8.2 Diversity and Ecology of Organic Solvent Tolerant Microorganisms

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The results of our investigation of solvent tolerance clarified that the growth of *Pseudomonas putida* strain IH-2000 differed depending on the organic solvent added to the culture medium. To determine how organic solvents affect the growth of microorganisms, the structural correlation between growth and the type of organic solvent was examined.

The physical basis of solvent toxicity is still poorly understood, and no physical parameter has been identified by which the relative toxicity of different solvents can be determined. Consequently, the solvent tolerance of microorganisms has not yet been correlated with organic solvent type. To investigate the correlation between the solvent tolerance of microorganisms and solvent toxicity, we isolated solvent-sensitive mutants using the replica-plating method in conjunction with penicillin selection after culture with 1-methyl-3-nitro-1-nitrosoguanidine (NTG). First, we constructed a Leu and Trp auxotroph from strain IH-2000 after two NTG treatments. We then isolated three toluene-sensitive mutants from 3,000 colonies of mutant cells. Remarkably, one of these (IH-2124T) was sensitive to toluene but resistant to xylene and cyclohexane.

We subsequently isolated one xylene-sensitive mutant (IH-2124TX) from 2,000 colonies of mutant cells derived from the toluene-sensitive strain. This strain was sensitive to both toluene and xylene, but resistant to cyclohexane. A cyclohexane-sensitive mutant (IH-2124TXC) that remained hexane resistant was isolated from strain IH-2124TX. Each of these mutants retained the Leu and Trp auxotrophic markers. The toluene-sensitive mutant, for which the frequency of back-mutation was less than 10^{-9}, was very stable. The xylene-sensitive and cyclohexane-sensitive mutants exhibited frequencies of back-mutation of 10^{-7} and 10^{-8}, respectively. In addition to toluene, these strains tolerated other toxic solvents including aliphatic hydrocarbons, alicyclic hydrocarbons, aromatic hydrocarbons, alcohols, and ethers.

By examining the partition efficient of an organic solvent, we can predict accurately the tolerance of microorganisms to other solvents in the polarity scale. It was found that the effects of solvents on the growth of toluene-tolerant strain IH-2000 and its toluene-tolerant mutants were quantitatively correlated by the parameter log P (Corwin and Anderson 1967; Rekker and de Kort 1979), where P is the partition coefficient of solvent dissolved in an equimolar mixture of octanol and water. Log P is also used as a quantitative index of solvent polarity in quantitative structure-activity relationship analysis (Hansch and Fujita 1964; Harnish et al. 1983). The correlations between log P values of solvents and the growth limits of strain IH-2000 and its three mutants are given in *Table 8.2.1*, together with those of other bacteria and yeasts. It is evident that although strain IH-2000 grows in solvents with log P values $\geq$2.4, such as heptanol (log P = 2.4), toluene (log P = 2.8), and *n*-octane (log P = 4.9), it does not grow in fuorobenzene, benzene, and butanol with log P values of 2.3, 2.1, and 0.8, respectively. Therefore, it appears that a critical point is reached between log P values of 2.4 and 2.3, at which the solvent is sufficiently polar to prevent growth. The concept of such a tolerance limit can be extended to predict the growth of other microbial species in solvent-saturated environments (Inoue and Horikoshi 1989).

These observations indicate that the nature of the interaction between the solvent and the cell surface which limits growth is at least partly determined by solvent polarity. The unusually high tolerance of strain IH-2000 to toluene and other solvents probably reflects the presence of cell surface components that are unique to the strain. In addition to mutant strains of *P. putida*, they can order other microorganisms in a similar hierarchy of solvent tolerance, in which growth in a solvent of a given polarity indicates tolerance to solvents of lower polarity.

Table 8.2.1
Solvent tolerance of bacteria and mutants

Solvent	Log P	IH-2000	IH-2124T	IH-2124TX	IH-2124TXC	*Escherichia. Coli* IFO 3806	*Pseudomonas. putida IFO 3738*	*P.fluorescens* IFO 3507	*Achromobacter delicatlus IFO 3058*	*Agrobacterium* tumefaciens IFO 3058	*Alcaligenes faecalis* JCM 1474	*Aeromonas hydrophila* JCM 1027	*B.Subtilis* AHU 1219	*Corynebacterium glutamicum* JCM 1318	*S. uvarum* ATCC 26602
Dodecane	7.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Decane	6.0	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Nonane	5.5	+	+	+	+	+	+	+	+	+	+	+	+	−	−
n-Hexylether	5.1	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Octane	4.9	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Isooctane	4.8	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Cyclooctane	4.5	+	+	+	+	+	+	+	+	−	+	+	−	−	−
Diphenylether	4.2	+	+	+	+	+	+	+	+	−	−	−	−	−	−
n-Hexane	3.9	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Propylbenzene	3.8	+	+	+	−	+	+	+	−	−	−	−	−	−	−
o-Dichlorobenzene	3.6	+	+	+	−	−	+	+	−	−	−	−	−	−	−
Cyclohexane	3.4	+	+	+	−	−	+	+	−	−	−	−	−	−	−
Ethylbenzene	3.3	+	+	−	−	−	+	−	−	−	−	−	−	−	−
p-Xylene	3.1	+	+	−	−	−	+	−	−	−	−	−	−	−	−

Table 8.2.1 (Continued)

Solvent	Log P	IH-2000	IH-2124T	IH-2124TX	IH-2124TXC	*Escherichia. Coli* IFO 3806	*Pseudomonas. putida* IFO 3738	*P.fluorescens* IFO 3507	*Achromobacter delicatlus* IFO 3058	*Agrobacterium tumefaciens* IFO 3058	*Alcaligenes faecalis* JCM 1474	*Aeromonas hydrophila* JCM 1027	*B.Subtilis* AHU 1219	*Corynebacterium glutamicum* JCM 1318	*S. uvarum* ATCC 26602
Styrene	2.9	+	+	–	–	–	–	–	–	–	–	–	–	–	–
Toluene	2.8	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Heptanol	2.4	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Benzene	2.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Limiting log P value for growth		2.4	2.9	3.4	3.9	3.8	3.1	3.4	3.9	4.8	4.5	4.5	4.9	7.0	7.0

+, growth; –, no growth

Diversity of Solvent-Tolerant Microorganisms Based on the Solvent Parameter log P

In previous systems, a direct correlation was observed between the solvent tolerance of microbes and the solvent parameter log P. It therefore appears that log P can serve as a quantitative index of the inherent ability of a solvent to limit the growth of microbes in solvent environment.

It was shown that the relative toxicities of different solvents are determined by their polarity. Limitations on the growth of microorganisms exerted by high concentrations of various organic solvents were shown to be correlated with the solvent parameter log P. This indicates that the solvent tolerance of microorganisms is determined by the log P value at water-saturated solvent concentrations.

To investigate the solvent-tolerant properties of bacteria, it is useful to elucidate the mechanisms conferring solvent tolerance. This section describes the solvent-tolerant limits of bacteria in terms of the log P parameter of solvents and compares the solvent tolerance of Gram-negative and Gram-positive bacteria and yeasts.

Type strains of Gram-negative bacteria belong to the genera *Pseudomonas*, *Escherichia*, *Aeromonas*, *Alteromonas*, *Achromobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Serratia*, *Proteus*, *Klebsiella*, *Flavobacterium*, and *Chromobacterium*, while Gram-positive bacteria belong to the genera *Bacillus*, *Micrococcus*, *Staphylococcus*, *Corynebacterium*, *Brevibacterium*, *Rhodococcus*, *Leuconostoc*, and *Lactobacillus* (Inoue and Horikoshi 1991a). Yeasts belonging to the genera *Endomycopsis*, *Saccharomycodes*, *Saccharomycopsis*, *Pichia*, *Hansenula*, *Shizosaccharomyces*, *Leucosporidium*, *Bellera*, *Sporidiobolus*, *Candida*, *Kloekera*, *Rhodotorula*, *and Torulopsis* were investigated by our group. The growth of bacteria type strains was examined in modified LB medium consisting of tryptone 10 g (Difco), yeast extract 5 g (Difco), NaCl 5 g, glucose 1 g, and $MgSO_4 \cdot 7H_2O$ 10 mM in 1 L of deionized water, pH 7. Yeast type strains were cultured in a malt extract medium consisting of malt extract 20 g (Difco), glucose 20 g, peptone 1 g (Difco) in 1 L of deionized water, pH 6.2.

The solvent tolerance of the Gram-positive and Gram-negative bacteria and yeast type strains was examined on agar plates overlaid with different solvents. Cells of each strain were streaked on the agar plate medium, directly overlaid with one solvent, and incubated at 37°C for bacteria or 30°C for yeast. The growth was based on the formation of colonies on the agar plates after 48-h incubation. The limiting log P values for growth were defined as the critical point at which the solvent was sufficiently polar to prevent the growth of the strains. Different solvents with log P values ranging from 2.1 (benzene) to 7.0 (dodecane) were examined. The solvent-tolerance limits of the Gram-negative and Gram-positive bacterial type strains and yeasts are shown in *Tables 8.2.2*, *8.2.3*, and *8.2.4*, respectively.

As shown in *Table 8.2.2*, the limiting log P value for the growth of the genera *Pseudomonas* varied from 3.2 to 3.4. It could grow in such solvents as *p*-xylene (log P = 3.1) and cyclohexane (log P = 3.4), but not in solvents with low log P values, such as styrene (log P = 2.9) and toluene (log P = 2.8). *P. putida* IFO 3738, *P. chlororaphis* IFO 3904, and *P. syringae* IFO 3310 had the greatest solvent tolerance among all *Pseudomonas* species investigated. Depending on the strain, *Escherichia coli* could grow in the presence of solvents with log P values greater than 3.4 or 3.8 (cyclohexane and hexane) but not in more polar solvents. Other microorganisms examined had similar characteristic limiting log P values for growth, as shown in *Table 8.2.2*. These bacteria could grow in solvents with greater than the growth-limiting log P value but not in more polar solvents with low log P.

Table 8.2.2

Solvent tolerance of Gram-negative bacteria

Type strains			Limiting log P values for growth
Pseudomonas aeruginosa	I FO	3924	3.4
Pseudomonas putida	I FO	3738	3.1
Escherichia coli	I FO	3806	3.8
Escherichia coli	I FO	3366	3.4
Aeromonas hydrophila	JCM	1027	4.5
Aeromonas hydrophila	I FO	3820	4.2
Alteromonas putrefaciens	I FO	3908	4.2
Achromobacter delicatulus	IAM	1433	3.9
Agrobacterium tumefaciens	I FO	3058	4.8
Alcaligenes faecalis	JCM	1474	4.5
Serratia marcescens	I FO	3406	3.4
Proteus vulgaris	I OF	3167	4.2
Proteus mirabilis	I FO	3849	3.8
Klebsiella pneumonia	I FO	3317	3.4
Flavobacterium lutescens	I FO	3084	4.0
Flavobacterium suaveolens	I FO	3752	5.1
Chromobacterium chocolatum	I FO	3758	7.0

Table 8.2.3

Solvent tolerance of Gram-positive bacteria

Type strains			Limiting log P values for growth
Bacillus subtilis	AHU	1390	4.5
Bacillus subtilis	I FO	3009	5.1
Bacillus circulans	I FO	3329	7.0
Micrococcus luteus	I FO	3333	4.8
Micrococcus roseus	I FO	3764	4.8
Staphylococcus epidermidis	I FO	3762	4.8
Staphylococcus faecalis	I FO	3826	5.1
Corynebacterium glutamicum	JCM	1318	7.0
Corynebacterium flavescens	IAM	1614	6.0
Brevibacterium ammoniagenes	I FO	12072	7.0
Brevibacterium roseum	ATCC	13825	6.0
Rhodococcus erythropolis	I FO	12320	6.0
Rhodococcus eque	I FO	3730	7.0
Leuconostoc mesenteroides subsp. *dextran*	I FO	3425	5.1

Table 8.2.4

Solvent tolerance of yeasts

Type strains			Limiting log P values for growth
Endomycopsis tibliglr	N I	7404	5.1
Saccharomycodes ludwigii	I FO	0798	5.1
Sacchromyces cerevisiae	I FO	0213	5.1
Sacchromyces uvaram	ATCC	26602	7.0
Sacchromycopsis lipolitica	I FO	0746	4.2
Pichia membranaefaciens	I FO	0989	5.5
Pichia farinose	I FO	1003	4.8
Hansenula americana	I FO	1368	3.9
Shizosaccharomyces octosporas	IAM	4842	4.8
Leucosporidium nivalis	I FO	1922	5.5
Bellera alba	I FO	1192	6.0
Sporidiobolus johnsonii	I FO	6903	4.8
Candida sake	I FO	0435	7.0
Candida tropicalis	I FO	0589	3.9
Candida utilis	I FO	0619	5.1
Kloekera africana	I FO	1155	4.9
Kloekera corticis	I FO	0633	5.1
Rhodotorula aurantiaca	I FO	0951	5.1
Rhodotorula glutimis var. *daireesis*	I FO	0415	4.8
Torulopsis famata	N I	7550	3.9

As shown in *Table 8.2.3*, the limiting log P value for the growth of *Bacillus* species ranged from 4.5 to 7.0. They could grow in the presence of cycloctane and dodecane but not in solvents with lower log P values, such as trichlorobenzene (log P = 4.3) and hexane (log P = 3.9). *Bacillus subtilis* AHU 1390 had the highest solvent tolerance among the *Bacillus* species examined. *Table 8.2.3* also shows that other Gram-positive bacteria have characteristic limiting log P values for growth.

Gram-negative bacteria showed greater solvent tolerance than Gram-positive ones. Among all the microorganisms examined, the *Pseudomonas* group showed the greatest solvent tolerance. On the other hand, the solvent tolerance of *Chromobacterium* and some species in the *Bacillus*, *Corynebacterium*, *Brevibacterium*, and *Rhodococcus* genera was low. Other genera showed moderate solvent tolerance as indicated by their limiting log P values, which ranged from 3.4 (cyclohexane) to 5.1 (hexylether).

Based on these results, the solvent tolerance of Gram-negative and Gram-positive bacterium examined was in the order *Pseudomonas* > *Escherichia*, *Serratia*, *Klebsiella* > *Achromobacter*, *Acinetobacter*, *Proteus* > *Alteromonas*, *Aeromonas*, *Flavobacterium* > *Alcaligenes*, *Bacillus* > *Agrobacterium*, *Micrococcus*, *Staphylococcus* > *Streptococcus* > *Leuconostoc*, *Lactobacillus* > *Coryerinebacterium*, *Chromobacterium*, *Brevibacterium*, *Rhodococcus*.

As shown in Table 8.2.4, the limiting log P value for the growth of the yeast group ranged from 3.9 to 7.0. Among all the yeasts examined, the *Hansenura*, *Candida*, and *Torulopsis* group showed the greatest solvent tolerance and were able to grow in the presence of *n*-hexane (log P = 3.9). On the other hand, the tolerance of *Sacchromyces uvarum* and *Candida sake* was the lowest in all the yeasts examined, although they grew in the presence of *n*-dodecane (log P = 7.0). Based on these results, the solvent tolerance of yeasts was in the order *Hansenura*, *Candida*, *Torulopsis* > *Saccharomycopsis* > *Shizosaccharomyces*, *Sporidiobolus*, *Rhodotorula*, *Pichia* > *Kloeckera* > *Endomycopsis*, *Sacchromycodes*, *Saccharomyces* > *Leucosporidium* > *Bullera*.

Figure 8.2.1 shows a comparison of the solvent tolerance of each micro population. The Gram-negative bacteria were able to grow in the presence of solvents with growth-limiting log P values ranging from 3.1 to 7.0. The limiting log P values for Gram-positive bacteria and yeasts ranged from 3.4 to 7.0. The solvent tolerance of Gram-negative bacteria was therefore greater than that of Gram-positive bacteria and yeasts, while that of the latter two groups was similar.

These cumulative results suggest that the polarity of solvents affects the cell surface characteristics and thus the growth of bacterial and yeast strains. It may therefore be possible that certain unique cell-surface properties control the growth of some strains in water-saturated organic solvents.

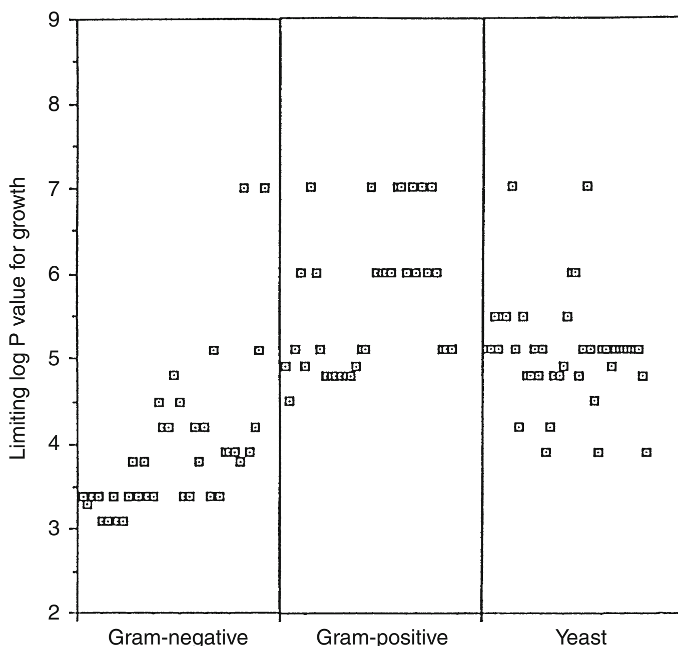

Fig. 8.2.1

Distribution map of limiting log P values of Gram-negative and Gram-positive bacteria, and yeasts

Diversity of Solvent-Tolerant Microorganisms in Deep-Sea Environments

The deep-sea bed is a unique environment that experiences extremely high pressures and low temperatures. Microorganisms living there have developed particular characteristics that allow them to thrive at such extremes. We have isolated and characterized a number of microorganisms from samples of deep-sea mud obtained by the manned submersible Shinkai 6500. This vehicle, which is operated by the Japanese Marine Science and Technology Center, (JAMSIEC), has the ability to submerge to a depth of 6,500 m. It was believed that unknown microbes we have not been able to isolate yet must be thriving in the deep-sea floor, and we attempted to isolate these microbes, especially those that are organic solvent tolerant, from the deep-sea mud samples. Such microorganisms could prove useful for new biotechnology application, such as two-phase (water/organic solvent) bioreactor systems.

Surprisingly, we observed that many organic solvent-tolerant microbes exist even in the deep-sea. Therefore, we are focusing on organic solvent-tolerant microbes and their industrial application.

The Isolation Frequency of Organic Solvent-Tolerant Microbes from Deep-Sea

To obtain information about the presence of solvent-tolerant microorganisms, we had tried to compare the isolation frequency of organic solvent-tolerant microorganisms from land soil and abyssal mud, respectively. As shown in *Table 8.2.5*, the isolation frequency in land soil was 1×10^{-8} to 3.6×10^{-8}. In contract, the isolation frequency in abyssal mud samples was 350×10^{-8} to 700×10^{-8}. From these experiments we found that 100 times as many organic solvent-tolerant microorganisms could be isolated from deep-sea mud samples as from soil samples taken from land.

As a result, we have discovered many useful organic solvent-tolerant strains from the deep-sea environment. Some of these isolates from deep-sea are shown in *Table 8.2.6*.

Benzene-Tolerant Hydrocarbon-Degrading Bacteria

Because various kinds of organic solvents (OSs), such as benzene, toluene, and *p*-xylene, are known to be components in petroleum, and the deep-sea is the ultimate receptor of polluted petroleum-hydrocarbons, we focused on deep-sea bacteria, which showed the characteristics of

Table 8.2.5

Comparison of the frequency of organic solvent-tolerant microbes from different sources

Source	Total microbes ($\times 10^5$ cells/g)	Solvent-tolerant microbes (cells/g)	Isolation frequency ($\times 10^{-8}$)
Land soil	2,800–11,500	7–16	1–3.6
Abyssal mud	54–109	19–52	350–700

Table 8.2.6

Organic solvent-tolerant microorganisms isolated from deep-sea

Strain	Property	Source	Reference
Organic solvent-tolerant bacteria			
DS-711	Degrades crude oil	Suruga Bay, 1,945 m	Moriya and Horikoshi 1993a
DS-944	Utilizes sulfur	Sagami Bay, 1,168 m	Moriya and Horikoshi 1993b
ST-1	Degrades cholesterol	Okushiri Ridge, 1,963 m	Moriya et al. 1995
DS-1906	Degrades polyaromatic hydrocarbons	Sagami Bay, 1,168 m	Abe et al. 1995
Organic solvent-tolerant yeast			
Y-40	Hydrocarbon-degrading yeast	Sagami Ridge, 1,200 m	

halotolerant growth in addition to OS tolerance (Moriya and Horikoshi 1993a, b). We also studied whether isolation of deep-sea bacteria having such characteristics could improve the degradation of polluting petroleum-hydrocarbons in marine environments.

We developed a novel, simple, efficient method for the isolation of a benzene-tolerant bacterium from deep-sea samples. To isolate benzene-tolerant bacteria, which also exhibited the characteristic of halo-tolerant growth, 1 ml of sediment samples was transferred to test tubes containing 3-ml artificial seawater. Benzene was then added at 1 ml (20% v/v), and then test tubes were plugged with silicone-rubber stoppers. The cultures were incubated at 4°C for a week in a test tube shaker. Then, 5 ml (50% v/v) of kerosene was added and the cultures were incubated for a further 5 days at 20°C. After incubation, the cultures were aseptically transferred to separating funnels and allowed to stand for 30 min. Then, the upper layers were carefully separated from the seawater layers, and a portion of each upper layer was directly spread onto M-I agar medium comprising 1 g of proteose peptone no. 3 (Difco), 0.5 g of phytone peptone (BBL), 1 g of $CaCl_2 \cdot 2H_2O$, 0.1g of $MgCl_2 \cdot 6H_2O$, 0.05 g of Na_2SO_3 and per liter of distilled water, at pH 7 and incubated for 2 day at 30°C. For microbial growth, M-II medium which contained 5 g of proteose peptone no. 3, 2.5 g of phytone peptone, 1 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $MgCl_2 \cdot 6H_2O$ and 0.05 g of Na_2SO_3 per liter distilled water, and a modified LB-medium (Inoue and Horikoshi 1991a) (tentatively named LB-1 medium in this study) were used. To prepare salt medium, the above media were supplemented with NaCl at 11.8% (w/v) unless otherwise stated. For preparation of agar medium, agar was added at 1.5%. The pH was adjusted at 6.5–7 by 1 N HCI or 1 N NaOH. Artificial seawater consisting of 35 g of NaCl, 10 g of $MgCl_2 \cdot 6H_2O$, 1 g of $CaCl_2 \cdot 2H_2O$, and 0.7 g of KCI per liter distilled water was used for the preparation of concentrated cell suspensions. As the *n*-alkane substrate for the microbial degradation experiment, kerosene which contained *n*-alkanes having 7–16 carbon atoms was used at the indicated concentration.

After treatment of the deep-sea sediment samples collected from a depth of 1,945 m in Suruga Bay with 20% v/v benzene followed by 50% (v/v) kerosene, 56 candidate colonies grown on selective medium that combined the characteristics of halotolerant growth and benzene tolerance were isolated as potential hydrocarbon degraders. These candidates belonged to various genera, including *Arthrobacter* sp., *Bacillus* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Flavobacterium* sp., and *Vibrio* sp. Finally, the bacterial strain that showed the

highest growth in the presence of 5% v/v benzene among above 56 isolates was selected as a hydrocarbon degrader that also exhibited the characteristics of halotolerant growth and benzene tolerance. This strain was identified based on its morphological and biochemical characteristics, as outlined in volume I of *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt 1984). Analysis of the G+C content of DNA was done by the method (Tamaoka and Komagata 1984). The growth temperature of selected isolates was determined using a temperature gradient bio-photo recorder (TN-112D5 Advaptec). Cells of the isolate had peritrichous flagella and showed motility. The isolate formed orange colonies on M-I and M-II salt media. It was Gram-negative, non-spore-forming, and strictly aerobic, oxidase-positive, and catalase-positive. The isolate showed resistance to many antimicrobials including streptomycin, penicillin, lincomycin, nalidixic acid, josamycin, and kanamycin and a susceptibility to novobiocin. Based on its morphological and biochemical characteristics, in addition to its characteristic of benzene tolerance the selected strain seemed to be a variant strain of *Flavobacterium* sp. and was tentatively named strain DS-711.

The OS tolerance of the *Flavobacterium* sp. strain DS-711 was compared with control strains, *E. coli* strain IFO 3806, *B. subtilis* strain IFO 3009, and the toluene-tolerant *P. putida* strain IH-2000. Tested strains were cultured on M-II salt medium and LB-I medium in the presence of OSs such as benzene, toluene, and *p*-xylene. Both, *E. coli* IFO 3806 and *B. subtilis* IFO 3009 were unable to grow not only in the presence of 1% benzene but also 10% toluene and 10% *p*-xylene, as shown in *Table 8.2.7*.

The toluene-tolerant strain IH-2000 showed considerable growth in the presence of 10% toluene, and 10% *p*-xylene, but it could not grow in the presence of more than 1% benzene. In contrast to IH-2000, DS-711 could grow in the presence of not only 10% toluene and 10% *p*-xylene but also 5% benzene. The tolerance of strain DS-711 to various toxic OSs in addition to benzene, toluene, and *p*-xylene, including saturated and unsaturated aliphatic hydrocarbons, alicyclic hydrocarbons, aromatic hydrocarbons, alcohol, and ethers, was further examined. DS-711 tolerated various toxic OSs, though others such as methoxytoluene, propanol, butanol, heptanol, octylalcohol, benzylether, cyclohexanone, and ethylacetate were not tolerated. Subsequently, we investigated the correlation between the log P value of the OSs and growth on the OSs using the OS-tolerant strains DS-711 and IH-2000.

Table 8.2.7

Comparison of organic solvent-tolerant between strain DS-711 and soil strains

	Growth (OD_{660})			
Solvent (log P)	Strain DS-711	*P.putida* IH-2000	*E.coli* IFO 3806	*Bacillus.subtilis* IFO 3009
No addition	1.36	2.98	3.05	2.95
Benzene 1% (2.1)	1.33	NG	NG	NG
Benzene 5%	1.08	NG	NG	NG
Toluene 10% (2.7)	1.18	2.88	NG	NG
p-Xylene 10% (3.1)	1.06	2.64	NG	NG

Growth was determined by measuring the optical density at 660 nm.
ND, no degradation; NG, no growth

Degradation of *n*-alkanes in kerosene and aromatic hydrocarbons by strain DS-711 was compared with that of the control strains, *Alteromouas* sp. DS-201 and *P. putida* IH-2000. To measure their ability to degrade *n*-alkanes in kerosene and aromatic hydrocarbons, one loop of strains was inoculated in 5,000-ml flasks containing 1,000 ml M-II salt medium and LB-I medium. Incubation was carried out for 2 days at 30°C. After incubation the cultures were centrifuged at 12,000 rpm and washed two times with artificial seawater and sterile distilled water to prepare a 15 ml concentrated cell suspension. The cell suspensions were inoculated into 100 ml fresh M-II salt medium and LB-1 medium at an initial concentration of 1×10^7 or 1×10^9 cells/ml and 10 ml (10% v/v) of kerosene and 1 ml (1% v/v) of aromatic hydrocarbon were overlaid individually. When the stepwise addition of the concentrated cell suspension was complete, the suspensions were added after 2 days and 3 days. An additional no inoculated control was run for measurement through evaporation. Inoculated samples and the additional control were incubated with shaking at 100 rpm for a week at 30°C. At the end of the incubation, cultures were transferred to separating funnels for extraction and analysis of hydrocarbons. The percentage degradation of hydrocarbons is given as the difference between the additional control and the inoculated samples. The total *n*-alkane content was made up of the total content of *n*-alkanes having 7–16 carbon atoms. Cultures on kerosene and aromatic hydrocarbons were centrifuged. Supernatants were transferred to separating funnels for hydrocarbon extraction. Residual kerosene and aromatic hydrocarbon oil droplets were separated from the lower aqueous layer. Then 50 ml of benzene containing 0.1 g of *n*-eicosane as an internal standard was added to the kerosene oil droplets. Kerosene was extracted with benzene as described in detail elsewhere (Walker and Colwell 1974; Walker et al. 1975). Residual aromatic hydrocarbon was extracted with 4-ml of *n*-decane. Any remaining water was removed by drying over Na_2SO_4. Anhydrous extracts were transferred to a vial and stored at −20°C until gas chromatography (GC) analysis was performed.

As shown in *Table 8.2.8*, all the strains used in this experiment could degrade *n*-alkanes in kerosene but did not degrade aromatic hydrocarbons. However, *n*-alkane degradation by strain DS-711 was elevated remarkably compared to that of the control strains, DS-201 and IH-2000. In particular, percentage degradation of *n*-decane and *n*-undecane by both of control strains was approximately 10%, whereas by the strain DS-711 it was 71.4% and 68%, respectively. DS-711 showed the highest *n*-alkane degradation among the strains used, with the degradation of *n*-alkanes by the bacterium reaching approximately 85% after 7 days.

The deep-sea bacterial strain *Flavobacterium* sp. DS-711, which was isolated by our novel, simple, efficient method, exhibited excellent benzene tolerance.

Because microorganisms that are able to grow in the presence of 5% benzene have not yet been found, it will be of interest to compare the cell surface components of the benzene-tolerant strain DS-711 with those of strain IH-2000. We tried to isolate a benzene-tolerant plasmid from DS-711, but no plasmid was obtained. As strain DS-711 harbors no plasmid DNA, the OS tolerance of this bacterium is thought to be dominated by information in genomic genes. Generally, Gram-negative bacteria show higher OS tolerance than Gram-positive ones (Inoue and Horikoshi 1989, 1991a). At present, we are investigating the mechanism of OS tolerance from two aspects: cell surface components and genetics. The discovery of a benzene-tolerant bacterium from the deep-sea is also valuable from the view point of microbial ecology.

Subsequently, hydrocarbon degradation by the benzene-tolerant DS-711 was compared with that of the control strains, DS-201 and the toluene-tolerant IH-2000. As shown in *Table 8.2.8*, with a shaking culture the degradation of *n*-alkanes in kerosene by the

Table 8.2.8

Hydrocarbon degradation by solvent-tolerant strains

Hydrocarbon	Degradation (%)		
	P.putida IH-2000	*Alteromonas* sp. DS-201	DS-711
n-Alkanes			
n-C_7	28	30.5	97
n-C_8	30.9	30.3	82.1
n-C_9	35.7	32.4	82.1
n-C_{10}	10.6	9.7	71.4
n-C_{11}	11.8	10.8	68
n-C_{12}	40.2	41.4	86.7
n-C_{13}	46.2	45.3	85.8
n-C_{14}	39.9	44.6	88
n-C_{15}	35.7	39	92.9
n-C_{16}	40.2	38.1	80.5
Aromatic hydrocarbons			
Benzene	NG	NG	0
Toluene	0	NG	0
p-Xylene	0	NG	0

Degradation (%) was calculated by the following equation.

$$\text{Degradation}(\%) = \frac{\begin{matrix}\text{Residual hydrocarbon conc.} \\ \text{of an addtional control}\end{matrix} - \begin{matrix}\text{Residual hydrocarbon conc.} \\ \text{of inoculated samples}\end{matrix}}{\text{Residual hydrocarbon conc. of an additional control}} \times 100$$

The residual *n*-alkane contents (mg) in 10 ml kerosene after treatment of additional control are : *n*-C_7, 34; *n*-C_8, 39; *n*-C_9, 190; *n*-C_{10}, 245; *n*-C_{11}, 270; *n*-C_{12}, 653; *n*-C_{13}, 501; *n*-C_{14}, 332; *n*-C_{15}, 188, *n*-C_{16}, 39.
NG, no growth.

benzene-tolerant DS-711 was greater than that of our selected control strains DS-201, and IH-2000. In particular, degradation of *n*-decane and *n*-undecane, which were hardly degraded by the control strains, were markedly elevated. However, these strains, including DS-711 showed a percentage degradation of *n*-decane and *n*-undecane lower than that of other *n*-alkanes such as *n*-heptane, *n*-octane, *n*-nonane, *n*-dodecane, *n*-tridecane, *n*-teiradecane, *n*-pentadecane, and *n*-hexadecane. This was thought to be due to lower activity of the emulsifying and solubilizing substance in regard to *n*-decane and *n*-undecane in kerosene. A study of the relationship between hydrocarbon degradation and hydrocarbon emulsification and solubilization is now in progress.

We are examining the identification and characterization of the hydrocarbon emulsifying and solubilizing substance in addition to elucidating OS tolerance and hydrocarbon degradation. Interestingly, neither DS-711 nor IH-2000 degraded aromatic hydrocarbons, such as benzene, toluene, and *p*-xylene. Hydrocarbon degradation is associated with assimilation. Accordingly, these results indicated that tolerance to aromatic hydrocarbons, such as benzene, toluene, and *p*-xylene, is distinguishable from tolerance to *n*-alkanes at least. The *n*-alkane

degrading ability of this bacterium will be useful for the bioremediation of oil spills, especially marine oil spills. Because of the OS tolerance of the bacterium is also available for commercial application in industrial biotransformation processes that involve the use of water insoluble organic substrates, such as biocatalysis in a water-OS two-liquid-phase system, discovery of the benzene-tolerant strain DS-71 1 is worthy of note.

Benzene-Tolerant Sulfur-Degrading Bacteria

We attempted to isolate an OS-tolerant bacterium from the deep-sea to degrade sulfur compounds in the presence of OS (Moriya and Horikoshi 1993b). Deep-sea sediment slurry samples were aseptically collected by sterile core samplers from a depth of 1,168 m in Sagami Bay. Screening the OS-tolerant sulfur degrading bacteria was done by a modification of the method described previously (Moriya and Horikoshi 1993a). Benzene (5 ml) was added to 5 ml of deep-sea sediment slurry, which was then incubated at 25°C for 7 days. After this preincubation the benzene layer was separated from the seawater layer, and the former was spread onto M-II agar medium (Moriya and Horikoshi 1993a) with 5.8% NaCl, 1% $Na_2S_2O_3$, and 0.002% phenol red. The plates were incubated at 37°C for 7 days. The sulfur degrading ability could be detected directly on the plates because of the clear zone that forms around colonies that produce sulfuric acid from $Na_2S_2O_3$, caused by a pH decrease. Thus, microorganisms in which a clear zone formed around colonies were selected and purified. One was designated strain DS-994. This bacterium was gram-positive, spore forming, aerobic, catalase-positive, oxidase-negative, gelatin-liquefying, and motile. It is rod-shaped (0.3–5 × 0.6–3 μm) and has flagella. From its morphological and physiological properties and its characteristics of halophilic growth, sulfur utilization, and OS tolerance, DS-994 seems to be a variant strain of *Bacillus* sp. Strain DS-994 displayed optimal growth at an initial pH 7, NaCl concentration of 12%, and 37°C.

To investigate the influence of OSs on the growth of strain DS-994, LB-II medium which contained 5 g of yeast extract (Difco,USA), 10 g of tryptone (Difco), 1 g of $CaCl_2 \cdot 2H_2O$, and 0.1 g of $MgCl_2 \cdot 6H_2O$ per liter of distilled water was used. The initial pH of the media was adjusted to 7, by NaOH. When incubated for 5 days, the strain showed good growth in the presence of 5% benzene, 10% toluene, and 10% *p*-xylene, respectively. In this OS tolerance experiment, *E. coli* strain IFO 3806, *B. subtilis* strain IFO 3009 and a representative OS-tolerant *P. putida* strain IH-2000 (Inoue and Horikoshi 1989, 1991a) were employed as controls and incubated on LB-I medium and LB-II medium supplemented with 0.5% NaCl in the presence of OS.

The *E. coli* and *B. subtilis* strains did not grow in the presence of any OS, where as the *P. putida* strain did grow in the presence of toluene and *p*-xylene but not benzene (*Table 8.2.9*).

Subsequently, the effect of benzene on the halophilic growth of this bacterium was further examined; it showed growth in the range of 0.01%–29.50% NaCl without influence from 5% v/v benzene. The toxicity of benzene (log P 2.1) is greater than that of the other organic solvents used in this study (toluene log P 2.7, *p*-xylene log P 3.1). Generally, Gram-positive bacteria have lower OS tolerance than Gram-negative ones (Inoue and Horikoshi 1989, 1991a). Therefore, the benzene tolerance of the Gram-positive strain DS-994 is particularly significant. Inoue and Horikoshi (1989, 1991a) also showed that OS-tolerant microorganisms, including the toluene-tolerant *P.putida* strain IH-2000, can grow in the presence of OSs whose log P is

Table 8.2.9

Bacterial growth on organic solvents

Organic solvent	Growth (OD_{660})			
	Bacillus sp. DS-994	*P.putida* IH-2000	*E.coli* IFO 3806	*B.subtilis* IFO 3009
No addition	+ (3.1)	+ (3.8)	+ (3.5)	+ (3.6)
Benzene 5%	+ (2.5)	–	–	–
Toluene 10%	+ (2.6)	+ (3.7)	–	–
p-Xylene 10%	+ (3)	+ (3.8)	–	–

Bacterial growth was determined by measuring the optical density at 660 nm, represented by the numbers in parentheses. Soil strains and strain DS-994 were incubated for 5 days at 37°C on LB-I and LB-II medium, respectively. +, growth; –, no growth.

higher than the specific value. However, our previous study (Moriya and Horikoshi 1993a) indicated that the mechanism of OS tolerance of a. benzene-tolerant bacterium was different from that of the toluene-tolerant *P.putida* strain IH-2000. Accordingly, investigations into the tolerance of strain DS-994 to various kinds of OSs, the mechanism of its OS tolerance, and the differences of OS tolerance between Gram-positive and Gram-negative bacteria are now in progress.

To confirm sulfuric acid production from $Na_2S_2O_3$ by strain DS-994, incubation was done on LB-II medium supplemented with 12% NaCl and 10% $Na_2S_2O_3$. When a 1-day fresh culture of DS-994 was inoculated into the medium and cultured aerobically for 10 days under the optimal growth conditions, the strain showed good growth, and the initial pH value of the culture broth gradually decreased below pH 5. Sulfuric acid from sulfur compounds was measured qualitatively by high-performance liquid chromatography (HPLC). The total sulfur content of organic sulfur compounds was measured on a Perkin-Elmer organic elemental analyzer 2400 II CHNS/O Analyzer (Perkin-Elmer, GmbH, Uberlingen, Germany). From the results of HPLC analysis of the culture broth at the end of the cultivation it was clear that strain DS-994 produced sulfuric acid from $Na_2S_2O_3$, and the sulfuric acid production seemed to cause the decrease of pH.

Because strain DS-994 showed OS tolerance and the ability to produce sulfuric acid from $Na_2S_2O_3$, its sulfur-degrading ability was further investigated in the presence of kerosene with or without 5% benzene. Kerosene and diesel oil were used as the model petroleum. When needed, the model petroleum was supplemented with OSs at the concentrations indicated. Strain DS-994 showed good growth in the presence of kerosene. Furthermore the results of HPLC analysis indicated that DS-994 changed the sulfur of $Na_2S_2O_3$ to sulfuric acid in the presence of kerosene with or without 5% benzene. When kerosene was supplemented with 10% of other organic solvents, sulfuric acid production was also observed. Thus, strain DS-994 has great ability to degrade $Na_2S_2O_3$ as an inorganic sulfur compound in the presence of an organic solvent.

To investigate its ability to utilize organic sulfur compounds such as dibenzothiophene, thiophene, and ethylmethyl sulfide, which are present in petroleum, strain DS-994 was incubated in a water-model petroleum two-liquid-phase system. Dibenzothiophene (DBT), thiophene (T), and ethylmethyl sulfide (EMS) were used as exogenous organic sulfur

Table 8.2.10

Utilization of organic sulfur compounds by strain DS-994

Organic sulfur compound	Total sulfur concentration (%)		
	Control 1	Control 2	Sample
Dibenzothiophene	0.2 (0)	0.195 (2.5)	0.175 (12.5)
Thiophene	0.2 (0)	0.195 (2.5)	0.180 (10)
Ethyl methyl sulfide	0.2 (0)	0.190 (5)	0.170 (15)

Numbers in parentheses represent the percent utilization.

compounds; 0.2% of each organic sulfur compound was added to the model petroleum as total sulfur content. Sterilized seawater (4.5 ml) was employed as the water layer. One-day fresh culture of DS-994 (500 ml) was centrifuged and washed two times with sterilized seawater. The concentrated cell suspension was prepared with 10 ml of sterilized seawater. To the water layer, 0.5 ml of concentrated cell suspension was added at approximately 1×10^9 cells/ml. Finally 5 ml diesel oil with or without 5% OS was overlaid above the water layer. Two additional controls were run: (1) without inoculation and (2) with organic sulfur compounds suspended in the water layer without model petroleum and OS. When incubated for 10 days under optimal growth conditions, strain DS-994 showed good growth on LB-II medium with 12% NaCl. As shown in *Table 8.2.10*, the initial total sulfur concentrations of DBT, T and EMS in the diesel oil decreased to 0.175%, 0.18%, and 0.17%, respectively. The percent sulfur degradation of the organic sulfur compounds solubilized in the diesel oil was higher than that of control 2. In this experiment, no difference in the percent utilization of organic sulfur compounds solubilized in the diesel oil was observed with or without OS.

Furthermore, when vigorous shaking was applied, marked emulsification of the model petroleum was observed. Probably these results indicated that strain DS-994 was in contact with diesel oil droplets, which dispersed in the growth medium and oxidized the sulfur component to sulfuric acid without significant influence of the OS in the model petroleum. Thus, sulfur degradation by this OS-tolerant bacterium was observed in a water-model petroleum two-liquid-phase system.

Organic Solvent-Tolerant Marine Cholesterol-Degrading Bacterium

Microbial cholesterol conversion occurs in aqueous media even when the reactants, such as steroids, are barely soluble in water. The reactants are often in a solid state in such media, and so the reaction rate is usually hindered by the limited availability of the substrate. For industrial microbial cholesterol degradation processes, some detergents, such as Tween and Span, have been mainly used to (1) form stable suspensions of the water-insoluble substrate during fermentation and (2) prevent simultaneous formation of solid particles. Although this detergent addition helps to increase the degradation rate, it does not prevent the simultaneous process of solid particle formation. Accordingly, as a reactant reservoir to keep the product and the substrate in a soluble form until the degradation is complete, the use of OSs is thought to be a logical way to prevent simultaneous formation of the solid particles.

We attempted cholesterol degradation in a water-OS two-liquid-phase system by an OS-tolerant marine bacterium isolated from the deep-sea (Moriya et al. 1995).

Screening OS-tolerant bacteria that possessed the ability to degrade cholesterol was done by modifying our previously described method (Moriya and Horikoshi 1993a). Deep-sea sediment slurry sample were aseptically collected by sterile core samplers from 1,963 m depth of the Okushiri Ridge. The samples (100 ml) were treated with the addition of 100 ml of benzene and kept for 7 days at ambient temperature. After treatment, the upper benzene layer was spread onto M-II agar medium (Moriya and Horikoshi 1993a) supplemented with 5% NaCl and 0.1% cholesterol. Incubation was for 5 days at 30°C. Fifty colonies that formed clear zones due to cholesterol degradation on M-II agar medium were picked up and purified as candidates for benzene-tolerant cholesterol degraders. The candidate that showed the best growth and cholesterol degradation in the presence of 5% (v/v) benzene containing cholesterol 1 mg/ml was selected and tentatively designated strain ST-1. This isolate was Gram-positive, non-spore-forming, strictly aerobic, catalase-positive, oxidase-positive, and nonmotile. It had a typical rodoccus growth cycle. Its cell wall peptidoglycan contains lysine. Optimal growth was at pH 6–8.6 and 35°C, respectively. The mole percent of C+G is 62. Based on the above morphological and biochemical characteristics (Jones and Collins 1984), ST-1 seemed to be a strain of *Arthrobacter* sp.

To compare the OS tolerance and cholesterol conversion ability of the selected isolate, *Arthrobacter symprex* strain ATCC 6946, *Flavobacterium dehydrogenans* strain ATCC 13930, *Mycobacterium* sp. ATCC 29472, and *Mycobacterium smegmatis* strain ATCC 12549 were employed as control cholesterol degraders. Strains ATCC 13930 (Bocren and Laane 1987) and ATCC 29472 (Flygare and Larsson 1987) were used as representative androstene producers. These five strains were individually inoculated into each 10 ml of modified M-II medium (Moriya and Horikoshi 1993a) at approximately 1×10^6 cells/ml and then incubated in the presence of 0.5 ml of various OSs with vigorous shaking for 3 days at 30°C. Six solvents including aromatic hydrocarbons benzene, toluene, and *p*-xylene and the aliphatic hydrocarbons *n*-hexane, *n*-decane, and *n*-dodecane, were used to measure the tolerance to and the effect of these OSs in a 1:20 (v/v) OS-water biphasic system. After incubation, cell growth was determined by measuring viable cell concentration by M-II and LB-II agar plate counts.

As shown in *Table 8.2.11*, the control strains could grow in either *n*-decane (log P 6.0) or *n*-dodecane (log P 7.0) but could not grow in the presence of *n*-hexane (log P 3.9) or aromatic hydrocarbons. In contrast to these controls, strain ST-1 could grow in benzene, toluene, *p*-xylene (log P 3.1), or *n*-hexane in addition to *n*-decane and *n*-dodecane. The control strains and strain ST-1 grew in the presence of 50% (v/v) *n*-decane and *n*-dodecane. Strain ST-1 also grew even in the presence of 10% (v/v) toluene, 10% *p*-xylene, and 10% *n*-hexane in addition to 5% benzene. The final viable cell concentration of strain ST-1 reached approximately 3×10^9 cells/ml with and without OSs. Strain ST-1 was halotolerant and showed good growth in the presence of OS without any effects of NaCl in the range 0–12%. The cholesterol degradation of strain ST-1 and control stains in a buffer-OS two-liquid-phase system was examined. Both cholesterol and androsta-1,4-diene-3,17-dione (ADD), which is one of the androstenes derived from cholesterol, were measured by HPLC. Three additional controls were run: (1) without inoculation; (2) with cholesterol suspended in buffer layer without OS; and (3) with cholesterol suspended in buffer containing 0.1% Tween 80.

The results of a batch experiment are shown in *Table 8.2.12*. When strain ST-1 was incubated in the presence of benzene, toluene, *p*-xylene, or *n*-hexane, the degradation of the initial cholesterol (1 mg/ml) was 22%, 22%, 24%, and 56%, respectively. In the experiments with control 2 and control 3, the cholesterol degradation was 68% and 78%, respectively. In these experiments we also detected ADD produced from cholesterol qualitatively. Therefore,

Table 8.2.11

Growth in the presence of various solvents

Strain	Organic solvents					
	Benzene (log P 2.1)	Toluene (log P 2.9)	*p*-Xylene (log P 3.3)	*n*-Hexane (log P 3.9)	*n*-Decane (log P 6.0)	*n*-Dodecane (log P 7.0)
ST-1	+	+	+	+	+	+
Arthrobacter symplex ATCC 6946	−	−	−	−	+	+
Flavobacterium dehydrogenans ATCC 13930	−	−	−	−	+	+
Mycobacterium sp. ATCC 29472	−	−	−	−	+	+
M. smegmatis ATCC 12549	−	−	−	−	+	+

Cell growth was determined by measuring viable concentration with and without organic.
+, growth; −, no growth.

Table 8.2.12

Cholesterol degradation in two-liquid phase system

Strain	Cholesterol degradation (%)				
	ST-1	*Arthrobacter symplex* ATCC 6946	*Flavobacterium dehydrogenans* ATCC 13930	*Mycobacterium* ATCC 29472	*Mycobacterium smegmatis* ATCC 12549
Water (control 2)	68 (22)	44	52 (12)	49 (10)	53
Tween (control 3)	78 (40)	55	60 (18)	59 (19)	62
Two-liquid system					
Benzene	22 (9)	5	7 (ND)	5 (ND)	4
Toluene	22 (9)	7	9	7	4
p-Xylene	24 (10)	8	9	8	6
n-Hexane	56 (54)	20	38 (44)	32 (40)	38
n-Decane	88 (70)	59	66 (60)	66 (62)	69
n-Dodecane	92 (78)	67	74 (65)	73 (66)	77

Values in parentheses indicate the percent production of anbrosta-1,4-dione −3.17 – dione (ADD) derived from cholesterol.
ND, not detected.

this bacterium had the ability to cleave the side chain of cholesterol presumably due to the activity of a cholesterol dehydrogenase. In contrast, the control strains could scarcely degrade cholesterol in the presence of aromatic hydrocarbons but could degrade cholesterol in an aqueous medium and in a medium supplemented with either *n*-hexane, *n*-decane, or

n-dodecane. However, the percentages of both cholesterol degradation and ADD production by the control strains were lower than those obtained by strain ST-1 in aqueous media and a two-liquid-phase system. When the batch experiment was successively repeated five times using *n*-dodecane, cholesterol degradation was 92% and 90% in the first and second batches, respectively. The degradation efficiency of cholesterol decreased gradually in the third to fifth batches. Changes in the viable cell concentration of the five strains used for batch experiments were measured by conventional agar-plate counts at daily intervals. When incubated in the presence of benzene, toluene, or *p*-xylene, 1×10^9 cells/ml of the initial viable cell concentration of strain ST-1 decreased gradually to approximately 3×10^8 cells/ml after a day and then remained constant. However, the viable cell concentrations of control strains decreased abruptly within a day, and no colony formation was observed on the agar plates after 2 days. When incubated in *n*-hexane, the cell viability, of strain ST-1 was more than 50%, but that of controls was less than 20% at the end of the incubation. After incubation in aqueous media and either *n*-decane or *n*-undecane, all strains tested remained at the initial viable cell concentration.

The marked effect of organic solvent on cholesterol degradation and ADD production might stem from the differences in cell viability. In general, a positive correlation is found between the hydrophobicity of organic solvents and the nontoxicity for the biocatalyst. Organic solvents with a log P value above 4 are highly hydrophobic and generally show no toxic effects on biocatalysts. The log P value is a measure for hydrophobicity suitable for characterization of organic solvents. Accordingly, biocatalysis derived from aromatic hydrocarbon-tolerant microorganisms are of interest for further study. Enzymes associated with cholesterol degradation of strain ST-1, which shows tolerance to aromatic hydrocarbons such as benzene, toluene, and *p*-xylene, are also an attractive object to study in detail.

OS-Tolerant Polyaromatic Hydrocarbon-Degrading Bacteria

We have focused on the deep-sea environment to obtain an OS-tolerant bacterium that possesses the useful characteristics for application in industry (Abe et al. 1995). We describe the polyaromatic hydrocarbon-degrading ability of an OS-tolerant bacterium isolated from the deep-sea.

Screening of OS-tolerant polyaromatic hydrocarbon-degrading bacteria was carried out by a slight modification of the method described previously (Moriya and Horikoshi 1993a). Deep-sea sediment slurry samples were aseptically collected from a depth of 1,168 m in Sagami Bay by the manned submersible Shinkai 2000 in 1992. Deep-sea sediment slurry (5 ml) was transferred to flasks containing 10 ml of artificial seawater. Benzene was overlaid on the aqueous layer (1:1), and these flasks were then closed with silicone-rubber stoppers. The cultures were incubated with shaking at 200 rpm for a week at 30°C. After incubation the benzene layer separated from the artificial seawater, and a portion of the benzene layer was spread directly onto plates of M-II and LB-II media (Moriya and Horikoshi 1993a, b) containing 1% naphthalene. The plates were. incubated for a week at 30°C. Microorganisms that formed clear zones around their colonies were selected and purified.

The isolate was selected as a polyaromatic hydrocarbon degrader that also exhibited organic solvent tolerance; it was named strain DS-1906. Strain DS-1906 was Gram-positive, spore forming, aerobic, oxidase and catalase-positive, gelatin-liquefying, and nonmotile. This strain also grew well at a wide range of pH 5–9, and optimum pH for growth was pH 5–6.

It grew well at NaCl concentrations over the range of 0–0.5 M. Based on the biochemical and morphological characteristics, in addition to its polyaromatic hydrocarbon-degrading ability and organic solvent tolerance, this strain appeared to be a strain of *Bacillus* sp.

The OS tolerance of the strain DS-1906 was compared with that of control strains *E. coli* IFO 3806, *B. subtilis* IFO 3009, and the toluene-tolerant. *P. putida* IH-2000. Various hydrocarbons, includin.g benzene, 1-heptanol, toluene, styrene, *p*-xylene, ethylbenzene, *o*-dichlorobenzene, *n*-propylbenzene, *n*-hexane, diphenylether, cyclooctane, isooctane, *n*-octane, *n*-hexylether, *n*-nonane, *n*-decane, and *n*-dodecane, were employed as organic solvents. Naphthalene, fluorene, phehanthrene, anthracene, pyrene, chrysene, and 1,2-benzopyrene were used as polyaromatic compounds. The results are shown in *Table 8.2.13*. When incubated for 3 days, the *E.coli* and *B.subtilis* strains did not grow in the presence of any of the organic solvents tested. *P. putida* strain IH-2000 grew in the presence of all the organic solvents except for benzene. In contrast to these strains, strain DS-1906 grew even in the presence of 10% benzene and some other solvents, but it could not grow in the presence of many other OSs, such as 1-heptanol, toluene, styrene, *p*-xylene, and cyclooctane.

Table 8.2.13

Comparison of organic solvent tolerance between strain DS-1906 and soil strains

Solvent	Tolerance				
	log P	*E.coli* IFO 3806	*B.subtilis* IFO 3009	*P.putida* IH-2000	DS-1906
n-Dodecane	7.0	–	–	+ (1.90)	+ (2.42)
n-Decane	6.0	–	–	+ (1.90)	+ (2.40)
n-Nonane	5.0	–	–	+ (1.88)	+ (2.35)
n-Hexylether	5.1	–	–	+ (1.90)	+ (2.30)
n-Octane	4.9	–	–	+ (1.83)	+ (2.30)
Isooctane	4.8	–	–	+ (1.80)	+ (2.27)
Cyclooctane	4.5	–	–	+ (1.80)	–
Diphenylether	4.2	–	–	+ (1.80)	+ (2.25)
n-Hexane	3.9	–	–	+ (1.78)	+ (2.25)
n-Propylbenzene	3.8	–	–	+ (1.75)	–
o-Dichlorobenzene	3.6	–	–	+ (1.75)	+ (2.10)
Cyclohexane	3.4	–	–	+ (1.70)	–
Ethylbenzene	3.3	–	–	+ (1.70)	–
p-Xylene	3.1	–	–	+ (1.65)	–
Styrene	2.9	–	–	+ (165)	–
Toluene	2.8	–	–	+ (1.58)	–
1-Heptanol	2.4	–	–	+ (1.53)	–
Benzene	2.0	–	–	–	+ (1.80)

The initial cell concentration was 1×10^5 cell/ml. Incubation was for 3 days at 30°C. Growth was determined by measuring the optical density at 660 nm (OD_{660}).
+, growth (>5×10^8 cells/ml); –, no growth.

Degradation of naphthalene by DS-1906 was compared with that by the control strains in a medium-OS two-liquid-phase system. Bacteria were incubated in 9 ml of LB-II medium with 1 ml of OSs containing 1% naphthalene. Incubation was for 1 week at 30°C with shaking at 200 rpm. At the end of the incubation, the remaining polyaromatic hydrocarbons were extracted and analyzed by gas chromatograph. The percent degradation of polyaromatic hydrocarbons was given as a difference between the control and the inoculated samples.

We observed a 1-day latency period before growth in the presence of *n*-hexane, and then DS-1906 grew rapidly. After the 1-day of latency period, residual naphthalene decreased gradually. After 7 days the value of residual naphthalene reached about 50%. As shown in *Table 8.2.14*, the control strains did not degrade polyaromatic hydrocarbons, whereas that by strain DS-1906 was 48.3% in the presence of *n*-hexane.

Furthermore, strain DS-1906 could degrade various polyaromatic compounds, such as fluorene, phenanthrene, anthracene, pyrene, chrysene, and 1,2-benzopyrene in a medium-OS (benzene) two-liquid-phase system (*Table 8.2.15*).

When polyaromatic hydrocarbons were solubilized in an organic solvent, the percent degradation was kept at a constant value, in contrast to the highly variable degradation in the absence of an organic solvent. The percent degradation in the presence of an organic solvent was higher than that obtained from cultures in which polyaromatic hydrocarbons were suspended in the medium without an organic solvent.

Table 8.2.14

Comparison of naphthalene degradation in the different OS by solvent-tolerant strains DS-1906 and soil strains

Solvent	Degradation (%)			
	E.coli IFO 3806	*B.subtilis* IFO 3009	*P.putida* IH-2000	DS-1906
n-Dodecane	NG	NG	NG	ND
n-Decane	NG	NG	NG	ND
n-Octane	NG	NG	NG	33
Isooctane	NG	NG	NG	28
Cyclooctane	NG	NG	NG	48
Diphenylether	NG	NG	NG	26
Toluene	NG	NG	NG	NG
Benzene	NG	NG	NG	17.2
No OS	NG	NG	NG	11.7–35.5

Degradation of naphthalene was examined in the medium-organic solvent (OS) two-liquid phase system. Bacteria were incubated in 9 ml of LB-II medium containing 1 ml of organic solvent (10%) containing 1% naphthalene for 7 days at 30°C.

Degradation was calculated by the following equation.

$$\text{Degradation}(\%) = \frac{\text{Residual polyaromatic hydrocarbons conc. of an additional control} - \text{Residual polyaromatic hydrocarbons conc. of inoculated samples}}{\text{Residual polyaromatic hydrocarbons conc. of an additional control}} \times 100$$

NG, no growth; ND, no degradation.

Table 8.2.15

Polyaromatic hydrocarbon degradation by solvent-tolerant strains DS-1906

Substrates	Degradation (%)	
	Without OS	With OS
Naphthalene	11.7–35.5	17.2
Fluorene	10.5–32	17.4
Phenanthrene	10.2–28.7	16.8
Anthracene	2.6–28.5	15.2
Pyrene	2.3–26.7	13.2
Chrysene	3–25.8	14.5
1,2, -Benzopyrene	2.7–24.8	12.6

Degradation was calculated by the following equation.

$$\text{Degradation}(\%) = \frac{\begin{matrix}\text{Residual polyaromatic hydrocarbons} \\ \text{conc. of an additional control}\end{matrix} - \begin{matrix}\text{Residual polyaromatic hydrocarbons} \\ \text{conc. of inoculated samples}\end{matrix}}{\text{Residual polyaromatic hydrocarbons conc. of an additional control}} \times 100$$

NG, no growth; ND, no degradation; OS, organic solvent (benzene).

When cultures were shaken, marked emulsification of the organic solvent was observed. This result possibly indicated that thy OS-tolerant bacterium was in contact with organic solvent dispersed in the medium, which could allow the bacterium to degrade the polyaromatic hydrocarbon substrates efficiently.

Although we tried to isolate OS-tolerant plasmids from this OS-tolerant bacterium, no plasmids were obtained. Accordingly, the OS tolerance is thought to be encoded by information in genomic genes.

Organic Solvent Hydrocarbon-Degrading Marine Yeast

We have reported the isolation, characteristics, and applications such as the degradation of polluting petroleum by microorganisms that tolerate organic solvents (Aono et al. 1991, 1992; Nakajima et al. 1992; Moriya and Horikoshi 1993a, b; Fukumaki et al. 1994). Some such microorganisms can degrade hydrocarbons and organic sulfur compounds in the presence of organic solvents (Moriya and Horikoshi 1993a, b).

We attempted to isolate and investigate marine yeast that tolerates organic solvents and explore its hydrocarbon-degrading ability. Deep-sea sediment samples were collected aseptically with sterile core samplers from a 1,200 m depth in Sagami Bay by a manned submersible vessel, the Shinkai 2000. For yeast growth, YPG medium, which contained 10 g of yeast extract (Difco), 20 g of Bacto-peptone (Difco), and 10 g of glucose per liter of distilled water; YPTC medium, which contained 0.5 g of yeast extract, 4 g of bactorpeptone, 1 g of trypticase peptone (BBL), 0.5 g of glucose, 3 g of $(NH_4)SO_4$, 0.1 g of K_2HPO_4, 0.5 g of $Mg_2SO_4 \cdot 7H_2O$, 1 g of $CaCl_2 \cdot 2H_2O$, and 30 g of NaCl per liter of distilled water; and yeast nitrogen base (YNB) medium (Difco) were used. For YPTC agar medium, agar was added at a concentration of 2% (w/v) to

YPTG medium. When needed, both penicillin C potassium (Wako, Osaka, Japan) and streptomycin sulfate (Nacalai Tesque, Kyoto, Japan) were added to these media at 0.010% each, and the pH was adjusted to 2.5.

For isolation of yeasts that could tolerate organic solvents, the deep-sea sediment samples were treated with 50% (v/v) *n*-hexane for 2 days by a slight modification of the described elsewhere method (Moriya and Horikoshi 1993a). Some organic solvent was spread directly on YPTG agar medium with penicillin and streptomycin. Incubation was at 20°C for 1 week. After incubation, yeast-like colonies growing on the medium were picked up and purified.

Altogether 43 yeast strains were isolated and purified from deep-sea sediment sample treated with *n*-hexane. Tolerance of organic solvents was examined on YPG medium. *n*-dodecane, *n*-decane, *n*-nonane, hexyl ether, *n*-octane, isooctane, cyclooctane, diphenylether, *n*-hexane, and kerosene were the organic solvents used.

One of these deep-sea isolates, strain Y-40 tolerated the aliphatic hydrocarbons *n*-dodecane, *n*-decane, *n*-nonane, *n*-octane, isooctane, and cyclooctane and the ethers, hexyl ether and diphenyl ether. This strain could grow even in the presence of 50% (v/v) organic solvents (*Table 8.2.16*). Some other deep-sea isolates and some type strains grew in the presence of diphenyl ether, which had the lowest log P value of the solvents used; but they could not grow in cyclooctane. Accordingly, we selected strain Y-40 as a possible hydrocarbon degrader. These yeast cells were short ovals and formed budding cells during growth in YPG medium. The strain fermented only D-glucose of the sugars tested. It grew in YNB medium, which contained trehalose, L-rhamnose, ribitol, D-mannitol, D-glucitol, or glycerol as the sole carbon source. Based on its morphological and biochemical characteristics, it seemed to be a variant strain of *Candida*. Strain Y-40 could not grow in YNB medium containing any of the organic solvents tested as the sole carbon source.

Table 8.2.16

Tolerance of organic solvents by marine yeast strain Y-40

Solvents	Concentration (%)	Cell growth (OD_{660})
None	–	21.5
n-Octane	10	16.3
	25	9.4
	50	20.1
Isooctane	10	22.2
	25	19.1
	50	17.4
Cyclooctane	10	12.7
	25	7.9
	50	7
Kerosene	10	20.5
	25	14.1
	50	17.8

After incubation at 30°C for 24 h, yeast growth was evaluated by measurement of the OD_{660}.

The ability of yeasts to assimilate *n*-alkanes and organic solvents was examined with 5 ml of YNB medium to which *n*-undecane, *n*-tridecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane, or one of the organic solvents listed above was added at 0.5% as the sole carbon source. Incubation was at 30°C. Strains that grew within a week were regarded as being hydrocarbon assimilators.

Degradation of *n*-alkanes in kerosene was examined in YPG medium under a mixture of organic solvents. A 10 μl aliquot from a 1-day culture was used to inoculate 4 ml of YPG medium in a test tube; 1 ml of an organic solvent that contained 10% kerosene was then overlaid on the medium. *n*-octane, isooctane, and cyclooctane were the organic solvents used. The control with no organic solvent and the other control without inoculation were used. Incubation was for 2 days at 30°C with shaking at 200 rpm. At the end of the incubation, the *n*-alkanes remaining in the kerosene were extracted and analyzed. The amount of degraded *n*-alkanes was recorded as the difference between one of the controls and the inoculated sample or control.

For a comparison of the hydrocarbon-degrading ability of strain Y-40 with those of two strains that assimilate *n*-alkane (*C. tropicalis* IFO 1400 and *Yarrowia lipolytica* IFO 1548), kerosene was used because it contains a mixture of *n*-alkanes. The results of degradation of *n*-alkanes are shown in *Table 8.2.17*. The three strains tested degraded *n*-alkane in the absence of an organic solvent. Of the three strains, *C.tropicalis* IFO 1400 degraded *n*-alkanes in kerosene without an organic solvent most. Neither *C.tropicalis* IFO 1400 nor *Y. lipolytipa* IFO 1548 degraded *n*-alkanes in the presence of any of the three organic solvents tested. Strain Y-40 degraded *n*-alkanes in the presence of *n*-octane. The amount of *n*-alkanes in kerosene with *n*-octane degraded by strain Y-40 was greater than that without *n*-octane. In the presence of isooctane or cyclooctane, strain Y-40 grew but did not degrade *n*-alkanes.

Table 8.2.17

Degradation of n-alkanes in organic solvents by yeasts

	n-Alkane degradation (mg)					
	Marin yeast Y – 40		*Candida tropicalis* IFO 1400		*Yarrowia lipolytica* IFO 1548	
n-Alkane	None	*n*-Octane	None	*n*-Octane	None	*n*-Octane
n-Nonane	0.59	1.04	1.20	ND	0.68	NG
n-Decane	1.15	1.92	2.40	ND	1.28	NG
n-Undecane	1.24	2.38	2.80	ND	1.34	NG
n-Dodecane	1.29	2.04	2.38	ND	0.85	NG
n-Tridecane	1.11	1.73	1.94	ND	0.94	NG
n-Tetradecane	0.65	1.02	1.12	ND	0.78	NG
n-Pentadecane	0.40	0.48	0.53	ND	0.30	NG
n-Hexadecane	0.12	0.16	0.24	ND	0.17	NG

Yeast was incubated in 4 ml of YPG with 1 ml (20%) octane containing 2% kerosene at 30°C for 3 days. The controls were incubated without *n*-octane.
ND, no degradation; NG, no growth.

Although *C.tropicalis* IFO 1400 and *Y. lipolytipa* IFO 1548 degraded n-alkanes in kerosene without other organic solvents, neither strain degraded *n*-alkanes in the presence of 20% (v/v) *n*-octane, isooctane, or cyclooctane, which have highly toxic effects on yeast growth. The degradation of *n*-alkanes in kerosene by strain Y-40 increased from 20% to 75% when *n*-octane was added at a concentration of 20% (v/v). The results showed that the metabolic system for *n*-alkanes in strain Y-40 was different from that in *C. tropicalis* IFO 1400 and *Y. lipolytipa* IFO 1548. The increase in *n*-alkane degradation by Y-40 might be due to induction of enzymes that degrade *n*-alkanes after the addition of *n*-octane. Strain Y-40 could grow in the presence of 20% (v/v) isooctane of cyclooctane but did not degrade *n*-alkanes in the presence of either organic solvent. This characteristic would be useful for the microbial conversion of water-insoluble compounds by fermentation in a two-phase system of organic solvent and aqueous medium.

Cross-References

- 8.3 Molecular Responses to Solvent Stress: Strategies for Living in Unpalatable Substrates
- 8.4 Genetics, Evolution and Applications

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