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Proteomic Analysis Reveals the Participation of Energy- and Stress-Related Proteins in the Response of *Pseudomonas putida* DOT-T1E to Toluene

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***Pseudomonas putida* DOT-T1E is tolerant to toluene and other toxic hydrocarbons through extrusion of the toxic compounds from the cell by means of three efflux pumps, TtgABC, TtgDEF, and TtgGHI. To identify other cellular factors that allow the growth of *P. putida* DOT-T1E in the presence of high concentrations of toluene, we performed two-dimensional gel analyses of proteins extracted from cultures grown on glucose in the presence and in the absence of the organic solvent. From a total of 531 spots, 134 proteins were observed to be toluene specific. In the absence of toluene, 525 spots were clearly separated and 117 proteins were only present in this condition. Moreover, 35 proteins were induced by at least twofold in the presence of toluene whereas 26 were repressed by at least twofold under these conditions. We reasoned that proteins that were highly induced could play a role in toluene tolerance. These proteins, identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry, were classified into four categories: 1, proteins involved in the catabolism of toluene; 2, proteins involved in the channeling of metabolic intermediates to the Krebs cycle and activation of purine biosynthesis; 3, proteins involved in sugar transport; 4, stress-related proteins. The set of proteins in groups 2 and 3 suggests that the high energy demand required for solvent tolerance is achieved via activation of cell metabolism. The role of chaperones that facilitate the proper folding of newly synthesized proteins under toluene stress conditions was analyzed in further detail. Knockout mutants revealed that CspA, XenA, and Tuf-1 play a role in solvent tolerance in *Pseudomonas*, although this role is probably not specific to toluene, as indicated by the fact that all mutants grew more slowly than the wild type without toluene.**

Organic solvents with a log P_{ow} (logarithm of the partition coefficient in a mixture of octanol and water) between 1.5 and 4 are extremely toxic for microorganisms because they partition in the cell membranes and disorganize them by removing lipids and proteins, eventually leading to cell death (8). However, since the report by Inoue and Horikoshi (19) of a bacterium able to thrive in the presence of high concentrations of organic solvents, other solvent-tolerant bacteria have been isolated. Among them, *Pseudomonas putida* DOT-T1E, a highly solvent-tolerant microbe, was isolated from a wastewater treatment plant in Granada, Spain (40). Solvent tolerance in *P. putida* DOT-T1E is an inducible phenomenon. When cells were shocked with 0.3% (vol/vol) toluene, only 1 out of 10,000 survived; however, if cells were preinduced with a small amount of toluene (i.e., 0.01% [vol/vol]), almost 100% of the cells survived the 0.3% (vol/vol) toluene shock.

The mechanisms underlying solvent tolerance are not yet fully understood, but a number of factors involved in the process have been characterized over the last 10 years (43). Several laboratories have identified efflux pumps belonging to the RND (resistance, nodulation, and cell division) family as being involved in solvent tolerance (23, 25, 36, 42, 45). In *P. putida* DOT-T1E, three of these efflux pumps have been found to participate in toluene tolerance. TtgABC is constitutively expressed and is able to extrude several organic solvents and antibiotics (10, 52). TtgDEF is not expressed under normal laboratory conditions, but it is induced by organic solvents such as toluene and styrene (36), and it is mainly involved in induced organic solvent tolerance. TtgGHI is constitutively expressed at relatively high basal levels, but the expression of the *ttgGHI* genes is also induced by organic solvents (16, 17, 46). As expected, *ttgB*, *ttgD*, and *ttgH* knockout mutants exhibited increased solvent sensitivity compared to the parental strain. Although extrusion of the solvent by efflux pumps is probably the most important mechanism leading to solvent resistance, some other factors have been identified as being involved in the solvent tolerance phenomenon. Among these factors are membrane rigidification via isomerization of the *cis* unsaturated fatty acids into the corresponding *trans* isomers (18, 22), flagellar export proteins (24, 50), and formation of membrane vesicles filled with toluene (26).

Apart from extruding aromatic hydrocarbons such as toluene, xylenes, and styrene (45), the three efflux pumps mentioned above are also able to remove aliphatic alcohols such as 1-hexanol and 1-octanol. In fact, *P. putida* DOT-T1E cells grown on Luria-Bertani (LB) medium tolerated the sudden addition of 0.3% (vol/vol) 1-octanol well: 1 out of 10 cells survived a 1-octanol shock if the culture had not been preinduced, and almost 100% of the cells managed to survive the shock when induced beforehand with toluene (i.e., 0.01% [vol/vol]) or with 1 mM 1-octanol (47). However, despite the fact that 1 mM 1-octanol was able to activate the main mechanisms

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of solvent resistance (the three efflux pumps and the *cis-trans* isomerization of the unsaturated fatty acids), the presence of small amounts of this aliphatic alcohol (6 mM) severely impeded growth (47). This is not the case for toluene since *P. putida* DOT-T1E not only survives a sudden shock with this organic solvent but is also able to grow in the presence of high concentrations of this aromatic hydrocarbon (40). Although *P. putida* DOT-T1E uses toluene through the toluene dioxygenase pathway, the strain's ability to grow in the presence of high concentrations of the solvent (up to 90% [vol/vol] toluene) is not a consequence of this catabolic property since a *todC1* gene (that encodes one of the components of toluene dioxygenase) knockout mutant which was unable to grow with toluene as a sole carbon source tolerated as much toluene as the wild-type strain (35). The above series of observations prompted us to hypothesize that a number of other proteins, in addition to the efflux pumps described above, could be induced in toluene-exposed *P. putida* cells to enable the cell to grow in the presence of the aromatic hydrocarbon. To investigate these factors, two-dimensional (2D) gel electrophoresis and mass spectrometry (MS) were used to identify proteins whose expression was affected in cultures grown in the presence of toluene. The biological significance of the proteins identified in this study is discussed.

MATERIALS AND METHODS

Bacterial strains, culture, and growth conditions. *P. putida* DOT-T1E was grown on M9 minimal medium (1) with 25 mM glucose as the sole carbon source in the presence or in the absence of toluene. Toluene was added via the gas phase. To this end, we introduced toluene in a U-shaped open glass tube which was placed inside the flask. The flask was then hermetically sealed. Toluene passed to the gas phase and dissolved in a concentration of 5.8 mM. Cultures were incubated at 30°C and shaken on an orbital platform operating at 200 strokes per min. For protein extraction, cells were incubated overnight with or without toluene and on the next day the cultures were diluted 1:100 and grown until the turbidity reached 0.8 at 660 nm. Three-hundred milliliter volumes of these cultures were harvested by centrifugation at 5,000 × *g* for 15 min at 4°C (Allegra 21R centrifuge C0650; Beckman Coulter, Madrid, Spain) and the pellets frozen in liquid nitrogen. The glucose concentration in the culture medium was measured with an Accutrend Sensor (Roche-Diagnostic).

Extraction of the cytosolic protein fraction, 2D gel electrophoresis, and image analysis. Ten milliliters of cold buffer (50 mM Tris-HCl [pH 7.6] with protease inhibitor cocktail at the concentration recommended by the manufacturer [Complete; Roche]) was added directly to the frozen pellets. The mixture was vortexed to homogeneity and subsequently frozen again at −80°C. Bacterial suspensions were then thawed and subjected to 10 pulses of sonication (full power, 0.5 s per pulse; IKASONIC U 200S control, ultrasonic processor). The extract was centrifuged for 15 min at 5,000 × *g* to precipitate cell debris and intact cells. The supernatant was carefully aspirated (to avoid disturbing the pellet) and transferred to Optiseal tubes (reference no. 361623; Beckman). The extracts were centrifuged again in a Beckman Ti70.1 rotor for 2 h at 56,700 × *g* at 4°C. The supernatant was collected, and the proteins were concentrated using Amicon columns (centrifuged at 4,000 × *g* for 10 min). The samples were then frozen after the protein concentration had been determined by the procedure described by Bradford (4). Extracted proteins were precipitated using the Ettan 2-D clean up kit (Amersham Biosciences) following the manufacturer's instructions and were subsequently resuspended in isoelectric focusing (IEF) buffer consisting of 7 M urea, 2 M thiourea, and 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS).

For the first dimension of protein separation, IEF was performed using 24-cm immobilized nonlinear pH gradient strips (pH 3 to 10; GE Healthcare, Little Chalfont, United Kingdom). The strips were rehydrated for 12 h at room temperature with IEF sample buffer-protein mixtures at 50 V. A 200-μg volume of protein was loaded for IEF into a Multiphor electrophoresis unit (Amersham Biosciences) using a program in which the voltage was set for 2 h at 150 V, 1 h at 500 V, 1.5 h at 1,000 V, and 8.5 h at 8,000 V.

Prior to the second-dimension electrophoresis, IPG gel strips were equilibrated for 10 min at room temperature in 1% (wt/vol) dithiothreitol to reduce the proteins and sulfhydryl groups were subsequently derivatized using 4% (wt/vol) iodoacetamide (both solutions were prepared in 50 mM Tris [pH 8.8]–6 M urea–30% [vol/vol] glycerol–2% [wt/vol] bromophenol blue). Strips were transferred to 1.5-mm-thick 12% (wt/vol) polyacrylamide gels (26 by 24 cm), and the second-dimension gels were run at 18 W/gel for 6 h. Gels were stained with the silver staining PlusOne kit (Amersham Biosciences) following the manufacturer's instructions. The gels were scanned with LabScan software (Amersham), and the images were analyzed with Phoretix 2D evolution software (Nonlinear Dynamics, Newcastle, United Kingdom).

MS analysis. Protein spots of interest were excised and destained. In-gel digestion with trypsin was carried out using a ProGest robotic instrument (Genomic Solutions) with reduction and alkylation performed using dithiothreitol and iodoacetamide, respectively. Following desiccation, the digestion products were redissolved in a freshly prepared solution of 50% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid. One-half microliter of each extract was spotted onto a matrix-assisted laser desorption ionization (MALDI) plate with 0.5 μl of freshly prepared α-cyanohydroxycinnamic acid (5 mg/ml in 50% [vol/vol] acetonitrile–0.1% [vol/vol] trifluoroacetic acid) as a matrix. Instrument calibration was done using the peptides angiotensin II, substance P, and melittin as standards. Samples were analyzed on an ABI4700 mass spectrometer with time of flight (TOF)-TOF optics (Applied Biosystems). Both MS and tandem MS were carried out and the results analyzed using MASCOT (http://www.matrixscience.com/cgi/index.pl?page=../home.html) to search the National Center for Biotechnology Information nonredundant database. Unidentified samples were further analyzed by tandem MS using NanoLC/MALDI-Q-TOF with a Q-Tof Ultima (Micromass).

For both sets of MS data, protein identification was done using MASCOT software with links to the National Center for Biotechnology Information and SwissProt protein sequence databases. Carbomethylation of Cys residues was set as a fixed parameter in database interrogation, and oxidation of methionine residues was set as an optimal modification. For protein identification, 0 to 1 missed trypsin cleavage site and a mass tolerance of 75 ppm were accepted.

Survival in response to toluene shock. Cells were grown overnight in 30 ml of LB medium. On the following day, cultures were diluted 1:100 and grown under the same conditions until the cultures reached a turbidity of about 0.8 at 660 nm. These cultures contained about 10^8 CFU/ml. The cultures were divided into two halves; 0.3% (vol/vol) toluene was added to one, and the other was kept as a control. The number of viable cells was determined before toluene was added and 10, 30, and 60 min later. Assays where the cultures were subjected to different treatments before toluene shock are described in Results.

Construction of *cspA2*, *xenA*, and *tuf-1* mutants. For the construction of a mutant strain carrying a knockout mutation of the cold shock *cspA2* gene, we amplified a fragment of *P. putida* DOT-T1E chromosomal DNA with primers CspA21 (5′-TCAAGGAGGTGGTGGTCA-3′; located 1.5 kb upstream from the *cspA2* gene sequence of *P. putida* KT2440) and CSma2 (5′-GGCTTCATC ACCCCGGGAGGCGGCGGC-3′; located within the *cspA2* sequence; underlined is the SmaI-recognized sequence). Another fragment was amplified with CSma1 (5′-GCCGCCGCCTCCCGGGGTGATGAAGCC-3′; complementary to CSma2; underlined is the SmaI recognition site) and CspA22 (5′-TATTCTT CGTGGTAGTACAG-3′; located 1.5 kb downstream from the *cspA2* gene). The amplification products were visualized in an agarose gel, and the bands were extracted from the gel using the Qiaex II gel extraction kit (QIAGEN) following the manufacturer's instructions. Each PCR product was cloned into pCR2.1 (Invitrogen) to obtain plasmids pANA186 (containing the CspA21-CSma2 amplification product) and pANA187 (amplification product CSma1-CspA22). Both plasmids were cut with SmaI and XbaI, and the insert of pANA187 was ligated into pANA186 to obtain plasmid pANA188. The insert of pANA188 was cut with EcoRI and ligated into pGEM-T Easy (Promega) previously cut with the same restriction enzyme. The resulting plasmid, pANA189, was cut with SmaI, and the 0.8-kb SmaI fragment from pBSK-*aphA* (32) that encodes kanamycin resistance was inserted into pANA189 to yield plasmid pANA192. About 600 ng of this suicide plasmid was electroporated into *P. putida* DOT-T1E cells, and the transformants that had integrated the pANA192 plasmid into the host chromosome via homologous recombination were selected on solid LB medium with kanamycin. To identify double recombinants, colonies were grown on LB medium plates with kanamycin and LB medium with kanamycin and piperacillin. Transformants that did not grow in the presence of piperacillin but remained kanamycin resistant were selected, and one random clone, *P. putida* DOT-T1E-PS113, was kept for further assays. The correct integration of the *aphA* cassette into the *cspA2* gene in the chromosome was checked by PCR and Southern blot hybridization (not shown). Using oligonucleotides cspA2-3 (5′-GTTGATGCAC GCGGGGGCAA-3′) and cspA2-4 (5′-ATTTCACCGACGCCGAGATT-3′),

TABLE 1. Growth rates of *P. putida* DOT-T1E and isogenic mutants in the presence and in the absence of toluene

Strain	Generation time (min)[a]	
	Without toluene	With toluene
Wild type	77 ± 5	75 ± 7
cspA2 mutant	90 ± 5	114 ± 3
xenA mutant	230 ± 19	300 ± 25
tuf-1 mutant	109 ± 8	184 ± 5

[a] The values reflect averages of at least four replicates.

we amplified a 690-bp fragment containing the complete *cspA2* gene. The band was extracted from the gel and sequenced.

To obtain a *P. putida* DOT-T1E mutant disrupted in *xenA*, the *xenA* gene (accession number AY957609) was first amplified by PCR from DOT-T1E chromosomal DNA using the XenA forward primer 5′-TTTGGATCCGCAGCGA ATTCATATGG-3′ and the XenA reverse primer 5′-TTTGGATCCGGTGCA GTATCTGAAACC-3′. The PCR product was ligated into the pGEM-T vector (Promega). The resulting construction (pDOTA3) was cut with EcoRV, and a 0.8-kb SmaI fragment containing the *aphA* gene from pBSK-aphA was ligated to yield pDOTA3apha. A 2.8-kb SpeI fragment containing the *xenA*::*aphA* allele was subcloned into pMRS101 (48) and cut with the same restriction enzyme to obtain pDOTA3apha101. This vector was then used for allelic exchange by introducing it into the *P. putida* DOT-TIE strain via triparental mating, and merodiploids were selected in citrate plus kanamycin and streptomycin. Sucrose toxicity was used to select the double recombination event. The correct insertion of the mutant allele of *xenA* into the chromosome was checked by PCR analysis and Southern blot hybridization (not shown).

A similar approach was used to construct a mutation in the *tuf-1* gene. The gene was amplified using oligonucleotides Tu-1A(+) (5′-GCTCGTGTGCTAT GGGGTGA-3′) and Tu-1A(−) (5′-AGCGACCGAAAATGAGCACC-3′) and cloned into the pCR2.1 vector to generate pPAT44. The insert of pPAT44 was cut with EcoRI and cloned into pUC18, thus obtaining plasmid pPAT48. pPAT48 was, in turn, cut with NruI, and the 0.8-kb *aphA* cassette described above was inserted to interrupt the gene. The resulting recombinant plasmid was introduced into *P. putida* DOT-T1E by electroporation, and kanamycin-resistant transformants were selected. Among these clones, those in which the double recombination event had taken place were selected based on the conservation of the kanamycin marker and the loss of ampicillin resistance. One of these clones was kept and the nature of the double recombination event in the mutant strain confirmed by PCR and Southern blot hybridization (not shown).

Nucleotide sequence accession number. The sequence of the *P. putida* DOT-T1E *cspA2* gene was deposited in the GenBank database under accession number AY957457.

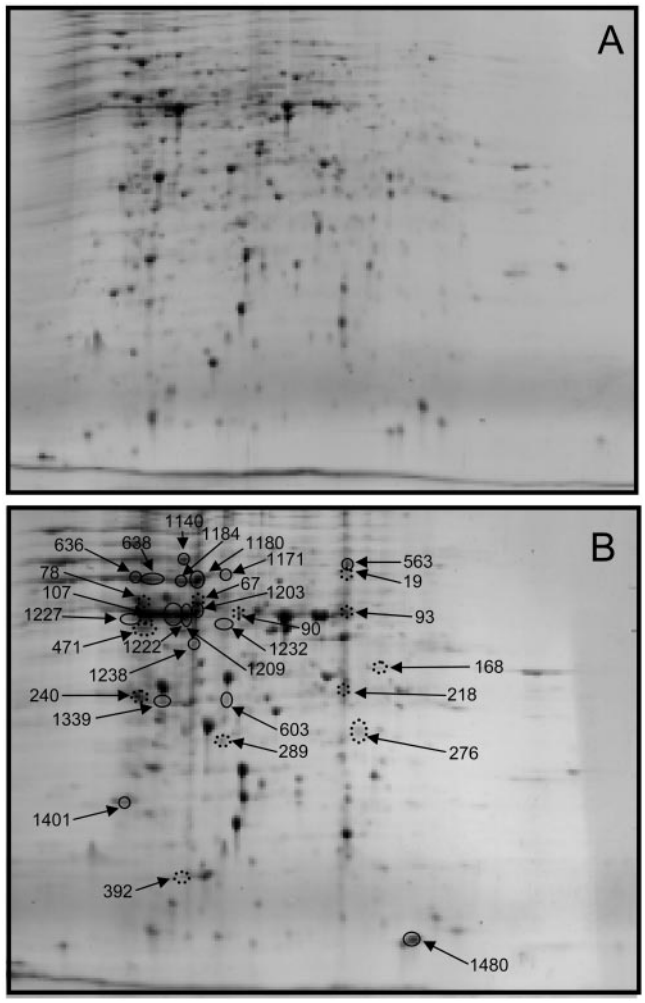

FIG. 1. 2D gels of protein extracts from cultures grown with glucose as the sole carbon source (A) and cultures grown with glucose plus toluene in the gas phase (B). Complete circles indicate spots only present in gels from extracts grown in the presence of toluene; dotted circles indicate spots induced more than twofold in the presence of toluene.

RESULTS

Protein variation between cells growing in the absence and in the presence of toluene in the vapor phase. *P. putida* DOT-T1E grows in minimal medium (M9) plus 25 mM glucose with a duplication time of around 77 min (Table 1). The presence of toluene in the medium in the vapor phase has no effect on the duplication time of the strain or on the culture yield since in both cases a turbidity at 660 nm of about 3 was achieved (Table 1). For protein analysis, cell cultures growing in the absence and in the presence of toluene were harvested in the exponential phase (optical density at 660 nm of 0.8), with a remaining glucose concentration in the culture medium of about 14 mM.

Cell extracts were then prepared as described in Materials and Methods, and soluble proteins were subjected to 2D electrophoresis. Two independent protein extracts were run on separate gels (Fig. 1). The two individual gel images for each condition were imported into the gel analysis software, and average gels were constructed to represent proteins present in both replicates for each condition. A total of 525 spots were detected in *P. putida* DOT-T1E cytosolic extracts from cells growing in minimal medium with glucose, whereas 531 spots were detected when the cells were grown in the presence of toluene. Two types of comparisons were made between the average 2D electrophoretic gel profiles: (i) spots present under only one of the conditions and (ii) spots whose relative intensity increased or diminished by twofold or more in cells growing in the presence of toluene. We found that 134 protein spots were present only in the gels with extracts from cells grown in the presence of toluene, whereas 117 spots were only present in gels from cells grown without toluene. The relative intensity of 35 protein spots was at least twofold higher in the presence of toluene than in its absence, whereas the relative intensity of 26 protein spots was half that under the same condition.

Identification of several proteins induced in the presence of toluene. To better understand the response of *P. putida* DOT-T1E to the presence of toluene in the medium, we decided to identify 30 of the spots. We analyzed 17 spots that appeared in

TABLE 2. Spots only present in gels from extracts of cultures grown in the presence of toluene

Spot(s)	Protein	Gene	Accession no.[a]	Protein mol wt (10^3)
638, 636, 1184, 1180	Toluene 2,3-dioxygenase (α subunit)	*todC1*	PPUY18245	51.3
1339	2-Hydroxy-6-oxo-2,4-heptadienoate hydrolase	*todF*	PPUY18245	30.8
603	Succinate-semialdehyde dehydrogenase	*gabD*	PP0213	51.8
1227	Succinyl-coenzyme A synthetase (β subunit)	*sucC*	PP4186	41.5
563	Oxoglutarate dehydrogenase, lipoamide dehydrogenase component	*lpdG*	PP4187	50.1
1232	Fructose-1,6-bisphosphate aldolase	*fda*	PP4960	38.7
1140	Phosphoenolpyruvate carboxykinase (ATP)	*pflu3720*[b]	Pflu3720	58.3
1401	Inorganic pyrophosphatase	*ppa*	PP0538	19.1
1808, 1222, 1203, 1209	Translation elongation factor Tu	*tuf-1*	PP0440	43.8
1238	ABC transporter, periplasmic binding protein		PP1726	38.6
1480	Cold shock protein CspA	*cspA2*	PP2463	7.7

[a] PP numbers are based on the locus of the genome of *Pseudomonas putida* KT2440. PPUY numbers are the accession numbers of the TodC1 and TodF nucleotide sequences of *P. putida* DOT-T1E deposited in GenBank.
[b] Based on the protein identified in *Pseudomonas fluorescens* PfO-1.

the extracts of cells grown in the presence of toluene and that were undetectable in extracts of cells grown without toluene (Table 2), as well as 13 spots whose relative spot intensity was at least twice as much as that in extracts prepared from cells grown in the absence of toluene (Table 3).

Based on their probable function, we established four groups. (i) Toluene degradation proteins made up the first group. *P. putida* DOT-T1E is able to use toluene as the sole carbon source through the toluene dioxygenase pathway (35). The *P. putida* DOT-T1E *tod* catabolic genes are organized in a single 10.3-kbp operon (*todXFC1C2BADEGIH*). Downstream from this operon and in the same transcriptional direction lies a two-gene operon (*todST*) that encodes a two-component regulatory system. Three enzymes of the pathway, the toluene 2,3-dioxygenase alpha subunit (TodC1), the 2-hydroxy-2,4-dienoate hydratase (TodG), and the 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase (TodF), were identified among the proteins that were induced by toluene (Tables 2 and 3). Interestingly, five different spots (638, 636, 1184, 1180, and 19) were identified as TodC1 and probably correspond to different posttranslational modifications of this protein. Four of these spots (638, 636, 1184, and 1180) were only observed when cells were grown on toluene, and spot 19 was detected under both culture conditions. Also of interest is the fact that TodF was detected as two different spots according to either the presence or the absence of toluene.

(ii) Proteins involved in general metabolism made up the second group. Five proteins identified as being induced in the presence of toluene participate in glucose metabolism and Krebs cycle metabolism (glucokinase, fructose-1,6-bisphosphate aldolase, phosphoenolpyruvate carboxykinase, dihydrolipoamide dehydrogenase [E3 component of 2-oxoglutarate dehydrogenase complex], and succinyl-coenzyme A synthetase). Three other enzymes are related to amino acid metabolism; two are involved in arginine degradation (arginine deiminase and the succinate semialdehyde dehydrogenase), and the third is involved in the biosynthesis of leucine from pyruvate (3-isopropylmalate dehydratase [small subunit]). We also identified 5,10-methylene-tetrahydrofolate dehydrogenase/cyclohydrolase (FolD2) and phosphoribosylformylglycinamide cyclo-ligase (PurM), two enzymes involved in purine biosynthesis. FolD2 is also involved in the tetrahydrofolate cycle. An inorganic pyrophosphatase, an enzyme implicated in the hydrolysis of the pyrophosphate formed in many biosyn-

TABLE 3. Proteins induced two-fold or more in protein extracts from cells growing in the presence of toluene

Spot	Protein	Gene	Accession no.[a]	Protein mol wt (10^3)	*n*-Fold increase
19	Toluene 2,3-dioxygenase (α subunit)	*todC1*	PPUY18245	51.3	3.85
240	2-Hydroxy-2,4-dienoate hydratase	*todG*	PPUY18245	28.3	24.75
218	2-Hydroxy-6-oxo-2,4-heptadienoate hydrolase	*todF*	PPUY18245	30.8	2.41
78	Arginine deiminase	*arcA*	PP1001	46.8	5.72
67	Arginine deiminase	*arcA*	PP1001	46.8	6.9
289	3-Isopropylmalate dehydratase, small subunit	*leuD*	PP1986	24.3	2.72
276	5,10-Methylene-tetrahydrofolate dehydrogenase/cyclohydrolase	*folD2*	PP2265	30.9	6.3
471	Phosphoribosylformylglycinamide cyclo-ligase	*purM*	PP1665	37.4	2.82
168	Glucokinase	*gck*	PP1011	34.2	3.35
93	Xenobiotic reductase A/xenobiotic flavin oxidoreductase A	*xenA*	PP1254	39.9	2.6
107	Translation elongation factor Tu	*tuf-1*	PP0440	43.8	3.54
90	Sugar ABC transporter, periplasmic sugar-binding protein		PP1015	45.5	5.75
392	Heat shock protein 10 (Cpn10)	*groES*	PP1360	10.2	3.9

[a] PP numbers are based on the locus of the genome of *Pseudomonas putida* KT2440. PPUY numbers are the accession numbers of the TodC1, TodG, and TodF nucleotide sequences of *P. putida* DOT-T1E deposited in GenBank.

thetic reactions (i.e., activation of fatty acids, synthesis of oligosaccharides, and the formation of aminoacyl-tRNA), was also found to be induced by toluene.

(iii) Transport proteins made up the third group. In gram-negative bacteria, ABC (ATP-binding cassette) transport systems consist of three or four proteins: the ATP-binding subunit, one or two permeases, and a periplasmic binding protein. Two of the proteins whose relative spot intensity was at least sixfold higher in cells growing in the presence of toluene corresponded to the ABC type of periplasmic binding proteins. One of these proteins was annotated as a sugar ABC transport protein. The corresponding gene is adjacent to the genes encoding an ATP-binding protein and porin B (an outer membrane protein involved in the uptake of sugars). These genes are clustered with those encoding a glucokinase (identified as being induced by toluene; Table 3) and the gluconate degradation genes. Our sequence analysis indicated that the *P. putida* DOT-T1E gene has similar neighboring genes as KT2440.

The second spot identified as a periplasmic binding protein of an ABC transport system has no assigned function. As above, the genes that encode the ATP-binding subunit and the permeases are adjacent to the gene that encodes the periplasmic protein. However, no function could be deduced from the neighboring genes.

(iv) Stress-related proteins made up the fourth group. Two spots induced by toluene are clearly related to stress responses. One of them is the heat shock protein GroES, and the other is the cold shock protein CspA2. Two other proteins induced by toluene are also related to stress responses. These two proteins are the translational elongation factor EF-Tu-1 and the xenobiotic reductase (XenA). EF-Tu-1 is mainly involved in the binding and transport of the appropriate codon specified by aminoacyl-tRNA to the aminoacyl site of the ribosome (6, 34), but it also acts as a molecular chaperone (7, 28). XenA is an oxidoreductase of unknown physiological function that has also been proposed to be a member of the oxidative stress response system (3, 27) and appears also in response to aromatic compounds.

Are stress proteins specifically involved in the toluene tolerance response? The identification of toluene degradation genes as being induced in response to toluene guaranteed that under our experimental conditions *P. putida* DOT-T1E cells were sensing toluene. The induction of sugar transport systems and the induction of enzymes involved in glucose metabolism and Krebs cycle enzymes are both in consonance with the presence of glucose in the culture medium and the need to generate energy for the RND efflux pumps to extrude a toxic chemical such as toluene. The set of stress-related proteins suggested that toluene imposes a stress response situation, which is in agreement with its toxic character. This study identifies for the first time stress proteins involved in tolerance to toluene, and for this reason we decided to further explore the potential role of the stress response proteins through a series of physiological and/or genetic assays.

(i) Physiological assays. The GroES protein has been identified in other microbes as a key chaperone in the response to heat stress (11, 12, 13, 14, 15), and it has been shown to be induced by cold shock (38), whereas CspA has been identified as a key chaperone under cold shock conditions (5, 21, 29). It has also been previously shown that *Pseudomonas* sp. strain DJ-12 cells pretreated with heat shock increased their resistance to ethanol (37). Based on this finding, we decided to test whether heat or cold treatment of *P. putida* DOT-T1E cells influences resistance to a toluene shock. We had previously established that *P. putida* DOT-T1E cells did not grow at 10°C or 42°C, although they did remain viable at these temperatures for at least 60 min without any loss of viability. *P. putida* DOT-T1E cells were grown at 30°C, and aliquots were then transferred for 30 min to baths that were kept at 42°C, 30°C, or 10°C and subsequently submitted to a toluene shock. Figure 2 confirms that incubation of DOT-T1E at any of the above temperatures in the absence of toluene did not produce a decrease in the number of CFU per milliliter with respect to the culture control samples without toluene. However, the survival rate of the heat-treated cells in response to toluene was 2 to 3 orders of magnitude lower (1 out of 10^6 to 10^7 CFU/ml) than that of the cells kept at 30°C (normal growth temperature for *P. putida* DOT-T1E). Interestingly, the survival rate of cells kept at 10°C was higher than for cells kept at 30°C. This suggested that heat-treated cells were more sensitive to toluene than cells grown at 30°C and therefore that induction of the heat shock response is not enough to overcome the toxicity of the solvent. On the other hand, the above results suggested that the CspA2 cold shock protein might have a role in solvent tolerance.

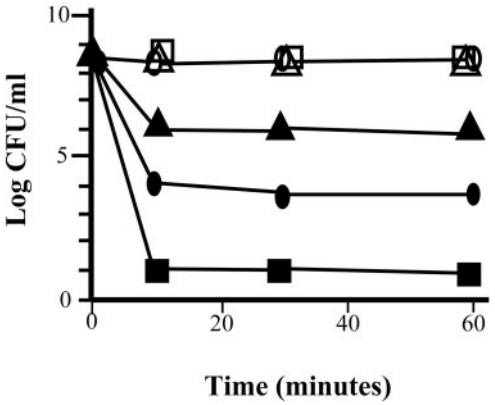

FIG. 2. Survival of *P. putida* DOT-T1E cells after the addition of 0.3% (vol/vol) toluene. Cells were grown at 30°C until they reached an optical density of 0.8; the cultures were then divided into three aliquots and maintained at 30°C, 42°C, and 10°C for 30 min before toluene addition. Symbols: circles, cultures kept at 30°C; squares, cultures at 42°C; triangles, cultures at 10°C. Open symbols, cultures without toluene; closed symbols, cultures with toluene.

(ii) Toluene tolerance and growth in the presence of toluene of CspA2, XenA, and Tuf-1 knockout mutants. Since no physiological assays were envisaged to test the roles of XenA and Tuf-1 in toluene tolerance, we decided to construct mutant strains deficient in the synthesis of these proteins as described in Materials and Methods. We also constructed a CspA2 protein mutant but failed in the construction of a *groES* mutant, and the effect of a deficiency in this protein could not be tested. In many bacteria, *groES* and *groEL* form an operon; in our strain, the two genes are separated by 48 bp, which indicates that they are probably also transcribed together (results not shown). GroES and GroEL work together, mediating the

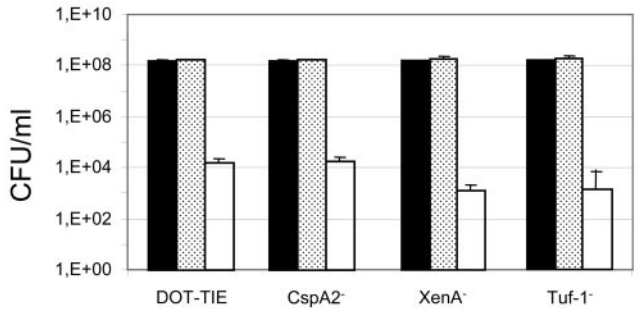

FIG. 3. Numbers of viable cells of the wild-type and mutant strains. Black bars indicate the number of viable cells before toluene addition ($t = 0$). Dotted bars, viable cells 10 min later in the culture without toluene; white bars, viable cells 10 min after 0.3% (vol/vol) toluene addition.

proper folding of proteins. It was previously reported that mutants deficient in these heat shock proteins were not viable in other organisms (11), which could also be the case for *P. putida* DOT-T1E. We tested the response of *cspA2* knockout mutant *P. putida* DOT-T1E-PS113 to toluene. First we tested the growth rate of the mutant in M9 minimal medium with and without toluene. We found that in the absence of toluene, the growth rate of the mutant was slightly slower than that observed for the wild-type strain (Table 1); however, the mutant deficient in CspA2 grew much more slowly when toluene was present in the medium (Table 1). This result confirms the potential participation of this protein in the response of DOT-T1E to the presence of toluene. We also submitted the CspA2 mutant to a sudden toluene shock and found that the mutant strain survived at a rate similar to that of the wild type (Fig. 3). The involvement of the CspA2 protein in the growth of *P. putida* DOT-T1E in the presence of toluene, though not in the survival of a sudden toluene shock, is not fully surprising. We have previously shown that although the solvent tolerance factors required for survival of a sudden 1-octanol shock (efflux pumps and *cis-trans* isomerization) were induced in cultures in the presence of the alcohol, the strain was not able to grow in the presence of this aliphatic alcohol (47). This new finding clearly indicates that *P. putida* DOT-T1E has different mechanisms for survival of solvent shocks and for growth in the presence of solvents.

The growth of the *xenA* knockout mutant in M9 minimal medium with glucose was considerably slower than that of the wild type, and it was exacerbated in the presence of toluene (Table 1). When the *xenA* knockout mutant was subjected to a solvent shock, its survival was at least 1 order of magnitude lower than that of the wild-type strain (Fig. 3).

In the *P. putida* DOT-T1E genome, we identified two highly similar EF-Tu factors, *tuf-1* and *tuf-2* (accession numbers AY957604 and AY957605), that differ in three amino acids. Despite this similarity, the spot induced in the 2D gels was clearly identified as EF-Tu-1. We constructed a *P. putida* DOT-T1E-*tuf-1*::*aphA* mutant as described above and tested its growth on M9 minimal medium with glucose with and without toluene. The mutant strain in the presence of toluene grew more slowly than the parental one (Table 1) and survived a sudden toluene shock at a rate of around 1 order of magnitude lower than the wild-type strain (Fig. 3). This series of results indicated that different chaperones play an active role in the response to stress imposed by an organic solvent such as toluene.

DISCUSSION

The presence of toluene in the culture medium in which *P. putida* DOT-T1E was being cultured provoked drastic changes in the protein pattern observed in 2D gels, indicating a complex response of DOT-T1E to this organic solvent. As part of this response, new proteins involved in solvent tolerance were synthesized and an increased expression of some preexisting proteins also took place, probably counteracting the decrease in activity due to structural damage caused by the presence of toluene inside the cell. In support of these changes in the protein pattern is the fact that we identified the induction of the Tuf-1 elongation factor involved in protein synthesis and an inorganic pyrophosphatase involved in providing the required energetic support for the reaction.

Although only a fraction of the proteins induced in response to toluene were identified by MALDI-TOF, our results are useful in understanding the process much better. Four groups of proteins were made; the first one included catabolic enzymes for toluene. These results were expected since it had been previously reported that the *todXFC1C2BADEGIH* operon was induced by toluene (36, 44), so the identification of three toluene degradation proteins among those induced at a high level by toluene confirmed previous results and indicated that the experimental procedure was correct. We detected low levels of TodC1, TodG, and TodF in protein extracts from cells not exposed to toluene. This reflects a low basal level of expression of the *tod* operon proteins, suggesting some basal expression of the *tod* genes in the absence of toluene, which was reported before (30; J. Lacal and J. L. Ramos, unpublished results). The 2D gels, however, revealed the existence of different isoforms of the α subunit of toluene dioxygenase, which most probably represents different isoforms resulting from posttranslational modifications. The nature of the modifications and the functionality of these isoforms remain to be assayed.

Under our experimental conditions, we cultured *P. putida* DOT-T1E on minimal medium with glucose in the absence and in the presence of toluene. Toluene extrusion is a highly energy-demanding process which is in part required for the operation of efflux pumps (20, 41). In consonance with this energy demand is the induction of sugar uptake, glucose catabolic enzymes, and Krebs cycle enzymes. The main catabolic pathway for glucose metabolism in *P. putida* involves its oxidation to 2-ketogluconate in a series of reactions that take place in the periplasm (Fig. 4). 2-Ketogluconate is then transported into the cytoplasm. *P. putida* can also convert glucose into glucose-6-phosphate via a glucokinase in the so-called phosphorylative pathway (although glucokinase activity is almost undetectable in *P. putida* when it is growing on glucose (31, 49, 56). Both pathways converge to form 6-phosphogluconate, which follows the Entner-Doudoroff pathway to render glyceraldehyde-3-phosphate and pyruvate (31) (Fig. 4). Our MALDI-TOF analysis revealed the induction of at least one sugar transport periplasmic binding protein and a glucokinase.

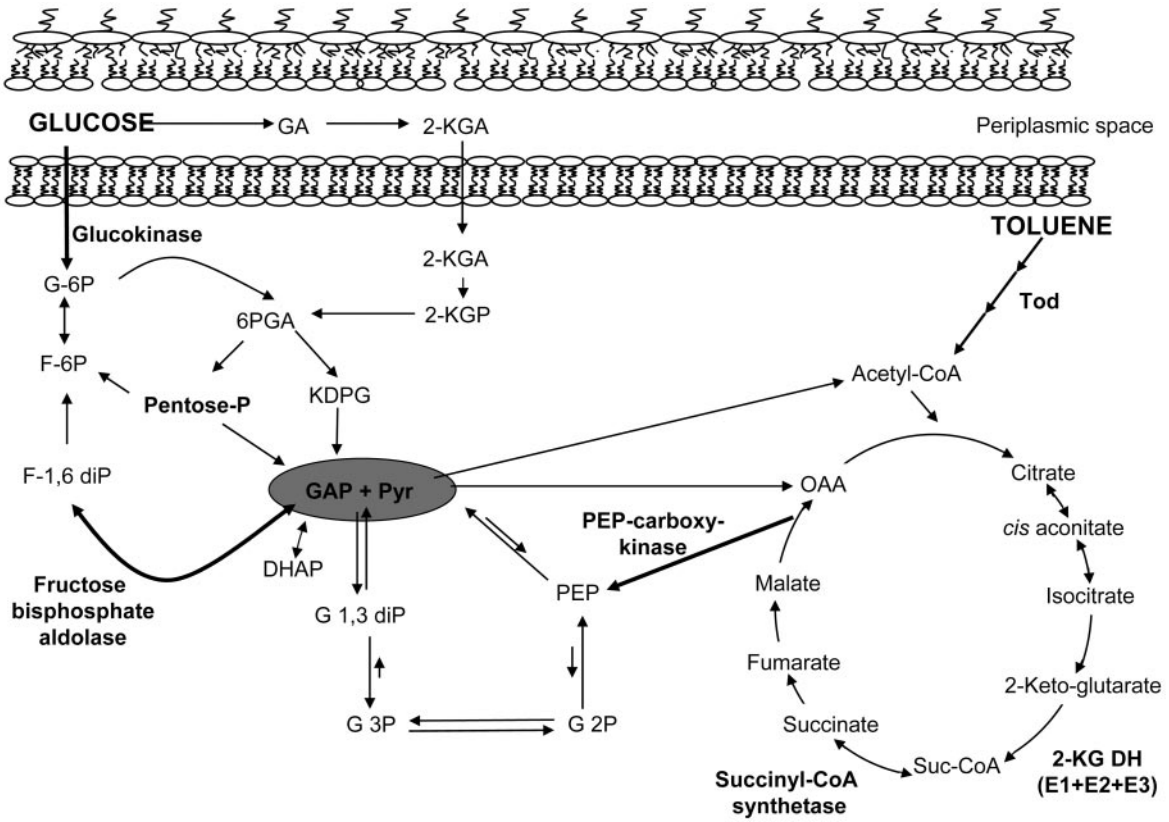

FIG. 4. Glucose catabolic pathways in *P. putida*. Enzymes induced by toluene are in boldface. Abbreviations: GA, gluconic acid; 2-KGA, 2-ketogluconic acid; 2-KGP, 2-keto-6-phosphogluconate; 6PGA, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone-3-phosphate; Pyr, pyruvate; F-1,6 diP, fructose 1,6-diphosphate; F-6P, fructose 6-phosphate; G-6P, glucose-6-phosphate; OAA, oxalacetic acid; PEP, phosphoenolpyruvate; G 2P, glycerate 2-phosphate; G 3P, glycerate 3-phosphate, G 1,3 diP; glycerate 1,3-diphosphate; 2KG-DH, 2-ketoglutarate dehydrogenase. CoA, coenzyme A; Suc, succinyl.

All of these proteins are involved in sugar metabolism, and the induction of glucokinase in cultures growing in the presence of toluene could particularly reflect an increase in the demand for energy to cope with the solvent. In line with the findings described above, Kim et al. (25) described an ABC transport mutant of *P. putida* GM73 as being involved in solvent tolerance based on the reduced survival rates after a 1% (vol/vol) toluene shock when growing on LB medium of a mutant strain deficient in this uptake system. However, the ABC transport system described by Kim et al. (25) was different from the ones identified here. Nonetheless, the set of results in both laboratories point toward an increase in the uptake of metabolites to support the high energy demands imposed by the need to extrude toluene. Also in line with the need to generate energy to cope with toluene sensitivity is the fact that two enzymes of the tricarboxylic acid cycle were induced, namely, dihydrolipoamide dehydrogenase (E3 component of 2-oxoglutarate dehydrogenase complex) and succinyl-coenzyme A synthetase.

The arginine deiminase pathway in *P. aeruginosa* is one of the major routes used by the cell to obtain energy under anaerobic conditions (33, 55); it generates 1 mol of ATP from 1 mol of L-arginine (53). The induction of this pathway in *P. putida* DOT-T1E could be related to the activation of another source to generate the necessary energy to extrude toluene (Fig. 5).

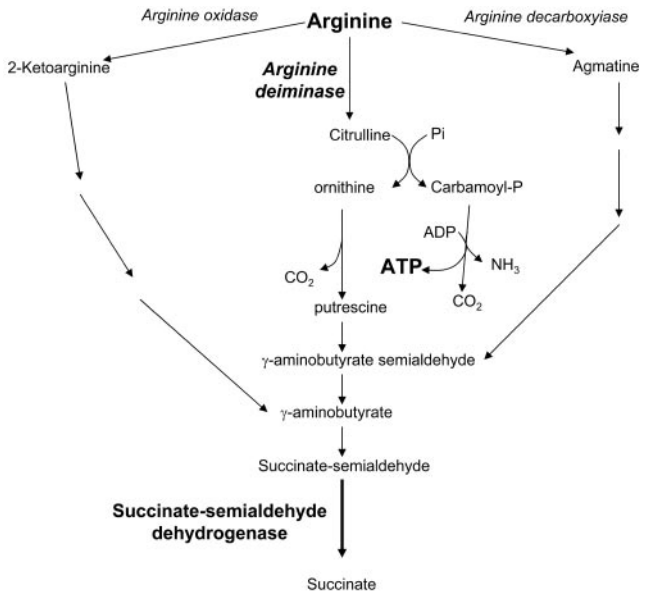

FIG. 5. Schematic representation of the three putative arginine degradative pathways in *Pseudomonas* (39) and synthesis of ATP from arginine. The first enzyme of each pathway is in italics. Enzymes induced by toluene in *P. putida* DOT-T1E are in boldface.

We found that the synthesis of fructose 1,6-biphosphate aldolase and phosphoenolpyruvate carboxykinase was induced in response to toluene. Two potential tasks are envisaged in this pathway; one is the need to replenish some of the glucose degradation intermediates, and the other is to divert oxaloacetate to the pentose-phosphate cycle so that the biosynthesis of purine required for ATP synthesis is enhanced. In line with this possibility is the fact that PurM and FolD2 were induced in response to toluene.

Toluene is a disturbing agent that disorganizes bacterial membranes (8, 51), and as such it was expected to trigger some kind of stress response. However, to date no stress-related proteins have been identified in the adaptive response of DOT-T1E to this solvent. We have identified four proteins; GroES and Tuf-1 function as chaperones and most probably increase the refolding of unfolded proteins, CspA2 favors mRNA translation under stress conditions, and XenA has been suggested to be involved in oxidative stress, but its specific physiological role remains unknown. In many microorganisms, GroES primarily serves to prevent protein aggregation and to assist in protein folding under different stress conditions, including heat stress (12, 13, 14, 15). Our results do not reveal any particular conclusive role of GroES in the toluene response because heat treatment of DOT-T1E cells did not result in an increase in toluene tolerance but rather led to a decrease in cell viability, probably because heat altered the cell membrane permeability barriers and allowed easier penetration by toluene with a subsequent increase in toxicity. It has previously been reported that overexpression of *groESL* in *Clostridium acetobutylicum* and in *Lactococcus lactis* and *Lactobacillus paracasei* NFBC 338 results in an increase in butanol tolerance (9, 54). Then the induction of GroES in DOT-T1E in response to toluene could be related to protein refolding.

Our results support a role for the other three stress-related proteins (CspA2, XenA, and Tuf-1) in the toluene response since the amounts of these proteins were not only influenced in response toluene, but single mutants deficient in the synthesis of one of these stress response proteins grew more slowly in the presence of toluene and/or were less tolerant of sudden toluene shocks than the parental strain. All of these proteins probably also have an important physiological role under normal growth conditions (this is obvious for the Tuf-1 protein). All of the mutant strains have longer duplication times than the wild type when growing on glucose. In *Escherichia coli*, the cold shock protein CspA activates the transcription of other cold shock genes (*hns* and *gyrA*) and can also selectively stimulate the translation of its own mRNA and possibly that of other cold shock mRNAs as well (5, 21, 29). Most cold shock proteins are involved in transcription and translation, suggesting that the cold shock response is an adaptive mechanism facilitating protein synthesis at low temperatures. Two *cspA* genes (*cspA1* and *cspA2*) have been identified in the genome of *P. putida* KT2440. Using PCR amplification with oligonucleotides based on these sequences, we were able to amplify the same two genes from *P. putida* DOT-T1E. Sequencing of the two amplification bands allowed us to conclude that there are also at least two *cspA* genes (*cspA1* and *cspA2*) in the *P. putida* DOT-T1E genome, but under toluene stress conditions we only detected the increased synthesis of CspA2. Since the CspA2 mutant strain grew more slowly than the parental strain in the presence of toluene, this protein could facilitate protein synthesis under toluene stress. The cytoplasmic Tuf-1 protein has been shown to be translocated to the periplasmic space of *E. coli* under certain stress conditions, where it can function as a molecular chaperone (2). Due to its log P_{ow}, toluene partitions preferably in cell membranes, increasing their permeability. Toluene is therefore expected to affect periplasmic proteins, and the increased synthesis of Tuf-1 could be related to its role as a chaperone in the periplasm.

Kohli and Massey (27) proposed XenA as one of the constituents of the antioxidant defense systems and suggested that it works in the protection of cells against various toxic compounds. The *P. putida* DOT-T1E XenA mutant strain grew more slowly than the wild type with glucose as the sole carbon source, and addition of toluene exacerbates this defect. This mutant was also less tolerant of the sudden addition of 0.3% (vol/vol) toluene than the parental strain. The role of XenA could derive from the generation of free oxygen radicals during respiration due to the increased electron flow derived from an increased feeding of chains from Krebs cycle intermediates and/or from futile oxygen reduction due to sublethal damage exerted at the membrane level by toluene.

In short, our proteomic analysis reveals valuable information on the cellular responses to the presence of toluene and that in its presence different chaperones contribute to the stabilization of proteins affected by this organic solvent. Cell metabolism is directed to increase energy generation, which is in turn needed to extrude this compound from the cell membranes, where it tends to accumulate.

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