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# Mechanism for phenol tolerance in phenol-degrading *Comamonas testosteroni* strain

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**Abstract** *Comamonas testosteroni* P15 and its mutant strain E23 can tolerate and utilize phenol as the sole source of carbon and energy at up to 15 mM and 20 mM, respectively. Compared to the wild type P15, mutant E23 showed higher values of $K_s$ and $K_i$ but a lower $\mu_{max}$ value, and had lower phenol hydroxylase and catechol 2,3-dioxygenase activities. Without phenol exposure, mutant E23 demonstrated a two-fold greater amount of cardiolipin than the wild type P15. Upon exposure to phenol, an increase in cardiolipin at the expense of phosphatidylethanolamine was observed in the wild type P15. However, there was no significant difference in major phospholipid contents between mutant E23 cells grown in the presence or absence of phenol. It was noted that the ratio of *trans*/*cis* fatty acids of phosphatidylethanolamine and cardiolipin in mutant E23 was 65–70% higher than that in the wild type P15. In the absence of phenol, the degree of saturation of cardiolipin in mutant E23 was 33% higher than that in wild type P15. In contrast to earlier findings, an increase in C16:1 9trans with a simultaneous decrease in C18:1 11cis instead of C16:1 9cis was observed in specific classes of phospholipids.

## Introduction

Phenolic compounds from coal gasification, chemical, and petrochemical industries are among the pollutants most ubiquitously distributed in industrial effluents (substituted by Swoboda-Colberg 1995; Klein and Lee 1978). Due to their generally unhealthy effects on humans, their degradation is of obvious interest. However, microbial degradation of phenolic compounds is often hampered by the toxicity exerted by high concentrations. Microorganisms such as bacteria, yeasts, and algae have been reported to degrade phenol mainly at low concentrations. Among them are the Pseudomonads (Hinteregger et al. 1992; Kotturi et al. 1991), *Bacillus* species (Gurujeyalakshimi and Oriel 1989), *Alcaligenes* species (Hughes et al. 1984), *Streptomyces* species (Antai and Crawford 1983), *Trichosporon* species (Neujahr and Varga 1970), *Candida* species (Neujahr et al. 1974), *Ochromonas* species (Semple and Cain 1996), and the most well-studied one, *Pseudomonas*.

The toxic action of phenol is always associated with loss of cytoplasmic membrane integrity (Heipieper et al. 1991, 1992; Keweloh et al. 1990). This results in disruption of energy transduction, disturbance of membrane barrier function, inhibition of membrane protein function, and subsequent cell death. In general, several mechanisms for decreasing membrane fluidity due to various environmental stress factors have been proposed for *Pseudomonas putida* (Heipieper et al. 1992), *Escherichia coli* (Keweloh et al. 1991) and *Vibrio* species (Okuyama et al. 1991). These include an increased degree of saturation of fatty acid, conversion of *cis*-unsaturated fatty acid to the *trans* isomer, and alteration of the polar head groups of phospholipids.

The first goal of this study was to isolate a phenol-degrading strain of *Comamonas testosteroni* and its mutant strain with elevated phenol tolerance. Subsequently, attempts were made to investigate the mechanisms of the increased phenol tolerance of the mutant strain, with an emphasis on the level of enzyme activity and membrane lipid composition. The present report gives details on the content of phospholipids and their fatty acid composition. It describes for the first time the mechanism of phenol tolerance in strains of the genus *Comamonas*.

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## Materials and methods

### Media and culture conditions

The composition of the basal minimal medium (MM) has been described previously (Hegeman 1966). The sole carbon source in the

culture medium was phenol. For the study of lipid composition, 4 g $l^{-1}$ of sodium succinate was used as the sole carbon source instead of phenol. All inocula were grown in MM with 5 mM phenol unless otherwise stated. The culture temperature was kept at 32 °C.

### Isolation and identification of phenol-degrading strains

*Comamonas testosteroni* strain P15 was first isolated from soil samples obtained from the DYNO industrial processing plant, by repeated transferring in MM agar plate containing different concentrations of phenol as the sole carbon source. The second strain used in this study is strain E23, which was isolated after ethylmethyl sulfonate (EMS) mutagenesis. The ability to utilize phenol was verified in liquid cultures by following the uptake of phenol and cell density. Identification of the isolated strains was carried out using the commercially available Analytical Profile Index (API) 20NE rapid test kit (bioMerieux Vitex, Inc., USA).

### Growth kinetics and batch degradation of phenol

Growth and phenol utilization by the isolated strains were studied in 2-l conical flasks containing 500 ml MM with varying concentrations of phenol (0.1–25 mM). Samples were taken from the cultures at designated times, and cell growth was evaluated by means of absorbance ($A$) measured at 580 nm. The amount of phenol uptake was determined by monitoring the disappearance of phenol in the medium, using a colorimetric method (Folsom et al. 1990). Average phenol uptake rates were calculated as total phenol consumed in a defined time period per dry weight. Flasks were incubated at 32 °C on a rotary shaker at 250 rpm.

### Enzyme assays

For assaying phenol hydroxylase (EC 1.14.13.7) in whole cells, the rate of $O_2$ uptake was measured polarographically using a Clark-type $O_2$ electrode as described by Sala-Trepat et al. (1972). Crude extracts were prepared by sonication of washed cells and then cell debris was removed by centrifugation at 16 000 $g$ for 30 min. at 4 °C. Activity of catechol 2,3-dioxygenase (C23O; EC 1.13.1.2.) in crude extract was assayed spectrophotometrically, according to Bayly and Wigmore (1973) by quantitating the formation of the yellow product 2-hydroxymuconic semialdehyde, which has a maximal absorbance at 375 nm.

### Phospholipid extraction and identification of phospholipid

Cells of 200-ml suspensions were sedimented 3 h after addition of phenol and washed with saline. Phospholipids were extracted with methanol–chloroform–saline (2:1:0.8, v/v) as described by Kates (1986). The lipid fractions were separated into various classes of phospholipids by two-dimensional thin-layer chromatography (TLC) on precoated silica gel $F_{254}$ plates. The first and second developing solvent system was chloroform-methanol-7N ammonium hydroxide (65:25:4, v/v) and chloroform–methanol–acetic-acid–water (170:25:25:4) respectively (Prasad 1996). Phospholipids were visualized with iodine vapor and confirmed with molydenum blue reagent (Dittmer and Lester 1964). Individual phospholipids were located by comparing the $R_f$ values of the sample lane with the $R_f$ values of known standards.

### Fatty acids analysis

Phospholipid fatty acid methyl esters were prepared by the direct transesterification method (Lepage and Roy 1984). Fatty acid composition was determined using capillary gas chromatography (GC) (AutoSystem XL Gas Chromatograph, Perkin Elmer) equipped with a fused silica capillary column (SP-2560, 100 m × 0.25 mm ID, 0.2 μm film; Supelco, USA) and a hydrogen flame ionization detector (FID). Individual fatty acids were identified by comparing the retention times with those of fatty acid methyl ester standards (Sigma Chemicals Company, St. Louis, Mo., USA). Quantitation of the fatty acids was based on comparison of peak areas to the peak area of known concentration of an internal standard (2.5 mg $ml^{-1}$ undecanoic acid).

The ratio of the relative amounts of saturated to unsaturated fatty acids was calculated as the degree of saturation. The *trans*/*cis* ratio was determined as the proportion of the C16:1 9trans unsaturated to the C16:1 9cis unsaturated fatty acids.

## Results

### Isolation of the phenol-degrading strains

By sequential subculturing on MM plates with ascending phenol concentrations, an isolate designated as P15 was found to grow on phenol at levels of up to 15 mM. The isolate P15 was identified as a strain of *Pseudomonas testosteroni*, which has been reclassified as *Comamonas* by Tamoaka et al. (1987). Subsequent characterization of this bacterium showed that it is aerobic, gram-negative, short-rod, and oxidase-positive.

Ten mutant strains which could grow in MM with an initial phenol concentration of 20 mM were obtained by mutagenesis of strain P15 with EMS. By further selection, a mutant strain designated as E23 that showed the shortest induction time for phenol degradation was selected for further study.

### Biomass growth and batch degradation at various phenol concentrations

Wild type P15 and mutant E23 showed a similar response towards phenol treatment. Degradation of phenol correlated well with growth of these strains. With rising phenol concentrations in the culture medium, the lag period of both cultures increased proportionally. Wild type P15 was able to degrade phenol completely in concentrations of up to 15 mM within 32 h, but was unable to grow in medium containing 20 mM phenol. Mutant E23, on the other hand, was able to grow when the initial phenol concentration in the medium was 20 mM. In this case, 82% of the phenol was consumed within 39 h.

The effects of phenol on the growth rate and average phenol uptake rate of the two strains studied are shown in Fig. 1. In general, mutant E23 demonstrated a lower growth rate and average phenol uptake rate for initial phenol concentrations ranging from 0.1 to 10 mM. Saturation constant ($K_s$) and inhibition constant ($K_i$) are the values of phenol concentration below and above the critical phenol concentration respectively when the specific growth rate is equal to half of the maximum value. The $\mu_{max}$, $K_s$ and $K_i$ of wild type P15 were found to be 0.52 $h^{-1}$, 0.066 mM and 10.6 mM respectively. Compared to wild type P15, mutant E23 showed a 1.7-fold lower $\mu_{max}$ (0.3 $h^{-1}$) but 2.7-fold and 1.8-fold higher values of $K_s$ (0.177 mM) and $K_i$ (19.3 mM) respectively.

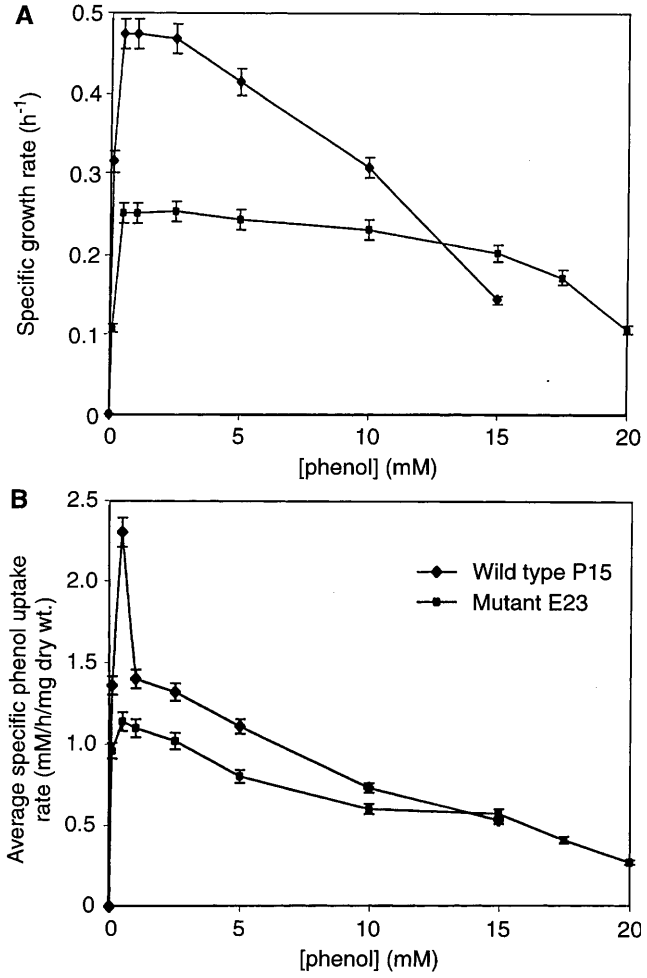


**Fig. 1** Comparison of (A) Biomass growth and (B) Phenol degradation of wild type P15 and mutant E23 at various phenol concentrations

Growth and phenol degradation assays were also carried out with cells of mutant E23 adapted to 20 mM phenol (data not shown). After the initial adaptation period, mutant E23 could degrade 20 mM phenol completely within 27 h. This mutant was capable of growing in medium with initial phenol concentrations of up to 23 mM, and degrading all the phenol completely within 46 h. A high-phenol-adapted mutant E23 had $\mu_{max}$, $K_s$ and $K_i$ values of 0.36 $h^{-1}$, 0.24 mM and 20.3 mM respectively.

## Activities of phenol degradative enzymes

Colonies of wild type P15 and mutant E23 cultured on phenol plates developed a yellow color when sprayed with catechol, indicating formation of 2-hydroxymuconic semialdehyde through a *meta*-cleavage pathway (Buswell 1974). The effects of phenol on the hydroxylating enzyme (phenol hydroxylase) and the ring-cleavage enzyme (catechol 2,3-dioxygenase) in phenol degradation were investigated. Activities of phenol hydroxylase and catechol 2,3-dioxygenase (C23O) could not be detected when both strains were grown in medium containing sodium succinate instead of phenol. Comparison of the enzyme-specific activities of the strains grown in media containing different phenol concentrations showed significant differences (Table 1). The phenol hydroxylase and C23O activities of the wild type P15 were proportional to the initial phenol concentration in the culture medium. For wild type P15, inhibition of phenol hydroxylase was observed at a phenol concentration of 10 mM, while no inhibition was observed for C23O. However, the specific activities of these enzymes in mutant E23 remained unchanged regardless of the initial phenol concentrations in the culture medium. The results revealed that the phenol

**Table 1** Specific activities of catabolic enzymes of wild type P15 and mutant E23 from cells grown at various concentrations of phenol

| Enzyme | Initial phenol concentration in the medium (mM) | Specific activities of enzymes[a] | |
|---|---|---|---|
| | | Wild type P15 | Mutant E23 |
| Phenol hydroxylase[b] | 0[d] | 0 | 0 |
| | 5 | 32.04 ± 2.5 | 22.53 ± 0.9[e] |
| | 10 | 45.39 ± 3.4 | 22.93 ± 1.2[e] |
| | 15 | 37.84 ± 2.1 | 23.78 ± 1.0[e] |
| | 17.5 | 0 | 21.94 ± 0.8[e] |
| | 20 | 0 | 21.68 ± 0.7[e] |
| Catechol 2,3-dioxygenase[c] | | | |
| | 0 | 0 | 0 |
| | 5 | 29.97 ± 0.4 | 22.01 ± 1.1[e] |
| | 10 | 34.19 ± 1.5 | 22.25 ± 0.5[e] |
| | 15 | 38.89 ± 0.9 | 24.58 ± 1.0[e] |
| | 17.5 | 0 | 22.61 ± 0.8[e] |
| | 20 | 0 | 23.75 ± 0.7[e] |

[a] Values are expressed as mean ± 1 standard deviation ($n = 3$)
[b] Assay substrate was 100 mM phenol. Specific activities of phenol hydroxylase were estimated on the basis of half of the oxygen uptake rate in micromoles of $O_2$ per minute per gram of dry weight (Pieper et al. 1988)
[c] Assay substrate was 100 mM catechol. Specific activities of catechol-2,3-dioxygenase were expressed as micromoles of product formed per minute per gram of dry weight
[d] Cells are grown in media containing sodium succinate as the sole carbon source
[e] Significant difference in comparison with the wild type P15 ($P < 0.05$)

hydroxylase and C23O activities of mutant E23 were generally lower than those of wild type P15.

## Phospholipid composition

Characterization of the phospholipid composition in both the wild type P15 and mutant E23 showed that the major classes of phospholipids were phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Concentrations of the other phospholipid components were too low to be detected. PE was the most abundant phospholipid in both strains and contributed about 50% of the total. As shown in Table 2, the PE and PG contents of both strains were very similar, while the mutant E23 had a two-fold greater percentage of CL than wild type P15. Upon exposure to phenol, alteration in phospholipid content was seen in wild type P15. The level of PG did not change significantly, whereas an elevation in the level of CL and a reduction in the level of PE were observed. However, there was no significant difference in the three major phospholipid contents between cells of mutant E23 grown in the presence or absence of phenol.

Considering the total content of the major phospholipids, wild type P15 demonstrated an overall decrease in the amount of phospholipid upon exposure to its maximum tolerable limit of phenol (15 mM). In contrast, the total content of the major phospholipid of mutant E23 did not change significantly after exposure to 15 mM phenol for 3 h.

## Fatty acid compositions of individual phospholipids

The fatty acid composition of the major phospholipids of wild type P15 and mutant E23 were similar. The predominant fatty acids were saturated (lauric acid, C12:0; palmitic acid, C16:0), *cis*-mono-unsaturated (palmitoleic acid, C16:1 9cis; *cis*-vaccenic acid, C18:1 11cis) and *trans*-mono-unsaturated (palmitelaidic acid, 16:1 9trans) fatty acids. In contrast to wild type P15, a third saturated fatty acid, myristic acid (C14:0) could be detected in a trace amount of between 0.7% and 1.0% in the mutant E23. No cyclopropane or branched-chain fatty acids were observed in either strain. In the three major phospholipids studied, cells of both strains showed a dramatic increase of C16:1 9trans upon exposure to phenol, while it was negligible when cells were grown in the absence of phenol.

Changes in the fatty acid composition of PE upon exposure to phenol were listed in Table 3. In the absence of phenol, the PE fraction of mutant E23 contained a lower level of C16:1 9cis but a higher level of C18:1 11cis than that of wild type P15. When 15 mM phenol was added to the exponentially growing cultures, an increase in C16:1 9trans at the expense of C18:1 11cis was observed in mutant E23. However, in wild type P15 the proportion of C16:1 9cis instead of C18:1 11cis was decreased. A higher degree of saturation for mutant E23 than for wild type P15 was observed in the presence of phenol. Mutant E23 showed a two-fold higher *trans*/*cis* ratio of fatty acids than that of wild type P15. This high value resulted from the high C16:1 9trans content in cells of mutant E23.

Table 4 shows the effect of phenol on the fatty acid composition of PG of the two strains studied. With sodium succinate as the sole carbon source, the proportion of C12:0 in wild type P15 was five-fold higher than that in mutant E23. In contrast, the contents of the other two saturated fatty acids, C14:0 and C16:0, were found to be higher in mutant E23 than in wild type P15. It was noted that both strains reacted to phenol with a similar modification of the fatty acid compositions of their PG. The level of C16:1 9trans increased markedly to as much as 15–17% of the total fatty acids. Simultaneously, a reduction of C18:1 11cis occurred. It was noted that the degree of saturation was slightly decreased in PG for both strains studied. The ratio of *trans*/*cis* unsaturated fatty acids was strongly affected by phenol, but there

**Table 2** Gram percentage of each class of phospholipid and total lipid obtained from wild type P15 and mutant E23 in the presence and absence of phenol

| Phospholipid class | Gram% | | | |
|---|---|---|---|---|
| | Wild type P15 | | Mutant E23 | |
| | 0 mM | 15 mM[a] | 0 mM | 15 mM |
| Phosphatidylethanolamine | 49 ± 0.6 | 30 ± 1.0[e] | 50 ± 2.0[f] | 52 ± 4.0[d] |
| Phosphatidylglycerol | 14 ± 0.1 | 16 ± 0.6[d] | 13 ± 0.6[f] | 15 ± 1.0[d] |
| Cardiolipin | 5 ± 0.4 | 15 ± 1.0[e] | 10 ± 0.6[g] | 12 ± 1.0[d] |
| Total lipid extracted[b] | 54.3 ± 2.8 | 45.1 ± 1.7 | 54.2 ± 3.5[f] | 46.4 ± 1.9[f] |
| Total major phospholipids[c] | 36.9 ± 1.7 | 27.5 ± 1.6[a] | 39.6 ± 3.1[f] | 37.1 ± 1.5[d] |

[a] 15 mM Phenol was added to cells for 3 h during the exponential growth phase
[b] Total lipid was extracted using the method of Kates (1986), and expressed as milligrams of lipid per gram of dry weight ± 1 standard deviation ($n = 3$)
[c] Sum of the amount of Phosphatidylethanolamine, phosphatidylglycerol and cardiolipin, expressed as milligrams of phospholipid per gram of dry weight ± 1 standard deviation ($n = 3$)
[d] No significant difference in comparison with the control (without phenol addition); $P < 0.05$
[e] Significant difference in comparison with the control (without phenol addition); $P < 0.05$
[f] No significant difference in comparison with the wild type P15; $P < 0.05$
[g] Significant difference in comparison with the wild type P15; $P < 0.05$

**Table 3** Fatty acid composition of phosphatidylethanolamine of wild type P15 and mutant E23 following exposure to phenol

| Retention time (min) | Fatty acid | Gram%[a] | | | |
|---|---|---|---|---|---|
| | | Wild type P15 | | Mutant E23 | |
| | | 0 mM | 15 mM[b] | 0 mM | 15 mM |
| 11.9 | 12:0 | ND | ND | ND | 4.4 ± 0.3 |
| 13.2 | 14:0 | ND | ND | 1.3 ± 0.06 | 1.5 ± 0.06 |
| 15.3 | 16:0 | 34.4 ± 0.5 | 33.4 ± 1.2 | 34.7 ± 0.8 | 33.8 ± 2.3 |
| 15.9 | 16:1 9trans | ND | 9.6 ± 0.3[c] | ND | 14.8 ± 0.4[c] |
| 16.4 | 16:1 9cis | 49.0 ± 0.6 | 38.8 ± 0.9[c] | 34.4 ± 2.5[d] | 35.3 ± 3.1 |
| 20.4 | 18:1 11cis | 16.6 ± 0.4 | 18.2 ± 0.3 | 29.6 ± 1.5[d] | 10.2 ± 0.4[c] |
| Degree of saturation | | 0.520 | 0.435 | 0.565 | 0.656 |
| *trans*/*cis* ratio | | 0 | 0.248 | 0 | 0.423 |

[a] Values are given as percentages of the total fatty acids, and are expressed as mean ± 1 standard deviation ($n = 3$)
[b] Phenol (15 mM) was added to cells for 3 h during the exponential growth phase
[c] Significant difference in comparison with the control (without phenol addition); $P < 0.05$
[d] Significant difference in comparison with the wild type P15; $P < 0.05$
*ND* Not detectable

was no significant difference in this ratio between wild type P15 and mutant E23.

CL was also referred to as diphosphatidylglycerol. There were significant differences in the fatty acid compositions of CL between wild type P15 and mutant E23 (Table 5). Besides the C14:0 which could only be found in mutant E23, a short-chain unsaturated fatty acid, C14:1 9cis was also observed in the CL of this mutant. Without exposure to phenol, the percentage content of C16:0 in mutant E23 was 66% higher than that in wild type P15. This resulted in a 34% higher degree of saturation in mutant E23 than in wild type P15. These two strains responded to phenol with different modifications of the fatty acid compositions of CL. For the wild type P15, an increase in C16:0 and C16:1 9trans was observed at the expense of the other three fatty acids, whereas an elevation of C16:1 9trans plus C18:1 11cis content and a reduction of C14:1 9cis plus C16:1 9cis contents was found in mutant E23. Although both strains had a similar content of C16:1 9trans after addition of phenol, the *trans*/*cis* ratio of mutant E23 was 65% higher than that of the wild type P15, due to the low amount of C16:1 9cis in the mutant E23.

## Discussion

The newly isolated *Comamonas testosteroni* P15 could utilize phenol of up to 15 mM as the sole source of carbon and energy. This tolerance limit of phenol was relatively high in comparison with *Pseudomonas putida* EKII (10.6 mM; Hinteregger et al. 1992), *Pseudomonas* sp. QT31 (10.6 mM; Masqué et al. 1987), *Pseudomonas putida* Q5 (10.6 mM; Kotturi et al. 1991) and *Rhodococcus opacus* (12.75 mM; Zaitsev et al. 1995). It was further observed that up to 23 mM phenol could be completely utilized by mutant E23 if the cells were previously grown in MM containing 20 mM phenol. This tolerable concentration of phenol was slightly higher than that reported for a mutant strain of *P. putida* WAS-2 (21.25 mM; patent J03067581) and of a fusant of *Candida tropicalis* (22 mM; Chang et al. 1995). Therefore, this mutant strain of *Comamonas testosteroni* E23

**Table 4** Fatty acid composition of phosphatidylglycerol of wild type P15 and mutant E23 following exposure to phenol

| Retention time (min) | Fatty acid | Gram%[a] | | | |
|---|---|---|---|---|---|
| | | Wild type P15 | | Mutant E23 | |
| | | 0 mM | 15 mM[b] | 0 mM | 15 mM |
| 11.9 | 12:0 | 18.7 ± 0.8 | 9.6 ± 0.9[d] | 3.8 ± 0.3[d] | 3.2 ± 0.3 |
| 13.2 | 14:0 | ND | ND | 6.9 ± 0.3[d] | 1.3 ± 0.04[c] |
| 15.3 | 16:0 | 31.5 ± 1.2 | 37.1 ± 1.3 | 42.3 ± 0.9[d] | 39.6 ± 1.7 |
| 15.9 | 16:1 9trans | ND | 15.5 ± 1.0[c] | ND | 17.3 ± 0.5[c] |
| 16.4 | 16:1 9cis | 30.1 ± 1.3 | 29.0 ± 1.0 | 32.3 ± 0.9 | 29.7 ± 2.1 |
| 20.4 | 18:1 11cis | 19.7 ± 0.3 | 8.8 ± 0.6[c] | 14.7 ± 0.7 | 8.9 ± 0.7[c] |
| Degree of saturation | | 1.00 | 0.878 | 1.13 | 0.787 |
| *trans*/*cis* ratio | | 0 | 0.531 | ND | 0.582 |

[a] Values are given as percentages of the total fatty acids, and are expressed as mean ± 1 standard deviation ($n = 3$)
[b] Phenol (15 mM) was added to cells for 3 h during the exponential growth phase
[c] Significant difference in comparison with the control (without phenol addition); $P < 0.05$
[d] Significant difference in comparison with the wild type P15; $P < 0.05$
*ND* Not detectable

**Table 5** Fatty acid composition of cardiolipin of wild type P15 and mutant E23 following exposure to phenol

| Retention time (min) | Fatty acid | Gram%[a] | | | |
|---|---|---|---|---|---|
| | | Wild type P15 | | Mutant E23 | |
| | | 0 mM | 15 mM[b] | 0 mM | 15 mM |
| 11.9 | 12:0 | 16.7 ± 0.8 | 10.0 ± 0.04[c] | 6.4 ± 0.4[d] | 9.8 ± 0.9 |
| 13.2 | 14:0 | ND | ND | 4.9 ± 0.3 | 4.5 ± 0.2 |
| 13.8 | 14:1 | ND | ND | 5.0 ± 0.1 | 0.7 ± 0.03[c] |
| 15.3 | 16:0 | 18.1 ± 0.7 | 30.0 ± 1.4[c] | 30.1 ± 2.1[d] | 28.3 ± 0.4 |
| 15.9 | 16:1 9trans | ND | 10.4 ± 0.3[c] | ND | 11.6 ± 0.6[c] |
| 16.4 | 16:1 9cis | 47.9 ± 0.6 | 37.4 ± 0.8[c] | 39.0 ± 2.2[d] | 25.3 ± 1.4[c] |
| 20.4 | 18:1 11cis | 17.3 ± 0.8 | 12.2 ± 0.8[c] | 14.6 ± 1.3 | 20.1 ± 0.3[c] |
| Degree of saturation | | 0.53 | 0.666 | 0.707 | 0.737 |
| *trans*/*cis* ratio | | 0 | 0.278 | 0 | 0.460 |

[a] Values are given as percentages of the total fatty acids, and are expressed as mean ± 1 standard deviation ($n = 3$)
[b] Phenol (15 mM) was added to cells for 3 h during the exponential growth phase
[c] Significant difference in comparison with the control (without phenol addition); $P < 0.05$
[d] Significant difference in comparison with the wild type P15; $P < 0.05$
*ND* Not detectable

can be regarded as the best phenol degrader of all phenol degrading strains reported up to date.

There were significant differences in the physiological constants between wild type P15 and mutant E23. Due to the high values of $K_s$ and $K_i$, it appears that mutant E23 develops an ineffective phenol uptake system to counteract the adverse effects of phenol inhibition. Consequently, mutant E23 became more resistant to the toxic effects of phenol.

There is a possibility that the toxicity of phenol to cells could be reduced if it is degraded rapidly by phenol-degradative enzymes produced by the cells. The results of the present study revealed that the production of phenol hydroxylase as well as the catechol 2,3-dioxygenase (C23O) of the two strains studied was inducible by phenol, which correlated well with previous findings reported by Hinteregger et al. (1992) and Gurujeyalakshmi and Oriel (1989). It is interesting to note that lower activities of phenol hydroxylase and C23O were displayed by mutant E23, which had a higher tolerance of phenol than the wild type P15. This observation was in agreement with the low specific growth rate, which might be caused by the lower rate of degradation of phenol. In contrast to wild type P15, the activities of phenol-degradative enzymes of mutant E23 were not affected by varying the phenol concentrations in the culture media. At an initial phenol concentration of above 15 mM, mutant E23 showed a higher growth rate and phenol uptake rate, but with lower activities of phenol-degradative enzymes than wild type P15. Since no accumulation of phenol was found in the cells of mutant E23 (data not shown), it seems likely that the increase in phenol tolerance of mutant E23 was at the level of the cell membrane.

The result from capsule staining revealed that there was no capsule formation to protect the cells of mutant E23 from phenol toxicity (data not shown). Hence, modification in lipid composition of the membrane is the most likely mechanism for mutant E23 to compensate for the increase in membrane fluidity commonly induced by phenol. Without phenol exposure, mutant E23 showed a two-fold greater amount of CL than the wild type P15. CL has the highest phase transition temperature ($T_M$) of all phospholipids (Boggs 1984), and so increased proportions of CL may result in a more rigid and stable bilayer structure of membrane. Upon exposure to phenol, wild type P15 demonstrated an increase in CL at the expense of PE, consistent with results observed in *Escherichia coli* (Ingram 1977, 1986), *Mycobacterium smegmatis* (Taneja and Khuller 1980), *Pseudomonas putida* DOT-T1 (Ramos et al. 1997) and *Zymomonas mobilis* (Carey and Ingram 1983; Becart et al. 1990). PE is a phospholipid with a relatively small headgroup area *a* compared to PG and CL. The decreased incorporation of PE and the increased incorporation of CL will thus increase the average phospholipid headgroup area *a*, which is expected to compensate for the increase in lipid volume *v* caused by the accumulation of phenol and thus stabilizes the bilayer structure of the membrane. As the $T_M$ of CL is about 10 K higher than PE (Keough and Davis 1984), the increased incorporation of CL will also have a rigidifying effect on the membrane.

On the other hand, there was no significant difference in the three major phospholipid (PE, PG, and CL) contents between cells of mutant E23 grown in the presence and absence of phenol. It seems that the biosynthesis of the major phospholipids in mutant E23 is a constitutive event, whereas it is an inducible event in the wild type P15. Another probable reason for this phenomenon is that a phenol concentration of 15 mM was not sufficient to induce a change in phospholipid content of mutant E23. These observations indicate that the amounts of PE and CL were the main factors in enhancing tolerance to phenol in mutant E23. Besides the increase in the level of CL (>10%), the level of PE had to be maintained at about 50% of the major phospholipids to enable the cells to grow in medium with high phenol concentration. Although the decreased incorpo-

ration of PE has a positive effect on the stability of the bilayer structure of the membrane, it will however disturb the bilayer to non-bilayer phospholipid ratio, which could possibly be unfavorable for cell growth. It can be concluded that an appropriate membrane lipid ordering ($T_M$) and bilayer stability ($T_{LH}$) of mutant E23 were achieved by maintaining a proper content of CL and PE respectively. The increase in PG was a common response to organic solvents in *P. putida* (Pinkart and White 1997), *E. coli* (Dombek and Ingram 1984) and *A. laidlawii* (Wieslander et al. 1986), but this was not shared by the two *C. testosteroni* strains studied here.

A large increase in *trans*/*cis* ratio in all fractions of phospholipids after addition of phenol into the cultures of *Comamonas testosteroni* was observed in this study. These results, in general, agree with those reported by Keweloh and Heipieper (1996). For wild type P15 and mutant E23, the *trans*/*cis* ratio of fatty acids of PG was the highest among the phospholipids studied. However, there was no significant difference in the *trans*/*cis* ratio of fatty acids of PG between these two strains. Therefore, the changes in the *trans*/*cis* ratio of fatty acids of PG do not play an important role for the increased phenol tolerance of mutant E23. Considering the ratio of *trans*/*cis* fatty acids of PE and CL, mutant E23 showed 65–70% higher ratios than the wild type P15. Apparently, mutant E23 has a greater ability than the wild type P15 to maintain a high ratio of *trans*/*cis* fatty acids.

In the absence of phenol, the degree of saturation of CL in mutant E23 was 33% higher than that of the wild type P15. Since two-fold higher levels of CL was found in mutant E23 than in the wild type P15, this resulted in a higher overall degree of saturation in mutant E23 than in the wild type P15. Upon exposure to phenol, the increase in saturation in mutant E23 occurred mainly in PE. The overall saturation of both the PE and the CL components of the phospholipids could explain the increase in membrane rigidity in mutant E23, thereby leading to a higher tolerance to phenol.

In all organisms studied up to now, the cells reacted to environmental stimuli with an increase in *trans* fatty acids and with a simultaneous decrease in the corresponding *cis* unsaturated fatty acids (Keweloh and Heipieper 1996). However, in the present study, it is puzzling to note that an increase in C16:1 9trans was observed in the PG of both strains and in the PE of mutant E23, accompanied by a decrease in the amount of C18:1 11cis. Further research is needed to verify the increase in C16:1 9trans fatty acid in relationship to the decrease in C18:1 11cis fatty acid.

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