Conversion of cis Unsaturated Fatty Acids to trans, a Possible Mechanism for the Protection of Phenol-Degrading Pseudomonas putida P8 from Substrate Toxicity

HERMANN-JOSEF HEIPIEPER, RUTH DIEFENBACH, AND HERIBERT KEWELOH*
Department of Microbiology, University of Münster, Corrensstrasse 3, D-4400 Münster, Germany

Received 10 October 1991/Accepted 16 March 1992

A trans unsaturated fatty acid was found as a major constituent in the lipids of Pseudomonas putida P8. The fatty acid was identified as 9-trans-hexadecenoic acid by gas chromatography, argentation thin-layer chromatography, and infrared absorption spectrometry. Growing cells of P. putida P8 reacted to the presence of sublethal concentrations of phenol in the medium with changes in the fatty acid composition of the lipids, thereby increasing the degree of saturation. At phenol concentrations which completely inhibited the growth of P. putida, the cells were still able to increase the content of the trans unsaturated fatty acid and simultaneously to decrease the proportion of the corresponding 9-cis-hexadecenoic acid. This conversion of fatty acids was also induced by 4-chlorophenol in nongrowing cells in which the de novo synthesis of lipids had stopped, as shown by incorporation experiments with labeled acetate. The isomerization of the double bond in the presence of chloramphenicol indicates a constitutively operating enzyme system. The cis-to-trans modification of the fatty acids studied here apparently is a new way of adapting the membrane fluidity to the presence of phenols, thereby compensating for the elevation of membrane permeability induced by these toxic substances.

The biodegradation of hazardous substances in wastewaters or contaminated soils is often limited by the antimicrobial action of the pollutants. An increase in cell tolerance against toxic substrates can crucially improve the degradability of the concerned microorganisms. Many studies demonstrate that degradation rates are diminished at high concentrations of the toxic substrates and lag phases of a considerable length precede the removal of these substances (2, 3, 20). The degradation process can often be strongly accelerated by an adaptation to the toxin, e.g., to phenol (28). Explanations for this phenomenon are the induction of degradative enzymes and growth-related biomass changes; alterations of the structure and dynamics of the cells themselves are also possible. It is known that many pollutants like phenolic substances affect the integrity and functioning of the cell membrane (10, 13). The tolerance of Escherichia coli cells to phenols can be increased by structural modifications of the membrane like the fatty acid composition of lipids (17). The influence of phenols on this essential cell component has never been studied with organisms like Pseudomonas putida P8, which have the capability of degrading these compounds. The dual character of phenol for this bacterium, as a source of growth and as a growth-inhibiting toxin, leads apparently to the acquisition of special mechanisms of cell protection as shown in this report.

MATERIALS AND METHODS

Microorganism and culture conditions. P. putida P8 was isolated from phenol-contaminated wastewater (3). The strain was cultivated in a minimal medium which contained (per liter): sodium succinate (4 g), (NH₄)₂SO₄ (1.06 g), MgSO₄ (0.1 g), KCl (0.74 g), K₂HPO₄ (2 g), FeSO₄ (1.6 mg), and 1 ml of a trace element solution (27). The pH was set to 6.0 before sterilization.

Cells were grown in 50-ml shake cultures at 30°C. In the case of adapted cells, strain cultivation was done on agar plates with minimal medium containing 0.5 g of phenol per liter as the sole carbon source instead of succinate. For the main cultures, the minimal medium with succinate was always used. Growing cells were harvested in the late-log phase. For the measurement of K⁺ efflux, potassium hydrogen phosphate was replaced by equimolar amounts of the sodium salt and the KCl concentration was reduced to 1 mM.

Preparation of nongrowing cells. Fifty milliliters of exponentially growing cells was harvested by centrifugation and suspended in the same volume of sodium phosphate buffer (50 mM, pH 7.0) with or without 4 g of sodium succinate per liter. The cell suspensions were gently shaken in a gyratory water bath shaker for 45 min before the experiments were started. Chloramphenicol (Sigma, Deisenhofen, Germany) was added shortly after the suspension of cells from an ethanolic stock solution to a final concentration of 50 mg/liter. This concentration was sufficient to inhibit the growth of P. putida P8 completely.

Lipid extraction and analysis. Cells of 45-ml suspensions (about 3 × 10¹⁰ cells) were sedimented 3 h after addition of the phenolic agent and washed with saline. The lipids were extracted with chloroform-methanol-water as described by Bligh and Dyer (4). Fatty acid methyl esters were prepared by a 15-min incubation at 95°C in boron trifluoride-methanol by the method of Morrison and Smith (24).

A determination of the fatty acid composition was performed by using gas chromatography (GC) (capillary column DB 225, 30 m and 180°C, with a flame ionization detector). The instrument used was a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan). The second identification of the trans unsaturated fatty acids employed a GC-9A gas chromatograph (Shimadzu) with an apolar capillary column (DB 5, 30 m and 180°C, with a flame ionization detector). The fatty acids were identified with the aid of standards. The relative amounts of the fatty acids were determined from the peak areas of the methyl esters by using a Chromatopak

* Corresponding author.
were withdrawn. Separation of these compounds was described as chloroform in nanomoles. The amount of chloroform layer was determined by using samples of Silica Gel 60 plates (Merck, Darmstadt, Germany) impregnated with silver nitrate. The plates were developed at −20°C with a solvent system composed of chloroform and ethanol (99:1, vol/vol). After drying, the entire plate was sprayed with 0.01% primuline in acetic-water (4:1, vol/vol) and the spots of fatty acid methyl esters were detected under UV light. The separated methyl esters were extracted from the appropriate regions of the silica gel by chloroform-methanol (1:1, vol/vol). The configuration of the double bond was determined by infrared absorption spectrometry (Perkin-Elmer 1420 infrared spectrophotometer) using samples prepared in potassium bromide discs.

**Argentation chromatography and infrared absorption spectrometry.** For the verification of trans and cis configurations of double bonds in the unsaturated fatty acids, thin-layer chromatography (TLC) was carried out on Silica Gel 60 plates. The plates were developed at −20°C with a solvent system composed of chloroform and ethanol (99:1, vol/vol). After drying, the entire plate was sprayed with 0.01% primuline in acetic-water (4:1, vol/vol) and the spots of fatty acid methyl esters were detected under UV light. The separated methyl esters were extracted from the appropriate regions of the silica gel by chloroform-methanol (1:1, vol/vol). The configuration of the double bond was determined by infrared absorption spectrometry (Perkin-Elmer 1420 infrared spectrophotometer) using samples prepared in potassium bromide discs.

**Analysis of de novo lipid synthesis.** In order to examine the de novo synthesis of lipids, 120 nCi of sodium [1-14C]acetate (48.9 mCi/mmol; Sigma) was added to 1 ml of growing or nongrowing cells containing equal amounts of cells. In case of the nongrowing cells, the labeled acetate was added at the same time that 4-chlorophenol was used to start the conversion of the fatty acids. After incubation for 2 h at 30°C, the cells were harvested and lipids were extracted as described above. The radioactivity of extracted lipids recovered in the chloroform layer was determined by liquid scintillation spectrometry. Amounts of lipids synthesized de novo were expressed in nanomoles of labeled acetate found in the chloroform layer.

**Measurement of cellular K⁺ content.** One-milliliter samples were withdrawn before and after addition of the phenolic compound. Separation of cells from the supernatant was carried out by rapid centrifugation through silicone oil as described by Bakker and Mangerich. The cell pellet was disrupted by boiling in 5% trichloroacetic acid, and debris was removed by centrifugation. The released K⁺ of the supernatant was measured by flame photometry in an SP9 atomic absorption spectrophotometer (Pye Unicam, Cambridge, Great Britain).

**RESULTS**

Identification of fatty acids in P. putida P8. The lipids of P. putida P8 were extracted, and the methyl esters of the fatty acids were subjected to silver nitrate TLC. We separated three fractions, which corresponded to saturated (fastest-running band), cis monounsaturated (slowest-running band), and trans monounsaturated fatty acids, as judged from a comparison of their K⁺ values with those of standards (data not shown).

To verify the trans and cis configurations, the fatty acid methyl esters were eluted from the corresponding regions of the silica gel and subjected to infrared absorption analysis (Fig. 1). The spectrum of the tentatively trans unsaturated fatty acid showed a peak at 966 cm⁻¹ which is characteristic of a trans double bond. This peak was missing in the otherwise very similar absorption spectrum of the fraction identified by TLC as fatty acids with a cis configuration.

The common existence of trans unsaturated fatty acids and the corresponding cis compounds in the lipids of P. putida P8 was documented by GC (Fig. 2). Palmitic acid (16:0), palmitelaidic acid (16:1 9trans), palmitoleic acid (16:1 9cis), and cis-vaccenic acid (18:1 11cis) were identified as major fatty acids of this bacterium. trans-Vaccenic acid (18:1 11trans), a second fatty acid with a trans configuration, and also myristic acid (14:0), stearic acid (18:0) and the cyclopropane fatty acids 17cyclo and 19cyclo could be detected in trace amounts of between 0.5 and 2%. Because of the fact that the occurrence of trans unsaturated fatty acids has been reported only relatively seldom, their identities were confirmed by using a second GC column and method as de-
scribed in Materials and Methods. GC analysis also showed that the fraction of the argentation chromatography identified as trans unsaturated fatty acids consisted of 16:1trans (99%) and 18:1trans.

Fatty acid compositions of cells adapted or not adapted to phenol. Figure 3 shows the relative amounts of the major fatty acids of cells adapted and nonadapted to phenol at different phenol concentrations. The proportion of 16:0 in the lipids of cells adapted to phenol as the sole growth substrate was significantly higher than in nonadapted cells, while the content of 18:1cis was decreased in adapted cells.

When various phenol concentrations were added to growing cells of *P. putida* P8, the fatty acid composition of these cells was changed in the following manner. The contents of 16:0 and 16:1trans increased and 16:1cis and 18:1cis decreased in cells in which the phenol concentration permitted continuation of a retarded growth.

Only after the addition of 1.25 g of phenol per liter to nonadapted cells was the growth completely blocked; these cells showed contents of 16:0 and 18:1cis, like the control cells. However, their content of 16:1trans was markedly increased and that of 16:1cis was decreased.

The fatty acid composition of the membranes was similarly modified when 4-chlorophenol instead of phenol was added to growing cells (data not shown). Cells of *P. putida* P8 can apparently change their cis-to-trans unsaturated fatty acid ratio by an unknown mechanism. A modification of the membrane composition by biosynthesis of new lipids cannot be expected when high concentrations of phenolic substances completely block the propagation of cells.

Membrane permeability of cells in the presence of phenol. Changes in the fatty acid composition induced by phenol may also affect the permeability of the cell membrane. This membrane property was studied by using K+ ions as an indicator. Phenol was added to adapted and nonadapted growing cells and caused a concentration-dependent K+ efflux. At lower concentrations of phenol, this efflux was reversible and the cells could restore their K+ gradients across the membrane in 30 to 60 min. At higher and therefore more toxic concentrations, the K+ efflux was irreversible, as shown in Fig. 4. Adapted cells which had a higher degree of saturation of their membrane lipids (Fig. 3) restored the K+ gradient across the membrane up to phenol concentrations of 750 mg/liter. On the other hand, the nonadapted cells were not able to do this at phenol concentrations higher than 250 mg/liter. Also, the K+ efflux rates of the adapted cells were smaller compared with those of the nonadapted cells (data not shown).

Conversion of cis-to-trans fatty acids in nongrowing cells. The following experiments were made by using 4-chlorophenol as the toxic substance. Since the change in the cis-to-trans ratio of the fatty acids apparently did not depend on the growth of the bacteria, as shown for cells with high phenol concentrations, the effect was further studied in nongrowing cells which were prepared as described in Materials and Methods. Also, in these cells, 4-chlorophenol induced an increase in the proportion of the 16:1trans fatty acid. Simultaneously, the content of the corresponding 16:1cis fatty acid decreased. The effect correlated with the 4-chlorophenol concentrations used (Fig. 5). All other fatty acids showed no change in their contents. The total amount of the two 16:1 fatty acids also stayed constant (38 to 39%) at all concentrations of 4-chlorophenol. The conversion of the fatty acids was also observed in the absence of an energy source like succinate, but to a slightly reduced extent (data not shown).

In these cells, de novo synthesis of lipids was completely absent as was documented by incorporation studies with [14C]acetate. Growing cells were able to incorporate 2.20 nmol of acetate per ml of cell suspension into the lipid fraction over the course of 2 h. If nongrowing cells were incubated with labeled acetate in the absence or in the presence of 300 mg of chlorophenol per liter, the incorporation was less than 0.001 nmol/ml (0.03% of growing cells).

The time course of conversion of the 16:1 unsaturated fatty acids induced by 4-chlorophenol is shown in Fig. 6. However, the formation of the trans fatty acid did not occur in cells boiled for 5 min previous to the addition of the phenolic substance (data not shown). This rules out a strictly chemical reaction and indicates an enzymatic involvement.
FIG. 3. Fatty acid composition of phenol-adapted (A) and -nonadapted (B) cells of P. putida P8. Phenol (concentrations as shown in grams per liter) was added to growing cells 3 h before they were harvested for fatty acid analysis.

The conversion of the double bond in the fatty acids is apparently catalyzed by a yet unknown cis-trans isomerase. When the cells were treated with chloramphenicol before the addition of 4-chlorophenol, the conversion of 16:1cis into 16:1trans fatty acids showed a similar course (Fig. 6). However, the cells already had an enhanced content of 16:1trans at the time of 4-chlorophenol addition. Also, in these experiments, the sum of the amounts of the two 16:1 fatty acids stayed constant. The formation of 16:1trans in the absence of fatty acid and protein biosynthesis indicates a constitutive enzyme system that directly isomerases the unsaturated fatty acids as constituents of phospholipids.

DISCUSSION

The change in fatty acid composition leading to an increase of the degree of saturation of the membrane lipids is a well-known reaction of bacteria against membrane-active substances like long-chain alcohols and aromatic compounds (15, 16). Also, phenols which are membrane active in bac-
steriostatic concentrations induce an increase in the degree of saturation of the membrane lipids of *Escherichia coli* K-12 (17). This membrane modification was found to correlate with an increase in cell tolerance against the toxic compounds. The reason for this may be that both the membrane changes and the presence of phenols affect the membrane fluidity, but in an opposite way, and, as a further consequence, the permeability of membranes (5, 19, 23). Another reaction of bacteria against phenol is a change in the lipid-to-protein ratio of the cytoplasmic membrane, which also influences the membrane viscosity (18, 29).

The constant contents of the fatty acids, including the total amounts of the 16:1 isomers at different concentrations of the toxin in nongrowing cells of *P. putida* P8, can be explained by the mechanism of procaryotes to change their membrane fluidity. Only growing bacteria were thought to be able to influence this membrane property by de novo synthesis of lipids and the incorporation into lipids of fatty acids in a changed saturated-to-unsaturated ratio (9). One exception to this restriction is the conversion of cis to trans unsaturated fatty acids found in this study for cells of *P. putida* P8. Also, the formation of cyclopropane fatty acids is known as a postsynthetic modification process of unsaturated fatty acids, but the function of this modification is unclear at present (9, 11) since the fluidity of the membrane is not significantly influenced by this conversion. The isomerization of the double bond is possibly a special mechanism of phenol degraders in reaction to high substrate concentrations under conditions not allowing growth and thereby inhibiting lipid de novo synthesis. However, the common occurrence of cis- and trans fatty acid isomers in the cellular lipids for some other strains of *Pseudomonas* (8, 12, 26), for *Vibrio* sp. (14, 26), and for methylotrophic bacteria (22) has also been reported. In *Vibrio* sp., probably a very similar mechanism of conversion of fatty acids is present (25). A cis-to-trans isomerization which is independent of lipid biosynthesis is induced in this psychrophilic strain after a sharp increase in the ambient temperature.

The cis configuration effects a strong increase in membrane fluidity by its bended steric structure. In contrast to this, the long, extended steric structure of the trans configuration is able to insert itself into the membrane similarly to saturated fatty acids, which are also mostly in all-trans conformations. Therefore, the conversion of cis-to-trans unsaturated fatty acids should reduce the membrane fluidity. Physicochemical investigations of the steric behavior of trans unsaturated fatty acids in membranes are in agreement with this argument (21). Moreover, different phospholipids containing a trans instead of a cis unsaturated fatty acid exhibited increases in phase transition temperatures of from 18 to 31°C, indicating a reduction in the membrane fluidity due to the changed double-bond configuration (25).

The function of the alterations in the membrane structure which change the fluidity of the lipid bilayer may be to reduce the permeability of the membrane. Adapted cells of *P. putida* P8, which had lipids with an enhanced degree of saturation, were characterized by maintenance of membrane gradients for K⁺ ions in the presence of high phenol concen-

---

**FIG. 4.** Influence of phenol on the K⁺ content of phenol-adapted (○) and -nonadapted (●) cells of *P. putida* P8. The K⁺ content of cells 1 h after the addition of the phenol which elicited a K⁺ efflux is shown. After this time, the K⁺ gradients were restored if possible.

**FIG. 5.** Content of 16:1cis (■) and 16:1trans (□) and the total 16:1 (▼) content in nongrowing cells of *P. putida* P8 at different 4-chlorophenol concentrations. Cells were harvested during the exponential growth phase and suspended in phosphate buffer (50 mM, pH 7.0) with 4 g of succinate per liter as the energy source. 4-Chlorophenol was added to the cells 2 h before the lipids were extracted for fatty acid analysis.

**FIG. 6.** Time course of formation of 16:1trans in nongrowing cells of *P. putida* P8 after the addition of 300 mg of 4-chlorophenol per liter as indicated by the arrow. Cells were treated with (○) or without (●) the addition of chloramphenicol, as described in the text.
trations. Thus, these cells could maintain limited growth at phenol concentrations at which nonadapted cells were totally inhibited in growth. Also, the conversion of cis-to-trans unsaturated fatty acids resulting in a diminution of the membrane fluidity should contribute to protection of cells from the toxicity of phenolic substrates.

The observation that, besides phenol and 4-chlorophenol, the aromatic compound chloramphenicol also induced cis-trans isomerization can be explained by an unspecific mechanism of enzyme regulation. The isomerase should be associated with the cell membrane due to gain access to the phospholipids as the probable substrates. Then one attractive possibility of regulation is that the system can directly sense the viscosity of the lipid bilayer. The conformation of the membrane protein, the displacement of the position inside the membrane, and the rate of lateral diffusion can be influenced by the viscosity of the lipid environment and can therefore be involved in the control of activity (29).

In contrast to the probable known enzyme activators, the system of isomerization apparently has a high specificity for C16 unsaturated fatty acids as substrates. The affinity to unsaturated fatty acids with a C18 chain is rather low, because 18:1trans was detected only in traces even when the amount of 18:1trans was higher than that of the corresponding cis fatty acid.

ACKNOWLEDGMENT

This study was supported by a grant from the Deutsche Forschungsgemeinschaft.

REFERENCES


