Biosynthesis of *trans* fatty acids from acetate in the bacterium *Pseudomonas atlantica*

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The *cis* configuration in fatty acids is the only known product of bacterial biosynthetic pathways of monounsaturated membrane fatty acids. The *trans* configuration is considered "nonphysiologic" or "unnatural." This research shows that *in vivo* synthesis of *trans*-monounsaturated membrane fatty acids from acetate occurs in the bacterium *Pseudomonas atlantica*. The saturated, *trans*, and *cis* phospholipid fatty acids (PLFA) of *P. atlantica* grown in the presence of [^{14}C]acetate were physically separated as the corresponding fatty acid methyl esters (FAME) and the presence of *trans* FAME was verified by infrared spectroscopy. The FAME isomers were quantified by gas chromatography and ^{14}C incorporation into these isomers was counted. The specific activities of *trans* and saturated PLFA were found to be equivalent, indicating that *P. atlantica* has the *in vivo* biosynthetic capabilities to synthesize *trans* PLFA from acetate. From these results, a biosynthetic pathway is hypothesized and the suggestion is made that *P. atlantica* would be a preferred test organism to elucidate this pathway as well as to test the ecological implications of these *trans* PLFA in terms of starvation survival and the initial biofouling–adhesion process.

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Chez les bactéries, les seuls produits connus des sentiers biosynthétiques relativement aux acides gras monoinsaturés de la membrane sont les acides gras de configuration *cis*. La configuration *trans* n'est pas considérée 'physiologique' ou 'naturelle'. La présente recherche montre que, chez la bactérie *Pseudomonas atlantica*, la synthèse *in vivo* des acides gras *trans*-monoinsaturés de la membrane se produit à partir d'acétates. Les acides gras phospholipidiques (PLFA) *cis*, *trans* et saturés de *P. atlantica* croissant en présence d'acétate [^{14}C] ont été séparés physiquement en leurs esters d'acides gras méthylés correspondants (FAME) et la présence de FAME *trans* a été vérifiée par spectroscopie à l'infrarouge. Les isomères FAME furent quantifiés par chromatographie en phase gazeuse et l'incorporation du ^{14}C dans ces isomères fut établie par comptages. Les activités spécifiques des PLFA *trans* et des PLFA saturés se sont révélé être équivalents, ce qui indique que *P. atlantica* a la capacité de synthétiser *in vivo* des PLFA *trans* à partir d'acétates. Ces résultats permettent d'avancer l'hypothèse d'un sentier biosynthétique et de suggérer que *P. atlantica* pourrait être utilisé de façon préférentielle pour élucider ce sentier et pour vérifier les implications écologiques de ces PLFA *trans* en termes de famine survivance et dans les processus initiaux de biopollution–adhésion.

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Introduction

The *cis* fatty acids are the only known products of bacterial biosynthetic pathways of monounsaturated membrane fatty acids (Harwood and Russell 1984; Fulco 1983). The *trans* configuration is considered "nonphysiologic" or "unnatural" by some researchers (Sommerfeld 1983).

The *trans* or *E* (from the German, *entgegen*, "opposite") configuration is more stable and tends to pack membrane fatty acids in a more ordered state, thus increasing the transition or melting temperature of the membrane, T_m. The *cis* or *Z* (from the German, *zusammen*, "together") configuration decreases the T_m of a membrane (Esfahani et al. 1969). Classical membrane fatty acid research has shown that as the incubation temperature decreases, the proportion of unsaturated (presumably *cis*) fatty acids increases to lower the T_m to keep the membrane in a fluid state (Marr and Ingraham 1962).

Although their source is unknown, *trans*-monounsaturated fatty acids have been found in geochemical investigations of marine (Boon et al. 1978; Perry et al. 1979), estuarine (Van Vleet and Quinn 1976, 1979; Volkman and Johns 1977; Volkman et al. 1980), mangrove (Gillan and Hogg 1984), and lacustrine (Cranwell 1982) sediments as well as isolates from these environments (Gillan et al. 1981, 1983). Other reports of *trans*-monounsaturated fatty acids include several species of methylotrophs (Makula 1978; Nichols et al. 1986*b*), *Vibrio cholerae* (Guckert et al. 1986), and *Pseudomonas atlantica* which has had three *cis*/*trans* monounsaturated pairs chemically verified (Nichols et al. 1986*a*).

A possible source of *trans* acids could be exogenous. Direct incorporation of an available *trans* fatty acid has been induced with unsaturated fatty acid auxotrophs (Beacham and Silbert 1973; Mavis and Vagelos 1972; Schairer and Overath 1969; Silbert et al. 1968, 1973). Rumen bacteria have been shown to synthesize *trans*-monounsaturated fatty acids, principally 18:1ω7*t*, by biohydrogenation of polyunsaturated media components such as linoleic acid (18:2ω6,9) and linolenic acid (18:3ω3,6,9) (Kemp et al. 1975; Verhulst et al. 1986), although neither the substrate nor the product has been found to be incorporated into cellular lipids (Kepler and Tove 1967).

There is evidence, however, which supports the possibility of *de novo* synthesis of *trans* fatty acids in bacteria. Although some of the isolates which have been reported to have significant levels of *trans* fatty acids could have a source of polyunsaturated fatty acids in their medium (i.e., yeast extract)

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(Gillan et al. 1981, 1983), the effect of these on an organism's fatty acid profile when it is grown in dilute media has been found to be minimal (Perry et al. 1979). The methyltrophs which have been shown to produce *trans* fatty acids of several positional isomers do so in an organic-free media utilizing methane as their sole carbon source (Makula 1978; Nichols et al. 1986*b*).

The purpose of this research was to show that *in vivo* synthesis of *trans*-monounsaturated membrane fatty acids from acetate does occur. *Pseudomonas atlantica* was used because of its high proportion of *trans* fatty acids (Nichols et al. 1986*a*). It was hypothesized that if *in vivo trans* synthesis occurs in *P. atlantica*, then when it is grown in the presence of [^{14}C]acetate, the specific activity of the *trans* fatty acids should be equivalent to that of the saturated and *cis* fatty acids.

After physical separation of the saturated, *cis*, and *trans* fatty acids, and after ^{14}C incorporation quantification, GC analysis of the quantity of each fatty acid isomer, and chemical verification of the *trans* configuration, this research shows that *in vivo trans* fatty acid biosynthesis from acetate occurs in *P. atlantica*. From these results, a biosynthetic pathway is hypothesized and the suggestion is made that *P. atlantica* would be a preferred test organism to elucidate this pathway as well as to test the ecological implications of these *trans* acids in starvation survival and the initial biofouling–adhesion process.

Materials and methods

Microorganism and culture medium

Pseudomonas atlantica, Humm (ATCC 19262, NCMD 301) was grown in a medium consisting of 5 g D-(+)-galactose (Eastman Kodak Co.), 2.5 g casamino acids (Difco), and 12.5 g artificial sea salts (Forty Fathoms) per 500 mL distilled water. The resulting salinity was approximately 32 parts per thousand (ppt). After thorough mixing to dissolve all components, the 500-mL batch was autoclaved.

Batch culture for phospholipid fatty acid (PLFA) profiles

Pseudomonas atlantica was grown in batch culture of the above medium for 18 h three separate times from different inocula. A fourth culture was allowed to grow for 48 h. The cells were harvested and the lipids analyzed as described below.

Experimental design

Fifteen 50-mL serum vials were stopped with cotton and autoclaved. To these, 9 mL of the above medium was aseptically added. Each was then inoculated with 1 mL of an 18-h starter culture of *P. atlantica* (same medium). The starter culture was also streaked onto a plate of the same media (plus agar) to check for culture purity. The vials were numbered 1–15 and were processed in this order for the rest of the experiment. Ten of these vials were randomly selected to receive 100 μL of filtered (0.2 μm) [^{14}C]acetate (specific activity = 30.7 μCi/mL; 1 Ci = 37 GBq) prior to inoculation. Five of the ^{14}C-labelled vials were randomly chosen to also receive 0.5 mL filtered (0.2 μm) formalin prior to adding [^{14}C]acetate. The treatment designations were therefore as follows: HOT, media + ^{14}C + cells; HOT/KILLED, media + formalin + ^{14}C + cells; and COLD, media + cells. Five independent replicates of each treatment were used. All vials were swirled slightly after all additions were complete to ensure a homogeneous suspension, and incubated without shaking at room temperature (25°C) for 18 h.

Culture harvesting

For each vial, the total culture volume (10 mL) was transferred to a screw-cap test tube with a Teflon-lined cap. The cells were concentrated by centrifugation (5000 × *g*, 10 min) and the supernatant was siphoned off. Five millilitres of autoclaved 32 ppt artificial seawater (ASW) was added to the serum vials and mixed. This serum vial wash was poured into the test tube with the corresponding cell pellet, the test tube was agitated with a vortex mixer to resuspend the pellet and the cells were reconcentrated by centrifugation, as before. The ASW wash was repeated once more.

Lipid extraction

The modified Bligh and Dyer method (White et al. 1979) was used. Five millilitres of 50 m*M* phosphate buffer (White et al. 1979) was added to each cell pellet (approximately 25 mg wet weight), the tube was agitated with a vortex mixer to evenly distribute the cells, and the resulting suspension was poured into a separatory funnel. This was repeated twice (total volume of buffer, 15 mL) before 37.5 mL methanol and 18.75 mL chloroform were added to the separatory funnel for an overnight (approx. 18 h) single-phase extraction. Equal volumes of chloroform and distilled water (18.75 mL) were then added and the phases allowed to separate for 24 h.

Lipid analysis

The total extractable lipids were separated by silicic acid column chromatography into the general lipid classes: neutral lipids, glycolipids, phospholipids. Mild alkaline methanolysis was used to prepare the fatty acid methyl esters of the phospholipid fraction (Christie 1973; Guckert et al. 1985).

Silver nitrate thin layer chromatography ($AgNO_3$ TLC)

Preparative $AgNO_3$ TLC was used to separate the saturated, *trans*-monounsaturated, and *cis*-monounsaturated fatty acid methyl esters (FAME) (Christie 1973). Commercially prepared Whatman K6 plates with vertical sample lanes designated by grooves cut into the silica were initially run in a solvent system composed of $AgNO_3$ – ethanol – distilled water (10 g: 60 mL – 30 mL) overnight in the dark. The plates were then air dried for 15 min and oven dried (100°C) for 15 min to remove water. Samples were run in the solvent system chloroform–ethanol (99:1, v/v). This system was found to adequately resolve authentic FAME standards with average R_f values for the three $AgNO_3$ TLC plates (±1 standard deviation) as follows: 19:0, R_f = 0.67 (±0.01); 16:1ω7*t*, R_f = 0.59 (±0.02); 16:1ω7*c*, R_f = 0.50 (±0.03).

The FAME of the HOT and COLD treatments were spotted on the TLC plate in chloroform such that two HOT and two COLD samples were alternatively spotted on each TLC plate. The two outer lanes were spotted with an equimolar mixture of the FAME standards: 19:0, 16:1ω7*t*, 16:1ω7*c* (Applied Science Laboratories and Nu-Chek Prep, Inc.). In addition, 16:1ω7*t* and 16:1ω7*c* were run separately to verify *cis*/*trans* resolution and check for $AgNO_3$-induced isomerization.

After $AgNO_3$ TLC development, the entire plate was lightly sprayed with 0.01% (w/v) Rhodamine 6G and the FAME spots were identified under an ultraviolet lamp. The TLC bands corresponding to the saturated, *trans*-monounsaturated, and *cis*-monounsaturated FAME (R_f values given above), as identified from the standards run in the outside lanes, were scraped and collected individually into test tubes. These were designated bands C, D, and E, respectively. In addition, equal size bands (1 cm wide) above band C and below band E were scraped, collected, and labelled bands B and F, respectively.

The scraped silica shavings collected in the screw-cap test tube were washed with 5 mL of chloroform–methanol (1:1, v/v). This suspension was agitated with a vortex mixer, centrifuged, and the solvent was removed. This elution was repeated once; the supernatants were combined and dried under nitrogen.

Gas chromatography (GC)

The five $AgNO_3$ TLC bands from the COLD treatments were analyzed by capillary GC using the nonpolar Hewlett-Parkard cross-linked methyl silicone (HP-CMS) 50 m column as previously described (Guckert et al. 1985) using a temperature program of 80 to 240°C at a rate of 4° per min. Peak identification was based on identical relative retention times with fatty acid standards obtained from Nu-Chek Prep (Elysian, MN) as well as previously analyzed *P. atlantica* PLFA which was structurally verified using gas chromatography – mass spectroscopy (GC–MS) (Guckert et al. 1985), and the dimethyl disulfide (DMDS) method (Nichols et al. 1986*a*), to determine double bond position and geometry.

Fourier transform infrared spectroscopy (FTIR)

Infrared analysis was used to verify the presence of *trans* FAME by

the absorbance at 966 cm^{-1} (Chapman 1965). A standard curve was developed to estimate the percent *trans* FAME composition of each $AgNO_3$ TLC band from the ratio of the peak absorbance at 966 cm^{-1} (*trans*-CH deformation) to that at 1170 cm^{-1} (ester C—O stretch) (Scholfield 1979). A calibration curve of percent *trans*-monounsaturated FAME versus the ratio of absorbance (966 cm^{-1} to 1170 cm^{-1}) was prepared with mixtures of 16:1ω7*t* and 19:0 FAME as well as 16:1ω7*t* and 16:1ω7*c* FAME.

The FTIR sampling was based on the methods developed for use with HPLC fractions of archaebacterial lipids (Mancuso et al. 1986). Infrared-grade potassium bromide (KBr; Mallinckrodt, Inc.) was ground for 1 min (Wig-L-Bug, Spectra Tech, Inc.), transferred to a 3 × 2 mm sample cup, and leveled (without compression, using a spatula) prior to diffuse reflectance FTIR analysis. FAME samples, either *P. atlantica* $AgNO_3$ TLC bands (both HOT and COLD) or standards, were dissolved in hexane (100 μL) and transferred to the sample cup. Background spectra were collected for each KBr-filled cup prior to FAME application. Details of the operating parameters for the FTIR instrumentation and software have been previously described (Nichols et al. 1985). After nondestructive FTIR analysis of the FAME, the KBr was poured back into the original test tube for that sample. The FAME were then eluted with solvent for further analysis.

Liquid scintillation counting (LSC)

Incorporation of ^{14}C was quantified by LSC. An appropriate aliquot (100–1000 μL) was dissolved in 10 mL Aquasol (New England Nuclear) and counted for 3 min on a LKB Wallac 1217 Rackbeta LSC. The computer system connected to the LSC calculated disintegrations per minute (dpm) based on the sample's counts per minute, the counting efficiency from an established quench curve, and an external standard ratio (^{226}Ra) measured for each sample.

Every fraction resulting from the lipid analysis was quantified with an appropriate dilution to attempt to account for all ^{14}C. The aliquots for the $AgNO_3$ TLC bands C, D, and E were 10% of the total sample and resulted in dpm at least two orders of magnitude above background (= 26 dpm). The final results were converted to microcuries (1 Ci = 37 GBq) for analysis of the specific activity of FAME.

Lipid nomenclature

Since the site of action by desaturase enzymes is defined by the distance from the carboxyl end (delta or Δ end) of the fatty acid molecule, the delta (Δ) nomenclature is used for these enzyme descriptions, i.e., *cis*-Δ9 desaturase. Once the double bond is formed, however, it is the distance of the site of unsaturation from the methyl end (omega or ω) of the fatty acid molecule which remains constant during further chain elongation as the 2-carbon units are added to the delta (Δ) end. Therefore, fatty acids are designated as the total number of carbon atoms : number of double bonds with the position of the double bond closest to the omega (ω) end of the molecule indicated with the geometry "*c*" for *cis* and "*t*" for *trans*, when known (i.e., 16:1ω7*c*).

Statistical analysis

Where appropriate, average values are expressed ± 1 standard deviation (SD) as an indication of the variability within a treatment for the independent replicates. For a derived variable such as specific activity, the ratio of two independent measures, there is no straightforward calculation of the ratio's variance (Sokal and Rohlf 1981). To estimate this variability for comparison of average ratios for the PLFA isomers, the measured data (recovery of ^{14}C and PLFA) were logarithmically transformed and a 95% confidence interval for the difference of means (log (^{14}C) − log (PLFA)) for the transformed data for each PLFA isomer fraction was obtained. Since subtraction of logarithms is equivalent to division of their antilogarithms, the back-transformed difference and corresponding confidence interval provides a 95% confidence interval about the ratio of the geometric means as a comparison of the specific activities for the saturated, *trans*, and *cis* fatty acids of *P. atlantica*, and the variability associated with each fraction (Sokal and Rohlf 1981).

TABLE 1. Phospholipid, ester-linked monounsaturated fatty acids of *Pseudomonas atlantica* grown on a galactose,casamino acids, and artificial sea salts medium. Times indicate when the culture was harvested for lipid analysis

Fatty acid	18-h culture*	48-h culture†
16:1ω9*c*	1.2(0.2)	0.8
16:1ω7*c*	23.1(2.3)	12.2
16:1ω7*t*	21.3(1.4)	30.2
17:1ω8*c*	1.2(0.2)	0.2
17:1ω8*t*	0.8(0.3)	1.4
18:1ω9*c*	0.5(0.1)	0.9
18:1ω7*c*	9.2(2.0)	13.6
18:1ω7*t*	0.6(0.2)	3.4
trans/*cis* ratios		
Total	0.68(0.10)	1.32
16:1ω7	0.93(0.13)	2.48
17:1ω8	0.70(0.12)	2.00
18:1ω7	0.07(0.01)	0.25

NOTE: All double bond positions were verified by DMDS GC–MS.
*Values are the results of three independent cultures and are expressed as the average mol% with 1 SD indicated in parentheses.
†Values are mol% from one determination.

Results

PLFA of P. atlantica

Table 1 shows the monounsaturated PLFA for *P. atlantica* (mole percent of the entire profile) grown on the same medium but at different times as well as from different inocula. Three cultures were harvested after 18 h, one was harvested after 48 h. The relative proportions of each isomer position (i.e., 16:1ω7) remained approximately the same from 18 to 48 h, however, the *trans*/*cis* ratio within each isomer type increased. The *trans*/*cis* ratio for 16:1ω7 and 17:1ω8 were always an order of magnitude greater than the ratio for 18:1ω7.

Controls

The recovery of label in whole cells from the HOT/KILLED (44 ± 99 dpm) was 0.004% of the HOT treatment results (990 000 ± 110 000 dpm). Since the HOT/KILLED was a formalin-killed 1-mL inoculum of an 18-h culture and the HOT was 10 mL of an 18-h culture from the same size inoculum, one would expect about 1–2 orders of magnitude more cells in the HOT than the HOT/KILLED. The actual ^{14}C recovery was over 4 orders of magnitude less for the HOT/KILLED, indicating no significant abiological incorporation of ^{14}C.

The samples were processed in numerical order such that the three treatments were randomly assigned across the order of processing. This was a control for cross contamination during lipid analysis. All COLD and HOT/KILLED samples were found to have an average level of ^{14}C below 1% of any corresponding HOT sample, indicating no cross contamination.

$AgNO_3$ TLC separation and GC results

Figure 1 indicates the GC results of the COLD samples for all bands of the $AgNO_3$ TLC separation. There were $n = 5$ replicates for each band. The histograms represent the average recovery of the saturated, *trans*-monounsaturated, and *cis*-monounsaturated FAME in nanomoles for each of these bands. The error bars indicate ± 1 SD.

Bands B and F, above and below the extent of FAME standard spots on the $AgNO_3$ TLC, had few FAME. Band C, where the saturated (19:0) FAME standard ran, was 94%

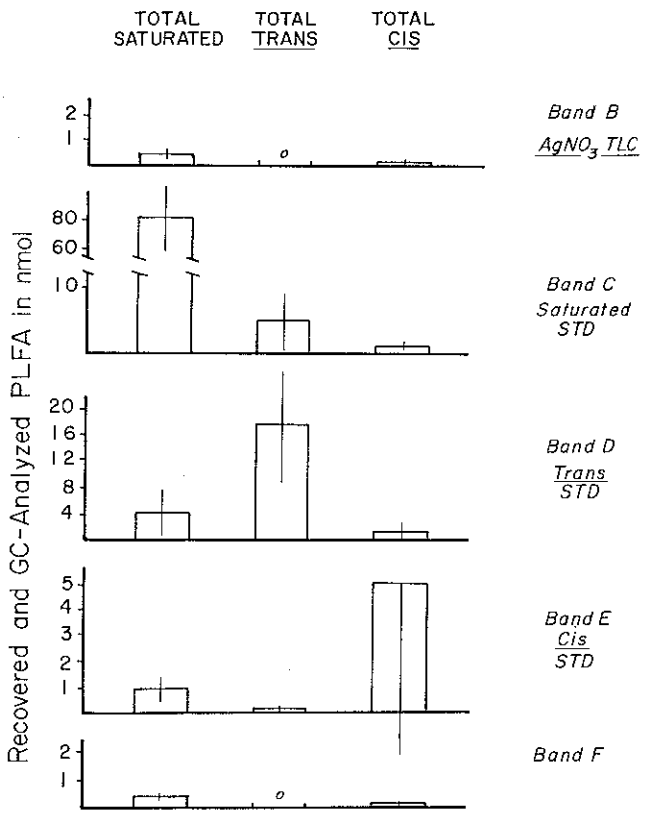

FIG. 1. Average recovery of saturated, *trans*-monounsaturated, and *cis*-monounsaturated *Pseudomonas atlantica* FAME in nmol for each of the $AgNO_3$ bands. The bands are labelled according to the fatty acid isomer standard (STD) which corresponded to that band. Bands B and F were above and below the standard bands. Histograms indicate the average of five independent replicates with the error bars indicating ± 1 SD. The average (± 1 SD) R_f values for the three $AgNO_3$ TLC plates used were as follows: band B, 0.73 (± 0.01); band C, 0.67 (± 0.01); band D, 0.59 (± 0.02); band E, 0.50 (± 0.03); band F, 0.42 (± 0.03).

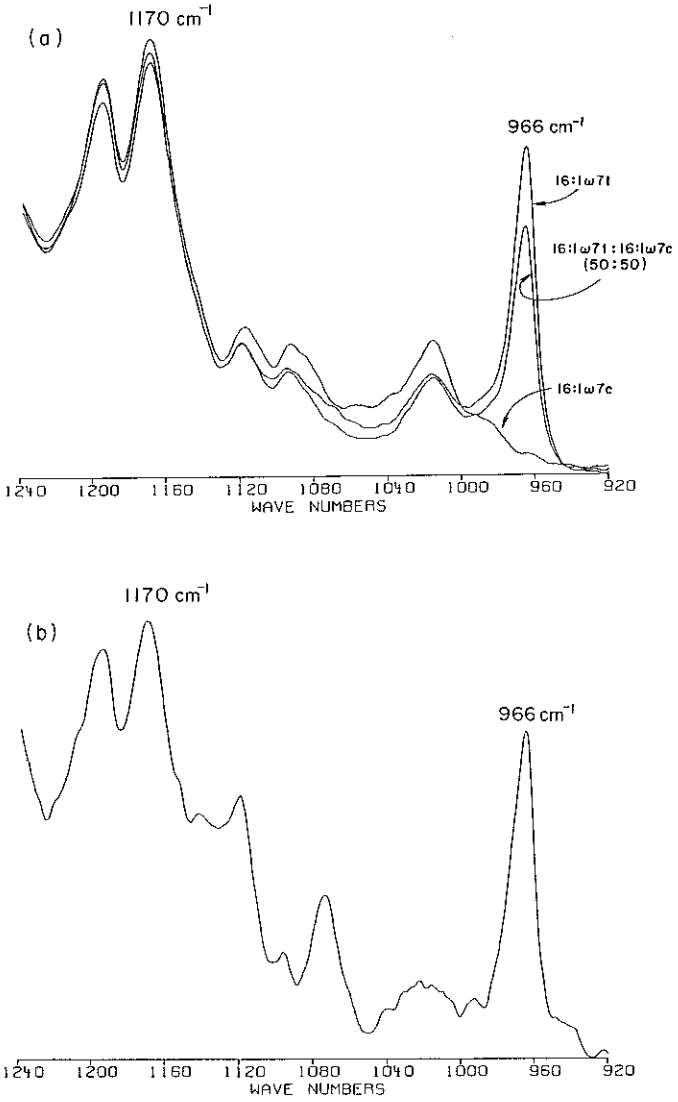

FIG. 2. (*a*) FTIR spectra of pure 16:1ω7*t*, an equimolar mixture of 16:1ω7*t* and 16:1ω7*c*, and pure 16:1ω7*c* FAME. All spectra are autoscaled to the methyl ester absorbance (1170 cm^{-1}). (*b*) FTIR spectrum of the *Pseudomonas atlantica* lipid recovered from band D of the $AgNO_3$ TLC plate for one of the COLD samples. The ratio of the *trans* (966 cm^{-1}) to the methyl ester (1170 cm^{-1}) absorbance is 0.867.

saturated FAME. Band D, where the *trans*-monounsaturated (16:1ω7*t*) FAME standard ran, was 77% *trans* FAME, and band E, where the *cis*-monounsaturated (16:1ω7*c*) FAME standard ran, was 81% *cis* FAME. The recovery of the *cis* FAME from band E was low in absolute terms (4.8 nmol) and quite variable over the five replicates (± 6.5 nmol) as compared with the recovery of saturated FAME from band C (82.4 ± 22.6 nmol) or the *trans* FAME from band D (17.4 ± 7.9 nmol).

FTIR verification of trans *acids*

FTIR analysis was used to verify the presence of *trans* fatty acids in band D and to independently estimate the percentage of *trans* fatty acids. Figure 2*a* shows the IR spectra of pure 16:1ω7*t*, an equimolar mixture of 16:1ω7*t* and 16:1ω7*c*, and pure 16:1ω7*c* FAME. The spectra are all autoscaled to the absorbance at 1170 cm^{-1} and showed a decreasing absorbance at 966 cm^{-1} with a decreasing quantity of *trans*-monounsaturated FAME. A spectra from one of the COLD $AgNO_3$ TLC band D *P. atlantica* samples is shown in Fig. 2*b* with the methyl ester absorbance of 1170 cm^{-1} and the *trans* absorbance at 966 cm^{-1} indicated. The ratio of these peaks (966 cm^{-1} to 1170 cm^{-1}) is 0.867.

The calibration curve of percent *trans* fatty acid in a mixture versus the ratio of the absorbances 966 cm^{-1} to 1170 cm^{-1} had a linear correlation of $r = 0.89$. The regression equation for all fatty acid mixtures used was $y = 0.006x + 0.196$, where x = percent *trans* in the mixture and y = the absorbance ratio. The results of the percent *trans* determinations by FTIR, as calculated using this regression equation, for $AgNO_3$ TLC bands C, D, and E for *P. atlantica* fatty acids (average of all replicates from both HOT and COLD treatments) are shown in Table 2 and are compared with the same determinations by GC analysis of the COLD treatments. The average FTIR estimates of *trans* content are higher than the GC estimates, but much more variable and, therefore, not significantly different from the GC estimates. The GC and FTIR analyses agree that the FAME found in $AgNO_3$ TLC band D is approximately 80% *trans*-monounsaturated.

^{14}C incorporation into FAME isomers

The comparison of specific activities for the PLFA isomer fractions of *P. atlantica* require a ratio of two independent measures, the recovery of ^{14}C analyzed by LSC and the

TABLE 2. Average calculated percent *trans* of *P. atlantica* PLFA for each $AgNO_3$ TLC band based on GC and FTIR as described in the text

$AgNO_3$ TLC band	Corresponding FAME standard	Analysis of *P. atlantica* lipids: GC, % *trans*	Analysis of *P. atlantica* lipids: FTIR, % *trans*
C	19:0	4.9(3.2)	15.6(35.0)
D	16:1ω7*t*	76.9(4.9)	86.9(35.5)
E	16:1ω7*c*	7.6(9.9)	23.3(18.8)

NOTE: Averages given are for five independent replicates with 1 SD indicated in parentheses.

recovery of total PLFA analyzed by GC for $AgNO_3$ TLC bands C (saturated PLFA), D (*trans*-monounsaturated PLFA), and E (*cis*-monounsaturated PLFA). The means (± 1 SD) of these measures for the independent replicates of each PLFA isomer fraction are given in Table 3. The specific activities of the *P. atlantica* PLFA isomer fractions are estimated by a ratio of the measured data's arithmetic means (Table 3). A direct comparison of these specific activities requires an estimate of the variability of these averages. As previously discussed, 95% confidence intervals were calculated about the ratio of the geometric means of the measured data to account for the variability of each of these independent measures.

The results shown in Table 3 indicate that the specific activities for the saturated (0.240 nCi/nmol) and *trans*-monounsaturated (0.239 nCi/nmol) PLFA isomers of *P. atlantica* were equivalent when the organism was incubated in the presence of [^{14}C]acetate. The higher specific activity of the *cis*-monounsaturated fraction was not significantly different from the specific activities of the other isomers, as indicated by the wide 95% confidence interval (0.208 to 2.10) which includes the specific activity estimates for the other isomers (Table 3).

Discussion

The saturated, *trans*, and *cis* fatty acids from the phospholipids of the bacterium *P. atlantica* (grown in the presence of [^{14}C]acetate) have been physically separated as the corresponding FAME, the presence of *trans* FAME has been directly verified by FTIR, and the *trans* fraction has been shown to be approximately 80% pure *trans* FAME by GC and FTIR (Table 2). The high variability of the FTIR estimates are probably due to the choice of diffuse reflectance of lipid applied to KBr powder. This infrared procedure is generally done in transmittance or attenuated total reflectance mode (Scholfield 1979). The described procedure was chosen, however, to decrease radioactive contamination of the FTIR and its sampling accessories from the HOT replicates and permit contaminant-free GC analysis of the COLD replicates. The coupling of gas chromatography with FTIR would provide an even better analysis for this type of project, taking advantage of separation power of GC and the unique ability of infrared spectroscopy to directly verify the double bond geometry of FAME. In addition, FTIR is a nondestructive detection technique, allowing further analysis of the separated samples with other detectors in series with the FTIR (Griffiths et al. 1983).

The results indicated in Table 3 confirm that the saturated and *trans*-monounsaturated PLFA of *P. atlantica* are synthesized by similar *in vivo* pathways. The more variable results for the *cis*-monounsaturated fraction may be due to the increased variability owing to incomplete elution from the $AgNO_3$. The *cis* configuration has the strongest interaction with the $AgNO_3$ stationary phase (Scholfield 1979). Significant amounts of [illegible] were counted on the silica shavings of band E (*cis*) after elution of lipid.

A comparison of the saturated and *trans* results, however, are sufficient to verify *in vivo* (and suggest *de novo*) synthesis of *trans* fatty acids from acetate in the membrane phospholipids of *P. atlantica*. This result, in conjunction with previously reported work on the changes of membrane fatty acid's *trans*/*cis* ratio during starvation–suvival of *Vibrio cholerae* (Guckert et al. 1986) indicate that the presence of *trans* fatty acids in bacterial membranes cannot be overlooked. Appropriate analytical separation techniques capable of resolving geometric isomers (e.g., capillary GC) must be used when reporting lipid profiles of microorganisms. Double bond positions can no longer be assumed to be of *cis*-configuration.

Bacteria synthesize *trans* fatty acids as intermediates in the anaerobic desaturase pathway. A branch in this pathway (generally around 10:0) involves the dehydration of the intermediate, D-β-hydroxydecanoyl-acyl carrier protein (Δ2OH-10:0-ACP). This dehydration may result in either a Δ3 *cis* double bond (10:1Δ3*c* = 10:1ω7*c*), which after chain elongation by 2-carbon units can produce the common 16:1ω7*c* and 18:1ω7*c*, or a Δ2 *trans* double bond (10:1Δ2*t* = 10:1ω8*t*), which is reported to be quantitatively reduced to yield the saturated analog (10:0) (Fulco 1983). If chain elongation could occur on the possible *trans* intermediates (8:1ω6*t*, 9:1ω7*t*, 10:1ω8*t*, 11:1ω9*t*, 12:1ω10*t*), the results could be *trans* PLFA. This could not be the mode of *trans* synthesis in *P. atlantica* since the ω7*t* PLFA would have to be odd chained and the ω[illegible] PLFA would have to be even chained. *Pseudomonas atlantica* has even chained ω7*t* and odd chained ω8*t* (Table 1).

Another possibility for *trans* acid biosynthesis is a direct, aerobic *trans* desaturase of a saturated product. A bacterial *trans*-Δ5 desaturase was hypothesized by Gillan et al. (1981) to explain the *trans* isomers found in his sediment isolates. *Pseudomonas atlantica* would require a *trans*-Δ9 desaturase to produce 16:1ω7*t* from 16:0 and 17:1ω8*t* from 17:0. Pseudomonads, however, are characterized by the anaerobic desaturase pathway (Lennarz 1966), and aerobic and anaerobic pathways have, thus far, been found to be mutually exclusive (Fulco 1983). *Pseudomonas atlantica* monounsaturated PLFA are consistent with the anaerobic pathway (Table 1), which makes it unlikely that aerobic *trans*-desaturase is the pathway of *trans* PLFA biosynthesis for *P. atlantica*.

Within the anaerobic desaturase pathway, the hypothesis most consistent with the evidence presented in this study is that an isomerization of the *cis*-monounsaturated end products of the anaerobic desaturase pathway produces the *trans* PLFA (Seltzer 1972). Several specific isomerases may be present in this bacterium, or a general isomerase able to utilize all the ω7*c* and ω8*c* fatty acids as substrates. There doesn't appear to be any isomerization of the ω9*c* position in this organism. The *trans*/*cis* ratios shown in Table 1 suggest that the isomerases for 16:1ω7 and 17:1ω8 have a higher activity than that for 18:1ω7 which is consistently an order of magnitude lower than that of 16:1ω7 or 17:1ω8.

For an organism to posses significant levels of *trans* PLFA[illegible] would follow there should be some benefit in their production. Increasing *trans*/*cis* ratios during long-term starvation of *Vibrio cholerae* has been discussed (Guckert et al. 1986).

TABLE 3. Comparison of specific activities of *P. atlantica* membrane fatty acids separated by $AgNO_3$ TLC

$AgNO_3$ TLC band	PLFA isomer fraction	Measured data		Specific activity estimates		
		^{14}C (nCi)	PLFA (nmol)	Ratio of measured arithmetic means (nCi/nmol)	Ratio of measured geometric means (nCi/nmol)	95% confidence interval about ratio of geometric means (nCi/nmol)
C	Saturated	21.0±4.6	87.6±24.3	0.240	0.243	0.171–0.347
D	*trans*-monounsaturated	5.4±1.3	22.6±10.3	0.239	0.250	0.161–0.389
E	*cis*-monounsaturated	2.3±1.2	6.0±6.9	0.383	0.661	0.208–2.100

During starvation, most of the membrane phospholipids are degraded in *V. cholerae* (Hood et al. 1986), but phospholipid utilization appears to be preferential for the *cis*-monounsaturated PLFA possibly owing to their rapid turnover (White and Tucker 1969) and relative ease of metabolism (Silbert et al. 1968). The importance of an increased proportion of *trans* PLFA during starvation may be related to the cell's inability to metabolize *trans* fatty acids as suggested from auxotroph research (Silbert et al. 1968). If the cell's membrane remained intact, owing to residual *trans* fatty acids acylated to the phospholipids which could resist starvation-induced breakdown, the chances of surviving a starvation event would be much greater. In addition, a higher *trans*/*cis* ratio during starvation survival may be related to the fact that *trans* PLFA will condense a higher temperatures (Esfanhani et al. 1969). This may provide a non sporeformer (like *V. cholerae*) a sporelike nonfluid coating around the cell which may assist starvation survival (Hood et al. 1986).

Pseudomonas atlantica will be a good organism to test some of these hypotheses. This organism is a common member of the natural microfouling community (Corpe 1974). Our current understanding of microbial biofouling and adhesion at hard surfaces is that free cells are in a nutrient-deficient state suspended in a solution of very dilute nutrients. A hard surface in this solution will physically and chemically concentrate these nutrients creating a favorable environment in oligotrophic waters (Kjelleberg et al. 1983; Marshall 1976). *Pseudomonas atlantica* is an organism found at such surfaces early in the microfouling sequence (Corpe 1974). This organism produces a sticky extracellular acidic polysaccharide glycocalyx (Corpe 1970) which it may use to adhere to hard surfaces. The greatest accumulation of these adherent polymers has been shown to occur late in stationary phase when its physiological status shows maximal stress, as measured by the cells' adenylate energy charge (Uhlinger and White 1983). The *trans*/*cis* ratio for this organism also increases as the culture ages (Table 1). *Pseudomonas atlantica* is, therefore, an important organism to test the interrelationships between starvation–survival, adhesion to surfaces in aquatic environments, glycocalyx production, and *trans* PLFA biosynthesis, turnover, and accumulation.

In conclusion, when a combination of $AgNO_3$ TLC, GC, FTIR, and LSC was used to separate FAME isomers, to directly verify the presence of *trans*-PLFA, to quantify the separated FAME isomers, and to measure ^{14}C incorporation into these isomers, the specific activities of *trans* and saturated PLFA were found to be equivalent when *P. atlantica* was incubated with [^{14}C]acetate. This indicates that *P. atlantica* has the *in vivo* biosynthetic capabilities to synthesize *trans* PLFA from acetate. The actual pathway is unknown at this time, but it is suggested that *P. atlantica* will be an organism of choice for the necessary work to elucidate this mechanism.

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BEACHAM, I. R., and SILBERT, D. F. 1973. Studies on the uridine diphosphate-galactose : lipopolysaccharide galactosyltransferase reaction using a fatty acid mutant of *Escherichia coli*. J. Biol. Chem. **248**: 5310–5318.

BOON, J. J., DE LEEUW, J. W., and BURLINGAME, A. C. 1978. Organic geochemistry of Walvis Bay diatomaceous ooze. II. Structural analysis of the monoenoic and polycyclic fatty acids. Geochim. Cosmo. Acta, **42**: 631–644.

CHAPMAN, D. 1965. Infrared spectroscopy of lipids. J. Am. Oil Chem. Soc. **42**:353–371.

CHRISTIE, W. W. 1973. Lipid analysis: isolation, separation, identification and structural analysis of lipids. Pergamon Press, Oxford.

CORPE, W. A. 1970. An acid polysaccharide produced by a primary film-forming marine bacterium. Dev. Ind. Microbiol. **11**: 402–412.

——— 1974. Periphytic marine bacteria and the formation of microbial films on solid surfaces. *In* Effect of the ocean environment on microbial activities. *Edited by* R. R. Colwell and R. Y. Morita. University Park Press, Baltimore. pp. 397–417.

CRANWELL, P. A. 1982. Lipids of aquatic sediments and sedimenting particulates. Prog. Lipid Res. **21**: 271–308.

ESFAHANI, M., BARNES, E. M., JR., and WAKIL, S. J. 1969. Control of fatty acid composition in phospholipids of *Escherichia coli*: response to fatty acid supplements in a fatty acid auxotroph. Proc. Natl. Acad. Sci. U.S.A. **64**: 1057–1064.

FULCO, A. J. 1983. Fatty acid metabolism in bacteria. Prog. Lipid Res. **22**: 133–160.

GILLAN, F. T., and HOGG, R. W. 1984. A method for the estimation of bacterial biomass and community structure in mangrove-associated sediments. J. Microbiol. Methods, **2**: 275–293.

GILLAN, F. T., JOHNS, R. B., VERHEYEN, T. V., VOLKMAN, J. R., and BAVOR, H. J., JR. 1981. *trans*-monounsaturated acids in a marine bacterial isolate. Appl. Environ. Microbiol. **41**: 849–856.

GILLAN, F. T., JOHNS, R. B., VERHEYEN, T. V., NICHOLS, P. D., ESDAILE, R. J., and BAVOR, H. J., JR. 1983. Monounsaturated fatty acids as specific bacterial markers in marine sediments. *In* Advances in organic geochemistry. *Edited by* M. Bjorny. John Wiley & Sons, New York. pp. 198–206.

GRIFFITHS, P. R., DE HASETH, J. A., and AZARRAGA, L. V. 1983. Capillary GC/FT-IR. Anal. Chem. **55**: 1361A–1387A.

GUCKERT, J. B., ANTWORTH, C. P., NICHOLS, P. D., and WHITE, D. C. 1985. Phospholipid, ester-linked fatty acids profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiol. Ecol. **31**: 147–158.

GUCKERT, J. B., HOOD, M. A., and WHITE, D. C. 1986. Phospholipid

ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. Appl. Environ. Microbiol. **52**: 794–801.

HARWOOD, J. C., and RUSSELL, N. J. 1984. Lipids in plants and microbes. George Allen and Unwin, London.

HOOD, M. A., GUCKERT, J. B., WHITE, D. C., and DECK, F. 1986. Effect of nutrient deprivation on lipid, carbohydrate, DNA, RNA, and protein levels in *Vibrio cholerae*. Appl. Environ. Microbiol. **52**: 788–793.

KEMP, P., WHITE, R. W., and LANDER, D. J. 1975. The hydrogenation of unsaturated fatty acids by five bacterial isolates from the sheep rumen, including a new species. J. Gen. Microbiol. **90**: 100–114.

KEPLER, C. R., and TOVE, S. B. 1967. Biohydrogenation of unsaturated fatty acids III. Purification and properties of linoleate Δ^{12} *cis*-Δ^{11} *trans*-isomerase from *Butyrivibrio fibtisolvens*. J. Biol. Chem. **242**: 5686–5692.

KJELLEBERG, S., HUMPHREY, B. A., and MARSHALL, K. C. 1983. Initial phases of starvation and activity of bacteria at surfaces. Appl. Environ. Microbiol. **46**: 978–984.

LENNARZ, W. J. 1966. Lipid metabolism in the bacteria. Adv. Lipid Res. **4**: 175–225.

MAKULA, R. A. 1978. Phospholipid composition of methane-utilizing bacteria. J. Bacteriol. **134**: 771–777.

MANCUSO, C. A., NICHOLS, P. D., and WHITE, D. C. 1986. A method for the separation and characterization of archaebacterial signature ether lipids. J. Lipid Res. **27**: 49–56.

MARR, A. G., and INGRAHAM, J. L. 1962. Effect of temperature on the composition of fatty acids in *Escherichia coli*. J. Bacteriol. **84**: 1260–1267.

MARSHALL, K. C. 1976. Interfaces in microbial ecology. Harvard University Press, Cambridge.

MAVIS, R. D., and VAGELOS, P. R. 1972. The effect of phospholipid fatty acid composition on membranous enzymes in *Escherichia coli*. J. Biol. Chem. **247**: 652–659.

NICHOLS, P. D., HENSON, J. M., GUCKERT, J. B., NIVENS, D. E., and WHITE, D. C. 1985. Fourier transform-infrared spectroscopic methods for microbial ecology: analysis of bacteria, bacteria–polymer mixtures and biofilms. J. Microbiol. Methods, **4**: 79–94.

NICHOLS, P. D., GUCKERT, J. B., and WHITE, D. C. 1986*a*. Determination of monounsaturated fatty acid double bond position and geometry for microbial monocultures and complex consortia by capillary GC–MS of their dimethyl disulphide adducts. J. Microbiol. Methods, **5**: 49–55.

NICHOLS, P. D., SMITH, G. A., ANTWORTH, C. P., HANSON, R. S., and WHITE, D. C. 1986*b*. Phospholipid and lipopolysaccharide normal and hydroxy fatty acids as potential signatures for methane-oxidizing bacteria. FEMS Microbiol. Ecol. **31**: 327–335.

PERRY, G. J., VOLKMAN, J. K., JOHNS, R. B., and BAVOR, H. J., JR. 1979. Fatty acids of bacterial origin in contemporary marine sediments. Geochim. Cosmo. Acta, **43**: 1715–1725.

SCHAIRER, H. U., and OVERATH, P. 1969. Lipids containing *trans*-unsaturated fatty acids change the temperature characteristic of thiomethylgalactoside accumulation in *Escherichia coli*. J. Mol. Biol. **44**: 209–214.

SCHOLFIELD, C. R. 1979. Analysis and physical properties of isomeric fatty acids. *In* Geometrical and positional fatty acid isomers. *Editea by* E. A. Emken and H. J. Dutton. American Oil Chemists' Society, Champaign, IL. pp. 17–52.

SELTZER, S. 1972. *Cis-trans* isomerization. *In* The enzymes. Vol. 6. 3rd ed. *Edited by* P. D. Boyer. Academic Press, New York. pp. 381–406.

SILBERT, D. E., RUCH, F., and VAGELOS, P. R. 1968. Fatty acid replacements in a fatty acid auxotroph of *Escherichia coli*. J. Bacteriol. **95**: 1658–1665.

SILBERT, D. E., ULBRIGHT, T. M., and HONEGGER, J.L. 1973. Utilization of exogenous fatty acids for complex lipid biosynthesis and its effect on *de novo* fatty acid formation in *Escherichia coli* K-12. Biochemistry, **12**: 164–171.

SOKAL, R. R., and ROHLF, F. J. 1981. Biometry. 2nd ed. W. H. Freeman, Co., San Francisco.

SOMMERFELD, M. 1983. *trans*-unsaturated fatty acids in natural products and processed foods. Prog. Lipid Res. **22**: 221–233.

UHLINGER, D. J., and WHITE, D. C. 1983. Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in *Pseudomonas atlantica*. Appl. Environ. Microbiol. **45**: 64–70.

VAN VLEET, E. S., and QUINN, J. G. 1976. Characterization of monounsaturated fatty acids from an estuarine sediment. Nature (London), **262**: 126–128.

——— 1979. Early diagenesis of fatty acids and isoprenoid alcohols in estuarine and coastal sediments. Geochim. Cosmochim. Acta, **43**: 289–303.

VERHULST, A., PARMENTIER, G., JANSSEN, G., ASSELBERGHS, S., and EYSSEN, H. 1986. Biotransformation of unsaturated long-chain fatty acids by *Eubacterium lentum*. Appl. Environ. Microbiol. **51**: 532–538.

VOLKMAN, J. K., and JOHNS, R. B. 1977. The geochemical significance of positional isomers of unsaturated acids from an intertidal zone sediment. Nature (London), **267**: 693–694.

VOLKMAN, J. K., JOHNS, R. B., GILLAN, F. T., and PERRY, G. J. 1980. Microbial lipids of an intertidal sediment I. Fatty acids and hydrocarbons. Geochim. Cosmochim. Acta, **44**: 1133–1143.

WHITE, D. C., and TUCKER, A. N. 1969. Phospholipid metabolism during bacterial growth. J. Lipid Res. **10**: 220–233.

WHITE, D. C., DAVIS, W. M., NICKELS, J. S., KING, J. D., and BOBBIE, R. J. 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia, **40**: 51–62.