanes ranging from C_{10} to C_{35} (94% of the fraction) and monounsaturated *n*-alkenes (C_{12}, C_{14}, C_{16} and C_{18}). The *n*-C_{10} to *n*-C_{22} alkanes had an even-carbon-number predominance, with a maximum at C_{16}. Longer chain alkanes were dominant but without an odd/even carbon number preference: they maximised at C_{27}. Minor branched C_{11} to C_{34} *iso* and *anteiso* alkanes were also identified. However, analyses of the salts used to prepare synthetic seawater for the growth medium of *M. hydrocarbonoclasticus* demonstrated that all these hydrocarbons were present at trace levels in the salts (ca 0.1 mg per litre of synthetic seawater, corresponding to ca 3 ppm, relative to the total salts) and exhibited exactly the same distribution as observed for the culture. Therefore, this finding indicated that the bacterium did not synthesise hydrocarbons, under the growth conditions used. In contrast, it would be able to efficiently sorb hydrocarbons, even if present in very low concentrations. Such a property is possibly related to its natural disposition to use hydrocarbons as a carbon source. However, in the presence of large amounts of acetate, these hydrocarbons would not be degraded. The distribution for the culture with *n*-icosane was similar to

Table 1
Abundance of the lipid pools isolated from *Escherichia coli* and *Marinobacter hydrocarbonoclasticus*[a]

Lipid pool	*E. coli* (strain K12)	*M. hydrocarbonoclasticus*	
		Ammonium acetate	*n*-Icosane
"Unbound"	4.8	3.5	5.6
"Ester bound"	1.9	1.1	1.5
"Amide bound"	0.5	0.6	0.7
Total	7.2	5.2	7.8

[a] % of dry biomass. "Unbound": total pool including the hydrocarbons. For *M. hydrocarbonoclasticus* grown on *n*-icosane, the value reported for the latter pool is overestimated by ca ×1.5, since it includes the unmetabolized alkane.

that with acetate, except for the non-metabolised *n*-C_{20} which strongly dominated the GC profile: the competition between this compound, furnished in very high amount, and the contaminant hydrocarbons explains the persistence of the latter in the culture medium.

The fatty acid and alcohol distributions in the "unbound", "ester bound" and "amide bound" lipids are listed in Table 2. Taken together, these products account for virtually all the GC-amenable components of each lipid pool and only a very low percentage remained unidentified. GC–MS analysis of the DMDS adducts of the unsaturated compounds allowed us to determine the carbon–carbon double bond positions. The C=C configuration in some of the parent compounds could be deduced from GC coelution with standards. The position of the hydroxyl groups was determined from the MS data and confirmed by analysis of the trimethylsilylated derivatives.

3.1. *Escherichia coli*

Most of the lipids isolated from strain K 12 of *E. coli* consisted of even-carbon-numbered straight chain ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$), cyclopropyl (C_{17}, C_{19}) and *n*-β-hydroxy ($C_{14:0}$) fatty acids, as previously reported from numerous studies on this species, and in particular for the analysed strain (for a review see Wilkinson, 1988). Important differences were noted in the three pools. The "unbound" lipids were dominated by $C_{16:0}$ and cyclopropyl C_{17} acids, followed by C_{19} cyclopropyl and $C_{14:0}$ acids; traces of *cis*-$C_{16:1}\omega7$ and *cis*-$C_{18:1}\omega7$ acids were the only unsaturated compounds detected. The "ester bound" pool had a quite different distribution. Indeed, components with shorter chains were now dominant ($C_{12:0}$ and $C_{14:0}$) together with the *n*-β-OH $C_{14:0}$ acid; lower (C_{12}) and higher (C_{16}) homologues of this hydroxy acid were also detected. Unusual fatty acids, such as *n*-β-OMe $C_{14:0}$ and *n*-β-OMe $C_{12:0}$ and the monounsaturated *n*-$C_{14:1}\omega12$ and *n*-$C_{12:1}\omega10$ acids were also identified. In the "amide bound" pool, the *n*-β-OH C_{14} component was dominant and accounted for 65% of the acids. It was accompanied by the *n*-β-OH C_{12} and the *n*-β-OMe C_{12} and C_{14} acids, by the unusual α-monounsaturated C_{12} and C_{14} acids already detected in the "ester bound" pool and by low amounts of $C_{12:0}$ and $C_{16:0}$.

The "unbound" lipids, recovered in the first step of the sequential extraction, correspond to neutral lipids and also, very likely, to polar diacylglycerol derivatives (Wilkinson, 1988). In the subsequent OH- treatment of the biomass, the "ester bound" lipids were released from the insoluble polar and/or macromolecular materials, mainly the LPS of the membranes, and to a lesser extent the lipoproteins. In *E. coli*, fatty acids occurring as esters in the LPS are the saturated *n*-C_{12}, *n*-C_{14}, *n*-C_{16}, and the *n*-β-hydroxy C_{14} (Seydel et al., 1984; Wollenweber et al., 1984; Wilkinson, 1988); these are the dominant compounds identified in the "ester bound" pool in this study. The *n*-β-OMe and *n*-α-monounsaturated C_{12} and C_{14} acids are certainly artifacts related to the corresponding *n*-β-hydroxy acids (Mendoza et al., 1987b). Cyclopropyl C_{17} and C_{19} fatty acids were also detected as minor compounds in the "ester bound" pool. They probably arise from non-LPS material, not extracted during the first step, or from acyl moities bound to lipoproteins by ester bonds. The "amide bound" lipids of the acid hydrolysate are characterized by the dominance of the β-hydroxy fatty acids (C_{12} and C_{14}) and of the closely related β-methoxy and α-monounsaturated analogues, preformed during the methanolysis, which together represent almost 90% of the "amide bound" pool. All of them derive from the amide-linked β-hydroxy C_{12} and C_{14} acids that occurred in the LPS. The other acids present in this fraction, the saturated C_{12} and C_{16} components, are unlikely to arise from LPS but more probably from lipoproteins which comprise amide-linked acyl moieties exhibiting no substitution by hydroxyl group (Lai and Wu, 1980; Wilkinson, 1988).

A qualitative comparison of the sequential extraction used in the present study with the one employed by Goossens et al. (1989a) for *Acinetobacter calcoaceticus*, which used a diethyl ether rather than a $CHCl_3$/MeOH, suggests that the present sequential extraction is more appropriate for identifying fatty acids occurring in LPS and in lipoproteins. Diethyl ether essentially extracts neutral lipids such as waxes, thus the second step of the scheme designed by Goosens et al. (1989a), i.e. methanolysis under basic conditions, would release not only fatty acids bound by ester bonds in LPS or lipoproteins, but also fatty acids from polar lipids such as diacylglycerol derivatives.

3.2. *Marinobacter hydrocarbonoclasticus*

Lipids recovered from *M. hydrocarbonoclasticus* cultured with ammonium acetate were mainly even-carbon-numbered straight chain fatty acids, ranging from C_{10} to C_{22}, and to β-oxygenated acids (C_{10}, C_{12} and C_{14} hydroxy acids and C_{12} methoxy acid; Table 2). The odd-numbered saturated *n*-C_{15} and *n*-C_{17} acids were minor components. Neither a C_{17} cyclopropyl, nor monounsaturated C_{17} fatty acids, previously reported to occur in this bacterium grown on complex media (Spröer et al., 1998; Huu et al., 1999), were detected. The very low abundance of fatty acids with odd carbon numbers was rather surprising because these compounds are generally considered as being specific to bacteria (Boon et al., 1977; Perry et al., 1979; Taylor and Parkes, 1983; Dowling et al., 1986; Saliot et al., 1991). The very low abundance of C_{15} and C_{17} fatty acids in *M. hydrocarbonoclasticus* grown on acetate may

Table 2
Fatty acid, hydroxy acid and fatty alcohol composition of the three lipid pools isolated from *Escherichia coli* and *Marinobacter hydrocarbonoclasticus*[a]

Lipids	*E. coli* (strain K12)			*M. hydrocarbonoclasticus*					
				Ammonium acetate			*n*-Icosane		
	"Unbound"	"Ester bound"	"Amide bound"	"Unbound"	"Ester bound"	"Amide bound"	"Unbound"	"Ester bound"	"Amide bound"
Fatty acids									
10:0	–[b]	–	–	–	3.5	–	–	17.6	–
12:0	tr[b]	34.4	5.6	–	20.3	tr	–	37.8	tr
12:1 ω7	–	–	–	–	0.6	–	–	–	–
12:1 ω10[c]	–	tr	0.9	–	1.2	6.1	–	0.9	3.9
14:0	4.4	20.8	–	3.0	1.8	tr	1.2	1.2	tr
14:1 ω5	–	–	–	–	0.6	–	–	–	–
14:1 ω7	–	–	–	–	0.4	–	–	–	–
14:1 ω9	–	–	–	–	13.5	–	0.5	9.3	–
14:1 ω12[c]	–	3.5	13.4	–	–	–	–	–	–
15:0	–	–	–	tr	0.1	–	0.3	tr	–
16:0	47.6	8.6	4.1	22.4	7.2	3.0	25.3	2.5	1.0
16:1 ω5	–	–	–	0.6	0.2	–	–	–	–
16:1 ω7 *cis*	0.3	2.2	–	7.3	2.1	–	8.9	0.5	–
16:1 ω9	–	–	–	10.6	2.3	–	16.2	1.2	–
17:0	–	–	–	tr	tr	–	0.4	–	–
17 ∇ω7,8	37.2	3.0	–	–	–	–	–	–	–
18:0	0.9	0.2	–	4.3	1.4	1.3	1.5	tr	tr
18:1 ω5	–	–	–	0.6	0.1	–	tr	tr	–
18:1 ω7 *cis*	0.6	–	–	5.5	1.4	–	–	0.3	–
18:1 ω9 *cis*	–	–	–	44.3	11.6	0.8	26.6	0.9	–
19 ∇ ω7,8	8.5	0.3	–	–	–	–	–	–	–
20:0	–	–	–	–	0.1	–	–	–	–
20:1 ω9 *cis*	–	–	–	0.6	0.1	–	4.2	–	–
22:0	–	–	–	–	0.1	–	–	–	–
22:1 ω9	–	–	–	0.6	tr	–	–	–	–
β-Oxygenated fatty acids									
10:0, OH	–	–	–	–	0.2	0.4	–	0.2	0.5
12:0, OH	–	1.0	4.3	tr	30.1	75.2	0.2	27.0	84.2
12:0, OMe[c]	–	0.1	0.4	–	0.2	12.3	–	0.2	9.5
14:0, OH	–	19.0	65.2	–	0.1	tr	0.1	tr	0.2
14:0, OMe[c]	–	5.9	5.1	–	–	–	–	–	–
16:0, OH	–	tr	tr	–	–	–	0.2	–	–
16:1 ω9, OH	–	–	–	–	–	–	0.1	–	–
18:0, OH	–	–	–	–	–	–	0.4	–	–
18:1 ω9, OH	–	–	–	–	–	–	0.3	–	–
20:0, OH	–	–	–	–	–	–	0.6	–	–
20:1 ω9, OH	–	–	–	–	–	–	0.3	–	–
Primary n-*fatty alcohols*									
16:0	–	–	–	tr	–	–	tr	–	–
18:1	–	–	–	tr	–	–	tr	–	–
20:0	–	–	–	–	–	–	1.3	–	–
20:1 ω9	–	–	–	–	–	–	10.2	–	–
Unidentified	0.5	1.0	1.0	0.2	0.8	0.9	1.2	0.4	0.7

[a] % of the total lipids for the considered pool; see Table 1 for the relative abundance of the three pools. "Unbound" corresponds to the hydrocarbon-free fraction for *M. hydrocarbonoclasticus* cultures.

[b] –Not detected; tr: trace.

[c] Probably correspond to artifacts derived from β-hydroxy acids.

be because this species is naturally poor in such compounds (Spröer et al., 1998; Huu et al., 1999), and/or the *de novo* synthesis of fatty acids starting from a C_2 carbon source would produce mainly even carbon-numbered acids. Another remarkable feature of this bacterium is its richness in *cis*-$C_{18:1}\omega9$ (ca 1.68% of dry biomass) and its low content of *cis*-$C_{18:1}\omega7$ (ca 0.21% of dry biomass). This leads to a *cis*-$C_{18:1}\omega9$/*cis*-$C_{18:1}\omega7$ ratio of 8. A similar, but less pronounced trend was observed for the $C_{16:1}$ fatty acids, with the dominance of the ω9 over the ω7 isomer. This 8:1 ratio, observed for *M. hydrocarbonoclasticus,* indicates the need for caution when considering *cis*-$C_{18:1}\omega7$ fatty acid as a bacterial biomarker (Perry et al., 1979; Volkman et al., 1980; Tronczynski et al., 1985). A relatively rare monounsaturated acid, $C_{14:1}\omega9$ (0.15% of dry biomass), of unknown stereochemistry, was also detected.

The ''unbound'' lipids of *M. hydrocarbonoclasticus* grown on acetate, are dominated by monounsaturated fatty acids (ca 70% of the whole pool) ranging from C_{16} to C_{22}, with *cis*-$C_{18:1}\omega9$ as the major compound. Tetradecanoic, hexadecanoic and octadecanoic acids were the sole saturated acids (Fig. 1). Two primary alcohols, *n*-*cis*-$C_{18:1}\omega9$ and *n*-$C_{16:0}$, were detected, but only at trace levels. As observed for *E. coli,* the ''ester bound'' lipids showed, by comparison, fatty acids, ranging from C_{10} to C_{22}, with an almost equal amount of saturates (C_{10}-C_{22}; 34.4%), monounsaturates (C_{12}-C_{22}; 34.2%) and β-hydroxy and related compounds (C_{10}, C_{12} highly dominant and C_{14}; 30.6%). Acids with ω9 unsaturation ranged from C_{14} to C_{22}, while those with a double bond at ω7 ranged from C_{12} to C_{18}, and at ω5 from C_{14} to C_{18}. Moreover, the proportion of β-hydroxy acids bound by ester bonds in this second extract (C_{10}–C_{14} plus their β-OCH_3 and α-unsaturated counterparts, due to artifact formation on methanolysis; C_{12} dominant; 31.8%) is similar to that observed for *E. coli* (29.5%). ''Amide bound'' lipids were almost exclusively β-hydroxy acids accompanied by their artifacts (94%; C_{12} dominant) released from LPS. As in *E. coli*, the non-hydroxylated $C_{16:0}$, $C_{18:0}$ and $C_{18:1}\omega9$ fatty acids, observed in this pool, could be derived from lipoproteins.

All the general features discussed above, for the culture grown on acetate, are retained for the *n*-icosane culture. For example, (i) the *n*-$C_{15:0}$ and *n*-$C_{17:0}$ acids only occur in low amounts and were the sole fatty acids with an odd carbon number, (ii) the rare $C_{14:1}\omega9$ acid accounted for a substantial amount of the dry biomass (0.17%) and (iii) in the ''amide bound'' lipids, the β-hydroxy acids were always dominant, maximising at C_{12}. However, some further compounds were also identified. In addition to trace amounts of *n*-*cis*-$C_{18:1}$ and *n*-$C_{16:0}$ alcohols, two long chain primary alcohols were detected in the ''unbound'' lipids (ca 11% of total pool). These were *n*-icosan-1-ol ($C_{20:0}$) and *n*-icos-11-en-1-ol ($C_{20:1}\omega9$), accounting for 0.07 and 0.57% of the dry biomass, respectively. In addition, C_{20}, C_{18} and C_{16}, β-hydroxy acids, in both saturated and ω9 monounsaturated forms, were also present (Fig. 1). These latter were identified only in the ''unbound'' lipid pool, with the alcohols corresponding to 0.64% of the dry biomass and the β-hydroxy acids to 0.12%: they were only present at trace levels in the ''unbound'' lipids from the acetate culture. In the ''ester bound'' and the ''amide bound'' lipids no new compound occurred, but some variations in composition were noted. Thus, in the ''ester bound'' lipids from the *n*-icosane culture, (i) short chain compounds, essentially $C_{10:0}$ and $C_{12:0}$, increased in relative abundance, while the C_{16} and C_{18} acids decreased, and (ii) ω5 and ω7 monounsaturated acids, which were minor compounds in the ''ester bound'' lipids from the acetate culture, were absent or present in trace amounts only.

Comparison of the data from the acetate and *n*-icosane cultures provided information on the metabolism of *n*-alkanes by *M. hydrocarbonoclasticus* and also on the use of long chain β-hydroxy acids as indicators of Gram-negative bacterial activity. Concerning the metabolism of *n*-icosane, it is likely that degradation proceeds via oxidation to the primary alcohol (Ratledge, 1978), which undergoes successive oxidations to the C_{20} fatty aldehyde, and then fatty acid, and further degradation via the classical β-oxidation cycle (Schulz, 1991). The desaturation of *n*-icosane and/or *n*-icosanol could then give *n*-icos-11-en-1-ol. Moreover, the absence in the ''unbound'' lipids of α,ω-diols, α,ω-diacids or β-hydroxy diacids suggests that oxidation of *n*-icosane occurs at only one end of the hydrocarbon chain.

Hydroxy acids often occur in particulate matter in the water column and in sediments (Perry et al., 1979; Mendoza et al., 1987b; Goossens et al., 1986, 1989b; Volkman et al., 1980; Cardoso and Eglinton, 1983; Fukushima et al., 1992a,b; Wakeham, 1999). As previously shown, ''unbound'' and ''bound'' (ester- and amide-) hydroxy fatty acids can be extracted from sediments and, for a given sample, exhibit different GC profiles (Goossens et al., 1986; Wakeham, 1999), which sometimes are very complex. For example, in particle and sediment samples originating from the Black Sea, Wakeham (1999), identified ''bound'' β-hydroxy fatty acids ranging from C_{10} to C_{22}, (odd- and even-carbon-numbered, normal and branched chains, with a maximum at C_{12}, C_{14} or C_{16}), while the ''unbound'' acids ranged from C_{14} to C_{22}, with a maximum at C_{16} or C_{17}. This general trend, for the presence in sediments of longer chain β-hydroxy acids in the ''unbound'' lipids, compared to the ''bound'' pools, has been considered to indicate that the former derive from oxidative degradation of fatty acids by bacteria. Our results on *M. hydrocarbonoclasticus* allow us to distinguish β-hydroxy acids from membranes from those deriving from the

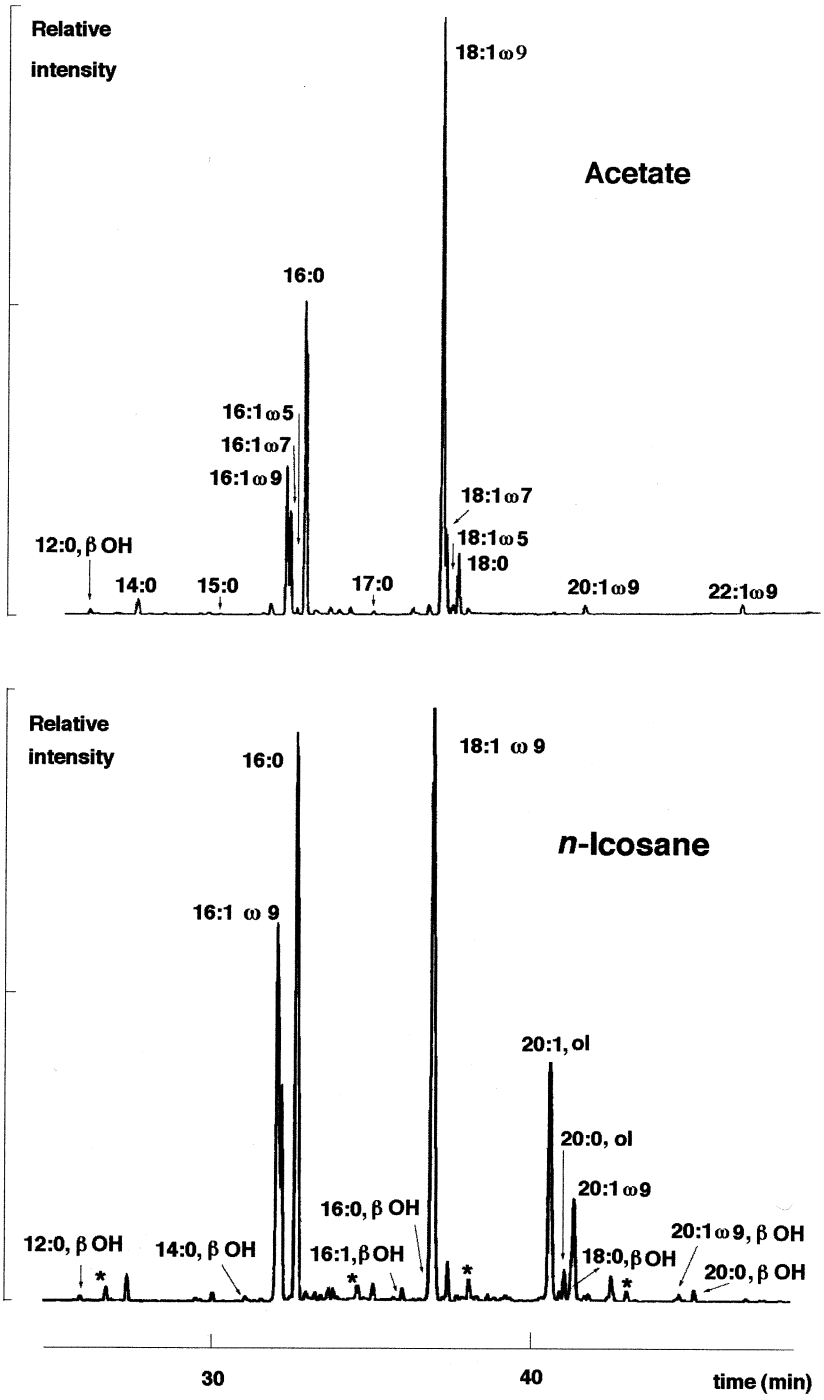

Fig. 1. Gas chromatographic profiles of the unbound lipid fractions obtained from *Marinobacter hydrocarbonoclasticus* grown on acetate and *n*-icosane and analyzed as methyl esters: ol, primary fatty alcohols; *, pollutants.

metabolism of organic compounds. The latter are not incorporated into the LPS and so are easily extracted by solvents. As a consequence, "unbound" long chain β-hydroxy acids in sediments are apparently the signature of bacterial metabolism of long chain lipids, through the β-oxidation process, as previously proposed by Cranwell (1981) from stereochemical analyses of "bound" and "unbound" β-hydroxy acids in sediments.

4. Conclusion

This study, which involved sequential extraction of the bacterial biomass of *M. hydrocarbonoclasticus*, grown on ammonium acetate or *n*-icosane, provides information on the origin of "unbound" β-hydroxy acids in Gram-negative bacteria. The degradation of *n*-icosane by *M. hydrocarbonoclasticus* shows that the "unbound" β-hydroxy acids, easily extracted with chloroform/methanol, originate from the initial oxidation of icosane to the $C_{20:0}$ alcohol. Mid-chain desaturation of icosan-1-ol probably leads to the formation of the $C_{20:1}$ alcohol and, thus, to the unsaturated series. These saturated and unsaturated C_{20} alcohols are then oxidised to the corresponding acids, which undergo β-oxidation in β-hydroxy acids. In contrast, as previously observed, the "bound" β-hydroxy acids, released under alkaline and acid conditions from solvent-extracted bacterial biomass, arise from ester- and amide-linked fatty acyl moieties of the LPS, respectively.

Therefore, whilst the present study is limited to one strain of a hydrocarbon-utilising bacterium, the observations have geochemical implications regarding the origin of "unbound" β-hydroxy acids in particulate matter and in sediments. It appears that such compounds, particularly those exhibiting long hydrocarbon chains and which are not, generally, specific of bacterial LPS, may arise from the bacterial reworking of organic materials such as long chain hydrocarbons and fatty acids.

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