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Toxicity of chlorobenzene on *Pseudomonas* sp. strain RHO1, a chlorobenzene-degrading strain

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Abstract

Pseudomonas sp. strain RHO1 able to use chloro- and 1,4-dichlorobenzene as growth substrates was tested towards sensitivity against chlorobenzene. Concentrations of chlorobenzene higher than 3.5 mM were found to be toxic to cells independent of pregrowth with chlorobenzene or nutrient broth. Below this concentration, sensitivity towards chlorobenzene depended on the precultivation of the cells, i.e. type of growth substrate (chlorobenzene or nutrient broth) and the concentration of chlorobenzene as the growth substrate. Cells grown in continuous culture were especially sensitive with a threshold concentration of 2.5 mM chlorobenzene. In addition to chlorobenzene, metabolites also seem to function as toxic compounds. 2-Chlorophenol and 3-chlorocatechol were isolated from cell extracts. Cleavage of 3-chlorocatechol by catechol 1,2-dioxygenase seems to be the critical step in the metabolism of chlorobenzene.

Introduction

Among the numerous chemicals that enter the environment with waste water and exhaust, a great number are benzene derivatives. Benzene, toluene and even chlorinated benzenes have been shown to function as the sole carbon source for several bacterial strains (Wagner 1914; Wieland et al. 1958; Marr & Stone 1961; Claus & Walker 1964; Gibson et al. 1968; Reineke & Knackmuss 1984; Schraa et al. 1986; Spain & Nishino 1987; Oltmanns et al. 1988). However, the cultivation of organisms on benzene, toluene or chlorobenzene was found to be difficult (Gibson et al. 1970; Reineke & Knackmuss 1984; Haigler et al. 1988). When substrates were directly supplied to the culture media, or even when they were supplied via the gas phase, inhibition of growth or inactivation of the organisms resulted.

The present report gives some information on the toxicity of chlorobenzene on *Pseudomonas* sp. strain RHO1 which is able to use the compound as the carbon and energy source.

Materials and methods

Continuous cultivation with chlorobenzene

Pseudomonas sp. strain RHO1 was cultivated in a 250-ml chemostat as described by Reineke & Knackmuss (1984). Fresh mineral medium was fed by a peristaltic pump at a rate of $D = 0.042\,h^{-1}$. Chlorobenzene was supplied by the incoming air (7.5 l/h) to give a concentration of 0.3 mM in the fermentor fluid without growing cells. At steady state in a continuously growing culture, the concen-

tration of chlorobenzene in the fermentor fluid was 0.01 mM or less.

Batch cultivation with chlorobenzene

Cells were grown at 30 °C in 500 ml sealed Erlenmeyer flasks containing 100 ml medium. Chlorobenzene was fed via the gas phase by injecting 20–25 μl of chlorobenzene in a side arm of the flask. Concentrations of chlorobenzene in the medium of about 2 mM or even more resulted when equilibrated. When the concentration reached about 0.01 mM additional portions of chlorobenzene were added.

Growth and induction of cells of Pseudomonas *sp. strain 39/D*

Pseudomonas sp. strain 39/D, a mutant strain lacking dihydroxycyclohexadiene dehydrogenase, was grown with mineral medium containing 10 mM glucose. Benzene as the inducing compound was supplied via the vapor phase at a final concentration of 1 mM. 39/D cells grown under these conditions were induced for benzene metabolism.

Incubation with chlorobenzene

Cell suspensions cultivated as described were diluted with phosphate buffer (50 mM, pH 7.2) to an optical density of 1 at 546 nm. Cells were incubated at 30 °C with various concentrations of chlorobenzene in the presence or absence of oxygen. For incubations in the absence of oxygen, cell suspensions were gassed with argon in Hungate tubes. Chlorobenzene was added directly to the medium as a liquid by use of a Hamilton syringe and quantified by GC/FID (United Technologie Packard 437 A, Delft, The Netherlands; column: 10% SE 30 methylsilicon, Supelchem, Sulzbach, Germany). Viability of cells was tested after an incubation period with chlorobenzene of half an hour by plating appropriate aliquots on nutrient agar or by introducing 2 ml of the treated cell suspensions into 25 ml nutrient broth medium (0.8% nutrient broth, 0.5% NaCl). In the second procedure the potential of the cells for growth was determined after 3 hours. Turbidities obtained with cells not treated with chlorobenzene were used as a control and were referred to as 100%. Since a linear correlation between viable cell count and the potential to start growth in liquid medium was observed the second procedure was used routineously.

Isolation of metabolites

Cell suspensions (50 ml at an optical density of 1 at 546 nm), from either batch or continuous culture, were incubated with 30 μl chlorobenzene for 30 min. The cells were concentrated 10-fold by centrifugation at 10 °C and 4500 × g for 20 min and resuspended in H_2O. The suspension was extracted with diethylether after acidification with H_3PO_4 (pH 2). The ether extract was dried with $CaCl_2$ and evaporated at 30 °C. The residue was dissolved in H_2O and analysed by high-pressure-liquid chromatography with a reversed phase RP8 column (Merck, Darmstadt, Germany). Vacuum-degassed 2% phosphoric acid containing 70% methanol was used as eluent.

Enzyme assay

Cell extracts were prepared as previously described (Reineke & Knackmuss 1984). All enzyme assays were done at 25 °C. *cis*-1,2-Dihydroxycyclohexa-3,5-diene (NAD^+) dehydrogenase (E.C.1.3.1.19) was measured by determination of NAD reduction at 340 nm in an analogous assay described by Reiner for the *cis*-1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase (1972). Catechol 1,2-dioxygenase (E.C.1.13.11.2) was measured as described previously (Dorn & Knackmuss 1978).

Protein estimation

The protein contents of cell-free extracts were determined by the method of Bradford (1976).

Chemicals

3-Chlorocatechol was prepared as previously described (Schreiber et al. 1980). 3-Chloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene was biologically prepared with *Pseudomonas* sp. strain 39/D. cis-1,2-Dihydroxycyclohexa-3,5-diene was obtained from Fluka AG (Buchs, Switzerland). All other chemicals were from commercial sources.

Results

Sensitivity of RHO1 cells towards chlorobenzene

The sensitivity of cells of strain RHO1 towards chlorobenzene was determined in the presence and absence of oxygen, which is the second substrate necessary for chlorobenzene degradation (Fig. 1). Besides cells grown with chlorobenzene in batch or continuous culture also non-induced cells (grown with nutrient broth) were tested. Identical results were obtained with cells which had been pregrown in batch culture with chlorobenzene independent from an aerobic or anaerobic incubation period. Non-induced cells showed the same behavior. Cells grown in continuous culture, however, were highly sensitive towards chlorobenzene when incubated in the presence of oxygen. In contrast, without any oxygen available those cells showed a sensitivity comparable to non-induced cells. These results indicate that in addition to chlorobenzene metabolites in the chlorobenzene degradation might have a toxic effect on the cells.

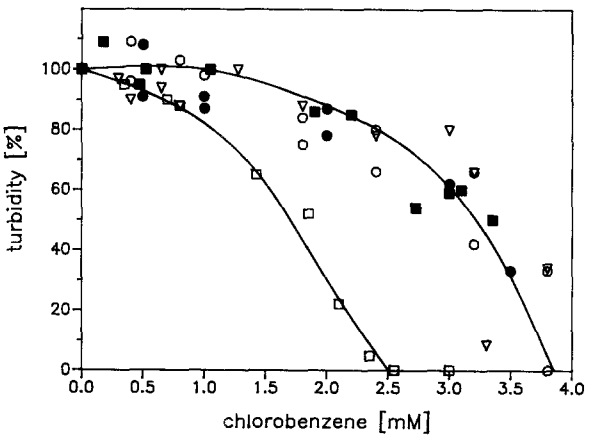

Fig. 1. Sensivity of strain RHO1 towards chlorobenzene when grown in the presence of chlorobenzene or nutrient broth. Incubation experiments were realized as described in 'Materials and methods'. Cell suspensions were obtained from batch (○,●) or continuous culture (□,■) with chlorobenzene or from batch cultures with nutrient broth medium (▽) as the growth substrate. The cell suspensions were incubated aerobically (open symbols) or anaerobically (filled symbols) for 30 min with various concentrations of chlorobenzene. The ability to grow on nutrient medium after incubation was tested. For details see 'Materials and methods'.

Activity of enzymes in cells grown at different conditions

We tried to find out why cells pregrown with chlorobenzene showed a different behavior when grown in batch or continuous culture. Measuring activities of enzymes involved in the degradation of chlorobenzene, some differences in the induction status were observed. Data were obtained by measuring activity with whole cells for the initial enzyme and in cell-free extracts for the *cis*-1,2-dihydroxycyclohexa-3,5-diene dehydrogenase and the ring cleaving dioxygenase. An interpretation is only possible by comparing the activities of the same enzyme in cells grown in batch or continuous culture. Chlorobenzene dioxygenase activity was determined by measuring the decrease of chlorobenzene concentration in the culture fluid by GC. Both type of cells (optical density of 1 at 546 nm) reduced the concentration from 2.5 to 1.3 mM within half an hour, indicating no difference with respect to the first enzyme in the degradation of chlorobenzene in both types of cells. However, while dehydrogenase activities were present at nearly identical levels, the activities of the catechol 1,2-dioxygenase was found to be only 40% in continuous grown cells compared to cells grown in batch culture (Table 1). Cells grown with nutrient broth did neither show activity to take up chlorobenzene nor activities of the dehydrogenase and catechol 1,2-dioxygenase.

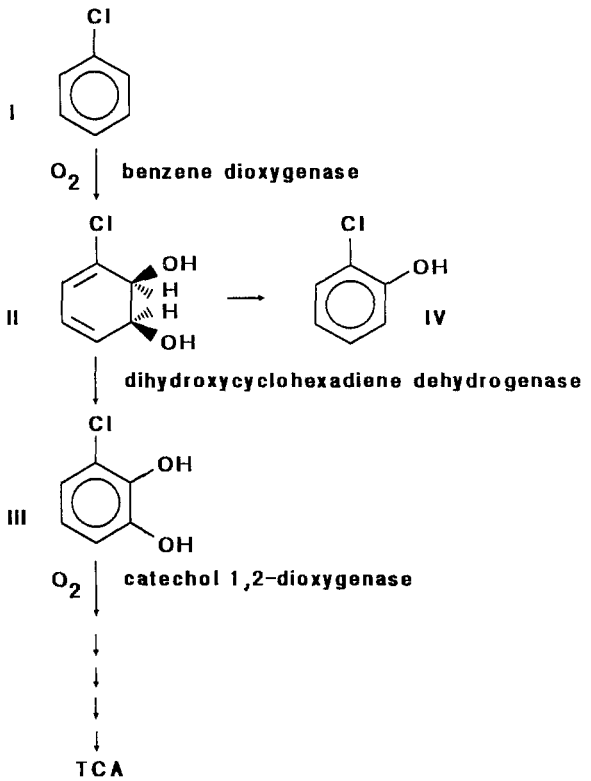

Fig. 2. Initial steps in the degradation of chlorobenzene. (I) chlorobenzene, (II) 3-chloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene, (III) 3-chlorocatechol, (IV) 2-chlorophenol.

Accumulation of metabolites during incubation with chlorobenzene

Metabolites could not be observed in culture fluids of RHO1 cells after incubation with chlorobenzene independent from modifications of culture condition, cell densities, addition of N-cetyl-N,N,N-trimethyl-ammonium bromide to permeate cells and variations of the concentration of chlorobenzene added. However, in extracts of the cells pregrown in continuous culture, 2-chlorophenol and 3- chlorocatechol were detected after incubation with chlorobenzene, which were absent in cells pregrown in batch culture.

Incubation of strain 39/D with chlorobenzene

A putative toxic effect due to the intracellular accumulation of 3-chloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene during metabolism of chlorobenzene was studied with mutant strain 39/D . Identical results were observed with induced and non-induced cells when incubated with chlorobenzene which excluded 3-chloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene to be a toxic metabolite. Overall, strain 39/D showed similar low sensitivity towards chlorobenzene as did strain RHO1 grown in batch culture.

Discussion

Strain RHO1 is an organism which is able to use chlorobenzene and 1,4-dichlorobenzene as growth substrates (Oltmanns et al. 1988). However, when chlorobenzene was used as the sole source of carbon and energy the cultivation was found to be difficult. The initial steps in the degradation of chlorobenzene are presented in Fig. 2.

There are different mechanisms which might be responsible for the sensitivity of strains such as

Table 1. Specific activities of catabolic enzymes in cells of strain RHO1 grown with chlorobenzene or nutrient broth.

Activity	Assay substrate	sp act. (U/mg Protein)		
		Chlorobenzene-grown		NB-grown batch culture
		Continuous culture	Batch culture	
cis-1,2-dihydroxycyclohexa-3.5-diene dehydrogenase	*cis*-1,2-dihydroxycyclohexa-3,5-diene	0.14	0.15	<0.001
catechol 1,2-dioxygenase	catechol	0.42	1 13	<0.001
	3-chlorocatechol	0.7	1.17	<0.001

Cell suspensions of RHO1 were obtained from continuous or batch culture with chlorobenzene or nutrient broth as growth substrate.

RHO1 towards chlorobenzene. One explanation of chlorobenzene being a toxic growth substrate can be derived from its highly lipophilic character. This effect might be similar to the effect observed with toluene on cell envelopes of gram-negative bacteria (Bucksteeg 1942; Jackson & DeMoss 1965; Woldringh 1973). Gibson et al. (1970) reported that addition of benzene directly to the medium did not result in growth of *Pseudomonas putida*. But even when benzene (Gibson et al. 1970) or chlorobenzenes (Reineke & Knackmuss 1984; De Bont et al. 1986; Schraa et al. 1986; Oltmanns et al. 1988) were added to the culture medium via the vapor phase, the benzenes had to be supplemented to a separate chamber in small portions. A second explanation for the difficulty in cultivating cells with chlorobenzene might be the toxicity of metabolites. Chlorocatechols have been discussed by other authors to be the toxic metabolites during degradation of chlorobenzenes (Reineke & Knackmuss 1984, Haigler et al. 1988, Oltmanns et al. 1988). Haigler et al. (1988) reported that the addition of 1,2-dichlorobenzene to suspensions of non-induced cells had to be started with low concentration in order to avoid an accumulation of chlorocatechol.

The toxic threshold concentration of the substrate chlorobenzene for strain RHO1 in general was actually determined to be above 3.5 mM. Here the lipophilic character of the substrate is assumed to be responsible for the inactivation of cells. Cooperative toxic effects of a lipophilic substrate and a metabolite have already been discussed by Jenkins et al. (1987) for toluene and its metabolite methyldihydroxycyclohexadiene. Toxic effects or cooperative toxic effects with chlorobenzene caused by 3-chloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene, the first metabolite from chlorobenzene degradation, were unlikely. This was shown with the chlorodihydroxycyclohexadiene accumulating mutant strain 39/D.

We did succeed to identify 3-chlorocatechol (Fig. 2, III) and 2-chlorophenol (Fig. 2, IV) as compounds in poisoned cells of RHO1. 3-Chlorocatechol is a metabolite in chlorobenzene degradation. 2-Chlorophenol can readily be interpreted as an artifact derived from chloro-*cis*-1,2-dihydroxycyclohexa-3,5-diene (Fig. 2, II). However, chlorophenol might result from enzymatic monooxygenation of chlorobenzene. Recently a toluene dioxygenase was demonstrated to function as a monooxygenase (Wackett et al. 1988).

Our results show that besides the toxic effect of chlorobenzene itself there might occur an additional toxic effect depending on the growth conditions of strain RHO1. Batch grown cells were accustomed to high concentrations of chlorobenzene and therefore highly induced for chlorobenzene degradation. These cells were poisoned by concentrations of chlorobenzene higher than 3.5 mM within half an hour. The continuously grown cells, adapted to very low chlorobenzene concentrations and therefore induced at a low level, lost viability during incubation with chlorobenzene concentrations of 2.5 mM. The latter cells have been poisoned by a metabolite in addition to chlorobenzene itself. In contrast non-induced cells, unable to produce a metabolite, were only poisoned by a chlorobenzene concentration higher than 3.5 mM. Comparing the chlorocatechol cleavage activity in batch-grown and continuously-grown cells it seems very likely that 3-chlorocatechol is the accumulating toxic metabolite in continuously grown cells.

The data presented indicate that undisturbed growth of organisms with compounds such as chlorobenzene can only be established when the induction of the enzymes involved in the degradation is well balanced to avoid the accumulation of toxic metabolites.

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