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Tributyl phosphate degradation by *Rhodopseudomonas palustris* and other photosynthetic bacteria

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Abstract

Tributyl phosphate (TBP) is widely used in nuclear fuel processing and other waste generating chemical industries. Although TBP is bacteriostatic, some microbes are resistant to it and may degrade it. Under dark aerobiosis, purple non-sulfur photosynthetic bacteria degraded up to 0.6 mM TBP, initially present at 2 mM, within 3 weeks and under photosynthetic conditions, *Rhodopseudomonas palustris* degraded 1.6 mM TBP within 3 weeks. The curing of the *Rhodopseudomonas palustris* endogenous plasmid demonstrated that the genes involved in the TBP degradation are chromosomal.

Introduction

Organophosphorus compounds are widely used as industrial additives and as chemical warfare agents. Exposure to organophosphates is a major concern for public health as it can cause delayed cholinergic toxicity and neurotoxicity (Raushel 2002). Increasingly, bioremediation is viewed as a safe, convenient and economically feasible method for the destruction of organophosphates. Although the molecular basis for the degradation of some organophosphates has been studied extensively (Kumar *et al.* 1996, Raushel 2002), biodegradation of tributyl phosphate (TBP) has been poorly investigated because of its low impact on mammals (Healy *et al.* 1995, McDonald *et al.* 2002). Therefore, TBP is a significant environmental contaminant and presents an acute toxicity to freshwater living organisms (Nakamura 1991, Michel *et al.* 2004). TBP can be degraded by bacteria as mixed cultures or as single isolates (Berne *et al.* 2004). However, the enzymatic mechanisms responsible for TBP elimination from the medium remain unknown.

This paper reports the behaviour of some photosynthetic bacteria grown in the presence of TBP. Photosynthetic bacteria were chosen for this study because they have a versatile metabolism and are known for their capability to degrade numerous xenobiotics. TBP degradation was investigated under two sets of growth conditions: dark aerobiosis and photosynthesis. As the genes encoding the degradation of xenobiotic compounds are often located on plasmids, we cured the endogenous plasmid (pRpal, 9.8 kb) of one of the photosynthetic strain (*Rps. palustris* CGA009) to determine its involvement in TBP degradation. We also examined cell-free extracts to determine whether TBP degradation is enzyme-dependent.

Materials and methods

Media

Photosynthetic strains were grown under photosynthetic conditions at 30 °C, in the light (10 W m^{-2}) in 100 ml air-tight bottles, in Hutner medium. Anaerobic conditions were obtained by out-gassing the medium before the inoculation (2% of a mid-late growth phase). A mineral medium (PM) was used for the growth of *Rps. palustris* CGA009 (12.5 mM Na_2HPO_4; 12.5 mM KH_2PO_4; 1 mM $(NH_4)_2SO_4$; 0.1 mM $Na_2S_2O_3 \cdot 5H_2O$; 1.45 mM *p*-aminobenzoic acid; 0.85 mM EDTA; 3.8 mM $ZnSO_4 \cdot 7H_2O$; 1.8 mM $FeSO_4 \cdot 7H_2O$, 0.9 mM $MnSO_4 \cdot 7H_2O$, 0.16 mM $CuSO_4 \cdot 5H_2O$, 86 μM $Co(NO_3)_2 \cdot 6H_2O$, 46 μM $Na_2B_4O_7 \cdot 10H_2O$; 0.1 M nitrilotriacetic acid; 0.24 M $MgSO_4$ anhydrous; 60 mM $CaCl_2$; 15 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.7 mM $FeSO_4 \cdot 7H_2O$; 10 mM succinic acid and Na_2CO_3 (the latter was only added for photosynthetic growth and was added aseptically after autoclaving). PM plates were made by aseptically mixing 2× agar in water (34 g l^{-1}) with an equal volume of 2× PM after autoclaving (C. Harwood, personal communication). After inoculation with *Rps. palustris*, the plates were incubated at 30 °C, in a GENbag Anaer (Biomérieux, France) under photosynthetic growth conditions. Dark aerobic cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml medium with shaking (150 rpm, 30 °C). Sterile tributyl phosphate (TBP) was added to culture media to give 2 mM.

Escherichia coli strains were grown on LB broth with shaking (150 rpm) or on LB agar (17 g l^{-1}) plates at 37 °C. When necessary, the media included kanamycin at 25 μg ml^{-1}.

Preparation of cell-free extracts

Rps. palustris cells, grown in Hutner medium in the presence or in the absence of 2 mM TBP, were harvested at mid- to late-growth phase by centrifugation (10 min, 8000 × *g*, 4 °C). Pellets were washed twice in KH_2PO_4/K_2HPO_4 buffer (50 mM, pH 7.2) and then suspended in 20 ml of the same buffer with the addition of a protease inhibitor cocktail (P8849, Sigma). Washed cells were broken by three passages through a French pressure cell at 16 000 psi. The exudate was centrifuged (20 min, 10 000 × *g*) to remove debris and unbroken cells.

Plasmid-curing experiments

pRpal was cured from *Rps. palustris* CGA009 by competition with a plasmid from the same incompatibility group (Suwanto & Kaplan 1992). A triparental mating was performed using 100 μl of *E. coli* DH5α *phe* (donor) (Eraso & Kaplan 1994) containing the pMG105 plasmid (Inui et al. 2000), 10 μl of HB101 (helper) (Boyer & Roulland-Dussoix 1969) and 1 ml of *Rps. palustris* (recipient). Bacteria were spread on PM plates + kanamycin and incubated under photosynthetic conditions. After 4 days, transconjugants bearing pMG105 were selected on PM + kanamycin plates. In most cases, the kanamycin-resistant bacteria had lost pRpal. The transconjugant strains were grown on a medium without kanamycin through several subcultures to eliminate plasmid pMG105. The resulting plasmid-free strain was designated *Rps. palustris* ΔpRpal. Each step of the curing procedure was monitored by plasmid isolation.

Enzyme assays

The reaction mixture (1 ml final volume) containing cell-free extract was diluted to 1 mg ml^{-1} in KH_2PO_4/K_2HPO_4 buffer (50 mM, pH 7.2). As controls to appreciate the abiotic disappearance of TBP, same experiments were performed with sterile buffer, heat treated crude extract of *Rps. palustris* (10 min, 95 °C) and *E. coli* DH5α crude extract.

Temperature, pH and protein concentration optima for cell-free TBP degradation systems

Assays were incubated with TBP for 20 h at 25 °C (except for the temperature dependence experiments). Optimum protein concentration was determined over a dilution range from 0 to 1.5 mg total proteins ml^{-1} in KH_2PO_4/K_2HPO_4 buffer (50 mM pH 7.2).

Kinetics analysis

Initial reaction velocities measured at various concentrations of TBP were fitted to the Lineweaver–Burk transformation of the Michaelis–Menten equation. Kinetic analyses by curve fitting were performed with the SigmaPlot program (Systat Software, Inc.). Because the enzyme activity was assayed

with crude extracts, the kinetic parameters are designated apparent concentration of TBP for half-maximal activity (Km_{ap}) and apparent maximal activity (Vm_{ap}).

Sample preparation for TBP assays and HPLC analysis

Sample preparation and HPLC analysis were performed as previously described (Berne *et al*. 2004).

Results

TBP degradation by photosynthetic bacteria under dark aerobic and photosynthetic conditions

A number of strains of purple non-sulfur photosynthetic bacteria were tested for the ability to degrade TBP: *Rhodobacter (Rba.) sphaeroides* strain 2.4.1, *Rba. capsulatus* strain Saint-Louis, *Rhodospirullum (Rsp.) rubrum* strain S1 and nine different strains of *Rhodopseudomonas (Rps.) palustris*. The bacteria were grown under dark aerobic or photosynthetic conditions in the presence of 2 mM TBP. The residual TBP was measured by HPLC after 3 weeks of growth. During dark aerobiosis, TBP decreased by 0.26 to 0.45 mM (Table 1). Under photosynthetic conditions, degradation by *Rps. rubrum* and all the *Rps. palustris* strains was markedly improved reaching more than 1.6 mM (Table 1). Because the genome of strain CGA009 has been completely sequenced (Larimer *et al*. 2004), we decided to focus on this strain.

Table 1. TBP degradation by purple non-sulfur photosynthetic bacteria.

Strains	TBP removal (mM)	
	Dark aerobiosis	Photosynthesis
Rba. sphaeroides 2.4.1[a]	0.36 ± 0.04	0.18 ± 0.01
Rba. Capsulatus S[t] Louis[a]	0.48 ± 0.04	0.64 ± 0.13
Rps. rubrum S1[a]	0.25 ± 0.03	1.60 ± 0.04
Rps. palustris CGA009[b]	0.36 ± 0.04	1.57 ± 0.09
Rps. palustris DSM 126[c]	0.35 ± 0.02	1.56 ± 0.04
Rps. palustris DSM 131[c]	0.34 ± 0.05	1.56 ± 0.07
Rps. palustris DSM 823[c]	0.30 ± 0.03	1.53 ± 0.1
Rps. palustris DSM 7375[c]	0.32 ± 0.04	1.51 ± 0.11
Rps. palustris LMG2953[d]	0.36 ± 0.04	1.48 ± 0.12
Rps. palustris LMG4314[d]	0.35 ± 0.02	1.52 ± 0.7
R. palustris LMG4316[d]	0.34 ± 0.05	1.58 ± 0.6
Rps. palustris LMG4317[d]	0.33 ± 0.02	1.59 ± 0.4
Rps. palustris CEA001[a]	0.35 ± 0.05	1.64 ± 0.6
Rps. palustris △pRpal[e]	0.36 ± 0.04	1.57 ± 0.04

TBP removal is calculated from the residual TBP in the medium after 3 weeks of culture. Values are means ± standard deviations of three replicates.
Sources of the different strains:
[a] our laboratory culture collection;
[b] Dr Harwood gift;
[c] Deutsche Sammlung von Mikroorganismen und Zelkulturen Braunschweig, Germany;
[d] Belgian Coordinated Collections of Microorganims, Belgium and
[e] this study.

Plasmid-curing experiments

According to a BLAST search, the genes *par*A and *rep*A from pRpal share 100% homologies with the kanamycin-resistant cloning vector pMG105. Thus, the two plasmids belong to the same incompatibility group. We exploited this property to cure pRpal by introduction of pMG105 by triparental mating. After mating, the endogenous plasmid was eliminated in bacteria bearing the pMG105 plasmid, selected on a kanamycin medium. The cloning vector was subsequently eliminated by growing the transconjugant cells in the absence of kanamycin, yielding the plasmid-cured strain *Rps. palustris* ΔpRpal (data not shown).

Under photosynthetic conditions, *Rps. palustris* ΔpRpal, and the wild-type strain were indistinguishable by growth as well as by colony morphology. *Rps. palustris* CGA009 is naturally resistant to tetracyclin (20 μg ml^{-1}) and gentamycin (50 μg ml^{-1}) and the plasmid-free strain grows in the presence of these antibiotics equally well as the wild-type. There was also no difference between the cured strain and the wild-type during growth on TBP (data not shown).

Optimal conditions for TBP degradation by Rps. palustris *CGA009 cell-free systems*

Cell-free systems were developed to elucidate the enzymatic dependence of TBP degradation by *Rps. palustris* CGA009. The TBP degradation yield increased with concentration up to 1 mg protein ml^{-1} and then reached a plateau (Fig-

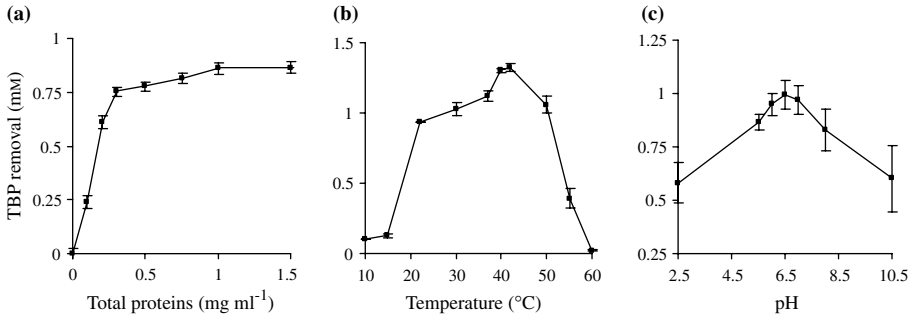

Fig. 1. Effects of protein concentration (a), temperature (b), and pH (c) on TBP removal over 20 h. pH was determined over a range from 2.5 to 10.5, using the following buffers: 100 mM Glycine HCl pH 2.5, 100 mM potassium acetate pH 5.5, 100 mM KH_2PO_4/K_2HPO_4 buffer pH 6, pH 6.5, pH 7 and pH 8, 100 mM CAPS pH 10.5. Values are means ± standard deviations of three replicates.

ure 1a). The temperature and pH dependences (protein at 1 mg ml^{-1}) are shown in Figures 1b and 1c, respectively. The optimal conditions for degradation were pH 6.5 and 40 °C for an assay time of 20 h.

TBP degradation by cell-free systems

TBP degradation by cell-free systems was studied with protein from *Rps. palustris* cultures grown in the presence ('induced cultures') or in the absence of 2 mM TBP ('non-induced cultures') and from *Rps. palustris* ΔpRpal. 1.4 mM TBP was removed by cell-free extracts at 40 °C in 20 h independent of the presence of TBP in the growth medium (data not shown). Same results were obtained from the cured strain extract. The negative controls failed to degrade TBP.

Kinetics of TBP degradation by cell-free systems

Cell-free systems were tested over a range of TBP concentrations (Figure 2): 2, 1.5, 1, 0.75, 0.5, 0.25, 0.1 and 0.05 mM. At TBP concentrations between 0.5 and 2 mM, degradation kinetics could be fitted to an exponential decay curve with a constant. At TBP concentrations below 0.5 mM, the profiles followed a single exponential decay curve.

Initial degradation rates decreased with the TBP concentration and the reaction velocity could be fitted to the Michaelis–Menten model, with a Vm_{ap} and a Km_{ap} values of 0.48 ± 0.04 mM h^{-1} and 2.6 ± 0.37 mM, respectively (Figure 3).

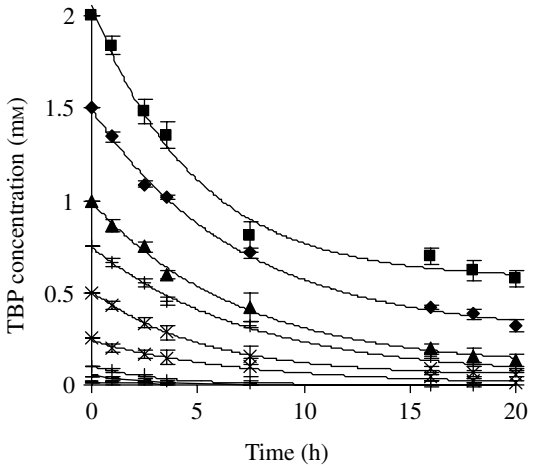

Fig. 2. Kinetics of TBP consumption by cell-free systems over 20 h at 40 °C at various TBP concentrations: 0.05 mM (–), 0.1 mM (*), 0.25 mM (+), 0.5 mM (×), 0.75 mM (●), 1 mM (▲), 1.5 mM (◆) and 2 mM (■).Values are means ± standard deviations of five replicates.

Discussion

We investigated the biodegradation of TBP under dark aerobic and photosynthetic conditions by a variety of purple non-sulfur photosynthetic bacteria, *Rba. sphaeroides*, *Rba. capsulatus*, *Rsp. rubrum* and *Rps. palustris*. Photosynthesis markedly enhanced TBP degradation, reaching more than 1.6 mM during a 3 week culture for *Rps. palustris* and *Rsp. rubrum*.

Different strains of *Rps. palustris* showed a comparable TBP degradation suggesting that the TBP degradation ability of these strains is widespread throughout the various *Rps. palustris*

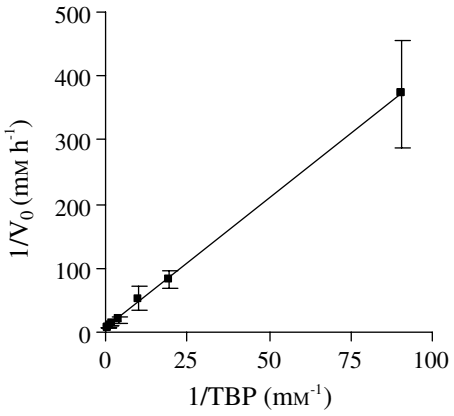

Fig. 3. Lineweaver–Burk plot for TBP degradation by cell-free extracts based on the equation $1/V_0 = (Km/Vm) \times (1/[S]) + (1/Vm)$, with V is the TBP degradation rate and S the TBP concentration. The kinetic parameters (Vm and Km) are given in the text. Values are means ± standard deviations of five replicates.

strains and is not specific to a particular strain. The genes involved in the degradation are likely present on the chromosomes of the bacteria as indicated by the comparable performances of *Rps. palustris* ΔpRpal and *Rps. palustris* CGA009 during TBP degradation. The result is different from that previously reported for bacterial TBP degradation by pseudomonads. Thomas et al. (1997a,b). reported TBP degradation by microbial isolates showing a strong correlation between the presence of a DNA band with cultures showing a high degradation capacity (up to 1.8 mM over 120 h); the degradation property was lost when the DNA was not detectable, suggesting that this property might be plasmid-borne.

Rps. palustris metabolizes nitrogen-containing compounds including amino acids and heterocyclic aromatic compounds, dehalogenates and degrades chlorinated benzoates and chlorinated fatty acids (Larimer et al. 2004). This present study is the first report to demonstrate the biodegradation of a non-cyclic organophosphorus compound by *Rps. palustris*. This strain can efficiently transform many pollutants and our cured strain is a potential tool to determine the plasmid or chromosomal locations of genes involved in different degradation pathways.

The Michaelis–Menten model provided a good description of the degradation kinetics in these experiments in which cell-free systems were used. A Km_{ap} of 2.6 mM indicates a moderate affinity of the enzymatic system for TBP. We note that with TBP between 2 and 0.5 mM, the rates slowed markedly as the reaction proceeds, which might be due to product inhibition. At TBP concentrations below 0.5 mM, the kinetics followed a single exponential decay in agreement with the above interpretation. The thermal stability of the metabolic enzymes was relatively high, as the half-life of this activity was 20 h at 55 °C. The pH optimum of 6.5 is in line with the physiological pH of the cell.

This study shows that the enzymatic reactions are still significant even under extreme conditions of pH (2 to 10.5) and temperature (10 °C to 55 °C). The enzyme(s) involved in such metabolism may be well suited for immobilization for biocatalytic applications. We are currently investigating the degradation pathway to identify the enzymes involved in this process.

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