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Evaluation of the growth inhibition strength of hydrocarbon solvents against *Escherichia coli* and *Pseudomonas putida* grown in a two-liquid phase culture system consisting of a medium and organic solvent

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Abstract The growth of microorganisms is often inhibited in a two-liquid phase culture system consisting of an aqueous medium and a large volume of hydrophobic solvent. *Escherichia coli* and *Pseudomonas putida* were cultured in a two-phase system containing a solvent with a log P_{ow} value in a range of 2.1 to 6.0. The increase in the cell mass was monitored by increase in turbidity of the medium phase. We devised a semiquantitative method to evaluate the growth inhibition strength of solvents based on the relative amount of bacterial growth occurring in the two-phase system. Analyses of growth of the bacteria by this method showed that the growth inhibition strength of a given solvent was usually but not always correlated inversely with its polarity. It is clear that growth inhibition strength is not determined simply by polarity of the solvent.

Key words *Escherichia coli* · *Pseudomonas putida* · Two-liquid phase culture system · Growth inhibition strength · Organic solvent · Solvent toxicity · Solvent resistance

Introduction

Microorganisms are sometimes incubated in two-phase systems consisting of a hydrophobic organic solvent and an aqueous medium. This method possibly serves as an effective means of biochemical conversion of hydrophobic compounds. For this purpose, it is necessary to understand both the toxicity of the solvent and the solvent resistance of the microorganism performing the corresponding biotransformation. In the past decade, the growth of bacteria has been investigated in two-phase systems. It is difficult to maintain a constant concentration of a hydrophobic solvent in the medium for a long period of incubation because some solvents have low solubility in the medium, are adherent to microbial cells, or are highly volatile. Thus, it is not convenient to measure the toxicity of such solvents by measuring the minimum inhibitory concentration against a given microorganism. Instead, solvent toxicity has been estimated from inhibition against microbial growth on a medium overlaid with a large volume of the solvent (Inoue and Horikoshi 1989; Aono et al. 1994; Ramos et al. 1995; Sikkema et al. 1995). From these experiments, an interesting empirical rule has been proposed, as follows: the toxicity of a solvent is correlated inversely with log P_{ow} of the solvent. Each bacterium is tolerant to a solvent having a log P_{ow} value greater than or equal to a certain value. Here, log P_{ow} is the common logarithm of P_{ow}, the partition coefficient of the solvent between *n*-octanol and water layers (Leo 1993).

This empirical rule has several weak points when applied to a two-phase liquid culture system. Even when a large volume of hydrophobic solvent is added to a medium, the concentration of the solvent does not exceed the solubility limit in the aqueous medium. Under these conditions, we can observe growth inhibition with the solvent at the saturation concentration. We cannot obtain any information on toxicity of the added solvent to the microbes, such as minimum inhibitory concentration. However, we can observe the extent of the growth inhibition strength of the solvents, which the empirical rule does not show.

Now, one aspect of the basis of the mechanisms of bacterial resistance to hydrophobic solvents has been resolved. The solvent resistance of *E. coli* is closely related with the multiple drug resistance (Aono et al. 1995). Toluene was maintained at a low intracellular level in *Pseudomonas putida* exposed to toluene by the active efflux system, although the concentration of toluene added was below the saturation concentration (Isken and de Bont 1996). Bioenergy production is indeed required for maintaining the intrinsic level of the solvent resistance in certain bacteria in

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the two-phase system (Noguchi et al. 1997). It is known that solvent efflux pumps are important for solvent resistance in several bacteria (White et al. 1997; Aono et al. 1998; Fukumori et al. 1998; Kieboom et al. 1998; Li et al. 1998; Ramos et al. 1998; Mosqueda and Ramos 2000).

It is necessary to know quantitatively the growth inhibition strength of each solvent for further detailed studies on solvent resistance mechanisms. We have devised a method to evaluate the growth inhibition strength of hydrophobic solvents on the basis of the extent of bacterial growth occurring in two-phase systems.

Inhibition of bacterial growth in two-phase systems

Bacteria differing in solvent resistance were grown overnight in LBGMg medium (1% Bacto tryptone (Difco), 0.5% Bacto yeast extract (Difco), 1% NaCl, 10 mM $MgSO_4$, and 0.1% glucose) in the absence of any solvent. Each bacterium was inoculated in a two-phase culture system consisting of LBGMg medium and one of various hydrocarbons listed in Table 1. The culture system (10 ml of the medium and 1 ml of solvent) was placed in a test tube (24×200 mm) and shaken at 150 rpm. Each tube was closed with a butyl rubber stopper after inoculum of each bacterium was added. Growth of each bacterium in the two-phase system was followed by measuring the optical density at 660 nm (OD_{660}). The bacteria tested were *P. putida* Px51T (toluene-resistant) (Noguchi et al. 1999), *E. coli* OST3410 (cyclohexane-resistant), *E. coli* JA300 (*n*-hexane-resistant), and *E. coli* JA300T (decane-resistant). OST3410 and JA300T are derivatives constructed from JA300 by *marR* mutation and *tolC* deletion, respectively (Aono 1998; Aono et al. 1998). The solvent resistance level of each bacterium shown was measured on the basis of growth on agar overlaid with the solvent.

E. coli JA300 grew with no obvious inhibition in the two-phase culture system containing a weakly polar solvent such as decane, nonane, octane, heptane, or cyclooctane. *n*-Hexane, *p*-cymene, or propylbenzene prolonged the lag phase of growth of JA300. Heptane and diethylbenzene lowered the growth rate of JA300. JA300 did not grow in medium saturated with highly polar solvents such as cyclohexane or *p*-xylene.

n-Hexane and cyclohexane lowered the growth yield of Px51T but not the initial growth rate. The growth rate of Px51T was lowered in the presence of toluene. The lag phase of growth of Px51T was greatly prolonged in the presence of 1-hexene. Px51T did not grow at all in the presence of benzene.

Strength of microbial growth inhibition in the two-phase culture systems

The profiles of growth inhibition caused by solvents differed, depending on the strain and the solvent. The harmful solvents mainly prolonged the lag phase or lowered the growth rate in the case of JA300, and reduced the growth yield or lowered the growth rate in the case of Px51T. Several authors have reported that solvents cause such bacterial growth inhibition. Despite the difference in profiles of growth inhibition, the observations suggest that the growth yield could be used as a scale representative of the growth inhibition strength of a solvent against a particular bacterium. We attempted to estimate the magnitude of growth inhibition caused by each solvent through comparison of the extent of growth of each bacterium in the two-phase culture systems.

Figure 1 shows the relative turbidity of the medium phase of the two-phase culture system in which Px51T was grown. The relative turbidity was calculated from OD_{660} of the medium phase in the absence of solvent. Some of the culture medium became somewhat turbid with solvent droplets dispersed in the medium. We tried to avoid this

Table 1. Organic solvents tested and their log P_{ow} values

Organic solvents	log P_{ow}[a]	Solvent number[b]
Saturated or unsaturated normal aliphatic hydrocarbons		
1-Pentene	2.86	1
n-Pentane	3.34	2
1-Hexene	3.38	3
n-Hexane	3.87	4
1,7-Octadiene	3.96	5
n-Heptane	4.40	6
1-Octene	4.44	7
n-Octane	4.93	8
n-Nonane	5.45	9
n-Decane	5.98	10
Alicyclic hydrocarbons and their alkyl derivatives		
Cyclopentane	2.79	11
Ethylcyclopentane	3.31	12
Cyclohexane	3.35	13
Methylcyclohexane	3.87	14
1,4-Dimethylcyclohexane	4.39	15
Ethylcyclohexane	4.40	16
Cyclooctane	4.47	17
Butylcyclohexane	5.46	18
Aromatic hydrocarbons and their alkyl derivatives		
Benzene	2.14	19
Toluene	2.64	20
p-Xylene	3.14	21
Ethylbenzene	3.17	22
Cumene	3.57	23
Mesitylene	3.64	24
p-Ethyltoluene	3.67	25
Propylbenzene	3.70	26
p-Cymene	4.07	27
p-Diethylbenzene	4.23	28
n-Butylbenzene	4.24	29

[a]The log P_{ow} values of the organic solvents were calculated by the addition rule by using log P_{ow} calculation software, ClogP version 4.0 (Bio Byte, Claremont, CA, USA)

[b]Solvent number is used to specify each solvent in Figs. 2 and 3

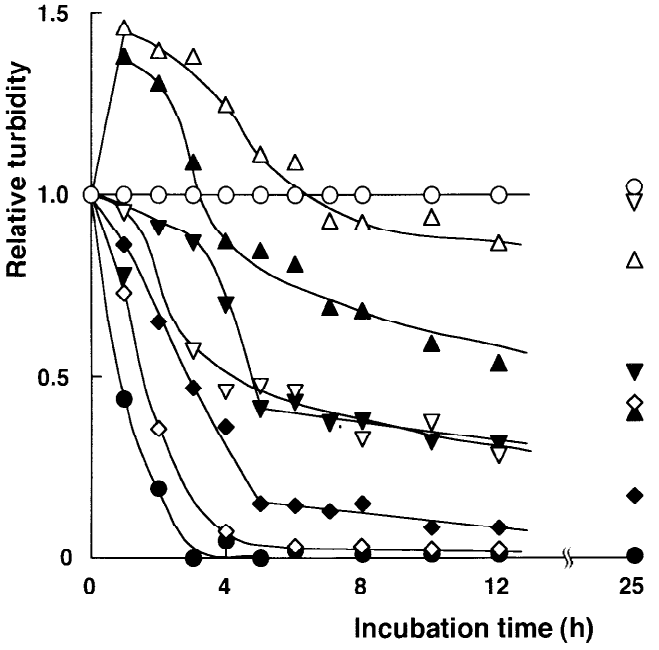

Fig. 1. Relative amount of growth of *Pseudomonas putida* Px51T in two-phase systems. *P. putida* Px51T was grown in LBGMg medium overnight. The culture was diluted 100 fold with the fresh medium. The subcultures were incubated aerobically after addition of a 10% volume of one of various organic solvents. Growth was followed by measuring the turbidity of the medium phase at 660 nm after more than threefold dilution with saline. The relative turbidity was calculated from the extent of OD_{660} of the medium phase, taking the turbidity in the absence of any solvent as 1.0. Symbols: *open circles*, control; *solid circles*, benzene (log P_{ow} 2.1); *solid diamonds*, toluene (2.6); *inverted solid triangles*, cyclohexane (3.4); *open diamonds*, 1-hexene (3.4); *inverted open triangles*, propylbenzene (3.7); *solid triangles*, *n*-hexane (3.9); *open triangles*, *p*-cymene (4.1)

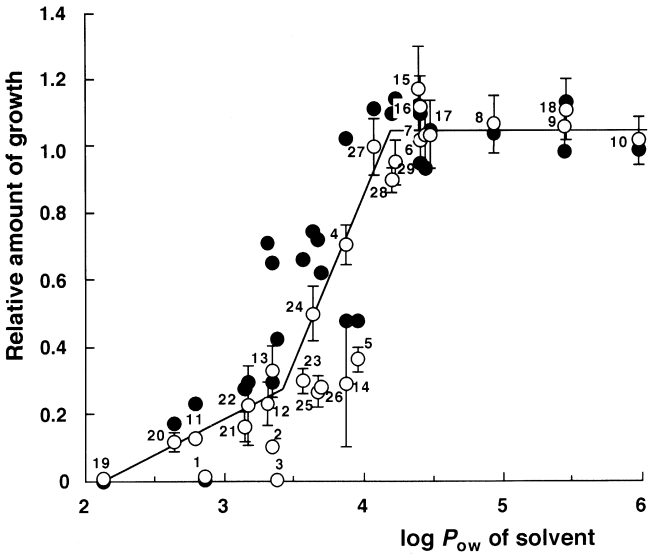

Fig. 2. Growth inhibition strength of solvents against *P. putida* Px51T in the two-phase culture systems. *P. putida* Px51T was grown at 30°C. The solvents used are listed in Table 1. The *numbers* shown in the figure indicate the solvent number listed in Table 1. The relative amount of growth was calculated periodically as shown in Fig. 1. The relative amount of growth versus log P_{ow} calculated for the solvent used is shown. Symbols: *open circles*, mean value of the relative amounts of growth for a 5- to 10-h period; *solid circles*, relative growth at 25 h. Standard errors were calculated from the relative growth for a 5- to 10-h period

pseudo-high turbidity, not attributable to the growth of Px51T, by measuring the OD_{660} of medium diluted with saline more than threefold. Thus, the turbidometric measurement contained some error at the beginning of the cultivation period because of low accuracy in measuring a low OD_{660}. For this reason, the pseudo-high turbidity was significant at the beginning. The relative turbidity almost reached a constant value during the late exponential phase of growth in the case of most solvents because the pseudo-high turbidity became negligible as the result of increased growth of Px51T. The relative turbidity became representative of the relative amount of growth during the late exponential phase of growth. In the presence of 1-hexene, the relative turbidity continued to increase after a long incubation period because of the growth of putative derivatives with increased resistance. Also, in the case of JA300, relative turbidity became constant in the late exponential phase of growth.

To demonstrate the growth inhibition strength of each solvent, we used the amount of growth of Px51T in the two-phase system while the bacterium was in the late exponential phase of growth in the absence of the solvent. Figure 2 shows the relative amount of growth calculated from the extent of growth of Px51T for a 5- to 10-h period in the presence of each solvent listed in Table 1. Also, Fig. 2 shows the relative growth of Px51T, calculated from the growth extent at 25 h. The relative amount of growth in the presence of certain solvents became great after 25 h of incubation because growth of Px51T had already ceased in the control culture. Such solvents included those causing prolongation of the lag phase period, such as 1-hexene. In addition, the relative amount of growth significantly fluctuated among the culture batches after 25 h because of the appearance of derivatives showing improved solvent resistance. The relative amount of growth calculated from the extent of growth for the 5- to 10-h period was correlated with the growth inhibition strength of the solvent, although the estimate might be inadequate for the strength of a solvent causing prolongation of the lag phase.

Comparison of the strength of microbial growth inhibition in the two-phase systems

Analysis of the magnitude of growth inhibition showed interesting characteristics in the case of Px51T. Solvents with log P_{ow} greater than 4.0 did not affect the growth of Px51T, indicating that Px51T is tolerant of such weakly polar solvents (see Fig. 2). The amount of growth was low in the presence of a solvent with log P_{ow} less than 3.9. Px51T

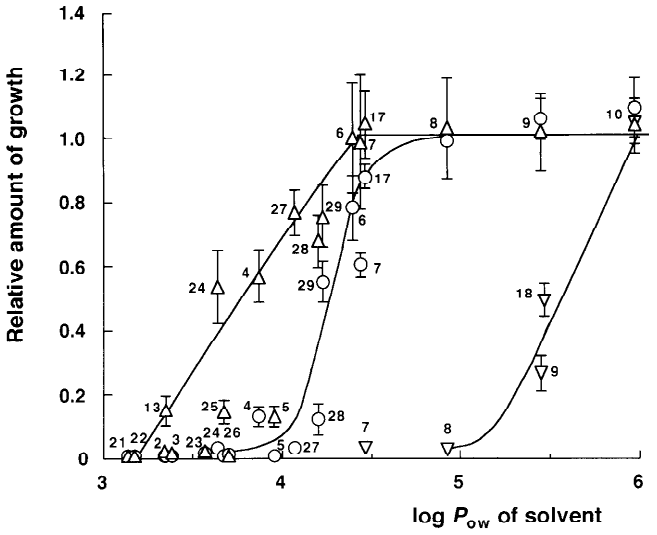

Fig. 3. Growth inhibition strength of solvents against *E. coli* JA300 and its derivatives in two-phase culture systems. *E. coli* JA300 (*open circles*), OST3410 (*open triangles*), and JA300T (*open inverted triangles*) were grown at 37°C in the two-phase systems, as shown in Fig. 2. The relative amount of growth in a 4- to 10-h period was calculated, and mean values are shown. Standard errors were calculated from the relative growth for a 4- to 10-h period. Each solvent used is indicated by the *solvent number* listed in Table 1

grows on agar overlaid with toluene or a solvent with log P_{ow} greater than 2.6. However, Fig. 2 shows that growth of Px51T is inhibited by such solvents with comparatively high polarity. The growth of Px51T was suppressed strongly by alkenes with log P_{ow} values greater than that of toluene, such as 1-pentene and 1-hexene. The growth inhibition strength of mesitylene, *p*-ethyltoluene, propylbenzene, methylcyclohexane, *n*-hexane, or 1,7-octadiene was not correlated with polarity of the solvent alone. The qualitative observation of growth on agar overlaid with a solvent did not show such a detailed difference in growth inhibition strength among the solvents.

Solvents with log P_{ow} values greater than 4.9 did not affect the growth of JA300, but those with log P_{ow} values less than 4.5 lowered the relative amount of growth of JA300 (Fig. 3). Also, JA300 was hypersensitive to alkenes, such as 1,7-octadiene and 1-octene. Although JA300 grows on agar overlaid with *n*-hexane, the relative amount of growth in the presence of *n*-hexane was remarkably low. JA300T was hypersensitive to the solvents tested. This result supports the view that the TolC protein is involved in maintaining the intrinsic solvent resistance of *E. coli* (Aono et al. 1998).

OST3410 showed elevated resistance to most solvents with log P_{ow} values in the range of 3.4 to 4.5. However, this strain was sensitive to 1-hexene, cumene, *p*-ethyltoluene, propylbenzene, or 1,7-octadiene. The elevated solvent resistance in the case of OST3410 is considered to result from a high level of the AcrAB-TolC pump, which is responsible for extruding hydrophobic compounds (Aono et al. 1998). Presumably, the solvents to which resistance is increased in OST3410 are good substrates for the efflux pump. In the case of OST3410, the magnitude of growth inhibition caused by solvents with log P_{ow} values in the range of 3.4 to 4.0 is not related to the log P_{ow} of the solvent. It is interesting that cumene, mesitylene, *p*-ethyltoluene, and propylbenzene differed in growth inhibition strength although these solvents have similar polarity and structure. These results suggest that these solvents differ in terms of suitability as substrate for the AcrAB-TolC efflux pump or in terms of their rate of influx into the membrane.

The relative amount of growth estimated for bacteria cultured in two-phase systems provides information of the growth inhibition strength of solvents at saturation concentrations. Comparison of inhibition strength showed that the magnitude of bacterial growth inhibition caused by a solvent was not always correlated with the log P_{ow} of the solvent.

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