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Formation of *trans* Fatty Acids Is Not Involved in Growth-Linked Membrane Adaptation of *Pseudomonas putida*

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Fatty acid compositions in growing and resting cells of several strains of *Pseudomonas putida* (P8, NCTC 10936, and KT 2440) were studied, with a focus on alterations of the saturation degree, *cis-trans* isomerization, and cyclopropane formation. The fatty acid compositions of the strains were very similar under comparable growth conditions, but surprisingly, and contrary to earlier reports, *trans* fatty acids were not found in either exponentially growing cells or stationary-phase cells. During the transition from growth to the starvation state, cyclopropane fatty acids were preferentially formed, an increase in the saturation degree of fatty acids was observed, and larger amounts of hydroxy fatty acids were detected. A lowered saturation degree and concomitant higher membrane fluidity seemed to be optimal for substrate uptake and growth. The incubation of cells under nongrowth conditions rapidly led to the formation of *trans* fatty acids. We show that harvesting and sample preparation for analysis could provoke the enzyme-catalyzed formation of *trans* fatty acids. Freeze-thawing of resting cells and increased temperatures accelerated the formation of *trans* fatty acids. We demonstrate that *cis-trans* isomerization only occurred in cells that were subjected to an abrupt disturbance without having the possibility of adapting to the changed conditions by the de novo synthesis of fatty acids. The *cis-trans* isomerization reaction was in competition with the *cis*-to-cyclopropane fatty acid conversion. The potential for the formation of *trans* fatty acids depended on the cyclopropane content that was already present.

The bacterial cytoplasmic membrane is an important target and/or receptor for stress factors. As a response to permanent physical and chemical changes in the cell environment, several protective mechanisms and metabolic adaptation reactions have evolved (10, 52, 56). At the level of membrane lipids and fatty acids, these processes are often referred to as homeoviscous adaptation (57). Cells control the fluidity of their membranes by altering the lipid composition to compensate for changes in fluidity induced by certain environmental factors, such as temperature or the presence of toxic, membrane-active compounds. In most cases, however, microorganisms are not able to compensate for externally induced fluidity changes with 100% efficacy (7, 37, 38). They can tolerate and maybe even need a wider range of different lipid compositions to establish homeostasis, especially during growth. Lipids can coexist in physically separated microdomains with more or less fluid- or gel-phase behavior, and membrane functions are locally influenced by many factors other than fluidity (20, 43). These are the reasons for attempts to enlarge the term homeoviscous adaptation (to maintain membrane fluidity) by use of the term homeophasic adaptation (to adjust membrane fluidity).

At the level of lipid membrane composition, the predominant response of many bacteria to environmental perturbations is the alteration of lipid acyl chain structures by changing the ratio of saturated to unsaturated fatty acids during growth (8, 15, 19, 26, 42, 53). Several other mechanisms have been found in some bacteria to adjust fluidity, such as shortening or growth-dependent elongation of fatty acid chain lengths (6, 8, 14, 53), growth-dependent changes in the ratio of terminally branched *iso* and *anteiso* fatty acids (28, 30), growth-dependent methylation of *cis* unsaturated fatty acids to methyl branched (31) or cyclopropane saturated ones (3, 29, 33, 42), and isomerization of *cis* to *trans* double bonds (11, 16, 18, 22, 25, 40, 47).

Pseudomonas putida is a ubiquitous gram-negative bacterium and a potent pollutant degrader. Strains of this species are of ecophysiological interest because most of them are able to use at least the following three adaptation mechanisms at the level of lipids to respond to physical and chemical stresses: (i) changes in the overall degree of saturation of fatty acids (22, 35, 37), (ii) the formation of cyclopropane fatty acids (35), and (iii) *cis-trans* isomerization with a double bond configuration (11, 22, 35, 36, 37, 46, 59). The kind and degree of the response determine the stability and resilience of the cells and decide their fate. Commonly, the different adaptation mechanisms of *P. putida* have been investigated independently. The purpose of this study was the integral examination of these three adaptation responses of *P. putida* and their dependence on different growth phases. To gain insights into strain-dependent differences and to verify the measured effects, we chose three well-known strains of this species for use in this study.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Three strains of *P. putida* were used for this study. *P. putida* P8 was kindly supplied by H. J. Heipieper (UFZ Centre for Environmental Research Leipzig-Halle, Leipzig, Germany), and *P. putida* NCTC 10936 and KT 2440 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). All three strains were maintained on Trypticase soy agar slants at 4°C and cultivated at 30°C. Shaking flasks containing 150 ml of growth medium were inoculated with cells from an overnight preculture (10% [vol/vol]). Standard mineral medium, designed for the development of 2 g of biomass/liter, was composed of the following reagents (in milligrams per liter): NH_4Cl, 761; KH_2PO_4, 681; K_2HPO_4,

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871; $CaCl \cdot 6H_2O$, 5.5; $MgSO_4 \cdot 7H_2O$, 71.2; $ZnSO_4 \cdot 7H_2O$, 0.4; $MnSO_4 \cdot 4H_2O$, 0.8; $CuSO_4 \cdot 5H_2O$, 0.8; $Na_2MoO_4 \cdot 2H_2O$, 0.3; and $FeSO_4 \cdot 7H_2O$, 5.0. Bacteria were grown in batches on sodium succinate (2 g/liter) as the sole carbon and energy source. For the study of growth-phase-dependent changes in the fatty acid composition, samples were harvested between the early and late exponential growth phases (3 to 8 h), during a transient phase of deprivation (24 h), and during stationary phase (48 h), as monitored by measuring the optical densities of the cultures at 700 nm (data not shown).

Sample preparation. During different growth phases, the cells were rapidly harvested without temperature changes by centrifugation at 4,000 × *g* for 5 min, washed in imidazole buffer (2 mM, pH 7), and recollected. This procedure was adhered to as far as possible with respect to the short centrifugation time and the constancy of temperature. From the data presented here, it seems that a brief spin at 4°C would be acceptable because it would minimize preparation artifacts. The pellets from different batches were processed in parallel in three different ways: (i) pellets were directly saponified to liberate the fatty acids without any changes in their physiological status quo, (ii) the pellets were incubated for 1 to 3 h at 30°C and analyzed immediately after incubation, and (iii) the biomass was put in a deep freezer at −20°C, stored for several days at this temperature, thawed for 1 or 15 min at 30°C, and then analyzed either immediately or after 1, 3, or 5 h of incubation in a shaking water bath at 30°C. To explore the effect of freezing and thawing, we modified the third procedure further. In some experiments, the cells were frozen twice, and in other experiments, the thawing temperature during the 2-h incubation period was set to 10, 20, 30, or 40°C prior to fatty acid analysis. Typically, two or more independent batches were pooled to collect samples for the analysis. Each experiment was performed at least twice, sometimes after a variation of the cultivation times to confirm observed trends of earlier results.

Fatty acid determination. Fatty acids were determined as methyl esters from whole-cell hydrolysates according to the procedure of the SHERLOCK microbial identification system (MIDI Inc., Newark, Del.). Samples of about 5 to 10 mg of cell dry mass were subjected to derivatization in Teflon screw-cap test tubes. For the preparation of esters, cells were saponified at 100°C for 30 min with 1 ml of reagent I (45 g of sodium hydroxide, 150 ml of methanol, 150 ml of water), methylated at 80°C for 10 min with reagent II (325 ml of 6 M hydrochloric acid, 275 ml of methanol), extracted with 1.25 ml of reagent III (200 ml of *n*-hexane, 200 ml of methyl *tert*-butyl ether), and base washed with 3 ml of reagent IV (10.8 g of sodium hydroxide, 40 g of sodium chloride, 900 ml of water). Methylation following saponification was preferred for total fatty acid analysis because transesterification procedures are suspected to cause underestimations of cyclopropane acids and are less effective at releasing cellular hydroxyl fatty acids (45). The separation and determination of the fatty acid methyl esters were performed with a 6890 GC gas chromatography system (Agilent, Palo Alto, Calif.) equipped with a flame ionization detector (GC-FID). An automatic sample inlet was used (split injection, split ratio of 100:1, 250°C), and the separation was carried out in an Ultra2 nonpolar capillary column (length, 25 m; inside diameter, 0.20 mm; film thickness, 0.33 μm; Agilent), with hydrogen as the carrier gas (9 lb of constant pressure/in^2). The temperature program was as follows: 170 to 260°C at 5 K min^{-1}, 260 to 310°C at 40 K min^{-1}, and 310°C for 1.5 min. Data acquisition and data analysis were controlled by ChemStation software (version 10.01; Agilent) and the Sherlock software package (version 4.5; MIDI). Fatty acid assignments were checked by comparisons of the retention times with those of known standards measured on columns with different polarities and were analyzed further after common derivatization procedures, including hydrogenation and silylation. The position of the double bond was determined by derivatization with pyrrolidine followed by an analysis of electron impact fragmentation by GC-mass spectrometry (GC-MS) (1). The *cis-trans* geometry of the double bonds was confirmed by comparisons of chromatographic data (retention times) and mass spectrometric data (double bond positions). The fatty acid composition was calculated from fractions of individual peaks of the total peak area. Replicate determinations indicated reproducible fatty acid profiles with relative errors of 2 to 5%, calculated as standard errors of the means (SEM).

The following fatty acid nomenclature was applied: the number preceding the colon indicates the total number of carbon atoms, the number following the colon indicates the number of double bonds, and the suffix designates the position of the double bonds (from the methyl end of the molecule, *cis* or *trans* indicates their configuration) or the position of hydroxyl groups (from the carboxyl end of the molecule). It was helpful for the sake of discussion to combine single fatty acids into groups with common structural characteristics. Such groups of interest are straight-chain saturated fatty acids (*n*:0), straight-chain unsaturated fatty acids with a *cis* (*n*:1 *cis)* or *trans* (*n*:1 *trans*) configuration, cyclopropane fatty acids (*n*:0 cyclo), and ratios of these groups. The degree of saturation, *S*, was defined as the ratio of straight-chain saturated fatty acids to the total amount of fatty acids. The average carbon chain length of fatty acids, ACCL, was expressed as $\Sigma\ (FA \times C)/100$, where *FA* is the percentage of each fatty acid and *C* is the number of carbon atoms in the straight chain.

TABLE 1. Total fatty acid compositions (wt/wt) of *P. putida* strains grown on 0.2% succinate at 30°C

Fatty acid(s)	% of total[a]					
	P8		NCTC 10936		KT 2440	
	6 h	48 h	6 h	48 h	6 h	48 h
10:0	1.3	0[b]	0.5	0.2	0.1	0.2
10:0 3OH	8.3	3.3	6.7	3.9	5.5	2.5
12:0	3.8	4.0	7.4	9.4	6.5	10.1
12:0 2OH	4.8	4.7	3.4	2.5	3.0	1.4
12:0 3OH	4.9	4.2	4.6	4.5	4.1	2.3
14:0	0.3	0.7	0.3	0.7	0.2	0.7
16:1ω7c	28.8	5.1	30.9	14.0	28.5	4.1
16:1ω7t	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]
16:0	27.3	36.1	25.4	28.7	26.8	31.4
17:0 cyclo	4.5	29.2	2.5	17.0	4.3	25.4
18:1ω7c	13.5	11.5	17.5	17.0	19.8	16.8
18:1ω7t	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]
18:0	0.6	0.6	0.8	1.0	0.7	0.8
19:0 cyclo	0[b]	0.4	0[b]	0.8	0.4	4.0
Total	98.1	99.8	100.0	99.7	99.9	99.7
Sum *n*:0[c]	33.3	41.4	34.4	40.0	34.3	43.2
Sum *n*:0 OH	18.0	12.2	14.7	10.9	12.6	6.2
Sum *n*:1 *cis*	42.3	16.6	48.8	31.0	48.3	20.9
Sum *n*:0 cyclo	4.5	29.6	2.5	17.8	4.7	29.4
Sum *n*:1 *trans*	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]
ACCL	15.1	15.5	15.3	15.5	15.5	15.7
S	52	54	49	51	47	50
trans/*cis* ratio	0[b]	0[b]	0[b]	0[b]	0[b]	0[b]
cyclo/*cis* ratio	0.11	1.79	0.05	0.57	0.10	1.40

[a] Samples were taken after 6 h (exponential growth phase) and 48 h (stationary growth phase) and were analyzed immediately. Comparable results were obtained in separate analyses on three occasions (typically with <5% SEM).
[b] Below limits of detection.
[c] *n* represents each carbon atom that was found.

RESULTS AND DISCUSSION

Fatty acid compositions of individual strains are very similar under comparable growth conditions. The fatty acid compositions of *P. putida* P8, NCTC 10936, and KT 2440 grown in batch cultures at 30°C on succinate as the sole source of carbon and energy were determined. Table 1 shows data obtained with exponentially grown cells (harvested after 6 h of cultivation) and cells from stationary phase (48 h). When the organisms were harvested during the same growth phase, their basic fatty acid profiles were qualitatively and quantitatively very similar and comprised saturated, *cis* (mono)unsaturated, hydroxyl, and cyclopropane fatty acids, mainly in the range of 10- to 18-carbon-atom chain lengths. Differences were found between the growth phases. At 6 and 48 h, saturated straight-chain fatty acids were in the ranges of 33 to 34% and 40 to 43%, respectively, whereas fatty acids originating from *cis* unsaturated ones (sum of *cis*, cyclo, and *trans* fatty acids) were in the ranges of 47 to 53% and 46 to 50%, respectively. The observed fatty acid patterns are typical for many gram-negative bacteria (34). Palmitic acid (16:0), palmitoleic acid (16:1ω7c), and vaccenic acid (18:1ω7c) were always the dominant fatty

TABLE 2. Percentage of *trans* fatty acids in *P. putida* P8 grown on complex medium or minimal medium

Growth conditions	% *trans* fatty acids of total fatty acids[a]		
	A	B	C
Growth on Trypticase soy broth (10 g/liter) for 4 h (exponential growth) at 30°C; sample processing as described in Materials and Methods		0	15
Growth on minimal medium with 4 g of sodium succinate/liter for 8 h (late exponential phase) at 30°C; sample processing as described by Heipieper et al. (22)	12	0	9
Growth on minimal medium with 0.2% succinate for 4 h (exponential phase) at 30°C; sample processing as described by Holtwick et al. (24)	6	0	12

[a] A, values cited from the literature; B, samples analyzed immediately; C, samples analyzed after freeze-thawing and a 3-h incubation in buffer at 30°C.

acid components of the cells. The occurrence of vaccenic acid indicated the presence of the anaerobic pathway of fatty acid biosynthesis and the absence of a fatty acid synthesis-independent desaturase system found in organisms possessing the aerobic pathway (e.g., *Acinetobacter calcoaceticus* [19]). Indeed, *P. putida* is known to be incapable of changing the saturation degree of existing fatty acids and rather relies on de novo biosynthesis of fatty acids (11).

No *trans* fatty acids are formed in exponentially growing cells or stationary-phase cells. *P. putida* is known for possessing *trans* unsaturated fatty acids in its outer membrane. Surprisingly, and in contrast to earlier reports with the same strain, *P. putida* P8 (11, 35), and related strains of *P. putida* (46, 51), *trans* unsaturated fatty acids were not found in this study, regardless of the time of sampling. As shown in Table 1, *trans* fatty acids were not formed during exponential growth or stationary phase by any of the strains, provided that the cells were analyzed immediately after sampling. This was all the more surprising since *trans* acids were also absent from cells of *P. putida* P8, which were grown in the presence of the inhibitor phenol at a concentration leading to 50% growth inhibition on succinate (data not shown). Only in long-term starved cells were small amounts of *trans* fatty acids detectable, as seen in an 11-day sample of *P. putida* P8 (2% 16:1ω7t). This phenomenon, already observed in cells of *Vibrio cholerae* that were starved for 30 days (25), will not be discussed here further because these *trans* fatty acids appeared in cells with a very undefined physiological state.

To exclude the possibility that the composition of the growth medium prevented the formation of *trans* fatty acids during growth, we alternatively cultivated strain P8 in a complex nutrient medium. The results were the same as those for minimal medium (Table 2). Moreover, growth experiments with *P. putida* P8 were repeated for media described by others (22, 24) that contained succinate as the sole carbon and energy source. In the past, such media had been chosen to investigate the impact of organic solvents on *cis-trans* isomerization. We performed our experiments under exactly the same growth conditions used by these laboratories, including the centrifugation, lipid extraction, and transesterification steps. In contrast to previous findings, we never found *trans* fatty acids in cells which had not been incubated with the toxicant if they were analyzed immediately (Table 2).

Cyclopropane fatty acids are predominantly formed during the transition from the exponential to the stationary growth phase. Cyclopropane fatty acids are typical for many gram-negative bacteria using the anaerobic pathway of fatty acid biosynthesis. Usually, only minor amounts of cyclopropane fatty acids are observed during exponential growth, and increasing amounts are detected during the transition to a starvation state, when growth ceases (3, 29, 33, 42). We were able to confirm these findings. As shown in Table 1 and in contrast

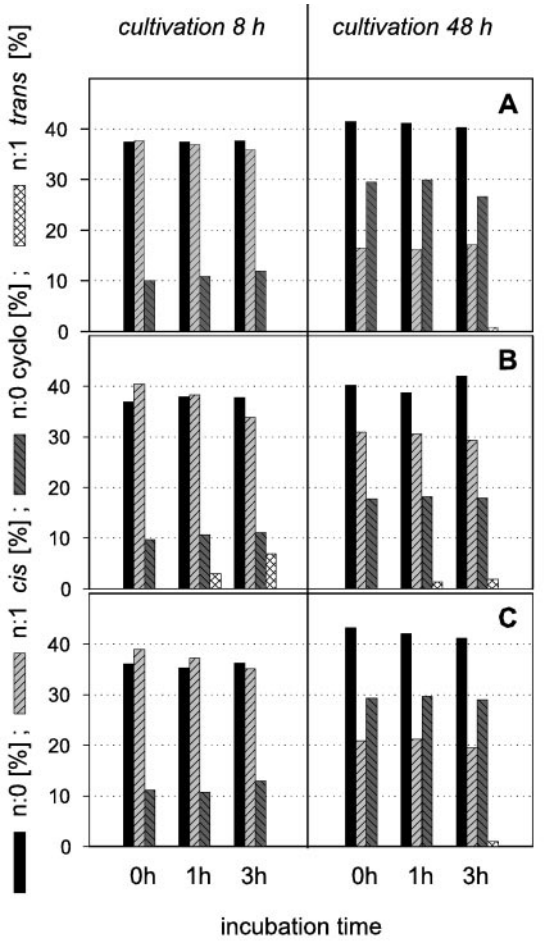

FIG. 1. Fatty acid compositions (wt/wt) of *P. putida* strains (P8 [A], NCTC 10936 [B], and KT 2440 [C]) grown on 0.2% succinate at 30°C. Groups of straight-chain saturated, cyclopropane, *cis*, and *trans* fatty acids are shown. Samples were taken after 8 h (exponential growth phase) and 48 h (stationary growth phase) and processed in different ways. One sample (control, 0 h) was analyzed immediately, and the other samples were incubated for 1 or 3 h in a nutrient-free buffer at 30°C before analysis. Representative results are shown. At least two independent experiments were performed (SEM, <5%).

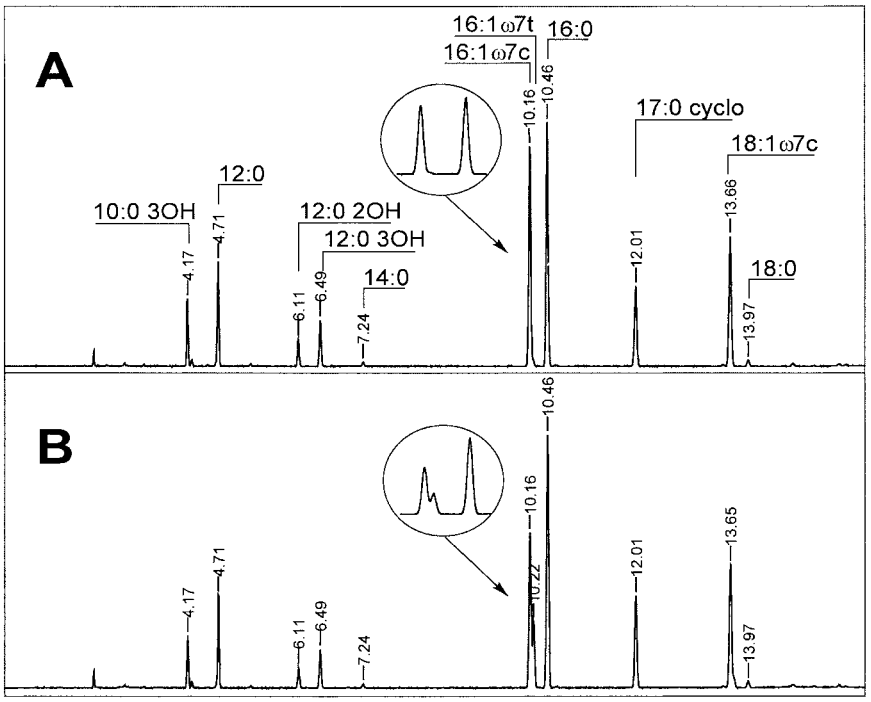

FIG. 2. GC-FID separation of fatty acid methyl esters of *P. putida* NCTC 10936 in a nonpolar column (HP Ultra2). Cells were grown on 0.2% succinate for 8 h. (A) The sample was analyzed immediately; (B) the sample was resuspended in imidazole buffer at 30°C for 3 h before analysis. Peaks representing 16:1 and 16:0 are enlarged.

to our findings for *trans* fatty acids, cyclopropane fatty acids were always present, although only in small amounts during the exponential growth phase. In stationary-phase cells, the amount of cyclopropane acids was up to 12 times higher than that during exponential growth. The cyclo/*cis* ratio was lower in *P. putida* NCTC 10936 (0.05 and 0.57 for 6 and 48 h, respectively) than in both *P. putida* KT 2440 (0.10 and 1.40 for 6 and 48 h, respectively) and *P. putida* P8 (0.11 and 1.79 for 6 and 48 h, respectively). Note that the increase in *n*:0 cyclo occurred at the expense of *n*:1 *cis*. It is known that the energy- and *S*-adenosyl-L-methionine-dependent cyclopropane synthase acts on phospholipid-esterified *cis* unsaturated fatty acids (32, 61). Although the molecular mechanisms controlling the formation of cyclopropane fatty acids as a function of the growth phase have been well established (5, 13, 58), the role of these fatty acids is not fully understood yet. Obviously, cyclopropane fatty acids stabilize lipids against turnover and degradation, so the energetic investment might be worthwhile because it minimizes membrane alterations that may lead to life-threatening changes in membrane fluidity during starvation (17, 33). There are reports that the presence of cyclopropane fatty acids seems to improve survival rates of acid-stressed cells (4), and it was established that especially large amounts of cyclopropane acids accumulated when starvation took place at high temperatures (9). Moreover, in comparison to *cis* unsaturated fatty acids, the presence of cyclopropane fatty acids should make the membrane somewhat more rigid because of their higher lipid melting points. This effect is small but significant (39) and may also contribute to stabilization of the lipid bilayer during the subsequent transition to starvation without much affecting the cells' ability to resume growth. The formation of cyclopropane fatty acids at the expense of *cis* unsaturated fatty acids toward the end of growth is favored and appears to represent an adaptation to starvation conditions.

Fatty acid chain length and hydroxy fatty acid content are only marginally affected by the growth phase. As shown in Table 1, the calculated ACCLs were very similar for all three strains. ACCLs ranged from 15.1 to 15.5 and 15.5 to 15.7 for 6 and 48 h of growth, respectively. The increasing chain lengths indicate a somewhat higher rigidity of membranes towards the end of exponential growth. However, the increase was only marginal compared with values found for *Micrococcus cryophilus* (53) and *Pseudomonas oleovorans* (6) and does not justify discussion in terms of adaptive strategies in *P. putida*. The proportion of hydroxy fatty acids was slightly decreased towards the beginning of stationary phase. The sum of hydroxy fatty acids ranged between 13 and 18% and 6 and 12% after 6 and 48 h of growth, respectively. The purpose of this decrease is unclear.

The saturation degree increases during transition from exponential to stationary growth phase. Growth-linked changes of the saturation degree (*S*) are the most frequently observed membrane adaptation to altering environmental conditions. The calculated saturation degrees were similar for all three strains provided that growth occurred under the same cultivation conditions (Table 1). For 6 and 48 h of growth, *S* ranged from 47 to 52% and 50 to 54%, respectively. This increase with prolonged cultivation and depletion of the growth substrate confirms earlier data obtained with *P. putida* P8 (35) and resembles data obtained with chemostat cultures of *Escherichia coli* at different dilution rates (2, 55). Increased contents of saturated fatty acids enhancing the rigidity of the cytoplasmic

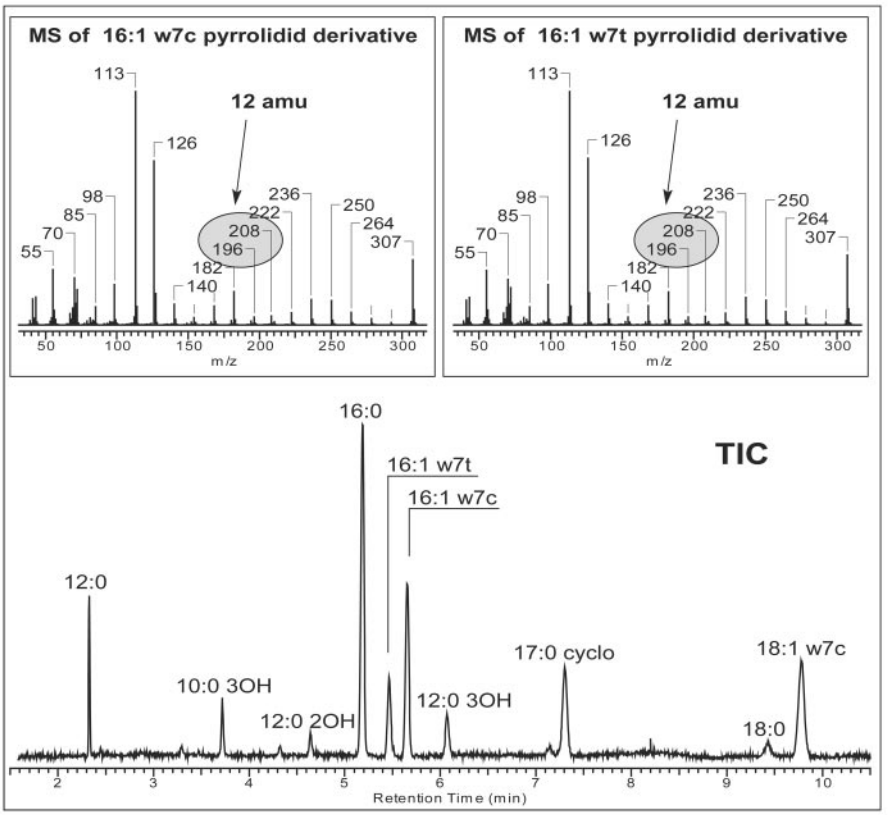

FIG. 3. GC-MS-total ion chromatogram (TIC) of fatty acid methyl esters of *P. putida* NCTC 10936 in a polar column (DB-23). The sample used was the same as that described for Fig. 2B. Mass spectra of the peaks representing 16:1ω7c and 16:1ω7t after derivatization of the fatty acid methyl esters to their corresponding pyrrolidide compounds are shown above the total ion chromatogram.

membrane are a well-known mechanism of adaptation to strong membrane-fluidizing factors such as a rise in temperature, growth on or in the presence of lipophilic organic solvents, etc. (26, 54). As shown here, an increase in saturation can also be the result of a transition to the starvation state. The variability of *S* along the growth curve was closely linked to the changing substrate supply, indicating that adaptive responses adjust rather than maintain membrane fluidity. Less saturation leading to higher membrane fluidity seemed to be optimal for growth. This may be explained by the role of membrane fluidity in the activity of membrane proteins involved in substrate uptake (8).

Reincubation of cells under nongrowth conditions leads to sudden formation of *trans* fatty acids. The extent to which the capacity of an organism to adapt is realized depends on its physiological state. We were interested in determining the possible changes in fatty acid composition in cells of *P. putida* under nongrowth conditions. To gain insights into the behavior of resting cells, we investigated variations in the fatty acid patterns of the strains after different treatments. The washed biomass was incubated in buffer (without growth substrates) for 1 to 3 h at 30°C before fatty acid analysis. Figure 1 shows the development of selected groups of fatty acids. Various combinations of cultivation times and incubation times as resting cells were studied. Whereas *P. putida* P8 and KT 2440 (8-h values) showed only marginal changes upon incubation as resting cells, a small percentage of palmitelaidic acid (16:1ω7t) appeared in resting cells of *P. putida* NCTC 10936 (grown for 8 h). The longer the incubation in buffer solution was, the more *trans* fatty acids formed at the expense of *cis* acids were found, whereas no further formation of cyclopropane acids was observed (Fig. 1). A similar behavior, but delayed and occurring to a somewhat lesser extent, was found for cells that had been harvested from stationary phase. This was consistent with a higher content of cyclopropane acids in stationary-phase cells, as it may be explained by the smaller amounts of *cis* acids available for *cis-trans* isomerization and the fact that the cells were already sufficiently adapted (via *cis*-cyclo conversion) at the time of incubation in buffer.

For verification of the analytical data, two original gas chromatograms of fatty acid methyl esters from exponentially growing cells of *P. putida* NCTC 10936 are shown as examples (Fig. 2). Palmitelaidic acid was only detectable in the sample representing resting cells (Fig. 2B). To prove that the peak at 10.22 min was really the *trans* compound and not an artifact or a *cis* fatty acid with another double bond position, we confirmed our findings by GC-MS, using a column with more polarity. For this experiment, the sample giving the chromatogram shown in Fig. 2B was analyzed in a polar column (DB 23 instead of HP Ultra2). Figure 3 shows the total ion chromatogram of the mass spectra. The elution order of fatty acid methyl esters changed as expected, and the peak interpreted as 16:1ω7t was found again and was baseline separated from 16:1ω7c. We verified the identities of both peaks by derivatization with

pyrrolidine (1). Figure 3 shows mass spectra typical for 16:1 pyrrolidide compounds. They are identical, thus proving the coexistence of two chromatographically separated 16:1 compounds with double bond ω7, as seen from the unique 12-atomic-mass-unit difference between *m*/*z* 196 and 208. They thus undoubtedly represent the *cis* and *trans* isomers. Moreover, in the past, the unambiguous occurrence of these *trans* fatty acids (16:1ω7t and, in minor amounts, 18:1ω7t) was proven in strain P8 by infrared spectrometry (22).

The surprising results that no *trans* fatty acids were formed during exponential and stationary growth but suddenly occurred during incubation in buffer may serve as a plausible explanation for the large amounts of *trans* acids previously found in *V. cholerae* (17) and in a marine bacterial isolate (41). Both organisms have in common the ability to form *trans* fatty acids, but possibly the *trans* acid contents measured were not characteristic for short-term-starved cells. For these starvation studies, the organisms were harvested and reincubated with an energy- and nutrient-free medium. Therefore, possibly the reincubation and not the short-term starvation was the cause for the large observed amounts of *trans* fatty acids.

Freeze-thawing of resting cells and increasing temperatures accelerate the formation of *trans* fatty acids. Figure 4 shows that the formation of *trans* fatty acids was promoted by a freeze-thaw procedure prior to fatty acid analysis. A combination of freeze-thawing and prolonged incubation of resting cells gave rise to the largest quantities of *trans* fatty acids. This observation is important because in analysis protocols from many laboratories freezing is a preferred technique for storing samples. Based on our results, this may be a source of erroneous data with regard to *trans* fatty acids. The occurrence of a small amount of *trans* fatty acids in some 0-h samples (Fig. 4) appears to be caused by the treatment prior to incubation (concentration of the biomass by centrifugation, freezing, and thawing without temperature control). To understand the influence of freeze-thawing on the formation of *trans* acids in more detail, we studied the effects of the temperature changes during these processes. Figure 5 shows two results obtained with *P. putida* KT2440. Sample freezing and thawing, even when repeated, did not lead to the formation of *n*:1 *trans* fatty acids provided that there was no increase in the temperature beyond the original growth temperature. When samples were incubated for 2 h after thawing at different temperatures, *cis-trans* isomerization was seen at the higher temperatures tested (30 and 40°C). At incubation temperatures that were lower than the cultivation temperature, no *trans* acids appeared (20 or 10°C). The strong temperature influence on the *trans* fatty acid formation is in accordance with earlier results (35). To verify our findings of the strong impact of sample treatment, we repeated some experiments that were done by other authors (22, 24). While no *trans* fatty acids could be found in samples cultivated according to the authors' specifications and analyzed immediately, the formation of *trans* fatty acids may be provoked by changing the sample treatment (Table 2).

Conclusions. Our results show for the first time that under conditions of undisturbed growth, no *trans* fatty acids are found in lipids of *P. putida*, regardless of the growth phase. However, upon a change in the environment of cells affecting membrane fluidity that was too abrupt to be compensated for by altered fatty acid synthesis, *trans* fatty acids were formed rapidly (21, 36, 59). The highest rate of *trans* fatty acid formation was found for cells that were harvested from the exponential growth phase and subsequently incubated for several hours in buffer at an elevated temperature. As expected, the contents of cyclopropane and saturated fatty acids remained largely constant during this treatment. The sudden appearance of *trans* fatty acids suggests that the cytoplasmic membrane was more fluid at the time of disturbance than was appropriate for functioning under the new conditions. Therefore, the formation of *trans* unsaturated fatty acids can be considered an adaptation response to increase the membrane rigidity, i.e., an

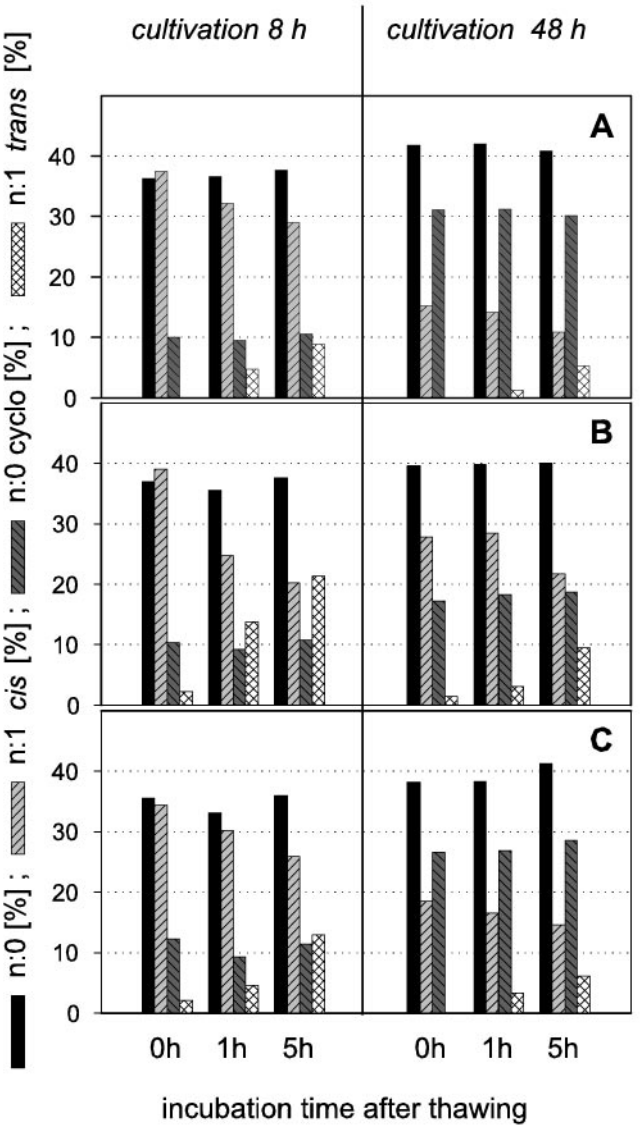

FIG. 4. Fatty acid compositions (wt/wt) of *P. putida* strains (P8 [A], NCTC 10936 [B], and KT 2440 [C]) grown on 0.2% succinate at 30°C. Groups of straight-chain saturated, cyclopropane, *cis*, and *trans* fatty acids are shown. Samples were harvested after 8 h (exponential growth phase) or 48 h (stationary growth phase), washed rapidly, and then frozen (−20°C). After thawing (30°C, 15 min), the samples were analyzed immediately (control, 0 h) or after an incubation time of 1 or 5 h in a nutrient-free buffer at 30°C. Representative results are shown. At least two independent experiments were performed (SEM, <5%).

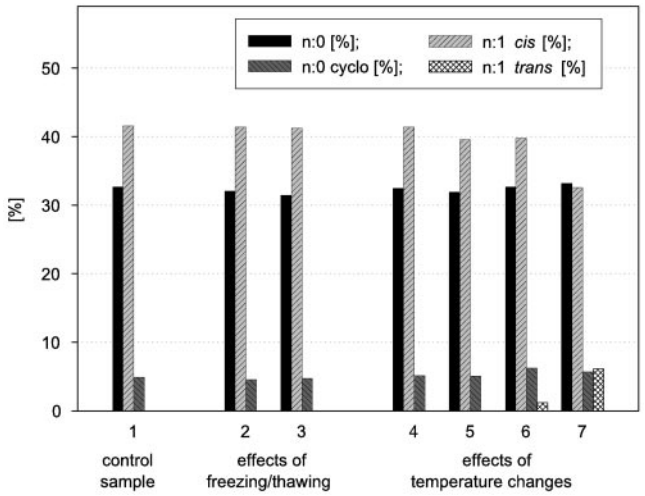

FIG. 5. Development of straight-chain saturated, cyclopropane, *cis*, and *trans* fatty acids of *P. putida* KT 2440 grown for 4 h on 0.2% succinate at 30°C. Samples were harvested, washed rapidly, and analyzed immediately after the following alternate treatments: 1, control sample without further treatment; 2, one freeze (−20°C)-thaw (30°C, 1 min) cycle; 3, two freeze-thaw cycles without a longer dwell time; 4 to 7, a freeze (−20°C)-thaw (10°C) cycle followed by a 2-h incubation at 10, 20, 30, and 40°C, respectively. At least two independent experiments were performed (SEM, <5%).

emergency reaction rather than a way to fine-tune (48) the membrane fluidity during growth and multiplication (23).

The formation of *trans* fatty acids is catalyzed by a nonreversible *cis-trans* isomerase without a shift in the double bond position and without a requirement of a cofactor or energy (12, 44, 50). The enzyme has been purified (49, 50), and the encoding gene was cloned and sequenced (24, 27). It has been verified that the isomerization reaction is possible without growth (22, 36). The fact that *trans* fatty acids are formed rapidly in cells incubated in an energy- and nutrient-free medium suggests that the *cis-trans* isomerase is already present (22, 47). If that is true, then the constitutively expressed enzyme exists in a nonactive state or is spatially separated to act on the phospholipid molecules of the membrane during the exponential and stationary growth phases. There is evidence that changes in the membrane fluidity are the primary signals eliciting the isomerization reaction (26, 46). However, if a high membrane fluidity is a precondition for the *cis-trans* conversion, it cannot be the only one. In fact, exponentially growing cells are characterized by lower degrees of saturation but do not contain *trans* acids.

The extent to which *trans* fatty acids can be formed obviously depends on the amounts of cyclopropane fatty acids already formed from the common precursor pool of *cis* unsaturated fatty acids. Only the remaining *cis* fatty acids can be used for the formation of *trans* acids. The relatively stable cyclopropane acids are unavailable for conversion into *trans* acids. Therefore, in contrast to a former hypothesis (17) and confirmative of later reports (11), the *trans*/*cis* ratio is a less robust indicator for low nutrient levels in ecosystems than the cyclo/*cis* ratio (60).

Theoretically, the content of *trans* fatty acids can serve as a stress indicator in ecosystems because *cis-trans* isomerization is a way for some bacteria to respond to a temperature rise or an exposure to toxic substances (59). However, the practical use of *trans* fatty acids as a quantitative biomarker of specific environmental stress is questionable because of their generally low quantities, their occurrence in only a small proportion of all bacteria, the competing cyclopropane fatty acid synthesis, and the fact that they are formed only under nongrowth conditions and upon shock impacts.

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