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Research in Microbiology 153 (2002) 263–268

Research in Microbiology

www.elsevier.com/locate/resmic

Mini-review

Tolerance of bacteria to organic solvents

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Received 11 December 2001; accepted 3 April 2002

First published online 14 May 2002

Abstract

Organic-solvent-tolerant bacteria are a relatively novel group of extremophilic microorganisms. They overcome the toxic and destructive effects of organic solvents due to the presence of various adaptive mechanisms. Extensive studies done on the toluene tolerance of certain *Pseudomonas* strains have led to an understanding of the mechanisms of organic solvent tolerance involving novel adaptations such as the toluene efflux pumps, *cis-trans* isomerisation of membrane fatty acids, rapid membrane repair mechanisms, etc. Organic-solvent-tolerant mutants of *Escherichia coli* have been constructed and genes enhancing such tolerance characterised. However, there is practically no information available on the tolerance mechanisms of the reported Gram-positive organic-solvent-tolerant bacterial strains like *Bacillus, Rhodococcus* and *Arthrobacter.* This review discusses the general aspects of organic-solvent-tolerant bacteria, their history, biodiversity, mechanisms of tolerance and proposes certain probable adaptations of Gram-positive bacteria in tolerance to organic solvents.

Keywords: Organic solvents; Toxicity; Tolerance; Efflux; Adaptation; Cell membrane

1. Introduction

Organic solvents are known to be extremely toxic to microbial cells, even at very low concentrations of 0.1% (v/v). Solvents are known to accumulate in and disrupt the bacterial cell membrane thus affecting the structural and functional integrity of the cell [18,37]. Although there are some microorganisms which can assimilate these toxic organic solvents, they do so only when the solvent concentration is very low. Any medium containing large volumes of organic solvents seems an extreme environment for microorganisms and hence for many years it was believed that no microorganism could withstand such a harsh environment [2,18]. The first report of an organic-solvent-tolerant bacterium was by Inoue and Horikoshi in 1989 [18]. They discovered a strain of *Pseudomonas putida* (IH-2000) which could actively grow and multiply in the presence of 50% (v/v) toluene. This surprising observation was confirmed by others [10,25,28,35] and the search to uncover the mechanisms behind this extraordinary characteristic began.

A large number of the reported organic-solvent-tolerant bacteria are *Pseudomonas* strains, especially *P. putida*. Organic-solvent-tolerant mutants, tolerant to p-xylene have been constructed from *E. coli* K-12 [2]. Since Gram-negative bacteria have an additional outer membrane made up of phospholipids and lipopolysaccharides compared to the single cytoplasmic membrane of Gram-positive bacteria, it was assumed that Gram-negative bacteria are better equipped to cope with solvent induced shock [19,32]. But recently, strains of Gram-positive bacteria like *Bacillus, Rhodococcus* and *Arthrobacter* tolerant to benzene have been reported [1,23,29,32].

2. Physiological basis of solvent toxicity and the concept of organic solvent tolerance

The primary site of action of organic solvents is the cell membrane. The cytoplasmic membrane of bacterial cells, a phospholipid bilayer, is a matrix in which various enzymes and transport proteins are embedded. It plays a vital role in solute transport, maintaining the energy status of the cell, regulation of the intracellular environment, turgor pressure, signal transduction and energy transducing

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0923-2508/02/$ – see front matter
PII: S0923-2508(02)01319-0

processes. Solvents partition into and disrupt the lipid bilayer, thus compromising cell viability [18,37,38]. It has been proved that it is not the chemical structure of the solvent, but the concentration to which it accumulates in the cell membrane that plays a crucial role in determining toxicity [11,20].

Physiological investigation of microbes has revealed a correlation between solvent toxicity and its log *P* value (Table 1). The parameter log *P* is defined as the partition coefficient of the given solvent in an equimolar mixture of octanol and water [18]. The greater the polarity, the lower the log *P* value and the greater the toxicity of the solvent. Generally, solvents with log *P* values below 4 are considered extremely toxic as their degree of partitioning into the aqueous layer (which contains cells) and from there into the lipid membrane bilayer is high. The greater the degree of accumulation of the solvent in the membrane, the higher its toxicity [11,20].

Each organism has its own intrinsic tolerance level for organic solvents, which is determined genetically and is also influenced by environmental factors [26]. Organic solvent tolerance is believed to be a strain specific property [17]. The tolerance level of each microorganism is represented by two terms, the index solvent and the index value. The index value is the log *P* value of the most toxic organic solvent (index solvent) among those that can be tolerated by the organism. Each bacterium can grow on agar media overlaid with any one of the organic solvents having a log *P* value greater than the index value. However, under such conditions, the growth of bacteria is suppressed by organic solvents having log *P* value near the index value [2].

Table 1
Organic solvents and their log *P* values

Solvent	log *P* value
n-Decane	5.6
Decalin	4.8
Diphenyl ether	4.3
Cyclooctane	4.2
Propyl benzene	3.8
Tetralin	3.8
Methyl cyclohexane	3.7
Hexane	3.5
Cyclohexane	3.2
Ethyl benzene	3.1
p-Xylene	3.0
Styrene	3.0
Octanol	2.9
Carbon tetrachloride	2.7
Toluene	2.5
Heptanol	2.4
Dimethyl phthalate	2.3
Fluorobenzene	2.2
Benzene	2.0
Chloroform	2.0
Cyclohexanol	1.5
n-Butanol	0.8

3. Isolation of organic-solvent-tolerant bacteria

Organic-solvent-tolerant *Pseudomonas* strains have been isolated from mud samples, garden, forest and humus soils [17,18,25]. Kato et al. report that organic-solvent-tolerant bacteria are over 100 times more abundant in deep-sea mud samples than in terrestrial soils. Most of the Gram-positive organic-solvent-tolerant microbial strains such as *Bacillus* DS-1906 and DS-994 and *Arthrobacter* ST-1 have been isolated from deep sea and marine mud samples. In addition, other organic-solvent-tolerant species like *Flavobacterium* DS-711 and a yeast (*Candida* Y-40) have also been obtained from marine mud, indicating a greater biodiversity of such bacteria in the marine environment [23]. Several laboratory strains have been found to adapt to high solvent concentrations, e.g., *P. putida* Idaho to p-xylene and *P. putida* S12 to styrene [10,42]. It has been found that *Pseudomonas* strains growing in the presence of short chain fatty acids like acetate have a lower membrane fluidity which prepares them for growth in the presence of supersaturating amounts of toxic non-metabolisable solvents like toluene. *P. putida* S12 could adapt to grow on styrene in a 2-phase styrene-water system. Acetate was toxic for *Pseudomonas* S12 but cells were able to adapt to higher concentrations of it [42]. *E. coli* mutants tolerant to cyclohexane and p-xylene have been constructed from parent strains having a solvent sensitive phenotype. The parent strain *E. coli* JA300 tolerated only n-hexane and diethyl ether. Spontaneous cyclohexane tolerant mutants were obtained at a frequency of one in a million after growth in an equivalent mixture of cyclohexane and p-xylene. A p-xylene tolerant mutant OST 3121 was isolated from the mixture after 1-methyl, 3-nitroso-guanidine treatment [2] (Table 2).

Kato et al. have described the following method for isolation of organic-solvent-tolerant bacteria that degrade crude oil, polyaromatic hydrocarbons or cholesterol or utilise sulphur compounds from deep sea sediment. Benzene was added to artificial sea-water containing deep-sea sediment to a concentration of 50% v/v and the cultures were incubated at room temperature on a rotary shaker for one week. After incubation, the benzene layers were carefully separated from the sea-water layers, and a portion of each benzene layer was spread on a suitable nutrient medium. Colonies that grow on the medium after incubation for 2 days at 25 or 30 °C were isolated and purified [23]. Growth in the presence of organic solvents in most organisms is known to lead to a decrease in growth rate and yield [21,36]. The reduction in yield is due to energy consuming adaptations as well as the uncoupling effects of solvents [21].

4. Organic solvent tolerance mechanisms in bacteria

Gram-negative bacteria, namely certain strains of *Pseudomonas* and some *E. coli* mutants, have devised various novel adaptive mechanisms which enable them to thrive in the

Table 2
Some reported strains of organic-solvent-tolerant bacteria and their characteristics

Strain	Organic solvent tolerance	Index value	Additional information
P. putida IH-2000	cyclohexane, xylene, styrene, heptanol	2.5	First organic-solvent-tolerant bacterium reported, does not metabolise toluene
P. putida DOT-T1E	90% toluene,	2.5	Degrades toluene
P. putida S12	cyclohexane, ethyl benzene, p-xylene, styrene, octanol, toluene, heptanol, dimethyl phthalate	2.3	Degrades styrene, octanol and heptanol
P. putida Idaho	p-xylene, m-xylene, toluene	2.5	Degrades p-xylene, m-xylene, toluene
E. coli JA300			
Mutants of JA 300			Solvent-tolerant mutants produced from a sensitive parent
OST3408	cyclohexane	3.2	
OST3301	n-pentane		
OST3121	p-xylene	3.0	
P. putida ST-200	p-xylene	3	Cholesterol degrader
Bacillus OS-1906	hexane, cyclohexane, benzene	2	Marine polyaromatic hydrocarbon degrader
Bacillus DS-994	p-xylene, toluene, benzene	2	Utilises sulphur compounds like thiophene in water petroleum 2-phase systems
Flavobacterium DS-711	p-xylene, toluene, benzene	2	Degrades crude oil
Arthrobacter ST-1	p-xylene, toluene, benzene	2	Marine origin, cholesterol degrader, accumulates 1,4 andro-stadiene 3,17 dione

Table 3
Organic solvent tolerance mechanisms of bacteria

Gram-negative bacteria: e.g., some strains of *Pseudomonas* and certain *E. coli* mutants	Known mechanisms:* 1) modifications in cell envelope to increase cell membrane rigidity and decrease permeability: a) *cis-trans* isomerisation of membrane fatty acids by *cis-trans* isomerase b) decreased cell surface hydrophobicity c) changes in chemical composition/proportions of membrane lipids and proteins 2) increased rate of membrane repair enzymes 3) special solvent-inactivating enzymes 4) active efflux of solvents by means of solvent efflux pumps (*tol C/mar/rob/sox S/acr AB* genes) 5) release of membrane vesicles with solvent molecules adhering to them 6) production of phage shock protein (stress protein in *E. coli*)
Gram-positive bacteria: e.g., some strains of *Bacillus*, *Rhodococcus*, *Arthrobacter*	Proposed mechanisms: 1) protection rendered by endospores? 2) induction of general stress regulon, leading to the production of general stress proteins? 3) solvent deactivating or emulsifying enzymes?

* [3–5,11,12,15,16,20,22,24,27,28,33,34,36,39,41,42].

presence of supersaturating amounts of toxic organic solvents. These mechanisms have been extensively reviewed (Table 3). While the Gram-negative bacteria show tolerance to high concentrations of toluene ($\log P = 2.5$), the reported Gram-positive organic-solvent-tolerant isolates like *Rhodococcus* sp., *Arthrobacter* ST-1, *Bacillus* DS-944 and DS-1906 show excellent tolerance to benzene ($\log P = 2.0$) which is much more toxic than toluene [1,11,23,29,30]. However, very little information is available on what makes these cultures tolerant to benzene. There is a difference between the cell membranes of Gram-positive and Gram-negative bacteria, and the additional outer membrane is missing in Gram-positive bacteria, which however possess a more extensively linked peptidoglycan [37]. Studies need to be undertaken to determine whether the molecular mechanisms of solvent tolerance elucidated in Gram-negative bacteria are also conserved in Gram-positive bacteria. It has been proposed that the mechanisms of solvent tolerance of the benzene tolerant *Bacillus* DS-1906 and the toluene tolerant *P. putida* IH-2000 are different, due to differences in cell surface components. Many of these benzene tolerant bacteria also have the potential to degrade this solvent. It is believed that organic solvent emulsifying/deactivating/solubilising enzymes/substances could play a very important role in diminishing solvent toxicity in Gram-positive bacteria [1].

With reference to *Bacillus* species, it would be interesting to compare the effect of organic solvents on spores and vegetative cells. Spores have been recognised as the hardiest life-forms on earth and display an almost unbelievable resistance to adverse conditions. *Bacillus* spores are known to withstand heat, ultraviolet and oxidative damage, dessication and chemical agents like acids, bases, phenols, alcohols, alkylating agents, etc. Factors important in spore resistance to chemicals are: impermeability of the spore coat to hydrophilic chemicals, low spore core water content which keeps the enzymes in an inactive state and protection of spore DNA by alpha-beta SASP proteins [31]. *Bacillus* endospores have survived in 95%(v/v) ethanol for prolonged periods. However, the growth rate of *B. subtilis* is found to be lowered but the final yield remains unchanged when ethanol is present in the growth medium. At concentrations allowing growth at half-maximal rate, practically no spores are formed. Post-exponential events such as excretion of certain enzymes and modification of RNA polymerase are altered or suppressed in the presence of ethanol. Sensitivity to ethanol is much greater for the sporulation process than growth, since a concentration of 0.7 M may reduce the yield of spores to the extremely low value of 10^{-5}, although it reduces growth rate only by half [8]. It is possible that similar effects on sporulation may be induced by organic solvents.

Another angle which needs to be investigated is the involvement of the general stress regulon in solvent tolerance of Gram-positive bacteria. It has been established that cells are under great stress in the presence of organic solvents and various genes undergo activation when subjected to solvent shock or other stress stimuli which threaten the cell membrane. This can be demonstrated by various instances.

In *E. coli*, organic solvent tolerance levels of strains are improved by overexpression of the stress response genes *marA, robA* and *soxS* [4–6,30]. The AcrAB efflux system which is triggered during solvent shock is also stimulated by other stress conditions like salt and ethanol exposure [39]. *E. coli* K12 strains express the phage shock protein A (PspA) under various stress conditions like heat shock, hyperosmotic shock, salt or ethanol treatment, prolonged stationery phase incubation, during inhibition of protein, fatty acid and ATP synthesis. PspA is believed to play a role in maintaining proton-motive force under stress conditions. It was found that introduction of a multi-copy plasmid vector carrying *psp* operon into *E. coli* improves the survival frequency of cells on sudden exposure to organic solvents like n-hexane, but not the growth rate [26].

Hence, the possibility that general stress proteins could be induced on solvent shock in Gram-positive bacteria also cannot be neglected. Like the sigma S of *E. coli, Bacillus subtilis* also controls a large stationery phase regulon called the sigma B regulon. Sigma B is a secondary sigma factor of *B. subtilis*. The RNA polymerase containing sigma B transcribed a subset of genes expressed after heat shock or on the onset of stationery phase. Initially it was believed that the sigma B regulon of *B. subtilis* is induced only in the stationery phase. However, it is now known that sigma B activity is induced both upon entry into the stationery phase and by environmental stress such as salt and heat stress and ethanol shock during logarithmic growth [9,13, 40]. Data suggest that the sigma B regulon, of *B. subtilis* together with the associated regulatory network provides a less extreme alternative to sporulation under growth limiting conditions. This may become critically important under environmental conditions that do not support sporulation [13]. Since ethanol represses sporulation in *B. subtilis*, [8] it is possible that organic solvents could exert a similar effect.

Extensive studies have been done on the general stress regulon of *B. subtilis* [7,9,13,40]. It is known that sigma B is required for the induction of approximately 100 genes after imposition of a whole range of stresses and energy limitations (exposure to heat, acid, ethanol, salt stress, deficiency of either glucose, phosphate or oxygen). Sigma B null mutants unable to induce the regulon following shock displayed at least a 50- to 100-fold reduction in survival after exposure to heat (54 °C), ethanol (9%), salt (10%), acid (pH 4.3) as well as freezing and desiccation compared to the wild type. Pre-loading cells with sigma-B-dependent general stress proteins prior to subjecting to the stress response confers considerable protection. Loss of sigma B reduces the viability of stationery phase cells grown in alkaline or acidic media and also decreases the cell yield in Luria broth containing high concentrations of ethanol. It is interesting to note that sigma B is very strongly induced by ethanol shock, and ethanol is known to have multiple effects on the cell membrane, hence the site of damage is the same as that of organic solvents. A large number of *csb* products appear to be associated with the cell envelope and it has been hypothesised that loss of sigma B function would become evident under environmental conditions that challenge envelope functions [13]. It is tempting to speculate that certain sigma B proteins induced strongly by ethanol shock, such as Gsp 9, 33, 67, 70, 71, 80 and Gta B, which confer a survival advantage on the cell when the membrane is affected, could play a vital role in protecting from organic solvent shock, which inflicts similar damage. Another interesting feature is that the extent of the sigma-B-dependent stress response varies from strain to strain, among specific strains used in analysis. For instance, *B. subtilis* 168 displayed a less pronounced induction of sigma B dependent stress genes than *B. subtilis* IS 58 [40]. It is possible the degree of solvent tolerance increases with the strength of the stress response. The crucial role of multidrug efflux transporters, whose expression is induced by structurally divergent compounds such as antibiotics, inhibitors and other toxic compounds in organic solvent tolerance of *E. coli* and *Pseudomonas* strains has been extensively researched [5,28,36]. In *B. subtilis*, the *bmrUR* operon, which encodes proteins that may contribute to resistance to multidrug compounds is regulated by sigma B [14]. It is highly probable that multidrug efflux proteins of *Bacillus* could also be involved in solvent tolerance.

5. Conclusions and future trends

It is evident that organic-solvent-tolerant bacteria are an interesting group of extremophiles with unique adaptations, cell components and enzymes capable of protecting the cells and allowing them to function in solvent-saturated environments. This property can be exploited in several industrial processes [11]. Studies done on the solvent tolerance mechanisms of bacteria like *E. coli* and *Pseudomonas* have enriched our understanding of what makes cells withstand such severe stress. However, there is a large void in available data on such mechanisms in Gram-positive bacteria. More studies need to be undertaken in this direction. Also, fresh habitats need to be explored to isolate other species displaying such tolerance.

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