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Isolation and characterization of novel organic solvent-tolerant bacteria

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Abstract Some organic solvents are extremely toxic to living organisms by virtue of their ability to partition into and disrupt the normal functioning of biological membranes. In recent years, several bacteria have been discovered that are more tolerant to these toxic solvents than most microorganisms. Using enrichment procedures, we have isolated new organic solvent-tolerant bacteria from both hyrdocarbon-contaminated and pristine soil samples. These organisms were characterized by several different experimental procedures including description of their cellular physiology, 16S rDNA homology, organic solvent tolerance range, and survival after solvent exposure. The results indicate that gram-positive bacteria can be isolated from the environment that are as tolerant to toxic organic solvents, if not more so, than the most organic solvent-tolerant gram-negative bacteria.

Keywords Solvent tolerance · Organic solvents · Bacterial cell envelope · Enrichment

Introduction

It is generally thought that organic solvents exhibit extreme toxicity toward living microorganisms because of their accumulation in hydrophobic biological membranes. Because of this toxicity, organic solvents have in the past been used as permeabilization agents, disinfectants, food preservatives, and industrial solvents (Davidson and Barnden 1981; de Bont 1998). The hydrophobicity of organic solvents can be expressed in terms of P_{ow}, which represents the ability of a compound to partition over an octanol/water two-phase system (Sikkema et al. 1994). It has been established that log P_{ow} is correlated with the toxicity of a specific organic solvent and that log P_{ow} values, especially between two and four, are highly toxic for microorganisms (Osborne et al. 1990). Whether a solvent is toxic to a bacterial cell depends upon its concentration in the membrane, which relates to its water solubility and its ability to partition from the water phase to the membrane (de Bont 1998). Solvents with log P_{ow}*s* below two are generally too hydrophilic to partition into membranes well, and solvents with log P_{ow}*s* above four are too hydophobic to have high water solubility (Kieboom and de Bont 2000). The mechanisms of membrane toxicity by organic solvents have been well studied and comprehensively reviewed (Sikkema et al. 1995). In gram-negative cells, extensive permeabilization is caused in the cytoplasmic membrane, while the outer membrane remains relatively intact. This preferential partitioning of organic solvents into the cytoplasmic membrane prevents normal functioning of the membrane as a selective barrier, allowing the leakage of such macromolecules as RNA, phospholipids, and proteins (Woldringh 1973). Perhaps more importantly, organic solvents produce a loss of ion gradients across the cytoplasmic membrane that destroys energy production and transduction via proton motive force (Sikkema et al. 1994). The accumulation of toxic organic solvents in the membrane increases membrane fluidity, increases membrane swelling, and reduces the normal functioning of membrane-associated proteins (Sikkema et al. 1995; Weber and de Bont 1996). The accumulation of organic solvents results in the disruption of bilayer stability and membrane structure, causing a loss of membrane function and ultimately cell death.

Despite this extreme toxicity, organic solvent-tolerant bacteria that are capable of growth in a two-phase water–toluene system have been isolated. Many of these tolerant bacterial species, including the first strain isolated, were gram-negative bacteria such as *Pseudomonas*

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putida (Inoue and Horikoshi 1989; Shima et al. 1991; Cruden et al. 1992; Weber et al. 1993; Ramos et al. 1995; Ohta et al. 1996; Kim et al. 1998) or closely related *Pseudomonas* sp. (Nakajima et al. 1992; Ogino et al. 1994). Gram-positive bacteria such as *Bacillus* (Moriya and Horikoshi 1993; Isken and de Bont 1998; Sardessai and Bhosle 2002b) and *Rhodococcus* (Paje et al. 1997) have also been found to be organic solvent tolerant, although limited investigation has occurred towards understanding the mechanisms of their organic solvent tolerance. Because the first interaction of a solvent with a cell occurs at the cell membrane, membrane modification was first studied as a mechanism of solvent tolerance in gram-negative bacteria (Sikkema et al. 1995). Several investigators have also been able to isolate solvent tolerant strains from solvent sensitive bacteria, suggesting that bacteria can adapt at least somewhat to the presence of organic solvents (Weber et al. 1993). More recently, it has been discovered that toxic organic solvents are a substrate for RND type multidrug efflux pumps found in both solvent-tolerant *Pseudomonas* sp. (Kieboom et al. 1998a; Ramos et al. 1998) and *Escherichia coli* (White et al. 1997). It is generally accepted that the relatively impermeable cell envelope works in conjunction with an energy-driven efflux pump to limit the entry of organic solvents into the cell and extrude any organic solvents that partition into the inner membrane through the outer membrane (Kieboom and de Bont 2000). However, for gram-positive bacteria, a generally more permeable cell envelope precludes the effectiveness of a specialized solvent efflux pump, suggesting that the mechanism of organic solvent tolerance is primarily membrane modification. In this paper, we present the isolation and characterization of several new gram-negative and gram-positive bacteria obtained through enrichment procedures that are extremely tolerant to toxic organic solvents.

Materials and methods

Isolation of bacteria

Cells were isolated from a mixed microbial population by culturing soil samples collected from various locations in rich Luria–Bertani (Lennox) media (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) or 1/2 LB media (5 g tryptone, 2.5 g yeast extract, and 2.5 g NaCl per liter) for several days at 25°C with shaking in sealed flasks with a known low level of organic solvent, typically toluene. Soil samples were obtained from both oil contaminated and pristine (rhizosphere) locations. Soil planted to marigolds was selected because of the potential presence of thiophenes (Christensen and Lam 1990). After allowing growth in the flask to proceed to opacity, an aliquot was removed and used as an inoculum in a new flask with fresh medium and an additional 5 mM of organic solvent. Although aqueous solutions should be saturated by toluene above 7 mM, at which point a two-phase system exists, we continued to add additional solvent in order to limit the effects of any potential solvent volatilization to the headspace. After repeated subcultures with up to 30 mM toluene, the cells were plated out under two conditions without organic solvent: a selective condition on *Pseudomonas* isolation agar (PIA) that selected for the preferential growth of pseudomonads, and a non-selective condition where cells were plated onto 1/2 LB. Four isolates from both selection conditions were retained for further testing.

Physiological characterization

The morphology of the gram-stained solvent-tolerant bacteria was examined by light microscopy using a Zeiss KF-2 light microscope. In order to examine changes to cellular morphology in the presence of organic solvents, cells were grown to log phase in LB medium containing either no toluene or 50 mM toluene. Samples were taken directly from the cultures and applied to grids that allowed the visualization of cells using a Philips/FEI (Morgagni) Transmission electron microscope with CCD camera in the department's microscopy unit. Initially, samples were negatively stained with 0.2% phosphotungstic acid before visualization. In order to specifically visualize the capsular structure, cells obtained from growth with or without solvent were labeled with polycationic ferritin to produce capsular stabilization followed by staining with ruthenium red (Sigma, MO, USA) and thin-sectioning prior to observation.

Biolog Microplates (Biolog, Hayward, CA, USA) were used to determine the ability of the strains to utilize any of a number of different carbon sources. Testing was performed according to the manufacturer's recommendations. Carbon utilization was scored positive if the corresponding well had reduction of tetrazolium dye producing a purple color. Gram-negative non-enteric bacteria were grown on Biolog universal growth (BUG) Agar with 5% sheep's blood at 30°C, and inoculated into the GN2 MicroPlate test wells with GN/GP-IF (0.4% NaCl, 0.03% Pluronic F-68 and 0.02% Gellum gum) according to the manufacturer's recommendations (Biolog). Gram-positive non-spore forming bacteria were grown similarly but inoculated using GN/GP-IF + 20% thioglycolate to reduce capsule formation, and tested in GP2 MicroPlates (Biolog). Gram-positive spore forming bacteria were grown in BUG media + 0.25% maltose + thioglycolate and inoculated into GP2 Microplates with GN/GP-IF without added thioglycolate. Microplates were read using Biolog's MicroLog3 4.20 software and GP 6.0 and GN 6.0 databases after 6 and 16 h of reaction time.

Taxonomic classification of bacterial strains

Genomic DNA was extracted from the newly isolated bacteria using a published protocol employing CTAB

(Ausubel et al. 1991). PCR reactions were performed using Invitrogen Pfx polymerase according to the manufacturer's recommendations (Invitrogen Corp., Carlsbad, CA, USA), and a standardized set of primers designed to amplify the bacterial 16S gene (Johnson 1994). To be consistent with other database entries, we attempted to obtain 16S DNA sequence in both directions of between 1,000 and 1,500 bp for each isolate. 16S rDNA sequence sizes obtained ranged from 1,160 bp for isolate ZZ5, to 1,498 bp for isolate ZZ7. In general, DNA was manipulated and stored as previously described (Sambrook et al. 1989). DNA sequencing of the PCR products was performed by our department's Molecular Biology Service Unit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), following purification with a QIAGEN PCR miniprep column (QIAGEN Inc., Mississauga, ON, USA). Sequences were edited and assembled using ABI's EditView and AutoAssembler programs (Applied Biosystems), and the sequences were compared to GenBank database entries using BLASTN (Altschul et al. 1990). Closely related sequences were selected for use in the construction of phylogenetic trees. The raw sequences were converted to LaserGene's EditSeq files and aligned using the CLUSTAL algorithm of LaserGene's MegAlign program (DNASTAR Inc., Madison, WI, USA).

Growth after solvent shock

In order to observe a cell's inherent ability to withstand toxic organic solvents without adaptation, cells were subjected to rapid solvent addition producing large-scale cell death. In this solvent shock growth condition, cells were initially cultured overnight in the absence of solvent to provide a fresh inoculum for a subculture. Cells were grown to early log phase in a sealed Nephelo flask, shocked with a set amount of solvent, and OD_{600} was measured as an indicator of cell growth.

Growth in two-phase solvent systems

Growth curves were initially performed in Nephelo flasks sealed with either a neoprene stopper or an aluminum foil-covered neoprene stopper to prevent solvent volatilization. Growth measurements were taken obtaining OD_{600} readings on a spectrophotometer without opening and resealing the flasks to prevent the loss of volatilized organic solvents. Cells were subcultured every 24 h to new media containing increasing amounts of toluene. An additional 5 mM toluene was added each day to the new flask and the experiment was carried out until the subcultured cells failed to grow in the new media overnight. Cell death at particular concentrations of organic solvent in solution indicates that appreciable amounts of solvent were not being lost to volatilization. Because solvent shock and solvent growth experiments suggested that cells were adapting to solvent concentrations well above the limit of solvent solubility, we attempted to compare the maximal solvent concentrations in which these cells could survive. In these experiments, cells were allowed to adapt to the presence of toxic organic solvents through any possible means including de novo gene expression or mutation. Overnight growth experiments with high concentrations of

Table 1 Characteristics of newly isolated solvent-tolerant bacteria

Strain name	16S Comparison—Closest Neighbor (GenBank accession number)	Original Sample	Toluene tolerance[a]
Staphylococcus sp. strain ZZ1	*Staphylococcus* sp. LMG-19417 (AJ276810) (1,187/1,187 bp identity)	Solvent contaminated media	100 mM [1% (v/v)]
B. cereus strain ZZ2	*B. cereus* strain ATCC 10987 (AJ577290) (1,166/1,166 bp identity)	Marigold soil (Edmonton, Alberta)	90 mM [0.96% (v/v)]
B. cereus strain ZZ3	*B. cereus* strain E33L (CP000001) (1,414/1,414 bp identity)	Marigold soil (Edmonton, Alberta)	100 mM [1% (v/v)]
B. cereus strain ZZ4	*B. cereus* strain LRN (AY138275) (1,366/1,366 bp identity)	Oil contaminated soil (Northern Alberta)	100 mM [1% (v/v)]
Pseudomonas sp. strain ZZ5[b]	*Pseudomonas* sp. (biodegradation) (Y13246) (1,482/1,482 bp identity)	Marigold soil (Southern Alberta)	20 mM [0.21% (v/v)]
Pseudomonas sp. strain ZZ6[b]	*Pseudomonas citronellolis* (Z76659) (1,156/1,160 bp identity; 99%)	Marigold soil (Southern Alberta)	20 mM [0.21% (v/v)]
S. maltophilia strain ZZ7[b]	*S. maltophilia* (AB180661) (1,487/1,498 bp identity; 99%)	Marigold soil (Edmonton, Alberta)	20 mM [0.21% (v/v)]
B. cepacia strain ZZ8[b]	*B. cepacia* complex strain ATCC 17759 (AY741334) (1,435/1,468 bp identity; 97%)	Marigold soil (Southern Alberta)	23 mM [0.24% (v/v)]

[a]Growth in LB media plus solvent
[b]Gram negative bacteria

organic solvents were performed with 10 ml of LB media in 125 ml Erlenmeyer flasks sealed with foil-covered neoprene stoppers, shaken at 220 rpm in a 30°C incubation chamber.

Results

Characterization of solvent-tolerant bacteria

The four isolates from the non-selective were gram-positive bacteria as determined by gram stain, whereas the four isolates bacteria from PIA were stained as gram-negative bacteria. The gram-positive bacteria were given the strain names ZZ1–ZZ4, and the gram-negative bacteria were named ZZ5–ZZ8. Initial analysis under a light microscope showed ZZ1 to be a gram-positive cocci found in irregular clusters, ZZ2 and ZZ3 to be a gram-positive rods existing as single cells or pairs, and ZZ4 to be a gram-positive rod existing in regular clusters. Light microscopy showed ZZ5 to be a gram-negative rod existing in clusters, and ZZ6, ZZ7, and ZZ8 to be gram-negative rods found in pairs or chains.

Taxonomic classification of these isolates was determined by both 16S rDNA comparison and carbon source utilization comparisons with other bacteria. As shown in Table 1 and Fig. 1a, the 16S rDNA sequence alignment of the newly isolated gram-negative bacteria with previously isolated organisms identified a novel *Pseudomonas* sp. (ZZ5) as well as a *Pseudomonas* sp. (ZZ6) that is similar to *P. citronellolis*, an organism frequently isolated from oil-contaminated soil (Bhattacharya et al. 2003). In addition, a *Stenotrophomonas maltophilia* isolate named ZZ7 was identified; an organism that has recently become a clinical problem due to its high levels of efflux mediated antibiotic resistance. Finally, we isolated a *Burkholderia cepacia* complex member named ZZ8 from soil planted to marigolds that could withstand the presence of slightly more toluene than the other bacteria isolated under selective conditions. Further taxonomic classification by 16S rDNA sequence comparison indicated that strain ZZ8 is a *B. cenocepacia* strain (genomovar IIIb).

In the non-selective culture condition, several gram-positive bacteria that exhibited higher solvent tolerances than the isolated gram-negative bacteria were isolated (Table 1). This was somewhat surprising given that gram-negative bacteria are generally more resistant to many toxic compounds because of their more impermeable double membrane cell envelope. By 16S rDNA analysis it was determined that we had isolated an environmental *Staphylococcus* sp. (ZZ1), two *Bacillus cereus* isolates (ZZ2 and ZZ4), and a *Bacillus* sp. (ZZ3). The partial 16S rDNA sequence of *Staphylococcus* sp. ZZ1 was shown to have 100% identity with *Staphylococcus* sp. strain LMG 19417 (AJ276810.1) and other related *Staphylococcus* sp. As shown in Fig. 1b, *Staphylococcus* sp. strain ZZ1 as well as the related *Staphylococcus* sp. (all reported to be involved in some form of organic hydrocarbon degradation), are more closely related to

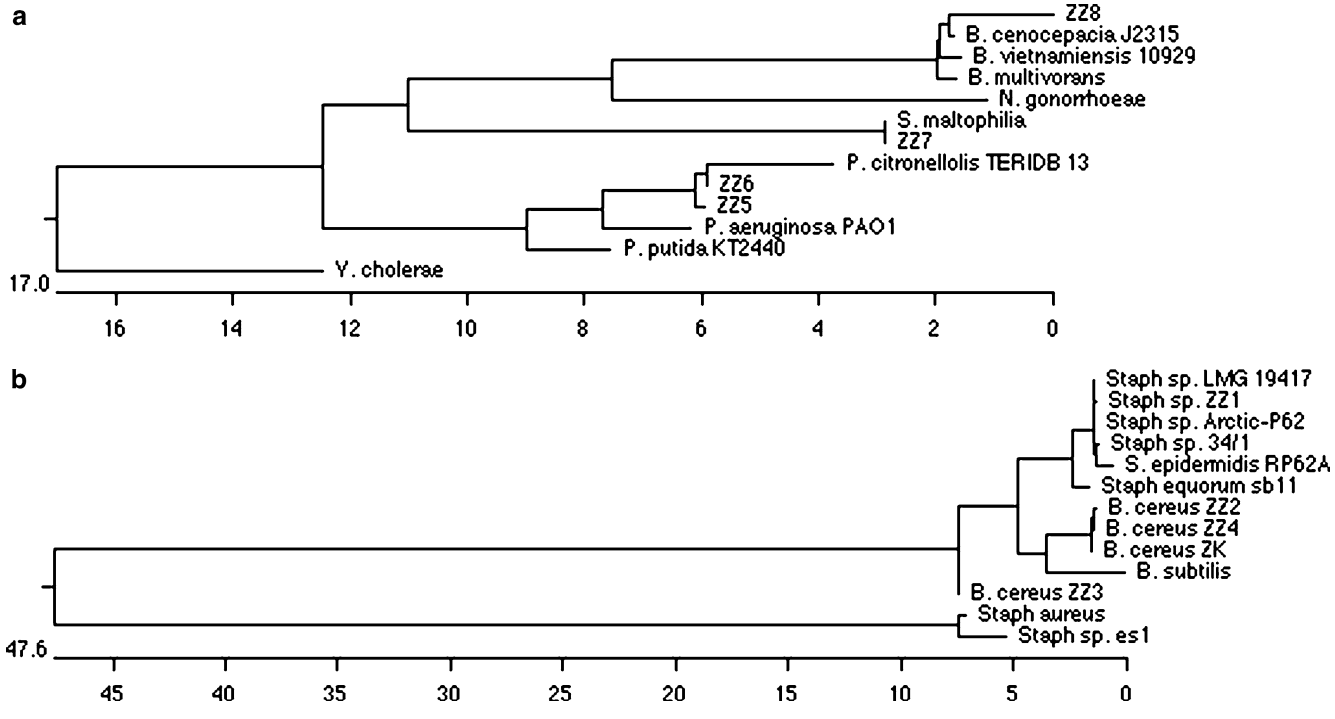

Fig. 1 **a** Phylogenetic tree of isolated gram-negative bacteria and closely related species. Branch distances represent relatedness between partial 16S sequences determined for each bacterial species. **b** Phylogenetic tree of isolated gram-positive bacteria and closely related species. The two major branches in each tree are used for outlier comparison purposes only and do not denote a close relationship

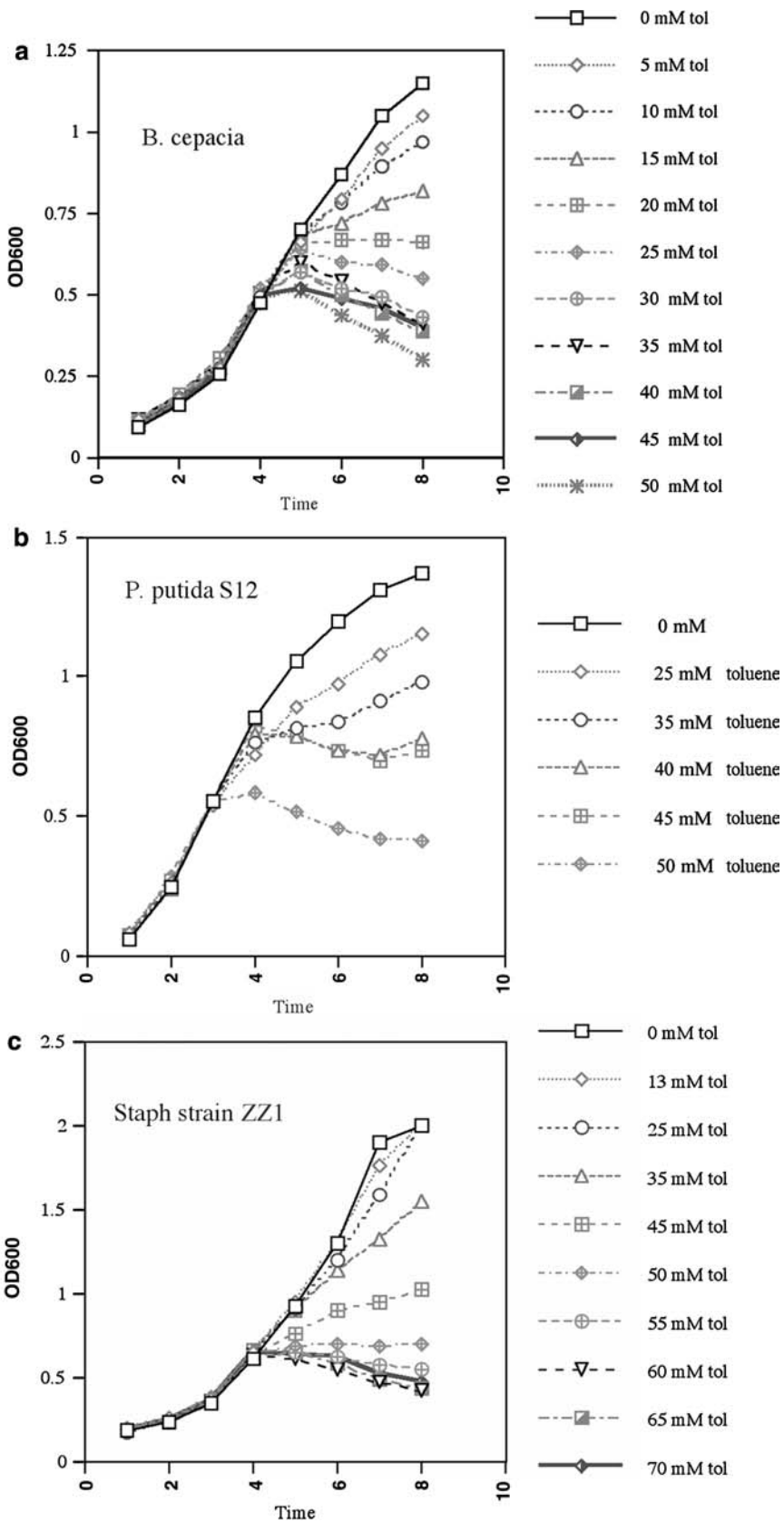

Fig. 2 Growth curves of isolated bacteria following solvent shock. Set quantities of organic solvents were added to cells growing in early lag phase at 4 h after subculture. Cell growth was assessed by measuring OD_{600} in sealed Nephelo flasks to reduce the loss of organic solvent by volatilization. Growth after different solvent shock quantities are displayed on one graph, showing an inverse relationship between the amount of solvent added to the media and the amount of cell growth. Shown are organic solvent shock growth curves for the, **a** newly isolated *B. cepacia* complex strain ZZ8, **b** the previously characterized *P. putida* S12 strain (19), and **c** the newly isolated *Staphylococcus* sp. strain ZZ1. The *y*-axis shows time elapsed in hours

Staphylococcus epidermidis RP62A (NC_002976) and the *Bacillus* clade of soil organisms than *Staphylococcus aureus*. The 16S rDNA sequences reported in this study have been submitted to the GenBank database with the respective accession numbers for strains ZZ1–ZZ8 deposited as DQ113448–DQ113455.

Table 2 Range of toxic organic solvent tolerance of gram-positive bacteria

Solvent (concentration)	log P_{ow}	*Staphylococcus* sp. strain ZZ1	*B. cereus* strain ZZ2	*B. cereus* strain ZZ3	*B. cereus* strain ZZ4
Hexane 100 mM [1.3% (v/v)]	3.5	+ + +	+ + +	+ + +	+ + +
Cyclohexane 100 mM [1% (v/v)]	3.2	+ + +	+ + +	+ + +	+ + +
p-Xylene 100 mM [1.2% (v/v)]	3.0	+ + +	-	±	-
Toluene 50 mM [0.53% (v/v)]	2.5	+ + +	+ + +	+ + +	+ + +
Toluene 100 mM [1% (v/v)]	2.5	+ + +	±	+ + +	+ + +
1-Heptanol 100 mM [1.4% (v/v)]	2.4	-	-	-	-
Dimethylphthalate 100 mM [2% (v/v)]	2.3	+ + +	-	+ + +	+ + +
Fluorobenzene 100 mM [1% (v/v)]	2.2	+ + +	+ + +	+ + +	+ + +
Benzene 100 mM [1% (v/v)]	2.0	+ + +	+ + +	+ + +	+ + +
Phenol 20 mM [0.18% (v/v)]	1.5	+ + +	-	+ + +	+ + +

+ + + growth overnight (16 h); ± minimal growth overnight; - no growth

Table 3 Range of toxic organic solvent tolerance of gram-negative bacteria

Solvent (concentration)	log P_{ow}	*Pseudomonas* sp. strain ZZ5	*Pseudomonas* sp. strain ZZ6	*S. maltophilia* strain ZZ7	*B. cepacia* strain ZZ8	*P. putida* strain S12
Hexane 100 mM [1.3% (v/v)]	3.5	+ + +	+ + +	+ + +	+ + +	+ + +
Cyclohexane 100 mM [1% (v/v)]	3.2	+ + +	+ + +	+ + +	+	+ + +
p-Xylene 100 mM [1.2% (v/v)]	3.0	±	±	-	-	+ + +
Toluene 30 mM [0.32% (v/v)]	2.5	-	-	-	-	+ + +
Toluene 100 mM [1% (v/v)]	2.5	-	-	-	-	+ + +
1-Heptanol 100 mM [1.4% (v/v)]	2.4	-	-	-	-	±
Dimethylphthalate 20 mM [0.33% (v/v)]	2.3	+ + +	+ + +	±	±	+ + +
Dimethylphthalate 100 mM [2% (v/v)]	2.3	+ + +	+ + +	-	-	+ + +
Fluorobenzene 100 mM [1% (v/v)]	2.2	-	-	-	-	-
Benzene 100 mM [1% (v/v)]	2.0	-	-	-	-	-
Phenol 20 mM [0.18% (v/v)]	1.5	-	-	-	-	-

+ + + growth overnight (16 h); ± minimal growth overnight; - no growth

General physiology of these newly isolated solvent-tolerant bacteria as determined by Biolog MicroLog3 metabolic analysis generally confirmed 16S comparisons. Physiological data using Biolog MicroLog3 MicroPlates predicted isolate ZZ1 to be a *Staphylococcus warneri* species with 100% probability. Isolate ZZ2 was predicted to be a novel *Bacillus* sp., though with strong similarity to *B. cereus* or *Bacillus thuringenesis* (which could not be distinguished using these tests). Isolates ZZ3 and ZZ4 were both predicted to be *B. cereus*/*thuringensis* strains with 100% probability. Even though 16S rDNA analysis predicted ZZ5 and ZZ6 to be closely related, isolate ZZ5 was predicted to be a new pseudomonad with the most similarity to *P. citronellolis*, but exhibited only 43% similarity in substrate metabolism, while isolate ZZ6 was predicted to be a *P. citronellolis* strain with 100% probability (84% similarity in substrate utilization). Isolate ZZ7 was predicted to be an *S. maltophilia* strain with 100% probability, while isolate ZZ8 was predicted to be a *B. cepacia* complex member (*Burkholderia multivorans*; genomovar II) with 100% probability (70% similarity in substrate utilization). The discrepancy between the ZZ8 16S rDNA comparison and the physiological data obtained through Biolog experiments suggests that ZZ8 is a *B. cenocepacia* strain with expanded metabolic capabilities similar to *B. multivorans*.

Bacterial growth characteristics in organic solvents

In terms of growth in organic solvents (shown in Table 1), it is apparent that the gram-positive mechanism of solvent tolerance is more effective than that seen with newly isolated gram-negative cells. Although solvent insolubility above a certain threshold concentration was expected to produce a cell death plateau, this was not observed. Instead, cell death increased with increasing solvent concentrations, in both solvent shock and adaptation conditions, even at solvent concentrations well above the known solvent solubility level. This effect was observed not only for the newly isolated gram-positive bacteria (Fig. 2c), but also for the gram-negative bacteria (Fig. 2a) including the previously well-characterized organic solvent-tolerant *P. putida* S12 (Fig. 2b, Weber et al. 1993). The finding that gram-positive organisms such as *Staphylococcus* sp. strain ZZ1 were tolerant to higher concentrations of toluene than the gram-negative organisms tested using the exact same conditions strongly suggests that these gram-positive bacteria can withstand the direct effects of toxic organic solvents better than most gram-negative bacteria. The detection of this killing effect at different concentrations above maximum solubility suggests that solvent toxicity is more complex than previously described.

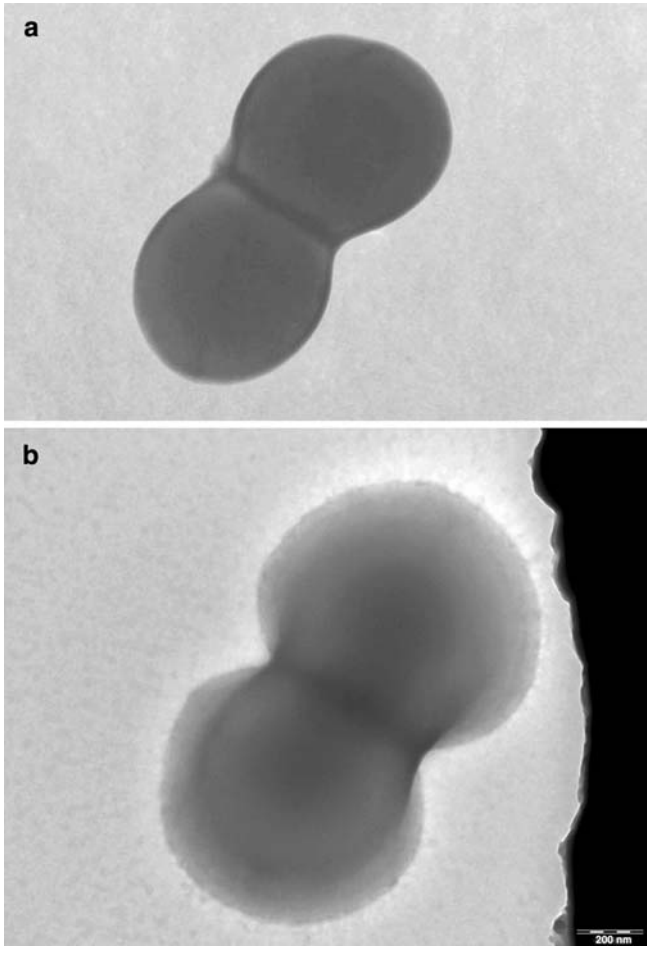

Fig. 3 Electron micrographs of the newly isolated organic solvent-tolerant *Staphylococcus* sp. ZZ1 grown in LB media under conditions, **a** without the organic solvent toluene, or **b** with 50 mM toluene in the growth medium. *Panel B* shows the modified membrane of *Staphylococcus* sp. ZZ1 at 44,000 times magnification as compared to a 200 nm scale bar. The black region observed in *panel b* is an electron micrograph grid section

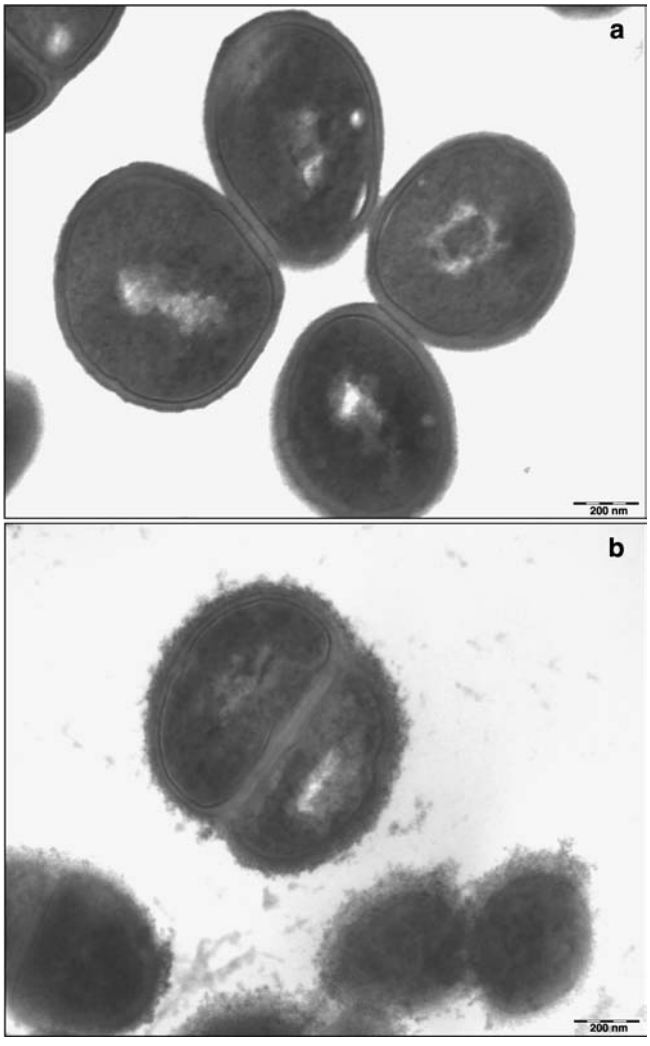

Fig. 4 Transmission electron micrographs of thin-sectioned *Staphylococcus* sp. ZZ1 for capsule detection. Isolate ZZ1 was grown in the absence (**a**) and presence (**b**) of 50 mm toluene in the growth medium before fixation, cross-sectioning, and staining with ruthenium red dye. The cells are shown in comparison to a 200 nm scale bar at 44,000 times magnification

In the adaptive growth condition, cells were repeatedly sub-cultured every 24 h into fresh media containing slightly increasing levels of one solvent such as toluene. This experimental procedure was less harsh than the solvent shock procedure illustrated in Fig. 2, and allowed the organisms an opportunity to adapt to the presence of solvents through mutation or gene expression. Using this procedure it was found that some cells, particularly the gram-positive cells, could withstand much higher levels of solvents than in the solvent shock experiments; for *Staphylococcus* sp. ZZ1 compare 100 mM toluene (Table 1) versus 50 mM toluene (Fig. 2c). In general, we found solvent shock was less well-tolerated than growth in solvents suggesting that cells can at least partially adapt to harsh growth conditions given time to either undergo mutation and/or express new cellular proteins or structures. In spite of the up-regulation of efflux pumps by some gram-negative bacteria to resist the presence of solvents in the solvent growth conditions (Kieboom et al. 1998b), the isolated gram-negative bacteria were less well adapted to the presence of high concentrations of solvents than were gram-positive bacteria (compare the horizontal lines representing no new growth in Fig. 2a–c).

In order to determine whether this high level of solvent tolerance seen in gram-positive bacteria was specific to toluene, we tested the new bacterial isolates in different toxic organic solvents. As shown in Table 2, all of the newly isolated gram-positive bacteria exhibited high tolerance in two-phase systems of organic solvents with log P_{ow} values approaching 2.0, including 20 mM phenol (except ZZ2), 100 mM benzene, and 100 mM fluorobenzene, while the gram-negative bacteria were killed at similar solvent concentrations (Table 3). The newly isolated gram-negative bacteria were also sensitive to 30 and 100 mM toluene as expected whereas the gram-positive

bacteria were tolerant to this solvent concentration. There was some solvent specificity exhibited by both gram-positive and gram-negative bacteria; the gram-negative cells were sensitive to toluene and solvents with log P_{ow} values near 2.0, and all bacteria were sensitive to 1-heptanol. The data obtained for *P. putida* S12 is similar to that previously obtained (Kieboom et al. 1998a).

Cellular adaptation to organic solvents

To examine the gram-positive mechanism of solvent tolerance more closely, we grew the newly isolated environmental *Staphylococcus* sp. ZZ1 in media containing solvent or no solvent and compared cellular morphologies under an electron microscope after staining with phosphotungstic acid. As compared to growth without solvent (Fig. 3a), cells grown in the presence of 50 mM toluene produce an extracellular capsule (Fig. 3b). This capsule gives the cells a stuffed or fuzzy appearance, and covers the coccoid *Staphylococcus* cells nearly completely, except at the attachment site to neighboring cells. It is apparent that this capsule extends some distance from the cell membrane as observed by the occlusion of background detail as shown in Fig. 3b. As shown in Fig. 4a and b, a more detailed visualization of this capsule was produced upon staining the organic solvent grown cells with ruthenium red and cross-sectioning the cells prior to EM visualization (Vanrobaeys et al. 1999).

Discussion

Bacterial tolerance to toxic compounds is thought to be the result of an interplay between a relatively impermeable membrane that limits the diffusion of these compounds into the cell, and energy-dependent efflux mechanisms that extrude any noxious agents out of the cell that have managed to bypass the membrane barrier. Since it has been demonstrated that RND efflux pumps from gram-negative cells can accept as substrates both organic solvents and multiple classes of antibiotics, it was anticipated that cells with the highest levels of antibiotic resistance would also have the highest levels of organic solvent tolerance. Therefore, it was somewhat surprising that gram-positive bacteria (with membranes more permeable to antibiotics) were found to be generally more tolerant to organic solvents than gram-negative bacteria. While bacteria with both types of cell envelope architecture seem to react similarly with respect to solvent growth or solvent shock (solvent shock conditions being more lethal), there is a clear difference in the ability of gram-positive cells to withstand higher concentrations of toxic organic solvents with log P_{ow} values of approximately 2.0.

By enriching for only those cells able to withstand extreme levels of toxic organic solvents, we anticipated that this selection would result in the isolation of bacteria best able to survive in organic solvents. Although the gram-negative bacteria we isolated were not as solvent tolerant as *P. putida* S12 (Weber et al. 1993), they were at least as tolerant as *E. coli* DH5α, which withstood 20–25 mM toluene using our experimental conditions (data not shown). *E. coli* is a moderately solvent tolerant bacterium due to its expression of the AcrAB-TolC efflux pump that is capable of both antibiotic and organic solvent efflux (White et al. 1997). In comparison, the data obtained for *P. putida* S12 in Table 3 is similar to that previously found (Kieboom et al. 1998a), and suggests that these newly isolated gram-negative bacteria do not contain a specialized solvent efflux pump like SrpABC (Kieboom et al. 1998a).

Other gram-positive bacteria tolerant to organic solvents have been previously identified. A *Rhodococcus* sp. isolated from a chemical-contaminated site in Australia has been shown to have exceptional tolerance to high levels of benzene (Paje et al. 1997). A *Bacillus* sp. strain has been isolated from soil that tolerates the organic solvent *n*-butanol (Sardessai and Bhosle 2002b), and a *Bacillus* sp. strain has been isolated from deep sea with a high level of tolerance to benzene (Moriya and Horikoshi 1993). Isken and de Bont (1998) describe the isolation of several *Bacillus* sp. from normal soil environments tolerant to toluene. One particular strain of *B. cereus* named R1 was subjected to further analysis (Matsumoto et al. 2002). In comparison with the results obtained for the *B. cereus* isolates described in this study, *B. cereus* R1 was less tolerant of solvents with low log $P_{o/w}$ values such as benzene at similar solvent concentrations (1% v/v). However, *B. cereus* R1 was tolerant to similar levels of hexane and toluene, and similarly sensitive to 1-heptanol. Some evidence was shown for the existence of an active transport system for the exclusion of solvents in *B. cereus* R1 (Matsumoto et al. 2002), however, it is unknown if such a system is involved or important to the tolerance observed in all *B. cereus* strains. While *Rhodococcus* sp. strain 33 is efficient at tolerating higher levels of benzene (2% v/v) in part through benzene metabolism (Paje et al. 1997), the gram-positive cells characterized in this study have been demonstrated to be almost as tolerant, withstanding at least 1% (v/v) benzene.

Although several mechanisms such as deactivating enzymes, endospore protection, or efflux pumps have been proposed for this tolerance in gram-positive bacteria, little information about these mechanisms has been described. We hypothesize that the observed solvent tolerance in some gram-positive bacteria is derived from the unusual capsular ultrastructure. While gram-positive bacteria have in the past been shown to use extracellular capsules to resist the effects of the immune system as well as penetration by antibiotics, to our knowledge this is the first observation of a capsule apparently being used to withstand the effects of noxious environmental chemicals. Since organic solvents preferentially partition into the hydrophobic portion of the phospholipid bilayer, it is anticipated that solvent tolerance can be achieved through the production of a hydrophilic carbohydrate

capsule that repels organic solvents and prevents them from reaching the cellular membrane. This capsule is evident in Fig. 3b, where the background detail is obscured by a capsule that is difficult to visualize experimentally. Ruthenium red staining of the cell surface prior to EM visualization produced somewhat better images of this structure (Fig. 4a, b). We are currently working to further characterize this gram-positive capsule and determine whether it is the sole mechanism responsible for the observed solvent tolerance in these bacteria. Because *Staphylococcus* strain ZZ1 exhibits tolerance to toxic organic solvents under solvent shock conditions where no prior adaptation such as capsule production has occurred, it suggests that there are also other physiological mechanisms that make these gram-positive bacteria solvent tolerant.

Organic solvents in two-phase systems are thought to only cause cellular toxicity when solubilized in the aqueous phase where cells are expected to exist. At concentrations above the threshold level of solvent solubility, it has been proposed that additional solvent will not cause increased toxicity or cell death. Our results appear to contradict these predictions. Our results (from both solvent shock and solvent growth conditions) with solvent concentrations in excess of solvent solubility may be the result of our growth conditions, where shaking flasks agitate the two-phase solutions such that cells are not always maintained in the aqueous phase. In this case, the addition of higher concentrations of solvents would cause the solvent phase to more frequently contact the cells directly with the expected outcome of membrane destruction and cell death. Provided that the experimental conditions are similar for each bacterium tested, accurate estimates can be made about the relative organic solvent tolerance of each bacterium at solvent concentrations above the threshold of solvent solubility (Bar 1988). Moreover, the ability of a bacterium to grow in solvents with different log P_{ow} values as an indicator of organic solvent tolerance (as shown in Tables 2, 3) mirror the results obtained in the solvent shock and solvent growth experiments for the new bacterial isolates versus previously characterized solvent-tolerant bacteria such as *P. putida* S12. This strongly supports the view that these new gram-positive bacteria are at least as tolerant, if not more tolerant than previously characterized solvent-tolerant gram-negative bacteria.

It has previously been shown that efflux pump genes can be transferred between gram-negative strains to confer increased solvent tolerance on the host (Kieboom et al. 1998a). Because the proper assembly of the tripartite RND efflux pump in the gram-negative cell envelope is essential to this increased solvent tolerance, it is unlikely that these genes could be transferred to gram-positive organisms to produce properly assembled efflux pumps in the single bacterial membrane. However, it may be possible to transfer the genes for an extracellular polysaccharide to a solvent-tolerant gram-negative bacterium and expect proper assembly on the surface of the cell. The combination of an extracellular capsule with an energy-dependent efflux system that can extrude solvents may confer additional organic solvent tolerance to modified bacteria that could be used industrially (Sardessai and Bhosle 2002a). Modified bacterial cells with enhanced solvent tolerance would be extremely useful in the fields of applied microbiology and biotechnology. Bacteria used in the environmental bioremediation of toxic wastes could be enhanced to withstand higher concentrations of the pollutants. In the industrial production of fine chemicals, organic solvent-tolerant bacteria would be better able to withstand extraction of the chemical end-product with a second phase of organic solvent. The further characterization of these specialized organic solvent-tolerant bacteria will provide us with the knowledge required for their use in biotechnological applications.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Ausubel FM, Brent R, Kingston RE, Moore DD, Seldman JG, Smith JA, Struhl K (1991) Current protocols in molecular biology. Greene Publishing Associates, NY

Bar P (1988) Effect of interphase mixing on a water-organic solvent two-liquid phase microbial system: ethanol fermentation. J Chem Tech Biotechnol 43:49–62

Bhattacharya D, Sarma PM, Krishnan S, Mishra S, Lal B (2003) Evaluation of genetic diversity among *Pseudomonas citronellolis*: strains isolated from oily sludge-contaminated sites. Appl Environ Microbiol 69:1435–1441

Cruden DL, Wolfram JH, Rogers RD, Gibson DT (1992) Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. Appl Environ Microbiol 58:2723–2729

Christensen LP, Lam J (1990) Acetylenes and related compounds in *Cynareae*. Photochem 29:2753–2785

Davidson PM, Barnden AL (1981) Antimicrobial activity of non-halogenated phenolic compounds. J Food Prot 44:623–632

de Bont JAM (1998) Solvent-tolerant bacteria in biocatalysis. Trends Biotechnol 16:493–499

Inoue A, Horikoshi K (1989) A *Pseudomonas* thrives in high concentration of toluene. Nature 338:264–266

Isken S, de Bont JA (1998) Bacteria tolerant to organic solvents. Extremophiles 2:229–238

Johnson J (1994) Similarity analysis of rRNAs. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. ASM Press, Washington, DC, pp 683–700

Kieboom J, Dennis JJ, de Bont JA, Zylstra GJ (1998a) Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J Biol Chem 273:85–91

Kieboom J, Dennis JJ, Zylstra GJ, de Bont JA (1998b) Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. J Bacteriol 180:6769–6772

Kieboom J, de Bont JA (2000) Mechanisms of organic solvent tolerance in bacteria. In: Storz G, Hengee-Aronis R (eds) Bacterial stress responses. ASM Press, Washington, DC, pp 393–402

Kim K, Lee L, Lee K, Lim D (1998) Isolation and characterization of toluene-sensitive mutants from the toluene-resistant bacterium *Pseudomonas putida* GM73. J Bacteriol 180:3692–3696

Ma D, Cook DN, Hearst JE, Nikaido H (1994) Efflux pumps and drug resistance in gram-negative bacteria. Trends Microbiol 2:489–493

Matsumoto M, De Bont JAM, Isken S (2002) Isolation and characterization of the solvent-tolerant *Bacillus cereus* strain R1. J Biosci Bioeng 94:45–51

Moriya K, Horikoshi K (1993) A benzene-tolerant bacterium utilizing sulfur compounds isolated from deep sea. J Ferment Bioeng 76:397–399

Nakajima H, Kobayashi H, Aona R, Horikoshi K (1992) Effective isolation and identification of toluene-tolerant *Pseudomonas* strains. Biosci Biotechnol Biochem 56:1872–1873

Ogino H, Miyamoto K, Ishikawa H (1994) Organic solvent-tolerant bacterium which secretes an organic solvent-stable lipolytic enzyme. Appl Environ Microbiol 60:3884–3886

Ohta Y, Maeda M, Kudo T, Horikoshi K (1996) Isolation and characterization of solvent-tolerant bacteria which can degrade biphenyl/polychlorinated biphenyls. J Gen Appl Microbiol 42:349–354

Osborne SJ, Leaver J, Turner MK, Dunnill P (1990) Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. Enzyme Microb Technol 12:281–291

Paje ML, Neilan BA, Couperwhite I (1997) A *Rhodococcus* species that thrives on medium saturated with liquid benzene. Microbiology 143:2975–2981

Ramos JL, Duque E, Huertas MJ, Haidour A (1995) Isolation and expansion of the catabolic potential of a *Pseudomonas putida* strain able to grow in the presence of high concentrations of aromatic hydrocarbons. J Bacteriol 177:3911–3916

Ramos JL, Duque E, Godoy P, Segura A (1998) Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. J Bacteriol 180:3323–3329

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sardessai Y, Bhosle S (2002a) Tolerance of bacteria to organic solvents. Res Microbiol 153:263–268

Sardessai Y, Bhosle S (2002b) Organic solvent tolerant bacteria in mangrove ecosystem. Curr Sci 82:622–623

Shima H, Kudo T, Horikoshi K (1991) Isolation of toluene-resistant mutants from *Pseudomonas putida* PpG1 (ATCC 17453). Agric Biol Chem 55:1197–1199

Sikkema J, de Bont JA, Poolman B (1994) Interactions of cyclic hydrocarbons with biological membranes. J Biol Chem 269:8022–8028

Sikkema J, de Bont JA, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201–222

Vandamme P, Holmes B, Vancanneyt M, Coenye T, Hoste B, Coopman R, Revets H, Lauwers S, Gillis M, Kersters K, Govan JR (1997) Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. Int J Syst Bacteriol 47:1188–1200

Vanrobaeys M, De Herdt P, Charlier G, Ducatelle R, Haesebrouck F (1999) Ultrastructure of surface components of *Streptococcus gallolytics* (*S. bovis*) strains of differing virulence isolated from pigeons. Microbiology 145:335–342

Weber FJ, Ooijkaas LP, Schemen RM, Hartmans S, de Bont JA (1993) Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. Appl Environ Microbiol 59:3502–3504

Weber FJ, de Bont JA (1996) Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim Biophys Acta 1286:225–245

White DG, Goldman JD, Demple B, Levy SB (1997) Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA*, *soxS*, or *robA* in *Escherichia coli*. J Bacteriol 179:6122–6126

Woldringh CL (1973) Effects of toluene and phenethyl alcohol on the ultrastructure of *Escherichia coli*. J Bacteriol 114:1359–1361