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Cells of *Pseudomonas putida* and *Enterobacter* sp. adapt to toxic organic compounds by increasing their size

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Abstract The phenol-degrading solvent-tolerant bacterium *Pseudomonas putida* P8 changed its cell shape when grown in the presence of aromatic compounds such as phenol and 4-chlorophenol. The sizes of cells that had been growing after addition of different concentrations of the toxic compounds were measured using a coulter counter that calculates the sizes of the rod-shaped bacteria to diameters of virtual spheres. The cells showed an increase in the diameter depending on the toxic effects of the applied concentrations of both solvents. The same effect was measured for an alkanol degrading bacterium, *Enterobacter* sp. VKGH12, in the presence of *n*-butanol. The reaction of the cells to different concentrations of *n*-butanol was examined by scanning electron microscopy. With this technique it could be shown that the size of the bacteria increased with increasing concentrations of *n*-butanol. These changes in cell size were dependent on the cellular activity and occurred only after addition of non-lethal concentrations. In the presence of lethal concentrations that completely inhibited cell growth, the cell sizes were similar to those of cells without intoxication. Taking into account the mathematical formula for spherical and cylindrical diameter and surface, respectively, the cells reacted to the presence of organic solvents by decreasing the ratio between surface and volume of the cells and therefore reducing their relative surfaces. As the cell surface and especially the cytoplasmic membrane are the major targets for the toxic effects of membrane-active compounds, this reduction of the relative surface represents an adaptive response to the presence of such compounds.

Keywords Microbial adaptation · Toxic organic solvents · Cell size · Coulter counter · Scanning electron microscopy

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Introduction

Organic solvents are known to be extremely toxic to microbial cells. The toxicity of these compounds to organisms is due to their preferential partitioning into membranes, causing an increase in the fluidity of the membrane that leads to its non-specific permeabilisation (Heipieper et al. 1994; Sikkema et al. 1995). The dose-dependent growth inhibition caused by organic solvents is very similar for a variety of aerobic microorganisms (Isken and Heipieper 2002).

However, microorganisms can adapt to different organic substances and other forms of environmental stress by several adaptive mechanisms (for review see: Ramos et al. 1997, 2002; Isken and de Bont 1998; Segura et al. 1999; Isken and Heipieper 2002). Here, especially bacteria of the species *Pseudomonas putida* have been shown to be the most solvent-tolerant strains. Among them, strain *P. putida* P8, a solvent-tolerant phenol-degrading bacterium, has been intensely investigated (Diefenbach et al. 1992; Heipieper et al. 1992; Diefenbach and Keweloh 1994). Thereby, changes in the membrane composition are known to play a crucial role in the mechanism contributing to solvent tolerance (Inoue and Horikoshi 1989; Ramos et al. 2001; Heipieper et al. 2003). Among them, the major mechanisms involved in solvent tolerance are changes in the degree of saturation of fatty acids (Pinkart et al. 1996), *cis*/*trans* isomerization of unsaturated fatty acids (Heipieper et al. 1992, 1995), composition of phospholipid head groups

(Ramos et al. 1997), and modification of lipopolysaccharides (Pinkart et al. 1996). Thus, the major adaptive responses of microorganisms to externally occurring changes in the environment are modifications of the cell envelopes. An open question remains whether these changes in the cell envelope are also connected with a change in the overall morphology of the cells.

In this study, we investigated the effect of toxic concentrations of solvents such as phenols and butanol on cell size and shape of two different bacteria, *P. putida* and *Enterobacter* sp.

Materials and methods

Strains and chemicals

P. putida P8 was isolated as a phenol-degrading bacterium and has previously been described (Bettmann and Rehm 1984). It was one of the first strains of *P. putida* in which solvent tolerance was investigated (Diefenbach et al. 1992; Heipieper et al. 1992). *Enterobacter* sp. VKGH12 was recently isolated as an alkanol-degrading organism tolerating high alkanol concentrations and was identified using the culture system BIOLOG. Additionally, the strain was identified as *Enterobacter* sp. by sequencing of 16S-rRNA and alignment using the BLAST algorithm with the BLAST server of the National Centre for Biotechnology (homology 99%, AY297785.1).

Culture conditions

P. putida P8 and *Enterobacter* sp. VKGH12 were cultivated in a mineral medium as described by Hartmans et al. (1989) with 20 mM Na_2-succinate or glucose as sole carbon source. Cells were grown in 50-ml shake cultures in a horizontally shaking water bath at 30°C. Growth was monitored by measuring the turbidity (optical density) at 560 nm (OD_{560}).

Incubation with toxic compounds

For the measurements of the toxic effects of the investigated compounds, they were added to exponentially growing cultures as described by Heipieper et al. (1995). Cultures were incubated in the presence of the compounds for 3 h in a shaking water bath at 30°C. Cells were then harvested and immediately used for the measurement of cell size. Growth inhibition caused by *n*-butanol was measured by comparing the differences in growth rate μ (h^{-1}) between intoxicated cultures ($\mu_{1,\ toxin}$) with that of control cultures ($\mu_{0,\ control}$). The growth inhibition of different concentrations of *n*-butanol was defined as the percentage of the growth rates of cultures grown with *n*-butanol and that of control cultures without toxin addition.

$$\text{Inhibition growth}\,(\%): \quad \frac{\mu_{1,\text{toxin}}}{\mu_{0,\text{control}}} \times 100.$$

Coulter counter

The cell size of *P. putida* cells was measured using a coulter counter Multisizer 3 (Beckman Coulter, High Wycombe, U.K). Before being measured, samples were diluted by factor 500 with an Isoton 2 standard solution (Beckman, High Wycombe, U.K.) using a Coulter diluter DD3 (Beckman Coulter, High Wycombe, U.K.). Cell sizes, given as sphere diameters, were calculated using the MS-Multisizer 3 software (Beckman Coulter, High Wycombe, U.K.).

As the Coulter counter calculates the measured cells to spheres, the volumes (V) and surface areas (a) of the cells were calculated as follows:

$$V\,(\mu\text{m}^3) = 4/3\pi r^3,$$

$$a\,(\mu\text{m}^2) = 4\pi r^2.$$

Scanning Electron Microscopy (SEM)

The effect of different *n*-butanol concentrations on the morphology of *Enterobacter* sp. VKGH12 was studied using SEM. After 3 h of incubation in the presence of different concentrations of *n*-butanol, bacterial cells were harvested and washed twice with 50 mM potassium phosphate buffer (pH 7.0). Bacteria were then fixed by immersion in 2.5 % glutaraldehyde (prepared in 50 mM potassium phosphate buffer) overnight at 4° C. Then the specimens were washed twice with phosphate buffer and dehydrated by an ethanol series of 30, 40, 50, 60, 70, 80, 90, and 100% (v/v) ethanol and stored in 100% ethanol. Specimens in 100 % ethanol were gold coated for 15 min using an IB-3 (Giko E. Co., Japan) sputter cotter and examined under an S-2500C (Hitachi Co., Japan) SEM. Cell dimensions of cylindrical bodies were directly measured from the SEM photographs to calculate cell volume and surface area by the following equations:

$$V\,(\mu\text{m}^3) = r^2\pi h,$$

$$a\,(\mu\text{m}^2) = 2r^2\pi + 2\pi rh,$$

where r is the radius and h the length of the cylindrical cells. Average cellular volumes and surface areas were calculated by using 30 individual bacteria per population. Cells showing deformations/depressions were not considered.

Statistics

All experiments were carried out 5-fold. For Coulter counter experiments, the mean values as well as the standard deviations were taken as calculated by the MS-Multisizer 3 software (Beckman Coulter, High Wycombe,

U.K.) of the Coulter counter equipment. For the SEM-method, the mean values were calculated from SEM photographs by taking 30 bacteria per population. For the SEM method, the mean values of the measures examined for 30 cells for each SEM photograph were calculated. Statistics were calculated using the Parametric Statistic programme, version 1.01 (Lundon Software, Inc., Chagrin Falls, OH, USA).

Results

Changes in the cell size of *P. putida* in the presence of phenol and 4-chlorophenol

Cells of *P. putida* were grown in a mineral medium with Na_2-succinate as energy and carbon source. The growth rate μ of the cells was about 0.65 h^{-1}, which corresponds to a doubling time (t_D) of about 1.08 h. Phenol and 4-chlorophenol were added in different concentrations during the exponential growth phase. The organisms continued to grow exponentially, but at reduced growth rates. After 3 h in the presence of the toxins, samples were taken to measure cell sizes using the coulter counter technique. Figure 1 shows the curves of the coulter counter measurements for *P. putida* cells for phenol (A) and 4-chlorophenol (B). In the presence of both solvents, a dose-dependent increase in the diameter of the cells can be observed. *P. putida* P8 can use phenol as carbon and energy source. This explains the slight increase in the cell number in the presence of 100 mg/l, whereas all other concentrations of phenol and 4-chlorophenol lead to a reduction in the cell number due to the toxic, growth reductive effect of the compounds.

In the presence of both toxic aromatic compounds, the cells increased their size in relation to the added concentration of the toxin but regardless of the fact that this compound was added as carbon and energy source (phenol) or as a non-degradable compound (4-chlorophenol). This effect was dependent on the growth inhibitory effect of the added non-lethal concentration of the specific solvent. Figure 2 shows the relation between the concentration-dependent growth inhibition caused by phenol (A) and 4-chlorophenol (B) and the change in the ratio between surface and volume of the cells. Depending on the concentration, a significant reduction of the surface-to-volume-ratio can be observed.

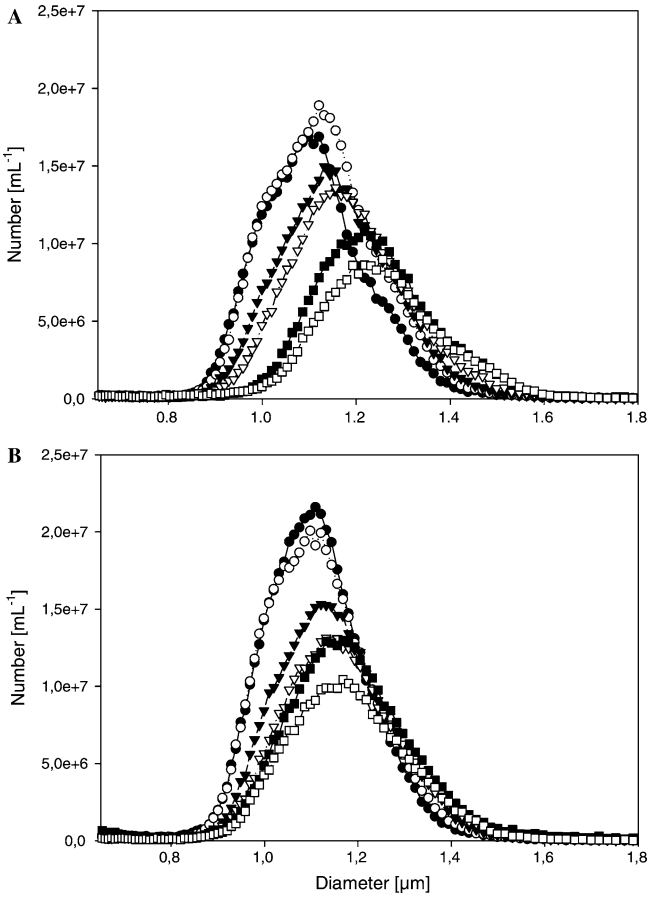

Fig. 1 Effect of toxic concentrations of phenol (**A**) and 4-chlorophenol (**B**) on the diameter of cells as well as cell number of *P. putida* P8 grown in mineral medium with Na_2-succinate as carbon source. Samples were taken after 3 h in the presence of the toxins and measured by Coulter counter. The concentrations added to the cultures were for (**A**) phenol (mg/l) 0 (*filled circle*), 250 (*open circle*), 500 (*inverted filled triangle*), 750 (*inverted open triangle*), 1000 (*filled square*), 1250 (*open square*); and for (**B**) 4-chlorophenol (mg/l) 0 (*filled circle*), 100 (*open circle*), 200 (*inverted filled triangle*), 300 (*inverted open triangle*), 400 (*filled square*), 500 (*open square*)

Changes in the cell size of *Enterobacter* sp. VKGH12 in the presence of *n*-butanol

Using the coulter counter technique, we could clearly show a dose-dependent effect of toxins on the cell size of *P. putida*. These coulter counter results were carried out with other bacteria and other toxins and always gave similar results (data not shown). To control these results and the applicability of the coulter counter, we used a second technique to measure the development of the cell size of a solvent-tolerant *Enterobacter* sp. VKGH12 grown in the presence of *n*-butanol. Therefore, the cell size was measured directly from SEM photographs that had been taken from cells growing in the presence of different concentrations of the toxin. This SEM technique has already been applied for studying the deleterious effects of stressful environment on the bacterial morphology (Shi and Xia 2003). The objects are clearly visualised and a three-dimensional view of the intact cells and their surface structure can be clearly observed. Cells of *Enterobacter* sp. VKGH12 were grown in a mineral medium with Na_2-succinate as energy and carbon source. The growth rate μ of the cells was about 0.55 h^{-1}, which corresponds to a doubling time (t_D) of about 1.33 h. *n*-butanol was added in different concentrations during the exponential growth phase. These organisms continued to grow exponentially as well, but at reduced growth rates. Figure 3 shows SEM photographs of *Enterobacter* sp. VKGH12 cells that had been

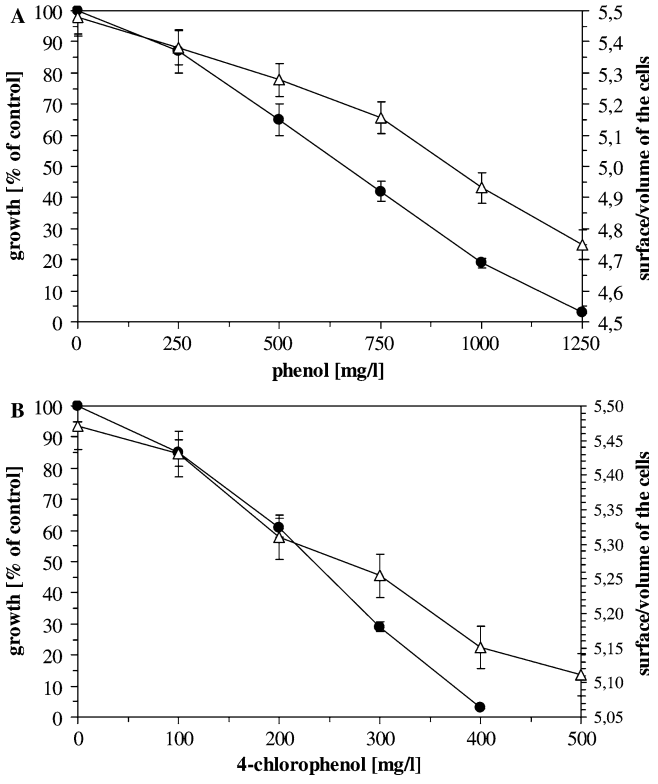

Fig. 2 Effect of phenol (**A**) and 4-chlorophenol (**B**) on growth (*filled circle*) and the ratio between cell surface and volume (*open triangle*) of cells of *P. putida* P8 measured by Coulter counter

grown for 3 h in the presence of different concentrations of *n*-butanol. The results are summarised in Table 1. In the presence of *n*-butanol, the cells increased their size in relation to the added non-lethal concentrations of the toxin. However, in the presence of lethal concentrations of each toxin (i.e. 2% v/v butanol), the cells show nearly the size of control cells without intoxication. Thus, the observed effect was strongly dependent on the fitness of the cells and is not just a chemico-physical effect of solvent addition. By increasing their size, the cells reduce the relative area of their cell envelope, which is known to be the major target for the toxic actions of aromatic compounds such as phenols that are also defined as membrane-active toxins. Figure 4 shows the relation between the growth inhibition caused by *n*-butanol and the change in the ratio between surface and volume of the cells.

Table 1 Effect of *n*-butanol on growth, cell shape, and the ratio between cell surface and volume of exponentially growing cells of *Enterobacter* sp. VKGH12

n-butanol (%)	Growth	Length	Radius	Surface	Volume	Surface/volume
0	100	1.29	0.199	1.862	0.160	11.60
0.1	93	1.40	0.211	2.136	0.196	10.91
0.25	87	1.43	0.219	2.269	0.215	10.53
0.5	75	1.44	0.221	2.306	0.221	10.44
0.75	45	1.45	0.222	2.332	0.224	10.39
1	20	1.69	0.225	2.707	0.269	10.07
1.25	2	1.85	0.234	3.064	0.318	9.63
1.5		1.93	0.242	3.302	0.355	9.30
1.75		1.55	0.205	2.260	0.205	11.05
2		1.35	0.201	1.959	0.171	11.43

Cell length and radius were estimated by measuring out SEM photographs. For calculations, cells shape was assumed to be cylindrical

Discussion

In eubacteria an external shell of peptidoglycan opposes internal hydrostatic pressure and prevents membrane rupture and death, as well as it gives each cell a certain size and shape that are also heritable, allowing the bacteria to adapt to changes in environmental condi-

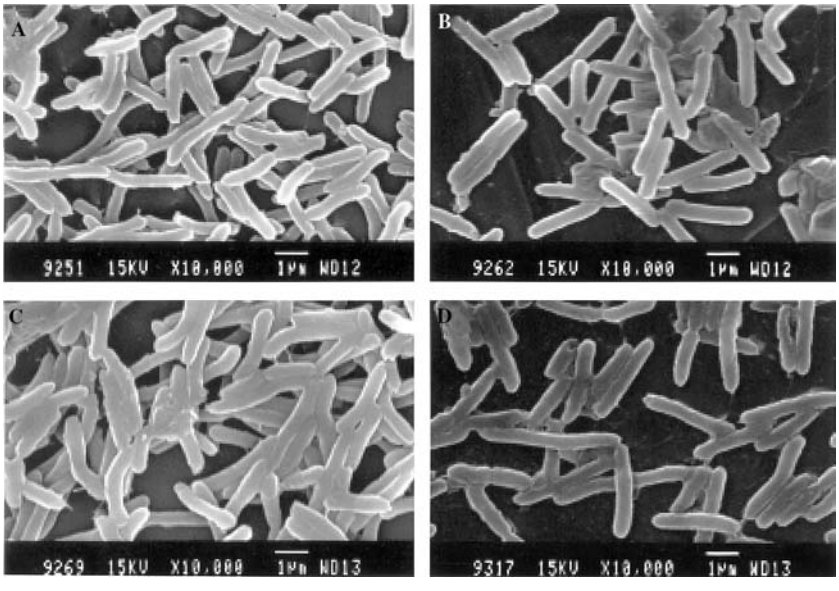

Fig. 3 SEM photographs of cells of *Enterobacter* sp. VKGH12 incubated for 3 h in the presence of 0% (**A**), 0.25% (**B**), 1.0% (**C**), and 1.5% (**D**) (v/v) *n*-butanol

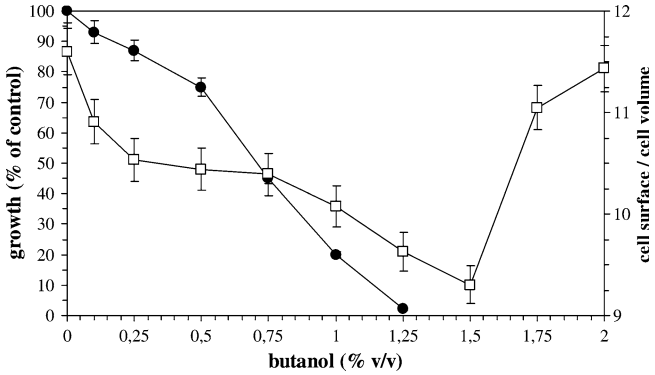

Fig. 4 Effect of toxic concentrations of *n*-butanol on growth (*filled circle*) and the ratio between cell surface and volume (*open square*) of cells of *Enterobacter* sp. VKGH12 measured from SEM photographs

tions (Young 2003). Indeed, changes in the morphology as an adaptive response have already been described for several bacteria and environmental conditions (stresses) (Chen et al. 1996; Rasanen et al. 2001; Ritz et al. 2001; Daniel and Errington 2003; Shi and Xia 2003).

The changes in cell morphology as a response to toxic organic solvents observed in our experiments can be explained by a relative reduction of cell surface with respect to its volume. By increasing the size, the volume of an organism increases with the cubic (for spheres, the volume $V=4/3\pi r^3$), whereas the surface only increases with the quadrate (for spheres, the surface $a=4\pi r^2$). This phenomenon is also known as Bergmann's law. Carl Bergmann, a nineteenth century's biologist, pointed out that amongst birds and mammals, individuals of the same species tend to be larger and heavier when they live in colder climates. Thus, the bigger a body is the smaller is its surface with respect to its volume. For homeothermic organisms such as birds and mammals, a smaller surface/volume ratio causes a relatively smaller loss of body heat in colder climates. This gives bigger animals an advantage in the competition with smaller conspecifics.

Transferring this law to microorganisms, this means that a bigger size reduces the relative surface and consequently reduces the attachable surface for toxic organic compounds. Therefore, bigger cells are better protected against toxic organic compounds than smaller cells of the same species. This relative reduction of the cell surface represents an effective mechanism of the cells to reduce the toxic effect of environmental stress factors just by reducing the attachable surface in relation to the whole cell volume. This reaction makes even more sense by taking into account that one of the major adaptive mechanisms to various environmental factors, including toxic organic solvents, is the active efflux of those compounds by ATP-driven efflux pumps (Lewis 1994; Nikaido 1994; Isken and de Bont 1996; Rojas et al. 2001). It is obvious that the functioning of such solvent efflux pumps is more effective if the overall membrane surface is reduced. This leads to a reduction in the area that allows diffusion and partitioning of solvents into the membrane where they are recognised and excluded by the efflux pump proteins (Rojas et al. 2001).

For starving cells, the opposite effect has been described. When entering the stationary growth phase, bacteria are known to reduce their size and shape to smaller, nearly coccoidal structures (Givskov et al. 1994; Moller et al. 1996). This observation can also be explained by the surface/volume ratio already used for solvent tolerance: under starvation the cells increase their relative surface, their membrane area, to allow a better uptake and consumption of nutrients under these limiting environmental conditions.

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