Transcriptional Tradeoff between Metabolic and Stress-response Programs in Pseudomonas putida KT2440 Cells Exposed to Toluene*

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When Pseudomonas putida KT2440 cells encounter toluene in the growth medium, they perceive it simultaneously as a potential nutrient to be metabolized, as a membrane-damaging toxic drug to be extruded, and as a macromolecule-disrupting agent from which to protect proteins. Each of these inputs requires a dedicated transcriptional response that involves a large number of genes. We used DNA array technology to decipher the interplay between these responses in P. putida KT2440 subjected to a short challenge (15 min) with toluene. We then compared the results with those in cells exposed to o-xylene (a non-biodegradable toluene counterpart) and 3-methylbenzoate (a specific substrate of the lower TOL pathway of the P. putida pWW0 plasmid). The resulting expression profiles suggest that the bulk of the available transcriptional machinery is reassigned to endure general stress, whereas only a small share of the available machinery is redirected to the degradation of the aromatic compounds. Specifically, both toluene and o-xylene induce the TOL pathways and a dedicated but not always productive metabolic program. Similarly, 3-methylbenzoate induces the expression not only of the lower meta pathway but also of the non-productive and potentially deleterious genes for the metabolism of (nonsubstituted) benzoate. In addition, toluene (and to a lesser extent o-xylene) inhibit motility functions as an unequivocal response to aromatic toxicity. We argue that toluene is sensed by P. putida KT2440 as a stressor rather than as a nutrient and that the inhibition by the aromatic compounds of many functions we tested is the tradeoff for activating stress tolerance genes at a minimal cost in terms of energy.

Pseudomonas putida KT2440 is a soil microorganism characterized by its metabolic versatility, which enables the strain to degrade a wide variety of natural and man-made aromatic compounds (1, 2). This strain can use toluene and m- and p-xylene via the pWW0 TOL plasmid-encoded pathways (3, 4). When the strain is confronted with these aromatic compounds, cells face an enticing paradox. On the one hand, these chemical species can be mineralized to yield carbon and energy for growth, allowing the strain to colonize niches refractory to other microbes. On the other hand, toluene and xylenes are toxic for the bacteria above a certain threshold, since they partition in the cell membrane and disorganize it by removing lipids and proteins, which eventually leads to cell death (5).

The mechanism of toluene, m-xylene, and p-xylene metabolism to CO2 and H2O in P. putida bearing pWW0 involves two sets of reactions and depends on the ability of two cognate regulatory proteins to become activated by certain aromatic compounds and to trigger the expression of specific pathways (summarized in Fig. 1) (4). But activation of metabolic genes is not the only effect of exposing P. putida cells to aromatic hydrocarbons, as these compounds also trigger a solvent tolerance response which involves a number of mechanisms that are not yet fully understood (6, 7). However, a number of factors involved in the process have been characterized over the last 10 years (for review, see Ref. 6). Several laboratories have identified efflux pumps belonging to the RND family as being involved in solvent tolerance (8–13), and the TtgABC efflux pump present in all strains that extrude several organic solvents and antibiotics is also present in Pseudomonas putida KT2440 (14, 15). Although extrusion of the solvent is probably the most important mechanism of solvent resistance, this phenomenon involves other factors such as membrane rigidification via cis to trans isomerization of the unsaturated fatty acids (16, 17) or the induction of a number of chaperones (18). Finally, exposure of P. putida cells to solvents and chaotropic agents triggers a heat-shock (HS) response that is likely to require a share of the available gene expression machinery of the cell (18, 19).

The issue at stake is, thus, how P. putida cells harboring a relatively stable transcriptional machinery are able to process all three different inputs from toluene; that is, a nutritional signal bound to trigger a large metabolic program, a toxic signal that triggers a solvent extrusion and tolerance response, and interference with the protein folding machinery leading to activation of the HS regulon. How the strain is able to manage all these tasks has not been examined because of the dearth of suitable technology (20). In this study we used a genome-wide DNA array setup to inspect in detail the immediate changes in global expression pattern of P. putida KT2440 in response to exposure to three different aromatic compounds; toluene, a metabolizable aromatic hydrocarbon (a carbon source and a stressor), o-xylene, a gratuitous counterpart known to induce the degradation pathway but which cannot be metabolized (21) (and, thus, acts only as a stressor), and 3MB, a specific inducer of the

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2 The abbreviations used are: RND, resistance nodulation cell division; HS, heat shock; 3MB, 3-methylbenzoate; RNAP, RNA polymerase; TIGR, The Institute of Genomic Research.
lower TOL pathway (primarily a C-source with lower toxicity). Our main objectives were to understand how the presence of all three compounds (i.e. of two contradictory signals) is differentially sensed, to detect the direct consequences of their presence, and to determine the short-term global outcome on the genetic program. Our results reveal that after exposure to aromatic compounds a strong and specific metabolic response at the level of aromatic pathways is concomitant with a general stress response at several levels, reflected by the induction of genes known to respond to membrane damage, oxidative stress, and misfolding of soluble proteins. The interplay between the HS response, the induction of catabolic pathways, the synthesis of efflux pumps, and the stimulation of amino acid biosynthesis resulted in distinct patterns of transcription under all three conditions. Interestingly, these patterns appear to reflect a shift of resources available to the transcriptional machinery from possibly more dispensable functions and toward these vital tasks. Furthermore, our results suggest that the regulatory network behind this metabolic and genetic interplay is not fortuitous but reflects how priorities in the use of the transcriptional machinery are established in different expression programs.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Culture, and Growth Conditions**—*P. putida KT2440 (pWW0)* was grown on M9 minimal medium (21) with 25 mM citrate as a carbon source. 500-ml flasks containing 100 ml of this culture medium were inoculated with *P. putida KT2440* to reach a turbidity of 0.03 and shaken at 30 °C and 200 rpm until the turbidity of the culture reached 0.7 at 660 nm. Then the cultures were exposed to a 1 mM concentration of the different organic compounds, a concentration that neither inhibits cell growth nor affects cell viability. After 15 min, cultures samples (15 ml) were harvested by centrifugation at 4 °C in tubes precooled in liquid nitrogen, and the cell pellets were immediately immersedin liquid nitrogen and stored at −80 °C. The expression of a number of genes, transcriptional fusions of the Pu and Pm promoter to reporter genes, and determination of the expression level of the leader mRNA in primer extension analyses (for review, see Ref. 4).

**Genomic DNA Microarrays of P. putida KT2440**—The genome-wide DNA chip used in this work has been described in detail (23). It consists of an array of 5539 oligonucleotides (50-mer) spotted in duplicate onto γ-aminosilane-treated slides and covalently linked with UV light and heat. The oligonucleotides represent the 5550 open reading frames annotated in the *P. putida KT2440* genome (1) along with the 140 open reading frames defined for the TOL plasmid pWW0 (24) and a suite of commonly used reporter genes and antibiotic resistance markers. The
chips are also endowed with homogeneity controls consisting of oligonucleotides as well as duplicate negative controls at 203 predefined positions (www.progenika.com).

RNA Isolation—RNA was extracted from cells with the hot acid-phenol isolation procedure (25). To this end the cell pellet was resuspended in 1.6 ml of LETS buffer prewarmed to 75 °C (100 mM LiCl, 10 mM EDTA, 1% (v/v) SDS, 10 mM Tris-HCl pH 8.0) and transferred to a prewarmed 15-ml tube containing 1 ml of glass beads (106-mm acid washed, Sigma) and 1.2 ml of acid phenol. The mixture was thoroughly vortexed for 3 min to complete cell lysis. Then 1.2 ml of chloroform was added, and tubes were vortexed for 30 s. The solution was centrifuged at 12,000 g for 15 min (4°C). The aqueous phase (0.7 ml) was added, and tubes were vortexed for 30 s. Then the mixture was vortexed for 15 s and left to stand for 5 min at room temperature. Then 0.24 ml of chloroform was added, and the mixture was shaken and left to stand for 3 min at room temperature. Finally, samples were centrifuged at 12,000 × g for 15 min (4°C). The aqueous phase (0.7 ml) was recovered and precipitated with 0.7 ml of isopropanol. The resulted pellet was washed with 1 ml of 75% (v/v) ethanol (4 °C), dried, and resuspended in 75 µl of diethyl pyrocarbonate/double distilled H2O. Then 1.2 ml of TRIzol (Sigma) was added to each sample, and the mixture was vortexed for 15 s and left to stand for 5 min at room temperature. The sample was thoroughly mixed for 3 min and centrifuged at 3200 × g at 4 °C for 10 min. Samples (1.6 ml) of the aqueous phase were recovered and transferred to a 15-ml tube containing 1.6 ml of acid phenol at 75 °C. The sample was thoroughly mixed for 3 min and centrifuged at 3200 × g for 10 min. Samples of the aqueous phase (2 × 0.7 ml) were recovered, precipitated with 0.7 ml of isopropanol at room temperature, and centrifuged at 12,000 × g for 25 min at 4 °C. The resulting pellet was washed with 1 ml of 75% (v/v) ethanol (4 °C), dried, and resuspended in 75 µl of diethyl pyrocarbonate/double distilled H2O. Then 1.2 ml of TRIzol (Sigma) was added to each sample, and the mixture was vortexed for 15 s and left to stand for 5 min at room temperature. When 0.24 ml of chloroform was added, and the mixture was shaken and left to stand for 3 min at room temperature. Finally, samples were centrifuged at 12,000 × g for 15 min (4°C). The aqueous phase (0.7 ml) was recovered and precipitated with 0.7 ml of isopropanol. The pellet was washed with 1 ml of 75% (v/v) ethanol (4 °C), dried, and resuspended in 15–25 µl of diethyl pyrocarbonate/double distilled H2O. Samples were heated to 55–60°C for 5 min and stored at −80 °C. The integrity and purity of the RNA were checked with agarose gel electrophoresis to rule out any traces of DNA. The concentration of RNA was measured spectrophotometrically at 260 nm.

Labeling and Hybridization Conditions—cDNA was generated from RNA samples by direct incorporation of Cy3- or Cy5-labeled dUTP into cDNA. Differentially labeled samples from two different conditions were mixed and hybridized to the P. putida microarrays. For each experiment three independent biological replicates were done. For each reaction, RNA (30 µg) was incubated with 0.5 µg of random hexamers (Amersham Biosciences) at 70 °C for 10 min and then chilled on ice for 2 min. A mix containing 1× reverse transcription buffer, 5 mM MgCl2, 10 mM dithiothreitol, deoxyxynucleotide triphosphates (0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, and 0.2 mM dTTP), 40 units of RNase OUT (Invitrogen), and either Cy3-dUTP or Cy5-dUTP (Amersham Biosciences) was added to the RNA primer mixture and incubated at 25 °C for 5 min. Superscript II reverse transcriptase (200 units, Invitrogen) was added, and the mixture was incubated at 25 °C for 10 min and then at 42 °C for 120 min. The reaction was stopped by heating the reaction mixture at 70 °C for 15 min. The RNA was then digested with RNase H (2 units, Invitrogen) at 37 °C for 20 min. Unincorporated nucleotides were removed through QiaQuick purification spin columns (Qiagen). The purified probes were mixed, dried, and reconstituted in 30 µl of hybridization buffer (3X SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 5× Denhardt’s solution, 0.2% (w/v) SDS, 5% dextran sulfate, 50% (v/v) formamide). Before hybridization, the probe was heated at 95 °C for 2 min, applied directly onto the microarray slide, and covered with a glass coverslip (24 × 60 mm). The array was hybridized overnight at 50 °C in a humidified hybridization chamber. After hybridization, the slides were washed for 5 min in 2× SSC and 0.1% (w/v) SDS (final volume 500 ml) followed by two 5-min washes in 50 ml of 1X SSC and 0.1× SSC. Before scanning, the slides were spun at 1600 × g for 5 min to remove residual salts. Finally, slides were scanned on a GMS 418 apparatus (Genetic Microsystems, Inc.). Separate images were acquired for Cy3 and Cy5 and then processed and analyzed with GenePix 4.0 software (Axon Instruments, Inc.).

Data Analysis—After background subtraction, signal intensities for each replica were normalized and statistically analyzed using the Lowess intensity-dependent normalization method (26) included in the Alma-zen System software (Alma Bioinformatics S.L.). p values were calculated with Student’s t test algorithm based on the differences between log 2 ratio values for each biological replicate. Genes were considered differentially expressed when they fulfilled the filter parameters of expression ratio ≥1.8 and p value ≤0.2. Cluster and Treeview software (27) were used to group and visualize genes whose expression varied in the same direction in response to all three aromatic compounds. Then a hierarchical clustering algorithm based on the average linkage method was used to identify genes that were expressed differentially under all three experimental conditions.

Western Blots—P. putida KT2440 (pWW0) was grown as described under “Bacterial Strains, Culture, and Growth Conditions”; after 15 min of challenge with aromatic compounds the cells (100 ml) were harvested by centrifugation, and the pellets were resuspended, washed once in 1× M9 buffer, and resuspended again in 2 ml of lysis buffer containing Tris 50 mM, pH 7.5, 50 mM NaCl, 2 mM EDTA, 4 mM β-mercaptoethanol, and 1× Complete™ protease inhibitor mixture (Roche Applied Science). Cells were lysed by sonication, and the insoluble fraction was discarded by centrifugation at 18,000 × g for 20 min. Aliquot fractions of the supernatant containing 70 µg of total protein were analyzed on SDS-PAGE (12.5% (w/v)) and transferred to a nitrocellulose membrane. The membranes were blocked for 3 h at room temperature with 5% nonfat dry milk in phosphate-buffered saline. Membranes were incubated overnight at 4°C with monoclonal antibodies against Escherichia coli σ7 subunit (Neocline), diluted 1:1000, washed with phosphate-buffered saline solution, and incubated with goat anti-mouse immunoglobulin G+L-conjugated with horseradish peroxidase (1:1000 dilution) for 1 h (Caltag Laboratories). The blots were developed with the SuperSignal® West Dura Extended Duration Substrate (Pierce). Chemiluminescent blots were exposed to autoradiographic film for 30 s to 2 min.

Swimming Assay—The medium used for assays was M9 citrate that contained 0.3% (w/v) agar and 0.05% (w/v) tetrazolium red. Swim plates were inoculated with bacteria from an overnight culture in M9 citrate agar (1.2% (w/v)) plates with a sterile toothpick. The plates were kept for 14–16 h at 30 °C in humidified containers to prevent dehydration. Plates were then photographed.

Hydrogen Peroxide Production—P. putida KT2440 (pWW0) was grown on M9 with citrate as a carbon source to a turbidity of 0.7 at 660 nm. The cultures were then exposed to 5 mM concentrations of different organic compounds. After 5 and 30 min of exposure, 10 ml of the culture was harvested by centrifugation, and the cells were broken as described above. We used 50 µl of the cell-free extract to determine the hydrogen peroxide concentration as described in Buege and Aust (28).

β-Galactosidase Assays—P. putida KT2440 (pMQ220EV) cells bearing or not bearing plasmid pWW0 were grown overnight on M9 citrate and diluted 1/100 in fresh medium, and cultures were divided in four aliquot fractions (10 ml each). After 1 h at 30 °C, one of the fractions was supplemented with 1 mM 3MB, another one with tolune, and a third one with o-xylene. The remaining fraction was kept without the addition of aromatics. Samples for β-galactosidase activity assays were taken 3 h after induction. β-Galactosidase activity was determined in permeabilized whole cells according to Miller (29).

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Supplemental Material—Complete microarray datasets are available as Microsoft Excel files. Supplemental Tables 1–11 show the most relevant changes.

RESULTS

P. putida KT2440 Genome-wide Microarrays Reveal a Massive Response to Aromatic Compounds—The DNA microarrays described previously (23) were used to study the global expression profile of P. putida (pWW0) in response to metabolizable (toluene and 3MB) and nonmetabolizable (o-xylene) aromatic compounds. P. putida (pWW0) grows in M9 minimal medium with citrate as the sole carbon source with a duplication time of about 70 ± 4 min. When cultures reached the mid-exponential growth phase (turbidity at 660 nm 0.7 ± 0.05) they were split into four aliquots. One was kept as a control, and 1 mM toluene, o-xylene, or 3MB was added to the other three. This concentration of aromatic compounds was sublethal, and 100% of the cells survived the addition of these chemicals. To analyze the early events in the response to these compounds, samples were collected 15 min after the addition of the tested aromatics, and total RNA was prepared from all cultures.

Our statistical analysis of the microarray data indicates low inter-experiment variance between replicates. On the basis of our data, we selected changes of 1.8-fold or more in mRNA levels between aromatic compound-treated and untreated preparations as significant. A total of 180, 185, and 64 genes were significantly up-regulated in response to toluene, o-xylene, and 3MB respectively, with o-xylene and toluene sharing 69 up-regulated genes (Fig. 2). On the other hand, 127, 217, and 69 genes were significantly down-regulated in toluene-, o-xylene-, and 3MB-treated cultures. All three treatments produced a similar down-regulation in 18 genes (Fig. 2), all of them related to membrane functions or energy transduction. Most up-regulated genes shared by all three aromatic treatments were related to the heat-shock response and general metabolism.

These genes were grouped as proposed previously according to the metabolic function of the corresponding products (30), e.g. metabolism of aromatic compounds, gene regulation, stress response, energy metabolism, transport of metabolites, chemotaxis, etc. (Fig. 3; see also below and supplemental Tables 1–11). Both up-regulated and down-regulated genes were found in most physiological groups with the exception of those groups grouped under “energy metabolism,” “pili-flagella,” and “protein metabolism,” where the presence of any of the aromatics caused repression in all cases. For most groups, o-xylene had the most pronounced effect. These general effects were concomitant with HS response, general stress endurance, and peripheral pathways for aromatic compound metabolism, among others (Fig. 3). These results and their significance are analyzed in detail below.

Specific Metabolic Response to Aromatics; Induction of Distinct Segments of the Aromatic Degradation Pathways—Of all the known effects caused by toluene, o-xylene, and 3MB on P. putida KT2440 (pWW0),
The best documented is induction of the promoters of TOL operons through two regulatory proteins specifically responding to the presence of aromatics (Fig. 1). We, therefore, used the output of xyl genes as a positive control to assess the performance of the array and then as an internal validation control. Fig. 4 highlights the changes in the expression of catabolic genes involved in the reaction of P. putida KT2440 (pWW0) to the three aromatic inducers we compared (see also supplemental Table 11). As expected, the whole of the TOL upper pathway was induced in response to both toluene and o-xylene. Although not identical, the results with the metabolizable substrate toluene were similar to those with o-xylene, which cannot serve as a substrate, although it is able to induce expression of the xyl genes by activating the XylR regulatory protein (21). Expression of the genes for the meta-cleavage pathway was also detected in response to both aromatic hydrocarbons and to 3MB (Fig. 4B). The differences in activation of the meta-cleavage pathway in response to toluene and o-xylene should be considered in terms of metabolism; whereas toluene yields benzoate which activates the XylS meta-cleavage regulatory, o-xylene cannot be metabolized, and internal production of the cognate XylS effector is prevented (Fig. 1).

The low levels of meta-cleavage pathway induction with this aromatic are most probably due to the so-called cascade activation through XylS overproduction (4) (Fig. 1).

Interestingly, the global analysis allowed us to detect differences in the induction levels of the different regions of the upper and meta operons (Fig. 4, A and B), suggesting additional regulatory steps in TOL gene expression regulation, e.g. changes in mRNA stability of different segments of the operon, which is transcribed as a single polycistronic mRNA (31, 32). Yet the results summarized in Fig. 4, A and B, not only confirmed data reported in the literature in the past years but also new features of this pathway.

The benABCDKEZF operon, present in the P. putida chromosome, specifies a conserved ortho-cleavage route for benzoate metabolism, which coexists with TOL plasmid pathways. Our data showed that 3MB, whose metabolism via the chromosomal ortho-cleavage pathway is nonproductive and leads to the accumulation of dead-end products (33), significantly induced this pathway (Fig. 4C). Toluene, which gives rise to unsubstituted benzoate through the action of the upper TOL pathway enzymes, induced the chromosomal ben route to lower levels than the induction caused by o-xylene, which is unable to act as a substrate for the TOL upper pathway enzymes. Our interpretation of this result is that XylS, which is overproduced in the presence of o-xylene, is responsible for this induction. The pWW0-encoded XylS regulator is highly homologous to the chromosomally encoded BenR protein, to the point that both regulators show a degree of cross-regulation (34, 35). To further test this hypothesis, we constructed a transcriptional P_{benA::lacZ} fusion and determined β-galactosidase levels in benR-proficient and benR-deficient backgrounds in P. putida with or without the TOL plasmid, which provides the xylS gene. We found that 3MB was recognized by BenR but induced higher levels of β-galactosidase expression from P_{benA::lacZ} when xylS was provided on the TOL plasmid (Table 1). In the presence of either toluene or o-xylene, we detected β-galactosidase expression only in the XylS-proficient backgrounds irrespective of the presence of BenR, a finding that supports the cross-regulation hypothesis.

Because both Pm and P_{benA::lacZ} are induced by 3MB and benzoate, cross-activation is translated into the simultaneous induction of competing pathways. The -fold induction of the TOL xylXYZ genes (encoding toluate dioxygenase) by 3MB is similar to that of the chromosomal benABC operons (Fig. 4, B and C). Enzyme affinities and activities not withstanding, the fact is that 3MB can enter either of these two routes, although biodegradation only becomes productive when the plasmid-encoded enzymes are involved.

All three regulators involved in TOL and ben pathway expression are constitutively present in the cell at low levels (4, 21, 32, 34). Thus, direct specific sensing of the aromatics may occur as a result of their physical interaction with these regulators to trigger the synthesis of a whole set of new enzymes. Although this would represent a considerable waste of
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energy (36), it would also allow an efficient use of the cell resources. Nevertheless, as for other known systems, genes induced for the utilization of new carbon sources constitute only a small fraction (5%) of the entire transcriptome reprogramming.

The Cell Envelope Is an Early Sensor of Aromatic Toxicity—The first defense against environmental stresses is the bacterial cell envelope, which tackles and senses toxic aromatics before they contact specific pathway regulators. Accordingly, in terms of the number of genes that change their expression pattern, the strongest response of the cell to aromatics is observed in functions related to the cell barrier; the cell envelope, lipid metabolism, transport, and pili-flagella synthesis accumulate a total of 124 genes that change their expression level (30 up-regulated and 94 down-regulated). Furthermore, 16 of 18 genes that were down-regulated in the presence of each of the three aromatics belong to this group (Fig. 2). We and others have shown before that P. putida cells exposed to a sudden shock of toxic chemicals maintain a balance between membrane fluidity and permeability by changing the ratio of cis to trans unsaturated fatty acids (37). Our genome-wide analysis revealed that genes for enzymes involved in fatty acid and phospholipid metabolism, particularly FadA, FadB, and β-ketothiolase, were repressed in the presence of toluene and o-xylene (see supplemental Table 1). In addition, we found that CfaB (PP5365), the major cyclopane fatty acid synthetase, behaved differently in response to different inducers. CfaB was recently identified as the major cyclic fatty acid synthase (38). With o-xylene, cfaB expression was reduced 4-fold, whereas with toluene and benzoate no significant change was found. This suggests that o-xylene is detected by the cells as the most toxic compound among those tested in this study. In accordance with this hypothesis we found that certain genes related to fatty acid composition (phaG, PP4030, and PP4975) were repressed in response to o-xylene but not to toluene or 3MB.

A number of membrane-related cell activities such as the synthesis of porins, peptidoglycans, and flagella (motor and hook proteins) were repressed as a consequence of the stress imposed by each of the three aromatic compounds tested (supplemental Table 1). In fact, unlike the situation in other functional groups, all motility-chemotaxis genes and pili-flagella-related genes influenced by the presence of the aromatics were repressed, and in no case was a change toward activation detected within this group. To confirm the actual consequence of this apparent absolute switch-off of motility on pili and flagella functionality, swimming motility assays were carried out in the presence and absence of each of the three aromatics. Fig. 5 clearly shows a diminished swimming halo when cells were incubated in the presence of the tested compounds. The effects were more pronounced with toluene, although o-xylene and 3MB also produced a quantifiable response.

The assembly and functioning of molecular machines for motility consume large amounts of ATP but do not provide traits essential for survival under these conditions, so repression could be a way to save energy for more useful stress endurance programs. By way of comparison, it has been estimated that the flagellum alone can represent as much as 8% of the total cell protein when flagellar operons are expressed optimally. Flagellar genes and chemotaxis are repressed in other organisms in response to a number of stresses, including osmotic or saline shock, alcohol, pH, and temperature upshift (39–43). The repression of a variety of genes involved in chemotaxis, pili biosynthesis, the flagellar basal body and flagellar assembly proteins (supplemental Table 2), thus, has precedents in other systems. The signals that trigger this response could involve the dinucleotide dGMPc, which has been shown to regulate cell transition to a sessile state after a number of stresses (44). The conserved GGDEF motif proteins are key elements in the synthesis of this messenger molecule in response to environmental signals (45). Interestingly, up to six GGDEF proteins (30% of all GGDEF-domain proteins annotated in P. putida) were induced more than 2-fold in response to the presence of all three aromatics (supplemental Table 5). We also observed a clear inhibition of the parA gene (the product of which mediates chromosome and plasmid segregation; supplemental Table 8) and of various penicillin-binding proteins (pabh genes), membrane proteins which participate in cell division. That these functions are all inhibited to some degree in cells exposed to the aromatics we tested could reflect a transient slowdown in cell division rate after sudden exposure to the aromatic chemicals, as is generally observed in response to other oxidative stress conditions (46). Further support for the idea of membrane damage is the finding that the expression of several extracytoplasmic σ factors known to sense stress at the membrane and periplasm level clearly changed in the presence of o-xylene (supplemental Table 6A).

We observed the induction of a set of genes corresponding to various RND efflux pump operons involved in the extrusion of toxic chemicals (6) as a defense mechanism against the toxicity of toluene and o-xylene (see also supplemental Tables 4 and 7). The induction of the itgABC operon (PP1384 through to PP1387) had been previously described in P.
Changes in enzymes related to Krebs cycle feeding and functioning

| TIGR accession no. | Gene Description | -Fold change |
|--------------------|-----------------|--------------|
| PP1022             | zwf-1           | -2.13        |
| PP1024             | eda             | -2.2         |
| PP0213             | gabD            | -2.06        |
| PP0386             | aceF            | -2.22        |
| PP0393             | aceE            | -1.84        |
| PP0356             | glcB            | -1.88        |
| PP0654             | mdh             | -2.36        |

The synthesis of tricarboxylic acid cycle enzymes, which is only compensated for when the toxic compound can be used as a carbon source. In fact, several tricarboxylic acid enzymes such as succinic semialdehyde dehydrogenase, malate dehydrogenase, and isocitrate lyase (PP4116) were also repressed (the latter only in response to o-xylene) (Table 3).

To experimentally confirm that oxidative stress was generated by the presence of the aromatic compounds, we followed hydrogen peroxide production after brief exposure to each of these chemicals. Further evidence that exposure to aromatic inducers caused oxidative damage came from the inhibition of iron-acquisition functions. The fpuA gene (PP4217), which codes for the iron-pyoverdine complex receptor, was tuned down in the presence of 3MB, like other TonB-dependent receptors (PP3612). It has been suggested that a decrease in iron acquisition may help compensate for the oxidative stress generated by exposure to the aromatics we tested (48).

Finally, we observed that several components of the OxyR regulon, known to be involved in the response to oxidative damage in P. aeruginosa and other bacteria, were substantially induced under our conditions. This was the case for the two annotated alkylhydroperoxidases (PP2439 and PP2440) and a new one identified as ahpB (PP1084) (Table 5) (49). In this connection it is worth noting that most of the changes we found in DNA metabolism related to genes that are associated with a defense response against oxidative stress (e.g. PP1974, PP5284; see supplemental Table 7) (50).

In summary, it appears that membrane damage is translated into oxidative stress, which is checked by slowing down or inhibiting the most productive step of aerobic metabolism, i.e. the Krebs cycle. In the short term this will inevitably cause a loss of energy, which must be compensated for somehow.

Aromatic-induced Membrane Damage Generates Oxidative Stress—As shown above, a relevant noxious effect of toluene and o-xylene occurs at the membrane level. The disruption of membrane functions and the ensuing impairment in electron transfer may lead to the generation of active oxygen species in the respiratory chains. That oxidative damage did occur is revealed by two distinct sets of genes. First, o-xylene clearly reduced the numbers of transcripts for components of the membrane-bound electron transport chain (PP3139, PP4264, PP4651) (see supplemental Table 3). This general repression was concomitant with the onset transcription for the synthesis of glutathione (gshA, PP2433) and glutathione S-transferase (gst, PP2023), which are generally involved in the metabolic detoxification of xenobiotics (19, 47). On the other hand the effects of all three inducers on the core step in aerobic metabolism (the Krebs cycle) showed that a number of enzymes involved in metabolic feeding (pyruvate dehydrogenase complex proteins PP0338 and PP0339, glyceraldehyde-3-phosphate dehydrogenase PP3443, and pyruvate kinase PP3632) were repressed when cells were exposed to toluene or o-xylene but not when they were exposed to 3MB (Table 3). Thus, it is likely that the sensing of membrane disruption induced by organic solvents is eventually translated into a slowdown in the synthesis of tricarboxylic acid cycle enzymes, which is only compensated for when the toxic compound can be used as a carbon source. In fact, several tricarboxylic acid enzymes such as succinic semialdehyde dehydrogenase, malate dehydrogenase, and isocitrate lyase (PP4116) were also repressed (the latter only in response to o-xylene) (Table 3).
and the fold induction were different with each compound (Fig. 3). In general, the intensity of the HS response (as judged from the number of genes involved and their expression) followed the order o-xylene > toluene > 3MB, which may reflect the relative toxicity of each of these chemicals. In fact, o-xylene and toluene are known to be more toxic than 3MB, as they can dissolve in the membrane, disrupt it, and release lipids and proteins (5). It, thus, makes sense that chaperones involved in the HS response be massively produced to compensate for this damage. As expected, proteins induced by the aromatics we tested belong to the stress response (18). Their induction in this trait (18). Their induction in this trait (18) but also corroborates that these gene products are involved in the response to toluene. A few other chaperones such as the universal stress protein (PP2187), XenB (PP1478), and the HS response protein 20 (PP3234) were repressed regardless of whether the cells were exposed to the aromatic hydrocarbons or to 3MB (Table 5).

**Effect of Aromatic Inducers on Amino Acid and Nucleotide Homeostasis and on Biosynthesis of Proteins and Nucleic Acids**—Any physiological response (whether metabolic or otherwise) to the presence in the medium of o-xylene, toluene, or 3MB involves de novo protein biosynthesis. It was, therefore, not surprising that major changes in the transcriptome were observed in genes involved in amino acid biosynthesis and in critical functions for protein production (Table 6). The most remarkable finding was the strong induction of methionine biosynthesis genes (every cistron in the pathway was induced) (Table 6). We also found that gltB and gltD, which encode glutamate synthase, were induced between 1.83- and 5.9-fold (Table 6). The increased production of glutamate may not only offset the possible impairment by this compound of the biosynthesis of other amino acids but may also enhance the selectivity of RNAP with alternative σ factors for their cognate promoters (54). But many other pathways of amino acid biosynthesis were also induced, in particular those for leucine, isoleucine, tryptophan, serine, and arginine (Table 6 and supplemental Table 5). We also found that the pathways for the catabolism of tryptophan and arginine were turned down, an effect that should have resulted in a further increase in the availability of these two amino acids.

The increase in amino acids in the short-term response to toluene probably reflects the need of these amino acids in the synthesis of the new proteome found in cells exposed to toluene (18). This hypothesis is supported by the induction of the Tuf-1 elongation factor for protein translation and the fold induction of the respective transcriptome were observed in genes involved in amino acid biosynthesis (Table 6).
TABLE 6
Changes in genes encoding enzymes related to amino acid metabolism

| TIGR accession no. | Gene | Description | Toluene Fold change | o-Xylene Fold change | 3MB Fold change |
|--------------------|------|-------------|---------------------|----------------------|-----------------|
| PP0675             | gdhA | Glutamate dehydrogenase | 1.7 | 1.81 |  |
| PP0840             | cySE | Serine O-acetyltransferase | 1.92 |  |  |
| PP1025             | leuA | 2-Isopropylmalate synthase |  |  |  |
| PP1079             | argF | Ornithine carbamoyltransferase | 1.87 | 2.43 |  |
| PP1346             | argJ | Glutamate N-acetyltransferase | 2.57 |  |  |
| PP1995             | trpE | N-(5-Phosphoribosyl)anthranilate isomerase | 1.7 | 2.36 |  |
| PP2375             | metH | 5-methyltetrahydrofuran-2-ol-homocysteine synthase | 3.49 | 1.99 |  |
| PP2698             | metE | 5-methyltetrahydrofurfurylglutamate-homocysteine methyltransferase | 5.23 | 3.25 | 3.77 |
| PP2776             | metS | Homocysteine 5-methyltransferase family protein | 2.25 | 1.87 |  |
| PP3571             | purU | Putative aromaticaminodehydrogenase | 2.10 |  |  |
| PP4680             | ilvB | Acetolactate synthase large subunit | 1.79 |  |  |
| PP4977             | metF | 5,10-Methenyltetrahydrofuran-2-one reductase | 1.81 |  |  |
| PP5075             | gltD | Glutamate synthase small subunit | 3.10 | 5.90 |  |
| PP5076             | gltB | Glutamate synthase large subunit | 1.83 | 2.37 |  |
| PP5078             | araB | 3-Dehydroquinate synthase | 2.32 | 1.91 | 1.53 |
| PP5079             | araK | Shikimate kinase | 2.32 | 2.02 |  |
| PP5097             | metX | Homoserine O-acetyltransferase | 1.84 |  |  |
| PP5098             | metW | Methionine biosynthesis protein | 2.40 |  |  |
| PP5149             | ilvA | 2-Threonine dehydrogenase | 1.82 | −1.59 | 1.55 |
| PP5185             | argA | N-Acetylglutamate synthase | 2.93 |  |  |
| PP4725             | dapB | Dihydricolipinic reductase | 3.58 | 5.64 | 1.35 |
| PP0420             | trpG | Anthranilate synthase component II | −1.84 | −2.00 |  |
| PP0421             | trpD | Anthranilate phosphoribosyltransferase | −1.84 | −2.53 |  |
| PP1001             | arcA | -arginine deiminase | −1.9 | −6.73 |  |

synthesis and an inorganic pyrophosphatase (PP0538), which is a major provider of energy for the cognate polypeptide-making reactions.

Exposure to o-xylene, toluene, or 3MB increased the level of expression of zwf-1, a pivotal enzyme in the synthesis of 6-phosphogluconate necessary to replenish some of the pentose phosphate intermediates needed for de novo nucleotide biosynthesis (see supplemental Table 5). This enzyme, which plays a key role in maintaining cellular reducing power, belongs to the SoxR regulon (55); therefore, the fact that it was induced in response to the aromatic compounds we tested is further evidence that oxidative stress is one of the driving forces in transcriptional reprogramming. Induction of the zwf-1 gene is also consistent with the fact that genes that encode other functions involved in nucleotide synthesis (purU, ushA-2, gmk-1, and others) were also up-regulated in response to the aromatic compounds we tested (supplemental Table 5).

DISCUSSION

Bacteria living in hydrocarbon-polluted soil are exposed to multiple environmental inputs that set in motion extensive gene expression programs. Three types of response are possible; they are metabolic programs consisting of the expression of gene sets required for the catabolism or anabolism of nutrients and intermediates. Stress-response programs for adaptation to suboptimal growth conditions, and morphological programs related to shape, transport, and surface chemistry of the bacterial cell. These three major programs are intimately connected, and their functioning determines the survival of a given population in specific niches or its displacement by a fitter organism. P. putida KT2440 (pWW0) provides an exceptional experimental system to examine how environmental bacteria manage the interplay between different gene expression programs, as some of this organism’s growth substrates (in particular toluene and m-xylene) also happen to be acute physiological stressors. We used DNA array technology to examine the response of P. putida KT2440 (pWW0) to toluene, a chemical that is a carbon and energy source for this strain but that also damages membranes and denatures proteins (for review, see Refs. 4 and 6). When cells face toluene in the medium, both new metabolic programs and stress endurance programs are activated. How such programs interact and influence each other is the subject of the work reported here. For comparison, we examined the response to o-xylene, a chemically close relative to toluene that is not a growth substrate for P. putida KT2440, and to 3MB, a substrate of the lower TOL pathway (Fig. 1) that lacks the considerable hydrophobicity and toxicity of the aromatic hydrocarbons.

Induction of the whole complement of the xyl genes borne in plasmid pWW0 was a useful criterion to establish the reliability of the DNA array technology in light of the availability of data on the regulation of catabolic operons (for review, see Ref. 4). Activation of the expression from the Pu promoter is a process assisted by the plasmid-encoded XylR protein activated by toluene or o-xylene, whereas the activation of the Pm promoter by toluene, o-xylene, and 3MB is a process mediated by the XylS regulator (see Fig. 1). With some minor refinements, our set of data on the xyl genes of the TOL plasmid is generally consistent with the gross expression profiling of the same cistrons reported earlier (21, 31, 56, 57). One remarkable aspect of these experiments is that the non-carbon source o-xylene was able to induce both the upper and the lower pathways of the TOL plasmid, albeit to a lesser extent than the true pathway substrates, toluene and 3MB.

However, an interesting finding in this respect was the very significant activation of the ben cluster for the metabolism of non-substituted benzoate through a competing chromosomal, ortho-cleavage pathway. Because benzoate is produced from toluene in a series of reactions catalyzed by the TOL upper pathway, it comes as no surprise that the ben genes are induced by toluene despite the competition between the meta and the ortho downstream routes. The intriguing thing about our findings is that non-substrates such as o-xylene and 3MB also induced the ben gene cluster. Although this can be explained by invoking the proven cross-activation of the benA promoter by XylS (35), the result of this gratuitous induction is misrouting of 3-methylcatechol into an ortho-cleavage route that yield dead-end products.

In terms of the number of regulated genes, the changes in expression observed in the genes of “peripheral” metabolic routes for toluene and 3MB represented only a minor fraction of the total number of genes, whose expression significantly varied upon exposure to each of the aromatic compounds we tested. The significant changes detected with the DNA array used here are summarized in Fig. 3, in which genes are arbitrarily grouped according to 17 general functions (see also supplemental Tables 1–11). Fig. 3 illustrates functions for almost 20% of all
Transcriptional Interplay Responses

genes in the P. putida KT2440 strain, and no significant changes in any of the remaining genes were found. The data in Fig. 3 suggest that the number of genes that were induced upon exposure to each of the three aromatics is roughly the same as the number of genes that were inhibited. Yet this general trend was not identical for the three chemical species. o-Xylene, which cannot be metabolized, acted mainly as a chemical stressor, whereas in the response to toluene and 3MB, more metabolic genes were induced than repressed. The same general pattern was observed when, instead of counting the number of genes, we scored the -fold induction of individual genes (not shown).

Our results also support that the gross effect of toluene on cells is much more similar to that of o-xylene (which acts as a strong stressor) than to 3MB (predominantly a substrate). In fact, the order of magnitude of both the number and the intensity of the changes was o-xylene > toluene ∼ 3MB. The Venn diagram shown in Fig. 2 illustrates that toluene- and o-xylene-dependent reprogramming share more than 55% of the up-regulated genes, but only 35% of the genes down-regulated by o-xylene were also repressed in the presence of toluene (Fig. 2). Genes down-regulated by all three compounds represent functions involved in primary sensing at the membrane level, whereas about half of the up-regulated genes under all three conditions are part of the HS regulon and metabolic functions. Cluster analysis of the response to each of the three aromatics clearly grouped o-xylene with toluene and distinguished the former from 3MB.

As deduced from the magnitude of the responses, it seems that the primary reaction of the cells to the presence of aromatic compounds takes place at the level of the cell envelope (membrane proteins, lipid metabolism, transport, etc.). Injury to the membranes leads to oxidative damage that is observed as a reduction in electron transport chain activity and increases in hydrogen peroxide production, which in turn produces a general response to oxidative stress. Finally, protein misfolding generated by the presence of aromatics triggers the classical HS response leading to induction of the HS regulon. Our results show that the induction of metabolic and stress-related functions by all three compounds was accompanied by the inhibition of motility and repression of enzymes involved in metabolic feeding or part of the Krebs cycle (see supplemental Tables 3 and 5). Although the specific regulatory mechanisms that switch off cell motility are not understood, inhibition of motility is a common response to almost any stress situation and may, thus, represent a general mechanism for saving energy. A group of recently characterized proteins containing the so-called GGDEF motif, involved in biosynthesis of the messenger molecule dGTPc, seems likely to be involved in signal transmission. In fact, six GGDEF motif proteins were induced under our experimental conditions.

Changes in ~20% of P. putida KT2440 genes occurred in response to the chemicals we tested within a short period of time (15 min), with no variation in the expression of RNAP components and only minor changes in the expression of transcriptional regulators. A plausible explanation for how the activation of genes occurred in the absence of changes in the transcriptional machinery is that stress caused by aromatic compounds led to a rapid reassignment of available transcriptional elements from dispensable functions and promoters to functions required for stress endurance. Depending on the relative affinities of RNAP for given promoters, one could argue that a fraction of the enzyme pool is permanently engaged in the transcription of basic housekeeping functions. But there may be a pool of roaming RNAP available to conditionally activate other sets of genes. The assignment of this pool to alternative promoters may depend on the presence of alternative σ factors, specific transcriptional factors, and specific promoter strength and on an appropriate intracellular environment. This hypothesis is supported by our finding that σ^{32} concentration increased in the presence of all three compounds (Fig. 6), reflecting the known mechanism of σ^{32} stabilization (51). We entertain the notion that exposure to o-xylene and toluene (and to a lesser extent, to 3MB) rapidly activates the HS σ^{32} protein (Fig. 6 and Table 5) (52, 53), which may then take over much of the roaming RNAP and redirect the response toward the defense against damage to the cell architecture (54, 58). In this scenario of limited RNAP availability, other metabolic and stress endurance functions can be expressed if other promoters are liberated. In other words, the number of genes that are not expressed in the presence of the aromatic compounds may not be specifically repressed but, rather, deprived of an otherwise engaged transcriptional apparatus which is reassigned to express functions that now become compulsory for survival.

How could this occur? It is possible that changes in general conditions such as DNA supercoiling or an increase in intracellular solutes (such as glutamate) trigger a shift in the binding of the available population of RNAP molecules among DNA sequences for which they have intrinsically low affinity. This possibility will be the subject of further studies but is supported by our finding that the levels of glutamate synthase increased in response to exposure to the toxic chemicals we tested.

Although these hypotheses may provide a retrospective explanation of the general response of P. putida to aromatic compounds, the issue is still whether these effects are simply a consequence of transcriptional economy at the single-cell level or whether the response has been selected via evolution as a survival strategy and might be common to other groups of microorganisms or even higher organisms. When E. coli cells are faced with a decrease in the growth potential of the environment, they seem to use a risk-prone foraging behavior (40). This strategy consists of increased motility and the massive induction of pathways for the metabolism of unavailable carbon sources (40). In contrast, we observed that stress caused by the aromatic hydrocarbons tested here led to the loss of motility functions and the inhibition of large portions of the basic metabolic machinery. This strategy of minimal energy expenditure, rather than expending energy in the pursuit of a better environment, may reflect a short-term response that forms part of the panoply of mechanisms by which bacteria adapt to hostile environments (59).

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