



Global Transcriptional Responses to Osmotic, Oxidative, and Imipenem Stress Conditions in *Pseudomonas putida*

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ABSTRACT Bacteria cope with and adapt to stress by modulating gene expression in response to specific environmental cues. In this study, the transcriptional response of *Pseudomonas putida* KT2440 to osmotic, oxidative, and imipenem stress conditions at two time points was investigated via identification of differentially expressed mRNAs and small RNAs (sRNAs). A total of 440 sRNA transcripts were detected, of which 10% correspond to previously annotated sRNAs, 40% to novel intergenic transcripts, and 50% to novel transcripts antisense to annotated genes. Each stress elicits a unique response as far as the extent and dynamics of the transcriptional changes. Nearly 200 protein-encoding genes exhibited significant changes in all stress types, implicating their participation in a general stress response. Almost half of the sRNA transcripts were differentially expressed under at least one condition, suggesting possible functional roles in the cellular response to stress conditions. The data show a larger fraction of differentially expressed sRNAs than of mRNAs with >5-fold expression changes. The work provides detailed insights into the mechanisms through which *P. putida* responds to different stress conditions and increases understanding of bacterial adaptation in natural and industrial settings.

IMPORTANCE This study maps the complete transcriptional response of *P. putida* KT2440 to osmotic, oxidative, and imipenem stress conditions at short and long exposure times. Over 400 sRNA transcripts, consisting of both intergenic and antisense transcripts, were detected, increasing the number of identified sRNA transcripts in the strain by a factor of 10. Unique responses to each type of stress are documented, including both the extent and dynamics of the gene expression changes. The work adds rich detail to previous knowledge of stress response mechanisms due to the depth of the RNA sequencing data. Almost half of the sRNAs exhibit significant expression changes under at least one condition, suggesting their involvement in adaptation to stress conditions and identifying interesting candidates for further functional characterization.

KEYWORDS KT2440, *Pseudomonas putida*, RNA-seq, antisense transcripts, differential expression, sRNA, transcriptomics

Bacteria commonly encounter stressful conditions during growth in their natural environments and in industrial biotechnology applications such as the biobased production of chemicals. As the coordinated regulation of gene expression is necessary to adapt to changing environments, bacteria have evolved numerous mechanisms to control gene expression in response to specific environmental signals. These include the activation of regulators, including alternative sigma factors (1) that direct RNA polymerase to specific promoters, of which the most abundant group comprises the extracytoplasmic function sigma factors (2). In addition, a wealth of two-component regulatory systems couples the sensing of environmental stimuli via a membrane-

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bound histidine kinase with a corresponding response regulator that modulates expression of specific genes (3).

Another class of regulators are the small regulatory RNAs, a heterogeneous group of molecules that are often expressed under specific conditions and in response to stress (4–6). Although some act by binding to protein targets and sequestering their function, the majority bind to mRNAs via base pairing and regulate their expression by modulating translation and/or stability. The base-pairing small RNAs (sRNAs) are divided into two groups according to their genomic location relative to their target(s). The *cis*-encoded or antisense sRNAs (asRNA) are encoded just opposite of, and have perfect complementarity with, their targets (7). The *trans*-encoded sRNAs are encoded in a different genomic location relative to, and typically exhibit limited complementarity with, their targets. Thus, they often have multiple targets and are incorporated into larger regulatory networks (8). In some bacteria, the RNA chaperone Hfq facilitates interactions between *trans*-encoded sRNAs and their targets (9).

Pseudomonas putida has served as a laboratory model organism for environmental bacteria and thrives in a variety of terrestrial and aquatic environments, including strains that colonize the rhizosphere and soil contaminated with chemical waste (10). Although some characteristics, including a versatile metabolism and general robustness toward stresses, are shared with other pseudomonads, *P. putida* lacks virulence factors (11) and has superior tolerance to organic solvents (12). These traits together with the availability of tools for genetic manipulation make it an attractive host for applications in industrial biotechnology and synthetic biology (10, 13, 14).

As bacteria are exposed to general stress conditions in their natural environments as well as in industrial bioprocessing applications, knowledge of stress response mechanisms is a prerequisite for understanding bacterial adaptation and optimizing bioprocesses to improve production yields. In order to obtain this information in *P. putida*, an RNA sequencing (RNA-seq) approach was used to investigate differentially expressed transcripts under osmotic, oxidative, and imipenem stress conditions in the well-characterized strain KT2440. A total of 440 sRNA transcripts were detected, consisting of both intergenic and antisense transcripts, of which over half are conserved within the *Pseudomonadaceae* family. Each type of stress was found to elicit a unique pattern of transcriptional changes with respect to both the extent and dynamics of the response. In all stress types, a general upregulation of genes encoding efflux pumps and other transporters, universal stress proteins, and redox enzymes was observed. The work identified several sRNAs with differential expression under multiple stress conditions that are interesting targets for further functional characterization.

RESULTS AND DISCUSSION

Stress conditions and experimental approach. *P. putida* KT2440 was grown in minimal medium in the presence of sodium chloride, hydrogen peroxide, or the cell wall-targeting antibiotic imipenem to induce osmotic, oxidative, and imipenem stress, respectively. In order to determine meaningful stress conditions, the aim was to apply the maximal stress without affecting cell viability. To this end, a series of growth experiments was carried out with a range of different compound concentrations to determine the pseudo-steady-state condition (15), where there was nearly no change in growth or viability relative to that at compound addition. Growth and survival after compound addition were monitored via the optical density at 600 nm (OD₆₀₀) and CFU counting, respectively (Fig. 1A to C). The final compound concentrations of 3% NaCl, 0.5 mM H₂O₂, and 0.1 μg/ml imipenem were chosen to induce pseudo-steady-state conditions. Cells were grown to mid-exponential phase, followed by compound addition, and harvested after 7 min (T1) of growth to investigate early transcriptional responses and after 60 min (T2) of growth to observe longer-term stress adaptation mechanisms. The control samples (T0) were harvested just prior to compound addition (Fig. 1D) in order to observe the changes within the cells after the introduction of a certain stress. Following RNA isolation and library preparation, the samples were sequenced on an Illumina HiSeq platform. A total of 225 million reads were obtained,

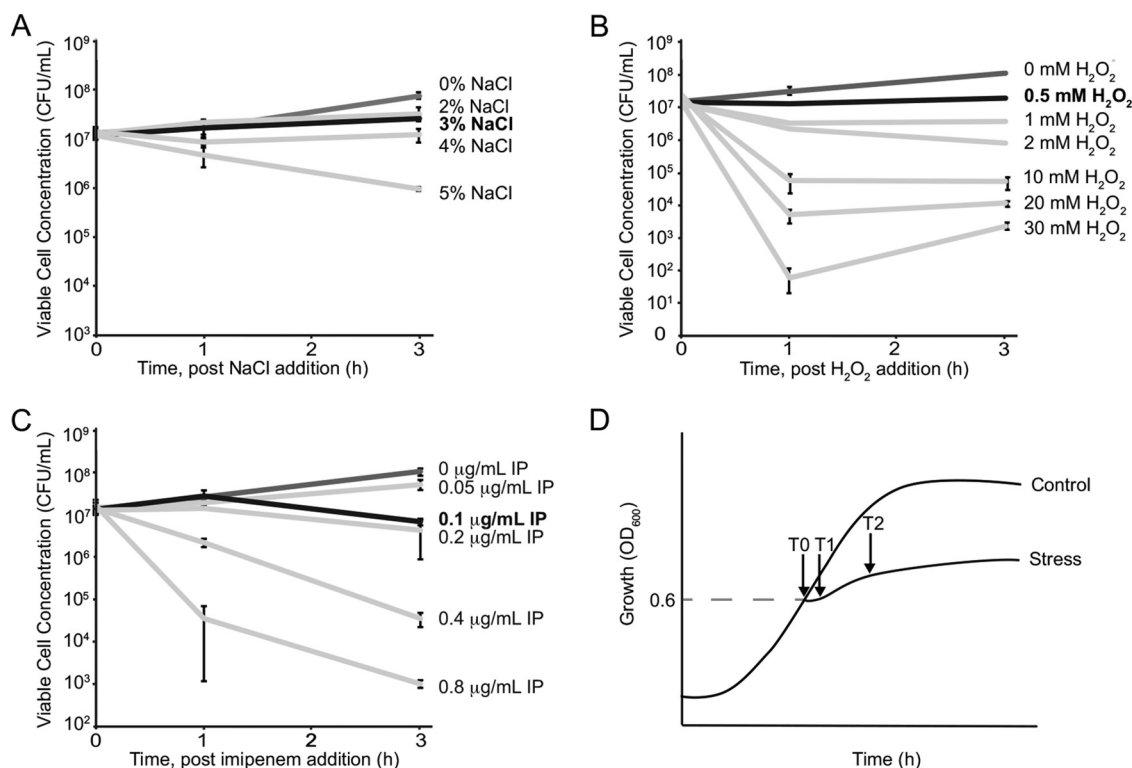


FIG 1 Effect of the addition of stressors on *P. putida* KT2440 survival as determined by viable cell concentration (CFU). Compounds were added to mid-exponential-stage cultures at different concentrations, as indicated to the right of each graph. The CFU count data after compound addition are shown. The chosen concentration of each compound is indicated in bold. (A to C) Effects of the addition of different concentrations of NaCl (A), H₂O₂ (B), and imipenem (C); (D) representative growth curves for the chosen conditions. The stress experiments were performed by the addition of the compounds in mid-exponential growth phase. Cells were harvested just before compound addition for the control (T0) and at 7 min (T1) and 60 min (T2) after compound addition for the stress samples. IP, imipenem.

of which 200 million reads mapped to the *P. putida* KT2440 genome (see Table S1 in the supplemental material).

Identification of sRNA transcripts and complementary sRNA pairs. For sRNA identification, transcripts detected by Rockhopper (16) were manually curated using Integrative Genomics Viewer (17). One group of transcripts located in intergenic regions and having independent expression profiles relative to flanking genes were classified as intergenic sRNA transcripts. A second group of transcripts encoded on the opposite strand relative to, and having either partial or complete overlap with, annotated genes were classified as *cis*-encoded antisense sRNAs. A total of 440 sRNA transcripts were identified in *P. putida* KT2440, significantly increasing the number of sRNA transcripts detected in this strain (18). A total of 45 sRNAs were either annotated or had homology to known RNA motifs (Rfam) (19) (Data Set S1). All homologous sRNAs in different *Pseudomonas* species (20) were detected. Seven copies of transcripts homologous to c4 antisense RNA (21, 22) and three cobalamin riboswitches were detected. Some annotated sRNAs, including 6S/SsrS and t44 RNA, were not detected by Rockhopper despite high expression profiles and were identified manually.

A total of 178 novel intergenic sRNA transcripts were identified (Data Set S2) and designated Pit001 to Pit178 (for *Pseudomonas putida* intergenic transcript) based on their genomic coordinates. The transcripts range in size from 24 to 1,790 nucleotides (nt), with an average length of 174 nt (Fig. 2A). Eight transcripts (Pit023, Pit053, Pit059, Pit062, Pit067, Pit098, Pit109, and Pit110) are putative 3'-untranslated region (3'-UTR)-derived sRNA candidates that overlap the 3' end of the gene or are in very close proximity to the stop codon (23). Five transcripts (Pit014, Pit054, Pit057, Pit102, and Pit108) are putative 5'-UTR-derived sRNA candidates or actuators (24). In a previous

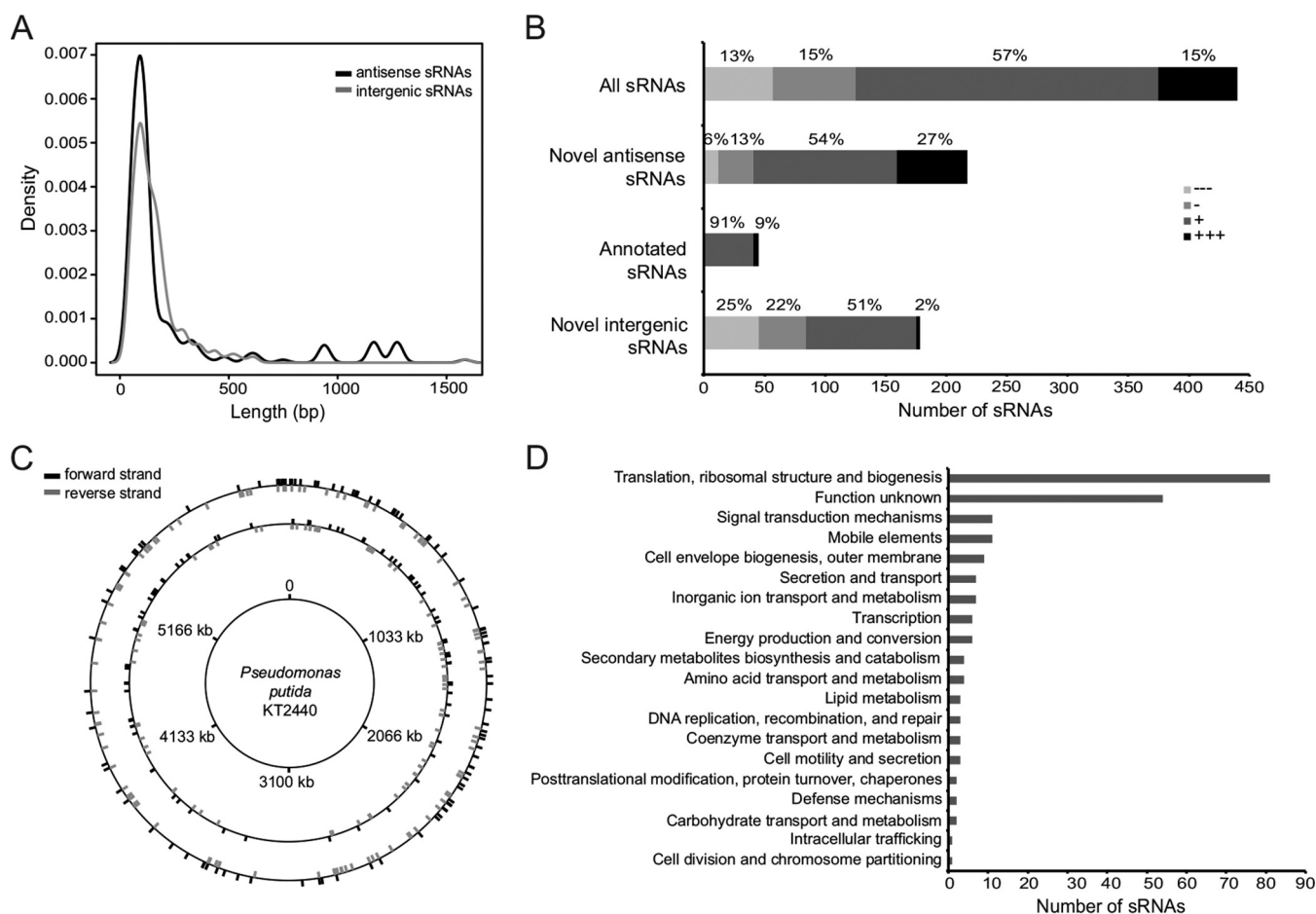


FIG 2 Properties of the sRNA transcripts identified in *P. putida* KT2440. (A) Length distribution of intergenic and antisense sRNA candidates. (B) Conservation of novel sRNA candidates: ---, no sequence conservation found outside the *P. putida* KT2440 strain; -, no sequence conservation found outside the *P. putida* species; +, sequence conservation primarily in *Pseudomonadaceae*; +++, sequence conservation in bacterial species outside the *Pseudomonadaceae* family. (C) Genomic distribution of intergenic sRNAs (outside circle) and antisense sRNAs (inside circle), with the sRNAs encoded on the positive and negative strands indicated on the outside and the inside of the circles, respectively. (D) The numbers of *cis*-encoded sRNA candidates encoded opposite of different functional classes of annotated genes.

study on *P. putida* KT2440, 36 intergenic transcripts were detected, of which 22 correspond to annotated sRNAs with homologs in other *Pseudomonas* species (18). The 45 annotated and 178 novel intergenic transcripts identified here are comparable to the 154 intergenic transcripts reported recently in the *P. putida* DOT-1TE strain (25).

A total of 217 *cis*-encoded RNA transcripts were identified (Data Set S3) and designated Pat001 to Pat217 (for *Pseudomonas putida* antisense transcript) based on their genomic coordinates. These transcripts range in size from 21 to 1,612 nt, with an average length of 223 nt (Fig. 2A), and represent antisense transcripts to 3.3% of the annotated genes in *P. putida* KT2440. In some cases, more than one antisense transcript is detected to the same gene. They overlap the 3' end, 5' end, middle, or entire gene on the opposite strand. The tRNA and rRNA genes had the largest number of antisense transcripts, followed by genes encoding hypothetical proteins (Fig. 2D). Many of the novel Pit and Pat RNA transcripts identified in this study have a rho-independent terminator or a palindrome at the 3' end (Data Sets S2 and S3). In *P. aeruginosa*, 232 and 380 *cis*-encoded RNAs have been detected in different studies (26, 27), and in *Pseudomonas syringae*, 124 genes had antisense transcripts (28). The numbers of genes having antisense transcripts or antisense transcription in other organisms ranges from 2 to 46% (7). In a recent study where transcription start sites (TSSs) were mapped in *P. putida* KT2440, 36% of genes had antisense TSSs, but in this study antisense transcripts were found only to 3.3% of the genes (29). This discrepancy has also been observed

previously in *Escherichia coli* (30) and is likely due to variations in experimental conditions, cDNA library preparation strategies, and data analysis pipelines and in the definition of an asRNA.

A total of 22 pairs of sRNA transcripts with complementarity in at least part of their sequences were identified (Table S2) and could potentially be acting as RNA sponges (31, 32). The P30 transcript (33, 34) that is antisense to CrcZ and two antisense transcripts to CrcY, Pit118 and Pit119, were found. Antisense transcripts were also detected to the transfer-messenger RNA (tmRNA)/SsrA (Pit157, Pit158), RsmZ (Pit063), RsmY (Pit020), 6S/SsrS (Pit164), P24 (Pat203), PrrF2 (Pit144), rnf RNA motif (Pit090), and SRP/4.5S RNA (Pit145). An antisense transcript to PrrF2 has been reported previously in *P. syringae* (28). For 20 of these sRNA pairs, the transcripts are encoded just opposite each other in the same genomic location, while in two cases the sRNA transcripts are encoded in distal genomic locations relative to each other (Pit146-Pit167 and Pit130-Pat180).

Conservation of novel sRNA transcripts. The novel sRNA transcripts found in this study were investigated for sequence conservation and homology in other bacteria by use of the Basic Local Alignment Search Tool (BLAST) (Fig. 2B). For both the intergenic and antisense transcripts, approximately half are shared among bacteria in the *Pseudomonadaceae* family. Most of the other intergenic transcripts are found either in the KT2440 strain or other *P. putida* strains, with only 2% being shared in other bacterial families. For the antisense transcripts, 19% are strain or species specific, while 27% are shared among many bacterial families (Table S3). The latter is not surprising, as a significant number of the antisense transcripts are located opposite essential genes, including rRNA genes that are present in multiple copies (Data Set S3). Of all the 440 sRNAs identified in this study, 13% are strain specific, 15% are species specific, 57% are found among different bacteria in the *Pseudomonadaceae* family, and 15% are found in other families. The chromosomal positions of the novel Pit (outer circle) and Pat (inner circle) transcripts are illustrated in Fig. 2C. Although the transcripts are found throughout the chromosome, there are two regions from around 0 to 1 and 1 to 2 Mb with a higher density of transcripts and a region with a dearth of transcripts around 3 Mb. The regions with higher density include 5 out of 7 copies of the rRNA genes and many genomic islands, where many novel sRNAs have been detected in this study. In order to search for homology among the novel RNA transcripts, the sRNA sequences were compared using BLASTN. Twenty-one groups of homologous sRNAs were identified (Table S4), including the previously known examples PrrF1-PrrF2 and CrcY-CrcZ (35). The majority of homologous intergenic sRNAs are related to transposases, and the homologous *cis*-encoded sRNAs are antisense to rRNA, tRNA, or transposase genes. These groups may be regarded as “sibling sRNAs” that can either be functionally redundant or exert nonredundant regulatory functions (36).

Two annotated sRNAs, P1 and P6, detected in a previous study on *P. putida* KT2440, were not detected here. In the earlier study, 14 possible novel sRNAs were predicted and named according to the intergenic region (IGR) in which they were located (18). Of these, only 5 were detected in the present data set (c4 antisense RNA 4, Pit104, Pit132, Pit140, and Pit148). There are several possible explanations for why all the annotated sRNAs were not detected here, including (i) different cDNA library construction methods lead to different transcripts detected, (ii) some RNAs may be defiant to reverse transcription in the cDNA library construction and are thus underrepresented in the final data set (37), (iii) the detection method (Rockhopper) did not detect some transcripts, and (iv) certain sRNAs are expressed only under specific conditions and are thus easily missed. One example is the characterized NrsZ sRNA in *P. aeruginosa* with sequence homology in the *P. putida* KT2440 genome (38). The NrsZ RNA was not expressed under the conditions used here, consistent with its activation by RpoN under nitrogen-limited conditions simulated by the use of nitrate but not ammonium as a nitrogen source.

General patterns of mRNA and sRNA differential expression under stress conditions. The stress conditions studied here induced extensive transcriptional changes in

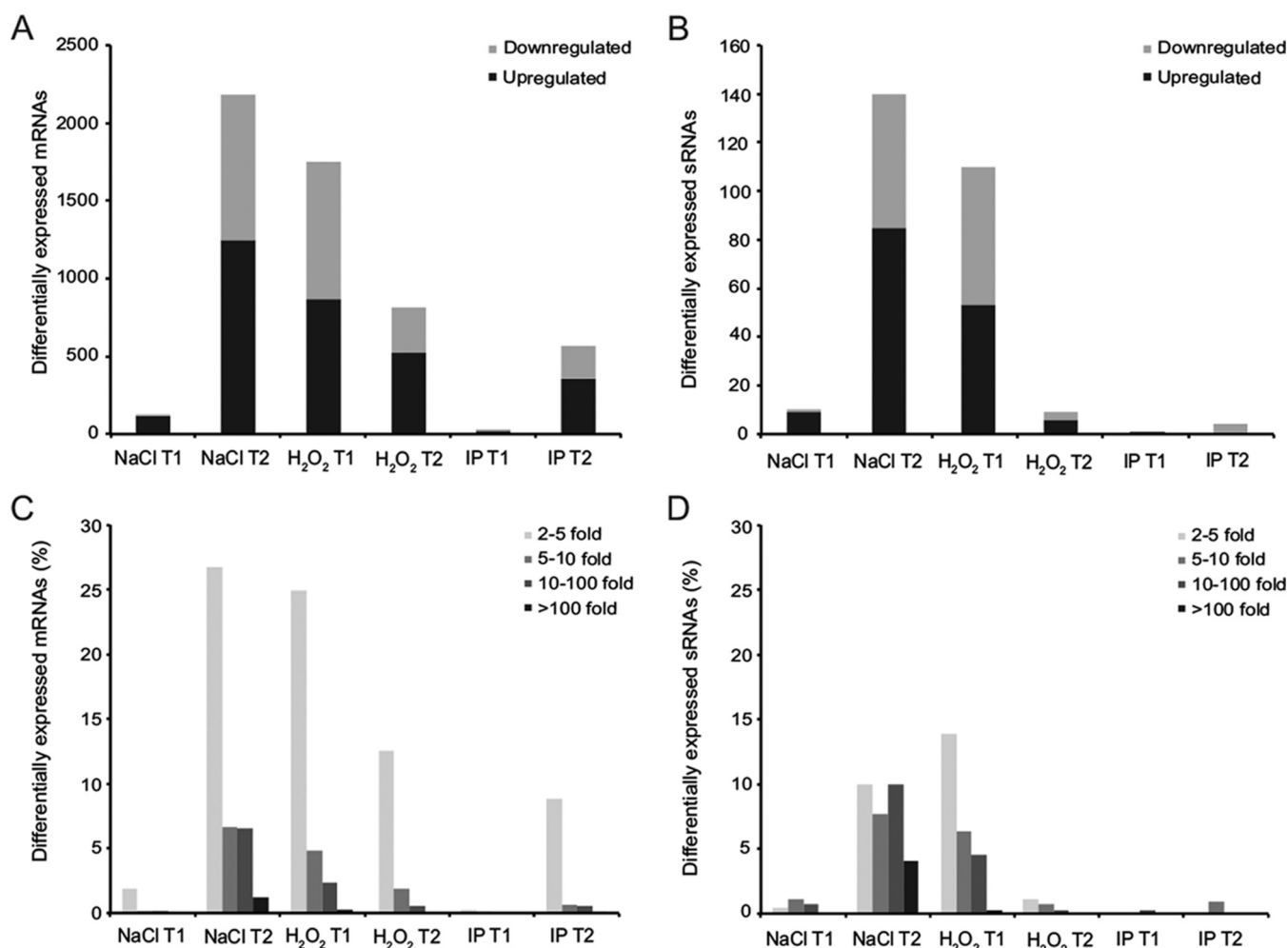


FIG 3 Overview of the differentially expressed mRNAs and sRNAs. (A and B) The numbers of differentially expressed mRNAs (A) and sRNAs (B) under osmotic (NaCl), oxidative (H₂O₂), and imipenem (IP) stress conditions at T1 (7 min) and T2 (60 min) compared to the control (without added stressor) are shown. (C and D) The percentages of transcripts exhibiting different fold changes in expression for mRNA (C) and sRNA (D) relative to the total number of 5,350 CDS and 440 sRNAs, respectively.

P. putida KT2440, with the numbers of differential expressed mRNA and sRNA transcripts summarized in Fig. 3A and B, respectively. Analysis of transcript levels at short and long stress exposure times provided a window into the dynamics of the responses, where osmotic and imipenem stresses elicited changes that increased over time while oxidative stress triggered rapid expression changes that decreased with time. The numbers of differentially expressed mRNA and sRNA transcripts followed similar trends under the different stress conditions. The highest numbers of differentially expressed mRNAs and sRNAs compared to those of the control were observed under osmotic stress after 60 min, followed by oxidative stress after 7 min.

The extents of the observed expression changes under different stress conditions for mRNA and sRNA transcripts are summarized in Fig. 3C and D, respectively. The majority of mRNA transcripts exhibited 2- to 5-fold expression changes under all conditions, and a larger proportion of mRNAs than sRNAs showed changes in this range. There was a larger proportion of sRNAs than mRNAs with >5-fold expression changes under all stress conditions. Very high changes (>100-fold) were observed for 4% of sRNA transcripts and 1% of mRNA transcripts during osmotic stress after 60 min. Taken together, the extent of differential expression observed is dependent on the specific stressor, the degree of stress applied, and the stress exposure time.

In general, there were relatively few common genes affected at both studied time points for all three conditions, suggesting that the response to each stressor is a highly

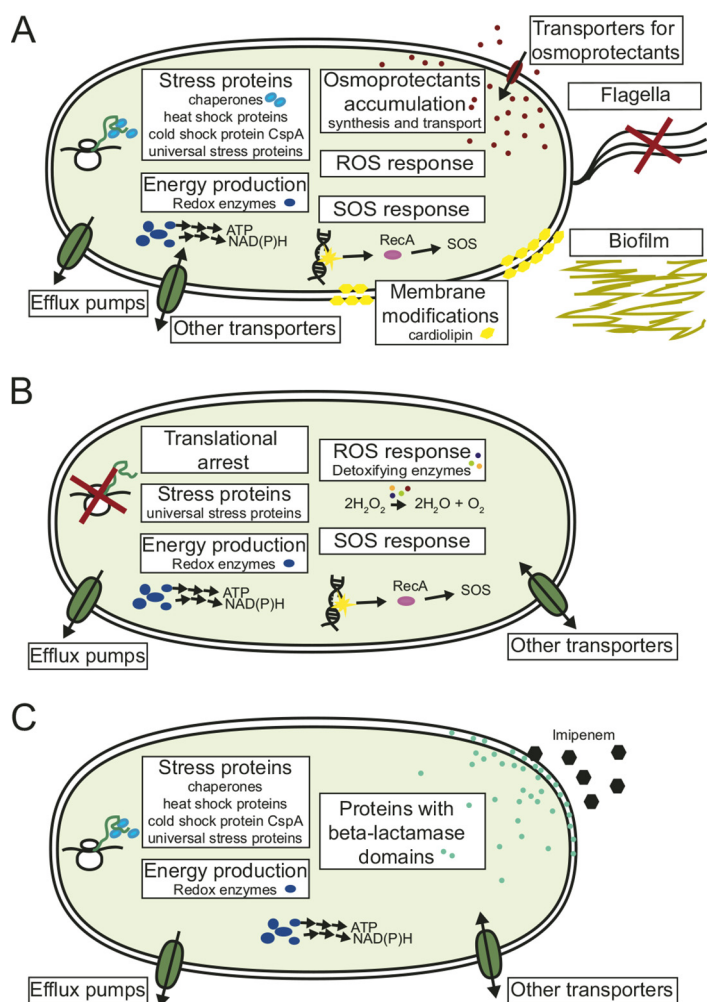


FIG 4 Overview of selected cellular functions and processes with differential expression under osmotic (A), oxidative (B), and imipenem (C) stress in *Pseudomonas putida* KT2440. In general, the depicted functions are upregulated except for those marked with X's, which are downregulated, as well as the differential expression of efflux pumps and other transporters, for which changes were observed in both directions for all stress conditions. The included processes are limited to those observed with RNA sequencing in this study on a transcriptional level and not changes observed in other studies, with other bacteria, or those regulated on other levels.

choreographed series of alterations to adapt to the changed environment. Previous studies of transcriptional responses to stress revealed large variations in the extent of observed differential expression. However, direct comparisons are not possible due to differences in the organism studied, stressor identity and exposure, and methodology.

The major physiological processes affected at the transcriptional level in *P. putida* KT2440 under the stress conditions studied are summarized in Fig. 4. Extrusion of stressor molecules has previously been shown to be an important response for *P. putida* survival (12, 25, 39, 40). Indeed, changed transcriptional levels in several permeases, ABC transporters, and RND efflux pumps were detected under all chosen conditions. The specific expression of transporters under stress conditions suggests that cells are very selective as to which molecules are transported across the membrane to facilitate survival.

Differential expression of mRNAs under osmotic stress. The RNA expression profile of *P. putida* KT2440 exposed to osmotic stress revealed a much stronger response at 60 min than at 7 min after NaCl addition, with 2,182 (40.8% coding sequences [CDS]) and 124 (2.3% CDS) differentially expressed genes, respectively (Fig. 3A; Data Set S4). In a study in which *P. aeruginosa* was subjected to osmotic stress, only 2.4% of genes were

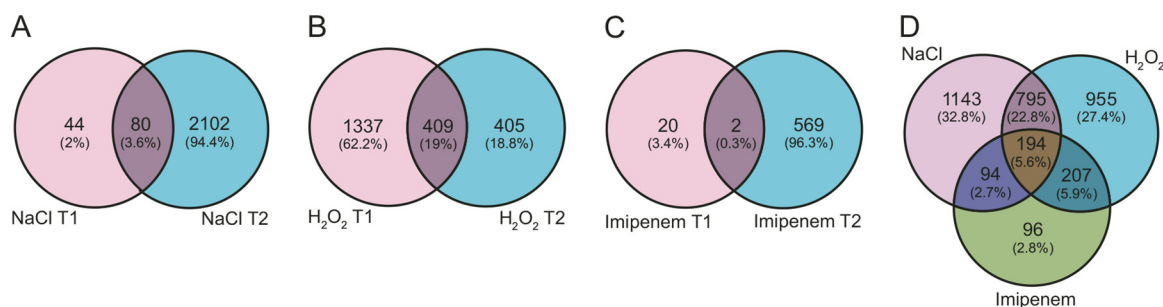


FIG 5 Venn diagrams illustrating the number of differentially expressed genes under osmotic stress (NaCl) (A), oxidative stress (H₂O₂) (B), imipenem (IP) stress at T1 (7 min) and T2 (60 min) (C), and under all three stress conditions (D). The proportions of differentially expressed genes under a certain type of stress condition are shown in parentheses.

differentially expressed with >3-fold changes (41), but a much lower salt concentration was used than in this work. In this study, only 80 genes (3.8% CDS) were common to both time points tested, including several transcriptional regulators, and over half the genes encode proteins of unknown function (Fig. 5A). The large number of differentially expressed genes at T2 is due in part to the differential expression of many sigma factors and transcriptional regulators, suggesting that many regulatory networks were affected.

Many observed responses are nonspecific (Table 1; Fig. 4), with increases in mRNAs encoding universal stress proteins, heat shock proteins, and other chaperones. Chaperones promote protein folding and prevent protein misfolding and aggregation that often occur under stress conditions (42). In addition, the induction of genes involved in the response to the presence of reactive oxygen species (ROS) was observed, pointing to increased levels of ROS during osmotic stress (Table 2).

Downregulation of flagellar genes and an upregulation of biofilm formation have been observed in our data (Table 1; Fig. 4). It has been reported that high salt concentrations inhibit flagellar biosynthesis in *E. coli* on the transcriptional level (43, 44). Motility reduction and biofilm formation seem to be general bacterial responses to osmotic stress in diverse organisms (45–49), and *P. putida* shows a similar osmoadaptive response. Significant energy is needed for flagellar biosynthesis, and bacteria are able to inhibit certain processes in cells to save energy in order to withstand stress.

Specific responses to osmotic stress include two main mechanisms: (i) accumulation by import (potassium, proline, and glycine betaine) or biosynthesis of osmoprotectants (trehalose, mannitol, *N*-acetylglutaminylglutamine amide [NAGGN]) in order to restore the osmotic balance in cells and (ii) alteration of membrane composition by fatty acid saturation and phospholipid composition to endure the changed turgor pressure (50–52). NAGGN, mannitol, and trehalose have been shown previously to be important compatible solutes in pseudomonads (41, 53, 54). The present data show that accumulation of glycine/proline betaine by the import uptake system and the biosynthesis of NAGGN, trehalose, mannitol, and also glycogen are important strategies for *P. putida* KT2440 to respond to osmotic stress (Table 1; Fig. 4). The two genes PP_1748 and PP_1750 show high similarity to *P. aeruginosa* NAGGN biosynthetic genes. From the current data, NAGGN seems to be a significant osmoprotectant, as genes for its biosynthesis were among the most upregulated genes at T2, similar to previous observations for *P. aeruginosa* (41).

Potassium glutamate is a common regulator of osmolarity in many organisms prior to the onset of long-term adaptation mechanisms (48, 55, 56). In a recent resequencing of the *P. putida* KT2440 genome, the potassium uptake transport systems Kdp and Trk were annotated and a novel subunit KdpF was identified (57). The uptake system is induced by the KdpDE two-component system in osmotic upshift and K⁺ limitation in *E. coli* (58). In our data, this system was not induced, while all other major protection mechanisms in osmotic stress were observed. This could be due to *P. putida* having a different mechanism of induction, or the system might be regulated on another

TABLE 1 Selected differentially expressed transcripts under osmotic stress

Gene function and locus tag	Gene name	Fold change ^a
Glycine betaine uptake		
PP_0868		250.5
PP_0869		363.4
PP_0870		389.1
PP_0871		140.3
PP_2914	<i>proP</i>	22.9
PP_3957		5.5
PP_0229		7.9
PP_1944		7.4
PP_0989	<i>gcvH</i>	−7.2
PP_5193	<i>gcvH</i>	−5.3
Trehalose and glycogen biosynthesis		
PP_2918		52.1
PP_4050	<i>glgA</i>	58.4
PP_4051		49.4
PP_4052	<i>malQ</i>	75.1
PP_4053		39.3
PP_4054		28.2
PP_4058	<i>glgB</i>	28.6
PP_4059		162
PP_4060		195.3
NAGGN and mannose biosynthesis		
PP_1748		1,030.5
PP_1750	<i>asnB</i>	1,082.8
PP_1277	<i>algA</i>	3.1
PP_5288		4.8
Cardiolipin production		
PP_3263		832.3
PP_3264		196.8
PP_3265		311.6
PP_3266		1,546.6
Biofilm		
PP_1281	<i>algL</i>	17.7
PP_1282	<i>algX</i>	144.2
PP_1283		194.7
PP_1284	<i>algE</i>	194.7
PP_1285	<i>algK</i>	177.8
PP_1286		200.3
PP_1287		172.2
PP_1288	<i>algD</i>	120
Flagella		
PP_4358	<i>fliM</i>	−5.3
PP_4369	<i>fliF</i>	−5.1
PP_4370	<i>fliE</i>	−6.7
PP_4385	<i>flgG</i>	−5.7
PP_4386	<i>flgF</i>	−6.6
PP_4389	<i>flgD</i>	−6.1
PP_4390	<i>flgC</i>	−6.8
PP_4391	<i>flgB</i>	−6.1
PP_4394	<i>flgA</i>	−5.4
Stress proteins		
PP_4727	<i>dnaK</i>	6.8
PP_2187		7.8
PP_4726	<i>dnaJ</i>	8.5
PP_3237		9
PP_4179	<i>htpG</i>	10.1
PP_3550		10.2
PP_1982	<i>ibpA</i>	13.3
PP_3970		309.8
PP_0866		−5.8
PP_0985		−6
PP_1522	<i>cspA-l</i>	−6.6
PP_3095		22.3

^aNumbers indicate fold changes for upregulated and downregulated (−) transcripts at T2 (60 min).

TABLE 2 Selected differentially expressed transcripts under oxidative and osmotic stress

Gene function or product and locus tag	Gene name	Fold change under indicated stress ^a		
		NaCl T2	H ₂ O ₂ T1	H ₂ O ₂ T2
General stress response (SOS)				
PP_1629	<i>recA</i>	3.1	4	
PP_4729	<i>recN</i>	2.2	5.2	2.4
PP_2143	<i>lexA1</i>	2.4	5.1	3.2
Catalases				
PP_0481	<i>katA</i>	3.4	936.5	7.7
PP_3668	<i>katB</i>		31.2	5.9
PP_0115	<i>katE</i>	281.4		
Peroxiredoxins				
PP_2439	<i>ahpC</i>		74.7	2.3
PP_2440	<i>ahpF</i>		203.4	3.5
PP_2441			247.3	4.4
OxyR-responsive genes				
PP_0786	<i>trxB</i>		8.7	−2.3
PP_5215	<i>trx-2</i>		2.8	
PP_0877			5.1	
Taurine				
PP_0230	<i>tauD</i>		6.7	
PP_0231	<i>tauC</i>		27	
PP_0232	<i>tauB</i>		38.7	
PP_0233	<i>tauA</i>		44.7	

^aNumbers indicate fold changes for upregulated and downregulated (−) transcripts, and the lack of a number indicates no differential expression under that condition. T1, 7 min; T2, 60 min.

level that was not captured in this study. Another possible reason could be the inherent difficulties of amplifying specific mRNAs and their underrepresentation in cDNA libraries.

The second protection strategy in osmotic stress by alteration of membrane composition to decrease permeability was confirmed in *P. putida*. Upregulation of the cardiolipin biosynthetic genes in osmotic stress was observed (Table 1; Fig. 4), similar to previous observations in *Bacillus subtilis* and *E. coli* (50). Also, transcriptional changes in a number of transporters were observed, including upregulation of RND efflux pumps (operon PP_5173–PP_5175, PP_3302–PP_3304, *tgt2* operon), permeases, and ABC transporters, as well as downregulation of several other transporter-related proteins (21 were downregulated >5-fold). The altered membrane composition is achieved mostly by increasing the cardiolipin content, but the induction of certain protein transporters is likely to affect membrane structure as well as selective transport in and out of the cells upon osmotic upshift. In addition, an upregulation of iron uptake mechanisms (siderophores) was observed here, as reported previously for *Sinorhizobium meliloti* (49). This strategy underscores the need for iron as a cofactor for various enzymes, which are activated during stress. Many of the iron-binding enzymes combat the presence of ROS (59), which is a side effect of osmotic stress, and thus these enzymes are in high demand.

Differential expression of mRNAs under oxidative stress. The RNA expression profile of *P. putida* KT2440 exposed to hydrogen peroxide revealed a much stronger response at 7 min than at 60 min after compound addition, with 1,746 (32.6% CDS) and 814 (15.2% CDS) differentially expressed genes at T1 and T2, respectively (Fig. 3A; Data Set S5). Almost one-fifth (409) of the differentially expressed genes at T1 also had changed transcriptional levels at T2 (Fig. 5B). A study in *P. aeruginosa* exposed to hydrogen peroxide after 10 min detected 33.7% differentially expressed genes (60), concurring with changes observed here and a similar study in *E. coli* (61). In another study, in which *P. putida* was subjected to the organic peroxide paraquat and cumene hydrogen peroxide, only 1.7% and 2.1% of genes were differentially expressed, respec-

tively (62), suggesting that addition of inorganic hydrogen peroxide causes a more extensive response and changes in transcript levels as observed here.

Bacteria have developed different mechanisms of oxidative stress sensing, regulation, and defense. They typically encode several enzymes that help in detoxification of ROS, such as superoxide dismutases (SODs), catalases, and peroxiredoxins. Their expression is controlled by several regulators, such as OxyR, FinR, and HexR, involved in protection against ROS. It has been shown previously that *E. coli* and *Pseudomonas* spp. have different regulatory networks combating oxidative stress (59). The two major oxidative stress regulators in *E. coli* and *Salmonella enterica* serovar Typhimurium are SoxR and OxyR (63). However, in *P. putida*, the SoxR regulator is not responsive to oxidative stress (62), and the oxidative stress defense genes of the SoxR regulon in enteric bacteria, such as *fpr*, *fumC-I*, *sodA*, and *zwf-1*, are independent of SoxR in *P. putida* (64). Although these *P. putida* genes have been shown to be responsive to superoxide and nitric oxide (64), they are not activated in the presence of cumene hydroperoxide (62) or hydrogen peroxide as shown in this study. The *sodA* and *sodB* superoxide dismutases, which catalyze the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide (59), were not differentially expressed in *P. putida* KT2440 after the addition of H_2O_2 . These enzymes are not required for the reduction of hydrogen peroxide, which is instead reduced to water and oxygen by catalases or alkyl hydroperoxide reductases.

The transcriptional levels of the transcriptional regulator OxyR, which has been shown to be constitutively expressed and activated by hydrogen peroxide, were not affected, whereas changes were observed in the transcript levels of its responsive genes encoding detoxification enzymes (*katA*, *katB*, *aphC*, *trxB*, *trx-2*) (Table 2; Fig. 4) (59). The levels of transcripts for these enzymes had the most drastic changes in the presence of hydrogen peroxide at T1, and their levels decreased at T2. The hydroperoxide reductase AphC has been shown to be inadequate for detoxification of high levels of peroxide (65), while the catalases are important for survival during oxidative stress (66, 67).

For the other regulators previously reported to have a role in oxidative stress response, the *finR* and *hexR* genes (59) did not have changed expression levels in our study, but the *ohrR* gene was slightly upregulated at both time points. The FinR regulator is essential for the induction of ferredoxin-NADP⁺ reductases (*fpr*) during exposure to superoxide stress in *P. putida* to mediate a reversible NADP⁺/NADPH reaction (68), but its transcription was not induced in the presence of hydrogen peroxide in this study. The DNA-binding transcriptional regulator HexR regulates intracellular energy and redox status by the Entner-Doudoroff (ED) pathway (69), but neither *hexR* levels nor ED pathway mRNA levels were affected in our study. On the other hand, there was an upregulation of transcript levels of several other redox enzymes (Fig. 4), including cytochrome and quinone carrier proteins, suggesting a high energy requirement for cells to combat the stress and produce detoxification enzymes.

Many ribosomal proteins were downregulated, whereas several membrane proteins and transporters were upregulated (Fig. 4). Strikingly, taurine transport and metabolism were upregulated at T1, consistent with the role of taurine as an antioxidant and membrane stabilizer (Table 2) (70). The upregulation of several SOS response genes (*lexA*, *recA*, and *recN*) was detected here at both time points during oxidative stress and after 60 min under osmotic stress (Table 2). The SOS regulon is probably upregulated indirectly by H_2O_2 and NaCl by oxidant-induced DNA damage and prolonged osmotic stress exposure. Similar changes have been observed in *P. aeruginosa* (60) and *E. coli* (61).

Differential expression of mRNAs under imipenem stress. The RNA expression profile of *P. putida* KT2440 exposed to imipenem showed a stronger response at 60 min than at 7 min after compound addition. A total of 593 genes (Fig. 3A; Data Set S6) were differentially expressed, including 22 (0.4% CDS) at T1 and 571 (10.7% CDS) at T2 (Fig. 5C). Imipenem is a bactericidal beta-lactam antibiotic that inhibits peptidoglycan synthetic enzymes with high affinity (71), and *Pseudomonas* spp. are sensitive to it (72,

73). A study on the transcriptional response of *P. putida* DOT-T1E to eight antibiotics, including the beta-lactam antibiotic ampicillin, suggested that each antibiotic elicited a unique transcriptional response, with ampicillin, chloramphenicol, and kanamycin eliciting responses most similar to those of the untreated control (25).

The genes with the highest fold changes at T1 are membrane proteins, including ABC and other transporters. Interestingly, a cluster of genes, PP_2663 to PP_2682, was highly upregulated, and included genes encoding a redox sensing protein, the AgmR regulator, an ABC efflux pump (regulated by AgmR), several redox-related proteins (quinoproteins and pyrroloquinoline quinone biosynthesis protein), and a beta-lactamase domain-containing protein (PP_2676). Another highly upregulated region (PP_0375 to PP_0380) is related to the *pqq* genes involved in coenzyme PQQ biosynthesis that are also regulated by AgmR. The upregulated gene cluster (PP_2663 to PP_2682) in cells exposed to imipenem was shown previously to be induced upon exposure to chloramphenicol (40), although these two antibiotics have different mechanisms of action. Antibiotics can induce oxidative stress in cells by increasing the levels of ROS, which inactivate various cell enzymes (59, 74, 75). This region was also upregulated in cells exposed to hydrogen peroxide at T2 (14- to 116-fold), whereas some of these genes were downregulated during osmotic stress (4- to 44-fold). Upregulation of the PP_2669 gene has also been observed in the rhizosphere due to oxidative stress caused by antimicrobials in the environment (76), where the *pqq* genes are a part of the cellular defense to redox changes (77). This genomic region seems to be important in the response to oxidative stress and antimicrobials causing oxidative stress. Upregulation was observed in genes related to the electron transfer chain (azurin, cytochrome *c* oxidase, and glycolate oxidase). In contrast, the housekeeping sigma factor σ^{70} was downregulated at T2.

Microarray studies in *P. putida* and *P. aeruginosa* showed that ampicillin activated oxidative-stress and SOS-inducible genes (78). In this study, with imipenem, induction of the SOS response was not observed. This could be due to a requirement for a longer exposure to the antibiotic for its induction or that imipenem does not induce the SOS response as some other antibiotics do (79).

The beta-lactamase genes *ampC*, *ampG*, and *ampD* were not upregulated in the presence of imipenem in this study. A longer exposure time may be needed to activate more pronounced changes in this specific response (80). However, a beta-lactamase domain-containing protein (PP_2676) was upregulated 60 min after imipenem addition, suggesting that the degradation of antimicrobials is an important strategy.

The numbers of differentially expressed genes that are either unique to a specific type of stress condition or common to two or three types of stress conditions are shown in Fig. 5D. Osmotic and oxidative stress conditions have the highest number of common differentially expressed genes (795 genes). There are 194 common differentially expressed genes found in all three studied stress conditions (Data Set S7) that likely represent the general response of *P. putida* KT2440 to stress. Among them are 18 transcriptional regulators from different families and hypothetical proteins representing a fraction of 40%. Other common genes encode membrane transport proteins, signal transduction proteins, cold shock protein CspD, heat shock proteins, coenzyme biosynthesis proteins (biotin, PQQ), and redox- and energy-related proteins (cytochromes), as well as DNA repair proteins. The induction of protection mechanisms against ROS was observed under all chosen conditions. ROS cause mutations and may thus promote bacterial evolution to produce a surviving mutant under stressful conditions, but since they most often cause lethal mutations, bacteria have to fight ROS (59).

Differential expression of sRNAs. A total of 198 out of 440 sRNAs identified in this study were differentially expressed under at least one condition (Table 3; Table S5). The differentially expressed sRNAs cluster into nine groups (Fig. 6; Data Sets S1, S2, and S3) based on their expression patterns under the different conditions. Three groups of sRNAs exhibit different extents of upregulation in osmotic stress after 60 min. Cluster 8 consists of four sRNAs with exceptionally high levels of upregulation (>2,000-fold),

TABLE 3 sRNAs with differential expression under at least three of six chosen conditions

sRNA	Fold change under indicated condition ^a					
	NaCl T1	NaCl T2	H ₂ O ₂ T1	H ₂ O ₂ T2	IP T1	IP T2
Pat107	−4.2	−13.5	−3.5	−3.5		−4.7
Pat044	8.7	7.0	71.5	7.6		
Pat077		−3.5	−2.9			−3.8
Pit020		−3.6	−3.8			−4.8
RsmY		−3.1	−3.7			−4.9
Pat110	6.8	6.1	4.2			
Pit116	5.5	5.8	4.0			
Pit087	5.0	8.1	2.9			
Pat181	4.8	4.7	7.6			
Pit082		−5.2	−3.0	−3.9		
Pit080		−12.8	−5.6	−4.0		

^aNumbers indicate fold changes for upregulated and downregulated (−) transcripts, and the lack of a number indicates no differential expression under that condition. IP, imipenem; T1, 7 min; T2, 60 min. All sRNAs with differential expression are shown in Table S5 in the supplemental material.

cluster 6 consists of sRNAs with 100- to 2,000-fold changes, and cluster 3 includes transcripts with <100-fold changes. Clusters 4 and 7 consist of sRNAs highly expressed under oxidative stress at T1, with some transcripts also being upregulated under other conditions (Table S5). The transcripts that are downregulated under all conditions group together in cluster 2. Pat092 comprises cluster 9 with high upregulation in osmotic stress at T2 and imipenem stress at T1. The other two clusters (1 and 5) are composed of sRNAs that exhibit mixed expression patterns under the different conditions.

The expression profiles of selected annotated and novel sRNA transcripts exhibiting differential expression patterns are shown in Fig. 7. The expression profiles of the two sRNAs, RsmY and ErsA, are shown in Fig. 7A and B, respectively. The ends of these transcripts are not visible, as the central portion of the transcripts had a higher number of reads. The profiles of four novel intergenic RNA transcripts are shown in Fig. 7C to F, and those of two novel antisense RNAs are shown in Fig. 7G and H.

Only Pat107 sRNA (Fig. 7H) was differentially expressed and downregulated under five out of six conditions. This sRNA is encoded opposite the *ttgR* gene (PP_1387), a

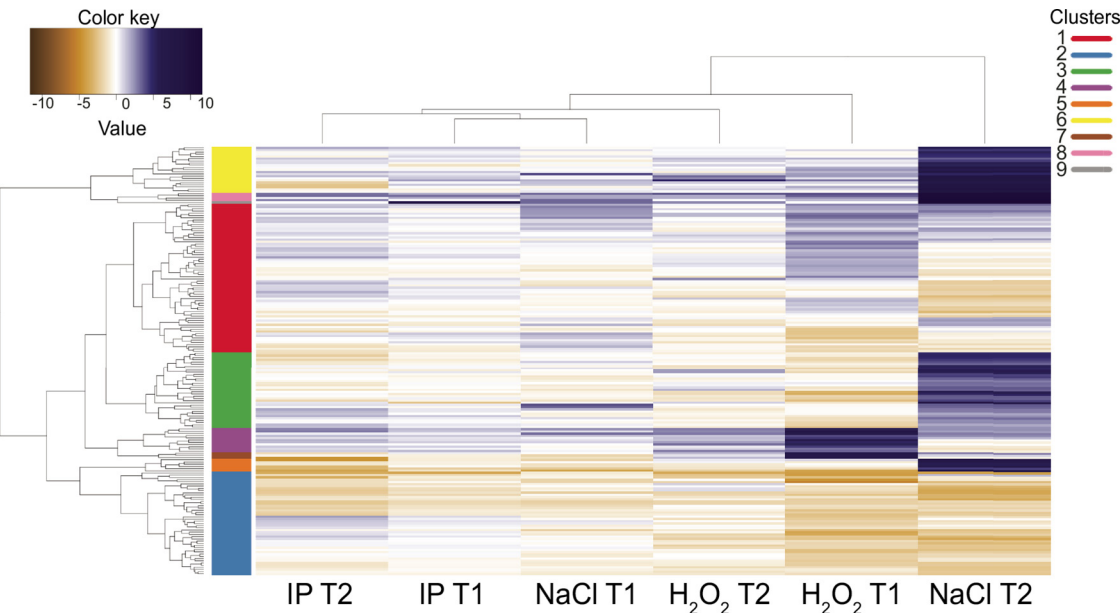


FIG 6 Heat map and hierarchical clustering of differentially expressed sRNAs under osmotic (NaCl), oxidative (H₂O₂), and imipenem (IP) stress conditions at T1 (7 min) and T2 (60 min) after exposure, in comparison to that of the control without added stressor (fold change of ≥2 and a *P* value of ≤0.05).

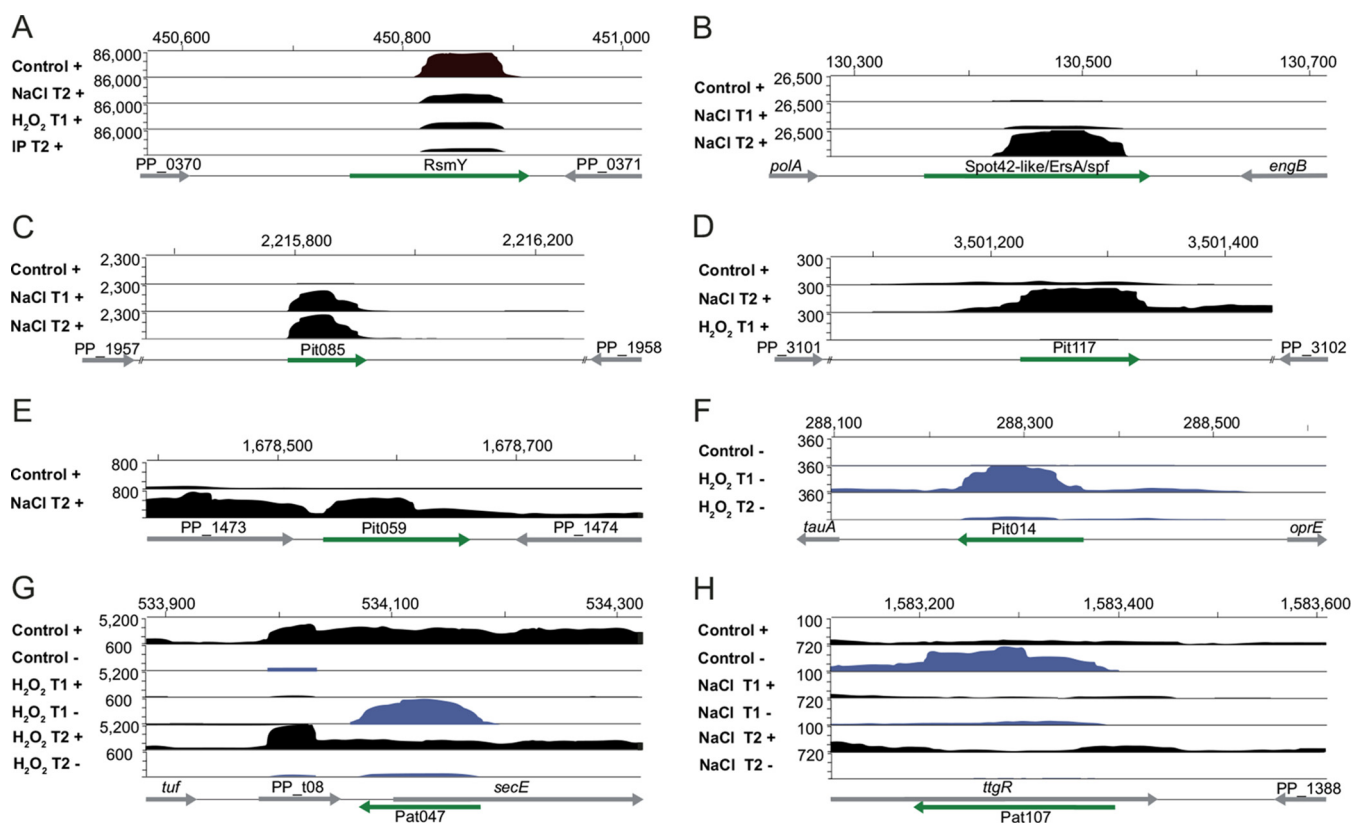


FIG 7 Expression profiles of sRNAs under different conditions. The profiles include two annotated sRNAs, RsmY (A) and Spot42-like/ErsA/spf (B), two novel intergenic sRNA candidates, Pit085 (C) and Pit117 (D), a putative 3'-UTR-derived sRNA candidate, Pit059 (E), a putative 5'-UTR-derived sRNA candidate or actuator, Pit014 (F), and two novel *cis*-encoded sRNA candidates, Pat047 (G) and Pat107 (H). Reads on the forward (+) and reverse (−) strands are indicated in black and blue, respectively. Note that the scales for the + and − strands differ. The sRNA transcripts are shown in green, and the flanking genes are in gray. The genomic location is shown at the top of each profile. The conditions include osmotic (NaCl), oxidative (H_2O_2), and imipenem (IP) stress at T1 (7 min) or T2 (60 min) after exposure, as well as without added stressor (Control).

transcriptional repressor of the TtgABC efflux pump, which has been shown to mediate resistance toward several antibiotics and organic solvents (81). This gene was upregulated 3.1-fold under osmotic stress at T2, where the highest down-expression for the *cis*-encoded sRNA Pat107 was observed (13.5-fold). The sRNA Pat077 was differentially expressed under three conditions and encoded opposite the *hexR* gene (PP_1021), also a transcriptional regulator that is responsive to oxidative stress. Although *hexR* levels were unchanged, it could possibly be regulated via sRNA binding on a translational level. RsmY (Fig. 7A) and Pit020 sRNA, which are antisense to each other, were both 4-fold downregulated in three conditions.

Nearly half of the sRNA transcripts identified in this study exhibit differential expression under at least one stress condition. The observed expression changes suggest that some of these transcripts may play roles in the adaptation to stress conditions. The ErsA (spf, Spot42-like) RNA was upregulated 14.8-fold after 60 min of osmotic stress. Recent work in *P. aeruginosa* and *P. syringae* has demonstrated that expression of ErsA is dependent on the envelope stress-responsive sigma factor σ^{22} /AlgU/RpoE (82, 83). This concurs with a 17-fold upregulation of *algU* observed under osmotic stress after 60 min in this study. In addition, deletion of the gene in *P. syringae* leads to increased sensitivity to hydrogen peroxide compared to that of the wild-type strain (83), although no expression changes were observed under the oxidative stress conditions used here. Of the differentially expressed sRNAs with characterized function in at least one pseudomonad, the CrcY, CrcZ, PhrS, and RsmY RNAs are part of cluster 2, where there is downregulation in one or more of the studied stress conditions. Although the functions of the differentially expressed sRNA transcripts are unknown, it is notable that many of the Pat transcripts that are found in clusters characterized by upregulation during osmotic and oxidative stress (3, 4, 6–8) are

located opposite genes encoding predicted transporters or membrane proteins. This concurs with the many observed changes in the expression of efflux pumps and transporters under the studied stress conditions and suggests that some of these may be regulated via mechanisms involving antisense transcripts.

Concluding remarks. In this work, extensive genome-wide changes in mRNA and sRNA transcript levels are documented in *P. putida* KT2440 exposed to osmotic, oxidative, and imipenem stress conditions. The results include many differentially expressed genes not described previously due to the depth of the RNA-seq data. This wealth of information is now available to the research community and adds rich detail to the understanding of stress responses in *P. putida*. Although each type of stress elicits a unique transcriptional response, there are notably 194 commonly differentially expressed genes in all stress types. The role of these genes, of which 40% have unknown function, and their involvement in a general stress response are an interesting area for future investigation. In addition, the insights gained here could be combined with transposon-insertion sequencing experiments under similar conditions to identify target genes that affect fitness during stress, with the aim of generating more robust strains as in several studies in *E. coli* (84, 85). Moreover, the transcriptomic data collected here combined with proteomic studies may yield important insights into regulation at the posttranscriptional level, including the involvement of sRNAs.

A total of 440 sRNA transcripts were detected, dramatically increasing the number of sRNAs reported in *P. putida* KT2440 and adding knowledge on antisense RNAs not described previously in this organism. Differential regulation of sRNAs under different stress conditions provides clues to their possible regulatory roles and will aid in the selection of relevant transcripts for functional characterization. Although characterization of a few *Pseudomonas* sRNAs has been carried out, there is a general dearth of knowledge on the specific functional roles of sRNAs in *P. putida*. Most studies have been performed in *P. aeruginosa*, and the identified targets are related to virulence, suggesting that sRNAs conserved in pseudomonads have additional targets and broader regulatory roles. Unraveling sRNA regulatory mechanisms in *P. putida* is an important next step and will yield insights into bacterial stress response mechanisms developed to adapt to changing environmental conditions. Depending on their specific functions and regulatory networks, their overexpression or deletion may have potentially useful applications in biotechnology to improve stress tolerance.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *P. putida* KT2440 strain (DSM6125) was cultivated in M9 medium (per liter, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 70 g; KH_2PO_4 , 30 g; NH_4Cl , 10 g; NaCl, 5 g) supplemented with 0.5% glucose and trace metals (per liter, H_3BO_3 , 300 mg; ZnCl_2 , 50 mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 30 mg; CoCl_2 , 200 mg; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mg; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 20 mg; and $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 30 mg) (86) at 30°C and 250 rpm in this study, unless otherwise indicated.

Single colonies were grown overnight in 5 ml M9 medium, and the cultures were diluted to a starting OD_{600} of 0.05 in 50 ml M9 medium in 250-ml shake flasks. At mid-exponential growth phase (OD_{600} ~0.6), different compounds were added at different concentrations, followed by monitoring of growth (OD_{600}) and survival. For osmotic stress, the following NaCl (Sigma) concentrations were tested: 0, 2, 3, 3.5, 4, 4.5, and 5%. For oxidative stress, the following H_2O_2 (Sigma) concentrations were tested: 0, 0.5, 1, 2, 5, 10, 15, 20, 25, and 30 mM. For imipenem stress, the beta-lactam antibiotic imipenem (Sigma) was used and final concentrations of 0, 0.05, 0.1, 0.2, 0.4, and 0.8 $\mu\text{g}/\text{ml}$ were tested. For monitoring survival, 1 ml of the culture was harvested before and 1, 3, and 24 h after compound addition. CFU were counted on LB chloramphenicol plates incubated at 30°C.

For RNA-seq experiments, the following compound concentrations were used: 3% NaCl, 0.5 mM H_2O_2 , and 0.1 $\mu\text{g}/\text{ml}$ of imipenem. The cultures grown in the same manner as described above were harvested 7 and 60 min after the addition of the stress compounds, and the control was a sample harvested just prior to compound addition. All experiments were carried out in three biological replicates.

Total RNA isolation. RNA extraction was performed as previously described (37). Briefly, 20 ml of harvested culture was mixed with 0.2 volumes of STOP solution (95% [vol/vol] ethanol, 5% [vol/vol] phenol). Cells were centrifuged, snap-frozen, and stored at -80°C . Total RNA was extracted with TRIzol (Invitrogen) and treated with DNase I (Fermentas) for DNA removal. Total RNA integrity and quality were validated by use of an Agilent 2100 Bioanalyzer (Agilent Technologies).

Library preparation and RNA sequencing. Transcriptome libraries were constructed as previously described (37) with some modifications. The total RNA sample was depleted of rRNA with the Ribo Zero

kit for Gram-negative bacteria (Illumina). cDNA libraries were prepared with the TruSeq stranded mRNA sample preparation kit (Illumina) by following the Low Sample LS protocol. Libraries were validated with a DNA 1000 chip on the Agilent 2100 Bioanalyzer, and concentration was measured using a Qubit 2.0 fluorometer (Invitrogen, Life Technologies). The concentration of each library was normalized to 10 nM in TE buffer (10 mM Tris-Cl, pH 7.0; 1 mM EDTA), and cDNA libraries were pooled for sequencing on the Illumina HiSeq 2000 platform at Beckman Coulter Genomics. The transcriptome libraries were single-end sequenced with 100-bp reads.

Data analyses. The RNA-seq data were trimmed using Trimmomatic (87) and analyzed with the open source software Rockhopper with the default settings, choosing reverse complement reads and strand-specific analysis (16) (version 2.0.3). The reads were mapped to the sequenced reference *P. putida* KT2440 genome (GenBank accession no. [NC_002947.3](https://doi.org/10.1093/nar/21.12.2440)). Using SAMtools (88), the mapped files were merged and the identification of novel transcripts was performed by visual inspection with Integrative Genomics Viewer (17), as Rockhopper detects many false positives. Differential gene and sRNA expression analyses were carried out with the Web server T-REx (89) using the RPKM values generated in the Rockhopper analysis, in which all the tested conditions were compared to the control, a sample harvested just prior to addition of the compound. Differential expression of genes was considered significant with a fold change of ≥ 2 and an adjusted *P* value of ≤ 0.05 . The Basic Local Alignment Search Tool (BLAST) with search criteria of query of $>80\%$, identity of $>60\%$, and E value of $<10^{-6}$ was used in sequence homology searches. The novel sRNA transcripts were analyzed for rho-independent terminators and palindromes with the Pseudomonas Genome Database (90) and ARNold tool (91).

Accession number(s). RNA-seq data have been deposited in the GEO database under accession number [GSE85475](https://doi.org/10.1093/bioinformatics/btt111).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.03236-16>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.05 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.07 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.07 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 6, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 7, XLSX file, 0.03 MB.

SUPPLEMENTAL FILE 8, XLSX file, 0.03 MB.

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There are no potential conflicts of interest to disclose.

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