Effects of octane on the fatty acid composition and transition temperature of Pseudomonas oleovorans membrane lipids during growth in two-liquid-phase continuous cultures

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Growth of Pseudomonas oleovorans GPol in continuous culture containing a bulk n-octane phase resulted in changes of the fatty acid composition of the membrane lipids. Compared to citrate-grown cells, the ratio of C18 to C16 fatty acids and the ratio of unsaturated to saturated fatty acids increased as a result of growth on octane. Trans-unsaturated fatty acids, which are rarely found in bacteria, were formed during continuous growth of P. oleovorans on octane. Moreover, the mean acyl chain length and unsaturated fatty acids also increased as the growth rates increased both in octane-grown and citrate-grown cells. Differential scanning calorimetry measurements of extracted lipids showed the transition temperature of membrane lipids from octane-grown cells increased from about 24°C to 32°C as the growth rate increased, whereas cells grown on citrate showed a constant transition temperature of about 6°C at all growth rates tested, indicating a decrease of membrane lipid fluidity in octane-grown cells. Because alkanes are known to increase bilayer fluidity by intercalating between lipid fatty acyl chains, the increased transition temperature of the lipids of cells grown on octane may be a physiological response of P. oleovorans to compensate for the direct effects of octane on its cellular membranes.

Keywords: Fatty acid composition; lipid transition temperature; octane; two-liquid-phase system; Pseudomonas oleovorans

Introduction

Pseudomonas oleovorans is capable of oxidizing aliphatic compounds. It can grow in two-liquid-phase systems using medium chain length (C8-C12) alkanes or alkenes as the sole energy and carbon source. The alkane hydroxylase system, which catalyzes the hydroxylation of alkanes and alkenes, consists of three components: a cytoplasmic membrane alkane hydroxylase, rubredoxin, and rubredoxin reductase. The enzyme system converts aliphatic compounds to oxidized intermediates, some of which are useful synths. To accumulate these compounds efficiently, a high activity of the alkane hydroxylase systems is required over a long period of time. This is difficult to achieve in batch cultures containing alkanes as a separate phase. Previous work from our laboratory has shown that when a culture of P. oleovorans enters the stationary phase, the cell membrane is damaged and the cells lose their viability and enzyme activity as a result of the presence of the apolar phase. However, when the same cells are grown in a continuous culture, the cells can maintain their membrane structure and viability for at least 200-300 h at growth rates between 0.05 h⁻¹ and 0.40 h⁻¹. Apparently, as long as P. oleovorans is
growing to some extent, it maintains its resistance to the apolar phase.

Electron microscopy of *P. oleovorans* cells grown under a variety of conditions suggests that apolar solvents have effects on the cytoplasmic and outer membranes of these cells. It is likely, therefore, that the resistance of *P. oleovorans* to apolar solvents is in some way related to the structure and physiological properties of the membranes of cells grown under different conditions.

Microorganisms can alter fatty acid compositions of membrane lipids to adapt to changing environmental conditions. A well-known example is the response of microorganisms to changes of growth temperature. At higher temperature, the saturated fatty acid content is increased to maintain a constant membrane fluidity. Microorganisms also change their membrane lipid composition in response to exposure to organic solvents. Thus, changes of membrane composition on exposure to alcohol and phenol are believed to be important in tolerance to these solvents.

To begin to understand the role of the cell membranes in cell survival in two-liquid-phase systems, we examined the composition and properties of the membrane lipids of *P. oleovorans* during continuous growth in two-liquid-phase media at different dilution rates (equivalent to the growth rates). For comparison, we did similar experiments for cells growing continuously in aqueous media, with citrate as a carbon source.

**Materials and methods**

**Bacterial strain and growth conditions**

*Pseudomonas oleovorans* GPol was used in all experiments, and all continuous culture experiments described subsequently were carried out using equipment and conditions developed and optimized previously. The mineral E₂-medium contained 3.5 g Na₂HPO₄ · 2H₂O, 0.246 g MgSO₄ · 7H₂O, and 1 ml MT (microelements) stock solution. The MT stock solution contained 1.0 g KH₂PO₄, 1.1 g (NH₄)₂SO₄, 0.246 g MgSO₄ · 7H₂O, 1.2 ml Fe³⁺ stock solution (1 ml Fe²⁺ stock solution contained 2.78 g FeSO₄ · 7H₂O per liter in 1N HCl), 1 ml modified MT, and 0.5 ml polypropylene glycol 2000 as antifoam.

**Extraction of lipids**

Cell lipids were extracted from whole cells by chloroform-methanol (1:2 v/v) as described. Whole cells and extracted lipids were saponified and methylated according to the method of Marvin et al. The methyl esters were identified by comparing with known standards. GLC analysis was performed on a 25-m CP-Sil SCB capillary column (Chrompack, The Netherlands). The column temperature program started at 60°C, which it was raised to 280°C at a rate of 2.5°C min⁻¹. Fatty acid contents were expressed as a percentage (mol/mol) of total lipid fatty acids. All data points were based on fatty acid determinations of whole cells, the latter analysis was used to determine the membrane lipid fatty acid compositions described subsequently.

**Continuous cultures**

All continuous growth experiments were performed in a 1000 ml tank fermenter with 1000 rpm stirring speed and 175-200 cm³ min⁻¹ air-flow rate as described previously. The aqueous medium contained 1.0 g KH₂PO₄, 1.1 g (NH₄)₂SO₄, 0.246 g MgSO₄ · 7H₂O, 1 ml Fe²⁺ stock solution (1 ml Fe²⁺ stock solution contained 2.78 g FeSO₄ · 7H₂O per liter in 1N HCl), 1 ml modified MT, and 0.5 ml polypropylene glycol 2000 as antifoam.

The modified MT stock solution contained (per liter water): 10 g ethylenediaminetetraacetic acid disodium dihydrate (EDTA disodi-
Differential scanning calorimetry (DSC)

Samples were prepared as follows: the lipid solution (see Lipid extraction) was dried under a stream of nitrogen and solvent traces were removed under vacuum until a constant weight was attained. The lipid film was suspended in ethylene glycol-water (1:1 v/v) to a concentration of about 0.1 g ml⁻¹. The lipid phase transition temperature was determined with a Perkin-Elmer DSC 7 apparatus. Samples were heated at 5°C min⁻¹ from -30°C to +50°C and cooled at the same rate after stabilizing for 4 min. Samples were scanned twice and the data were taken from the second scan. DSC scans of the standard lipid dimyristoyl-phosphatidylglycerol (DMPG) (a gift from Dr. T. A. A. Fonteijn, Organic Chemistry Department, University of Groningen) showed that the transition temperature was 26 ± 0.9°C under those conditions.

Results

Lipid fatty acid composition of P. oleovorans during steady-state growth on n-octane in a two-liquid-phase medium

The composition of membrane lipid fatty acids in P. oleovorans was determined after growth of the cells on octane for about 200-300 h at low (μ = 0.065 h⁻¹) and high (μ = 0.43 h⁻¹) growth rates. The higher cell density observed at the low growth rate was due to the formation of substantial amounts of poly-(R)-hydroxyalkanoate (PHA), an intracellular polyester that accumulates when P. oleovorans grows under ammonium limitation. The fatty acids found in the membrane lipids of P. oleovorans included tetradecanoic acid (14:0), hexadecanoic acid (16:0), 9-cis-hexadecenoic acid [16:1(9c)], 9-trans-hexadecenoic acid [16:1(9t)], cis-9,10-methylene-hexadecanoic acid (17:cy), octadecanoic acid (18:0), 11-cis-octadecenoic acid [18:1(11c)], and 11-trans-octadecenoic acid 18:1(11t). Figure 1 shows that the fatty acid composition and the cell density remained constant during the entire cultivation for both growth rates as expected, indicating that these continuous two-liquid-phase cultures provided stable fatty acid composition data. The total amount of octadecenoic acid (18:1) was much higher in rapidly growing cells than in slowly growing cells, at the expense of hexadecanoic acid (16:0) and hexadecenoic acid (16:1), which were present in lower amounts in rapidly growing cells. Fatty acids 14:0, 17:cy, and 18:0 never accounted for more than 5% in either slowly or rapidly growing cells, and are therefore not presented in Figure 1.

Effects of the growth rate on the lipid fatty acid composition of P. oleovorans during continuous growth on n-octane

Figure 2 shows the fatty acid composition of the membrane lipids of P. oleovorans during continuous growth on n-octane. Between μ = 0.072 h⁻¹ and μ = 0.30 h⁻¹, 18:1 increased from about 27 to 37%, whereas 16:0 and 16:1 decreased from 33 to 30% and 32 to 27%, respectively. From μ = 0.30 h⁻¹ to μ = 0.46 h⁻¹, 18:1 dropped slightly, 16:1 rose slightly, and 16:0 remained unchanged (Figure 2a). Fatty acid 18:0 fluctuated around 4%, fatty acid 17:cy decreased from 2% to ≤0.4%, and 14:0 was present in trace amounts only (≤0.3%) (data not shown).

It is interesting that trans-unsaturated fatty acids, which have not been found in most bacteria, accounted for a constant 10% of the total fatty acids for 16:1(9t), whereas 18:1 (11t) increased linearly from 2% at the lowest to 8% at the highest growth rate (Figure 2b). The cis-isomers remained the major constituents: 18:1(11c) accounted for 23–31%, whereas 16:1(9c) accounted for 17–22%.

These changes resulted in increases of the mean acyl chain length and overall unsaturated fatty acids content of the membrane lipids (see Figure 4). The C₁₄/C₁₆ ratio increased from 0.45 to about 0.75 at growth rates between μ = 0.07 h⁻¹ and μ = 0.30 h⁻¹, and then decreased to 0.67 as the growth rate reached a maximum of μ = 0.46 h⁻¹. The degree of unsaturation increased from 62 to 66% as the growth rate increased. This slight increase was entirely due to an increase in the trans-isomers.

Lipid fatty acid composition of P. oleovorans during continuous growth in an aqueous medium

To compare these results to those seen for cells grown in an aqueous medium, P. oleovorans was grown continuously on citrate. The membrane lipids of the cells contained fatty acids 14:0, 16:0, 16:1(9c), 17:cy, 18:0, and 18:1(11c). In contrast to octane-grown cells, citrate grown cells contained little or no trans-unsaturated fatty acids: The amount of 16:1(9t) never exceeded 1.5% and no 18:1(11t) was detected. Figure 3 shows that major fatty acid 16:0 decreased from 44 to 36%, whereas the other major fatty acid, 16:1, increased from 33 to 41% as the growth rate increased from μ = 0.045 h⁻¹. The composition of membrane lipid fatty acids in P. oleovorans shows that major fatty acid 16:0 decreased from 44 to 36%
Figure 2. The effects of growth rate on the fatty acid composition of octane-grown *Pseudomonas oleovorans*. The cells were grown at 30°C in continuous culture with n-octane, as described for Figure 1. Samples were taken during successive steady states for fatty acid assays. Different data points are taken from three to four different independent continuous cultures. The fatty acids that accounted for <2% of total fatty acids are not shown. a: Major saturated (16:0, 18:0) and unsaturated fatty acids (16:1, 18:1). b: Distribution of cis- and trans-unsaturated fatty acids.

Figure 3. The effects of growth rate on the fatty acid composition of *Pseudomonas oleovorans* grown continuously in an aqueous minimal medium containing 2.1% trisodium citrate dihydrate as the carbon source. Different data points are taken from three different independent continuous cultures.

The changes of fatty acid composition resulted in a linear increase of the mean acyl chain length with increasing growth rates. The absolute levels of the C18/C16 ratio were much lower in citrate-grown cells than in octane-grown cells. (Figure 4a). The unsaturated fatty acid content of citrate-grown cells was also lower than that of octane-grown cells (Figure 4b). Here, however, the differences were smaller, and at higher growth rates the unsaturated fatty acid content approached that of octane grown cells.

**Differences in the transition temperature of the membrane lipids between octane and citrate-grown *P. oleovorans***

Compared to the citrate-grown cells, growth of *P. oleovorans* on octane resulted in an increase of the mean acyl chain length (Figure 4a), which was expected to increase the transition temperature of the membrane lipids, whereas the higher ratio of unsaturated to saturated fatty acids (Figure 4b) was expected to do the opposite. To determine the net effect of the observed changes, we measured the phase transition temperature of the isolated lipids by DSC.

*Pseudomonas oleovorans* was grown in continuous cultures with 15% (v/v) octane or 2.1% (w/v) citrate at slow, medium, and fast growth rates. The highest growth rate in the aqueous medium was set to 0.29 h⁻¹ rather than 0.40 h⁻¹ because the μmax of *P. oleovorans* during growth in citrate medium is 0.39 h⁻¹.

Figure 5 shows typical DSC thermograms of extracted lipids from these steady-state cultures. In general, the observed lipid phase transition occurred over a very wide temperature range. When the cells were grown on octane, the transition started below and finished above the growth temperature. There was only one obvious transition peak, the position of which varied with the growth rate. When lipids were isolated from cells grown on citrate, the lipid phase transition occurred almost completely below the growth temperature. There was a major transition peak at about 6°C with an extended shoulder around 24°C.

The transition temperatures of lipids from cells grown at different dilution rates on octane or citrate are shown in Figure 6. For octane-grown cells, the lipid transition temperature increased as the growth rate increased. For citrate-grown cells, the major lipid transition temperature remained...
Effect of octane on membrane lipids: Q. Chen et al.

**Figure 4** Changes in fatty acid mean length and degree of unsaturation with growth rates for both octane- and citrate-grown *Pseudomonas oleovorans*. a: Ratio $C_{16}/C_{18} = (18:1 + 18:0)/(16:1 + 16:0)$. b: Percentage of unsaturated fatty acids relative to total lipid fatty acids. 17:cy was counted as a cis-unsaturated fatty acid.

constant at 6°C at different growth rates, whereas the shoulder fluctuated slightly with a maximum between 22.7°C and 26.3°C.

**Discussion**

We found that the fatty acid composition of the membrane lipids of *P. oleovorans* is affected by the growth rate during continuous cultivation. For citrate grown cells, the mean acyl chain length as well as the cis-unsaturated fatty acids increased when the growth rate increased. These changes did not alter the transition temperature of the membrane lipids, indicating that the increase of membrane lipid fluidity that might be expected from an increased content of cis-unsaturated fatty acids was completely compensated for by the simultaneous increase of the mean acyl chain length. When the cells were grown in the presence of n-octane, the transition temperature of the membrane lipids increased as the growth rates increased. This can be accounted for by the increase of the mean acyl chain length, which was not compensated for by a corresponding increase of cis-unsaturated fatty acids. Instead, there was an increase of trans-unsaturated fatty acids (Figure 4). Trans-unsaturated fatty acids resemble saturated fatty acids in that they possess an extended conformation and small molar volume. Therefore, the effect of converting cis-unsaturated fatty acids to trans-unsaturated fatty acids is similar to that seen for the substitution of unsaturated fatty acids by saturated fatty acids, resulting in a decrease of the membrane lipid fluidity, although the effect of the former conversion is less than that of the latter. In contrast to the unsaturated-to-saturated conversion, the conversion of cis-to-trans fatty acids is independent of de novo synthesis of lipids and proteins. In our studies, the positions of the trans–double bonds were always identical to those of the cis–double bond [16:1(9t) versus 16:1(9c) and 18:1(11t) versus 18:1(11c)], suggesting that there is simple isomerization of cis–to-trans–double bonds. Thus, *P. oleovorans* may be capable of adjusting its...
membrane fluidity meticulously and rapidly by conversion of cis-unsaturated fatty acids to trans-isomers.

Changes in the fatty acid composition of the lipids of *P. oleovorans* during growth on octane may be a response of the cells to growth in the presence of *n*-alkanes. *n*-Alkanes are taken up by membrane lipid bilayers by dissolving in the presence of octane, and *n*-alkanes with 6 to 12 carbons have been shown to decrease the transition temperature of membrane lipids. Therefore, during growth in the presence of octane, *P. oleovorans* may alter the composition of its fatty acids to compensate for the increase of membrane fluidity caused by the intercalation of octane in lipid bilayers. Apparently, *P. oleovorans* cells do so not only by increasing the mean acyl chain length of the membrane lipids, but also by increasing the ratio of trans- to cis-unsaturated fatty acids.

The strategy to modulate membrane fluidity via isomerization of cis- to trans-unsaturated fatty acids has recently been observed in several genera. Diefenbach *et al.* reported that *Pseudomonas oleovorans* uses this route to modify its membrane fluidity when cell growth is partly or completely inhibited, conditions under which neither the ratio of unsaturated to saturated fatty acids, nor the mean acyl chain length of the membrane lipids can be changed sufficiently to attain the desired change of membrane fluidity, because of a decrease of phospholipid and protein synthesis. In the experiments reported here, the conversion of cis- to trans-fatty acids cannot be triggered by growth inhibition because *P. oleovorans* grows at least as well on octane as on citrate, and the cis-to-trans conversion increases with growth rate. This suggests that each of the three strategies—increasing chain length, altering the degree of saturation, and cis-to-trans-isomerization—is used to modulate the fluidity of the membranes of cells growing on octane.

Finally, in contrast to growth in aqueous media, when *P. oleovorans* was grown in the presence of octane, two separate processes occurred. First, the cell membranes were exposed to a bulk apolar octane phase. Second, alkane hydroxylase, which is located in the cytoplasmic membrane, was induced to use octane as carbon source. Because both organic solvents and membrane proteins can influence membrane lipid composition and properties, we are currently examining whether the differences in the lipid fatty acid composition and fluidity between octane and citrate grown cells is due to the effects of octane on the membrane as an organic solvent, or an inducer of alkane hydroxylase, or both.

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**References**