JOURNAL OF BACTERIOLOGY, June 2006, p. 4079–4092 Vol. 188, No. 11
0021-9193/06/$08.00+0 doi:10.1128/JB.00101-06

Functional Genomics of Stress Response in *Pseudomonas putida* KT2440

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Received 19 January 2006/Accepted 15 March 2006

The metabolically versatile soil bacterium *Pseudomonas putida* has to cope with numerous abiotic stresses in its habitats. The stress responses of *P. putida* KT2440 to 4°C, pH 4.5, 0.8 M urea, and 45 mM sodium benzoate were analyzed by determining the global mRNA expression profiles and screening for stress-intolerant non-auxotrophic Tn*5* transposon mutants. In 392 regulated genes or operons, 36 gene regions were differentially expressed by more than 2.5-fold, and 32 genes in 23 operons were found to be indispensable for growth during exposure to one of the abiotic stresses. The transcriptomes of the responses to urea, benzoate, and 4°C correlated positively with each other but negatively with the transcriptome of the mineral acid response. The CbrAB sensor kinase, the cysteine synthase CysM, PcnB and VacB, which control mRNA stability, and BipA, which exerts transcript-specific translational control, were essential to cope with cold stress. The *cyo* operon was required to cope with acid stress. A functional PhoP, PtsP, RelA/SpoT modulon, and adhesion protein LapA were necessary for growth in the presence of urea, and the outer membrane proteins OmlA and FepA and the phosphate transporter PstBACS were indispensable for growth in the presence of benzoate. A lipid A acyltransferase (PP0063) was a mandatory component of the stress responses to cold, mineral acid, and benzoate. Adaptation of the membrane barrier, uptake of phosphate, maintenance of the intracellular pH and redox status, and translational control of metabolism are key mechanisms of the response of *P. putida* to abiotic stresses.

Its metabolic versatility, degradative potential, and ability to colonize bulk soil and the rhizosphere make *Pseudomonas putida* an ideal candidate for genetic engineering and applications in biotechnology, bioremediation, and agriculture (10, 29, 30, 33, 58, 68, 80, 98, 99, 102, 112). Strain KT2440, whose genome has recently been sequenced (85), is one of the best-characterized pseudomonads and has been optimized as a "laboratory workhorse" for many years, but it has retained its ability to survive and function in the environment. This strain has been certified as a biosafety strain (32), which means that it can be used as a host strain for containment systems (79) for applications in biotechnological production and release into the environment. The successful persistence of *P. putida* in its natural habitats, as well as its use as a host strain in biotechnology and agriculture applications, requires that it adheres to surfaces or copes with limited nutrients and also has mechanisms for tolerating various environmental stresses (28). Several types of stresses may occur in soil, particularly in the rhizosphere; these stresses include temperature stress, pH stress, water stress, oxidative stress, and stress caused by competition with other microorganisms (18, 46, 50, 92).

Free-living bacteria are frequently exposed to temperature shifts and nonoptimal growth temperatures. In order to grow at low temperatures, an organism must overcome the growth-limiting effects of these stress conditions, such as decreased membrane fluidity, altered redox status, and increased stability of RNA and DNA secondary structures and thus reduced efficiency of replication, transcription, and translation (92). Cold shock proteins, such as the CspA family proteins, are widely distributed among bacteria (18, 50, 92, 118). Regulated by DNA or mRNA stability, these proteins are expressed immediately after a temperature downshift and act mainly as chaperones of DNA or RNA. Cold shock acclimation proteins (i.e., proteins that exhibit a high level of expression at low temperatures) have been identified in *Pseudomonas* species and might be responsible for the psychrotrophic phenotype of these organisms (45, 72).

High and low pH values can damage membrane proteins and severely affect cytoplasmic processes. Short-chain fatty acids, acetate, and benzoate cause more severe damage than inorganic acids cause because they can easily enter the cytosol and release protons (38, 53). A general mechanism that bacteria use to persist and grow at extreme pH values is retention of pH homeostasis (77, 100). The charged membrane surface and the buffering capacity of the cellular proteins contribute passively to a constant pH. The activity of proton pumps and the transport of potassium ions lead to alkalinization of the cytoplasm at a low pH, whereas a sodium ion circuit, including an Na^+/H^+ antiporter, maintains the cytosolic pH in alkaline media (107). Changes in intracellular pH values can also affect gene expression. Amino acid decarboxylase is expressed at

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TABLE 1. Stress assay conditions, growth conditions, and media

Stress	Assay condition(s)	Medium
Osmotic stress/chaotropic	0.8 M urea, 26°C	Liquid ABC minimal medium with 15 mM sodium benzoate in 96-well plates
Organic acid stress	45 mM sodium benzoate, 26°C	Liquid ABC minimal medium in 96-well plates
Acid stress	pH 4.5 (with HCl), 26°C	LB medium in 96-well plates
Low-temp stress	4°C	ABC minimal medium agar plates with 15 mM sodium benzoate

higher levels at a low pH, and the polyamines generated are secreted and increase the pH in the environment of the cells. At an alkaline pH, the expression of deaminases increases. The by-products of this expression, weak acids, are secreted and decrease the pH outside the cells. Recent work has demonstrated that chaperones (70) and lipopolysaccharides (LPS) (6) are also involved in the response and resistance to short-chain fatty acid stress.

Chaotropic solutes, such as urea, phenol, ethylene glycol, benzyl alcohol, and many other noxious organic compounds, cause nonturgor water stress in *P. putida* (46). These chaotropic compounds do not affect turgor, but they reduce water activity and perturb macromolecule-water interactions and thus destabilize cellular macromolecules and inhibit growth (46). Bacteria respond to this type of water stress by adaptive adjustment of the cytoplasmic membrane fluidity mediated by changes in the fatty acid composition and the degree of saturation (95, 104).

In this work, we examined ability of the *P. putida* KT2440 genome to cope with the stresses caused by low temperature, chaotropic solutes, and organic and mineral acids. Using a genome-wide approach, we analyzed the growth of several thousand transposon mutants of strain KT2440 under extreme conditions in order to identify the genes and gene products that are essential for the responses to the stresses. During the initial annotation process, we predicted or identified numerous genes that are related to general and specific stress responses, including several cold shock proteins, transporters, ion channels, and global stress regulators (85). However, a number of unknown key players in stress response might be hidden in the large number of unclassified and unknown proteins. Furthermore, the availability of the complete genome sequence permitted analysis of the functional and phylogenetic context of candidate genes. It also provided the opportunity to complement and perform a search for stress-related genes by monitoring the effects of individual stress conditions and the effects of the candidate genes on the global gene expression that can be monitored on DNA microarrays.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. putida* KT2440 (= DSM 6125) (5) was obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany. *P. putida* cells were incubated at 26°C in LB (Oxoid) medium or in ABC minimal medium containing 2.0 g/liter $(NH_4)_2SO_4$, 6.0 g/liter Na_2HPO_4, 3.0 g/liter KH_2PO_4, 3.0 g/liter NaCl, and 15 mM sodium benzoate as the sole carbon source. The inorganic salts were "purum" quality. *Escherichia coli* DH5α [F^- ϕ80*lacZΔM15* Δ(*lacYZA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1* (Nal^r)] was used as the plasposon donor for triparental mating and general carrier of plasmids. *E. coli* HB101 [F^- *supE44 hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1*) carried the helper plasmid pRK2013 (37) for triparental mating. *E. coli* Top10 competent cells (Invitrogen) were used as recombinant plasmid carriers. *E. coli* cells were grown at 37°C in LB medium. In order to prepare selective media for genetically recombinant strains, the following concentrations of antibiotics (SERVA) were used: tetracycline, 100 μg/ml and 30 μg/ml (for *P. putida* and *E. coli*, respectively); kanamycin, 50 μg/ml; and gentamicin, 30 μg/ml.

Generation of a signature-tagged transposon KT2440 mutant library. *P. putida* mutants were generated by signature-tagged mutagenesis with plasposon pTnMod-OGm-ST (21), which was modified by cloning an 80-bp sequence that included a 40-bp variable signature tag into the unique KpnI site of the vector (121). A total of 21 pTnMod-OGm-ST variants with differential tags were generated. The three strains utilized for generation of transposon mutants were grown in 5 ml LB media overnight as follows: the acceptor strain *P. putida* KT2440 was grown at 26°C in 5 ml LB medium, the helper strain *E. coli* HB101 with pRK2013 was grown at 37°C in 5 ml LB medium containing 10 μg/ml kanamycin, and the donor strain *E. coli* DH5α with pTnMod-OGm-ST was grown at 37°C in LB medium containing 15 μg/ml gentamicin. Then 1 ml of the *P. putida* cells was incubated at 42°C for 4 h. The turbidity of all three cell cultures was determined at 578 nm, and the strains were diluted to obtain a donor/helper/acceptor ratio of 10:10:1 by mixing 0.5 ml of the donor strain, 0.5 ml of the helper strain, and 0.05 ml of the acceptor strain. The mixture was centrifuged at 3,000 × *g* for 5 min, and the pellet was resuspended in 100 μl LB medium containing 10 mM $MgSO_4$ and incubated on an LB agar plate at 30°C for 16 h. In order to select for transposon mutants, the cell layer from each half of a triparental mating plate was resuspended in 5 ml of a 10 mM $MgSO_4$ solution. Then 50 μl of this suspension was transferred onto an M9 minimal medium agar plate containing 40 μg/ml gentamicin and 15 mM sodium benzoate and incubated at 26°C for 2 days. To avoid redundant mutants, only a small fraction (less than 5%) of the colonies from each selection plate (121) were selected and stored on fresh M9 medium plates (40 μg/ml gentamicin, 15 mM sodium benzoate) for several weeks at 4°C. After this, the mutants were sorted into 96-well plates (LB medium) and finally stored as glycerol stocks at −80°C.

Stress assay protocol. The library of *P. putida* transposon mutants was screened for survival under the stress conditions shown in Table 1. To monitor growth under urea, benzoate, and inorganic acid stress conditions in 96-well plates, the cells were precultured in 200 μl LB medium for 4 h, and aliquots (40 μl) of the initial culture suspension were transferred to stress assay plates with 160 μl of medium. The turbidity (I) of each mutant at 578 nm was determined with an Elisa reader (MWG) initially, after 2, 3, and 4 h of growth in the preculture, and after 2, 3, 4, 5, 6, 20, 24, 28, 36, 40, and 46 h of incubation under the stress conditions. The multiwell plates were shaken every hour for 30 s, which ensured that the growth rates in all wells of the plate were similar. The results of the turbidity measurements were uploaded as single files into a computer, and then the growth coefficient (μ) was calculated for each of the 96 mutants as follows: $\mu = [\ln(I_1) - \ln(I_0)]/(t_1 - t_0)$. A histogram of growth coefficients showed that the distribution was Gaussian, and hence 99% confidence intervals were calculated from the means and variance of the data for each 96-well plate. Mutants whose growth coefficient was consistently below the 99% confidence interval in three independent experiments were considered to be growth deficient under the stress conditions examined. Next, in order to exclude auxotrophic mutants and to compare the growth of the selected mutants with the growth of the wild-type strain, mutants and the wild-type strain were grown in parallel both in the absence and in the presence of the stressor. If the wild-type strain grew significantly better ($P < 0.01$) than the mutant in the presence of the stressor but the growth was similar in the absence of the stressor, the transposon mutant was subjected to sequencing of the insertion site (see below). Cold stress was tested on agar plates. After an initial incubation at 26°C for 4 h, one plate was incubated at 4°C for 3 days, and a control plate was maintained at 26°C for 16 h. If no macroscopic colonies were visible by day 3 in all five independent experiments, the mutant was considered cold sensitive.

Identification of transposon insertion sites. Genomic DNA from *P. putida* and *E. coli* cells was isolated by using a protocol introduced by Chen and Kuo (14). In order to identify the genomic sequences adjacent to the insertion sites of the transposed elements, the Y-linker method (69) was used. The Y-linker was

prepared by annealing two oligonucleotides, Y1 (5′-TTTCTGCTCGAATTCA AGCTTCTAACGATGTACGGGGACACATG) and Y2 (5′-TGTCCCCGTAC ATCGTTAGAACTACTCGTACCATCCACAT). Genomic DNA of the mutants (1-μg aliquots) was cut with 5 U NlaIII (New England Biolabs) for 3 h and ligated to the Y-linker. The resulting product was used as a template in a PCR with a pTnMOD-specific primer (5′-GCGTTCGGTCAAGGTTCTGG) and the Y primer (5′-CTGCTCGAATTCAAGCTTCT). The resulting PCR products were purified by agarose gel electrophoresis and extracted using a QIAGEN gel extraction kit. Sequencing was done by QIAGEN using the pTnMOD-specific primer. The raw sequences were analyzed by a blastn search against the sequences of the predicted genes, as well as the complete genome sequence of *P. putida* KT2440 (85).

KT2440 cosmid library preparation. For construction of the cosmid library from the chromosomal DNA of *P. putida*, we used procedures described in detail elsewhere (47). Briefly, cosmid Lawrist-4 was linearized with ScaI and dephosphorylated, and this was followed by cleavage of the BamHI cloning site. The genomic DNA was partially cut with MboI and treated with phosphatase prior to ligation to the vector. The average insert size was 37 kb. Approximately 3,800 clones, representing 24-fold genome coverage, were picked into 360-well plates.

Complementation in *trans*. A gene of interest without promoter was amplified by PCR with *Pfu* DNA polymerase (Promega) from a recombinant cosmid or KT2440 chromosomal DNA. Primers were designed to contain unique restriction sites for cloning into the vector plasmid. A list of templates and primer sequences is available at http://www99.mh-hannover.de/kliniken/kinderheilkunde/kfg/pub.htm. PCR products were purified with a QIAGEN PCR purification kit, restricted, dephosphorylated with alkaline phosphatase (Boehringer Mannheim), and ligated into the multicopy plasmid vector pME6010. The plasmid was introduced into *E. coli* OneShotTOP10 chemically competent cells (Invitrogen) by transformation and subsequently into a competent *P. putida* mutant by electroporation with a Bio-Rad pulser (0.2-mm gap, 400 Ω, 25 μF, 2.5 kV). To prepare competent cells, a 25-ml *P. putida* culture grown overnight in LB medium at 26°C was put on ice and washed three times (5 min each) by centrifugation. The presence of the plasmid in the recombinant *P. putida* transposon mutant was verified with a FastPrep kit (Eppendorf).

Transcriptome analyses. Three 25-ml liquid *P. putida* KT2440 cultures were grown in either the absence or presence of the stressor to the logarithmic phase (turbidity at 600 nm [T_{600}], 0.8) or to the stationary phase (T_{600}, 3.0) (for the medium used see Table 1). Total RNA was prepared from each culture and transcribed into cDNA as described by von Götz et al. (117). To label the cDNA used for transcriptome analyses, three mRNA samples from each stress condition, as well as three control samples, were pooled, and random priming (33) was performed with 500 ng cDNA in the presence of 15 mM ATP, 15 mM GTP, 15 mM TTP, and 25 mM Cy3- or Cy5-labeled dCTP (Amersham-Pharmacia, Amersham, United Kingdom).

The microarrays for genomic fragments of *P. putida* KT2440 (109) were hybridized as described previously (23). For each condition, four arrays were hybridized independently, two with Cy3-labeled cDNA from the stress condition and Cy5-labeled control cDNA and two with Cy5-labeled cDNA from the stress condition and Cy3-labeled control cDNA. Fluorescence signals were detected with a ScanArray5000 unit (Packard, Billerica, MA) and were analyzed with the GenePix software package (Axon Instruments, Union City, CA).

Assessment of data quality, normalization, and subsequent analyses were performed by using procedures that meet or exceed the MIAME criteria for microarray analysis (9). Data analysis was performed with the data warehouse and interpretation software package M-CHiPS (http://www.mchips.org). If the correlation coefficient for normalization was less than 0.8, the hybridization result was not used for further analysis. Due to the relatively low number of experiments for each condition, significantly differentially expressed fragments were identified by applying the highly stringent min-max separation criterion (7), followed by correspondence analysis of the intensity ratios (34, 35).

A subset of mRNA transcripts that were interpreted to be differentially expressed during growth at pH 4.5 or in the presence of 0.8 M urea by transcriptome analysis were reassessed by real-time quantitative SYBR Green I-based PCR performed according to the manufacturer's real-time PCR protocols (QIAGEN AG, Hilden, Germany), using an Applied Biosystems model 7700 sequence detector. Primer sequences for the selected genes are available at http://www99.mh-hannover.de/kliniken/kinderheilkunde/kfg/pub.htm. Assays were performed in duplicate. Melting curve analyses were performed to verify the amplification specificity. The levels of expression of the *rplF* (PP0469), *gcdH* (PP0158), *relA* (PP1656), and *speB* (PP2196) genes in the absence of a stressor were used to determine reference cycle threshold values for variable amounts of input RNA template (50, 100, 200, 500, 1,000, and 1,500 ng).

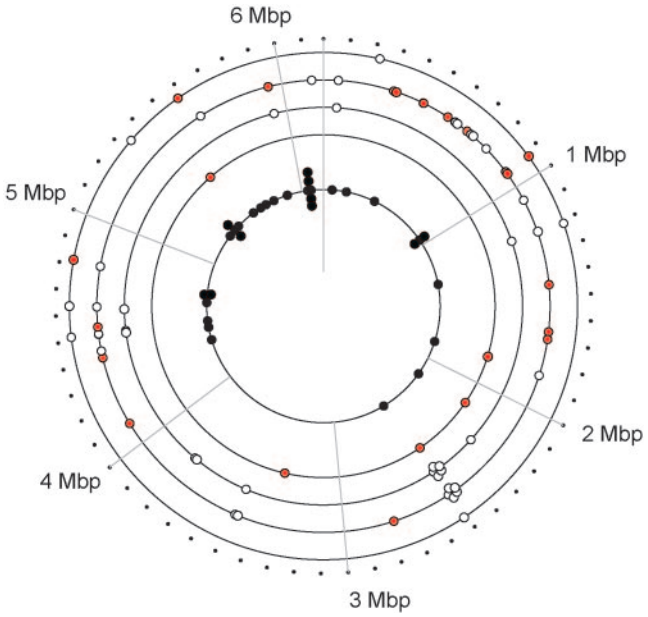

FIG. 1. Chromosomal map positions of stress response genes. Circle 1 (outermost circle) to circle 4 indicate expression of chromosomal loci that are up-regulated (red circles) and down-regulated (open circles) by >2.5-fold upon exposure to stress conditions. Circle 1, organic acid stress (45 mM sodium benzoate); circle 2, chaotropic stress (0.8 M urea); circle 3, cold stress (growth at 4°C); circle 4, acid stress (growth at pH 4.5 [HCl]). The positions of the STM-disrupted genes are indicated in the innermost circle.

Transcriptome data accession number. The transcriptome data have been deposited in the GEO database under accession no. GSE4048.

RESULTS AND DISCUSSION

Screening for stress response genes. A signature-tagged mini-Tn*5* transposon (STM) library (51) was generated in *P. putida* KT2440 by triparental mating with 21 differentially tagged plasposons that yielded mutants with comparably high efficiency. Auxotrophic mutants were counterselected by growth on benzoate as the single carbon source. Cell division of the plasposon mutants led to about 10 CFU per transposition event on a mating plate. Hence, at most 5% of the CFU from a conjugation experiment were included in the STM library to avoid redundant clones. A subsequent Southern hybridization analysis of representative, randomly selected subsets of mutants with a pTnMod-OGm-ST-derived probe confirmed that all 21 differentially tagged plasposons had inserted randomly in the chromosome and that more than 90% of the mutants in the library were single clones.

In order to identify essential and nonredundant genes, processes, and regulation circuits for the response and adaptation to environmental stresses, 2,880 STM mutants in a 96-well format were grown under the following conditions: low-temperature stress (4°C); acid stress caused by high concentrations of the strong acid HCl (pH 4.5) and the weak organic acid benzoate (45 mM); and osmotic stress caused by a high concentration of urea (0.8 M), a chaotropic compound that increases the entropy of water and destabilizes interactions between nucleic acids, proteins, and lipids. A mutant was consid-

TABLE 2. Isogenic *P. putida* KT2440 mutants deficient in growth under abiotic stress conditions

Gene	Insert position(s)[a]	Mean normalized growth coefficient(s) (%) under the following stress conditions[b]:				Encoded protein
		Urea	Benzoate	HCl	4°C	
PP0063[c]	539		9	3	I[d]	Lipid A biosynthesis fatty acid acyltransferase
PP0168	23, 658	26, 33				Surface adhesion protein
PP0368	1748				I	Acyl-CoA dehydrogenase, putative
PP0813	544			39		CyoB cytochrome *o* ubiquinol oxidase, subunit I
PP0814	454			14		CyoC cytochrome *o* ubiquinol oxidase, subunit III
PP0816	273, 379, 495, 778		16, 26	19, 31		CyoE-2 protoheme IX farnesyltransferase
PP1186	423	12				PhoP transcriptional regulatory protein
PP1654	92				I	CysM cysteine synthase
PP1910	95				I	Conserved hypothetical protein
PP2242	1829, 1831		<0.1, <0.1			FepA outer membrane ferric enterobactin receptor
PP3820	222				I	Group II intron-encoding maturase
PP3951[c]	689		<0.1			PcaI 3-oxoadipate-CoA transferase, subunit A
PP4002[c]	69			10		AAA family ATPase
PP4129	840				I	NADH dehydrogenase I, L subunit
PP4185[c]	772		<0.1			SucD succinyl-CoA synthetase, alpha subunit
PP4186[c]	71		<0.1			SucC succinyl-CoA synthetase, beta subunit
PP4646	762			4	I	Ferredoxin $NADP^+$ reductase
PP4695	263				I	CbrA sensor histidine kinase
PP4696	948				I	CbrB response regulator
PP4697	731				I	PcnB poly(A) polymerase
PP4731	394		0.5			OmlA outer membrane lipid protein
PP4880	970, 1482				I	VacB RNase R
PP4941	186		3			Conserved hypothetical protein
PP4982	382		18			Conserved hypothetical protein
PP5044	358, 505, 845, 1110, 1032				I	BipA GTP-binding protein
PP5145[c]	218, 741	33, 31				PtsP phosphoenolpyruvate-protein phosphotransferase
PP5302	1453	27				SpoT guanosine-3,5-bis(diphosphate) 3-pyrophosphohydrolase
PP5322	412, 413, 425			4, 4, 6	I	CBS domain protein
PP5326	561		<0.1			PstB-PstA-PstC-PstS phosphate ABC transporter operon
PP5327	262		7			PstB-PstA-PstC-PstS phosphate ABC transporter operon
PP5328	1310		<0.1			PstB-PstA-PstC-PstS phosphate ABC transporter operon
PP5329	596		<0.1			PstB-PstA-PstC-PstS phosphate ABC transporter operon

[a] Insertion position of the plasposon in the gene relative to the start codon.

[b] Normalized growth coefficients (μ_{norm}): $\mu_{norm} = \mu_{mutant}/\mu_{wild\text{-}type\ KT2440}$. Growth coefficients were determined as follows: $\mu = [\ln(T_1) - \ln(T_0)]/(t_1 - t_0)$. μ_{norm} values of <0.1% could not be further differentiated, and the growth was indistinguishable from no growth. If the mean μ_{norm} was <0.1%, the individual μ_{norm} values of the three growth curves were each also <0.1%. If the mean μ_{norm} was >0.1% for a mutant and stress condition, the individual μ_{norm} values of the independent experiments differed by 1.1-fold to 8-fold (median, 1.6-fold; inner quartiles, 1.4- to 3.7-fold). Stress conditions: Urea, growth with 0.8 M urea; Benzoate, growth with 45 mM sodium benzoate; 4°C, low-temperature stress; HCl, growth at pH 4.5 in medium with hydrochloric acid.

[c] Mutations were complemented; complementation in *trans* restored the growth in the presence of the stressor to the KT2440 wild-type phenotype.

[d] I, growth of the mutant was impaired at 4°C (there were no visible macroscopic colonies after 72 h of incubation).

ered to be affected by a stress when its growth coefficient was consistently below the 99% confidence interval in three independent experiments. By using this stringent criterion, a total of 44 STM mutants with mutations in 32 different genes were found to have impaired growth during exposure to these abiotic stress conditions (Fig. 1 and Table 2). Two significant nonrandom associations were observed. First, the genes that were identified as genes that are indispensable for normal growth cluster around the origin of replication ($P < 0.01$, as determined by Monte Carlo simulation). Second, four genes (PP0063, PP0816, PP4646, and PP5322) were identified as genes that are vital for more than one stress condition ($P < 10^{-6}$ for the hypothesis that this finding is due to random association), suggesting that these genes play a more global role in the stress response. The causative relationship between transposon inactivation of a gene and susceptibility to stress was verified for six nonredundant STM mutants by complementation in *trans* with plasmid-borne wild-type genes and by reversion to normal growth in the appropriate bioassay.

Parallel to the STM approach, in which we searched for the essential nonredundant genes involved in stress responses, the global mRNA responses of *P. putida* KT2440 to urea, benzoate, inorganic acid, and low temperature in the exponential and stationary phases were assessed by transcriptome analyses. cDNA preparations were hybridized on a *P. putida* microarray of genomic PCR fragments that had been constructed from a minimal tiling path of the shotgun plasmid library used for sequencing of the *P. putida* KT2440 genome (85, 109). The 4,600 fragments representing the minimal tiling path cover more than 90% of the genome. On average, each fragment covers 2.5 genes. In contrast to oligonucleotide-based microarrays that have also been produced for the KT2440 strain (26, 122), the differential expression signals of our microarray refer to clones and cannot be assigned to single genes. Only when there were overlaps of genes and/or operons on adjacent gene fragments and there was a coordinated change in expression levels in the same direction was assignment to individual genes or operons feasible. Nevertheless, the statistical evaluation (see below) identified a major portion of the genetic repertoire

TABLE 3. Differential expression of *P. putida* KT2440 mRNA transcripts upon exposure to abiotic stresses

Gene(s)[a]	Clone or RT/PCR[b]	Up-regulation or down-regulation (fold) under the following stress conditions[c]:							Annotation
		4°C		Benzoate		Urea		HCl	
		Log phase	Stationary phase	Log phase	Stationary phase	Log phase	Stationary phase	Stationary phase	
PP0055-PP0056	FJ44	−2.7	−3.0			−2.6	−3.2		LysR family transcriptional regulator, GMC family oxidoreductase
PP0168-PP0169	HC88			−2.7					Surface adhesion protein, TauD/TfdA family dioxygenase
PP0255-PP0256	AZ28						2.7		Conserved hypothetical protein, molybdopterin oxidoreductase alpha subunit
PP0256	JP83						2.5		Molybdopterin oxidoreductase alpha subunit
PP0485-PP0486	JZ96						2.6		Single-stranded DNA-binding protein, GntR family transcriptional regulator
PP0525	DX87						−2.7		B12 family TonB-dependent receptor
PP0526	AH03						−3.0		ISPpu10 transposase
PP0578	DP32						2.6		Methyltransferase
PP0596-PP0597	GO91					−3.8	−3.8		Beta-alanine-pyruvate transaminase, *mmsA-1* methylmalonate-semialdehyde dehydrogenase
PP0596	RT/PCR					−5.2	−30		Beta-alanine-pyruvate transaminase
PP0597	CU21					−2.5	−2.6		*mmsA-1* methylmalonate-semialdehyde dehydrogenase
PP0674	ES55	−2.5	−2.2				−2.6		ABC transporter, ATP-binding protein
PP0799	FM30					2.7			Porin
PP0801-PP0802	HZ04			2.5		2.5	2.8		Hypothetical protein, chemotaxis protein CheV
PP0923-PP0924	EF34					−2.6			Acyltransferase, conserved hypothetical protein
PP1063-PP1064	GQ02		−3.0		−2.6		−4.0		MetR transcriptional activator, alpha/beta fold family hydrolase
PP1270-PP1271	HQ74						2.6		LysR family transcriptional regulator, multidrug efflux MFS transporter
PP1450	DO75						3.1		TPS family activation/secretion protein
PP1486-PP1487	HD74						2.7		Polyamine ABC transporter, conserved hypothetical protein
PP1656	KA10						−3.6	2.5	RelA GTP pyrophosphokinase
PP1656	RT/PCR					−1.8	−4.7	5.4	RelA GTP pyrophosphokinase
PP1891	DK62							2.7	FimL type 1 pilus subunit
PP2001-PP2002	FY14	−3.7							*O*-Succinylhomoserine sulfhydrylase, short-chain dehydrogenase/reductase
PP2194-PP2195	GN24		−2.5				−3.1		LysR family transcriptional regulator, periplasmic polyamine-binding protein
PP2196-PP2198	DF39	−2.9	−3.1			−3.8	−6.4	2.8	SpeB agmatinase, conserved hypothetical proteins
PP2196-PP2198	FG42		−2.9			−2.8	−5.6		SpeB agmatinase, conserved hypothetical proteins
PP2195	RT/PCR					−9	−22	6	Periplasmic polyamine-binding protein
PP2196	RT/PCR					−8	−4.4	4.0	SpeB agmatinase
PP2198	RT/PCR					−1.2	−19	4.5	Conserved hypothetical protein
PP2208-PP2209	HA62	−2.6		−2.5		−3.6			PhnX 2-phosphonoacetaldehyde hydrolase, PhnW 2-aminoethylphosphonate:pyruvate aminotransferase
PP2209-PP2210	FS72	−2.5				−2.6			PhnW 2-aminoethylphosphonate:pyruvate aminotransferase, LysR family transcriptional regulator
PP2432	GI59					2.8			Oxygen-insensitive NAD(P)H nitroreductase
PP2914	AS96							5.5	ProP proline/betaine MFS transporter
PP3091	EM20		−2.8			−2.5	−4.3		Conserved hypothetical protein
PP3091-PP3092	ED21						−4.4		Conserved hypothetical proteins
PP3336-PP3338	HC16	−2.5							Conserved hypothetical proteins, ubiquinol oxidase subunit II-related protein
PP3342-PP3343	IA41	−4.2							NikAB nickel ABC transporters
PP3607-PP3608	FW80						2.6		Conserved hypothetical protein, LysR family transcriptional regulator
PP3893	DP94					2.5			Phage DNA helicase
PP3934	DK15					−2.8	−4.2		LysR family transcriptional regulator
PP4004	FO78	−2.5				−3.7	−4.7		FtsK cell division protein
PP4004-PP4005	CC31	−2.7		−2.8		−5.6	−5.6		FtsK cell division protein, leucyl/phenylalanyl-tRNA-protein transferase

Continued on following page

TABLE 3—*Continued*

Gene(s)[a]	Clone or RT/PCR[b]	Up-regulation or down-regulation (fold) under the following stress conditions[c]:							Annotation
		4°C		Benzoate		Urea		HCl	
		Log phase	Stationary phase	Log phase	Stationary phase	Log phase	Stationary phase	Stationary phase	
PP4004	RT/PCR					−4.1	−1.3		FtsK cell division protein
PP4034	GL77					3.8	5.3		*N*-Carbamoyl-beta-alanine amidohydrolase
PP4034	RT/PCR					1.9	4.0		*N*-Carbamoyl-beta-alanine amidohydrolase
PP4097	DH50	−2.5		−2.5		−3.0			PgsA CDP-diacylglycerol–glycerol-3-phosphate 3-phosphatidyltransferase
PP4235-PP4236	ID29			2.5		−2.8	−4.2		DsbDE thiol:disulfide interchange proteins
PP4695-PP4696	AJ22				−3.4				Sensory box histidine kinase, CbrB response regulator
PP4809-PP4812	AS95							2.7	Conserved hypothetical proteins, ProA gamma-glutamyl phosphate reductase, DNA-3-methyladenine glycosylase
PP4910	DN26			2.6	2.8				HAMP domain protein
PP4936	DE71						−2.7		Lipopolysaccharide core biosynthesis protein
PP5203-PP5205	CJ53	−3.1							5-Formyltetrahydrofolate cyclo-ligase family protein, conserved hypothetical proteins
PP5207	HR60						2.7		ABC transporter, ATP-binding protein
PP5378-PP5379	JX59						−6.5		Cytochrome *c* family protein, CopB copper resistance protein B

[a] Hybridization of *P. putida* KT2440 cDNA preparations on the KT2440 clone contig (109). The genes are the genes found in the clones.
[b] For some genes that belong to fragments with >2.5-fold differential expression, the expression of the individual gene was reinvestigated by quantitative real-time PCR kinetics of cDNA (indicated by RT/PCR).
[c] The values indicate the mean fold up-regulation (positive values) and down-regulation (negative values) of gene expression of gene fragments under the stress conditions compared to the expression in the growth control in the absence of a stressor. The table includes all significantly differentially expressed fragments ($P < 0.01$) with >2.5-fold changes, which were identified by applying the highly stringent min-max separation criterion (7), followed by correspondence analysis of the intensity ratios (34, 35). The correspondence analysis plot displayed the significance of differences both among the genes and among the hybridizations in terms of the χ^2 statistic. Stress conditions: Urea, growth with 0.8 M urea; Benzoate, growth with 45 mM sodium benzoate; 4°C, low-temperature stress; HCl, growth at pH 4.5 in medium with hydrochloric acid.

of *P. putida* KT 2440 (392 gene fragments) that was significantly differentially regulated upon exposure to at least one stress condition. In other words, a substantial portion of the changes in the expression profile were detected by our type of microarray.

The initial hybridization experiments revealed rather low sensitivity; the intensities varied only within 2 orders of magnitude. As a consequence, we applied rather stringent evaluation criteria, including filtering by MinMax separation of condition and control (exclusion overlap) (7) to focus on the robust and strong changes in expression levels. We found 392 significantly differentially expressed gene fragments, and the up-regulated genes clustered in the auxotroph-rich region of the chromosome (54, 120) ($P = 0.002$ for Monte Carlo simulation), while no reliable preferences were detected for the down-regulated genes ($P = 0.432$). Figure 1 and Table 3 provide an overview of the 47 gene fragments that responded to stress conditions by >2.5-fold differential expression. The results were confirmed by real-time PCR for seven genes (Table 3). Sixteen gene fragments were found to be regulated by more than one stress ($P < 10^{-6}$ for the hypothesis that this association occurred by chance), indicating that these genes play a more general role in stress responses.

The PP0168 and PP4695-PP4696 genes encoding the surface adhesion protein LapA and the CbrAB two-component system were the only genes that were identified as important stress response elements by both the mutant screening (Table 2) and a >2.5-fold change in the mRNA level (Table 3). The stresses applied, however, were different, implying that there was not a single case in which a gene was detected by both approaches. Hence, we concluded that the mRNA expression of all the essential nonredundant genes that were identified by screening the plasposon library was not significantly regulated upon exposure to the stress conditions used. Redundant genes show up only in the transcriptome and never in a library of single mutants. The observed lack of overlap confirmed a posteriori the usefulness of the study design to search for major stress response genes by complementary approaches.

General stress response. Screening the STM library for survival at a low temperature and in the presence of high concentrations of sodium benzoate, urea, and protons revealed only a few stress-sensitive mutants (Table 2), which can be explained by the high level of paralogy of "fitness" genes in the versatile bacterium *P. putida* (27, 28). In other words, our approach identified a subset of nonredundant genes. All but one mutant were sensitive to either one or two unfavorable conditions (Table 2).

Expression analysis at the transcriptional level revealed that the various stresses resulted in similar response patterns. The transcriptome to sodium benzoate and urea correlated by 0.6 (Table 4). The transcriptional profiles obtained at a low temperature correlated with the transcriptional profiles obtained with both urea and benzoate more strongly in the logarithmic growth phase than in the stationary growth phase (Table 4). In contrast, opposite regulation of *P. putida* was observed when the exposure to hydrochloric acid was compared to the other six conditions, and the most negative correlation coefficient, −0.5, was obtained for the comparison between mineral acid

TABLE 4. Correlation of transcriptome data[a]

Conditions	Correlation coefficient						
	Urea		Benzoate		Low temp		pH 4.5 (Stationary phase)
	Log phase	Stationary phase	Log phase	Stationary phase	Log phase	Stationary phase	
Urea, log phase	1						
Urea, stationary phase	0.8	1					
Benzoate, log phase	0.6	0.4	1				
Benzoate, stationary phase	0.6	0.6	0.6	1			
4°C, log phase	0.4	0.3	0.3	0.1	1		
4°C, stationary phase	0.3	0.3	0.1	0.1	0.5	1	
pH 4.5, stationary phase	−0.4	−0.5	−0.2	−0.3	−0.1	−0.1	1

[a] Pearson correlation coefficients were calculated for the set of 392 gene fragments, each of which showed at least once a >1.5-fold change in the normalized and filtered fluorescence cDNA hybridization signal (34) compared to the signal of the nonperturbed control.

and urea in the stationary phase (Table 4). The response of *P. putida* was influenced by the growth phase. The correlation coefficients for the expression profiles in the logarithmic and stationary phases were 0.8, 0.6, and 0.5 for the urea, sodium benzoate, and low-temperature stresses, respectively (Table 4).

The gene fragments that exhibited >2.5-fold differential expression in response to the stressors are listed in Table 3. Expression of the FtsK cell division gene (PP4004) was down-regulated under all conditions, although not always by >2.5-fold, corresponding to the prolonged doubling times of KT2440. Among the transcriptional regulators, the stress was sensed predominantly by members of the LysR family. Of the 101 LysR homologs, 14 genes were differentially expressed. Low temperature, urea, and benzoate had similar effects on the expression of LysR regulators that corresponded to positive correlations with the global transcriptional profile; the opposite type of regulation was never observed. Growth of *P. putida* at low pH, however, did not affect expression of the LysR regulators. The other differentially expressed regulator genes were the GntR regulator gene PP0486, the TonB-dependent receptor gene PP0525, the MetR transcriptional activator gene PP1063, and two-component CbrB response regulator genes (PP4695-PP4696).

The bacterial cell envelope is the major barrier between the cell and the environment and hence is essential for the response to osmotic stress, pH stress, and toxic compounds. *P. putida* balances membrane fluidity and permeability in response to many physical and chemical stresses by changing the length, degree of saturation, and *cis*/*trans* ratio of fatty acids (65, 96), and key enzymes of LPS biosynthesis and lipid metabolism were identified as major elements of the bacterial stress response. The phosphatidylglycerophosphate synthase PgsA (PP4097), which is essential for the biosynthesis of phospholipids (110), was down-regulated during exponential growth under all conditions tested except acid stress. LPS, the major constituent of the outer cell membrane, is important for buffering, cell protection, and membrane fluidity. One LPS biosynthesis gene, PP0063, was identified in the STM screening to be essential for *P. putida* to cope with cold, benzoate, and acid stresses. *Pseudomonas* sp. lipid A typically is penta-, hexa-, or heptaacylated; up to five fatty acid and/or R-3-hydroxy fatty acid residues are attached directly to the glucosamine disaccharide backbone, and another two fatty acids are attached to the R-3-hydroxy groups of the R-3-hydroxy fatty acid residues in the distal unit (67, 114). In the later steps of the lipid A biosynthetic pathway, the key intermediate $(KDO)_2$-lipid IV_A is sequentially acylated with fatty acids. The lipid A fatty acid composition of pseudomonads is variable and depends on the strain and the environmental conditions (64, 65). The *P. putida* KT2440 genome contains two homologous genes, PP0063 and PP1735, that encode lipid A fatty acyl transferases, and each of these genes has orthologs in related taxa. However, according to the results of the STM screening the product of the PP0063 gene, but not the product of the PP1735 gene, is essential for adapting the lipid A fatty acid composition to the environmental stress conditions.

Expression of the PP3091 gene is down-regulated by low temperature, urea, and benzoate stresses (Table 3). This gene, annotated as a conserved hypothetical gene, encodes a membrane IcmF domain protein that is typically involved in modeling the cell surface structure (17). There are three other paralogous genes in the *P. putida* genome, PP2627, PP3090, and PP4071; however, only PP3091 is regulated under stress conditions.

PP0168 encodes LapA, which is among the largest bacterial proteins known to date (85, 119). The multimodular large adhesion protein LapA is essential for biofilm formation (52), for adhesion to abiotic surfaces and seeds (31), and for coping with urea stress (Table 2). The hallmarks of this protein are large threonine-rich repeats with no defined secondary structure. Hence, LapA generates a hydrophilic micromilieu of hydrated random coil proteins around the bacterial cell that is suitable for incorporation of urea into its mesh and hence for neutralization of the water stress caused by this chaotropic solute and for prevention of its partitioning into the cell membrane (46). LapA-deficient cells are therefore more sensitive to urea-mediated water stress and membrane perturbation.

The various abiotic stresses affected the central metabolism involving the key energy-rich intermediate phosphoenolpyruvate and polyamine metabolism. First, enzymes (PP2208 and PP2209) which convert phosphoenolpyruvate to acetaldehyde and to intermediates of aminophosphonate metabolism (61) were down-regulated at the transcriptional level at a low temperature and in the presence of high concentrations of sodium benzoate and urea (Table 3). Inhibition of these enzymes may preserve the energy-rich phosphoenolpyruvate for other energy-consuming processes to cope with the stressors. Second, SpeB (PP2196), which hydrolyzes the polyamine agmatine to putrescine and urea (84), was down-regulated at a low temperature and in the presence of a high concentration of urea and

was up-regulated at a low pH. By either limiting or stimulating the production of the chaotropic compound urea, the cell apparently tries to minimize the deleterious effects of the stressors on intracellular water activity, ion strength, and solvation of cytosolic constituents.

Urea stress. Exposure to urea revealed the smallest number of indispensable genes in the STM screening but the largest number of regulated genes in the transcriptome among all the conditions tested (Tables 2 and 3). We concluded that *P. putida* is protected against noxious chaotropic solutes by numerous redundant defense mechanisms probably because it is commonly confronted with water stress in its natural ecotopes (46). First, *P. putida* copes with urea by minimizing entry via diffusion. The secreted large adhesion protein LapA may reduce the amount of free urea by incorporating it into its solvation shell. The DsbD and DsbE membrane thiol-disulfide interchange proteins (93) were down-regulated at the transcriptional level, suggesting that the diffusion barrier of outer membrane proteins is stabilized by disulfide bonds. Down-regulation of genes for an acyltransferase (PP0923) and an LPS biosynthesis protein (PP4936) suggests that there is adaptive modification of the cell envelope fatty acids and lipopolysaccharides. Second, many transmembrane transporters are activated under urea stress conditions (Table 3). These transporters include porin PP0799, the multidrug efflux MFS transporter PP1271, the TPS family activation/secretion protein PP1450, and the ABC transporter PP5207. These proteins may remove urea and possible noxious products of the cellular stress response. Third, urea is sensed by the PhoP-PhoQ two-component system (Table 2), which in *Pseudomonas aeruginosa* regulates resistance to cationic antimicrobial peptides in response to low concentrations of Mg^{2+} (74). The other sensor that is essential for *P. putida* to survive urea stress is PtsP (Table 2). Complementation of *ptsP* in *trans* restored the natural resistance to urea. PtsP is a member of the phosphotransferase family, but in contrast to the paradigm in many other bacteria, in which the phosphotransferase system is required for carbohydrate transport (76, 94), PtsP in *P. putida* has another function that has not been determined yet (2, 12).

The RelA/SpoT alarmone modulon is also necessary for *P. putida* to cope with urea stress. A *spoT* mutant did not grow in the presence of urea, suggesting that the control of (p)ppGpp alarmone synthesis by SpoT is crucial for the bacterial cell to respond to a urea overload and the effects of urea on carbon metabolism and growth. Typically, only a *relA-spoT* double mutant is not able to cope with prolonged amino acid and carbon starvation (100, 108). Exposure to urea had a greater effect, because the intact *relA* gene could not rescue the *spoT* mutant phenotype. The RelA mRNA transcript was down-regulated in the wild-type KT2440 strain in the presence of urea (Table 3), implying that a stringent response to urea stress is deleterious for the bacterial cell. Moreover, the intermediate metabolism adapted to the external urea overload by minimizing endogenous production of this chaotropic solute. Amino acid metabolism was reorganized to funnel amide groups into the de novo synthesis of pyrimidine precursors (up-regulation of PP4034 and down-regulation of PP0596 and PP0597) (Table 3).

In summary, *P. putida* KT2440 responds to a high concentration of urea with a concerted action involving intuitively plausible mechanisms, including extracellular capture of urea by LapA, prevention of urea uptake, export from the cell, activation of anabolic pathways, and a reduction in endogenous synthesis of the stressor.

Benzoate stress. Sodium benzoate is a widely used food preservative (83). Aqueous sodium benzoate enters cells by diffusion of uncharged benzoic acid. The subsequent acidification severely damages and kills the cells (15). *P. putida* KT2440 grew at similar rates with 15, 30, and 45 mM sodium benzoate as the sole carbon source in our experimental setup; however, growth was delayed and impaired with 60 mM sodium benzoate, and no growth was observed with 75 and 90 mM benzoate. The bacterium catabolizes this chaotropic organic acid via catechol and the β-ketoadipate pathway (55, 58, 63, 81) and contains a number of other known systems to cope with organic acid stress, including proton pumps and lipopolysaccharides (27, 28, 62). Mutations in either *pcaI* (PP3951) or *sucDC* (PP4185-PP4186) made the cells sensitive to 45 mM benzoate. PcaIJ catalyzes the penultimate step in the degradation of benzoate and protocatechuate, the transfer of coenzyme (CoA) to β-ketoadipate (49, 58, 63, 89). The succinyl-CoA synthetase is a key enzyme of the tricarboxylic acid cycle and converts the product of the final step of the β-ketoadipate pathway, succinyl-CoA, in a three-step reaction into succinate and nucleoside triphosphate (60). Complementation of the mutants in *trans* restored the initial growth rate of the wild type. Interestingly, the three mutants could grow with 15 mM sodium benzoate as the sole carbon source but could not grow with 45 mM benzoate. The KT2440 genome includes biochemically uncharacterized paralogs of *pcaIJ*, PP3122-PP3123, annotated as acyl-CoA:acetate/3-keto acid CoA transferase (85). Orthologs of PP3122-PP3123 are present in all *Pseudomonas* genomes that have been sequenced, and their products exhibit substantial amino acid sequence homology with PcaIJ, the acetoacetate acetyl-CoA transferase of *E. coli* (57) and higher eukaryotes, and the only bacterial succinyl-CoA:acetoacetate CoA transferase that has been characterized (16). PP3122-PP3123 may be responsible for the residual activity that allows the mutants to grow on low concentrations of benzoate. We suspect that PP3122-PP3123 allows the *pcaI* and *suc* mutants to bypass blocks in either the β-ketoadipate or tricarboxylic acid pathway by replacing the missing enzyme (PcaI) or redirecting the flow of substrate into the ketone body metabolism pathway (PcaI, SucDC). A recent proteomic analysis demonstrated that growth of *P. putida* KT2440 with benzoate increased not only the cellular levels of succinyl-CoA synthetase but also the cellular levels of succinate semialdehyde dehydrogenase (DavD, PP0213) (63). Hence, PP0213 and PP3724, the paralog of the succinyl-CoA synthetase gene, may compensate for the inactivation of SucDC in *suc* mutants.

Two outer membrane proteins (PP2242 and PP4731), a protein of the *cyo* operon (Cyo-E2, PP0816), a phosphate transporter (PP5326-PP5329), and two proteins whose functions are unknown (PP4941 and PP4982) were also identified as essential elements for coping with benzoate stress (Table 2). PP4731 encodes the lipoprotein OmlA, which is known to be important for maintaining the integrity of the cell envelope in related taxa (87), and PP2242 encodes the outer membrane ferric enterobactin receptor FepA, which, based on analogy with its ortholog in *E. coli* (75, 82), could bind aromatic acids. The finding that most indispensable key elements that cope with toxic

levels of the nutrient benzoate are located in the cell envelope is in accordance with the outcome of a recent transcriptome analysis which showed that the primary reaction of *P. putida* to the presence of aromatic compounds takes place at the level of the cell envelope (26).

The uptake of phosphate is important for most cellular processes. In the presence of high concentrations of benzoate, intracellular phosphate is a buffer for neutralizing the organic acid and is necessary for the synthesis of membrane constituents and energy-rich intermediates, to mention just the evident rescue mechanisms for overcoming benzoate stress. The *P. putida* genome contains three clusters of genes that encode ABC transporters for phosphate uptake, the PP0824-PP0827, PP2656-PP2659, and PP5326-PP5329 operons. Despite this apparent redundancy, the PP5326-PP5329 operon is indispensable for cell growth in the presence of benzoate.

The pattern of gene expression under benzoate stress conditions correlated with the response to urea stress (Table 4); however, on average, the differential regulation was weaker (Table 3). The single benzoate-specific response was up-regulation of PP4910 encoding a conserved hypothetical protein that contains a HAMP sensor domain (1, 36).

In summary, the defense of *P. putida* KT2440 against benzoate involves the decomposition of the stressor, prevention of its uptake, and neutralization of the acid by phosphate.

Low-temperature stress. *P. putida* KT2440 grew at 4°C at a pH range of 5.5 to 10 in ABC medium and at a pH range of 4 to 10 in LB medium (doubling time of a planktonic culture in ABC medium at pH 7, 10.7 h), in contrast to *E. coli* K-12 strains, which exhibited no significant growth at 4°C (5). The KT2440 genome encodes various cold shock proteins, homologs of which were identified in *E. coli* (78, 97). In none of the 14 mutants that were identified in our STM screening (Table 2) was one of these known cold shock proteins inactivated, probably because *P. putida* has numerous paralogs that can complement each other for growth at 4°C. For CspA, for example, the major cold shock protein of *E. coli* (111), two paralogs are encoded in the genome of *P. putida* KT2440 (85).

The PP4695 (*cbrA*) and PP4696 (*cbrB*) genes encode a sensory box histidine kinase and a response regulator, respectively. The very similar orthologs of this two-component system in *P. aeruginosa*, designated CbrA and CbrB, were found to control the utilization of carbon and nitrogen sources (86). Mutants of *P. aeruginosa* deficient in *cbrA* and *cbrB* grew poorly on carbon sources such as glucose, citrate, or pyruvate and were not able to utilize several amino acids and polyamines (86). The latter compounds play an important role in enhancing translation efficiency (3, 20, 113) and ribosome assembly (59). Considering the paramount role of CbrAB in central metabolism, it is reasonable that knockout of orthologs in *P. putida* severely impairs growth at a low temperature. Disruption of PP4697 downstream of *cbrAB* also eliminated the ability to grow at 4°C (Table 2). This gene organization in the chromosome is conserved in all *Pseudomonas* genomes that have been sequenced, indicating that there may be functional interplay between the gene products. PP4697 encodes the poly(A) polymerase PcnB governing polyadenylation at the 3′ ends of mRNAs. The functions of bacterial RNA polyadenylation range from modulation of mRNA stability to mRNA degradation, and polyadenylation may play a role in mRNA translation (105). A recent study demonstrated that polyadenylation occurs in the mRNAs of all the major functional classes of genes in *P. aeruginosa* (103). In *E. coli*, polyadenylation of some genes depends on the growth rate (56) and the growth phase (11), and increased polyadenylation is observed in slowly growing cells. Our data pinpoint a similar role of polyadenylation in the mesophilic organism *P. putida*, in which lower temperatures inherently lead to lower growth rates. The functionally related 3′,5′-exoribonuclease VacB (PP4880) was also found to be necessary for *P. putida* to cope with cold stress. VacB cleaves poly(A), poly(U), and rRNAs in vitro (8, 19, 20). It contains a so-called cold shock domain (Pfam accession number PF00313), which is typical of cold shock proteins, whose expression is increased when cells are shifted to low temperatures (39). Our STM data demonstrate that both PcnB and VacB, with their antagonistic actions on mRNA polyadenylation, are essential for *P. putida* to adapt to low temperatures.

Five different mutants that carried an insertion in the *bipA* gene (PP5044) were identified in the assay (Table 2). BipA is a widespread and evolutionarily conserved member of the ribosome binding GTPase superfamily in bacteria and plants (88). BipA shares the binding site on 70S ribosomes with the four major G proteins involved in protein biosynthesis. BipA has been shown to function as a master regulator by transcript-selective translational control (88). In *E. coli*, it is a master regulator of growth at low temperatures (91) and of the expression of genes in pathogenicity islands (43). Considering the conservation of domain arrangement and the 71% amino acid sequence identity between *E. coli* and *P. putida* BipA, we concluded that in *P. putida* BipA has a key role in adaptation to cold stress and growth at 4°C due to transcript-selective translation control.

Global expression analyses of the response to cold stress in other bacteria (44) have revealed numerous ways that structural constituents and the intermediary metabolism adapt to low temperatures. According to our STM screening (Table 2), two genes are central for the maintenance of the redox status of *P. putida*: PP4129, which encodes a NADH dehydrogenase, and PP4646, which encodes a ferredoxin NADP reductase. Second, sulfur homeostasis is crucial for *P. putida* to grow at 4°C. The genome contains two paralogs encoding cysteine synthetases, *cysK* and *cysM* (116), the latter of which has been found to be indispensable for survival of the KT2440 strain at low temperatures (Table 2). Both enzymes can utilize sulfide as a nucleophile, but only CysM can also use thiosulfate as a substrate (106, 116). Third, the bacterial cell membranes must adapt to low temperatures, and two genes, one involved in fatty acid metabolism (PP0368) and one involved in LPS biosynthesis (PP0063) (see above), were found to be essential for *P. putida* to grow at 4°C. Moreover, two mutants with mutations in the PP1910 and PP5322 genes, which encode conserved hypothetical proteins, were identified. The genomic context suggests that these proteins have roles in lipid metabolism (PP1910) and uptake of phosphate (PP5322). PP5322 is localized downstream of the genes encoding the PhoB-PhoR two-component system (PP5320 and PP5321) in a phosphate uptake operon (PP5329-PP5320) that is conserved in pseudomonads. PhoB is a key regulator of phosphate uptake and controls the membrane phospholipid composition under phosphate limitation conditions that was demonstrated for *Rhizobium meliloti* (40), and

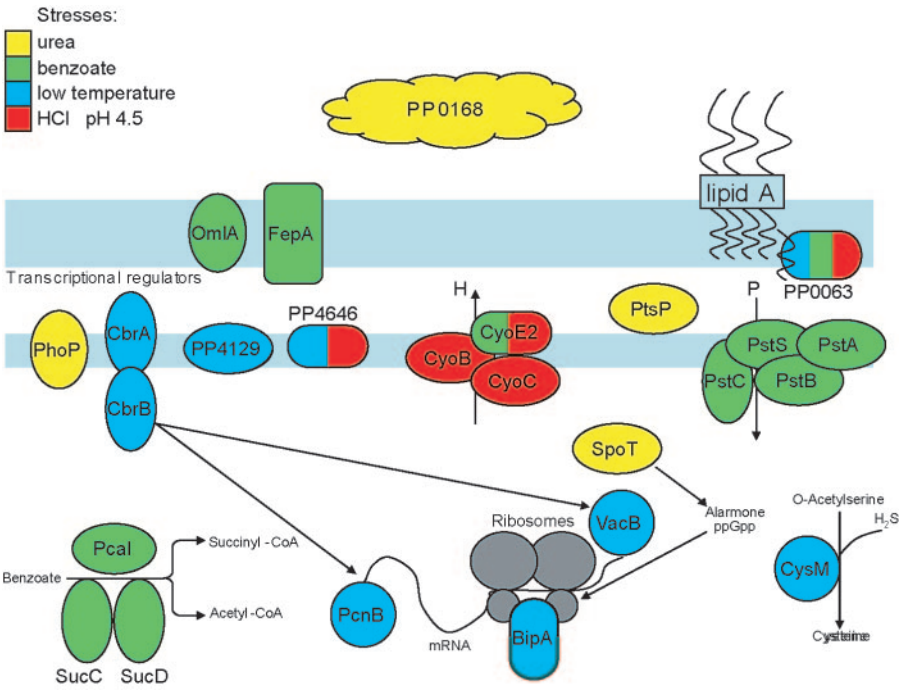

FIG. 2. Roles and cellular localization of the indispensable nonredundant key elements of the *P. putida* KT2440 response to abiotic stresses.

according to our data, the CBS domain protein encoded by PP5322 plays a crucial role in these processes at 4°C.

In summary, the genes that were found to be essential for survival at low temperatures can be assigned to three major processes: (i) maintenance of transcription, translation, and ribosomal activity, (ii) maintenance of membrane integrity and fluidity, and (iii) maintenance of the redox status of the cell.

Acid stress. The STM screening revealed that the PP4002 gene product has a striking role in bacterial growth at low pH. PP4002 encodes an AAA family ATPase and is part of a gene contig that is highly conserved in proteobacteria. The gene contig encompasses genes encoding the cell division protein (*ftsK*), an outer membrane protein (*lolA*), the AAA+ protein, the putative adaptor protein (*crcB*) that in *E. coli* checks DNA topology (101), and a seryl-tRNA synthetase. In a bacterial cell, several vital cellular processes, including cell division, cell differentiation, and the regulation of the heat shock response, are known to be controlled by members of the AAA+ superfamily (29). Substrate specificity is often due to a specific adaptor protein. The PP4002 gene product conferred a complex phenotype, susceptibility to acid in an unstirred solution. *P. putida* KT2440 did not grow in acidic LB medium with vigorous shaking, but the PP4002 transposon mutant thrived under these conditions and did not grow at pH 4.5 without shaking. Complementation of the mutation in *trans* restored the initial properties of the wild type; i.e., the complemented strain grew in LB broth at pH 4.5 without agitation, but it did not survive with shaking. In an unstirred medium gradients of metabolites and oxygen become established, whereas during stirring the milieu is homogeneous. It seems that at a low pH the PP4002 gene product controls growth that is dependent on the supply of nutrients and oxygen and it suppresses growth in the presence of high oxygen tension and a homogeneous concentration of metabolites. The only known regulon that controls carbon metabolism depending on the oxygen tension is the *cyo* operon (24). The STM screening identified four mutants with mutations in three genes of the *cyoABCDE* operon encoding cytochrome *o* ubiquinol oxidase, the main terminal oxidase of the electron transport chain under highly aerobic conditions (42). The activity of the electron transport chain reflects the global physiological status of the cell. Inactivation of the *cyo* genes severely impairs the ability of *P. putida* to maintain the redox status in an acidic environment and its ability to transmit global transcriptional signals of metabolic control (24, 25, 90).

The transcriptome at pH 4.5 in the presence of mineral acid exhibited no correlation or a negative correlation with the global expression profiles under the other stress conditions (Table 4). Thus, in contrast to the other conditions, *speB* agmatinase (PP2196) was up-regulated, which was confirmed by quantitative real-time PCR (Table 3). Active synthesis of polyamines may explain the observed alkalinization of the medium from pH 4.5 to pH 8.0 during the growth of *P. putida* for 16 h at 26°C. In contrast to what occurs under urea stress conditions, *relA* (PP1656) was activated at a low pH. RelA GTP pyrophosphokinase is the major synthetase of the alarmone pppGpp, which triggers the stringent response of growth control that is associated with the accumulation of buffering metabolites (13). Accordingly, the *proP* proline/betaine MFS transporter (PP2914) was up-regulated to stimulate uptake of the amino acid proline.

Key elements of the *P. putida* stress response. The soil bacterium *P. putida* is used as a biocontrol agent in plants and for bioremediation of polluted habitats (27, 28, 30, 41, 98). *P. putida* preferentially colonizes the rhizosphere of plants,

where it is exposed to steep gradients of pH, solute, and nutrient concentrations (66, 73, 98). Concomitantly, the bacterium has to cope with water stress and acidic stress, the latter predominantly caused by organic acids (46). In other words, the stress is greatest at sites where the nutrient supply is maximal, which are also the sites where biotechnology applications have been developed. We chose chaotropic solutes and acid (i.e., urea, sodium benzoate, and mineral acid) to mimic the common stresses that *P. putida* has to deal with. This mesophilic bacterium does not grow at temperatures greater than 37°C (48), but it is able to survive at temperatures close to the freezing point of water. Thus, we decided to identify the essential elements of adaptation to low temperatures.

In its habitat this bacterium is typically confronted simultaneously with organic acids and chaotropic agents, and we obtained a high correlation value for the global expression profiles after exposure to urea and benzoate. In general, one major outcome of this study was the similarity of the responses of the bacterium to the different stresses. A striking observation resulting from the screening of the *P. putida* transposon library was the low number of nonredundant genes that were found to be essential for coping with all the different stresses examined. Just 32 different genes located in 22 operons were identified. A few genes probably escaped our attention because either they were not represented by a transposon mutant in the library or their phenotypes did not match the stringent criterion used, growth consistently below the 0.5% growth centile. The hypothesis that in all likelihood the screening was not comprehensive, however, does not contradict the general conclusion of this study that the repertoire of indispensable stress response genes in *P. putida* is small.

Figure 2 shows a summary of the cellular roles and localization of the products of the essential genes. Three outer membrane constituents were found to be indispensable for coping with one or more of the stresses tested; two of these outer membrane constituents sense the stress from the environment, and one is a key enzyme of LPS biosynthesis which adjusts outer membrane integrity and fluidity. The large adhesion protein LapA (PP0168) apparently neutralizes chaotropic solutes and protects the bacterial cell from entry of these compounds. The ABC transporter encoded by PP5326-PP5329 in the inner membrane supplies phosphate to the cell to maintain the intracellular pH and provides an essential precursor for the biosynthesis of membrane constituents and energy-rich compounds. Of the 135 two-component signal transduction systems, just two sensors, PhoPQ and CbrAB, were found to be essential for *P. putida* to cope with urea and cold stress, respectively. These two sensors are complemented by PtsP, which is known to be involved in the control of amino acid metabolism in *E. coli* (71). The cytochrome *o* ubiquinol oxidase and the product of another gene (PP4646) are involved in the maintenance of redox status and energy metabolism and are necessary to cope with acid stress. Posttranscriptional control is necessary for *P. putida* to cope with stress, particularly to adapt to low temperatures. CbrAB is involved in translational control and ribosome assembly. PcnB and VacB, which control polyadenylation and the half-life of mRNA transcripts, are two other indispensable elements of posttranscriptional control at low temperatures. Furthermore, *P. putida* needs an intact RelA/SpoT modulon to adapt anabolic pathways and macromolecule biosynthesis to the constraints of external stress. The ribosome binding G protein BipA exerts transcript-selective translational control that also is necessary for *P. putida* to survive in the presence of cold stress. BipA and PhoPQ are major regulons for the expression of virulence and pathogenicity islands in enterobacteria (4, 19, 43), but in the case of the nonvirulent soil bacterium *P. putida* they are master regulators of metabolism and stress response. This observation provides further evidence for the hypothesis based on analyses of PhoPQ in *Salmonella* (4, 19) and BipA in *Bordetella* (22, 115) and pathogenic *E. coli* (43) that pathogens expanded the use of major regulators, such as PhoPQ and BipA, which exert global metabolic control in commensals, to a second role as master regulators of virulence in pathogens.

In summary, the adaptation of the membrane barrier, the maintenance of intracellular pH and redox status, and the translational control of metabolism are the indispensable key mechanisms of the *P. putida* stress response. If recombinant *P. putida* strains are developed as bioremediation and biocontrol agents, the integrity of these key stress response elements and pathways should be ensured to maintain the fitness of the bacteria.

ACKNOWLEDGMENTS

O.R. was the recipient of a postgraduate stipend from the DFG-sponsored European Graduate College "Pseudomonas: Pathogenicity and Biotechnology." Financial support by the Bundesministerium für Bildung und Forschung and the European Commission (grant QLK3-CT2000-00170, MIFRIEND) is gratefully acknowledged.

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