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Small RNA-Controlled Gene Regulatory Networks in *Pseudomonas putida*

PhD Thesis

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August 2016

Small RNA-Controlled Gene Regulatory Networks in *Pseudomonas putida*

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When you tread your way,
Always go to the end.
In spring, to a flower so sweet,
In summer, to a shower of wheat,
In autumn, to pantries that glow,
In winter, to the lady of snow,
In life, to the truth that is thine,
Until color leaks into your cheeks.
And if you don't climb the first time,
To the top and reap the best crop,
Try it once more
And over and over again.

Ko hodiš, pojdi zmeraj do konca.
Spomladi do rožne cvetice,
poleti do zrele pšenice,
jeseni do polne police,
pozimi do snežne kraljice,
v knjigi do zadnje vrstice,
v življenju do prave resnice,
v sebi do rdečice čez eno in drugo lice.
A če ne prideš ne prvič,
ne drugič do krova in pravega kova
poskusi: vnovič in zopet in znova."

– Tone Pavček
(Slovenian poet)

Preface

This thesis is written as a partial fulfillment of the requirements to obtain a PhD degree at the Technical University of Denmark. The work presented in this thesis was carried out from September 2013 to August 2016 at the Novo Nordisk Center for Biosustainability, Technical University of Denmark in Hørsholm. The work was supervised by Associate Professor Katherine S. Long. Funding was provided by the Novo Nordisk Foundation and an ITN grant from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme (FP7-People-2012-ITN), under grant agreement No. 317058, Bactory.

The thesis was evaluated by Rebecca M. Lennen, Senior researcher at DTU (Denmark); Birgitte Hahr Kallipolilis, Associate Professor at Syddansk Universitet (Denmark); and Professor Claudio Valverde from Universidad Nacional de Quilmes (Argentina).

Klara Bojanovič

Lyngby, August 2016

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Isotta you have been a special character in this chapter. When we were lost in the data and the terminal commands, feeling 'different' at the Czech course, under the rain in Mexico with your head broken, looking puzzled at the results or at our cultures growing 'weird' – I am so happy we were always shoulder to shoulder. Thank you for always being there and letting me bother you. The rest of my group – thank you Mikkel for keeping up with me and patiently answering countless questions about R, terminal commands, Danish translations, etc., as well as Xioachen for going for dinners and for sometimes pissing me off so I felt like you were my brother (meant in the most positive way) ☺. Mafalda, thank you for always being there on the long working days, prepared to have fun at any moment, and for having such a strong personality. Patricia, Sofie and Henrique being part of the Bactory Summer School organizers was a special experience trying our skills outside of science and I am happy we did it so well together. 'Best-office-ever'– Isotta, Mafalda, Patricia, Sofie, Xiaochen, Mikkel, Dario, and Mr. Kim thank you everyone! It was a true highway to laughter, culture-mixing, trouble-sharing, and colorful emotions. Being part of that office was one of the best things in these three years – I'll keep all the chocolate treats, BBQs, Friday music afternoons, and the cemetery in my memory. Also I am grateful to be one of the Bactory students (those not yet mentioned – Ivan, Songyuan, Stephanie, Christina, Eric, and Sonia) because it was very nice to be a part of a group, sharing the problems and the happiness – together everything was much easier.

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The best thing that happened to me on this journey is you, Henrique. Thank you for being supportive, loving, open-minded, adventurous, and strong-willed. With you everything is better and easier. I am grateful to have amazing friends and family whose support and presence is the foundation of me being myself. Ana Ž., Maruša, Ana H., Neja, Lea, Ana Š., Didi, Neža, Mojca… Thank you for the all the fun, craziness, Skype conversations, and keeping me down to Earth. Thank you to my family, especially to mom, dad, Katja, and Miha – without a solid base no one can be great. Thank you for the support, without which I would never be where I am. Also thank you for the packages of Milka and Slovenian specialties that kept me going.

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Abstract

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. In addition to two-component systems, small regulatory RNAs (sRNAs) have emerged as major regulators of gene expression. The majority of sRNAs bind to mRNA and regulate their expression. They often have multiple targets and are incorporated into large regulatory networks and the RNA chaperone Hfq in many cases facilitates interactions between sRNAs and their targets. Some sRNAs also act by binding to protein targets and sequestering their function.

In this PhD thesis we investigated the transcriptional response of *Pseudomonas putida* KT2440 in different conditions via identification of differentially expressed mRNAs and sRNAs. *P. putida* is a soil bacterium with a versatile metabolism and innate stress endurance traits, which makes it suitable as future cell factory for the production of valuable compounds.

Detailed insights into the mechanisms through which *P. putida* responds to different stress conditions and increased understanding of bacterial adaptation in natural and industrial settings were gained. Additionally, we identified genome-wide transcription start sites, and many regulatory RNA elements such as sRNAs and riboswitches. Further, the sRNAome during the growth of bacteria was investigated and compared to the strain without Hfq protein. Hfq has a big impact on

sRNAs and gene expression in *P. putida*, hence many Hfq-associated sRNAs and mRNAs were found.

Together, the results reported here significantly increase the knowledge of adaptation mechanisms in *P. putida*, as well as its transcriptome and regulatory networks. This will likely benefit the design and optimization of future cell factories.

Dansk resumé

Bakterier møder ofte stressfyldte betingelser ved vækst i deres naturlige miljøer og i industrielle bioteknologiapplikationer som biobaseret produktion af kemikalier. Da koordineret regulering af genekspression er nødvendig for tilpasning til skiftende miljøer, har bakterier udviklede talrige mekanismer til at regulere genekspression i respons til specifikke miljøsignaler. Udover tokomponentsystemer, har små regulatoriske RNA'er (sRNAs) vist sig som store regulatorer af genekspression. Størstedelen af sRNA'er binder til mRNA og regulerer deres ekspression. De har ofte flere mål og er inkorporerede i større regulatoriske netværk, og RNA chaperonen Hfq fremmer i mange tilfælde interaktionen mellem sRNA'er og deres mål. Nogle sRNA'er virker også ved at associere med proteiner og ændre deres funktion.

I denne Ph.d.-tese undersøger vi det transkriptionelle respons af *Pseudomonda putida* KT2440 til forskellige betingelse via identifikation af differentielt udtrykte mRNA'er og sRNA'er. *P. putida* er en jordbakterie med en alsidig metabolisme og medfødte stressudholdenhedsegenskaber, og er derfor anset som en potentiel fremtidig cellefabrik til produktion af værdifulde kemiske forbindelser.

Detaljeret indsigt i mekanismerne, hvorved *P. putida* reagerer på forskellige stressbetingelser, og øget forståelse af bakteriel tilpasning til naturlige og industrielle miljøer blev opnået. Endvidere identificerede vi helgenom transkriptionsstartsteder og mange regulatoriske RNA

elementer som sRNA'er og 'riboswitches'. Ydermere, blev sRNAomet under væksten af bakterier undersøgt og sammenlignet med en stamme uden Hfq-proteinet. Hfq har en stor indflydelse på sRNA'er og genekspression i *P. putida*. Derfor blev mange Hfq-associerede sRNA'er og mRNA'er fundet.

Resultaterne, der her rapporteres, øger signifikant kendskabet til *P. putida*'s tilpasningsmekanismer, såvel som dens transkriptom og regulatoriske netværk, hvilket vil gavne udviklingen og optimeringen af fremtidige cellefabrikker.

Publications

1 Bojanovič K., D'Arrigo I., Long K. S. (2016) **Global transcriptional responses to oxidative, osmotic, and membrane stress conditions in *Pseudomonas putida*.** (submitted to Appl. Environ. Microbiol.)

2 Bojanovič K., Long K. S. (2016) **Investigation of the *Pseudomonas putida* sRNAome reveals growth phase specific expression and insights into the Hfq regulon** (in preparation)

3 D'Arrigo I., Bojanovič K., Yang X., Rau M. H., Long K. S. (2016) **Genome-wide mapping of transcription start sites yields novel insights into the primary transcriptome of *Pseudomonas putida*.** Environ Microbiol. [Epub ahead of print] doi:10.1111/1462-2920.13326.

Publications not included in this thesis:

4 Rau M. H., Bojanovič K., Nielsen A. T. and Long K. S. (2015) **Differential expression of small RNAs under chemical stress and fed-batch fermentation in *E. coli*.** BMC Genomics 16:1051.

5 Calero P., Jensen S. I., Bojanovič K., Koza A., Lennen R. M., Nielsen A. T. (2016) **Genome-wide identification of mechanisms for the tolerance of *P. putida* KT2440 towards *p*-coumaric acid. (**in preparation)

6 Machado H.*, Cavaleiro A.M.*, D'Arrigo I., Bojanovič K., Nørholm M.H.H. and Gram L. (2016) **Exploring marine environments to unravel tolerance mechanisms to relevant compounds and discover new microbial cell factories**. (in preparation)
*equal contribution

Table of Content

Introduction

Environmental awareness and the knowledge that petroleum-based sources are coming to an end have put focus into sustainable and bio-based production. Therefore, the chemical and pharmaceutical industries are focusing on microorganisms as cell factories for production of added-value compounds. For microbial production to be successful and triumph over the classical chemical manufacturing routes, it needs to be economically efficient (1).

Synthetic biology focuses on designing and constructing an rewired cell capable of performing desired traits, such as decontaminating water and soil or producing valuable compounds like antibiotics, biofuels, bioplastics, and building-blocks (2, 3). *Escherichia coli* has been the pioneering host for recombinant protein production followed by yeast *S. cerevisae*, filamentous fungi, molds, diverse mammalian cell lines, insect cells, and whole plants and animals (as transgenic systems). To name just a few successful microbial cell factories widely used: *E. coli* producing anti-malarial drug precursors, recombinant human insulin, human growth hormone, and gasoline; *Bacillus subtilis* producing various antibodies and amylases; and *Saccharomyces cerevisiae* producing insulin analogues and hepatitis B virus vaccine (1, 4, 5).

A bacterial chassis is a cellular container that accommodates and executes the necessary cellular functions that can be edited and rationally engineered into desired traits. Unfortunately, biological systems are complex, subject to evolution, and still vastly unknown. An ideal bacterial chassis encodes in its genome basic biological functions of self-maintenance and stress endurance, is robust and stable. They have to be easily amenable to genetic manipulations in order to 'plug-in' desirable and 'plug-out' undesirable genetic circuits. At the same time they have to lack undesirable traits such as virulence factors (6–8).

The production of biofuels and other valuable molecules as well as the biodegradation of chemicals are usually metabolized via various feedstocks and intermediates that are toxic for cells. At the same time the over-production of unnatural compounds to the host causes stress in the microbial cells and lowers the productivity, hence the knowledge is missing to overpass such scenarios in the design of efficient cell factories (1).

On the other hand there are many microorganisms in addition to the most commonly used bacteria *E. coli* and *B. subtilis* with innate metabolic pathways, stress endurance and other features required for an ideal platform strain or microbial cell factory (9). One of such examples are some species of *Pseudomonas* spp. (6).

The recent developments in high-throughput techniques and bioinformatics tools have enabled the decoding of genomes, transcriptomes, proteomes, metabolomes, and fluxomes and expanded the possibilities of metabolic engineering (10). Using systems biology-based tactics involving '–omics' technologies (genomic, transcriptomics, proteomic, and metabolomics) to learn about multiple layers of information and regulation is required in order to acquire a full picture of living microorganisms. This information will allow us to learn about and improve host strains for biotechnological applications (1, 2).

Thesis outline

The PhD thesis is divided into three parts where Chapter 1 discusses the alternative cell factory *Pseudomonas putida* with the emphasis on the *P. putida* KT2440 strain and its properties. Chapter 2 focuses on regulatory RNAs as an important layer of the regulatory networks in the cells that carry a useful additional panel of possible modifications and can be used as a valuable tool when designing a cell factory. Chapter 3 explains the role of the RNA chaperone Hfq, which is in many cases needed for the riboregulation and is one of the global bacterial post-transcriptional regulators. Finally, the thesis concludes with the manuscripts presenting the work done over the three years of PhD studies in an effort to contribute to the expansion of the pool of scientific knowledge. Hopefully it will shed light onto the multi-layered regulatory networks in *P. putida* KT2440 and assist in the design of an optimal microbial cell factory.

1 *Pseudomonas putida*

1.1 General characteristics of *Pseudomonas putida*

Pseudomonas putida is a Gram-negative rod-shaped γ-Proteobacteria bacterium with polar flagella. γ-Proteobacteria members share features such as the ability to thrive in hostile conditions and adapt to different environments, to degrade a variety of chemicals as well as to synthesize various bioactive compounds. Their metabolic versatility enables them to be ubiquitous microorganisms found also in soil contaminated with heavy metals and organic compounds (11, 12). They are also found in rhizosphere, where they promote plant growth by synthesis of growth-promoting hormones and helping in the defense against pathogens. To the contrary some species are plant and/or human pathogens (13–15).

Pseudomonas putida strain mt-2 was isolated from soil in Japan by its ability to use 3-methylbenzoate as the sole carbon source due to the presence of the TOL plasmid pWW0. *P. putida* KT2440 is a derivative of this strain not carrying the plasmid (16–18). *P. putida* KT2440 is one of the best characterized pseudomonads and generally recognized as safe (GRAS-certified). *P. putida* is genetically accessible and genome-wide pathway models have been constructed (19, 20). It is used as a 'workhorse' for genetics and physiology studies as well as for cloning and expression of heterologous genes (18, 21).

The *P. putida* KT2440 genome was first sequenced in 2002 and consists of 6.18 Mbp with 62% of GC content. *P. putida* metabolizes glucose and other hexoses via the Entner-Doudoroff pathway because its lacks 6-phosphofructokinase (*pfk* gene) for Embden–Meyerhof–Parnas glycolysis

(20, 22). Different from *E. coli* and *B. subtilis*, glucose is not the preferential carbon source for *P. putida* that prefers organic acids (such as succinate). The underlying mechanism that reduces the uptake of glucose and increases the preferential carbon source is called carbon catabolite repression (23, 24). The *P. putida* KT2440 genome is closely related to pathogenic *P. areuginosa* since they are sharing 85% of predicted coding regions. *P. putida* is missing key virulence traits, such as exotoxin A, phospholipase C, enzymes for synthesis of rhamnolipids, and type III secretion systems (22). Recently the genome has been re-sequenced and slightly re-annotated which resulted still in 21% of the genes with still unknown functions (20).

1.2 Stress tolerance of *P. putida*

P. putida KT2440 is exceptionally versatile in nutrient uptake due to the unusual number of nutrient acquisition systems such as oxido-reductases, dehydrogenases, mono- and dioxygenases, transferases, ferredoxins and cytochromes, and ferric siderophore transport systems. In addition it carries many extracytoplasmatic function sigma factors, two-component systems, regulators and stress response systems. Its genome encodes for 370 membrane transport systems such as ABC transporters and efflux pumps (13). *P. putida* has many multidrug efflux systems for extrusion and inactivating enzymes for toxic compounds in the environment, such as heavy metals, organic solvents, and antibiotics (25–27). The sigma factor σ^{70} is controls the expression of housekeeping genes while alternative sigma factors are responsive to various external and internal signals. There is an impressive high number of 24 sigma factors in the *P. putida* KT2440 genome (13).

P. putida KT2440 tolerates various heavy metals (28), carries many metabolic pathways for degradation of aromatic compounds (22, 29, 30), and tolerates the presence of various antibiotics, disinfectants, and detergents (13, 18). Its genome encodes 10 universal stress proteins, six cold shock proteins, five heat shock proteins, and 15 starvation-related proteins, which contribute to cell tolerance to stressors in the environment, such as the presence of xenobiotics and other toxic chemicals, temperature and pH changes, and limiting nutrient accessibility (13).

The *P. putida* KT2440 genome encodes a high number of 36 conserved IS elements (insertion sequences) with the majority being present in multiple copies. The IS elements ISPpu8, ISPpu9, ISPpu10, ISPpu11, and ISPpu13 are unique to the *P. putida* genome (11). IS elements are usually acquired via horizontal gene transfer and are associated with resistance and accessory functions. They cause genome rearrangements and mutations, which can be lethal or produce a beneficial mutation and a surviving mutant (31). *P. putida* KT2440 also has 61 putative genomic islands carrying many resistance and stress response genes. The abundance of IS and other mobile elements might be connected to the versatile metabolism of the KT2440 strain, which is able to adapt to various environments compared to other strains having many less of mobile elements and thriving in more specialized niches (11, 32).

Oxidative stress

Pseudomonas putida strains are able to thrive in conditions that are associated with oxidative stress, such as the rhizosphere or soil rich with metals and intermediate molecules generated during the breakdown of aromatic compounds (11). Oxidative stress can be also generated by antibiotics (33, 34) and during normal aerobic metabolism (35). Reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide

(H_2O_2), and hydroxyl radicals ($HO^\bullet$) cause oxidative stress and are dangerous to the cells because they cause mutations in the genome, inactivate enzymes, and disrupt cell membranes. As part of the defense against ROS bacteria encode for various stress sensing and regulatory proteins and detoxifying enzymes (36). *P. putida* encodes for two superoxide dismutases catalyzing superoxide (SODs: *sodA* and *sodB*); four catalases (*katA*, *katB*, *katE,* and PP_2887) and peroxiredoxin (*ahpC*) degrading hydrogen peroxide.

The stress responses are controlled through complex regulatory networks (37). Oxidative stress in *P. putida* KT2440 is regulated via stress-sensing proteins OxyR, FinR, and HexR, which activate oxidative stress defense genes, such as detoxifying enzymes, DNA repair mechanisms, and enzymes for NADPH production. The responses of *P. putida* differ from the ones of *E. coli* and *Salmonella* spp. (36).

Osmotic stress

P. putida is often found in polluted environments where it has to deal with different concentrations of various osmolytes. To tolerate osmotic stress and prevent cell lysis, the *P. putida* KT2440 genome encodes various systems for accumulation of osmoprotectants via either biosynthesis or transport (38–40). *P. putida* encodes uptake systems for compatible solutes such as glycine betaine or proline betaine and six members of the choline/carnitine/betaine transporter family (13). It also synthesizes various osmoprotectants *de novo* such as trehalose, mannitol (41), and N-acetylglutaminylglutamine amide (NAGGN) (42). Trehalose is electroneutral and stabilizes proteins and is therefore a major osmoprotectant in bacterial cells (43, 44). *P. putida* encodes two pathways for the synthesis of trehalose either from glycogen or maltose (20). Part of the cellular defense to osmotic stress is membrane composition alterations with increased production of cardiolipin and extrusion systems (such as RND efflux pumps, permeases, and transporters) (45).

Stress caused by antibiotics

Cells exposed to different antibiotics respond with induction of extrusion systems (transporters, efflux pumps, or permeases), oxidative stress defense mechanisms, specific degradation of the antimicrobials, altered targets of the inhibitor, and changed membrane permeability (46). A study on the transcriptional response of *P. putida* DOT-T1E to eight different types of antibiotics including the beta-lactam antibiotic ampicillin suggested that each antibiotic elicited a unique transcriptional response, where ampicillin, chloramphenicol and kanamycin were most similar to the untreated control (47).

1.3 Industrial potential of *P. putida*

P. putida exhibits a high biotechnological potential due to its high intrinsic resistance to various stressors, amenability to genetic modifications, fast growth on various substrates, and metabolic versatility. In addition *P. putida* KT2440 is generally recognized as safe (GRAS-certified) (Figure 1) (6, 19).

In the past, *P. putida* gained attention as a bacterium able to degrade oil and therefore as a potential bioremediation actor of petrol spills and as a promoter of plant growth due to production of siderophores, biosurfactants and antibiotics (48). In addition, different *P. putida* strains can metabolize various aromatic compounds, pesticides, herbicides, and explosives (6). It also stores excess carbon in intracellular polyester granules – polyhydroxyalkanoates (PHAs), which are biodegradable and have potential as a tissue engineering material and replacing the plastic derived from oil especially for packaging purposes (49).

Currently *P. putida* is becoming an efficient cell factory for production of industrially relevant compounds, such as biopolymers (PHA), industrially relevant enzymes, pharmaceuticals (antibiotics and antitumor compounds), plant-promoting compounds (biosurfactants and siderophores), and aromatic compounds (phenol, *t*-cinnamate, *p*-coumarate, *p*-hydroxybenzoate, phenylalanine, etc.), which are building blocks for valuable bioactive small molecules, resins, and polymers (9, 21, 50).

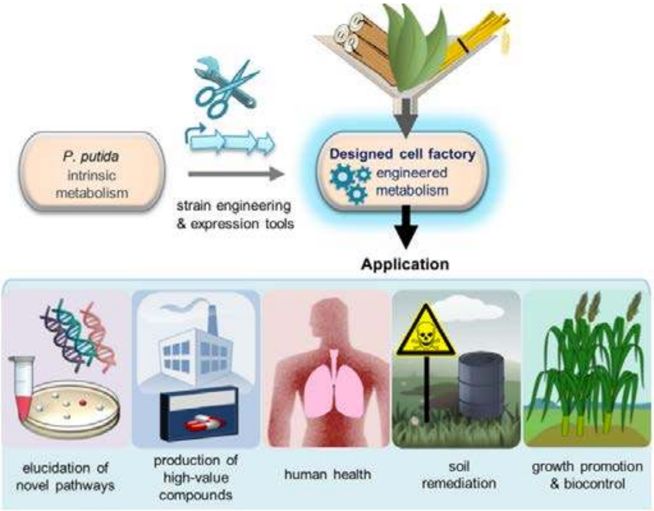

Figure 1: Perspectives in *P. putida* research and applications. Future improvements in toolbox and strain engineering will enable *P. putida* to become an efficient cell factory, which will use renewable substrates to produce added-value compounds (9).

The limiting factor of *P. putida* as a more widespread chassis is the lack of knowledge of its behavior under industrial and environmental conditions as well as the limited toolbox for genetic manipulation. The rational design of *P. putida* strains and expansion of the toolbox as well as in-depth analysis of its metabolism and regulatory networks raise possibilities for a wide application range of *P. putida* in the future (21, 51).

2 Small regulatory RNAs

2.1 RNA and the central dogma

The central dogma of molecular biology claims that the flow of genetic information goes from 'DNA to RNA to protein.' Such a unidirectional hierarchy has DNA on the top, which guides the functioning and adaptation of the biological systems (52). By controlling the DNA it has been believed that the biological system can be manipulated and dominated with the use of genetic engineering tools or direct DNA synthesis (53, 54). This approach has been widely used in engineering of the perfect cell factory for the production of the future building blocks (2). But such systems often fail or are difficult to maintain. A way to approach these problems is to influence the biological systems on the transcriptional and post-transcriptional levels using oscillators, toggle-switches, light-sensing, etc. (53).

RNA has been in recent years recognized as more than just a mere molecule in the middle of information transfer from DNA to protein (mRNA) or an actor in protein synthesis (transfer RNA – tRNA or ribosomal RNA – rRNA). Discovery of riboswitches, regulatory RNA molecules (in prokaryotes small regulatory RNAs and in eukaryotes snRNAs, siRNAs, miRNAs, hnRNA, piRNAs, lncRNAs, etc.), ribozymes, CRISPR, etc. together with the development of high-throughput sequencing have expanded the known roles of RNA. It has been established that RNA also carries biological functions. RNA can store information, catalyze reactions, and regulate gene expression and protein activity. There is a hypothesis that regulatory RNAs could also be spread between cells and generations (55, 56).

2.2 Regulatory RNAs in bacteria

Small regulatory RNAs (sRNAs) are RNA molecules, which together with regulatory proteins co-ordinate the cell machinery to cause the necessary changes and fine-tune bacterial physiology in response to environmental changes. sRNAs can modulate protein activity or base pair with mRNAs and regulate their stability and/or translation or, and in some cases mimic other nucleic acids. sRNAs are involved in various adaptation processes and influence many different aspects of bacterial physiology, virulence and behavior in cells. They are regulatory actors in transcription reprogramming, carbon metabolism, iron homeostasis, cell envelope homeostasis, quorum sensing, biofilm formation, motility and virulence (57, 58). Some sRNAs can also encode for small proteins and therefore carry dual functions. Some examples are SgrS in enteric bacteria (59), SR1 in *B. subtilis* (60), RNAIII in *Staphylococcus aureus* (61), or PhrS in *Pseudomonas aeruginosa* (62).

sRNAs vary in size with the majority being between 50-400 nt and having variable secondary structures (63). Base pairing sRNAs can be *cis*- or in *trans*-encoded. *Trans*-encoded transcripts are encoded at distant loci on the genome relative to their targets and regulate mRNAs by short and imperfect base pairing interactions (*cis*-encoded are described in the next section). In Gram-negative bacteria the RNA chaperone Hfq is often required for the activity and/or stability of this family of sRNAs. Hfq often protects sRNAs from degradation by ribonucleases and helps sRNA and mRNA anneal into a duplex (64, 65). It has been shown that the interaction region between sRNA and mRNA varies from 5 to 20 bases (66).

sRNAs are regulated on the level of their abundance, either via their synthesis and/or stability (57). They have a wide range of half-lives (<2 to

>32 min) indicating that generalizations cannot be made about their metabolic stability. On the other hand housekeeping RNAs (tRNAs, rRNAs) have longer half-lives and are more stable (67). sRNAs can base pair with their targets via stretches accessible in the loops or single stranded starches of the molecule. The region of base pairing is called seed region (Figure 2). Many sRNAs in enteric bacteria have been shown to have Rho-independent terminators (Rho IT) on their 3′ ends, which carry a hairpin structure with a loop followed by polyU stretch (68) but several sRNAs have also been found to be terminated by the transcriptional terminator Rho (69).

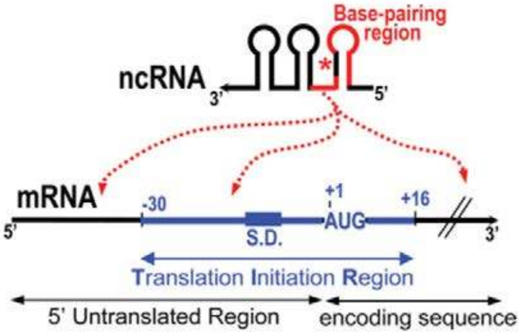

Figure 2: Different regions of mRNA regions can be targeted by sRNAs. The part of sRNAs base pairing to mRNA is indicated in red. The base pairing region contains parts (*) that do not interact with mRNA confirming the mismatches in the seed region. The sRNAs can base pair to the translation initiation region (usually from -30 to +16 relative to the start codon) or upstream of it, even deep in the coding regions or on the 3′ ends (57).

Base pairing sRNAs can regulate gene expression either negatively (70) or positively (71) (Figure 3). Negative regulation is often due to direct inhibition of translation initiation by binding close to the ribosome binding site (RBS) and thus inhibiting assembly of the translation initiation complex, which requires accessibility of a sequence stretch located between -35 to +19 relative to the start codon. sRNAs can also bind to the ribosome stand-by site or translation enhancer elements. Alternatively, binding of sRNA anywhere in the mRNA can promote endoribonuclease-mediated degradation of a target. On the other hand, sRNAs can also activate gene expression by stabilizing the mRNA and/or stimulating its translation. sRNAs can prevent formation of the inhibitory intramolecular structures in the 5′UTR of the mRNA. This mechanism is called an 'anti-antisense mechanism' and activates target translation. In addition, sRNA binding to the mRNA target can hide ribonuclease cleavage sites and thereby prevent mRNA degradation and promote mRNA translation. (71).

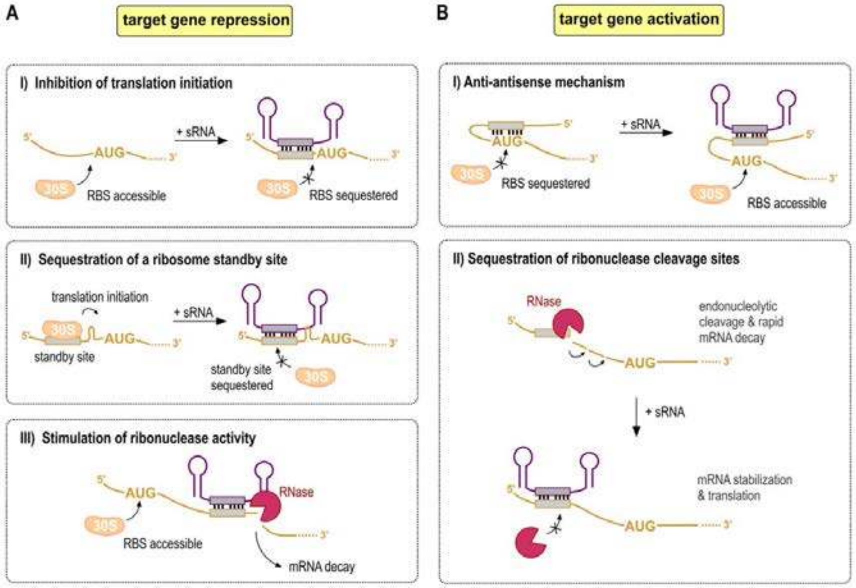

Figure 3: Mechanisms of gene regulation by base pairing sRNAs. (A) Mechanisms of repression of gene expression by sRNAs. (B) Mechanisms of activation of gene expression by sRNAs (63).

In some cases it is only mRNA being affected in the degradation process, yet other cases show sRNA being degraded together with mRNA. Translation can be affected as well and in that case both transcripts stay stable. The degradation of RNA or processing into stable transcripts occurs by RNase E, PNPase, or RNase III ribonucleases. (63, 68). RNase E is an endoribonuclease aiming for single-stranded RNA stretches. RNase III is also an endoribonuclease but cleaves double-stranded RNA duplexes. The decay of mRNA together with the sRNA with RNase III resembles the eukaryotic RNAi system. Exoribonuclease PNPase has also emerged as a regulator of sRNAs levels, often degrading sRNAs that do not have their 3′-ends protected by Hfq (72).

The synthesis of RNA is a lower metabolic burden to the cells than synthesis of proteins. It can be regulated faster and includes additional levels of regulation. RNA-mediated regulation has unique regulatory properties such as the fact that sRNA can be degraded together with the target. Regulation with sRNAs offers advantages over protein-based regulation (68, 73). Many mRNAs of the transcriptional regulators seem to be regulated by sRNAs, thus sRNAs regulatory networks can be vast. Such examples are *rpoS* encoding stress sigma factor (74), *csgD* regulating curli genes (75), and *lrp* involved in amino acid biosynthesis in *E. coli* (76); as well as *luxR* and *aphA* quorum sensing regulators in *Vibrio* spp. (77).

A few examples of characterized sRNA regulatory networks in different microorganisms are explained in more detail below.

Spot 42 is a highly abundant sRNA in *E. coli*, which regulates at least 15 genes connected to secondary metabolism, redox balancing and consumption of non-preferred carbon sources. Its transcription is inhibited when cAMP activates the cAMP receptor protein CRP, which in turn activates genes from transport and metabolism of non-preferred carbon sources (78). At the same time some of the Spot 42 mRNA targets are known to be regulated by other sRNAs, such as *maeA*, encoding NADH-dependent malate dehydrogenase being repressed by the sRNA FnrS (79), and *dppA*, encoding for an amino acid transporter that is repressed by the sRNA GcvB (80). This example shows how sRNAs can have wide regulons and impact many targets at the same time as well as how a single mRNA can be a target of several sRNA, adding to the complexity of the regulatory networks.

In *Pseudomonas* there is ErsA sRNA in the same genomic context as Spot 42 in *E. coli*, but does not function in carbon catabolite repression. ErsA reaches its highest level in stationary phase and is Hfq-bound just as Spot 42. It is under the transcriptional control of the envelope stress response σ^{22} and negatively influences the translation of *algC* mRNA. AlgC is a virulence-associated enzyme important for production of the exopolysaccharide alginate in *P. aeruginosa* (81).

Under nitrogen limitation, the intracellular levels of glutamine decrease and the two-component system NtrB/C induces the transcription of RpoN. RpoN is a global regulator involved in nitrogen metabolism, amino acid transporters, and carbon assimilation in *P. putida* (82), as well as in motility, quorum sensing, and virulence traits in *P. aeruginosa* (83). The NrsZ RNA is induced under nitrogen limitation by NtrB/C and RpoN. It is a processed transcript conserved among pseudomonads. NrsZ post-transcriptionally controls the *rhlA* gene in *P. aeruginosa*, involved in rhamnolipids synthesis. Rhamnolipids are surfactants and virulence factors needed for swarming. NrsZ and *rhlA* mRNA form a kissing-complex in the 5′UTR, which leads to activation of mRNA translation (84).

The ferric uptake regulator Fur is a transcriptional repressor and is essential for maintaining iron homeostasis (85). In *E. coli* Fur represses sRNA RyhB when iron is not limited. In iron-limiting conditions RyhB base pairs with target mRNAs and causes their degradation. Its targets are genes for bacterioferritins and some metabolic genes, as well as *sodB* mRNA, encoding a superoxide dismutase (86).

When there are more than one sRNAs with highly similar sequences in the same bacterium, they are called 'sibling sRNAs.' They can be redundant and exhibit identical regulatory functions or not (87). In *P. aeruginosa* two redundant sRNAs PrrF1 and PrrF2 are involved in iron homeostasis, central carbon and quorum-sensing regulation. They are also synthetized during iron-limiting conditions, where they base pair with RBS of mRNAs (eg. *sodB*, *katA*, etc.) and cause their degradation. PrrF sRNAs are functional homologs of RyhB although their nucleotide sequence is not similar. PrrF sRNAs are found only in pseudomonads (88, 89).

PhrS is expressed in stationary phase and is an Hfq-associated sRNA. It is under the positive control of the ANR regulator in oxygen-limiting conditions. PhrS activates PqsR synthesis, one of the key quorum-sensing regulators in *P. aeruginosa*. PhrS binds to the RBS of *uof*, which is translationally coupled to *pqsR*, and activates their translation. PqsR further activates gene expression for several virulence genes such as quinolone signal (PQS) and pyocyanin (PYO) (62).

Some sRNAs can modulate protein activity rather than base pair with RNA molecules (Figure 4). Some examples are 6S RNA, CsrB/RsmZ family of sRNAs, and CrcZ/CrzY. CsrB sRNA in *E. coli* has been shown to contain 22 GGA binding sites for the CsrA protein, encoding the carbon storage regulator. Csr and its homolog repressor of secondary metabolites Rsm either repress or activate expression of target mRNAs post-transcriptionally. They participate in central carbon flux, production of extracellular products, cell motility, biofilm formation, quorum sensing, and/or pathogenesis. CsrB sRNA sequesters CsrA's activity by acting as a direct competitor for CsrA target mRNAs (90, 91).

A homologous mechanism is present in *Pseudomonas* species with redundant sRNAs RsmX, RsmY, RsmZ sequestering the RsmA/E protein. In P. *fluorescens* there are all three sRNAs (92), while in *P. aeruginosa* and *P. putida* there are only RsmZ and RsmY (93, 94). The GacS/GacA two-component system is needed for activation of transcription of RsmX/Y/Z sRNAs. These sRNAs also carry GGA motifs as CsrB sRNAs and sequester RsmA/E proteins and its regulation of the mRNA targets (95, 96) Since these sRNAs are able to sequester, store and release RsmA/E, they act as ideal protein 'sponges' (97). In *P. aeruginosa* this system is involved in a switch between an acute to chronic state of infection, while in *P. fluorescens* the system is involved in the regulation of secondary metabolites and extracellular enzymes protecting plant roots (98).

6S/SsrS in *E. coli* forms a complex with housekeeping sigma factor σ^{70} and stabilizes the connection between RNA-polymerase (RNAP) and σ^{70} when it accumulates in stationary phase. When bound to a holoenzyme complex, 6S RNA mimics the open complex structure of promoter DNA. Hence, the transcriptional activity of the cells is changed and only a subset of σ^{70}-dependent promoters is being transcribed. Thereby 6S is inhibiting transcription of specific genes and indirectly favoring the transcription of RNAP-σ^{S}-dependent genes. The 6S sRNA is highly abundant and conserved across divergent bacteria, and it is likely that the mechanism is ubiquitous (99).

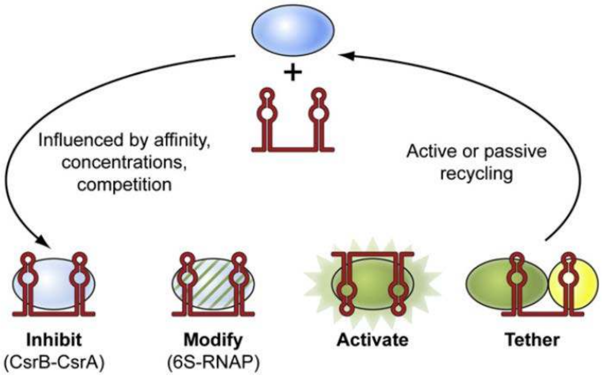

Figure 4: Mechanisms of action for protein-modulating sRNAs. They have been shown to inhibit and/or modify protein activity. It is also proposed that sRNA binding to proteins can bring more proteins together (68).

2.3 Antisense sRNAs

Antisense sRNAs (asRNAs) are encoded on the opposite DNA strand of their targets (*cis*-encoded) with which they share extensive complementarity. asRNAs have been found to impact mRNAs translation and/or stability and they usually range in size from ten to thousands of nt (100–102). Initially they were found encoded on plasmids, phages, and transposons (103). asRNAs have been shown to repress the synthesis of transposases and toxic proteins, regulate levels of transcription regulators, and impact metabolism and virulence (100).

asRNAs can overlap the target in the 5′- or 3′-end, in the middle or through the entire gene. They can alter transcription of the mRNAs, impact its stability by promoting or blocking cleavage sites for ribonucleases, or influence translation of the target. Bacterial asRNAs show similarities to *trans*-acting sRNAs regarding the mechanisms of action when base pairing with their target mRNAs with the difference that asRNAs can form more stable RNA duplexes due to longer complementarity shared with the target (100, 102). Recently, the excludon paradigm has been described in *Listeria* spp., where many unusually long asRNAs have been found. Excludons are an unusually long asRNA inhibiting the expression of one group of genes while enhancing the expression of a second group of genes (Figure 5) (104–106).

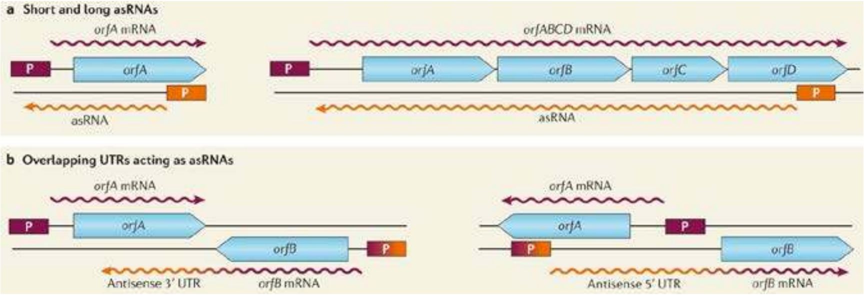

Figure 5: Various types of bacterial antisense sRNAs. (a) asRNAs can exist as autonomous transcripts of various sizes where they overlap one ORF or several ORFs. (b) Some mRNAs have very long 3′ and 5′UTRs thus they result as an asRNA to a neighbouring gene - excludons (105).

The numbers of asRNAs reported in bacteria vary extensively. Several have been characterized even though less focus has been put on them. The ranges of genes having antisense transcripts varies from 2-49% in the so far studied microorganisms of different species from *Bacillus, Pseudomonas, Escherichia, Helicobacter, Mycoplasma, Vibrio, Chlamydia, Staphylococcus, Salmonella* and *Sinorhizobium* (with the minimum detected in *Sinorhizobium meliloti* and maximum in *Staphylococcus aureus*) (107–119). One of the reasons for such differences could be due to the artifacts introduced by cDNA synthesis and amplification in cDNA library preparations (more reasons are described below in section 2.5). Such high numbers of antisense transcripts have to be taken with caution, since only several were confirmed by independent experiments, and even less characterized. It is possible that some of the antisense transcripts are byproducts of nonspecific transcription or read-through from flanking genes and are thus just noise or are experimental artifacts (100).

2.4 Regulatory RNAs come in many more flavors

Most of the sRNAs so far identified are independently expressed RNAs from intergenic regions (IGR) but there are several known cases where they originate from larger transcripts by processing (Figure 6). Primary transcripts carry 5′-triphosphate (5′ PPP), whereas processed transcripts possess a 5′ P (or 5′ OH, which is less common) (67, 120–122). During recent years many fragments derived from tRNAs, rRNAs, mRNAs, and riboswitches have been detected and shown to carry biological functions (123). Here various examples of RNA elements that play specific cellular roles are described.

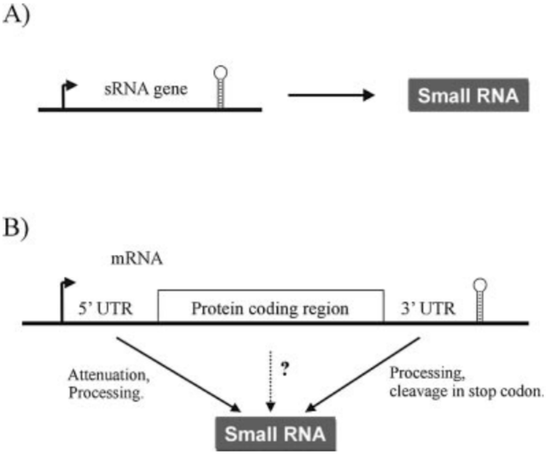

Figure 6: *Trans*-encoded sRNAs can originate from (A) their own sRNA gene in intergenic regions or (B) through a parallel transcriptional output with mRNA (67) among other options.

mRNA-derived fragments

mRNAs have been shown to be a source of various RNA fragments, which can carry regulatory roles in cells. They can derive from within mRNA (120), 5′-untranslated regions (UTR) or 3′UTRs and can be acting as normal *trans*-encoded sRNAs or have another mechanism of action. 3′UTR-derived transcripts can be functional RNAs, which has been observed in eukaryotes (124) and in prokaryotes (121). They can be independently transcribed (type I) or are processed from mRNAs post-transcriptionally (type II) (Figure 7). Many mRNA 3′ regions have been found to be enriched in co-immunoprecipitations (coIP) with the RNA chaperone Hfq in *Salmonella* and *E. coli* (125) as well as in *Vibrio cholera* (126). DapZ sRNA is a primary transcript abundant in the transition growth phase in *Salmonella* and is Hfq-associated. It is transcribed from a promoter upstream of the stop codon of its adjacent mRNAs. This sRNA acts in *trans* and represses the synthesis of ABC transporters during the invasion of the host cells (121). Another 3′UTR-derived sRNA is MicL in *E. coli*. It is transcribed from an independent promoter within the coding region of its adjacent gene and is further processed into an active sRNA. It downregulates an outer membrane lipoprotein Lpp and thus helps in reducing envelope tension under membrane stress conditions (127).

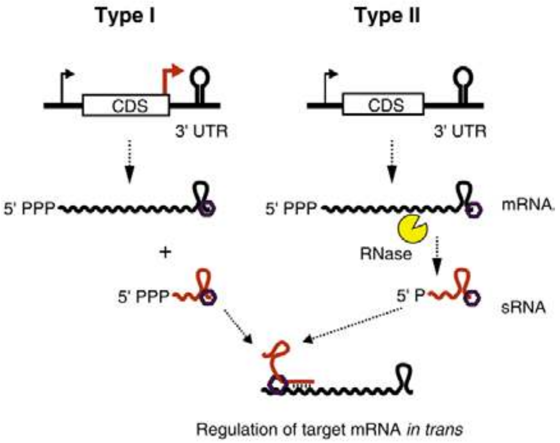

Figure 7: Two general pathways of biogenesis of sRNAs from the 3′ region of mRNA loci. The sRNA can be either transcribed from an mRNA-internal promoter (type I) or processed from its parental mRNA (type II). The sRNA and mRNA share Rho ITs and associate with Hfq (125).

One of the more exciting examples is found in *S. aureus* where a long 3′UTR region base pairs with the 5′UTR of its own mRNA *icaR* in Shine–Dalgarno sequence (SD). IcaR is a repressor of biofilm development, hence when *icaR* mRNA 3′UTR is bound to 5′UTR, the mRNA is exposed to RNaseIII degradation, and thereby induces biofilm formation (128).

In 5′UTRs the possible regulatory elements can be riboswitches, RNA thermometers and 5′UTR-derived sRNAs. An interesting example of a 5′UTR acting in *trans* as a regulator is found in *Streptococcus mutans*. Only a 5′UTR of mRNA *irvA* is needed to stabilize *gbpC* mRNA by base pairing in its coding-region. Therefore the *gbpC* mRNA encoding the surface lectin is protected from RNase-mediated degradation and virulence is induced. This mechanism is an example of a mRNA that not only encodes a protein but can also act in regulatory networks (129). Recently, the term actuaton was coined for sRNAs encoded in 5′UTRs of mRNA, where mRNA is transcribed as a read-through from the sRNA due to incomplete termination of transcription (130).

RNA thermometers are riboregulators that mediate temperature-responsive regulation of a downstream open reading frame (ORF). At low temperatures they form a secondary structure encompassing a RBS, thereby it is inaccessible to ribosome-binding. Upon raising the temperature the secondary structure melts and allows for translation of the gene. The majority of RNA thermometers control the synthesis of heat shock proteins and virulence (131). RNA thermometers can also induce the translation only at low temperatures, usually regulating cold shock proteins (132).

Riboswitches

Riboswitches are regulatory RNA elements present in the 5′UTR that regulate the expression of downstream genes in *cis* by changing their structural conformation upon presence or absence of the ligand. Riboswitches bind diverse ligands including metabolites such as glucosamine-6-phosphate, lysine, and glycine; coenzymes such as B_{12} and flavin mononucleotide; and ions such as magnesium and fluoride. They can either induce transcription termination or inhibit translation initiation in the presence of a ligand when acting as repressors. On the other hand when acting as activators the binding of a ligand induces the gene expression (Figure 8) (133, 134).

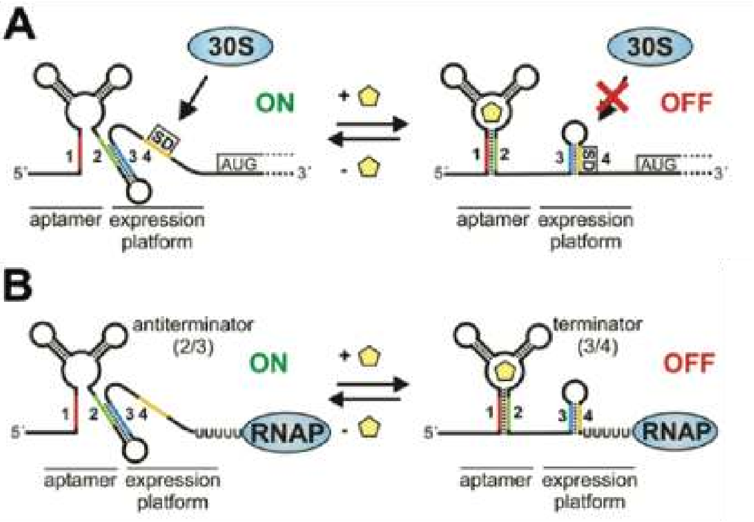

Figure 8: Mechanisms of riboswitches with the example of negative regulation upon metabolite binding. (A) Regulation of translation initiation when the metabolite is absent and a stem loop structure is formed, thus the SD is accessible and translation occurs. Upon binding the metabolite, the formation of the alternative stem loop structure sequesters the SD sequence and translation is inhibited. (B) Regulation of transcription termination when metabolite binding provokes the formation of the terminator structure, thereby terminates transcription. Noteworthy riboswitches that activate transcription and translation upon metabolite binding have the opposite effect (134).

Recently, ‘marooned’ riboswitches have been described in *Firmicutes*. They are ‘marooned’ in the genome without any associated gene to regulate. They can be antisense-oriented and instead regulate the transcription of an antisense RNA, which further regulates the expression of the genes antisense to it (135, 136). Furthermore, riboswitches can be located far from any ORF and regulate *trans*-acting sRNAs (137, 138). Additionally, riboswitches can influence the regulation of a nascent RNA with proteins such as exposing or hiding RNaseE cleavage sites (139) or promoting transcription termination with transcription termination factor Rho (140).

A novel and exciting level of regulation has been shown in *Listeria monocytogenes*, where two *cis*-acting riboswitches SreA and SreB when bound to its ligand S-adenosylmethionine (SAM) result in a premature termination, therefore preventing expression of downstream ORFs. But surprisingly, this terminated RNA fragment plays a regulatory role by acting in *trans* as sRNA on distant targets. It binds to the 5'UTR of a virulence regulator gene *prfA* and downregulates its expression. This way the same fragment is simultaneously regulating metabolism and virulence in *L. monocytogenes* (141).

tRNA derived fragments

Recently, RNA fragments excised during the tRNA maturation process have been found to have biological functions as sRNAs. They base pair with the sRNA RyhB in *E. coli* and inhibit its activity by acting as a sponge to absorb transcriptional noise of the sRNA. Hence, its mRNA targets are efficiently expressed (142). Similar mechanisms have been found in human cells (143).

2.5 sRNA discovery (prediction, detection, and characterization)

Initially, sRNAs were discovered by chance due to their high abundance in cells, such as 4.5S RNA, tmRNA, 6S RNA, RnaseP RNA, and Spot42 RNA. The first systematic searches for sRNAs were based on bioinformatics predictions by homology and structural conservation at the RNA level. Further IGRs were examined for specific elements that many sRNAs have in common, essentially orphan promoters, Rho ITs, and inverted repeat regions (144, 145). Although these approaches were very fruitful in enteric bacteria, they do have limitations because many sRNAs are conserved only in closely related species, and therefore not useful in more distant organisms where not much is known. Also many sRNAs do not have predictable promoters or terminators or have Rho terminators, which are difficult to predict (69). Additionally, many sRNAs are longer than the set size limits (usually up to 400 bp) or their antisense position of to ORF would fail to meet given criteria and could thus not be predicted (145).

Many sRNAs have been discovered during transcriptomic studies using microarrays, which have DNA probes for a defined set of genomic regions. Further tilling arrays were developed carrying up to thousands of DNA oligonucleotides systematically covering the sense and antisense strand of a genome, as well as IGRs, from where most known sRNAs are expressed. Such assays were used for many organisms and were able to successfully detect many predicted sRNAs under different conditions. Nevertheless, these assays have certain limitations, such as issues of probe labeling and cross-hybridization. In addition, tiling arrays are very expensive to be produced, are organism-specific, and have limited resolution (146, 147).

The recent developments of high-throughput technologies have revolutionized sRNA discovery (Figure 9). RNA sequencing (RNA-Seq) allows high-resolution assays of transcriptional changes and has revealed hundreds of regulatory RNAs in IGRs and also overlapping with the coding sequences in bacteria. When looking for sRNAs in RNomics approaches the RNA samples are often size-selected to enrich for the transcripts smaller than 500 nt by gel extraction. The protocols have been optimized during the years by depleting the RNA samples of small 5S rRNA and tRNAs, which represent the majority of RNA transcripts in the cells (147). Size-selected RNA is further reverse transcribed into cDNA and amplified by added adapters. cDNA library is sequenced (146) using any of the currently available high-throughput technologies such as 454 pyrosequencing (Roche), SOLEXA (Illumina) or SOLiD (ABI) (148, 149).

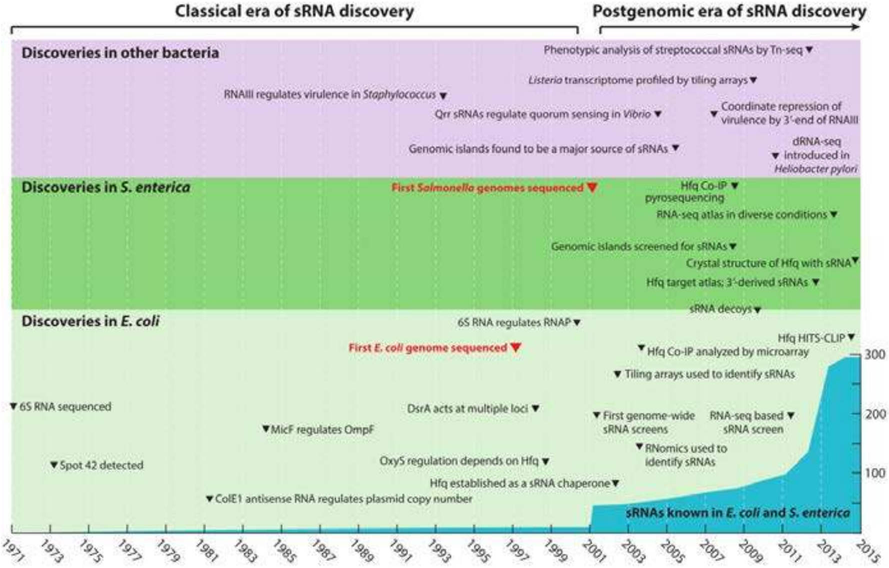

Figure 9: Discovery of sRNAs in the two most studied bacterial species *E. coli* and *S. enterica* with the timeline of influential studies is sRNA field. The y-axis shows the approximate accumulation of detected sRNAs in either *E. coli* or *S. enterica* over time (147).

Furthermore, differential RNA-Seq (dRNA-Seq) has been developed to identify the primary transcripts and distinguish them from processed ones (Figure 10a). This approach enables the genome-wide identification of transcription start sites (TSS). The 5′ monophosphate-dependent terminator exonuclease TEX is used to degrade processed transcripts and enriching for the primary transcripts. dRNA-Seq also allows to identify sRNAs (107)

Another approach to identify sRNAs is via the co-purification with proteins, since many cellular RNAs are associated with proteins. The most common bait for sRNA discovery has been the RNA chaperone Hfq (Figure 10b). Some of the first studies used polyclonal antisera against Hfq followed by hybridization to tiling arrays (150) or RNA-Seq (89, 151). This approach was further developed to tag the Hfq protein with a triple FLAG tag epitope on the chromosome (152) and analyze Hfq-associated RNA after co-immunoprecipitation (coIP) with a commercial monoclonal anti-FLAG antibody by RNA-Seq. Comparing coIP of the FLAG-tagged Hfq to control immunoprecipitation in a wild-type strain enabled the discovery of many sRNAs not detected by other methods as well as potential mRNA targets *in vivo* (153). The drawbacks of coIP with tagged-Hfq are possible nonspecific binding and unstable protein-RNA interactions during the experiments. Therefore further protocols to UV-crosslink RNA to the protein were developed (147).

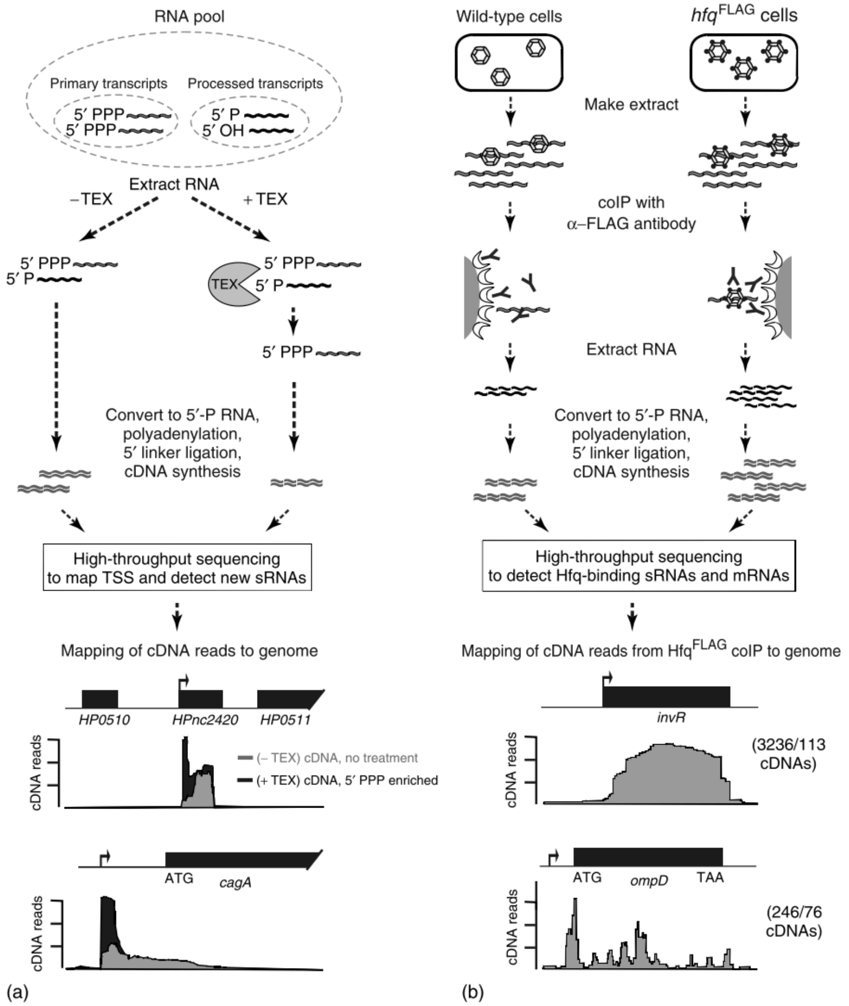

Figure 10: Detection of sRNAs using dRNA-Seq and Hfq coIP followed by RNA-Seq. (a) Cell transcripts are mostly either primary (5′ PPP) or processed (5′ P). Pimary transcripts can be enriched by TEX treatment (black) when comparing to untreated control (grey). (b) Identification of sRNAs and mRNAs by coIP to Hfq-FLAG-tagged protein with anti-FLAG antibodies, where a control sample is the untagged strain (154).

With recent developments many sRNAs have been predicted and detected by RNA-Seq. The next challenge is to validate the identified transcripts and determine their functions. Northern blot analysis has been the gold standard to independently experimentally validate sRNAs. Northern analysis is also able to provide the information about the size and potential processing events. Some clear limitations of Northern blots are that some sRNAs are expressed only in specific growth conditions or at very low levels. Further they can have complex secondary structures and prevent attachment of the probes, therefore in these cases sRNAs may not be detected (68, 145). sRNAs can also be detected by RT-PCR, primer extension or RNA protection (146).

Further it needs to be determined if the transcripts carry any biological functions in the cells or are some of them just noise. To date, very few candidate sRNAs have been functionally characterized. In addition there is a surprising difference between numbers of sRNAs reported and low overlap of them even in the same organism. The reasons likely contributing to this are the different conditions tested, cDNA library preparations and sequencing platforms used, different parameters and analysis pipelines incorporated (66–68, 155).

Small RNA targets can be found bioinformatically or experimentally. Often experiments include overexpression and deletion of sRNAs, but such experiments cannot distinguish between direct and indirect targets and can have downstream effects (such as toxicity, or over titrating proteins). However, some phenotypes associated with increased or decreased expression of a sRNA are subtle and can only be noticed under specific conditions, therefore many different conditions usually need to be tested in screenings (68, 146)

Bioinformatics can help to define an initial pool of target candidates that can then be experimentally validated (156). Experimentally, transcriptomic analysis of pulse-expressed sRNAs has become the standard to identify putative mRNA targets. Here sRNAs are induced for a short time (up to 15 min), just long enough to affect direct target mRNAs. Its drawback is that the targets need to be transcribed in the tested conditions and that it can only detect targets whose stability is affected by base pairing with sRNA (146). Further target verifications need to be validated through compensatory mutations in sRNA and its target sequence using a reporter system (like GFP or l*acZ*).

Thus far, the characterization of either base pairing or protein modulating sRNAs has been done on the individual sRNAs, therefore it will take many years to elucidate their roles (68).

2.6 sRNAs in *Pseudomonas* spp.

sRNAs exert many important regulatory roles in pseudomonads. Classical and highly abundant sRNAs such as 6S RNA, tmRNA, 4.5S RNA, and Rnase P are present and characterized in enteric bacteria and believed to have analogous functions in pseudomonads. Other sRNAs of *Pseudomonas* spp. have little or no sequence similarities to enteric bacteria (94, 98).

There have been some genome-wide searches for sRNAs in different species of this genus. In *P. aeruginosa* PAO1 and PA14, 573 and 233 sRNAs have been reported, respectively with 126 sRNAs overlapping in both strains (155, 157, 158). In *P. putida* KT2440 36 intergenic sRNAs have been previously detected out of which 22 are annotated sRNAs with homology in other *Pseudmonas* species (159). In *P. putida* DOT-T1E strain

154 *trans*-encoded sRNAs have been found in a RNA-seq study with 16 annotated (47). In *P. syringae* DC3000 strain 25 sRNAs have been detected (160) and in *P. extremaustralis* 14-3b 156 intergenic sRNAs have been reported (94, 161). There has been a gap in the number of transcripts observed in the reference strain *P. putida* KT2440 comparing to other pseudomonads, which has been addressed in this PhD thesis.

Some studies also focused on identifying asRNAs in pseudomonads. In *P. aeruginosa* 232 and 380 cis-encoded RNAs have been detected in different studies (110, 158) and in *P. fluorescens* 10 antisense transcripts have been reported (162). In *P. syringae* 124 genes had antisense transcription (160).

The sRNAs characterized in *Pseudomonas* species so far are: RsmY/RsmX/RsmZ, CrcZ/CrcX, PrrF1/PrrF2, PhrS, NrsZ, and ErsA (81, 84, 98, 163). Further there were some experiments made with PrrH and RgsA sRNAs but their regulatory networks are not well known. PrrH in *P. aeruginosa* is possibly having a role in iron storage and oxidative stress protection (89), while RgsA is associated with Hfq and may contribute to survival under oxidative stress in *P. aeruginosa* and also heat stress in *P. syringae* (164, 165).

The only functional characterization of the annotated sRNAs in *P. putida* KT2440 has been done with CrcZ/CrcY sRNAs (23, 166–168) These have been shown to bind and titrate Hfq, thereby preventing it from repressing the target mRNAs in *P. aeriginosa* PAO1 (169). For the rest of the sRNAs only a homology to known motifs does not necessary mean that they carry the same function in this strain. The majority of characterized sRNAs have been shown to have a function in pathogenic *P. aeruginosa* and are connected to its virulence, while *P. putida* KT2440 is an avirulent strain (22). For example, sRNA PhrS is an activator of PqsR synthesis, one of the key quorum-sensing regulators in *P. aeruginosa* but the PqsR protein is found only in *P. aeruginosa* strains (62). PhrS sRNA must have different targets in other strains and possibly also in *P. aeruginosa*.

3 RNA chaperone Hfq

3.1 General properties of RNA chaperone Hfq

Hfq protein has been first described as **h**ost replication **f**actor for the bacteriophage **Q**β in *E. coli* (170) and its importance was recognized when its deletion was shown to have severe phenotypic outcomes (171). Hfq is a homo-hexameric ring-shaped protein bearing similarities to eukaryotic Sm and Sm-like proteins, which carry RNA processing functions and primarily recognize U-long stretches (172). It is an abundant protein, estimated to be present at 10.000 Hfq-hexamers per cell with the majority being affiliated with ribosomes (173). The Hfq monomer is a small polypeptide ranging from 8-11 kDa in different microorganisms.

The Hfq protein is a highly conserved protein present in many bacteria and archaea and it is involved in modulating multiple cellular functions, including stress responses (Figure 11). The Hfq protein is a very influential global regulator of gene expression in bacteria but it is not essential. Homologs of *hfq* are lacking in ε-proteobacteria, like *Helicobacter pylori* and *Campylobacter jejuni* and in actinomicetales like *Frankia* and *Streptomyces*. As these organisms have active sRNAs but no Hfq homolog, it could be that there are other so far unidentified proteins in play or their sRNAs may also function via different mechanisms. Also some homologs may be less conserved and not identified via *in-silico* searches (174).

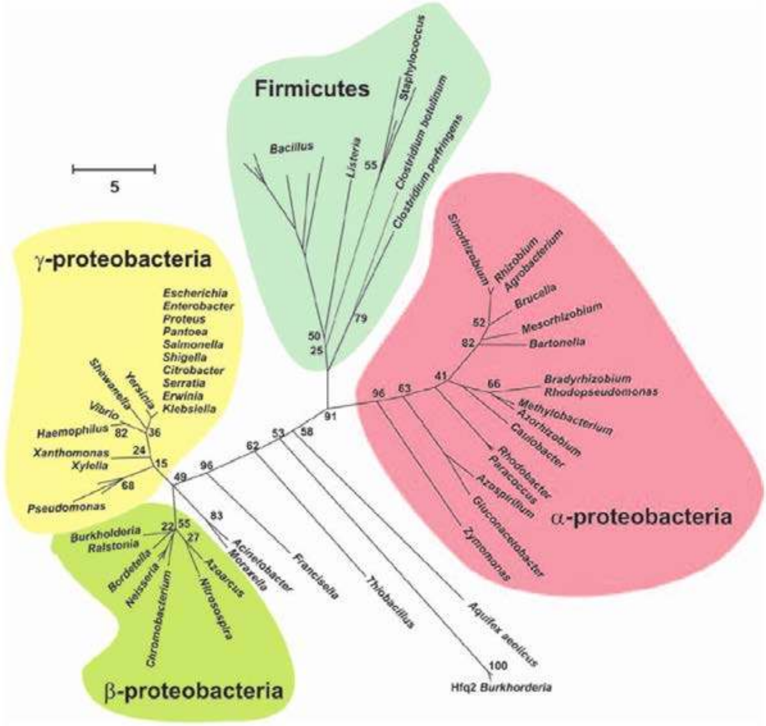

Figure 11: The presence of Hfq in bacterial taxa and the phylogenetic relationships among the Hfq proteins (174).

Apart from affecting the activity of transcription factors σ^E and σ^S (175, 176), the absence of Hfq results in pleiotropic phenotypic changes in various microorganisms, such as *E. coli, Vibrio cholera*, *Brucella abortus*, *Legionella pneumophila*, *L. monocytogenes*, *P. aeruginosa*, *P. putida*, *S. typhimurium*, *Francisella tularensis*, *Burkholderia cepacia*, *Shigella sonnei*, and *S. flexneri*. The absence of Hfq decreases the fitness of bacteria, stress tolerance against environmental changes, attenuates virulence, and impairs motility and quorum sensing (177–187). These defects are at least in part due to the fact that Hfq is required for function of many sRNAs (150).

Hfq has been shown to autoregulate its own synthesis at the translational level in *E. coli* and *S. meliloti*. Hfq binds to 5′UTR of its own mRNA and inhibits formation of the translation initiation complex and thus autorepressing its translation (188, 189). Also in the coIP experiments, the *hfq* mRNA has been found among the RNAs bound to Hfq in *Rhodobacter sphaeroides* also indirectly suggesting an auto-regulation (190).

The majority of Hfq studies have been done in enterobacterial models, thus there is a need to expand research on Hfq function in other taxons. For example Hfq does not seem to have the same function in *Firmicutes* as it does in enterobacteria. The Hfq absence does not affect growth in *L. monocytogenes* and *S. aureus* although it does somewhat reduce the stress tolerance and virulence in *L. monocytogenes* (177, 191).

3.2 Hfq in the game with sRNAs in regulation of gene expression

The RNA chaperone Hfq has been widely accepted as an essential RNA chaperone for the function of many base pairing sRNAs in numerous bacteria but detailed mechanism by which it promotes the pairing of RNAs remains ambiguous (192). There is evidence that Hfq (1) increases the stability of sRNAs *in vivo* and *in vitro*; (2) binds mRNA and sRNA and facilitates their base pairing by bringing them in the proximity; (3) changes structures of RNAs upon binding; (4) stabilizes sRNA-mRNA interactions; and (5) promotes negative sRNA-mediated regulation on gene expression by delivering the sRNA-mRNA pair to the degradosome (65, 70, 193). Hfq binds both base-pairing sRNAs and their target mRNAs in a random order (194, 195).

Hfq protein can contact with RNAs at four sites: proximal face, distal face, rim and C-terminal tail (Figure 12). Different RNA species bind to different parts of Hfq chaperone (193). In *S. aureus*, *E. coli*, and *L. monocytogenes* it was found that the proximal face of Hfq binds polyU sequences (172, 196, 197). PolyU is present in Rho ITs found in all sRNAs binding to Hfq (198, 199) thus uridine-binding pocket is a conserved characteristic of proximal face in Gram-negative and Gram-positive bacteria (193).

The distal face binds A-rich sequences (200) although there are differences in exact motifs in *E. coli* and *S. aureus* (201). A-rich sequences have been primarily found in Hfq-binding mRNAs, and the position of the A-rich motif on mRNA relative to the base pairing region is very important (202–204). Since many sRNAs also carry A-rich regions, they can as well bind to the distal face of Hfq. It has been also shown that rim is a secondary binding site for UA-rich sequences of sRNAs (199, 205–209) and some mRNAs (210). In addition also C-terminal tail seems to be important for interaction with some sRNAs (209). Altogether, Hfq is an active player in positioning the RNAs for optimal base pairing.

Thus the sRNAs binding Hfq are divided in two classes: class I sRNAs binding to proximal and rim domains of Hfq and base pairing with mRNAs binding to distal face; and class II sRNAs binding the proximal and distal faces of Hfq and base pairing with mRNAs binding on rim site of Hfq. The majority of sRNAs are in class I (205).

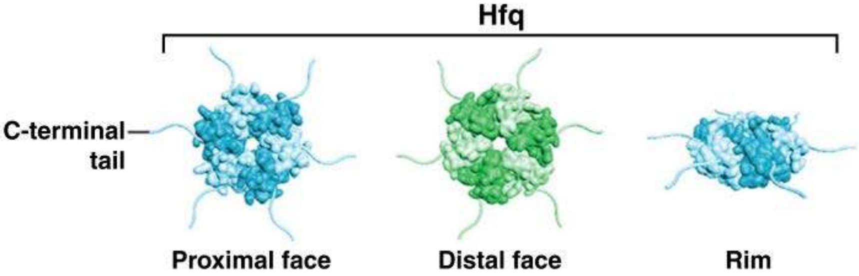

Figure 12: Structure of Hfq with proximal face, distal face, rim and C-terminal tail (193).

In *Enterobacteriaceae* Hfq stabilizes sRNAs and facilitates their pairing with mRNAs while in pseudomonads sRNAs and mRNAs coIP with Hfq, no evidence has been presented for Hfq involvement in the sRNA-mRNA interactions (84). On the other hand, pseudomonads have added flavor to Hfq's functions by pointing at its new role as a translational repressor of several catabolic genes. Two redundant sRNAs CrcZ and CrcY RNAs in *Pseudomonas* have been shown to be a part of a regulatory network in carbon catabolite repression, where cells adapt to changed nutrient availability. Previously it has been thought that these sRNAs bind to catabolite repression control protein Crc (168, 211), but the protein has been shown not to possess RNA-binding activity (212). Recently it has been shown that the main post-transcriptional regulator in carbon catabolite repression is actually the RNA chaperone Hfq. Hfq binds to A-rich sequences within the ribosome binding site and inhibits their translation. When sRNAs CrcZ is present, it sequesters Hfq and abolishes its translational repression on the catabolic genes (169). Furthermore Crc protein has been shown to cooperate by facilitating a stable complex of Hfq with its targets (213). This shows a novel function of Hfq as a global and direct post-transcriptional regulator of genes, where the sRNA target is Hfq and not mRNA and highlights the need of looking into various organisms to learn new aspects of sRNAs and Hfq.

Several bacterial proteins other than Hfq may have roles in sRNA-mediated regulation. Such proteins could act as RNA chaperones in addition to Hfq or could be implemented in riboregulation in species not carrying Hfq homolog. ProQ protein in *E. coli* has been suggested to be a RNA chaperone (214) as well as YbeY, which is ubiquitous in bacteria. YbeY shares structural similarities to the eukaryotic Argonaute protein and in *S. meliloti* influences gene expression similarly to Hfq (215).

4 Industrial possibilities of sRNAs

Synthetic biology has been using a variety of available technologies such as cloning, modulation of metabolic pathways, alterations of protein amino acid sequence, codon optimization, and more in order to construct cell factories (216). The vast majority of genetic systems engineered to-date have utilized protein-based transcriptional control strategies (3) but since sRNAs have been recognized for their role in important sensing functions and regulatory power in changing conditions there has been a growing interest in the design and implementation of synthetic RNA (Figure 13) (217).

RNA has many positive aspects to be used in synthetic biology, for example they are independently controllable and possible to be tightly fine-tuned. Additionally, their structures are easily manipulated; they are portable among different organisms, as well as modular and can affect any level of gene expression. They also represent a smaller energetic burden to the cell comparing to proteins and in addition RNA-mediated regulation acts generally faster than the protein-based (218).

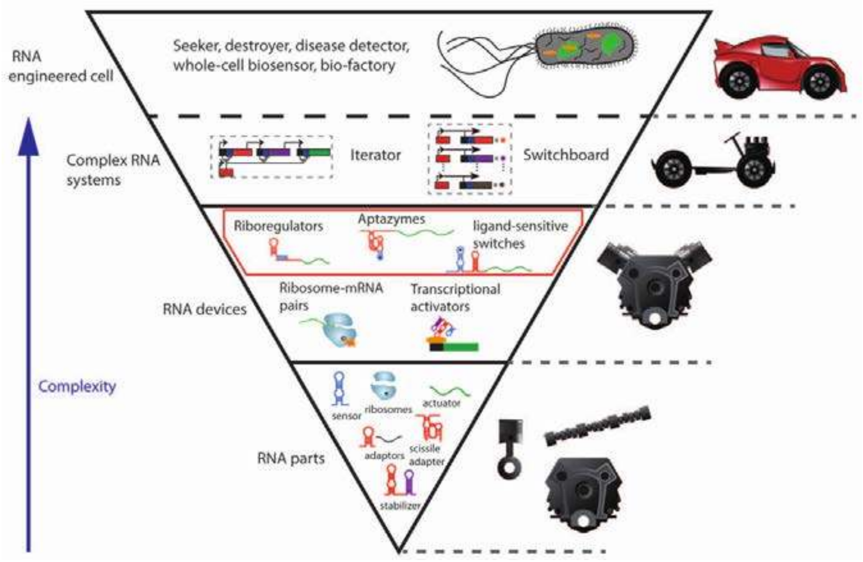

Figure 13: Use of synthetic RNA devices and parts in all kingdoms of life (216).

The first RNA elements exploited were riboswitches, where a variety of mechanisms have been discovered (133). The idea came from using natural mechanisms discovered and enhancing their roles. Riboswitches are highly sensitive on the ligands and can often distinguish between molecules with small differences, which can be exploited for the detection of various molecules and stimuli (217). Additionally, synthetic riboswitches can be constructed through aptamer selection to be responsive to ligands of choice and can be used to detect metabolites *in vivo* (133). Also RNA thermometers are interesting because they do not need a ligand for their activity. They have been exploited with the aim to be used as natural thermosensors and control gene expression (219). Furthermore RNA thermometers have been used as modular elements in synthetic RNA biology to produce thermozymes, able to modulate ribozyme activity, which shuts off gene expression at high temperatures (220).

A key property of sRNAs is that they can regulate multiple targets and thus switch on/off many metabolic pathways and responses to environmental cues at the same time. They are very precise in their target mRNA or protein recognition. sRNAs can also have many interactions and bind multiple proteins (216). sRNAs have thus been first used for the inhibition of target genes. In metabolic engineering antisense RNA strategies have been already used in many applications to inhibit growth when targeting essential genes, help in unraveling mechanisms of action of potential new drugs. In industrial scale they are useful to alter bacterial gene expression in order to optimize chemical and protein production and produce less byproducts (100). As such they have already been used to increase production of acetone and butanol or to reduce carbon flux to acetate and thus heterologous gene expression was increased. sRNAs are important in stress tolerance of the cells and can be exploited to improve strain tolerance in bioprocessing applications for example in prolonged fermentations or in toxic intermediates and/or products presence (221, 222).

Artificial sRNAs can be used as an alternative strategy for gene knock-outs, and can provide a wide range of regulation of gene expression (223). sRNAs can also be used in bioremediation and agriculture to seek and turn on the metabolic pathways of compound degradation (216).

sRNAs can be used as diagnostic tools as living sensors seeking disease sites. The sRNA promoters are very sensitive and responsive to any particular stress and could serve as reporters of conditions encountered by a cell (224). sRNAs can be exploited as antimicrobial therapies via their capability to base pair with basically any target in the cells, and such could interfere with pathogenesis by modulating the expression of virulence genes. Also many sRNAs have been found to be essential for survival of pathogens in the hosts or the adaptation to changed conditions. Taking advantage of these observations can be exploited for the use of sRNAs in medicine but are so far in the early stages with an additional major bottleneck in use of synthetic RNAs in the delivery to the host cells (68, 132, 216).

Looking at the possibilities of modular combinations of using RNA parts and their mechanisms in regulation of gene expression and further the capabilities to construct *de novo* RNA devices has vast biotechnical opportunities, which are limited only by our imagination. With further knowledge of new RNA elements, mechanisms of action and interactions, we will be able to rationally engineer RNA devices to benefit the human needs in the future.

5 Conclusions and future perspectives

P. putida is emerging as a future microbial cell factory for the production of added-value compounds but there is still a lot unknown about the behavior of the cells in stressful conditions and its regulation. This PhD work has used RNA-Seq technologies to investigate the transcriptome of *P. putida*. We gained detailed insights into the mechanisms and RNA elements through which *P. putida* KT2440 responds to different stress conditions and increased understanding of bacterial adaptation in natural and industrial settings.

In research article 1 the transcriptome of *P. putida* was investigated under osmotic, oxidative and membrane stress conditions, which are often encountered in the nature as well as in production bioprocesses. We tested the cellular response at the two time points of 7 and 60 min after the stress addition and identified many response mechanisms enabling survival of *P. putida*. In addition, many sRNAs were identified with differential expression in the chosen conditions, thus pointing that they could exert regulatory roles.

In research article 2 the sRNAome during the growth of bacteria was mapped and compared to the corresponding strain without Hfq protein. We found out that Hfq has a large impact on sRNAs and gene expression in *P. putida*, thus indicating dependency of RNA transcripts on the Hfq RNA chaperone. 199 sRNAs and 924 mRNAs (reperesenting 17.3 % of the genes) were found to be associated with Hfq *in vivo*.

In research article 3 dRNA-Seq technology was used to map transcription start sites in *P. putida*. Further 5′UTRs were investigated and many unusually long 5’UTRs were detected as well as several riboswitches. This approach allowed us to predict novel intergenic sRNAs not found in previously published studies.

Studies of sRNAs highlight that very little goes to waste in bacteria with mRNA cleavage products, tRNA processed fragments and terminated riboswitches having second lives as regulatory RNAs. Altogether these discoveries suggest that many other RNA fragments, pseudogenes, and cleavage products may be important regulatory elements yet to be discovered. Increasing numbers of sRNAs are being detected with the fast pace of high-throughput technology coupled with advancements in bioinformatics and many more are expected to keep emerging. We identified many sRNAs and mechanisms of stress responses in *P. putida* KT2440, which will help the design of a future cell factory. The next challenge lies in understanding their functions and roles in regulatory circuits, as this might unravel new functions or mechanisms of action. Such knowledge could provide important insights for potential biotechnological and therapeutic application of sRNA. Omics methodologies allow genome-wide insights and will in the future help in strain engineering with sRNAs, which can when combined with the traditional metabolic engineering approaches produce efficient cell factories.

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PAPER 1

Global transcriptional responses to oxidative, osmotic, and membrane 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 oxidative, osmotic, and membrane stress conditions at two time points was investigated via identification of differentially expressed mRNAs and sRNAs. A total of 440 small RNA transcripts were detected, where 10% correspond to previously annotated sRNAs, 40% are novel intergenic transcripts and 50% are 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 in at least one condition, suggesting possible functional roles in the cellular response to stress conditions. The data show a higher fraction of differentially expressed sRNAs with greater than 5-fold expression changes compared with mRNAs. 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 is to our knowledge the first investigation of the complete transcriptional response of *P. putida* KT2440 to oxidative, osmotic and membrane stress conditions including both short and long exposure times. A total of 440 small RNA transcripts are detected, consisting of both intergenic and antisense transcripts, increasing the number of previously identified sRNA transcripts in the strain by a factor of ten. 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 in at least one condition, suggesting their involvement in adaptation to stress conditions and identifying interesting candidates for further functional characterization.

Introduction

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, where the most abundant group is comprised of the extracytoplasmic function sigma factors (2). In addition, a wealth of two-component regulatory systems couples the sensing of environmental stimuli via a membrane-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 sRNAs are divided into two groups according to their genomic location relative to their target(s). The *cis*-encoded or antisense sRNAs 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 towards 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).

In this work, the complete transcriptional response of the well-characterized *P. putida* strain KT2440 to oxidative, osmotic and membrane stress conditions is mapped with RNA-sequencing. A total of 440 small RNA transcripts are detected, consisting of both intergenic and antisense transcripts, where over half are conserved within the *Pseudomonadaceae* family. Each type of stress is 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 as well as redox enzymes is observed. Specific alterations include an upregulation of beta-lactamase domain proteins under imipenem stress, induction of the SOS response and translational arrest under oxidative stress, and the accumulation of osmoprotectants and increased cardiolipin production under osmotic stress. The work identifies several small RNAs with differential expression in multiple stress conditions that are interesting targets for further functional characterization.

Materials and Methods

Bacterial strains, media and growth conditions

The *P. putida* KT2440 strain (DSM6125) was cultivated in M9 medium (per liter: $Na_2HPO_4 \cdot 12H_2O$, 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; $MnCl_2·4H_2O$, 30 mg; $CoCl_2$, 200 mg; $CuCl_2·2H_2O$, 10 mg; $NiCl_2·6H_2O$, 20 mg; and $NaMoO_4·2H_2O$, 30 mg) (15) 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 membrane 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 μg/mL were tested. For monitoring survival, 1 mL of the culture was harvested before as well as 1, 3 and 24 hours after compound addition. Colony forming units (CFU) were counted on LB chloramphenicol plates incubated at 30° C.

For RNA-seq experiments, the following compound concentrations were used: 3% NaCl, 0.05 mM H_2O_2 and 0.1 μg/mL of imipenem. The cultures grown in the same manner as described above were harvested 7 and 60 minutes 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 3 biological replicates.

Total RNA isolation

RNA extraction was performed as previously described (16). Briefly, 20 mL of harvested culture was mixed with 0.2 volumes of STOP solution (95% [v/v] ethanol, 5% [v/v] 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 Agilent 2100 Bioanalyzer (Agilent Technologies).

Library preparation and RNA sequencing

Transcriptome libraries (LIB>100) were constructed as previously described (16) 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) 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 and cDNA libraries were pooled together 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 was trimmed using Trimmomatic (17) and analyzed with the open source software Rockhopper with the default settings, choosing reverse complement reads and strand specific analysis (18) (version 2.0.3). The reads were mapped to the sequenced reference *P. putida* KT2440 genome (GenBank accession no. NC_002947.3). Using SAMtools (19) the mapped files were merged and the identification of novel transcripts was performed by visual inspection with Integrative Genomics Viewer (20), as Rockhopper detects many false positives. Differential gene and sRNA expression analysis were carried out with the webserver T-REx (21) using the RPKM values generated in the Rockhopper analysis, where 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 ≥ 2 and adjusted p-value ≤ 0.05. The Basic Local Alignment Search Tool (BLAST) with search criteria of query >80%, identity >60%, and e-value $<10^{-6}$ were used in sequence homology searches. The novel sRNA transcripts were analyzed for Rho-independent terminators and palindromes with the Pseudomonas genome database (22) and ARNold tool (23).

Accession numbers

RNAseq data has been deposited at the GEO Database under accession numbers: GSE85475.

Results

Experimental strategy

As bacteria are exposed to general stress conditions such as oxidative, osmotic and membrane stresses 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*, a RNA sequencing approach was used to investigate differentially expressed transcripts under oxidative, osmotic and membrane stress conditions in the well-characterized strain KT2440. *P. putida* KT2440 was grown in minimal medium in the presence of hydrogen peroxide, sodium chloride, or the cell wall-targeting antibiotic imipenem to induce oxidative, osmotic, and membrane stress, respectively. With the aim of applying the maximal stress without affecting cell viability, a series of growth experiments were carried out with a range of different compound concentrations to determine the pseudo-steady state condition (24), 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 OD_{600} and CFU counting, respectively (Fig. 1A-C). The final compound concentrations of 3% NaCl, 0.5 mM H_2O_2 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 minutes (T1) of growth to investigate early transcriptional responses and 60 minutes (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 the Illumina HiSeq platform. A total of 225 million reads were obtained, of which 200 million reads mapped to the *P. putida* KT2440 genome (Table S1).

Identification of small RNA transcripts

For small RNA identification, transcripts detected by Rockhopper (18) were manually curated using Integrative Genomics Viewer (20). One group of transcripts located in intergenic regions and having independent expression profiles relative to flanking genes was 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 was classified as *cis*-encoded antisense sRNAs. A total of 440 small RNA transcripts were identified in *P. putida* KT2440, significantly increasing the number of small RNA transcripts detected in this strain (25). A total of 45 sRNAs were either annotated or had homology to known RNA motifs (Rfam) (26) (Dataset 1). All homologous sRNAs in different *Pseudomonas* species (27) were detected. Seven copies of transcripts homologous to c4 antisense RNA (28, 29) 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 (Dataset 2) and denoted Pit001 to Pit178 for *Pseudomonas* ***p**utida* **i**ntergenic **t**ranscript based on their genomic coordinates. The transcripts range in size from 24 to 1790 nt, with an average of 174 nt in length (Fig.

2A). Eight transcripts (Pit023, Pit053, Pit059, Pit062, Pit067, Pit098, Pit109, and Pit110) are putative 3′-UTR-derived sRNA candidates that overlap with the 3′-end of the gene or are in very close proximity of the stop codon (30). Five transcripts (Pit014, Pit054, Pit057, Pit102, Pit108) are putative 5′-UTR-derived sRNA candidates or actuatons (31).

A total of 217 *cis*-encoded RNA transcripts were identified (Dataset 3) and denoted Pat001 to Pat217 for *Pseudomonas* ***p****utida* **a**ntisense **t**ranscript based on their genomic coordinates. These transcripts range in size from 21 to 1612 nt, with an average of 223 nt in length (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 the 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 (Dataset 2 and 3).

A total of 22 pairs of small RNA transcripts with complementarity in at least part of their sequences were found (Table S2) and could potentially be acting as RNA sponges (32, 33). The P30 transcript (34, 35) that is antisense to CrcZ and two antisense transcripts to CrcY, Pit118 and Pit119, were found. Antisense transcripts were also identified to the tmRNA/SsrA (Pit157, Pit158), RsmZ (Pit063), RsmY (Pit020), 6S/SsrS (Pit164), P24 (Pat203), PrrF2 (Pit144), rmf RNA motif (Pit090) and SRP/4.5S RNA (Pit145). An antisense transcript to PrrF2 has been reported previously in *P. syringae* (36). For 20 of these small RNA pairs, the transcripts are encoded just opposite to each other in the same genomic location, while in two cases the small RNA transcripts are encoded in distal genomic locations relative to each other (Pit146-Pit167 and Pit130-Pat180).

The novel sRNA transcripts found in this study were investigated for sequence conservation and homology in other bacteria using 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 (Dataset 3). Of all the 440 small RNAs 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 sRNAs transcripts are illustrated in Fig. 2C and show that the Pit (outer circle) and Pat (inner circle) transcripts are evenly distributed on the genome. In order to search for homology among the novel RNA transcripts, the small RNA 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 (37). 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 non-redundant regulatory functions (38).

General patterns of mRNA and sRNA differential expression under stress conditions

Each induced stress elicited a specific transcriptional response regarding the dynamics and extent of the gene expression changes (Fig.

3A-B). Osmotic and membrane stresses induced expression changes that increased with time while oxidative stress induced a strong immediate response that decreased after one hour. The numbers of differentially expressed mRNA and sRNA transcripts followed similar trends in the different stress conditions, The highest numbers of differentially expressed mRNAs and sRNAs compared to the control were observed under osmotic stress after 60 minutes, followed by oxidative stress after 7 minutes.

The extent of the observed expression changes under different stress conditions for mRNA and sRNA transcripts are summarized in Fig. 3C-D. The majority of mRNA transcripts exhibited 2-5 fold expression changes in all conditions and a higher proportion of mRNAs showed changes in this range compared to sRNAs. There was a higher fraction of sRNAs with above 5-fold expression changes compared to mRNAs in all stress conditions. Very high changes (above 100-fold) were observed for 4% of sRNA and 1% of mRNA transcripts during osmotic stress after 60 minutes.

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 minutes compared to 7 minutes after NaCl addition, with 2182 (40.8% coding sequences CDS) and 124 (2.3% CDS) differentially expressed genes, respectively (Fig. 3A, Dataset 4). Only 80 genes (3.8% CDS) are common to both time points, including several transcriptional regulators, and over half encode proteins of unknown function (Fig. 4A). 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.

As for the non-specific response to osmotic stress, the chaperones *groES*, *dnaK* and *dnaJ* (310-, 7-, 9-fold, respectively), heat shock proteins *hsp20* and *hsp90* (13- and 10-fold, respectively), and two universal stress proteins (PP_3237, PP_2187) were upregulated (around 8-fold). In addition, expression of *recA* and the catalases *katA* and *katE*, involved in the general response to the presence of reactive oxygen species (ROS), was increased (3-, 3- and 281-fold, respectively), while, interestingly, the cold shock protein *cspA* was decreased 6-fold. Moreover, 12 genes related to biofilm formation were >5-fold upregulated and flagella genes were downregulated (between -16 and -47-fold).

Specific responses to osmotic stress include the accumulation and biosynthesis of osmoprotectants as well as alterations in membrane composition (39, 40). The osmoprotectant operon *opuBC-BB-BA* for glycine/proline betaine uptake, the proline betaine MFS transporter *proP*, and two members of the choline/carnitine/betaine transporter family were significantly upregulated (above 5-fold). The trehalose synthesis pathway PP_4051-4054 (predicted *treZY*) and PP_4058-4059 (predicted *treS*) operons, the single-gene PP_4060 (alpha-amilase) and the glycogen metabolism genes PP_2918 and PP_4050 were highly expressed in osmotic stress. The two genes PP_1748 and PP_1750 with similarity to *P. aeruginosa* N-acetylglutaminylglutamine amide (NAGGN) biosynthetic genes (41) were highly upregulated at T2. Moreover, mannose synthesis was activated, with phosphomannomutase (PP_5288) and *algA* (PP_1277) genes upregulated 5- and 3-fold, respectively. The operon including the cardiolipin synthase 2 (PP_3264) involved in membrane alteration was strongly increased. Transcriptional changes in a number of transporters were observed including upregulation of RND efflux pumps (operon PP_5173-5175, PP_3302-3304, *ttg2* operon), permeases, and ABC transporters, as well as downregulation of several other transporter-related proteins (21 were downregulated > 5-fold).

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 minutes compared to 60 minutes after compound addition, with 1746 (32.6% CDS) and 814 (15.2% CDS) differentially expressed genes at T1 and T2, respectively (Fig. 3A, Dataset 5). Almost one-fifth (409) of the differentially genes at T1 also had changed transcriptional levels at T2 (Fig. 4B). The data show upregulation of several enzymes involved in ROS detoxification. The major catalase gene *katA* (PP_0481) was upregulated more than 900-fold at T1 and more than 20-fold at T2, while *katB* (PP_3668) was upregulated more than 30-fold at T1 and almost 6-fold at T2. In addition, the expression of the two hydroperoxide reductases *ahpC* (PP_2439) and *ahpF* (PP_2440) was very high at T1 (247- and 334-fold) and then decreased at T2 (2- and 4-fold). The *katA*, *katB*, and *ahpC* genes as well as genes encoding two thioredoxin reductases (*trxB*, *trx-2*) are under the control of the OxyR redox-sensing regulator (42). The data is consistent with the activation of the OxyR regulon in the presence of H_2O_2 (43).

Other notable changes are the upregulation of transcript levels of several redox enzymes, including cytochrome and quinone carrier proteins. Many ribosomal proteins were downregulated, whereas several membrane proteins, transporters, and DNA repair mechanisms were upregulated. Strikingly, taurine transport and metabolism was upregulated in T1, consistent with the role of taurine as an antioxidant and membrane stabilizer.

Differential expression of mRNAs under imipenem stress

The RNA expression profile of *P. putida* KT2440 exposed to imipenem showed a stronger response at 60 minutes compared to 7 minutes after compound addition. A total of 593 genes (Fig. 3A, Dataset 6) were differentially expressed, including 22 (0.4% CDS) at T1 and 571 (10.7% CDS) at T2 (Fig. 4C). The genes with the highest fold changes at T1 are membrane proteins including ABC and other transporters. At T2, 43 genes are upregulated and 12 are downregulated with above 5-fold changes. Interestingly, a cluster of genes PP_2663-PP_2682 was upregulated more than 5-fold, including 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_0375-0380) is related to the *pqq* genes involved in coenzyme PQQ biosynthesis that are also regulated by AgmR. 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 6-fold at T2.

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. 4D. 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 (Dataset 7) 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), redox and energy related proteins (cytochromes) as well as DNA repair proteins.

Differential expression of small RNAs

A total of 198 out of 440 sRNAs identified in this study were differentially expressed in at least one condition (Table 1; Table S5). The differentially expressed sRNAs are clustered into nine groups (Fig. 5;

Dataset 1, 2, 3) based on their expression patterns in the different conditions. Three groups of sRNAs exhibit different extents of upregulation in osmotic stress after 60 minutes. Cluster 8 consists of four sRNAs with exceptionally high levels of upregulation (greater than 2000-fold), cluster 6 consists of sRNAs with 100-2000 fold changes, and cluster 3 includes transcripts with less than 100-fold changes. Clusters 4 and 7 consist of sRNAs highly expressed under oxidative stress at T1, with some transcripts also being upregulated in other conditions (Table S5). The transcripts that are downregulated in 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 comprised of sRNAs that exhibit mixed expression patterns in the different conditions.

The expression profiles of selected annotated and novel sRNA transcripts exhibiting differential expression patterns are shown in Fig. 6. The expression profiles of the two sRNAs RsmY and ErsA are shown in Fig. 6A and 6B. 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. 6C-6F and two novel antisense RNAs are shown in Fig. 6G and 6H.

Only Pat107 sRNA (Fig. 6H) was differentially expressed and down-regulated in five out of six conditions. This sRNA is encoded opposite to the *ttgR* gene (PP_1387), which is a transcriptional repressor of the TtgABC efflux pump, which has been shown to mediate resistance towards several antibiotics and organic solvents (44). This gene was upregulated 3.1-fold in 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 in three conditions and encoded opposite to 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. 6A) and Pit020 sRNA, which are antisense to each other were both 4-fold down-regulated in three conditions.

Discussion

The stress conditions studied here induced extensive transcriptional changes in *P. putida* KT2440. Analysis of transcript levels at short and long stress exposure times provided a window into the dynamics of the responses, where osmotic and membrane stresses elicited changes that increased over time while oxidative stress triggered rapid expression changes that decreased with 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 choreographed series of changes 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, as well as methodology. A study in *P. aeruginosa* exposed to hydrogen peroxide after 10 minutes detected 33,7% differential expression (45), concurring with changes observed here and a similar study in *E. coli* (46). In another study where *P. putida* was subjected to the organic peroxides paraquat and cumen hydrogenperoxide, only 1.7% and 2.1% of genes were differentially expressed respectively (42), suggesting that addition of inorganic hydrogen peroxide causes more extensive changes in transcript levels as observed here. In a study where *P. aeruginosa* was subjected to osmotic stress, only 2.4% of genes were differentially expressed with >3-fold changes (41), but a much lower salt concentration was used compared to this work. Finally 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, where ampicillin, chloramphenicol and kanamycin were most similar to the untreated control (47). Taken together the extent of differential expression observed is dependent on the specific stressor, the degree of stress applied and the stress exposure time.

The major physiological processes affected in *P. putida* KT2440 under the different stress conditions studied are summarized in Fig. 7. Extrusion of molecules causing stress has previously been shown to be an important response for *P. putida* survival (12, 47–49). Indeed, changed transcriptional levels in several permeases, ABC and RND efflux pumps were detected in 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.

The present data show that the accumulation of glycine/proline betaine by import uptake system, and the biosynthesis of NAGGN, trehalose, mannitol, and glycogen are important strategies for *P. putida* KT2440 to respond to osmotic stress. NAGGN, mannitol and trehalose have been shown previously to be important compatible solutes in pseudomonads (41, 50, 51). The osmoprotectant NAGGN is notable as the genes for its biosynthesis were among the most upregulated genes in T2, supporting similar observations made previously for *P. aeruginosa* (41). In addition, an upregulation of iron-uptake mechanisms (siderophores) was observed here (15-46 fold), as reported previously for *Sinorhizobium meliloti* (52). The alteration of membrane composition by increasing cardiolipin content was confirmed in *P. putida* as these genes were highly upregulated. Upregulation of the cardiolipin biosynthetic genes has been observed previously in *B. subtilis* and *E. coli* (39). Finally, a downregulation of flagellar genes and an upregulation of biofilm formation was reported in salt-stressed *P. putida* (52–56). Motility reduction and biofilm formation seem to be a general bacterial response to osmotic stress.

P. putida has developed different mechanisms of oxidative stress sensing, regulation, and defense (43), among which upregulation of the detoxifying enzymes seems to be the most drastic change in the presence of hydrogen peroxide. Their expression is controlled by several regulators, such as OxyR, FinR and HexR, involved in protection against ROS. The two major oxidative stress regulators in *E. coli* and *S. typhimurium* are SoxR and OxyR (57). However, in *P. putida* SoxR regulator is not responsive to oxidative stress (42) and the oxidative stress defense-genes of the SoxR regulon in enteric bacteria such as *fpr*, *fumC-1*, *sodA*, and *zwf-1* are independent of SoxR in *P. putida* (58). Although these *P. putida* genes have been shown to be responsive to superoxide and nitric oxide (58) they are not activated in the presence of cumen hydroperoxide (42) or hydrogen peroxide as shown in this study. This suggests that their induction is dependent upon the specific compound causing oxidative stress.

The transcriptional levels of the transcriptional regulator OxyR that is constitutively expressed and activated by hydrogen peroxide were not affected, whereas changes were observed in the transcript levels of its responsive genes (*katA*, *katB*, *aphC*, *trxB*, *trx-2*, *hslO*) (43). The hydroperoxide reductase AphC has been shown to be inadequate for detoxification of high levels of peroxide (59), while the catalases are important for survival during oxidative stress (60, 61).

The upregulation of several SOS response genes (*lexA*, *recA*, and *recN*) was detected here at both time points during oxidative stress and after 60 minutes with osmotic stress. The SOS regulon is probably upregulated indirectly by H_2O_2 and NaCl by oxidant-induced DNA damage and a prolonged osmotic stress exposure. Similar changes have been observed in *P. aeruginosa* (45) and *E. coli* (46).

Antibiotics can induce oxidative stress in cells by increasing the levels of ROS, which inactivate various cell enzymes (43, 62, 63). Microarray studies in *P. putida* and *P. aeruginosa* showed that ampicillin activated oxidative-stress and SOS inducible genes (64). The upregulated gene cluster (PP_2663-2682) in cells exposed to imipenem was shown previously to be induced upon exposure to chloramphenicol (49), although these two antibiotics have different mechanisms of action. This region was also upregulated in cells exposed to hydrogen peroxide at T2 (14-116-fold), whereas some of these genes were downregulated during osmotic stress (4-44-fold). Upregulation of the PP_2669 gene has also been observed in the rhizosphere due to oxidative stress caused by antimicrobials in the environment (65), where the *pqq* genes are a part of the cellular defense to redox changes (66). This genomic region seems to be important in the response to oxidative stress and antimicrobials causing oxidative stress.

The beta-lactamase genes *ampC*, *ampG*, and *ampD* were not upregulated in the presence of the imipenem in this study. A longer exposure time may be needed to activate more pronounced changes in this specific response (67). On the other hand a beta-lactamase domain-containing protein (PP_2676) was upregulated 60 minutes after imipenem addition, suggesting that the degradation of antimicrobials is an important strategy.

This study reports the detection of 440 small RNA transcripts in *P. putida* KT2440, increasing the number of documented transcripts in this strain by over an order of magnitude. In a previous study on *P. putida* KT2440, 36 intergenic transcripts were detected, of which 22 correspond to annotated sRNAs with homologs in other *Pseudomonas* species (25). 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 (47). This is the first report of *cis*-encoded RNA in *P. putida*, with 217 asRNAs detected. In *P. aeruginosa* 232 and 380 *cis*-encoded RNAs have been detected in different studies (68, 69), and in *P. syringae* 124 genes had antisense transcripts (36). The numbers of genes having antisense transcripts or antisense transcription in other organisms ranges from 2-46% (7). In a recent study where transcription start sites (TSS) were mapped in *P. putida* KT2440, 36% of genes had antisense TSSs, but in this study antisense transcripts were only found to 3.3% of the genes (70). This discrepancy has also been observed previously in *E. coli* (71) and is likely due to variations in experimental conditions, cDNA library preparation strategies, data analysis pipelines and in the definition of an asRNA.

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) they were located in (25). Of these only 5 were detected in the present dataset (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: (1) different cDNA library construction methods lead to different transcripts detected; (2) some RNAs may be defiant to reverse transcription in the cDNA library construction and are thus underrepresented in the final dataset (16); (3) the detection method (Rockhopper) did not detect some transcripts; (4) certain sRNAs are expressed only in specific conditions and are thus easily missed. One example is the characterized sRNA NrsZ in *P. aeruginosa* with sequence homology in the *P. putida* KT2440 genome (72). 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 nitrogen source.

Nearly half of the small RNA transcripts identified in this study exhibit differential expression in at least one stress condition and can be divided into nine clusters depending on their expression pattern. 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 minutes 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 (73, 74). This concurs with a 17-fold upregulation of *algU* observed under osmotic stress after 60 minutes in this study. In addition, deletion of the gene in *P. syringae* leads to increased sensitivity to hydrogen peroxide compared to the wild type strain (74), 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 small RNA 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 (3,4,6,7,8) are located opposite to 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 membrane 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, where 40% have unknown function, and their involvement in a general stress response is an interesting area for future investigation. Moreover, the transcriptomic data collected here combined with proteomic studies could yield important insights into regulation at the posttranscriptional level, including the involvement of small RNAs.

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 in different stress conditions provides clues to their possible regulatory roles, and will aid 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.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Tables

Table 1: Small RNAs with differential expression in at least three out of six chosen conditions. The numbers indicate fold-changes for upregulated (+) and downregulated (-) transcripts, and lack of a number denotes no differential expression in that condition. IP stands for imipenem. All sRNAs with differential expression are shown in Table S5.

Name	NaCl T1	NaCl T2	H_2O_2 T1	H_2O_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		

Figures

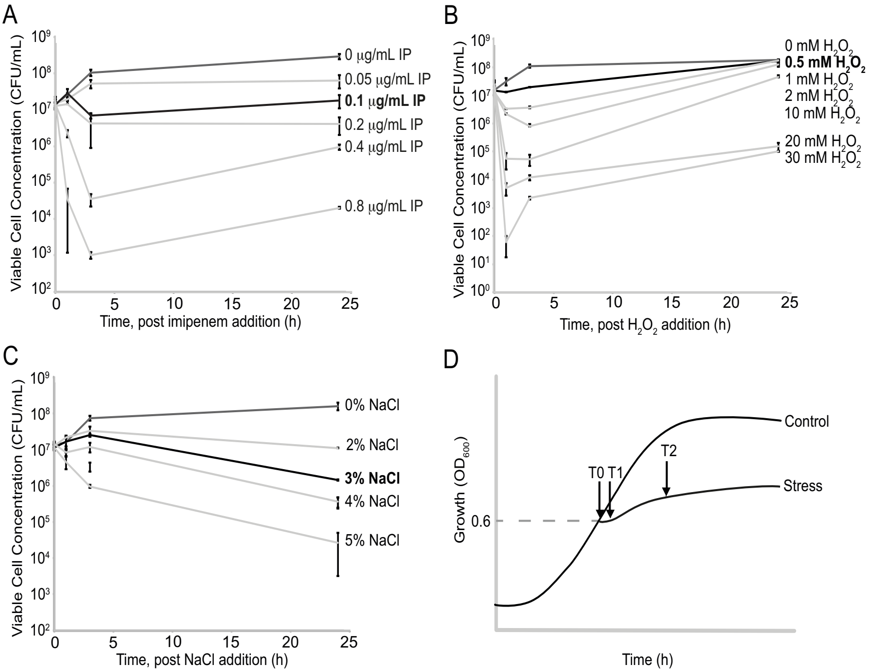

Fig. 1: Effect of the addition of stressors on *P. putida* KT2440 survival as determined by viable cell concentration (CFU). Compounds were added to mid-exponential stage cultures in different concentrations, as marked on the right of each graph. The CFU count data after compound addition is shown. The chosen concentration of each compound is indicated in bold. Effects of the addition of different concentrations of (A) imipenem, (B) H_2O_2, and (C) NaCl. (D) Representative growth curves for the chosen conditions. The stress experiments were performed by addition of the compounds in mid-exponential growth phase. Cells were harvested just before compound addition for the control (T0) and 7 minutes (T1) and 60 minutes (T2) after compound addition for the stress samples.

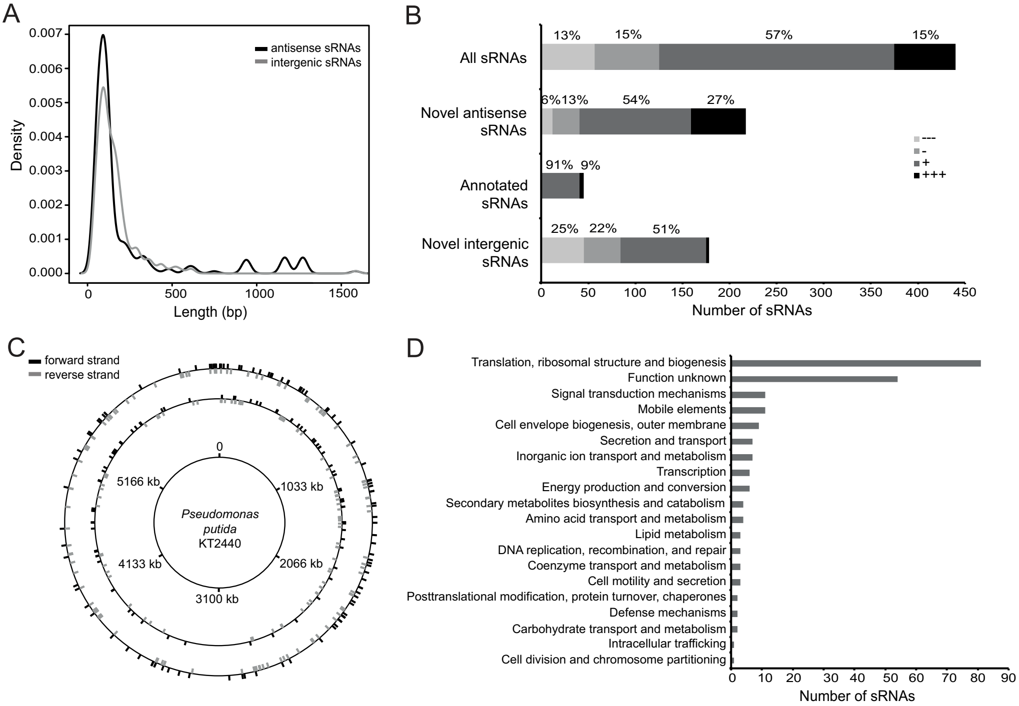

Fig. 2: Properties of the small RNA transcripts identified in *P. putida* KT2440. (A) Length distribution of intergenic and antisense sRNA candidates. (B) Conservation of novel sRNA candidates: (---) no sequence conservation found outside of the *P. putida* KT2440 strain; (-) no sequence conservation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

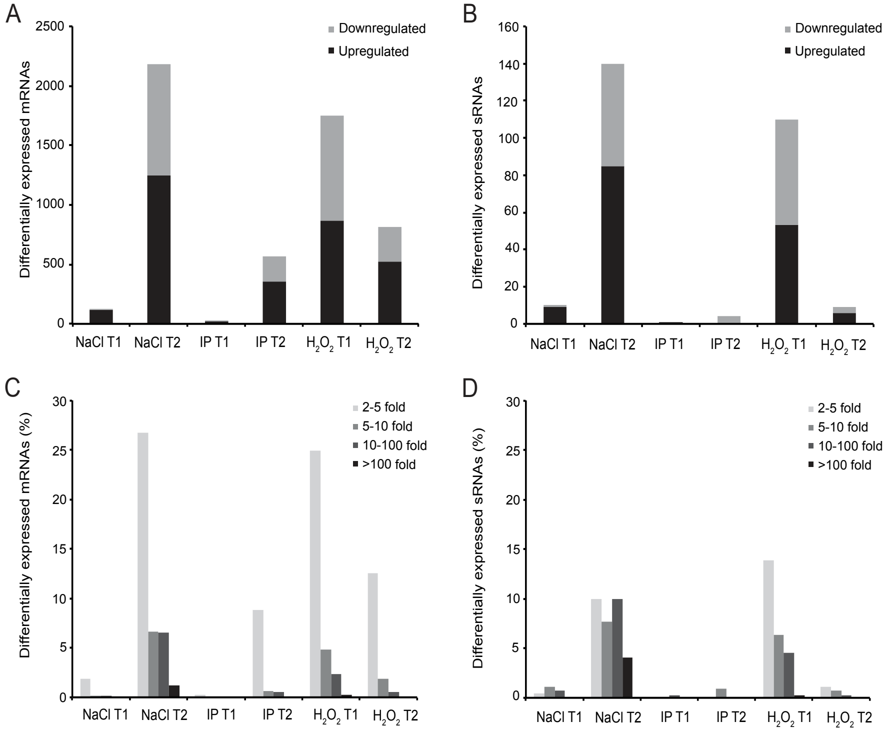

Fig. 3: An overview of the differentially expressed mRNAs and sRNAs. The number of differentially expressed mRNAs (A) and sRNAs (B) in osmotic (NaCl), imipenem (IP) and oxidative (H_2O_2) stress conditions at T1 (7 minutes) and T2 (60 minutes) compared to the control (without added stressor) are shown. The percentages of transcripts exhibiting different fold-changes in expression for (C) mRNA and (D) sRNA relative to the total number of 5350 CDS and 440 sRNAs, respectively.

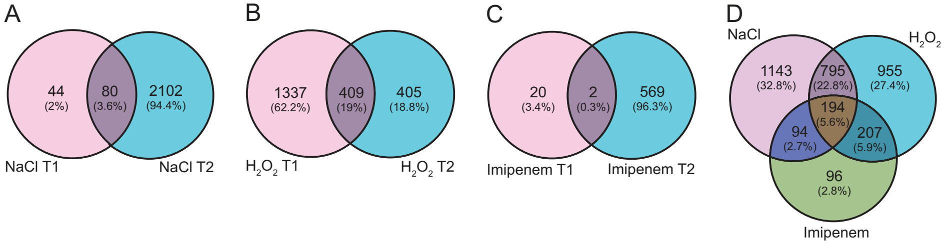

Fig. 4: Venn diagrams illustrating the number of differentially expressed genes under (A) osmotic stress (NaCl), (B) oxidative stress (H_2O_2), (C) imipenem (IP) stress and (D) in all three stress conditions. The proportions of differentially expressed genes in a certain type of stress condition are shown in parentheses.

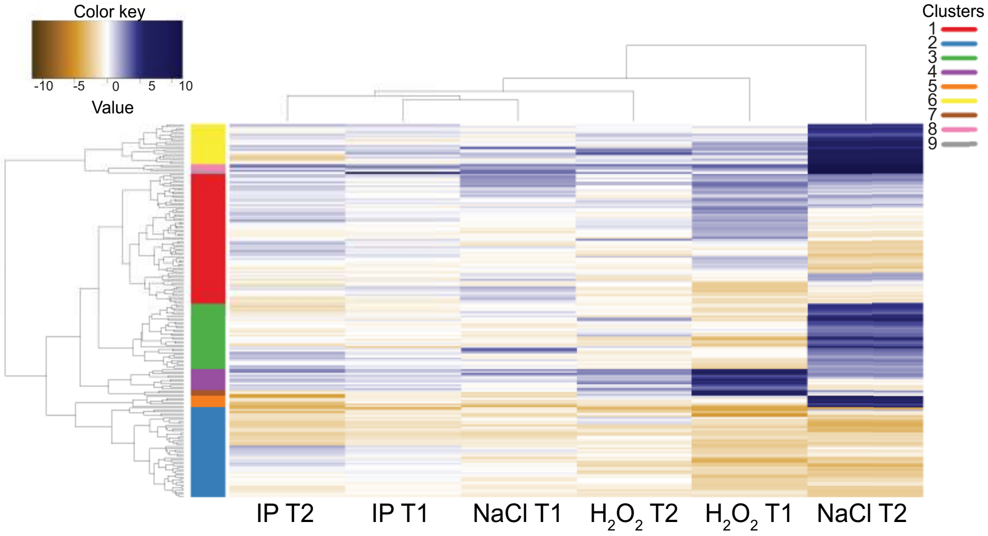

Fig. 5: Heat map and hierarchical clustering of differentially expressed sRNAs in osmotic (NaCl), oxidative (H_2O_2) and imipenem (IP) stress conditions at T1 (7 minutes) and T2 (60 minutes) after exposure compared to the control without added stressor (fold change ≥ 2 and a *p*-value ≤ 0.05).

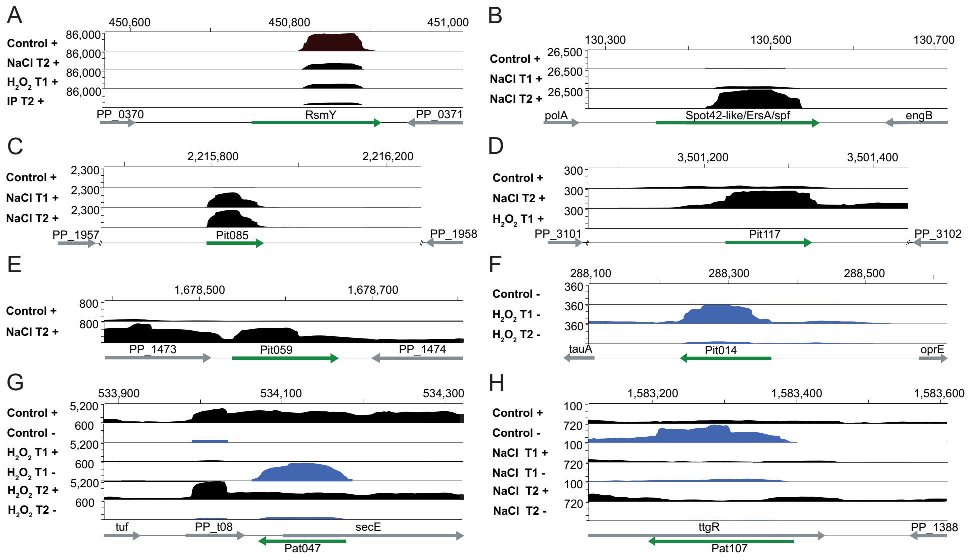

Fig. 6: Expression profiles of sRNAs in different conditions. The profiles include two annotated sRNAs RsmY (A) and Spot42-like/ErsA/spf (B), two novel intergenic sRNA candidates Pit085 (C) and Pit117 (D), a putative 3′UTR-derived sRNA candidate Pit059 (E), a putative 5′UTR-derived sRNA candidate or actuaton Pit014 (F), and two novel *cis*-encoded sRNA candidates Pat047 (G) and Pat107 (H). Reads on the forward (+) and reverse (-) strands are denoted 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 on the top.

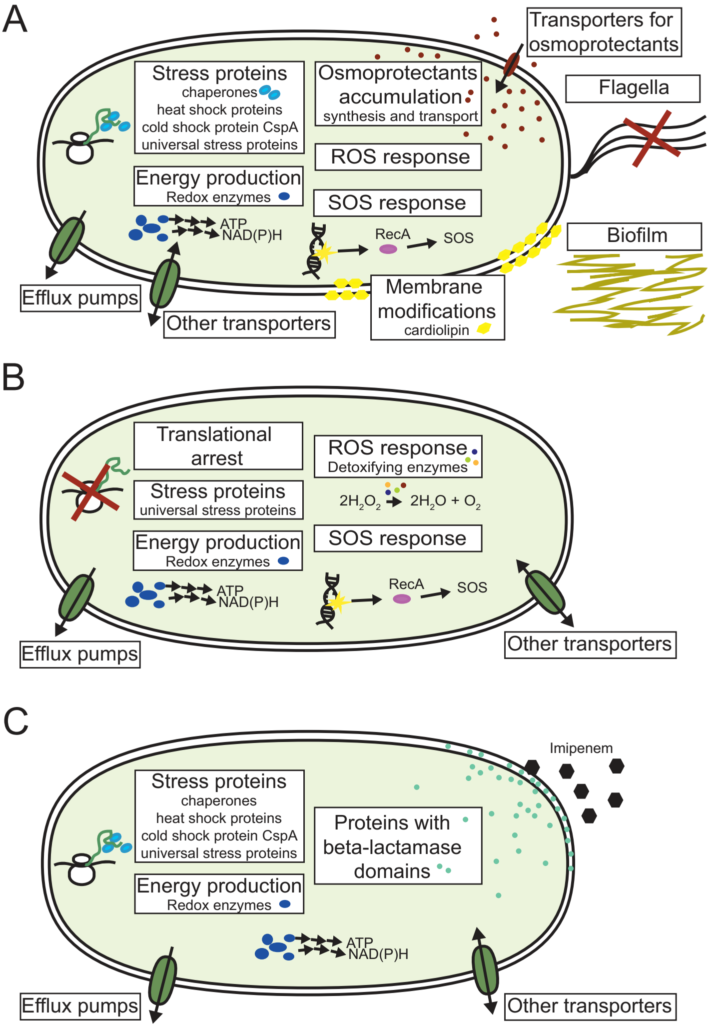

Fig. 7: Overview of selected cellular functions and processes with differential expression under (A) osmotic, (B) oxidative and (C) imipenem stress in *Pseudomonas putida* KT2440.

Supplementary Information

Table S1: Summary of cDNA libraries and read mapping.

Condition	Number of biological replicates	Library name	Total number of reads	Number of mapped reads
Exponential growth (control)	3	C_14_1	16,318,328	6696361 (41%)
		C_15_1	12,758,072	12587486 (99%)
		C_16_1	14,776,447	14671936 (99%)
H2O2 7 min	3	H2O2_9_1	10,230,319	9402826 (92%)
		H2O2_11_1	7,344,725	5472477 (75%)
		H2O2_12_1	8,416,513	5602970 (67%)
H2O2 60 min	3	H2O2_9_2	9,232,187	9179552 (99%)
		H2O2_11_2	21,055,607	17251667 (82%)
		H2O2_12_2	10,250,336	9144734 (89%)
Imipenem 7 min	3	IP_5_1	9,554,118	9493735 (99%)
		IP_7_1	11,684,566	11641443 (100%)
		IP_8_1	8,926,964	8896613 (10%)
Imipenem 60 min	3	IP_5_2	2,557,260	2518848 (98%)
		IP_7_2	6,985,922	6661039 (95%)
		IP_8_2	4,002,625	3960712 (99%)
NaCl 7 min	3	NaCl_1_1	11,575,480	10522745 (91%)
		NaCl_2_1	15,715,295	14079601 (90%)
		NaCl_3_1	11,906,584	10619489 (89%)
NaCl 60 min	3	NaCl_1_2	10,750,141	10668542 (99%)
		NaCl_2_2	11,697,539	11306426 (97%)
		NaCl_3_2	9,269,100	9190989 (99%)
Total			225,008,128	199,570,191

Table S2: Complementary sRNA transcripts in *P. putida* KT2440.

Nr.	Name	Strand	Name	Strand
1	Pit032	-	Pit031	+
2	Pit128	-	Psr2/CrcY	+
3	Pit129	-	Psr2/CrcY	+
4	Pit157	-	SsrA tmRNA	+
5	Pit158	-	SsrA tmRNA	+
6	Pit063	-	RsmZ	+
7	Pit164	-	6S/SsrS	+
8	Pit146*	-	Pit167*	-
9	Pit020	-	RsmY	+
10	Pit019	-	Pit018	+
11	Pit038	-	Pit037	+
12	P24	-	Pat203	+
13	Pit046	-	Pit045	+
14	Pit178	-	Pit177	+
15	Pit130*	-	Pat180*	-
16	Pit071	-	Pit070	+
17	Pit003	-	Pit002	+
18	P30	-	CrcZ	+
19	Pit144	-	Prrf2	+
20	Pit176	-	Pit175	+
21	rmf	-	Pit090	+
22	SRP/4.5S rRNA	-	Pit145	+

* These transcripts are antisense to each other but encoded in different genomic locations (all other pairs of transcripts are encoded opposite each other in the same genomic location)

Table S3: Novel sRNA transcripts conserved in organisms outside the *Pseudomonadaceae* family.

Nr.	Name	Orders	Classes
1	Pit138	Pseudomonadales/Methylococcales/Neisseriales	Gammaproteobacteria/Betaproteobacteria
2	FMN_RS	Pseudomonadales/Vibrionales/Pelagibacterales/Desulfuromonadales/Rhizobiales/Burkholderiales/Neisseriales/Xanthomonadales/Rhodocyclales/Oceanospirillales	Gammaproteobacteria/Deltaproteobacteria/Alphaproteobacteria/Betaproteobacteria
3	RNA21	Pseudomonadales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria
4	2_group_II	Pseudomonadales/Alteromonadales/Enterobacteriales/Alteromonadales/Oceanospirillales/Vibrionales/Burkholderiales/Desulfovibrionales/Desulfuromonadales/Rhodocyclales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria/Deltaproteobacteria
5	TPP_RS_1	Pseudomonadales/Rhodobacterales/Rhizobiales	Gammaproteobacteria/Alphaproteobacteria
6	Pit103	Pseudomonadales/Myxococcales	Gammaproteobacteria/Deltaproteobacteria
7	Pat004	Pseudomonadales/Alteromonadales	Gammaproteobacteria
8	Pat014	Pseudomonadales/Xanthomonadales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria
9	Pat017, Pat024, Pat039, Pat057, Pat086, Pat136, Pat199	Pseudomonadales/Myxococcales	Gammaproteobacteria/Deltaproteobacteria
10	Pat019, Pat026, Pat041, Pat059, Pat088, Pat139, Pat197	Pseudomonadales/Rubrobacterales	Gammaproteobacteria/Actinobacteria
11	Pat021, Pat028, Pat029, Pat043,	Pseudomonadales/Alteromonadales/Cellvibrionales/Burkholderiales/Enterobacteriales/Chromatiales	Gammaproteobacteria/Betaproteobacteria

	Pat061, Pat090, Pat141, Pat195		
12	Pat032	Pseudomonadales/Xanthomonadales	Gammaproteobacteria
13	Pat036	Pseudomonadales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria
14	Pat045	Pseudomonadales/Cyanobacteria/Flavobacteriales	Gammaproteobacteria/Nostocales/Flavobacteria
15	Pat049	Pseudomonadales/Enterobacteriales/Aeromonadales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria
16	Pat056, Pat127, Pat128, Pat129, Pat135, Pat200	Pseudomonadales/Enterobacteriales/Pasteurellales/Bacillales/Oceanospirillales	Gammaproteobacteria/Bacilli
17	Pat063	Pseudomonadales/Chromatiales/Neisseriales/Enterobacteriales	Gammaproteobacteria/Betaproteobacteria
18	Pat068	Pseudomonadales/Enterobacteriales/Burkholderiales/Deinococcales	Gammaproteobacteria/Betaproteobacteria/Deinococci
19	Pat093, Pat094, Pat095	Pseudomonadales/Enterobacteriales	Gammaproteobacteria
20	Pat104	Pseudomonadales/Aeromonadales	Gammaproteobacteria
21	Pat121, Pat122, Pat123, Pat172	Pseudomonadales/Enterobacteriales/Pasteurellales/Cytophagales/Bacteroidales	Gammaproteobacteria/Cytophagia/Bacteroidetes
22	Pat124	Pseudomonadales/Xanthomonas	Gammaproteobacteria
23	Pat141	Pseudomonadales/Cellvibrionales	Gammaproteobacteria
24	Pat147	Pseudomonadales/Rhodocyclales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria
25	Pat156	Pseudomonadales/Enterobacteriales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria
26	Pat157	Pseudomonadales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria
27	Pat158	Pseudomonadales/Enterobacteriales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria

28	Pat159	Pseudomonadales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria
29	Pat166	Pseudomonadales/Enterobacteriales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria
30	Pat176	Pseudomonadales/Alteromonadales	Gammaproteobacteria
31	Pat188	Pseudomonadales/Xanthomonadales	Gammaproteobacteria
32	Pat205	Pseudomonadales/Enterobacteriales	Gammaproteobacteria
33	Pat207, Pat208	Pseudomonadales/Rhodospirillales/Caulobacterales/Sphingomonadales/Actinomycetales/Fimbriimonadales/Spirochaetale/Rhizobiales	Gammaproteobacteria/Alphaproteobacteria/Actinobacteria/Fimbriimonadia/Spirochaetes/Actinobacteria
34	Pat215	Pseudomonadales/Alteromonadales/Burkholderiales/Xanthomonadales	Gammaproteobacteria/Betaproteobacteria
35	Pat216	Pseudomonadales/Chromatiales	Gammaproteobacteria
36	Pat216	Pseudomonadales/Burkholderiales	Gammaproteobacteria/Betaproteobacteria
37	Pat217	Pseudomonadales/Chromatiales	Gammaproteobacteria

Table S4: Homologous sRNAs transcripts in *P. putida* KT2440.

Nr.	Homologous sRNA transcripts								
1	Psr2/CrcY	CrcZ							
2	Prrf1	PrrF2							
9	2 group II 1	2 group II 2							
7	C4 AS RNA 3	C4 AS RNA 1							
3	Pit017	Pit126							
4	Pit024	Pit064	Pit092	Pit127	Pit153	Pit163	Pit169		
5	Pit105	Pit137	Pit049	Pit056	Pit124	Pit132	Pit154	Pit162	Pit106
6	Pit048	Pit055	Pit107	Pit125	Pit133	Pit155	Pit161		
8	Pit052	Pit051							
10	Pat121	Pat122	Pat123	Pat172					
11	Pat056	Pat127	Pat128	Pat129	Pat135	Pat200			
12	Pat207	Pat208							
13	Pat019	Pat026	Pat041	Pat059	Pat088	Pat139	Pat197		
14	Pat021	Pat028	Pat029	Pat043	Pat061	Pat090	Pat141	Pat195	
15	Pat017	Pat024	Pat039	Pat057	Pat086	Pat136	Pat199		
16	Pat015	Pat022	Pat037	Pat054	Pat084	Pat133	Pat202		
17	Pat083	Pat100	Pat154	Pat160	Pat193				
18	Pat018	Pat025	Pat040	Pat058	Pat087	Pat137	Pat138	Pat198	
19	Pat020	Pat027	Pat042	Pat060	Pat089	Pat140	Pat196		
20	Pat016	Pat023	Pat038	Pat055	Pat085	Pat134	Pat201		
21	Pat093	Pat094	Pat095						

Table S5: Differentially expressed sRNAs (fold change ≥ 2, p-value ≤ 0.05) in multiple stress conditions.

Nr.	Name	NaCl T1	NaCl T2	H_2O_2 T1	H_2O_2 T2	IP T1	IP T2
1	Pat107	-4.2	-13.5	-3.5	-3.5		-4.7
2	Pat044	8.7	7	71.5	7.6		
3	Pat077		-3.5	-2.9			-3.8
4	Pit020		-3.6	-3.8			-4.8
5	RsmY		-3.1	-3.7			-4.9
6	Pat110	6.8	6.1	4.2			
7	Pit116	5.5	5.8	4			
8	Pit087	5	8.1	2.9			
9	Pat181	4.8	4.7	7.6			
10	Pit082		-5.2	-3	-3.9		
11	Pit080		-12.8	-5.6	-4		
12	Pat190	6.6		8.8			
13	Pit085	18.2	30.8				
14	Pat126	13.5	11.6				
15	Pit046	10.4	10.2				
16	Pat092		3034.3			313.2	
17	Pat106		419.4		28.5		
18	Pat173			10.1	6.7		
19	Pat047			32.7	4.4		
20	Pat158		10.7		3.2		
21	Pat069		141.9	-14.9			
22	Pat215		55.9	4.9			
23	Pat102		49.2	-8.8			
24	Pat182		33	-3.7			
25	Pat149		32.3	-11.4			
26	Pat066		20.5	-3.3			
27	Pat104		18.4	-6.4			
28	Pat213		11.9	-3.2			

29	Pit119	11.8	3.2
30	Pit117	11.2	-3.9
31	Pit159	10.2	6.8
32	Pat209	6.7	97.8
33	Pat081	6	14.8
34	Pit118	6	-3.3
35	Pat101	5.5	45.7
36	Pit004	4.2	2.9
37	Pit122	3.8	-3.4
38	Pit034	3.6	10.1
39	Pit045	3	4.6
40	Pit171	3	20.6
41	Pit038	-2.4	3.6
42	Pat214	-2.6	2.6
43	Pit172	-2.9	2.3
44	2_group_II_1	-2.9	-3.6
45	2_group_II_2	-3.1	-4
46	Pat147	-3	3.1
47	Pit128	-3.2	-2.8
48	Pit073	-3.7	-12.2
49	Pit148	-3.8	3.3
50	Cobalamin_RS_1	-4.1	-6.1
51	Cobalamin_RS_2	-5	-3.9
52	Pat114	-4.1	2.4
53	Pat097	-4.5	-4.1
54	Pat115	-4.7	2.7
55	Pat098	-4.9	-4.1
56	Pit074	-5.1	-14.2
57	CrcZ	-5.3	-3.2
58	Pat169	-5.4	-2.6
59	Pat145	-6	-7
60	Pit094	-6.3	-6.3

61	Psr2/CrcY	-7.1	-3.2
62	Pit025	-7.2	-45.5
63	Pit079	-7.7	-5.1
64	Pat004	-7.8	-3
65	Pat205	2546	
66	Pat131	2143.1	
67	Pat171	2055.8	
68	Pat151	2014.5	
69	Pat008	606.5	
70	Pat073	398.2	
71	Pat109	357.2	
72	Pat119	345.6	
73	Pat148	292.2	
74	Pat211	290.7	
75	Pit057	199.7	
76	Pat186	197.3	
77	Pat053	127.3	
78	Pit059	117.2	
79	Pat067	109	
80	Pat070	77.5	
81	Pit123	68.9	
82	Pat156	52	
83	Pat165	47.1	
84	Pat010	46.2	
85	Pat157	44.2	
86	Pat206	41.8	
87	Pat071	40.1	
88	Pat014	39.1	
89	Pat117	37.4	
90	Pat091	30.2	
91	Pat068	26.9	
92	Pat204	26.8	

93	Pat194	26.2
94	Pat082	24.2
95	Pit022	21
96	Pat013	19.4
97	Pat180	19.1
98	Pit008	19
99	Pat116	17
100	Spot42-like/spf/ErsA	14.8
101	Pat144	12
102	Pit021	10.3
103	Pat009	10.3
104	Pat174	9
105	Pat183	9
106	Pat074	8.2
107	Pit086	7.3
108	P32	7.1
109	Pit066	6.9
110	Pit065	5.8
111	Pat096	5.5
112	Pat168	4.9
113	Pit047	4.7
114	Pit147	4.4
115	Pit102	4.2
116	Pit121	4
117	Pit089	3.8
118	Pat033	3.7
119	Pit143	-2.2
120	Pat178	-3
121	Pat078	-3
122	Pit002	-3.4
123	Pit033	-3.4

124	Pit113	-3.4	
125	Pat150	-3.5	
126	Pit052	-3.8	
127	P30	-4.1	
128	Pit069	-4.3	
129	Pat177	-4.3	
130	Pit139	-4.5	
131	Pat035	-4.5	
132	Pat099	-4.7	
133	Pat012	-5.2	
134	Cobalamin_RS_3	-5.3	
135	Pit053	-5.4	
136	Pit081	-7.1	
137	Pit006	-7.5	
138	TPP_RS_1	-8.4	
139	TPP_RS_2	-9	
140	Pat203	-10.7	
141	Pit129	-12.6	
142	P24	-13.7	
143	Pit035	-16.9	
144	Pit012		182.6
145	Pit013		79.8
146	Pat210		76.8
147	Pit096		74.7
148	Pat088		42.5
149	Pit014		19.1
150	Pit156		8.7
151	Pat075		8.4
152	Pit037		7.6
153	Pit115		7
154	Pit170		6.9
155	Pat001		6.8

156	Pat152	5.4
157	Pat064	5.1
158	Pit050	5
159	Pat207	4.6
160	Pat153	4.5
161	Pit099	3.9
162	Pat103	3.9
163	Pat034	3.8
164	Pit060	3.7
165	Pat048	3.7
166	Pat170	3.6
167	Pit030	3.1
168	Pit173	3.1
169	Pat079	3
170	Pat003	3
171	Pit044	2.8
172	Pit011	-2.4
173	Pit078	-2.7
174	Pat006	-3
175	Pit090	-3.2
176	C4_AS_RNA_1	-3.2
177	PhrS	-3.3
178	Pat175	-3.3
179	Pat062	-3.6
180	Pit039	-3.8
181	Pit005	-3.9
182	Pit068	-4.5
183	Pat105	-4.5
184	Pat185	-4.7
185	Pat007	-5.3
186	Pat124	-5.4
187	Pit003	-5.5

188	gyrA			-5.6			
189	Pit028			-5.7			
190	SAH_RS			-5.8			
191	Pat142			-6.4			
192	YybP-YkoY			-6.4			
193	Pat130			-6.6			
194	Pseudomon-groES			-19.1			
195	Pat094			-24.2			
196	Pit040				6.3		

* Upregulated transcripts are highlighted in red and downregulated transcripts are highlighted in blue. IP stands for imipenem. Empty spaces indicate no differential expression in that condition.

These datasets are too big to be shown in the thesis, but can be sent upon request.

Dataset 4: Differentially expressed genes (fold change ≥ 2, p-value ≤ 0.05) under osmotic stress conditions (T1 and T2).

Dataset 5: Differentially expressed genes (fold change ≥ 2, p-value ≤ 0.05) under oxidative stress conditions (T1 and T2).

Dataset 6: Differentially expressed genes (fold change ≥ 2, p-value ≤ 0.05) under imipenem stress conditions (T1 and T2).

Dataset 1: *Pseudomonas putida* KT2440 annotated sRNAs and candidate sRNAs with homologies in the Rfam database.

Legend:

Rfam - matches with known RNAs in the Rfam database are indicated

Blast - the sequence conservation of candidate sRNAs in other microbial organisms was investigated using BLASTN algorithm:

(---) no sequence conservation found outside of P. putida KT2440 strain; (-) no sequence conservation found outside of P. putida species;

(+) sequence conservation primarly in Pseudomonadaceae; (+++) sequence conserved in bacterial species outside the Pseudomonadaceae family

Cluster - number of the cluster from differential expression analysis of sRNAs (for more info see Figure 5)

Nr.	Name	Start	Stop	Length	Strand	Upstream flanking gene	Downstream flanking gene	Orientation	Rfam	Blast	Reference	Cluster
1	Spot42-like/spf/ErsA	130362	130561	200	+	PP_0123	PP_0124	> > <	Pseudomon-1	+	1, 2, 3, 4	4
2	gabT	264769	264873	105	+	PP_0213	PP_0214	> > >	gabT	+	4, 14	
3	c4 antisense RNA 1	335696	335870	175	+	PP_0277	PP_0278	< > >	C4	+	14	1
4	RsmY	450752	450916	165	+	PP_0370	PP_0371	> > <	RsmY	+	2, 3, 4, 7, 8, 1	2
5	P26	537405	537502	98	+	PP_0446	PP_0447	> > >	P26	+	2, 3,4, 8	
6	rpsL leader	546001	546170	170	+	PP_0448	PP_0449	> > >	rpsL_psuedo	+	10	
7	Alpha RBS	561399	561492	94	+	PP_0475	PP_0476	> > >		+	11	
8	FMN riboswitch	616507	616373	135	-	PP_0530	PP_0531	< < >	FMN	+++	2, 3	
9	c4 antisense RNA 2	759513	759682	170	+	PP_0651	PP_0652	> > <	C4	+	14	
10	YybP-YkoY	876097	875944	154	-	PP_0760	PP_0761	< < <		+	2, 3	2
11	PhrS	1316293	1316402	110	+	PP_1148	PP_1150	> > >	PhrS	+	5, 8, 13	1
12	2 group II 1	1425775	1425975	201	+	PP_1249	PP_1250	> > >	group-II-D1D4-3	+++	6	2
13	RnpB/P28/RNase P RNA	1512683	1513072	390	+	PP_1326	PP_1328	> > >	RNaseP_bact_a	+	2, 3, 4, 5, 13	
14	Pseudomon-groES RNA	1549132	1549255	124	+	PP_1359	PP_1360	> > >	Pseudomon-groES	+	14	2
15	t44	1785119	1785225	107	+	PP_1590	PP_1591	< > >	t44	+	2, 3, 4	
16	RsmZ	1822011	1822181	171	+	PP_1624	PP_1625	> > <	PrrB_RsmZ	+	2, 2, 4, 13	
17	Cobalamin riboswitch 1	1866975	1867159	185	+	PP_1671	PP_1672	< > >	Cobalamin	+	2, 3	2
18	gyrA	1970946	1970997	52	+	PP_1766	PP_1767	> > >		+	14	1
19	2 group II 2	2069323	2069493	171	+	PP_1845	PP_1846	> > >	group-II-D1D4-3	+++	6	2
20	RgsA/P16	2229834	2229726	109	-	PP_1967	PP_1968	> < >	P16	+	2, 3, 5, 8	
21	c4 antisense RNA 3	2303002	2302769	234	-	PP_2026	PP_2027	< < <	C4	+	14	
22	rmf RNA motif	2388741	2388343	399	-	PP_2095	PP_2096	> < >	rmf	+	3	
23	Cobalamin riboswitch 2	2765195	2765043	153	-	PP_2418	PP_2419	< < <	Cobalamin	+	14	2
24	c4 antisense RNA 6	2855911	2855757	155	-	PP_2507	PP_2508	> < >	C4	+	14	
25	P15	3466252	3466082	171	-	PP_3080	PP_3081	< < <		+	3, 4, 8	
26	TPP riboswitch 1	3613951	3614033	83	+	PP_3184	PP_3185	< > >	TPP	+++	6	2
27	Cobalamin riboswitch 3	3981922	3981816	107	-	PP_3508	PP_3509	< < >	Cobalamin	+	14	2
28	CrcY/Psr2	4013165	4013581	417	+	PP_3540	PP_3541	> > <	CrcZ	+	2, 3, 4	
29	PrrF2	4595123	4595325	203	+	PP_4069	PP_4070	> > >	PrrF	+	2, 4	
30	sucA-II RNA	4735743	4735637	107	-	PP_4189	PP_4190	< < <	sucA-II	+	14	
31	c4 antisense RNA 7	4856709	4856553	157	-	PP_4270	PP_4271	> < >	C4	+	14	
32	Bacteria_small_SRP/4.5S rRNA	4858513	4858392	122	-	PP_4273	PP_4274	> < <	Bacteria_small_SRP	+	2	
33	c4 antisense RNA 4/IGR 4535	5149065	5148926	140	-	PP_4534	PP_4535	< < <	C4	+	14	
34	PrrF1	5325394	5325493	100	+	PP_4685	PP_4686	> > <	PrrF	+	2, 3, 5	
35	CrcZ	5338210	5338622	413	+	PP_4696	PP_4697	> > >	CrcZ	+	2, 3, 4, 12	2
36	P30	5338614	5338287	328	-	PP_4696	PP_4697	> < >	CrcZ (-)	+	4, 8, 13	1
37	P31	5373151	5373213	63	+	PP_4724	PP_4725	< > <	P31	+	3, 8	
38	P32	5373351	5373255	97	-	PP_4724	PP_4725	< < <		+	3, 8, 13	4
39	SsrA tmRNA	5389943	5390415	473	+	PP_4738	PP_4739	> > >	tmRNA	+	2, 3, 4, 13, 15	
40	c4 antisense RNA 5	5390629	5390766	138	+	PP_4738	PP_4739	> > >	C4	+	14	
41	P24	5437810	5437675	136	-	PP_4775	PP_4776	< < >	P24	+	2, 4	2
42	TPP riboswitch 2	5596316	5596174	143	-	PP_4922	PP_4923	< < >	TPP	+	2, 3	2
43	SAH riboswitch	5667848	5667999	152	+	PP_4975	PP_4976	< > >	SAH_riboswitch	+	14	2
44	6S/SsrS	5934663	5934842	180	+	PP_5202	PP_5203	> > >	6S	+	2, 3, 4	
45	Pseudomon-Rho	5948619	5948465	155	-	PP_5214	PP_5215	< < <	Pseudomon-Rho	+	14	

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Dataset 2: Novel intergenic sRNA transcripts in *P. putida* KT2440 (Pit).
Legend:
Rfam - matches with known RNAs in the Rfam database are indicated
Blast - the sequence conservation of candidate sRNAs in other microbial organisms was investigated using BLASTN algorithm:
(---) no sequence conservation found outside of P. putida KT2440 strain; (-) no sequence conservation found outside of P. putida species;
(+) sequence conservation primarily in Pseudomonadaceae; (+++) sequence conserved in bacterial species outside the Pseudomonadaceae family
Cluster - number of the cluster from differential expression analysis of sRNAs (for more info see Figure 5)
Rho IT/palindrome - ** predicted Rho-independent terminator, * palindrome at the end or close to the end. Analysis was done using pseudomonas.com and ARNold tool.

Name	Start	Stop	Length	Strand	Upstream flanking gene	Downstream flanking gene	Orientation	Rfam	Blast	Rho IT/palindrome	Cluster
Pit001	9148	9338	191	+	PP_0009	PP_0010	< > >		+		
Pit002	16174	16490	317	+	PP_0013	PP_0014	> > >		+		1
Pit003	16401	16097	305	-	PP_0013	PP_0014	> < >		+		3
Pit004	32395	32468	74	+	PP_0028	PP_0029	< > >		+		1
Pit005	56009	55915	95	-	PP_0048	PP_0049	> < <		+		2
Pit006	58399	58537	139	+	PP_0049	PP_0050	< > <		+	**	1
Pit007	81289	81240	50	-	PP_0070	PP_0071	> < >		-	**	
Pit008	144095	144281	187	+	PP_0136	PP_0137	> > >		+		5
Pit009	193949	193866	84	-	PP_0167	PP_0168	< < >		-		
Pit010	194292	194042	251	-	PP_0167	PP_0168	< < >		-		
Pit011	220667	220867	201	+	PP_0168	PP_0169	> > >		+		2
Pit012	252160	252093	68	-	PP_0201	PP_0202	> < <		+		4
Pit013	252625	252294	332	-	PP_0201	PP_0202	> < <		+		
Pit014	288361	288232	130	-	PP_0233	PP_0234	< < >		-		4
Pit015	321949	321999	51	+	PP_0266	PP_0267	> > <		+		
Pit016	343759	343854	96	+	PP_0284	PP_t03	> > <		-		
Pit017	400370	400292	79	-	PP_0333	PP_0334	> < >		+		7
Pit018	410719	410971	253	+	PP_0339	PP_0340	< > >		+		
Pit019	410974	410866	109	-	PP_0339	PP_0340	< < >		+	*	
Pit020	450911	450813	99	-	PP_0370	PP_0371	> < <	RsmY (-)	+		2
Pit021	453808	453857	50	+	PP_0373	PP_0374	> > >		-		3
Pit022	453971	453994	24	+	PP_0373	PP_0374	> > >		+		6
Pit023	584075	584149	75	+	PP_0494	PP_0495	> > <	tRNA-Sec	+++	*	
Pit024	611072	610911	162	-	PP_0525	PP_0526	< < >		+	*	
Pit025	624137	623992	146	-	PP_0536	PP_0537	< < <		+	**	2
Pit026	*703168*	*703055*	114	-	PP_5SD	PP_0598	> < >		-	**	
Pit027	730413	730314	100	-	PP_0624	PP_0625	> < >		+	*	
Pit028	733317	733203	115	-	PP_0625	PP_t11	> < >		+	*	2
Pit029	736807	736761	47	-	PP_0628	PP_0629	< < >		+		
Pit030	750933	750980	48	+	PP_0640	PP_0641	< > <		---		1
Pit031	751819	752405	587	+	PP_0640	PP_0641	< > <		---	*	
Pit032	752374	752336	39	-	PP_0640	PP_0641	< < <		+	*	
Pit033	763543	763489	55	-	PP_0655	PP_0656	> < <		-		1
Pit034	813340	813376	37	+	PP_t17	PP_0700	< > <		+		1
Pit035	867937	868029	93	+	PP_0750	PP_0751	> > <		+	**	2
Pit036	1017579	1017521	59	-	PP_0877	PP_0878	< < <		-	*	
Pit037	1017648	1017873	226	+	PP_0877	PP_0878	< > <		-	**	1
Pit038	1017858	1017740	119	-	PP_0877	PP_0878	< < <		+		1
Pit039	1105372	1105328	45	-	PP_0965	PP_0966	> < >		-		2
Pit040	1142630	1142533	98	-	PP_1002	PP_1003	< < >		+		1
Pit041	1168594	1168633	40	+	PP_1024	PP_1025	> > <		-	**	
Pit042	1274834	1274769	66	-	PP_1115	PP_1116	< < <		---		
Pit043	1275799	1275106	694	-	PP_1115	PP_1116	< < <		---	**	
Pit044	1276407	1276534	128	+	PP_1115	PP_1116	< > <		---		1
Pit045	1278359	1278571	213	+	PP_1116	PP_1117	< > <		---		1
Pit046	1278408	1278264	145	-	PP_1116	PP_1117	< < <		---		3
Pit047	1280602	1281105	504	+	PP_1117	PP_1118	< > <		---		1
Pit048	1296787	1296600	188	-	PP_1132	PP_1133	< < >		+	**	
Pit049	1298337	1298477	141	+	PP_1132	PP_1133	> > <		+	*	
Pit050	1316513	1316462	52	-	PP_1149	PP_1150	> < >		-	*	1
Pit051	1349057	1349159	103	+	PP_1173	PP_1174	< > <		-	*	
Pit052	1349586	1349848	263	+	PP_1174	PP_1175	< > <		---		1
Pit053	1385249	1385161	89	-	PP_1205	PP_1206	< < <		+		2
Pit054	1388606	1388390	217	-	PP_1209	PP_1210	< < >		+		
Pit055	1440302	1440115	188	-	PP_1259	PP_1260	< < >		+		
Pit056	1441853	1441991	139	+	PP_1260	PP_1261	> > <		+		
Pit057	1474620	1474398	223	-	PP_1288	PP_1289	< < <		+		6
Pit058	1626891	1627080	190	+	PP_1426	PP_1427	< > >		+		
Pit059	1678539	1678662	124	+	PP_1473	PP_1474	> > <		+	**	6
Pit060	1749031	1749276	246	+	PP_1548	PP_1549	< > <		+		1
Pit061	1777458	1777309	150	-	PP_1584	PP_1585	> < <		---	**	
Pit062	1804568	1804667	100	+	PP_1607	PP_1608	> > >		-	*	
Pit063	1822122	1822033	90	-	PP_1624	PP_1625	> < <	PrrB_RsmZ (-)	+		
Pit064	1847248	1847030	219	-	PP_1652	PP_1653	> < >		+	*	
Pit065	1884018	1883852	167	-	PP_1691	PP_1692	< < >		+		3
Pit066	1915700	1915801	102	+	PP_1714	PP_1715	> > >		+	**	3
Pit067	1970749	1970818	70	+	PP_1766	PP_1767	> > >		+	*	
Pit068	2005781	2005913	133	+	PP_1788	PP_1789	< > <		---		2
Pit069	2016842	2016692	151	-	PP_1795	PP_1796	< < >		---		1
Pit070	2034336	2034551	216	+	PP_1808	PP_1809	> > >		-		
Pit071	2034605	2034311	295	-	PP_1808	PP_1809	> < >		-		
Pit072	2034963	2034866	98	-	PP_1808	PP_1809	> < >		-		
Pit073	2035721	2035682	40	-	PP_1809	PP_1810	> < >		-		2
Pit074	2038067	2038012	56	-	PP_1810	PP_1811	> < <		+	*	2
Pit075	2087886	2087954	69	+	PP_1865	PP_1866	> > <		---		
Pit076	2140113	2140023	91	-	PP_1896	PP_1897	> < >		+		
Pit077	2151206	2150992	215	-	PP_1905	PP_1906	< < >		+		
Pit078	2164081	2164133	53	+	PP_1919	PP_1920	> > <		---		1
Pit079	2182192	2182334	143	+	PP_1935	PP_1936	< > <		---		2
Pit080	2182483	2182584	102	+	PP_1935	PP_1936	< > <		---		2
Pit081	2182663	2182994	332	+	PP_1935	PP_1936	< > <		-		1
Pit082	2183421	2183690	270	+	PP_1935	PP_1936	< > <		-		2
Pit083	2183930	2184046	117	+	PP_1935	PP_1936	< > <		---		
Pit084	2188900	2188412	489	-	PP_1936	PP_1937	< < >		-		
Pit085	2215785	2215916	132	+	PP_1957	PP_1958	> > <		---		6
Pit086	2216128	2216221	94	+	PP_1957	PP_1958	> > <		---		3
Pit087	2217102	2217166	65	+	PP_1957	PP_1958	> > <		---		1

Pit088	2256062	2256155	94	+	PP_1989	PP_1990	> > >		+	*	
Pit089	2273192	2273308	117	+	PP_2003	PP_2004	> > <		+		1
Pit090	2388431	2388527	97	+	PP_2095	PP_2096	> > >		+		2
Pit091	2427927	2427816	112	-	PP_2127	PP_2128	< < >		+		
Pit092	2435428	2435259	170	-	PP_2133	PP_2134	> < >		+	*	
Pit093	2547203	2546845	359	-	PP_2238	PP_2239	> < <		+	**	
Pit094	2622635	2623183	549	+	PP_2294	PP_2295	> > >		---	**	2
Pit095	2624356	2624262	95	-	PP_2295	PP_2296	> < >		---	*	
Pit096	2650792	2650612	181	-	PP_2322	PP_2323	< < >		+		4
Pit097	2672585	2672433	153	-	PP_2339	PP_2340	< < >		+		
Pit098	2674735	2674968	234	+	PP_2343	PP_2344	> > <		---	**	
Pit099	2796697	2796638	60	-	PP_t55	PP_t56	> < >		-	*	1
Pit100	2817932	2817852	81	-	PP_t57	PP_2473	> < >		+		
Pit101	2821661	2821620	42	-	PP_2474	PP_2475	< < >		+		
Pit102	2842055	2841960	96	-	PP_2492	PP_2493	< < <		---		1
Pit103	2851516	2851994	479	+	PP_2504	PP_2505	> > <		+++		
Pit104/IGR 2510	2858064	2858010	55	-	PP_2509	PP_2510	< < <		+	*	
Pit105	2925584	2925722	139	+	PP_2563	PP_2564	< > <		+		
Pit106	2937772	2937890	119	-	PP_2569	PP_2570	> < <		-		
Pit107	2939075	2939249	175	+	PP_2570	PP_2571	< > <		+	*	
Pit108	3023082	3023194	113	+	PP_2638	PP_2639	> > >		+	*	
Pit109	3261540	3261455	86	-	PP_2858	PP_2859	< < <		-		
Pit110	3275596	3275812	217	+	PP_2873	PP_2874	> > >		+	**	
Pit111	3342221	3342292	72	+	PP_2938	PP_2939	< > <		-		
Pit112	3411775	3411973	199	+	PP_3024	PP_3025	> > <		+		
Pit113	3448540	3447964	577	-	PP_3066	PP_3067	> < >		---		1
Pit114	3450217	3450305	89	+	PP_3067	PP_3068	> > >		+		
Pit115	3450542	3450479	64	-	PP_3067	PP_3068	> < >		+		1
Pit116	3500093	3499994	100	-	PP_3101	PP_3102	> < <		+		1
Pit117	3501227	3501326	100	+	PP_3101	PP_3102	> > <		+		3
Pit118	3502297	3502584	288	+	PP_3101	PP_3102	> > <		-		3
Pit119	3506280	3506114	167	-	PP_3103	PP_3104	< < >		---	*	6
Pit120	3519553	3519669	117	+	PP_3109	PP_3110	> > >		-		
Pit121	3520224	3520278	55	+	PP_3109	PP_3110	> > >		---		3
Pit122	3521227	3521358	132	+	PP_3109	PP_3110	> > >		-	*	3
Pit123	3703160	3703832	673	+	PP_3269	PP_3270	> > <		-		5
Pit124	3826446	3826129	318	-	PP_3380	PP_3381	< < <		---		
Pit125	3827997	3828171	175	+	PP_3381	PP_3382	< > <		+	*	
Pit126	3967924	3967830	95	-	PP_3497	PP_3498	> > >		---		
Pit127	3971965	3971766	200	-	PP_3501	PP_3502	> < >		+	*	
Pit128	4013318	4013260	59	-	PP_3540	PP_3541	> < <		+		1
Pit129	4013566	4013474	93	-	PP_3540	PP_3541	> < <		+		2
Pit130	4022652	4022461	192	-	PP_3547	PP_3548	< < <		+	*	
Pit131	4032268	4032208	61	-	PP_3554	PP_3555	> < >		-	*	
Pit132/IGR 3586	4073883	4073626	258	-	PP_3585	PP_3586	< < <		---	**	
Pit133	4075434	4075611	178	+	PP_3586	PP_3587	< > <		+	*	
Pit134	4197786	4197169	618	-	PP_3688	PP_3689	< < >		---		
Pit135	4199554	4199599	46	+	PP_3689	PP_3690	> > <		---		
Pit136	4224252	4224620	369	+	PP_3703	PP_3704	< > <		---	*	
Pit137	4302533	4302370	164	-	PP_3774	PP_3775	> < <		+		
Pit138	4371672	4371631	42	-	PP_3848	PP_3849	> < >		+++		
Pit139	4413211	4415000	1790	+	PP_3898	PP_3899	> > <		---	**	1
Pit140/IGR 3917	4425381	4425259	123	-	PP_3916	PP_3917	> < >		-	**	
Pit141	4430160	4430105	56	-	PP_3924	PP_3925	> < >		-	*	
Pit142	4484802	4484744	59	-	PP_3976	PP_3977	> < >		---	*	
Pit143	4488900	4488999	100	+	PP_3981	PP_3982	> > <		---		1
Pit144	4595452	4595115	338	-	PP_4069	PP_4070	> < >	PrrF (-)	+		
Pit145	4858396	4858468	73	+	PP_4273	PP_4274	> > <	Bacteria_small_SRP (-)	+		
Pit146	4945337	4945242	96	-	PP_4351	PP_4352	< < <		-	*	
Pit147	5045239	5045192	48	-	PP_4448	PP_4449	> < <		---	*	1
Pit148/IGR 4451	5047208	5047425	218	+	PP_4450	PP_4451	> > <		-		1
Pit149	5103204	5103399	196	+	PP_4491	PP_4492	> > >		+	*	
Pit150	5132806	5132618	189	-	PP_4518	PP_4519	> < >		+		
Pit151	5140624	5140398	227	-	PP_4524	PP_4525	> < <		+	**	
Pit152	5152468	5152149	320	-	PP_4535	PP_4536	< < >		---	**	
Pit153	5219069	5218914	156	-	PP_4598	PP_4599	> > >		+	*	
Pit154	5222750	5222607	144	-	PP_4602	PP_4603	< < <		+		
Pit155	5224318	5224502	185	+	PP_4603	PP_4604	< > <		+	*	
Pit156	5237100	5237532	433	+	PP_4613	PP_4614	> > >		+	**	1
Pit157	5390140	5390072	69	-	PP_4738	PP_4739	> < >	tmRNA (-)	+		
Pit158	5390374	5390212	163	-	PP_4738	PP_4739	> < >	tmRNA (-)	+		
Pit159	5392106	5392005	102	-	PP_4739	PP_4740	> < >		-		1
Pit160	5401042	5400960	83	-	PP_4743	PP_4744	> < <		-		
Pit161	5453316	5453130	187	-	PP_4790	PP_4791	> < >		+	*	
Pit162	5545506	5545227	280	-	PP_4877	PP_4878	< < <		---		
Pit163	5756969	5756780	190	-	PP_5049	PP_5050	> < >		+	*	
Pit164	5934772	5934682	91	-	PP_5202	PP_5203	> < >		+		
Pit165	5941446	5941310	137	-	PP_5208	PP_5209	> < <		+	**	
Pit166	5971945	5971848	98	-	PP_5237	PP_5238	> < >		---		
Pit167	5988840	5988892	53	-	PP_5246	PP_5247	> > >		-		
Pit168	5989901	5989792	110	-	PP_5247	PP_5248	> < <		-	**	
Pit169	6038999	6039220	222	+	PP_5290	PP_5291	< > <		+	*	
Pit170	6128020	6128117	98	+	PP_5375	PP_5376	< > >		+	*	1
Pit171	6128565	6128460	106	-	PP_5375	PP_5376	< < >		+	*	4
Pit172	6137122	6137302	181	+	PP_5384	PP_5385	> > >		+		1
Pit173	6150429	6150673	245	+	PP_5394	PP_5395	< > >		+		1
Pit174	6158691	6158385	307	-	PP_5401	PP_5402	> < <		---		
Pit175	6158908	6158983	76	+	PP_5401	PP_5402	> > <		---	**	
Pit176	6159167	6158772	396	-	PP_5401	PP_5402	> < <		---		
Pit177	6166492	6166571	80	+	PP_5406	PP_5407	< > >		---		
Pit178	6166629	6166446	184	-	PP_5406	PP_5407	< < >		---	*	

Dataset 3: Novel antisense sRNAs transcripts in *P. putida* KT2440 (Pat).

Legend:

Antisense gene Rfam - matches between antisense genes and RNAs in the Rfam database are indicated

Blast - the sequence conservation of candidate sRNAs in other microbial organisms was investigated using BLASTN algorithm:

(---) no sequence conservation found outside of P. putida KT2440 strain; (-) no sequence conservation found outside of P. putida species;

(+) sequence conservation primarly in Pseudomonadaceae; (+++) sequence conserved in bacterial species outside the Pseudomonadaceae family

Cluster - number of the cluster from differential expression analysis of sRNAs (for more info see Figure 5)

Rho IT/palindrome - ** predicted Rho-independent terminator, * palindrome at the end or close to the end. Analysis was done using pseudomonas.com and ARNold tool.

Name	Start	Stop	Length	Strand	Antisense gene	Antisense gene Rfam	Blast	RhoIT/palindrome	Antisense gene product description
Pat001	19854	19799	56	-	PP_0015		+		ATPase AAA
Pat002	25912	25834	79	-	PP_0021		+	*	hypothetical p.
Pat003	27029	27095	67	+	PP_0022		+		hypothetical p.
Pat004	27627	27738	112	+	PP_0023		+++		hypothetical p.
Pat005	53372	53308	65	-	PP_0046		+		porin
Pat006	107948	107880	69	-	PP_0102		+	*	hypothetical p.
Pat007	108440	108329	112	-	PP_0102		+		hypothetical p.
Pat008	122230	122418	189	+	PP_0116		+		lipoprotein
Pat009	122903	122987	85	+	PP_0116		+	*	lipoprotein
Pat010	130949	131000	52	+	engB (PP_0124)		+		ribosome biogenesis GTP-binding protein YsxC
Pat011	131093	131307	215	+	engB (PP_0124)		+		ribosome biogenesis GTP-binding protein YsxC
Pat012	135236	135130	107	-	PP_0129		+	*	diguanylate cyclase
Pat013	137953	137907	47	-	PP_0131		+	*	diguanylate phosphodiesterase
Pat014	138504	138345	160	-	PP_0131		+++		diguanylate phosphodiesterase
Pat015	171488	171391	98	-	PP_16SA	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat016	172902	171611	1292	-	PP_16SA	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat017	173474	173377	98	-	PP_23SA	LSU_rRNA_bacteria	+++		23S ribosomal RNA
Pat018	174507	173558	950	-	PP_23SA	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat019	174626	174561	66	-	PP_23SA		+++		23S ribosomal RNA
Pat020	175933	174752	1182	-	PP_23SA	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat021	176340	176250	91	-	PP_5SA	5S_rRNA	+++		5S ribosomal RNA
Pat022	176918	176821	98	-	PP_16SB	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat023	178332	177041	1292	-	PP_16SB	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat024	178904	178807	98	-	PP_23SB	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat025	179937	178991	947	-	PP_23SB	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat026	180056	179991	66	-	PP_23SB		+++		23S ribosomal RNA
Pat027	181363	180182	1182	-	PP_23SB	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat028	181770	181680	91	-	PP_r01	5S_rRNA	+++		23S ribosomal RNA
Pat029	181991	181905	87	-	PP_5SB	5S_rRNA	+++	**	5S ribosomal RNA
Pat030	188380	188271	110	-	PP_0165		+		diguanylate cyclase
Pat031	195257	195164	94	-	PP_0168		+		surface adhesion protein
Pat032	255290	255320	31	+	PP_t01		+++		tRNA-Arg
Pat033	305677	305996	320	+	PP_0251		+		hypothetical p.
Pat034	335087	335148	62	+	PP_0277		+		hypothetical p.
Pat035	472859	472990	132	+	rpoD (PP_0387)		+		RpoD sigma 70
Pat036	479389	479470	82	+	cca (PP_0394)		+++		multifunctional tRNA nucleotidyl transferase
Pat037	525048	524951	98	-	PP_16SC	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat038	526462	525172	1291	-	PP_16SC	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat039	527034	526937	98	-	PP_23SC	LSU_rRNA_bacteria	+++		23S ribosomal RNA
Pat040	528067	527120	948	-	PP_23SC	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat041	528186	528121	66	-	PP_23SC		+++		23S ribosomal RNA
Pat042	529493	528312	1182	-	PP_23SC	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat043	529889	529797	93	-	PP_5SC	5S_rRNA	+++		5S ribosomal RNA
Pat044	532323	532136	188	-	PP_0439		+		hypothetical p.
Pat045	532422	532382	41	-	PP_t05		+++		tRNA-Tyr
Pat046	532631	532594	38	-	PP_t07		+		tRNA-Leu
Pat047	534177	534072	106	-	secE (PP_0441)		+		preprotein translocase subunit SecE
Pat048	536522	536319	204	-	rplJ (PP_0445)	P27	+		50S ribosomal protein L10
Pat049	548301	548265	37	-	fusA (PP_0451)		+++		elongation factor G
Pat050	561852	561807	46	-	rpsM (PP_0476)		+		30S ribosomal protein S13
Pat051	609690	609789	100	+	PP_0525		+		B12 family TonB-dependent receptor
Pat052	612235	612442	208	+	dxs (PP_0527)		+		1-deoxy-D-xylulose-5-phosphate synthase
Pat053	678778	678860	83	+	fabG (PP_0581)		+		3-ketoacyl-ACP reductase (fatty acid synthesis)
Pat054	697923	697826	98	-	PP_16SD	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat055	699337	698047	1291	-	PP_16SD	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat056	699581	699540	42	-	PP_t10		+++		tRNA-Ala
Pat057	700121	700025	97	-	PP_23SD	LSU_rRNA_bacteria	+++		23S ribosomal RNA
Pat058	701155	700209	947	-	PP_23SD	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat059	701274	701209	66	-	PP_23SD		+++		23S ribosomal RNA
Pat060	702581	701400	1182	-	PP_23SD	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat061	702995	702897	99	-	PP_5SD	5S_rRNA	+++		5S ribosomal RNA
Pat062	707267	707354	88	+	rpsT (PP_0600)		+		30S ribosomal protein S20
Pat063	741374	741335	40	-	PP_t16		+++		tRNA-Thr
Pat064	744868	744967	100	+	PP_0637		---		ISPpu15, ORF 2
Pat065	839819	839856	38	+	ipk (PP_0723)		+		4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
Pat066	856576	856967	392	+	PP_0738		+		hypothetical p.
Pat067	857114	857158	45	+	PP_0738		-		hypothetical p.
Pat068	892064	892149	86	+	pta (PP_0774)		+++		phosphate acetyltransferase
Pat069	916931	916983	53	+	PP_0798		+	*	diguanylate cyclase
Pat070	917372	917465	94	+	PP_0798		+	*	diguanylate cyclase
Pat071	917792	917833	42	+	PP_0799		+		porin
Pat072	968920	968962	43	+	PP_t19		+		tRNA-Leu
Pat073	1007430	1005819	1612	-	PP_0867		+		FecA-like outer membrane receptor
Pat074	1086738	1086836	99	+	PP_0941		+		hypothetical p.
Pat075	1101445	1101393	53	-	ttg2B (PP_0959), ttg2C (PP_0960)		+		hypothetical p.
Pat076	1137380	1137511	132	+	PP_0998		+		hypothetical p.
Pat077	1164619	1164804	186	+	hexR (PP_1021)		+		HexR transcriptional regulator
Pat078	1164872	1165210	339	+	hexR (PP_1021)		+		HexR transcriptional regulator
Pat079	1175692	1175666	27	-	guaB (PP_1031)		+		inosine 5'-monophosphate dehydrogenase
Pat080	1282211	1282278	68	+	PP_1118		+		recombinase-like protein
Pat081	1292418	1292348	71	-	estC (PP_1127), PP_1128		+		beta-lactamase and OmpA/MotB domain-containing protein
Pat082	1294422	1294509	88	+	PP_1130		+		hypothetical p.
Pat083	1298229	1298131	99	-	PP_1133		-	*	ISPpu9
Pat084	1325602	1325505	98	-	PP_16SE	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat085	1327016	1325725	1292	-	PP_16SE	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat086	1327588	1327491	98	-	PP_23SE	LSU_rRNA_bacteria	+++		23S ribosomal RNA
Pat087	1328621	1327674	948	-	PP_23SE	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat088	1328740	1328675	66	-	PP_23SE		+++		23S ribosomal RNA
Pat089	1330047	1328866	1182	-	PP_23SE	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat090	1330455	1330363	93	-	PP_5SE	5S_rRNA	+++		5S ribosomal RNA
Pat091	1360285	1360233	53	-	oprH (PP_1185)		+		outer membrane protein H1
Pat092	1364743	1364849	107	+	PP_1189		-		hypothetical p.

Pat093	1380558	1380598	41	+	PP_t20		+++		tRNA-Met
Pat094	1380769	1380809	41	+	PP_t21		+++	*	tRNA-Met
Pat095	1380980	1381020	41	+	PP_t22		+++		tRNA-Met
Pat096	1397712	1397539	174	-	tolA (PP_1221)		+		biopolymer transport protein TolA
Pat097	1423669	1423734	66	+	PP_1247		-		hypothetical membrane p.
Pat098	1424002	1424025	24	+	PP_1247		-	*	hypothetical membrane p.
Pat099	1424309	1424526	218	+	PP_1247		-	**	hypothetical membrane p.
Pat100	1441746	1441645	102	-	PP_1260		-	*	ISPpu9
Pat101	1503253	1503158	96	-	rplM (PP_1315)		+		50S ribosomal RNA L13
Pat102	1512674	1512566	109	-	PP_1326		+	*	uroporphyrin-III C/tetrapyrrole methyltransferase
Pat103	1530263	1530166	98	-	ftsZ (PP_1342)		+		cell division protein FtsZ
Pat104	1544598	1545180	583	+	ampG (PP_1355)		+++		AmpG-related permease/muropeptide transporter
Pat105	1555536	1555582	47	+	PP_1366		+		transcriptional regulator MvaT, P16 subunit
Pat106	1556978	1556912	67	-	purU (PP_1367)		-		formyltetrahydrofolate deformylase
Pat107	1583396	1583196	201	-	ttgR (PP_1387)		+		TetR family transcriptional regulator
Pat108	1606565	1606660	96	+	phaG (PP_1408)		+		alpha/beta hydrolase
Pat109	1678796	1678894	99	+	PP_1474		+		hypothetical p.
Pat110	1719007	1718951	57	-	PP_1514		-		hypothetical p.
Pat111	1745877	1746196	320	+	PP_1544		---		hypothetical p.
Pat112	1747946	1747838	109	-	PP_1547		-	*	hypothetical p.
Pat113	1751008	1750827	182	-	PP_1551		+		phage replication protein O
Pat114	1848631	1848538	94	-	cysM (PP_1654)		+	*	cysteine synthase B
Pat115	1912271	1912295	25	+	PP_1712		---		hypothetical p.
Pat116	1936869	1936758	112	-	PP_1736		+		Patatin_and_cPLA2; Patatins and Phospholipases
Pat117	1957336	1957283	54	-	PP_1754		-		hypothetical p./alginate lyase A1
Pat118	2005998	2006080	83	+	PP_1789		---		HAD superfamily hydrolase
Pat119	2057695	2057675	21	-	PP_1832		+		oxidase
Pat120	2102641	2102585	57	-	PP_t31		+		tRNA-Glu
Pat121	2102771	2102733	39	-	PP_t32		+++		tRNA-Gly
Pat122	2103020	2102979	42	-	PP_t34		+++		tRNA-Gly
Pat123	2103181	2103137	45	-	PP_t35		+++		tRNA-Gly
Pat124	2158648	2158583	66	-	acpP (PP_1915)		+++		acyl carrier protein
Pat125	2162730	2162594	137	-	PP_1919		---		thymidylatekinase (frame shift)
Pat126	2186461	2186510	50	+	PP_1936		---		hypothetical p.
Pat127	2241583	2241541	43	-	PP_t37		+++	*	tRNA-Ala
Pat128	2241858	2241816	43	-	PP_t39		+++		tRNA-Ala
Pat129	2242113	2242071	43	-	PP_t41		+++	*	tRNA-Ala
Pat130	2271412	2271374	39	-	PP_t43		+	*	tRNA-Val
Pat131	2343129	2343071	59	-	PP_2058		+		outer membrane porin
Pat132	2434134	2434171	38	+	PP_2132		-		universal stress protein
Pat133	2548788	2548691	98	-	PP_16SF	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat134	2550202	2548911	1292	-	PP_16SF	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat135	2550446	2550405	42	-	PP_t49		+++		tRNA-Ala
Pat136	2550987	2550890	98	-	PP_23SF	LSU_rRNA_bacteria	+++		23S ribosomal RNA
Pat137	2551322	2551073	250	-	PP_23SF	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat138	2552020	2551391	630	-	PP_23SF	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat139	2552139	2552074	66	-	PP_23SF		+++		23S ribosomal RNA
Pat140	2553446	2552265	1182	-	PP_23SF	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat141	2553854	2553762	93	-	PP_5SF	5S_rRNA	+++		5S ribosomal RNA
Pat142	2633298	2633261	38	-	hupB (PP_2303)		-		histone family protein DNA-binding protein
Pat143	2650205	2650281	77	+	oprI (PP_2322)		+		outer membrane lipoprotein OprI
Pat144	2809210	2808901	310	-	PP_2464		+		hypothetical p.
Pat145	2817094	2817059	36	-	PP_t57		+		tRNA-Pro
Pat146	2819979	2819853	127	-	PP_2473		+		hypothetical p.
Pat147	2837590	2837724	135	+	PP_2489		+++		xenobiotic reductase, YOE, oxidative stress
Pat148	3353431	3353479	49	+	PP_2948		-		GntR family transcriptional regulator
Pat149	3496203	3496242	40	+	PP_3099, PP_3100		-		hypothetical p., suspected component of type VI protein sectretion
Pat150	3547424	3547363	62	-	PP_3132		+	*	polysaccharide biosynthesis protein
Pat151	3675791	3675761	31	-	PP_3239		-		Tn4652, cointegrate resolution protein T
Pat152	3730030	3729933	98	-	PP_3296		---		hypothetical p., predicted sulphur transporter
Pat153	3732205	3732267	63	+	PP_3299		-		outer membrane lipoprotein
Pat154	3826555	3826653	99	+	PP_3381		-	*	ISPpu9
Pat155	3865605	3865639	35	+	PP_3414		-		methyl-accepting chemotaxis transducer/sensory box protein
Pat156	3892778	3892670	109	-	rarD-2 (PP_3436)		+++		multidrug resistance efflux transporter RarD protein, DMT superfamily transporter
Pat157	3892965	3892897	69	-	rarD-2 (PP_3436)		+++		multidrug resistance efflux transporter RarD protein, DMT superfamily transporter
Pat158	3893352	3893119	234	-	rarD-2 (PP_3436)		+++		multidrug resistance efflux transporter RarD protein, DMT superfamily transporter
Pat159	4071000	4071052	53	+	PP_3584		+++		RNA efflux transporter (frame shift)
Pat160	4073992	4074091	100	+	PP_3586		-	*	ISPpu9
Pat161	4179621	4179473	149	-	PP_3677		---		hypothetical p.
Pat162	4220537	4220459	79	-	PP_3699		-		hypothetical p.
Pat163	4278924	4278869	56	-	PP_3750		+		GntR family transcriptional regulator
Pat164	4321413	4321883	471	+	PP_3792		+		hypothetical p., Y-family of DNA polymerases
Pat165	4364360	4364120	241	-	PP_3840		-		hypothetical p.
Pat166	4367195	4367492	298	+	PP_3844		+++		D-aminopeptidase
Pat167	4369747	4369872	126	+	PP_3846		+		carbon-nitrogen hydrolase
Pat168	4373493	4373388	106	-	PP_3849		---		calcium-binding protein, hemolysin-type
Pat169	4404517	4405269	753	+	PP_3885		---		hypothetical p.
Pat170	4410796	4410903	108	+	PP_3894		-		phage replication protein O
Pat171	4586179	4586503	325	+	PP_4063		+		AMP-binding protein
Pat172	4632567	4632614	48	+	PP_t61		+++		tRNA-Gly
Pat173	4636357	4636428	72	+	PP_4101		+		acetyltransferase
Pat174	4724692	4725037	346	+	PP_4182		+	*	hypothetical p.
Pat175	4735193	4735262	70	+	sucA (PP_4189)		+		2-oxoglutarate dehydrogenase E1
Pat176	4736516	4736591	76	+	sdhB (PP_4190)		+++		succinate dehydrogenase iron-sulfur subunit
Pat177	4743871	4743975	105	+	PP_4197		+		GntR family transcriptional regulator
Pat178	4767567	4767470	98	-	fpvA (PP_4217)		-		outer membrane ferripyoverdine receptor
Pat179	4840791	4840693	99	-	ccoN-2 (PP_4255)		+		cbb3-type cytochrome c oxidase subunit I
Pat180	4909807	4909544	264	-	PP_4318		-		ISPpu8
Pat181	4942779	4942680	100	-	PP_4350		-		aminotransferase
Pat182	4965169	4965769	601	+	fleQ (PP_4373), PP_4374		+		hypothetical p. and Fis family transcriptional regulator
Pat183	4965937	4966035	99	+	fliS (PP_4375)		+		flagellar protein FliS
Pat184	4967114	4967182	69	+	fliD (PP_4376)		+		flagellar cap protein FliD
Pat185	4969542	4969602	61	+	fliC (PP_4378)		---		flagellin FliC
Pat186	5011449	5011239	211	-	PP_4415		---		DNA binding hypothetical p.
Pat187	5027669	5027599	71	-	PP_4431		-		amino acid MFS transporter
Pat188	5032245	5032150	96	-	dadA-1 (PP_4434)		+++		D-amino acid dehydrogenase small subunit
Pat189	5048355	5048427	73	+	PP_4451		+		hypothetical p.
Pat190	5079808	5080019	212	+	PP_t68, csrA (PP_4472)		+	*	csrA - carbon storage regulator
Pat191	5172865	5173223	359	+	fadD (PP_4549)		+	*	long-chain-fatty-acid--CoA ligase
Pat192	5208453	5208715	263	+	PP_4589, PP_4590		+		D-isomer specific 2-hydroxyacid dehydrogenase, hypothetical p.
Pat193	5222876	5222974	99	+	PP_4603		-	*	ISPpu9
Pat194	5238157	5238205	49	+	PP_4615		+		phosphate-starvation-inducible E
Pat195	5307443	5307544	102	+	PP_5SG	5S_rRNA	+++		5S ribosomal RNA
Pat196	5307855	5309036	1182	+	PP_23SG	LSU_rRNA_bacteria	+		23S ribosomal RNA

Pat197	5309162	5309229	68	+	PP_23SG		+++		23S ribosomal RNA
Pat198	5309280	5310230	951	+	PP_23SG	LSU_rRNA_bacteria	+		23S ribosomal RNA
Pat199	5310314	5310411	98	+	PP_23SG	LSU_rRNA_bacteria	+++		23S ribosomal RNA
Pat200	5310845	5310887	43	+	PP_t69		+++		tRNA-Ala
Pat201	5311164	5312455	1292	+	PP_16SG	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat202	5312578	5312675	98	+	PP_16SG	SSU_rRNA_bacteria	+		16S ribosomal RNA
Pat203	5437678	5437810	133	+	PP_4775	P24 -	+		hypothetical p.
Pat204	5477211	5477366	156	+	PP_4814		+		ATP-dependent protease La
Pat205	5523140	5523254	115	+	PP_4857		+++		AsmA family protein
Pat206	5529022	5529642	621	+	PP_4863		+		branched chain amino acid ABC transporter ATP-binding protein
Pat207	5549400	5549096	305	-	PP_t73	tRNA	+++		tRNA-Leu
Pat208	5549575	5549517	59	-	PP_t74		+++	*	tRNA-Leu
Pat209	5666531	5666367	165	-	PP_4974		+		sodium/hydrogen exchanger
Pat210	5667141	5666660	482	-	PP_4974		+		sodium/hydrogen exchanger
Pat211	5679447	5679385	63	-	bioA (PP_4984)		+		adenosylmethionine-8-amino-7-oxononanoate aminotransferase
Pat212	5719290	5719192	99	-	PP_5020		-		methyl-accepting chemotaxis sensory transducer
Pat213	5723393	5723478	86	+	PP_5024		+		amino acid ABC transporter substrate-binding protein
Pat214	5744212	5744260	49	+	glgP (PP_5041)		+		glycogen/starch/alpha-glucan phosphorylase
Pat215	5954495	5954732	238	+	PP_5219		+++		hypothetical p., fatty acid hydoxylase - sterol desaturrase
Pat216	5977316	5977226	91	-	PP_5239		+++		magnesium chelatase subunit D/I family protein
Pat217	6011820	6011724	97	-	rep (PP_5264)		+++		ATP-dependent DNA helicase Rep

Dataset 7: Commonly differentially expressed genes in osmotic, oxidative, and membrane stress conditions.

NA - Not available

Nr.	Locus tag	Name	Product name	Function
1	PP_0032	-	hypothetical protein	NA
2	PP_0036	-	LysR family transcriptional regulator	Transcription
3	PP_0048	-	hypothetical protein	NA
4	PP_0086	-	hypothetical protein	Function unknown
5	PP_0088	-	luciferase	Energy production and conversion
6	PP_0089	osmC	OsmC family protein	Posttranslational modification, protein turnover, chaperones
7	PP_0090	-	hypothetical protein	NA
8	PP_0174	-	FecR anti-FecI sigma factor	General function prediction only
9	PP_0181	-	surface adhesion protein	NA
10	PP_0186	-	TonB-dependent siderophore receptor	Coenzyme metabolism
11	PP_0197	gcdH	acyl-CoA dehydrogenase	Cell envelope biogenesis, outer membrane
12	PP_0363	bioF	8-amino-7-oxononanoate synthase	Coenzyme metabolism
13	PP_0364	bioH	carboxylesterase	General function prediction only
14	PP_0365	bioC	biotin biosynthesis protein BioC	General function prediction only
15	PP_0375	-	prolyl oligopeptidase	Lipid metabolism
16	PP_0378	pqqC	pyrroloquinoline quinone biosynthesis protein PqqC	Coenzyme metabolism
17	PP_0379	pqqB	pyrroloquinoline quinone biosynthesis protein PqqB	General function prediction only
18	PP_0482	-	bacterioferritin	Inorganic ion transport and metabolism
19	PP_0528	ispA	polyprenyl synthetase	Coenzyme metabolism
20	PP_0536	-	hypothetical protein	NA
21	PP_0549	-	hypothetical protein	General function prediction only
22	PP_0620	-	GntR family transcriptional regulator	Transcription / Amino acid transport and metabolism
23	PP_0621	-	hypothetical protein	NA
24	PP_0641	-	hypothetical protein	NA
25	PP_0745	uraA	uracil-xanthine permease	Nucleotide transport and metabolism
26	PP_0757	-	hypothetical protein	NA
27	PP_0762	hprA	glycerate dehydrogenase	Amino acid transport and metabolism
28	PP_0770	-	PemI-like protein	Signal transduction mechanisms
29	PP_0784	-	hypothetical protein	Function unknown
30	PP_0787	nadC	nicotinate-nucleotide pyrophosphorylase	Coenzyme metabolism
31	PP_0794	fruK	1-phosphofructokinase	Cell envelope biogenesis, outer membrane
32	PP_0798	-	diguanylate cyclase	Signal transduction mechanisms
33	PP_0832	queA	S-adenosylmethionine--tRNA ribosyltransferase-isomerase	Translation, ribosomal structure and biogenesis
34	PP_0877	-	hypothetical protein	NA
35	PP_0885	-	peptide ABC transporter substrate-binding protein	General function prediction only
36	PP_0951	rpoX	sigma 54 modulation protein/ribosomal protein S30EA	Translation, ribosomal structure and biogenesis
37	PP_0981	-	hypothetical protein	NA
38	PP_1014	-	hypothetical protein	Carbohydrate transport and metabolism
39	PP_1059	-	amino acid permease	Amino acid transport and metabolism
40	PP_1111	-	synthetase	Translation, ribosomal structure and biogenesis
41	PP_1116	-	resolvase site-specific recombinase	DNA replication, recombination, and repair
42	PP_1143	-	3-hydroxyisobutyrate dehydrogenase	Carbohydrate transport and metabolism
43	PP_1177	nrdB	ribonucleotide-diphosphate reductase subunit beta	Nucleotide transport and metabolism
44	PP_1215	ruvC	Holliday junction resolvase	DNA replication, recombination, and repair
45	PP_1267	-	hypothetical protein	NA
46	PP_1291	-	PhoH family protein	Signal transduction mechanisms
47	PP_1305	-	Pyocin S-type immunity protein	NA
48	PP_1306	-	pyocin S-type Killer domain-containing protein	NA
49	PP_1354	-	major facilitator superfamily transporter	Carbohydrate transport and metabolism
50	PP_1361	groEL	molecular chaperone GroEL	Posttranslational modification, protein turnover, chaperones
51	PP_1364	-	type IV pilus assembly PilZ	NA
52	PP_1400	-	metabolite/H+ symportermajor facilitator superfamily metabolite/H+ symporter	Carbohydrate transport and metabolism
53	PP_1408	phaG	alpha/beta hydrolase	General function prediction only
54	PP_1425	-	hypothetical protein	NA
55	PP_1458	-	metabolite-proton symporter	NA
56	PP_1477	recJ	single-stranded-DNA-specific exonuclease RecJ	DNA replication, recombination, and repair
57	PP_1503	-	hypothetical protein	NA
58	PP_1514	-	hypothetical protein	NA
59	PP_1550	-	Cro/CI family transcriptional regulator	NA
60	PP_1576	-	immunity protein	NA
61	PP_1631	-	hypothetical protein	General function prediction only
62	PP_1662	-	hypothetical protein	NA
63	PP_1676	cobC	threonine-phosphate decarboxylase	Amino acid transport and metabolism
64	PP_1742	-	hypothetical protein	Function unknown
65	PP_1743	actP	acetate permease	General function prediction only
66	PP_1754	-	hypothetical protein	NA
67	PP_1787	-	hypothetical protein	NA
68	PP_1788	-	hypothetical protein	NA
69	PP_1814	-	hypothetical protein	NA
70	PP_1840	-	hypothetical protein	NA
71	PP_1864	-	hypothetical protein	NA
72	PP_1923	-	hypothetical protein	NA
73	PP_1933	-	hypothetical protein	NA
74	PP_1938	-	hypothetical protein	NA
75	PP_1960	-	hypothetical protein	NA
76	PP_1961	-	hypothetical protein	NA
77	PP_1980	-	thioesterase	General function prediction only
78	PP_1997	folC	bifunctional folylpolyglutamate synthase/ dihydrofolate synthase	Cell envelope biogenesis, outer membrane
79	PP_1998	-	sporulation domain-containing protein	Function unknown
80	PP_2029	-	von Willebrand factor A	Coenzyme metabolism
81	PP_2062	-	hypothetical protein	NA
82	PP_2063	-	hypothetical protein	NA
83	PP_2155	lolD	lipoprotein releasing system, ATP-binding protein	Defense mechanisms
84	PP_2245	-	Cro/CI family transcriptional regulator	Transcription
85	PP_2292	-	hypothetical protein	NA
86	PP_2296	-	hypothetical protein	NA
87	PP_2297	-	integrative genetic element Ppu40, integrase	DNA replication, recombination, and repair
88	PP_2396	-	hypothetical protein	NA
89	PP_2417	-	transport system permease	Inorganic ion transport and metabolism
90	PP_2422	-	alkylhydroperoxidase	Function unknown
91	PP_2446	-	hypothetical protein	NA
92	PP_2447	-	hypothetical protein	NA
93	PP_2452	-	hypothetical protein	NA
94	PP_2462	-	hypothetical protein	NA
95	PP_2551	-	LysR family transcriptional regulator	Transcription
96	PP_2580	-	hypothetical protein	NA
97	PP_2644	-	hypothetical protein	NA
98	PP_2665	agmR	LuxR family transcriptional regulator	Transcription / Signal transduction mechanisms
99	PP_2669	-	hypothetical protein	General function prediction only
100	PP_2674	qedH	quinoprotein ethanol dehydrogenase	Function unknown

101	PP_2681	-	pyrroloquinoline quinone biosynthesis protein PqqD	NA
102	PP_2682	-	iron-containing alcohol dehydrogenase	Energy production and conversion
103	PP_2722	-	hemerythrin HHE cation binding domain-containing protein	NA
104	PP_2739	-	sensory box protein	Signal transduction mechanisms
105	PP_2938	-	OsmC family protein	Secondary metabolites biosynthesis, transport and catabolism
106	PP_2940	-	hypothetical protein	Cell division and chromosome partitioning
107	PP_2947	-	transcriptional regulator MvaT, P16 subunit	NA
108	PP_2951	-	TetR family transcriptional regulator	Transcription
109	PP_3025	-	amino acid transporter LysE	Amino acid transport and metabolism
110	PP_3026	-	phage recombinase	NA
111	PP_3033	-	transcriptional repressor pyocin R2_PP	Transcription
112	PP_3102	-	hypothetical protein	NA
113	PP_3105	-	hypothetical protein	NA
114	PP_3115	-	ISPpu13, transposase Orf3	NA
115	PP_3126	-	polysaccharide export protein	Cell wall/membrane/envelope biogenesis
116	PP_3300	-	TetR family transcriptional regulator	Transcription
117	PP_3312	-	heat shock protein	Posttranslational modification, protein turnover, chaperones
118	PP_3313	-	heat shock protein	Posttranslational modification, protein turnover, chaperones
119	PP_3332	-	cytochrome c-type protein	Energy production and conversion
120	PP_3368	-	major facilitator family transporter	Carbohydrate transport and metabolism
121	PP_3416	gnuK	thermoresistant glucokinase carbohydrate kinase	Carbohydrate transport and metabolism
122	PP_3431	-	ThiJ/PfpI domain-containing protein	General function prediction only
123	PP_3434	-	hypothetical protein	NA
124	PP_3443	-	glyceraldehyde-3-phosphate dehydrogenase	Energy production and conversion
125	PP_3453	-	integral membrane sensor signal transduction histidine kinase	Signal transduction mechanisms
126	PP_3454	-	winged helix family two component transcriptional regulator	Cell motility and secretion / Signal transduction mechanisms
127	PP_3455	-	RND family efflux transporter MFP subunit	Defense mechanisms
128	PP_3456	-	hydrophobe/amphiphile efflux-1 (HAE1) family transporter	Inorganic ion transport and metabolism
129	PP_3507	cobN	cobaltochelatase subunit CobN	Coenzyme metabolism
130	PP_3536	-	hypothetical protein	NA
131	PP_3567	-	LysR family transcriptional regulator	Transcription
132	PP_3589	sdaC	aromatic amino acid ABC transporter permease	Amino acid transport and metabolism
133	PP_3611	-	hypothetical protein	NA
134	PP_3676	-	hypothetical protein	NA
135	PP_3677	-	hypothetical protein	NA
136	PP_3678	-	hypothetical protein	NA
137	PP_3700	-	hypothetical protein	Cell division and chromosome partitioning
138	PP_3704	-	hypothetical protein	NA
139	PP_3745	glcD	glycolate oxidase subunit GlcD	Cell envelope biogenesis, outer membrane
140	PP_3747	glcF	glycolate oxidase iron-sulfur subunit	Energy production and conversion
141	PP_3772	-	phage repressor	Transcription
142	PP_3775	-	sarcosine oxidase	Amino acid transport and metabolism
143	PP_3781	-	oxygen-independent coproporphyrinogen III oxidase	Energy production and conversion
144	PP_3782	-	hypothetical protein	NA
145	PP_3785	-	hypothetical protein	NA
146	PP_3851	-	hypothetical protein	NA
147	PP_3852	-	hypothetical protein	NA
148	PP_3900	hicA-2	hicA protein	NA
149	PP_3909	-	hypothetical protein	NA
150	PP_3921	-	hypothetical protein	NA
151	PP_3963	-	hypothetical protein	NA
152	PP_3982	-	hypothetical protein	DNA replication, recombination, and repair
153	PP_3991	-	hypothetical protein	NA
154	PP_3992	-	xanthine/uracil permease	Nucleotide transport and metabolism
155	PP_4009	clpS	ATP-dependent Clp protease adaptor protein ClpS	Function unknown
156	PP_4010	cspD	cold-shock protein CspD	Transcription
157	PP_4028	-	hypothetical protein	NA
158	PP_4033	-	ribonuclease Z	General function prediction only
159	PP_4047	-	hypothetical protein	NA
160	PP_4054	-	hypothetical protein	NA
161	PP_4136	-	LuxR family transcriptional regulator	Transcription
162	PP_4170	-	hypothetical protein	NA
163	PP_4183	-	hypothetical protein	Function unknown
164	PP_4292	-	hypothetical protein	NA
165	PP_4303	-	hypothetical protein	NA
166	PP_4327	ccmA	cytochrome c biogenesis protein CcmA	Inorganic ion transport and metabolism
167	PP_4345	-	GntR family transcriptional regulator	Transcription / Amino acid transport and metabolism
168	PP_4362	-	Hpt protein	Signal transduction mechanisms
169	PP_4364	-	anti-sigma-factor antagonist	NA
170	PP_4387	-	hypothetical protein	NA
171	PP_4391	flgB	flagellar basal-body rod protein FlgB	Cell motility and secretion
172	PP_4410	-	hypothetical protein	NA
173	PP_4468	-	Cro/CI family transcriptional regulator	NA
174	PP_4487	acsA	acetyl-CoA synthetase	Secondary metabolites biosynthesis, transport, and catabolism
175	PP_4537	-	carboxylate/amino acid/amine transporter	NA
176	PP_4557	-	hypothetical protein	NA
177	PP_4593	-	hypothetical protein	NA
178	PP_4614	-	hypothetical protein	NA
179	PP_4653	-	xanthine/uracil/vitamin C permease	Nucleotide transport and metabolism
180	PP_4669	-	OmpA/MotB domain-containing protein	Cell motility and secretion
181	PP_4707	-	transport-associated protein	General function prediction only
182	PP_4813	-	PAP2 family protein/DedA family protein	Lipid metabolism
183	PP_4817	-	acyl dehydratase MaoC	Lipid metabolism
184	PP_4943	-	glycosyl transferase family protein	Cell envelope biogenesis, outer membrane
185	PP_4959	-	response regulator receiver modulated diguanylate cyclase/phosphodiesterase with PAS/PAC sensor(s)	Signal transduction mechanisms
186	PP_4975	-	thioesterase	Lipid metabolism
187	PP_4985	-	16S ribosomal RNA methyltransferase RsmE	Function unknown
188	PP_5008	-	poly(hydroxyalkanoate) granule-associated protein	NA
189	PP_5088	priA	primosome assembly protein PriA	Transcription / DNA replication, recombination, and repair
190	PP_5283	-	peptide ABC transporter substrate-binding protein	General function prediction only
191	PP_5298	-	peptidase C26	Nucleotide transport and metabolism
192	PP_5319	-	hypothetical protein	Function unknown
193	PP_5377	-	hypothetical protein	General function prediction only
194	PP_5395	-	hypothetical protein	General function prediction only

PAPER 2

Investigation of the *Pseudomonas putida* sRNAome reveals growth phase specific expression and insights into the Hfq regulon

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Abstract

The RNA-chaperone Hfq is a global post-transcriptional regulator in bacteria, important especially for fine-tuning of gene expression when environmental conditions change and cells have to adapt. The sRNAome was investigated during growth of *Pseudomonas putida* KT2440 and compared to the corresponding *hfq* deletion strain. Numerous sRNAs were upregulated at a specific point during growth at exponential, transition or stationary phase, thus pointing to their possible regulatory roles in fast-growing cells, adaptation stage when nutrients become limiting or in nutrient-limited conditions, respectively. In the absence of Hfq the levels of 170 sRNAs were affected, suggesting that Hfq either directly or indirectly impacts their stability. Further, Hfq-binding RNA transcripts were identified *in vivo* with coimmunoprecipitation. A total of 131 novel sRNAs were detected only in the coIP experiment, emphasizing the need to test different conditions, growth phases, and enrichment methods to get a thorough picture of the sRNAome and its dynamics in the cells. 199 sRNAs are directly binding to Hfq in *P. putida*. In addition 924 mRNAs were found to bind to Hfq *in vivo*, representing 17.3% of the genes being Hfq-associated. Together, 202 antisense and 355 intergenic sRNAs have been detected in this study, several of them being 3′UTR- or 5′UTR-derived, and IS-related and 17 were experimentally validated.

Introduction

In recent years, regulation of gene expression in bacteria by small RNA molecules (sRNA) has been recognized as pivotal for bacterial survival during stress. sRNAs are relatively short (50-400 nt) transcripts, which together with regulatory proteins co-ordinate the cell machinery to cause the necessary alterations and fine-tune bacterial physiology when cells encounter changing environmental conditions. sRNAs can modulate protein activity or regulate mRNA stability and/or translation or, in some cases mimic other nucleic acids (1). Base pairing sRNAs are either *trans*-encoded when they regulate a distant mRNA target and share short stretches of complementarity with it, or *cis*-encoded (antisense) when they regulate the target on the opposite strand, to which they usually share extended complementarity. sRNAs can regulate multiple targets and fine-tune several genes and operons at the same time as well as one mRNA can be targeted by several sRNAs. The coordination of the sRNA regulatory networks is regulated by the presence of the transcripts in the cells with modulation of the transcription or stability of the sRNAs (2). Functional sRNAs can be primary transcripts or be formed through processing events, in addition some are derived from mRNAs or tRNAs (3).

Base pairing sRNAs can bind to the translation initiation region (TIR) of mRNAs, upstream of the TIR or in coding region, and sRNAs can modulate translation either positively or negatively. In addition, RNA duplex formation of sRNA-mRNA impacts mRNA stability either by targeting them for endonuclease-mediated degradation or protecting them from it (4).

A large number of base pairing sRNAs associate with the RNA chaperone Hfq, a major global post-transcriptional regulator (5). Hfq protein is an abundant Sm-like family protein and is widely conserved in many bacterial and archaeal genomes. Hfq is most often involved in promoting sRNA-mRNA interactions. Hfq has been investigated in many bacteria and although it is not essential, Hfq is a very important global gene regulator. The Hfq mutants showed pleiotropic phenotypes such as attenuated virulence, impaired growth, and increased sensitivity to stressful conditions, pointing at a global role of Hfq in the cells (6). Many of the pleiotropic phenotypes observed have also been connected to poor translation of the general stress sigma factor RpoS in the absence of Hfq. Recently, a Δ*hfq* mutant of *Pseudomonas putida* KT2440 has been described and it showed low stress endurance, slower growth, impaired motility and higher sensitivity to various compounds (7).

Pseudomonas putida is a soil and rhizosphere bacterium with a versatile metabolism and several innate stress-endurance traits from the *Gammaproteobacteria* class (8). It can degrade various inhibiting xenobiotic compounds and grow at various temperatures. *P. putida* is used in biotechnology for production of bio-based compounds, bioremediation and agriculture and as a model organism for soil bacteria (9). Strain KT2440 is one of the best characterized pseudomonads and has been pronounced as a biosafety strain. It is closely related to pathogenic *P. aeruginosa* (sharing 85% of its genomic content) but is lacking the virulence traits (10).

Hfq has been shown to be a key regulator of catabolite repression in *P. aeruginosa* (11) and *P. putida* (12). And although there are many studies on *P. putida* and its tolerance, diverse metabolism, and regulation in stressful conditions, knowledge about sRNA-mediated regulatory networks is lacking in *P. putida* (7). Most of the understanding of Hfq and its interactions with RNAs come from a small set of organisms but a lot of questions about the mechanisms are still unanswered (13). In this study RNA-sequencing (RNA-Seq) has been used to learn about the dynamics of the sRNAome during the growth of *P. putida* and shed light into Hfq-associated regulation. 220 sRNAs with changing levels during the growth were detected. Such sRNAs could be interesting to further investigate their role potentially regulating growth-related mechanisms and adaptation to availability of nutrients and oxygen. Also many sRNAs were affected by the absence of Hfq thus pointing at the importance of Hfq in sRNA-mediated regulation. In addition Hfq-binding RNA transcripts *in vivo* were identified in this study, pointing at an immense Hfq-regulon in *P. putida* KT2440.

Results

Transcriptomic profiling of sRNA expression in different growth phases

In order to gain insights into sRNA expression and the role of Hfq in pseudomonads, the sRNAomes of the *Pseudomonas putida* KT2440 wild type (wt) and corresponding *hfq* deletion (Δ*hfq* mutant) (7) strains were characterized. The strains were grown in triplicate in LB medium and cells were harvested in exponential, transition and stationary phases of growth (Figure 1). The extracted RNA was size-selected (up to 500 nt) to enrich for sRNA transcripts. A total of 188 million reads were obtained in RNA-sequencing (RNA-Seq) for cDNA libraries constructed (pooled cDNA libraries designated as KB1 library), of which 153 million mapped to *P. putida* KT2440 genome. The depletion of rRNA with MICROBExpress kit was not very efficient but still 11% of the reads mapped to unannotated regions, showing the depth was sufficient for sRNA identification (Table S1).

For small RNA identification, transcripts detected by Rockhopper (14) were manually curated using Integrative Genomics Viewer (15). The transcripts located in intergenic regions and having independent expression profiles relative to flanking genes are classified as intergenic and transcripts encoded on the opposite strand and having an overlap with annotated genes were classified as antisense sRNAs. Altogether, 262 intergenic (Table S2) and 153 antisense sRNAs (Table S3) were detected in the KB1 cDNA library. Many among them are novel transcripts and were named Pit (*Pseudomonas* ***p****utida* **i**ntergenic

transcript; Pit179-Pit246) and Pat (*Pseudomonas **p**utida* **a**ntisense **t**ranscript) and for the easier understanding of the data we continued the numbering system from the previous studies performed in our group (Bojanovič *et al.*, submitted).

Growth phase specific expression of sRNAs

During cell growth there are different phases, where cells behave differently. We sought out to see how the growth shapes the sRNAome of *P. putida* KT2440. Very particular expression profiles during cell growth were observed, with different groups of sRNAs exhibiting high expression in each of the three phases (Table S4). As seen on the heat map (Figure 2) the expression of sRNAs shows distinct profiles with a group of sRNAs upregulated only in each of the three growth points. sRNAs with such expression profiles are potentially exerting important regulatory roles at a specific growth phase. Several sRNAs differentially expressed in the RNA-Seq data have been confirmed to be growth dependent by Northern blots. Such examples are Pit245 and Pit165 being strongly upregulated in exponential phase; Pit192 having the peak of its expression in transition phase; and Pit217, Pit051, and Pit052 with high expression in stationary phase.

106 sRNAs exhibit high expression only in exponential phase comparing to other two time points, including RsmY, t44, P15, P24, P26, P32 and some riboswitches such as YybP-YkoY, groES, cobalamin, TPP, etc. (Table S4-1). 28 sRNAs were induced only in transition phase, among them being CrcY sRNA, which is 2.6-fold induced in this time point (Table S4-2). This is a specially interesting observation with specific profiles different from exponential and stationary phase are observed – most likely representing the transition phase, where the growth is becoming limiting and cells need to reshuffle their metabolism in order to adapt to the new conditions. In this phase the regulation of the cellular functions is especially important and these sRNAs are

upregulated in transition phase and not in stationary phase. 40 sRNAs were significantly upregulated only in stationary phase, including PrrF2 (6.5-fold) and RsmZ (30-fold), while 6S RNA was 2.7-fold downregulated (Table S4-3). In addition, there are 37 sRNAs in common upregulated in transition and stationary phase comparing to exponential phase.

Hfq affects the abundance of numerous sRNAs

Hfq deletion has been shown to have severe effects on cell phenotypes, because the Hfq chaperone is needed for tuning the regulation of translation in the cells. Deletion of *hfq* in *P. putida* KT2440 has been previously shown to influence the general performance of this strain, such as increased susceptibility to tolerate various stress conditions, slower growth, and attenuated motility (7). In order to investigate the impact of Hfq on the sRNAome, sRNA expression in the wt strain and deletion mutant was compared in the same growth phases.

During exponential phase 47 sRNAs had changed expression levels in Δ*hfq* mutant pointing at the influence on their stability either directly or indirectly by the presence of Hfq (Table S4-4). Among them some were more represented than in the wt while others were less represented in the absence of the Hfq. PhrS, Spot42-like/spf/ErsA, PrrF1, PrrF2, CrcY and CrcZ are among those whose presence decreased in the deletion strain in exponential phase. In the transition phase 84 sRNAs had a changed expression in the absence of Hfq (Table S4-5). Some of the annotated RNA elements and riboswitches had higher expression in the Hfq deletion mutant such as groES, YybP-YkoY, SAH, TPP, gyrA, rpsL etc. This was also observed for the sRNAs RsmZ and PhrS, whereas downregulation was observed for PrrF1, PrrF2, CrcZ, CrcY and the rmf RNA motif. The highest number of sRNAs affected in the Hfq absence is found in stationary phase (Table S4-6). Among 129 such sRNAs again several RNA motifs and riboswitches were upregulated while sRNAs such as RgsA/P16, RsmY, PrrF2, CrcZ and CrcY decreased the transcript levels.

There were 21 sRNAs that showed differential expression in all the three growth stages tested (Table 1). The characterized sRNAs CrcZ, CrcY and PrrF2 are among them, including also several novel intergenic and antisense sRNA transcripts. All three characterized sRNAs are downregulated in the Δ*hfq* mutant.

Coimmunoprecipitation with Hfq reveals a broad spectrum of RNA molecules

As the differential expression of RNA transcripts could also be due to indirect effects of the deletion of the *hfq* gene on the chromosome, the transcripts bound to Hfq were further investigated with coimmunoprecipitation (coIP) experiments followed by deep sequencing (coIP/RIP-seq). For this purpose a *P. putida* KT2440 strain was constructed with a C-terminal 3xFLAG tagged version of Hfq. The tagged Hfq strain exhibited growth that was indistinguishable for the wt strain (not shown), suggesting that the 3xFLAG insertion in the strain did not affect functionality of the Hfq protein. For the coIP the RNAs bound to the tagged Hfq were compared to the control coIP of the RNA from the untagged wt strain. Both strains were grown in duplicate in LB medium and cells were harvested at the same exponential, transition and stationary growth phases as described above (Figure 1). The coIP samples were subjected to Western blot analysis, where the coIP showed enrichment of the Hfq tagged protein (~12.1 kDa) comparing to the total sample and the flow-through, showing that the chosen experimental conditions were sufficient and specific for extraction of the tagged Hfq from the cell lysates (Figure S1). The coIPed RNA from both strains was used for cDNA libraries preparation (pooled cDNA libraries designated as KB4 library). For the pooled cDNA libraries 104 million reads were received, with 90 million mapping to the *P. putida* KT2440 genome

(Table S1). While the majority of the RNA species in the control sample are rRNAs, the majority bound to Hfq originate from intergenic regions confirming that the coIP experiment enriched for specific RNAs via binding to the tagged Hfq (Figure 3A).

A total of 298 intergenic and 187 antisense sRNAs were detected in this dataset. Among them were 83 intergenic (Pit247-Pit329) and 48 antisense sRNAs that were only detected in the KB4 dataset (coIP enriched library). The naming and numbering system of these has been applied as described above.

In the exponential growth phase 310 sRNAs had changed expression in coIP, while 313 and 307 were changed in transition and stationary growth phases, respectively. There were 133 common overrepresented sRNAs in the coIPed samples from all the three growth phases (Table S5, Figure 3B). Among them Spot42-like/spf/ErsA, CrcZ, CrcY, PrrF1, RgsA/P16, PhrS, and the YybP-YkoY riboswitch were overrepresented in coIP with the tagged Hfq, suggesting that they bind Hfq. On the other hand 6S, tmRNA, and RsmZ were in the group of downregulated RNAs in all the growth stages, indicating they are not bound to Hfq.

Hfq-associated mRNAs

There were 920, 1202, and 948 genes with differential abundance in coIP vs. control in exponential, transition and stationary growth phases, respectively (Table S7). In common to all the three time points were changed levels of 393 mRNAs with 290 genes being enriched in the Hfq tagged strain (Figure 3C). Nearly 100 of these are hypothetical proteins but the rest are genes encoding various functions, such as membrane proteins and transporters, cell division and motility proteins, and genes that are part of energy production systems (such as cytochromes). Many of the overrepresented mRNAs are connected to metabolism of lipids, nucleotides, amino acids, carbohydrates, inorganic ions and coenzymes.

As sRNAs often have big regulons, it is not surprising that 38 of the most overrepresented mRNAs in the coIP samples were transcriptional regulators, including the sigma factors *rpoS* and *rpoH*.

There are 924 mRNAs enriched in the coIP with Hfq detected at least in one time point, which represents 17.3% of the genes (5350 CDS in total) being bound to Hfq in *Pseudomonas putida* KT2440 at some point during the growth.

Furthermore the highly expressed 5′UTRs of the genes in cases where only the 5′UTR was upregulated were examined. The 5′UTRs of 13 genes showed 2-55 fold enrichment in the coIP with Hfq (PP_0298, PP_0489, PP_0917, PP_0927, PP_1132, PP_1841, PP_2042, PP_2230, PP_2428, PP_4495, PP_4782, PP_4883, PP_5264). This suggests that these mRNAs may be bound to and/or regulated by Hfq in their 5′UTR regions.

Diversity of sRNAs in *Pseudomonas putida* KT2440

As previously shown sRNAs can originate from 3′UTRs (16) or 5′UTRs, also called actuatons (17) and with Northern blot their presence was confirmed in *P. putida*. Out of 36 candidates chosen for Northern analysis, 17 were confirmed. A total of 202 antisense and 355 intergenic sRNAs were detected in this study. The presence of seven Pit sRNAs (Pit003, Pit032, Pit051, Pit052, Pit165, Pit200, Pit235) encoded in the integenic regions was confirmed (Figure 4). For the Pit sRNAs, 52 are possibly 3′UTR-derived and 21 are possibly 5′UTR-derived or actuatons, whereas three are either one or the other (due to the flanking genes in the opposite directions this is difficult to predict). Northern blot analysis on selected candidates, confirming 17 sRNAs and showed the variability of the biogenesis of the sRNAs.

The Pit245 RNA has been detected as a 49 or 85 nt long 3′UTR-derived transcript in the KB1 and KB4 libraries, respectively. With the Northern blot the size of 85 nt is confirmed and additionally, there is a band at 150 nt in exponential phase in both wt and *hfq* deletion strains as well as bands for the mRNA transcript (Figure 5A). This RNA could be a processed transcript from the *aspA* mRNA (PP_5338), which encodes aspartate ammonia lyase and they share a common Rho independent terminator. The blot also confirms the RNA-Seq expression data – the sRNA is highly expressed in exponential phase and downregulated in transition and stationary phases in the wt. In the Δ*hfq* mutant it is downregulated in exponential and stationary phases (Table 1), where it is undetectable in the latter, suggesting it could be dependent upon the presence of Hfq in the cell. In the coIP with Hfq Pit245 has been upregulated 14-36 fold (Table S5) in all three tested conditions showing that it is primarily bound to Hfq and it possibly protects it from degradation, while the mRNA *aspA* was slightly upregulated (2-3 fold) in exponential and stationary phases.

The Pit192 RNA has been detected as a possible 3'UTR-derived 49 nt long transcript and this is confirmed by Northern blot (Figure 5B). This sRNA does not have a band for mRNA, so it is probably a transcript originating from an independent promoter from PP_0884, which encodes peptide ABC transporter substrate-binding protein and is not processed from its mRNA. As the intensities of the blot confirm, the transcript is not present in exponential phase but in the later growth phases in the wt, with the highest expression during the transition phase. In the Δ*hfq* mutant it is upregulated in all time points studied (48.4-fold in exponential phase) (Table 1). This points to a higher stability of the transcript in the absence of Hfq. On the other hand Pit192 was not present in the samples of coIP with Hfq, showing it is not primarily bound to the RNA chaperone but suggests there is another player involved in its regulation.

The Pit023 RNA has been detected as a 94 nt long transcript, possibly 3'UTR-derived from *selB* (PP_0494) encoding selenocysteine-specific translation elongation factor. On Northern blot its length has been confirmed and no mRNA band was observed (Figure 5C). The sRNA likely originates from an independent promoter within the gene. Its expression profile also concurs with RNA-Seq data showing that Pit023 is most highly expressed in the stationary phase and the presence of Hfq has no impact on its abundance. Moreover, it was not detected in the coIP experiment, indicating it is not dependent upon or bound to Hfq.

Actuatons are 5′UTR-derived sRNAs and 21 possible cases are found in this study in *P. putida*. Pit217 is derived from a 5′UTR of the *galE* (PP_3129) gene, encoding UDP-glucose 4-epimerase but no mRNA has been detected on the Northern blot (Figure 6A) In addition, the size predicted with the coIP experiment of 104 nt has been confirmed. This sRNA is not present in exponential phase but its expression is increasing during the growth with the peak in the stationary phase. Although no differential expression of Pit217 was found in the Δ*hfq* mutant compared to wt in the RNA-Seq expreiment, the Northern blot analysis shows that the sRNA is not as abundant in stationary phase in the *hfq* deletion strain as in the wt. Furthermore it was not upregulated in the coIP experiment and is probably not Hfq-bound.

Similarly, Pit211, Pit229, and Pat223 were detected by Northern blot as possible 5′UTR-derived transcripts and they all have mRNAs expressed too (Figure 6B-D). Interestingly, Pat223 is an antisense sRNA to PP_0085 and at the same time partly a 5′UTR and a 5′ end of PP_0086 encoded on the opposite strand. This transcript was detected on Northern blot together with the mRNA bands of the operon PP_0086-87 (Fig. 6D). Interestingly, in Δ*hfq* mutant in stationary phase there is a strong band at around 250 bp nt but no differential expression was detected in KB1 library nor in the coIP experiment. This transcript could be a processed product of the mRNA.

In KB4 library 7 sRNAs (Pit017, Pit089, Pit099, Pit126, Pit142, Pit221 and RNA2) have been found to be longer than previously described resulting in the transcript overlapping a part of the ORF of some genes.

In the case of Pit221 from the Northern blot results it is clear that the transcript is indeed longer as found in the coIP library (76 nt) and antisense to PP_3851 (Figure 7A), which is a hypothetical protein. This sRNA was strongly decreased in the Δ*hfq* mutant but in the coIP was not overrepresented compared to the control (Table 1). As illustrated by Northern blot, Pit221 expression is increasing during growth, with the highest expression in stationary phase, but in the *hfq* deletion strain it is undetectable. As the coIP results argue against direct binding to Hfq, the decreased stability of Pit221 in the *hfq* deletion strain is probably a secondary effect.

Similarly, Pit142 was detected as a longer transcript in the coIP experiment (75 nt) and antisense to PP_3977 but had no differential expression in any of the experiments in this study. In the Northern blot analysis there is a band around 75 nt but it seems to be even longer than detected with the RNA-Seq or representing its precursor, resulting in bands of around 80 and 100-110 nt (Figure 7B). Pit142 and Pit221 are probably antisense transcripts but were left with the nomenclature as when first detected as intergenic transcripts (Pit).

The Pit017 and Pit126 are homologous RNAs encoded antisense to the 5′UTR of the ISPpu11 transposase (PP_0334 and PP_3498) and were detected as previously reported (Bojanovič *et al.*, submitted). In the coIP experiment longer versions were predicted but Northern blot analysis showed a 79 nt long transcript (Figure 7C). These sRNAs were both 4.6-fold upregulated in the wt and hfq deletion strains in stationary phase as supported by the blot. These experiments confirmed the presence of intergenic, antisense, 3′UTR-derived sRNAs, actuatons and transposase-related sRNAs in *P. putida* KT2440.

Discussion

In exponential growth phase the bacterial population is doubling due to favourable and nutrient rich conditions. When the nutrients become scarce and/or the waste products accumulate, the growth becomes inhibiting and the population enters the stationary phase. In between these phases cells have to fine-tune the regulation of the gene expression in order to survive the changing conditions – transition phase (18). In addition to small RNAs, there are many regulatory proteins and two-component signal transduction systems involved in cell growth and responses to environmental changes (19). Novel twists in sRNA mechanisms of regulation (20, 21) and Hfq roles as essential post-transcriptional regulator of several catabolic genes (11) have been uncovered in pseudomonads. This study was performed in order to shed light on the sRNAome dynamics during growth and the Hfq-mediated regulation of the sRNAome.

In this study we have altogether detected 557 sRNAs in *P. putida* KT2440. 221 of them (40%) (Table S4) have been differentially expressed during growth pointing at potential regulation of the whole metabolic rearrangements during growth and changing conditions. There are many sRNAs described being upregulated only in certain growth conditions where they exert their regulatory function. Just some examples are RgsA and PhrS in stationary phase in *P. aeruginosa* (22, 23), DapZ in transition phase in *Salmonella* (16).

sRNAome changes in the Δ*hfq* mutant in comparison to the wt provide clues about the transcript dependence upon the RNA chaperone Hfq. The experiments with the *hfq* deletion strain indicated that there are 171 sRNAs with changed expression, representing 31% of sRNAs being affected in the absence of Hfq. This could be due to direct or indirect effects of Hfq on their transcription and/or stability. The *hfq* knock-outs in many bacteria have resulted in pleiotropic phenotypes that have in common reduced fitness and attenuated responses against stressful conditions (24). Δ*hfq* mutant of *P. putida* KT2440 is slower in growth comparing to the wt in minimal and rich media, suggesting weakened metabolism due to the absence of Hfq. In general the strain was more sensitive to many stress conditions such as antibiotic and oxidizing agents presence, pH extremes, and lost some metabolic versatility (7). Hfq has been described to protect sRNAs from the ribonucleases or promote their degradation (5) and many resulting phenotypes in Hfq absence are possibly connected to its downstream effects on sRNAs regulatory networks. To learn how an RNA chaperone Hfq mediates gene regulation in pseudomonads, its direct targets need to be determined. This is the first study aiming to identify direct RNA transcripts bound to Hfq with co-immunoprecipitation in *P. putida*. 199 sRNAs (36%) have been significantly increased in the coIP experiment with Hfq, pointing that those sRNAs are Hfq-bound.

PhrS, a characterized sRNA in *P. aeruginosa*, which activates the translation of the key quorum sensing transcriptional regulator PqsR, is upregulated in stationary phase under oxygen limitations (22). In *P. putida* KT2440 we have not observed the same, PhrS has not been differentially expressed during the growth at any of the time points but has been downregulated 3-fold in exponential phase and slightly upregulated (2.5-fold) in transition phase in the Δ*hfq* mutant. *P. putida* is missing key virulence traits (10) and PqsR is only found in *P. aeruginosa* strains (21). In fact, PhrS has been found 3.3-fold downregulated in oxidative stress in a recent study in *P. putida* KT2440 (Bojanovič *et al.*, submitted). This points to a different role of PhrS in *P. putida* or maybe to the presence of additional targets of PhrS in pseudomonads.

Small RNA RgsA is expressed in stationary stage in *P. aeruginosa*, *P. fluorescens*, and *P. syringae* and its absence increases cells sensitivity to oxidative stress. This sRNAs is under RpoS regulation and has been reported to be Hfq-bound (23, 25). Our data confirms (Table S4) the increase of its levels during different growth phases (3-fold in transition and 9-fold in stationary phases) and Hfq-binding properties also in *P. putida*. In *hfq*-less strain RgsA was slightly downregulated, indicating that Hfq could be partly responsible for its stability.

RsmY and RsmZ are two functionally redundant sRNAs, which control secondary metabolism, carbon storage, virulence and stress responses in pseudomonads by sequestering RsmA protein (26). Only RsmY has been shown to be binding to Hfq, which protects it from the RNase E-mediated degradation (27). In our data RsmZ was upregulated transition phase in Δ*hfq* mutant and underrepresented in coIP experiment, confirming that it is not Hfq bound. On the other hand RsmY was downregulated in Δ*hfq* mutant but it has not been significantly enriched in the coIP. For transcripts whose levels are not found to be higher in coIP experiment it is unclear whether they are Hfq-bound or not. It has been shown that some RNA molecules can get degraded during the steps of the coIP protocol (28) and this might be the case with RsmY.

CrcZ and CrcY sRNAs bind and titrate Hfq and prevent it from repressing the target mRNAs of catabolite repression in *P. aeriginosa* PAO1 with the help of Crc protein (11). Both of them have been downregulated in Δ*hfq* mutant and overrepresented in the coIP confirming their Hfq dependence and binding properties. PrrF1 and PrrF2 are involved in iron homeostasis, regulation of central carbon metabolism and quorum-sensing. They have been shown to be Hfq-bound (29) and our data confirms it by both of them being downregulated in the Δ*hfq* mutant and overrepresented in coIP with Hfq.

ErsA/spf/Spot42-like sRNA is involved in regulation of the virulence-associated gene *algC* in *P. aeruginosa* PAO1 but does not target the same mRNA in PA14. Hfq has been shown to influence ErsA levels and mode of action (30) and our data confirms it's Hfq dependence and binding characteristics.

RIP-seq enables to identify RNAs bound to the tagged protein but indeed also has its limitations with possible nonspecific binding and unstable protein-RNA interactions during the experiments (31). A large number of sRNAs was detected only in coIP experiment setting, which is probably enriching sRNAs that are lower expressed and missed otherwise.

Different lengths of some sRNAs were detected in different experiments. The biases could come from the differences in the cDNA library preparations or because different processed RNA versions are present in different conditions due to unstable precursors as shown for SraH sRNA in *Salmonella* in the coIP experiment (32). Some of the cases were resolved with the Northern blot analysis (such as Pit052, Pit221, Pit142, Pit245, Pit229, etc). For example, Pit052 has been detected in both recent studies in *P. putida* KT2440 (33) (Bojanovič *et al.,* submitted) but each time a different length was predicted. As seen in the Northern blot, we detected two bands of 100 nt and 75 nt. The shorter band is probably a processed transcript that was detected in the coIP library while in the KB1 dataset we detected a full-length transcript. This shows how different experiments and library preparations lead to detection of various transcript versions and illustrates that RNA-Seq is not well-suited for mapping exact transcript lengths.

sRNAs can originate from genic as well as intergenic regions of the genome. They arise from antisense transcription, marooned riboswitches, tRNAs, and mRNAs (3). 5′UTR-derived transcripts or actuatons (17) as well as 3′UTR-derived sRNAs in eukaryotes (34) and in prokaryotes (16, 35). The latter could be independently transcribed or processed from mRNAs post-transcriptionally. We confirm that such cases also exist in *P. putida* with Pit023 and Pit192 probably being independent transcripts, while Pit245 seems to be a processed one.

Interesting cases are transcripts derived from insertion sequences (IS) Pit017 and its homologue Pit126. Similarly, stable sRNAs originating from close to or within transposable elements have been shown on Northern blot in *Salmonella* (32). ISPpu11 is part of the IS110 family of IS. IS are a group of transposable elements, which are dynamic in the host genome and are thought to contribute to the genetic variation and evolution. Such events of insertions, deletions and rearrangements on the chromosomes need to be well regulated because they can either provide lethal mutations or fitter mutants being better in adaptation to the environment encountered (36). Due to that IS are probably essential for the survival only in specific conditions and dispensible in more stable times (37). We have detected more sRNAs IS-derived from ISPpu9 and ISPpu10 (Table S2). These sRNAs could be horizontally acquired together with the IS element (4) or *de-novo* made during the rearrangements in the transposition, which came to a regulatory function (36). IS-derived sRNAs could have an important role to regulate horizontally-acquired sequences or they could also regulate core genome components (4, 38).

The Northern blot analyses have also demonstrated that several sRNAs go through processing events as some sRNAs showed band patterns that suggest specific cleavage. We have used Northern blot detection method for experimental validation of some sRNAs and although the method is able to shed light on length and processing events of the sRNA, it has several limitations. From 36 sRNAs tested in this study about only half of them were confirmed. The transcripts could not be detected if they are not sufficiently expressed, are present only a short time in a certain condition so they can be missed or they get degraded during the detection process. Also the detection would fail in case of highly structured RNAs and hybridization with probes is not successful (39).

The absence of Hfq leads to changes in 10% of the proteome in *P. putida* but it is unclear which effects are direct or indirect. Several loss-of-function phenotypes could be connected to Hfq involvement in regulation of the steady-state levels of RpoS, a general stress response regulator (7). In *E. coli*, RpoS is regulated on transcriptional, translational and post-translational levels and Hfq together with some sRNAs are important players in its regulation (40). We have found *rpoS* mRNA overrepresented in the Hfq coIP pointing at a similar mechanism in pseudomonads as described in *E. coli*. However, the involved sRNAs in *P. putida* needs to be studied further. In *S. typhimurium* around 25% of mRNAs have been found to be binding Hfq *in vivo* (32) and in *P. putida* this number is a bit higher – 35%.

Among the Hfq-bound mRNAs is *hfq* itself, as it has been previously described in some other bacteria (24, 41). In *E. coli* Hfq has been described to autorepress its own translation (42) and the same autoregulatory mechanism could be acting in pseudomonads. Additionally Pat337, an antisense sRNA to the *miaA* gene has been detected, which starts 36 nt in front of the *hfq* translation start site and could possibly add another level of regulation of the Hfq protein.

The numbers of detected sRNAs in bacteria have risen sharply in recent years as a result of development of various RNA-Seq methodologies. In *P. putida* KT2440 there have been 388 intergenic and 335 antisense sRNAs detected altogether (in this and previous studies) (Frank *et al*., 2011; D'Arrigo *et al*., 2016, Bojanovič *et al*., submitted). Out of these 33 intergenic and 133 antisense sRNAs have not been detected in this work. This could be due to the use of different conditions, cDNA library preparations, enrichment methods of RNA transcripts, sequencing technologies, and analysis pipelines.

Taken together, our data provide insights into the impact of Hfq on sRNA and gene expression and various origins of sRNAs in *P. putida*. Many of the sRNAs have been detected in at least two independent experiments and show differential expression during growth or in stress conditions (Bojanovič *et al*., submitted). Such transcripts probably exert various biological functions, which need further characterization in order to be unraveled. With more tested conditions and different detection methods, even more sRNAs might be detected in the future.

Materials and Methods

Bacterial strains and growth conditions

P. putida KT2440 was grown at 30 °C with shaking at 250 rpm and routinely cultured in LB medium in the shake flasks according to standard protocols (44) unless otherwise stated. *E. coli* DH5*α λpir* was used for cloning and plasmid maintenance and was grown in LB at 37 °C.

For all experiments the overnight culture of *P. putida* was diluted to a starting OD_{600} of 0.1 in 100 mL (for KB1 library) or 200 mL (for KB4 library) of LB. Cells were harvested at three time points on the growth curve: mid-exponential (OD_{600}~0.5), transition (OD_{600}~2.5), and stationary phase (twice doubling time after the transition phase). For the size-selected library KB1 three biological replicates of wt and Δ*hfq* strains were grown and for coIP experiments two biological replicates of wt and KL3 (*P. putida* KT2440 *hfq*_3xFLAG) were used.

Hfq-tagging

The *hfq* gene was C-terminally tagged with 3xFLAG epitopes on the chromosome. DNA fragments were amplified by PCR using the PfuX7 polymerase (45) and the primers listed in Table S7. The 3xFLAG sequence (46) was inserted with primers containing long tails carrying a sequence for 3xFLAG. Plasmid pKB1 was constructed with the Uracil excision-based cloning system (USER). The backbone pSIJ218 and two homologous regions of about 800 bp of *P. putida* KT2440 genome (upstream and downstream of the insertion – stop codon of *hfq* gene) were amplified with primers containing uracil, forming single-stranded overhangs compatible among each other to form the plasmid pKB1 in a USER reaction as described previously (47). The plasmid was transformed into chemically competent *E. coli* DH5*α λpir* for plasmid replication. The correct assembly of pKB1 was checked by colony PCR and sequencing. pKB1 was further transformed into *P. putida* KT2440 by electroporation as previously described (48). The *hfq* gene was replaced with the *hfq*::3xFLAG allele forcing two recombination events by conditionally expressing a homing endonuclease I-*Sce*I introduced in plasmid pSW-I, as previously described (49). Correct genomic insertions were confirmed by sequencing and the resulting strain with the 3xFLAG tag on the *hfq* gene was named KL3.

Immunoprecipitation assays

For coIP experiments wt and KL3 strains were used. An equivalent number of cells to OD_{600} = 50 was harvested in three points on the curve by centrifugation at 4000 x *g* at 4 °C for 10 min. Pellets were washed with 1 mL of ice-cold lysis buffer (20 mM Tris pH 8, 150 mM KCl, 1 mM MgCl2, 1 mM DTT), snap frozen and stored on -80 °C. Cell pellets were resuspended in 0.8 mL of lysis buffer, supplemented with 200 U of RiboLock RNase inhibitor (Thermo Scientific) and cOmplete mini protease inhibitor (Roche). Cells were lysed by vortexing with glass beads (diameter 150-212 μm, Sigma) twice for 20 s, with a 1 min incubation on ice in between. Lysis buffer (0.4 mL) was added, followed by centrifugation at 16.000 x *g* for 30 min at 4 °C. The cleared lysate (0.9 mL) was used for immunoprecipitation with Hfq combined with 80 μL of ANTI-FLAG M2 Magnetic Beads (Sigma, M8823) for 4 h at 4 °C with rotation. Beads with precipitated antibody-protein-RNA complexes were washed five times with TBS and finally resuspended in 0.5 mL of TBS. RNA was extracted by phenol:chlorophorm:isoamyl alcohol and ethanol-precipitated. Following DNaseI treatment (Fermentas), the RNA was analysed with a Bioanalyzer (RNA 6000 Pico Kit, Agilent). During the immunoprecipitation assay aliquots were saved and used for Western blot analysis.

Western blot

Cell lysates equivalent to OD_{600} = 50 were used for Western blot. Protein samples were mixed with NuPAGE LDS sample buffer without reducing agent and heated at 70 °C for 10 min, followed by separation on 10% Bis-Tris NuPAGE gels (Life Technologies). Proteins were electro-blotted with iBlot™ (Invitrogen). Membranes were blocked in 5% non-fat milk in TBST for 1 h at room temperature. The membranes were then incubated with monoclonal ANTI-FLAG M2-Peroxidase M2 antibodies (Sigma, #A8592) and developed using ECL™ Prime reagent (GE Healthcare).

Total RNA isolation

RNA extraction for cDNA library KB1 preparations and Northern blots was performed as previously described (50). Briefly, 10 mL of harvested culture was mixed with 0.2 volumes of STOP solution (95% [v/v] ethanol, 5% [v/v] phenol). Cells were centrifuged, 1 mL of Trizol (Invitrogen) was added and the samples were snap frozen. Total RNA was extracted and treated with DNase I (Fermentas). Total RNA integrity and quality were validated by Bioanalyzer (Agilent).

cDNA library construction

For the KB1 cDNA library, RNA was size-selected (up to 500 nt) as described previously (50) using 10% polyacrylamide-urea gels containing urea (Bio-Rad) with some changes. The samples were depleted of rRNA using the MICROBExpress Kit (Ambion) and treated with Tobacco Acid Pyrophosphatase TAP (Epicentre). Following the fragmentation with RNaseIII, the libraries were prepared using the TruSeq Small RNA Sample Preparation Kit (Illumina).

For the KB4 cDNA library, coIPed RNA was treated with RNA 5′ Polyphosphatase (Epicentre) instead of TAP. The fragmentation was omitted and cDNA libraries were prepared using the same Illumina kit.

Deep sequencing and data analyses

The KB1 cDNA libraries were sequenced on the Illumina HiSeq2000 platform (pair-end sequencing with read lengths of 100 nt). The KB4 cDNA libraries were sequenced on the Illumina NextSeq 500/550 platform (single-end sequencing with read lengths of 75n nt). RNA-seq data was analysed with the open source software Rockhopper (version 2.0.3) (14). For read mapping the sequenced reference genome *P. putida* KT2440 was used (GenBank accession no. NC_002947.3) and the results are summarized in Table S1. For the novel transcript identification the data was visually inspected with Integrative Genomics Viewer (15). Differential gene and sRNA expression analysis were carried out with the webserver T-REx (51) using the RPKM values generated in the Rockhopper analysis. Differential expression of genes was considered significant with a fold change ≥ 2 and adjusted p-value ≤ 0.05.

Northern blot

For Northern blot analysis 10 μg of total RNA from wt and Δ*hfq* strain were treated with DNaseI (Fermentas) and separated on 15% Mini-PROTEAN TBE-Urea gels (Bio-Rad). Prior to loading on the gel, RNA was mixed with Gel Loading Buffer II (Ambion) and incubated on 95 °C for 5 min. RNA was transferred to the nylon membranes using iBlot DNA Transfer Stacks (Life Technologies). After UV-crosslinking, the membranes were probed with DNA probes (Table S7) in Amersham Rapid-hyb buffer (GE Healthcare) following the manufacturer's protocol. DNA probes were labelled with [γ-32P]-ATP using T4 PNK (Thermo Scientific) per user manual.

Accession numbers

RNAseq data has been deposited at the GEO Database under accession numbers: GSE85578 (KB1) and GSE85581 (KB4).

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Table

Table 1: Common sRNAs affected in all three growth stages in the Δ*hfq* mutant.

Nr.	sRNA	Δ*hfq* Ex : wt Ex	Δ*hfq* Tr : wt Tr	Δ*hfq* St : wt St
1	Pit221	-2226.7	-220.6	-3826.2
2	Pit222	-547.2	-564.3	-307
3	Pit139	-269.4	-28.8	-29.8
4	CrcZ	-98.6	-297.4	-358.2
5	Pit140/IGR 3917	-73.3	-23.2	-20.6
6	Pat170	-68.1	-191.6	-67.7
7	CrcY	-63.2	-194.5	-146.3
8	Pit245	-6.3	2.4	-25.5
9	PrrF2	-6.3	-5.3	-3.9
10	Pit049	2	2.2	2.6
11	Pit056	2	2.2	2.6
12	Pit105	2	2.2	2.6
13	Pit124	2	2.2	2.6
14	Pit132/IGR 3586	2	2.2	2.6
15	Pit154	2	2.2	2.6
16	Pit213	3.5	-2.9	-3.5
17	Pit218	3.5	23.4	2.3
18	Pat182	5.2	6.2	3.2
19	Pit207	5.5	8.1	4
20	Pit118	8.8	13.1	7
21	Pit192	48.6	9.5	4.4

Figures

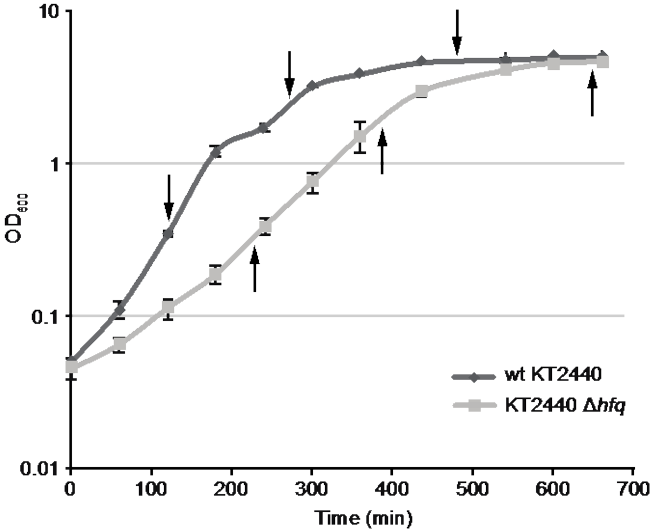

Figure 1: Growth curves of the *P. putida* KT2440 wild type and *hfq* deletion (Δ*hfq*) strains in LB medium at 30°C. The points of cell harvest are marked with arrows.

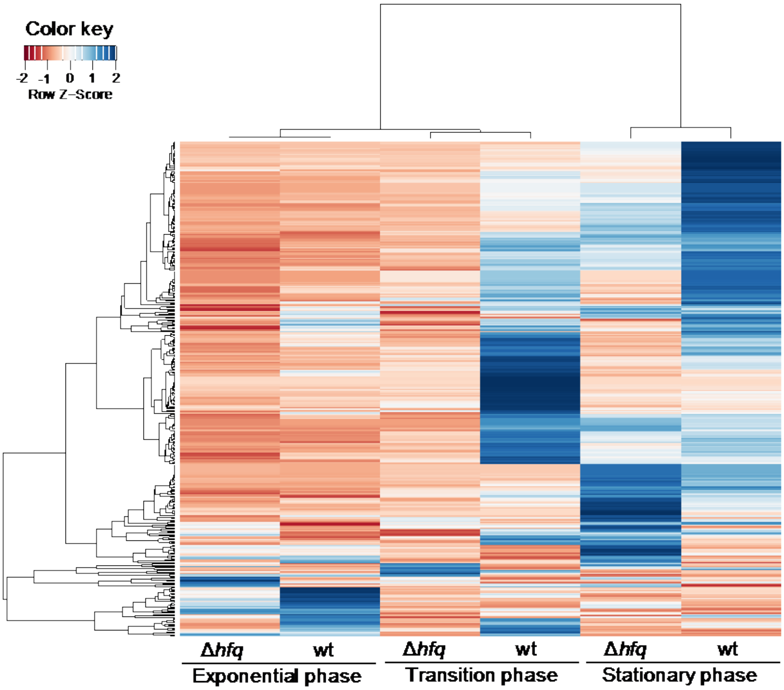

Figure 2: Heat map showing relative sRNA expression levels in *P. putida* KT2440 wild type and Δ*hfq* mutant strains in exponential, transition and stationary growth phases.

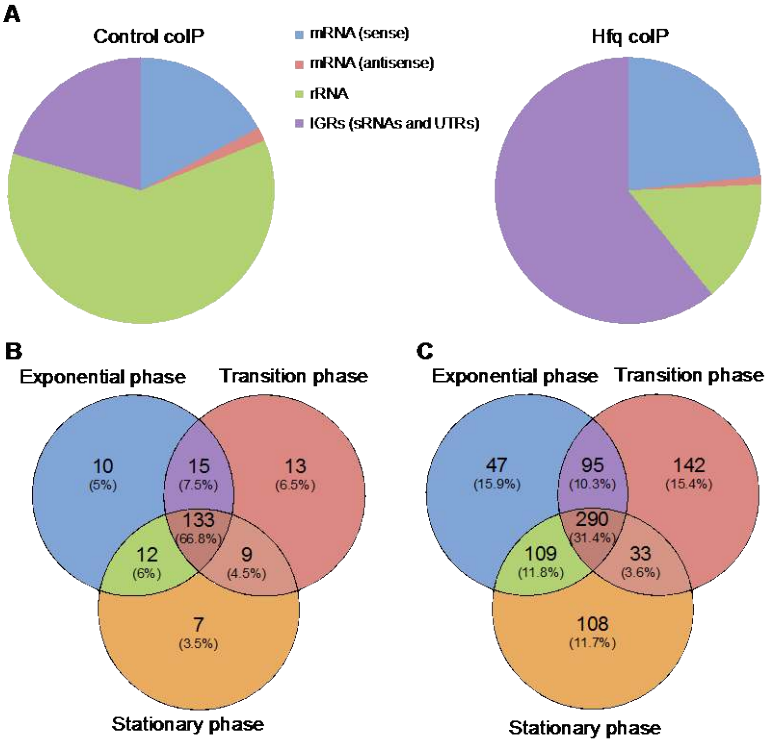

Figure 3: Results of the co-immunoprecipitation experiment. (A) Pie chart of sequencing results of the KB4 library. Venn diagrams of the overrepresented sRNAs (B) and mRNAs (C) in the coIP samples in different growth phases.

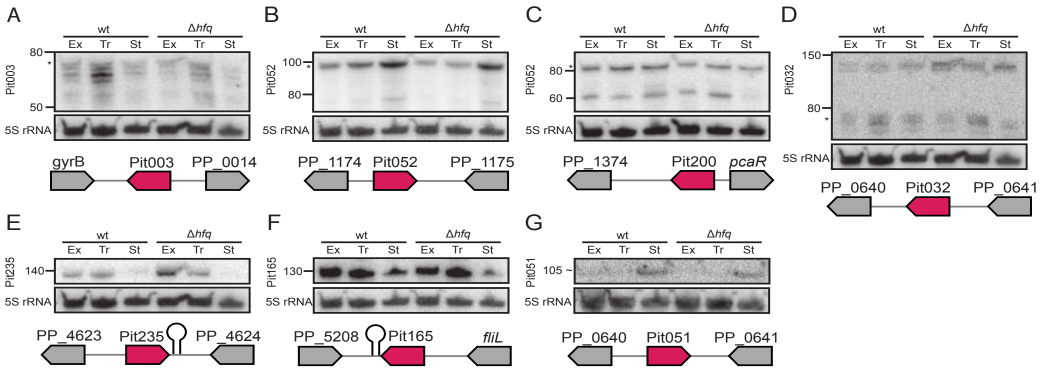

Figure 4: Identification and verification of intergenic sRNAs in *P. putida*. Total RNA was extracted at three time points from the wild type and Δ*hfq* mutant (Ex – exponential, Tr – transition, St – stationary phase) and analysed by Northern blot. (A) Pit003 – a 72 nt long transcript, (B) Pit052 – a 94 nt long transcript and a processed version of 75 nt as detected in coIP, (C) Pit200 – 93 nt long transcript (D) Pit032 – 77 nt long transcript, (E) Pit235 – 138 nt long transcript, (F) Pit165 – 128 nt long transcript, (G) Pit051 - 105 nt long transcript. RNA transcripts are indicated (*).

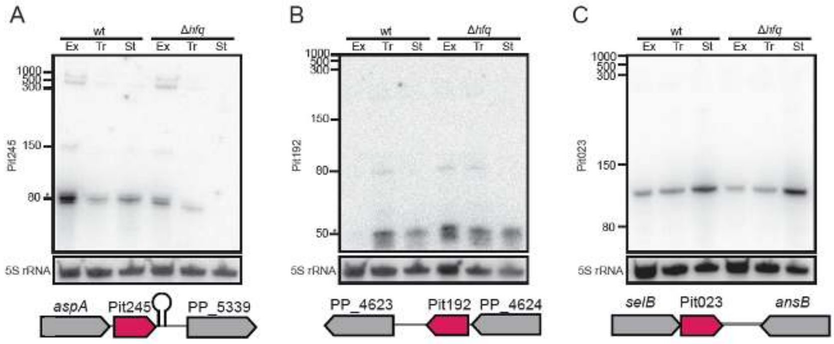

Figure 5: Identification and verification of 3'UTR-derived sRNAs in *P. putida*. Total RNA was extracted at three time points from the wild type and Δ*hfq* mutant (Ex – exponential, Tr – transition, St – stationary phase) and analysed by Northern blot. (A) Pit245 – a 85 nt long transcript processed from mRNA of the *aspA* gene sharing a common Rho IT with the mRNA, (B) Pit192 – a 49 nt long transcript, which is not processed from mRNA but is probably expressed from an independent promoter, (C) Pit023 – 94 nt long transcript processed from mRNA of the *selB* gene. RNA transcripts are indicated (*).

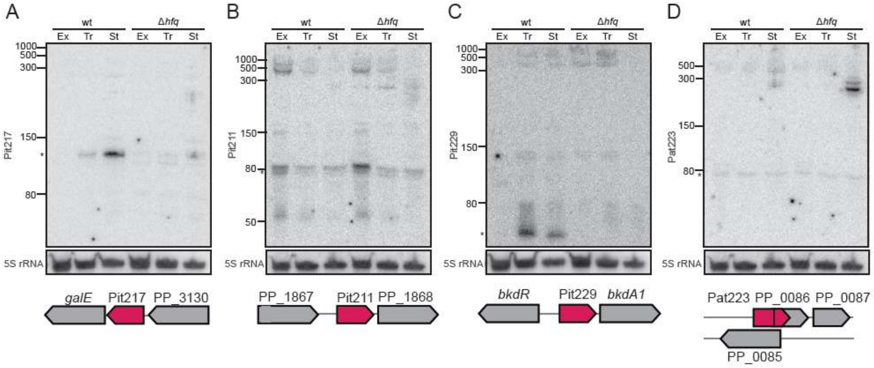

Figure 6: Identification and verification of 5'UTR-derived sRNAs in *P. putida*. Total RNA was extracted at three time points from the wild type and Δ*hfq* mutant (Ex – exponential, Tr – transition, St – stationary phase) and analysed by Northern blot. (A) Pit217 – a 102 nt long transcript derived from 5'UTR of mRNA of the *galE* gene, (B) Pit211 – a 72 nt long transcript derived from 5'UTR of mRNA of the PP_1868, (C) Pit229 – 76 nt long transcript derived from 5'UTR of mRNA of the *bkdA1* gene, (D) Pat223 – 76 nt long transcript derived from 5'UTR and 5'end of mRNA of the PP_0086, and antisense to PP_0085. RNA transcripts are indicated (*).

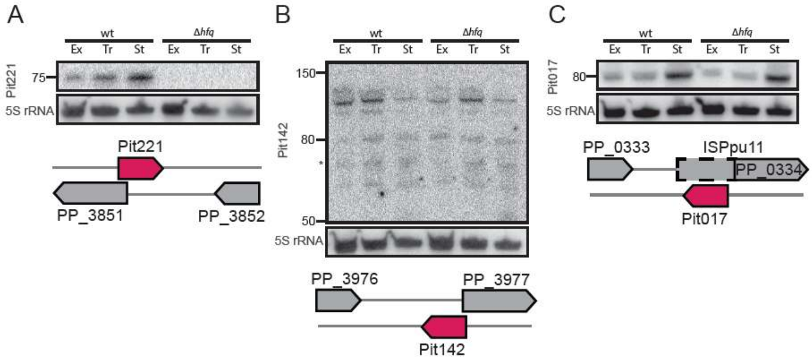

Figure 7: Identification and verification of antisense and transposase-related sRNAs in *P. putida*. Total RNA was extracted at three time points from the wild type and Δ*hfq* mutant (Ex – exponential, Tr – transition, St – stationary phase) and analysed by Northern blot. (A) Pit221 – a 76 nt long transcript and (B) Pit142 – 75 nt long transcript with processed species and possibly a longer precursor. Both A and B were primarily identified short and intergenic but were found longer in coIP and antisense, which was confirmed by Northern blot. (C) Pit017 – 79 nt long transcript antisense to the 5′UTR of the transposase ISPpu11 (PP_3977). The same situation is observed in homologous transcript Pit126 antisense to 5′UTR of the ISPpu11 (PP_3498) (representative Pit017 is shown). RNA transcripts are indicated (*).

Supplementary Material

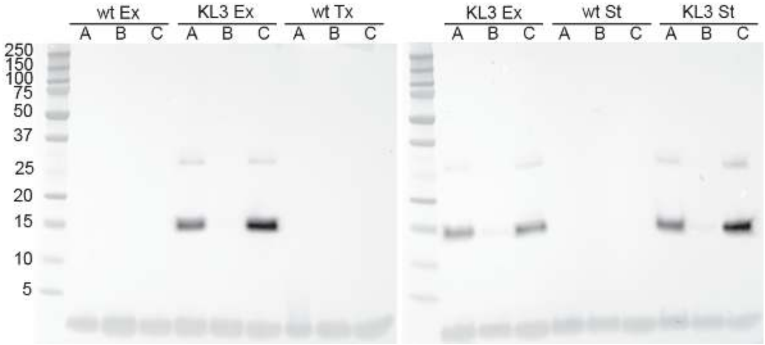

Figure S1: Western blot analysis of the co-immunoprecipitation of Hfq in the wild type (wt) strain and C-terminal tagged-Hfq strain (KL3) in exponential (Ex), transition (Tr) and stationary (St) phases. The loaded samples are total protein (A), flow-through fraction (B), and the coIP fraction (C).

Table S6: Fold enrichment of mRNA binding in the co-immunoprecipitation with Hfq protein (KB4 dataset). (The table is too big to be shown.)

Table S1: RNA sequencing results from the pooled cDNA sequencing libraries KB1 and KB4.

Legend:
Ex - exponential phase
Tr - transition phase
St - stationary phase
Wt - wild type, control
Δhfq - P. putida KT2440 Δ*hfq*
KL3 - P. putida KT2440 *hfq*::3xFLAG

Library KB1	**WtEx1**	**WtEx2**	**WtEx3**	**WtTr1**	**WtTr2**	**WtTr3**	**WtSt1**	**WtSt2**	**WtSt3**	**Δ*hfq*_Ex1**	**Δ*hfq*_Ex2**	**Δ*hfq*_Ex3**	**Δ*hfq*_Tr1**	**Δ*hfq*_Tr2**	**Δ*hfq*_Tr3**	**Δ*hfq*_St1**	**Δ*hfq*_St2**	**Δ*hfq*_St3**	**Summary**
Total reads:	8,422,199.00	10,196,287.00	9,871,015.00	9,748,490.00	11,287,219.00	9,459,885.00	11,530,076.00	12,086,137.00	14,238,702.00	9,991,334.00	10,760,347.00	9,549,860.00	11,931,603.00	9,201,781.00	7,420,271.00	11,931,485.00	9,469,629.00	10,649,019.00	187,745,339.00
Successfully aligned reads:	6,851,404.00	8,280,492.00	8,187,055.00	8,177,506.00	9,314,981.00	7,625,646.00	9,306,441.00	9,741,250.00	11,367,593.00	8,465,039.00	8,722,839.00	7,926,093.00	9,936,708.00	7,510,529.00	6,147,969.00	9,770,532.00	7,520,217.00	8,524,882.00	153,377,176.00
Successfully aligned reads (%):	81%	81%	83%	84%	83%	81%	81%	81%	80%	85%	81%	83%	83%	82%	83%	82%	79%	80%	
Aligning (sense) to protein-coding genes:	2%	2%	1%	2%	2%	2%	2%	2%	3%	1%	2%	1%	1%	2%	1%	2%	2%	2%	
Aligning (sense) to ribosomal RNAs:	83%	83%	91%	85%	86%	83%	79%	79%	75%	94%	82%	92%	92%	88%	88%	86%	81%	82%	
Aligning (sense) to transfer RNAs:	2%	3%	1%	2%	3%	2%	3%	3%	4%	1%	2%	1%	1%	1%	1%	2%	3%	3%	
Aligning to unannotated regions:	13%	12%	6%	11%	10%	12%	15%	16%	19%	5%	14%	6%	6%	9%	9%	10%	14%	12%	

Library KB4	**WtEx1**	**WtEx2**	**WtTr1**	**WtTr2**	**WtSt1**	**WtSt2**	**KL3_Ex1**	**KL3_Ex2**	**KL3_Tr1**	**KL3_Tr2**	**KL3_St1**	**KL3_St2**	**Summary**
Total reads:	10,331,667	6,150,201	8,718,618	7,995,165	5,217,614	3,798,588	14,581,613	9,715,656	11,142,763	6,559,685	11,659,048	8,584,736	104,455,354
Successfully aligned reads:	8,697,959	5,025,841	7,456,311	6,798,016	4,359,985	3,002,654	13,151,097	8,846,431	9,727,146	5,716,817	9,413,459	7,386,900	89,582,616
%	84%	82%	86%	85%	84%	79%	90%	91%	87%	87%	81%	86%	
Aligning (sense) to protein-coding genes:	15%	14%	21%	18%	20%	15%	32%	32%	19%	18%	20%	18%	
Aligning (antisense) to protein-coding genes:	1%	1%	2%	2%	2%	2%	1%	1%	1%	1%	1%	1%	
Aligning (sense) to ribosomal RNAs:	67%	69%	53%	59%	53%	62%	16%	18%	7%	9%	18%	21%	
Aligning to unannotated regions:	16%	16%	24%	21%	25%	21%	50%	49%	73%	71%	61%	59%	

Table S2: Intergenic sRNAs detected in this study.

Table legend:

3'UTR - Possibly 3'UTR-derived transcript

5'UTR - Possibly 5'UTR-derived transcript

antisense - these transcripts were detected longer in KB4 library and are therefore antisense to an ORF

3'UTR/5'UTR - the transcript is either 3'UTR- or 5'UTR-derived, it is not clear

		KB1				KB4							
Nr.	Name	Start	Stop	Length	Stand	Start	Stop	Length	Stand	Upstream flanking gene	Downstream flanking gene	Orientation	Comment
1	Pit001	9148	9338	191	+	9129	9251	123	+	PP_0009	PP_0010	< > >	
2	Pit002	16274	16419	146	+	16160	16468	309	+	PP_0013	PP_0014	> > >	
3	Pit003	16419	16348	72	-	16419	16339	81	-	PP_0013	PP_0014	> < >	
4	Pit004	32378	32468	91	+					PP_0028	PP_0029	< > >	
5	Pit005	56009	55915	95	-	56019	55944	76	-	PP_0048	PP_0049	> < <	
6	Pit006	58359	58537	179	+	58364	58439	76	+	PP_0049	PP_0050	< > <	
7	Pit007	81305	81222	84	-				-	PP_0070	PP_0071	> < >	
8	Pit247					81212	81316	105	+	PP_0070	PP_0071	> < >	
9	Pit248					105103	104991	113	-	PP_0099	PP_0100	> < >	
10	Pit249					123114	123039	76	-	PP_0116	PP_0117	< < <	3'UTR
11	Spot42-like/spf/ErsA	130370	130542	173	+	130302	130570	269	+	PP_0123	PP_0124	> > <	
12	Pit008	144096	144230	135	+	144096	144230	135	+	PP_0136	PP_0137	> > >	
13	Pit009	193949	193796	154	-	193949	193796	154	-	PP_0167	PP_0168	< < >	
14	Pit179	194402	194465	64	+	194336	194445	110	+	PP_0167	PP_0168	< < >	5'UTR/actuaton
15	Pit250					220526	220601	76	+	PP_0168	PP_0169	> > >	3'UTR
16	Pit251					251841	252054	214	+	PP_0201	PP_0202	> > <	3'UTR
17	Pit252					252110	252184	75	+	PP_0201	PP_0202	> > <	
18	Pit253					263055	263130	76	+	PP_0212	PP_0213	> > >	3'UTR
19	Pit180	264705	264744	40	+					PP_0213	PP_0214	> > >	3'UTR
20	Pit014					288416	288341	76	-	PP_0233	PP_0234	< < >	
21	Pit181	289902	289946	45	+					PP_0234	PP_0235	> > >	3'UTR
22	Pit015	321886	321952	67	+	321898	321950	53	+	PP_0266	PP_0267	> > <	
23	Pit182	324548	324644	97	+	324549	324624	76	+	PP_0267	PP_0269	< > >	5'UTR/actuaton
24	C4_AS_RNA_1	335696	335870	175	+	335673	335795	123	+	PP_0277	PP_0278	< > >	
25	Pit183	353109	353079	31	-					PP_0293	PP_0294	> < >	3'UTR
26	Pit254					358250	358325	76	+	PP_0297	PP_0298	> > >	
27	Pit184	372596	372674	79	+	372596	372674	79	+	PP_0310	PP_3011	> > >	3'UTR
28	Pit185	379511	379465	47	-	379512	379433	80	-	PP_0315	PP_0316	< < >	5'UTR/actuaton
29	Pit017	400370	400292	79	-	400353	400166	188	-	PP_0333	PP_0334	> < >	antisense: PP_0333
30	Pit186	403301	403270	32	-	403331	403256	76	-	PP_0336	PP_0337	< < <	5'UTR/actuaton
31	Pit255					406127	406202	76	+	PP_0337	PP_0338	< > <	
32	Pit329					406325	406258	68	-	PP_0337	PP_0338	< < <	3'UTR
33	Pit019	410974	410872	103	-	411029	410949	81	-	PP_0339	PP_0340	< < >	
34	RsmY	450781	450944	164	+	450790	450913	124	+	PP_0370	PP_0371	> > <	
35	Pit020	450911	450813	99	-					PP_0370	PP_0371	> < <	
36	Pit256					453377	453469	93	+	PP_0372	PP_0373	> > >	3'UTR
37	Pit021_Pit022	453746	453994	249	+	453757	453899	143	+	PP_0373	PP_0374	> > >	
38	Pit257					465214	465322	109	+	PP_0383	PP_0384	< > <	
39	Pit187	478279	478332	54	+					PP_0393	PP_0394	> > <	
40	Pit258					506992	507062	71	+	PP_0417	PP_0418	> > <	3'UTR
41	P26	537405	537502	98	+	537436	537463	28	+	PP_0446	PP_0447	> > >	
42	rpsL	546001	546170	170	+	546085	546170	86	+	PP_0448	PP_0449	> > >	
43	Pit259					557460	557384	77	-	PP_0467	PP_0468	> < >	
44	Alpha_RBS	561399	561492	94	+	561411	561492	82	+	PP_0475	PP_0476	> > >	
45	Pit023	584059	584152	94	+					PP_0494	PP_0495	> > <	3'UTR
46	Pit024/RNA4	611076	610907	170	-	611021	610919	103	-	PP_0525	PP_0526	< < >	5'UTR/actuaton
47	FMN RS	616507	616373	135	-					PP_0530	PP_0531	< < >	
48	Pit025	624137	623992	146	-	624144	624069	76	-	PP_0536	PP_0537	< < <	
49	Pit188	650883	650851	33	-					PP_0560	PP_0561	< < <	5'UTR/actuaton
50	Pit260					658314	658390	77	+	PP_0565	PP_0566	> > >	5'UTR/actuaton
51	Pit026	703218	703134	85	-	703218	703143	76	-	PP_5SD	PP_0598	> < >	
52	Pit027	730413	730314	100	-	730440	730366	75	-	PP_0624	PP_0625	> < >	
53	Pit261					747254	747179	76	-	PP_0638	PP_0639	< < <	5'UTR/actuaton
54	Pit262					748289	748364	76	+	PP_0638	PP_0639	< > <	
55	Pit030	750949	751020	72	+	750910	750985	76	+	PP_0640	PP_0641	< > <	
56	Pit031	751819	752405	587	+	751778	752271	494	+	PP_0640	PP_0641	< > <	
57	Pit032	752374	752336	39	-	752292	752216	77	-	PP_0640	PP_0641	< < <	
58	Pit263					753588	753514	75	-	PP_0641	PP_0642	< < >	
59	Pit264					755299	755237	63	-	PP_0641	PP_0642	< < >	
60	C4_AS_RNA_2	759536	759686	151	+	759628	759704	77	+	PP_0651	PP_0652	> > <	
61	Pit034					813322	813357	36	+	PP_t17	PP_0700	< > <	
62	Pit035	867940	867989	50	+	867986	868040	55	+	PP_0750	PP_0751	> > <	
63	Pit265/IGR 0752					870123	869954	170	-	PP_0751	PP_0752	< < >	
64	YybP-YkoY	876097	875931	167	-	876111	875931	181	-	PP_0760	PP_0761	< < <	
65	Pit189	894566	894609	44	+	894553	894625	73	+	PP_0776	PP_0777	> > >	3'UTR
66	Pit266	1011422	1011502	81	+	1011427	1011502	76	+	PP_0871	PP_0872	< > >	
67	Pit190	1013214	1013248	35	+					PP_0872	PP_0873	> > <	3'UTR
68	Pit191	1017441	1017395	47	-					PP_0877	PP_0878	< < <	
69	Pit036	1017579	1017521	59	-	1017578	1017478	101	-	PP_0877	PP_0878	< < <	
70	Pit037					1017890	1018038	149	+	PP_0877	PP_0878	< > <	
71	Pit038	1017904	1017824	81	-	1017904	1017827	78	-	PP_0877	PP_0878	< < <	
72	Pit192	1025337	1025289	49	-					PP_0883	PP_0884	< < <	3'UTR
73	Pit193/IGR 0886	1028970	1029065	96	+	1028976	1029057	82	+	PP_0885	PP_0886	< > <	
74	Pit039	1105372	1105328	45	-	1105428	1105353	76	-	PP_0965	PP_0966	> < >	
75	Pit194	1105351	1105428	78	+	1105285	1105359	75	+	PP_0965	PP_0966	> > >	5'UTR/actuaton
76	Pit040	1142630	1142533	98	-	1142629	1142554	76	-	PP_1002	PP_1003	< < >	
77	Pit041	1168594	1168633	40	+					PP_1024	PP_1025	> > <	
78	Pit267					1232821	1232891	71	+	PP_1073	PP_1074	< > <	
79	Pit195	1236310	1236261	50	-	1236306	1236187	120	-	PP_1076	PP_1077	< < >	5'UTR/actuaton
80	Pit268					1243022	1242947	76	-	PP_1083	PP_1084	< < >	
81	Pit042					1274821	1274745	77	-	PP_1115	PP_1116	< < <	
82	Pit043	1275830	1275106	725	-	1275828	1275071	758	-	PP_1115	PP_1116	< < <	
83	Pit044	1276407	1276534	128	+	1276318	1276631	314	+	PP_1115	PP_1116	< > <	
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85	Pit046					1278474	1278352	123	-	PP_1116	PP_1117	< < <	
86	Pit047	1280602	1281105	504	+	1280582	1281127	546	+	PP_1117	PP_1118	< > <	
87	Pit048	1296787	1296602	186	-	1296786	1296618	169	-	PP_1132	PP_1133	< < >	
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89	PhrS	1316221	1316433	213	+	1316226	1316432	207	+	PP_1148	PP_1150	> > >	
90	Pit050	1316513	1316462	52	-	1316528	1316411	118	-	PP_1149	PP_1150	> < >	

91	Pit269					1348785	1348689	97	-	PP_1173	PP_1174	< < <	5'UTR/actuaton
92	Pit051	1349036	1349140	105	+	1349048	1349216	169	+	PP_1173	PP_1174	< > <	
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94	Pit270					1352090	1352015	76	-	PP_1177	PP_1178	< < <	
95	Pit271					1355848	1355923	76	+	PP_1180	PP_1181	> > >	
96	Pit196	1360848	1360881	34	+					PP_1185	PP_1186	> > >	5'UTR/actuaton
97	Pit053	1385249	1385161	89	-					PP_1205	PP_1206	< < <	3'UTR
98	Pit272					1386801	1386723	79	-	PP_1206	PP_1207	< < >	
99	Pit054	1388606	1388390	217	-	1388618	1388420	199	-	PP_1209	PP_1210	< < >	
100	Pit197	1420292	1420355	64	+	1420198	1420459	262	+	PP_1244	PP_1245	< > >	5'UTR/actuaton
101	Pit055	1440302	1440115	188	-	1440300	1440133	168	-	PP_1259	PP_1260	< < >	
102	Pit056	1441836	1441995	160	+	1441832	1441997	166	+	PP_1260	PP_1261	> > <	
103	Pit198	1436067	1436031	37	-					PP_1256	PP_1257	< < <	
104	Pit199	1478795	1478827	33	+					PP_1291	PP_1292	> > >	3'UTR
105	RnpB/P28	1512685	1513072	388	+	1512690	1513092	403	+	PP_1326	PP_1328	> > >	
106	groES	1549132	1549255	124	+	1549116	1549255	140	+	PP_1359	PP_1360	> > >	
107	Pit273					1560111	1560210	100	+	PP_1370	PP_1371	< > <	
108	Pit200	1566223	1566131	93	-	1566223	1566131	93	-	PP_1374	PP_1375	< < >	
109	RNA6					1607683	1607566	118	-	PP_1409	PP_1408	< < <	5'UTR/actuaton
110	Pit058	1626891	1627080	190	+					PP_1426	PP_1427	< > >	
111	Pit202	1644838	1644877	40	+					PP_1443	PP_1444	< > <	
112	Pit059	1678539	1678624	86	+	1678528	1678628	101	+	PP_1473	PP_1474	> > <	3'UTR
113	Pit274					1685134	1685208	75	+	PP_1480	PP_1481	< > <	
114	Pit203	1744940	1745074	135	+	1744942	1745067	126	+	PP_1542	PP_1543	< > <	
115	RNA7	1748828	1748728	101	-	1748902	1748827	76	-	PP_1549	PP_1548	< < <	5'UTR/actuaton
116	Pit060	1749031	1749276	246	+	1748972	1749339	368	+	PP_1548	PP_1549	< > <	
117	Pit204	1750629	1750589	41	-					PP_1550	PP_1551	> < >	
118	Pit061	1777484	1777421	64	-	1777482	1777396	87	-	PP_1584	PP_1585	> < <	
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120	Pit062	1804568	1804667	100	+	1804550	1804624	75	+	PP_1607	PP_1608	> > >	3'UTR
121	Pit205	1810254	1810320	67	+	1810259	1810334	76	+	PP_1612	PP_1613	> > >	3'UTR
122	Pit206	1811425	1811473	49	+	1811425	1811500	76	+	PP_1614	PP_1615	> > <	3'UTR
123	Pit275					1818735	1818661	75	-	PP_1622	PP_1623	> < >	
124	RsmZ	1822011	1822190	180	+	1822011	1822169	159	+	PP_1624	PP_1625	> > <	
125	Pit063	1822122	1822033	90	-					PP_1624	PP_1625	> < <	
126	Pit064	1847250	1847088	163	-	1847225	1847101	125	-	PP_1652	PP_1653	> < >	
127	Cobalamin_RS_1	1866975	1867299	325	+	1867140	1867301	162	+	PP_1671	PP_1672	< > >	
128	Pit207	1883402	1883348	55	-					PP_1690	PP_1691	< < <	3'UTR
129	Pit065	1884034	1883852	183	-	1884089	1883799	291	-	PP_1691	PP_1692	< < >	
130	Pit276					1898998	1898929	70	-	PP_t29	PP_1703	> < >	
131	Pit066	1915656	1915801	146	+	1915661	1915736	76	+	PP_1714	PP_1715	> > >	5'UTR/actuaton
132	Pit277					1936847	1936922	76	+	PP_1736	PP_1737	> > <	3'UTR
133	gyrA	1970946	1971021	76	+	1970908	1971043	136	+	PP_1766	PP_1767	> > >	
134	RNA1	1995669	1995846	178	+	1995617	1995693	77	+	PP_1781	PP_1782	< > <	
135	Pit068	2005781	2005913	133	+	2005751	2005936	186	+	PP_1788	PP_1789	< > <	
136	Pit070	2034336	2034551	216	+	2034323	2034481	159	+	PP_1808	PP_1809	> > >	
137	Pit071	2034605	2034311	295	-	2034674	2034242	433	-	PP_1808	PP_1809	> < >	
138	Pit278					2035173	2035098	76	-	PP_1808	PP_1809	> < >	
139	Pit279					2036030	2035962	69	-	PP_1809	PP_1810	> < >	
140	Pit074	2038126	2037940	187	-	2038123	2038048	76	-	PP_1810	PP_1811	> < <	
141	Pit208	2046745	2046804	60	+	2046691	2046766	76	+	PP_1819	PP_1820	> > <	3'UTR
142	Pit209	2050632	2050588	45	-					PP_1824	PP_1825	< < <	
143	Pit210	2064131	2064174	44	+	2064098	2064172	75	+	PP_1840	PP_1841	> > <	3'UTR
144	Pit211	2090459	2090530	72	+	2090462	2090631	170	+	PP_1867	PP_1868	> > >	5'UTR/actuaton
145	Pit076	2140113	2139959	155	-	2140049	2139973	77	-	PP_1896	PP_1897	> < >	
146	Pit077	2151137	2150965	173	-	2151141	2151075	67	-	PP_1905	PP_1906	< < >	
147	Pit078	2164081	2164204	124	+					PP_1919	PP_1920	> > <	
148	Pit280					2168560	2168635	76	+	PP_1921	PP_1922	> > <	
149	Pit079					2182077	2182404	328	+	PP_1935	PP_1936	< > <	
150	Pit080					2182561	2182636	76	+	PP_1935	PP_1936	< > <	
151	Pit081	2182944	2183081	138	+	2182948	2183055	108	+	PP_1935	PP_1936	< > <	
152	Pit083	2183916	2184046	131	+	2183835	2183987	153	+	PP_1935	PP_1936	< > <	
153	Pit084	2188973	2188488	486	-	2188973	2188661	313	-	PP_1936	PP_1937	< < >	
154	Pit281					2195994	2195919	76	-	PP_1940	PP_1941	< < <	
155	Pit085	2215785	2215947	163	+	2215770	2215987	218	+	PP_1957	PP_1958	> > <	
156	Pit087	2217066	2217303	238	+					PP_1957	PP_1958	> > <	
157	RgsA/P16	2229834	2229726	109	-	2229832	2229713	120	-	PP_1967	PP_1968	> < >	
158	Pit088	2256062	2256155	94	+	2256046	2256121	76	+	PP_1989	PP_1990	> > >	
159	Pit089	2273175	2273376	202	+	2273151	2273402	252	+	PP_2003	PP_2004	> > <	antisense: PP_2004 (AraC reg)
160	C4_AS_RNA_3	2303002	2302769	234	-	2302966	2302891	76	-	PP_2026	PP_2027	< < <	
161	Pit090	2388431	2388527	97	+					PP_2095	PP_2096	> > >	
162	rmf	2388735	2388318	418	-	2388749	2388336	414	-	PP_2095	PP_2096	> < >	
163	Pit212	2389001	2389044	44	+	2389020	2389095	76	+	PP_2095	PP_2096	> > >	5'UTR/actuaton
164	Pit213	2418777	2418821	45	+	2418780	2418855	76	+	PP_2119	PP_2120	> > >	5'UTR/actuaton
165	Pit091	2427935	2427606	330	-	2427935	2427606	330	-	PP_2127	PP_2128	< < >	
166	Pit092	2435428	2435259	170	-	2435391	2435279	113	-	PP_2133	PP_2134	> < >	
167	Pit214	2456848	2456817	32	-	2456880	2456805	76	-	PP_2149	PP_2150	> < >	
168	Pit215	2501511	2501544	34	+					PP_2194	PP_2195	< > >	5'UTR/actuaton
169	Pit093	2547203	2546845	359	-	2547229	2547164	66	-	PP_2238	PP_2239	> < <	
170	RNA2	2608246	2608116	131	-	2608288	2608213	76	-	PP_2284	PP_2285	> < >	antisense: PP_2285
171	Pit282					2621613	2621688	76	+	PP_2294	PP_2295	> > >	3'UTR
172	Pit283					2621834	2621909	76	+	PP_2294	PP_2295	> > >	
173	Pit094	2622569	2623025	457	+	2622571	2622924	354	+	PP_2294	PP_2295	> > >	
174	Pit097	2672585	2672433	153	-	2672557	2672471	87	-	PP_2339	PP_2340	< < >	
175	Pit098	2674717	2674937	221	+	2674725	2674799	75	+	PP_2343	PP_2344	> > <	3'UTR
176	RNA3	2710973	2710798	176	-					PP_2373	PP_2374	> < <	
177	Cobalamin_ RS_2	2765195	2765043	153	-	2765150	2765073	78	-	PP_2418	PP_2419	< < <	
178	Pit284					2794981	2794914	68	-	PP_2447	PP_2448	> < <	3'UTR
179	Pit099	2796745	2796671	75	-	2796745	2796671	75	-	PP_t55	PP_t56	> < >	antisense: PP_t56
180	Pit285					2817574	2817499	76	-	PP_t57	PP_2473	> < >	
181	Pit100	2818047	2817981	67	-	2818072	2817997	76	-	PP_t57	PP_2473	> < >	
182	Pit101					2821656	2821598	59	-	PP_2474	PP_2475	< < >	
183	Pit102	2842055	2841960	96	-	2842043	2841970	74	-	PP_2492	PP_2493	< < <	5'UTR/actuaton
184	Pit286					2848947	2848807	141	-	PP_2501	PP_2502	< < <	
185	Pit103	2851516	2851865	350	+					PP_2504	PP_2505	> > <	
186	C4_AS_RNA_6	2855911	2855831	81	-					PP_2507	PP_2508	> < >	
187	Pit287					2856837	2857080	244	+	PP_2509	PP_2510	< > <	
188	Pit288					2857635	2857809	175	+	PP_2509	PP_2510	< > <	
189	Pit104/IGR 2510	2858085	2857977	109	-					PP_2509	PP_2510	< < <	

190	Pit105	2925567	2925726	160	+	2925563	2925727	165	+	PP_2563	PP_2564	< > <	
191	Pit106	2937772	2937890	119	-					PP_2569	PP_2570	> < <	
192	Pit107	2939075	2939260	186	+	2939077	2939244	168	+	PP_2570	PP_2571	< > <	
193	Pit108	3023065	3023212	148	+	3023070	3023247	178	+	PP_2638	PP_2639	> > >	5'UTR/actuaton
194	Pit109	3261566	3261498	69	-	3261585	3261510	76	-	PP_2858	PP_2859	< < <	3'UTR
195	Pit110	3275596	3275812	217	+	3275579	3275736	158	+	PP_2873	PP_2874	> > >	
196	Pit111	3342194	3342361	168	+	3342187	3342321	135	+	PP_2938	PP_2939	< > <	
197	Pit113	3448540	3447964	577	-	3448551	3447980	572	-	PP_3066	PP_3067	> < >	
198	Pit114	3450208	3450305	98	+	3450156	3450322	167	+	PP_3067	PP_3068	> > >	
199	Pit115	3450542	3450479	64	-	3450593	3450527	67	-	PP_3067	PP_3068	> < >	
200	P15	3466266	3466159	108	-	3466264	3466186	79	-	PP_3080	PP_3081	< < <	
201	Pit116	3500093	3499994	100	-	3500121	3500046	76	-	PP_3101	PP_3102	> < <	
202	Pit117	3501227	3501326	100	+	3501067	3501322	256	+	PP_3101	PP_3102	> > <	
203	Pit118	3502441	3502541	101	+	3502420	3502495	76	+	PP_3101	PP_3102	> > <	
204	Pit289					3503072	3502998	75	-	PP_3101	PP_3102	> < <	
205	Pit119	3506317	3506114	204	-	3506113	3506067	47	-	PP_3103	PP_3104	< < >	
206	Pit290					3516006	3516085	80	+	PP_3108	PP_3109	> > >	
207	Pit120	3519553	3519690	138	+	3519503	3519696	194	+	PP_3109	PP_3110	> > >	
208	Pit121	3520224	3520278	55	+	3520274	3520380	107	+	PP_3109	PP_3110	> > >	
209	Pit291					3525478	3525366	113	-	PP_3115	PP_3116	> < >	
210	Pit216	3534665	3534775	111	+	3534722	3534797	76	+	PP_3123	PP_3124	> > >	3'UTR
211	Pit217	3542804	3542720	85	-	3542826	3542723	104	-	PP_3129	PP_3130	< < <	5'UTR/actuaton
212	TPP_RS_1	3613938	3614036	99	+	3613938	3614013	76	+	PP_3184	PP_3185	< > >	
213	Pit292					3671159	3671084	76	-	PP_3233	PP_3234	> < >	
214	Pit123					3703142	3703591	450	+	PP_3269	PP_3270	> > <	
215	Pit218	3772211	3772133	79	-					PP_3331	PP_3332	> < >	
216	Pit293					3784545	3784626	82	+	PP_3346	PP_3447	> > <	
217	Pit124	3826463	3826304	160	-	3826465	3826303	163	-	PP_3380	PP_3381	< < <	
218	Pit125	3827997	3828182	186	+	3827999	3828166	168	+	PP_3381	PP_3382	< > <	
219	Pit126	3967947	3967869	79	-	3967943	3967791	153	-	PP_3497	PP_3498	> < >	antisense: PP_3497
220	Pit127	3971968	3971799	170	-	3971909	3971811	99	-	PP_3501	PP_3502	> < >	
221	Cobalamin_RS_3	3981991	3981816	176	-	3981991	3981729	263	-	PP_3508	PP_3509	< < >	
222	Psr2/CrcY	4013242	4013505	264	+	4013153	4013537	385	+	PP_3540	PP_3541	> > <	
223	Pit128	4013318	4013260	59	-					PP_3540	PP_3541	> < <	
224	Pit129	4013566	4013474	93	-					PP_3540	PP_3541	> < <	
225	Pit130	4022643	4022482	162	-	4022526	4022492	35	-	PP_3547	PP_3548	< < <	
226	Pit131	4032313	4032178	136	-	4032291	4032131	161	-	PP_3554	PP_3555	> < >	
227	Pit132/IGR 3586	4073900	4073742	159	-	4073896	4073740	157	-	PP_3585	PP_3586	< < <	
228	Pit133	4075434	4075619	186	+	4075436	4075603	168	+	PP_3586	PP_3587	< > <	
229	Pit294					4076529	4076459	71	-	PP_3587	PP_3588	< < >	
230	Pit295					4079514	4079439	76	-	PP_3589	PP_3590	< < <	3'UTR
231	Pit296					4088590	4088721	132	+	PP_3598	PP_3599	> > >	3'UTR
232	Pit297					4126034	4125945	90	-	PP_3629	PP_3630	< < <	3'UTR/5'UTR
233	RNA9	4170053	4170166	114	+	4169995	4170070	76	+	PP_3668	PP_3669	< > >	5'UTR/actuaton
234	Pit298					4190453	4190318	136	-	PP_3685	PP_3686	> < <	
235	Pit299					4192439	4192514	76	+	PP_3686	PP_3687	< > >	
236	Pit300					4196377	4196453	77	+	PP_3688	PP_3689	< > >	
237	Pit134	4197786	4197169	618	-	4197071	4196996	76	-	PP_3688	PP_3689	< < >	
238	Pit301					4199219	4199295	77	+	PP_3689	PP_3690	> > <	3'UTR
239	Pit135	4199586	4199690	105	+					PP_3689	PP_3690	> > <	
240	Pit219	4221110	4221200	91	+	4221116	4221197	82	+	PP_3699	PP_3700	> > <	3'UTR
241	Pit136	4224280	4224620	341	+	4224240	4224414	175	+	PP_3703	PP_3704	< > <	
242	Pit302					4224597	4224397	201	-	PP_3703	PP_3704	< < <	
243	Pit137	4302526	4302368	159	-	4302442	4302365	78	-	PP_3774	PP_3775	> < <	
244	Pit220	4321155	4321231	77	+	4321159	4321234	76	+	PP_3791	PP_3792	< > <	
245	Pit303					4321710	4321644	67	-	PP_3791	PP_3792	< < <	
246	Pit138	4371672	4371622	51	-					PP_3848	PP_3849	> < >	
247	Pit221	4375974	4376030	57	+	4375952	4376027	76	+	PP_3851	PP_3852	< > <	antisense: PP_3851
248	Pit222	4411329	4411368	40	+					PP_3894	PP_3895	< > <	
249	Pit139	4413261	4413551	291	+	4413195	4413576	382	+	PP_3898	PP_3899	> > <	
250	Pit304					4414130	4414292	163	+	PP_3898	PP_3899	> > <	
251	Pit305					4414971	4415047	77	+	PP_3898	PP_3899	> > <	
252	Pit306					4422976	4422900	77	-	PP_3913	PP_3914	> < >	
253	Pit307					4424674	4424749	76	+	PP_3916	PP_3917	> > >	
254	Pit140/IGR 3917	4425476	4425240	237	-	4425448	4425373	76	-	PP_3916	PP_3917	> < >	
255	Pit141	4430160	4430105	56	-	4430180	4430105	76	-	PP_3924	PP_3925	> < >	
256	Pit308					4473358	4473433	76	+	PP_3961	PP_3962	< > >	
257	Pit142	4484802	4484744	59	-	4484825	4484751	75	-	PP_3976	PP_3977	> < >	antisense: PP_3977
258	Pit143	4488900	4488999	100	+	4488854	4489030	177	+	PP_3981	PP_3982	> > <	
259	Pit309					4564088	4564163	76	+	PP_4049	PP_4050	< > >	
260	RNA10	4564472	4564630	159	+	4564472	4564547	76	+	PP_4049	PP_4050	< > >	5'UTR/actuaton
261	Prrf2	4595123	4595325	203	+	4595087	4595315	229	+	PP_4069	PP_4070	> > >	
262	Pit144	4595381	4595275	107	-	4595378	4595099	280	-	PP_4069	PP_4070	> < >	
263	Pit223/IGR 4095	4630733	4630507	227	-	4630632	4630557	76	-	PP_4094	PP_4095	> < >	
264	Pit310					4730005	4729926	80	-	PP_4186	PP_4187	< < <	
265	sucA-II RNA	4735743	4735637	107	-	4735774	4735698	77	-	PP_4189	PP_4190	< < <	
266	Pit224	4779455	4779389	67	-	4779454	4779377	78	-	PP_4219	PP_4220	< < <	3'UTR
267	Pit311					4812464	4812753	290	+	PP_4238	PP_4239	> > <	
268	Pit312					4812864	4812789	76	-	PP_4238	PP_4239	> < <	
269	Pit225	4832097	4832137	41	+					PP_4244	PP_4245	> > <	3'UTR
270	C4_AS_RNA_7	4856709	4856553	157	-					PP_4270	PP_4271	> < >	
271	Pit145	4858396	4858468	73	+					PP_4273	PP_4274	> > <	
272	SRP	4858513	4858392	122	-	4858501	4858380	122	-	PP_4273	PP_4274	> < <	
273	Pit226	4861135	4861194	60	+					PP_4274	PP_4275	< > <	
274	Pit227	4904981	4904910	72	-	4904980	4904878	103	-	PP_4312	PP_4313	< < <	5'UTR/actuaton
275	Pit313					4938795	4938870	76	+	PP_4345	PP_4346	< > >	
276	Pit146	4945337	4945242	96	-					PP_4351	PP_4352	< < <	
277	Pit228	4963832	4963785	48	-	4963878	4963802	77	-	PP_4372	PP_4373	< < <	3'UTR
278	Pit314					4970367	4970509	143	+	PP_4378	PP_4379	< > <	
279	Pit229	4991979	4992029	51	+	4991955	4992030	76	+	PP_4400	PP_4401	< > >	5'UTR/actuaton
280	Pit230	4996973	4997048	76	+	4996989	4997063	75	+	PP_4404	PP_4405	> > <	3'UTR
281	Pit315					5007658	5007583	76	-	PP_4410	PP_4411	> < >	
282	Pit316					5030911	5030842	70	-	PP_4433	PP_4434	> < >	
283	Pit147	5045239	5045192	48	-	5045285	5045217	69	-	PP_4448	PP_4449	> < <	
284	Pit148/IGR 4451	5047175	5047425	251	+	5047180	5047255	76	+	PP_4450	PP_4451	> > <	
285	Pit317					5051245	5051170	76	-	PP_4451	PP_4452	< < >	
286	Pit318					5070924	5070998	75	+	PP_4466	PP_4467	< > >	
287	Pit319					5075334	5075492	159	+	PP_4468	PP_t62	> > <	3'UTR
288	Pit231	5091133	5091053	81	-	5091112	5091037	76	-	PP_4480	PP_4481	< < <	3'UTR

289	P6					5097000	5097075	76	+				
290	Pit149	5103204	5103410	207	+	5103183	5103445	263	+	PP_4491	PP_4492	> > >	
291	Pit232	5116768	5116803	36	+					PP_4502	PP_4503	> > >	3'UTR
292	Pit233	5125104	5125176	73	+	5125099	5125174	76	+	PP_4511	PP_4512	> > >	5'UTR/actuaton
293	Pit150	5132806	5132618	189	-					PP_4518	PP_4519	> < >	
294	Pit151	5140624	5140398	227	-	5140665	5140431	235	-	PP_4524	PP_4525	> < <	3'UTR
295	C4_AS_RNA_4/IGR 4535	5149012	5148931	82	-	5149148	5148950	199	-	PP_4534	PP_4535	< < <	
296	Pit152	5152482	5152218	265	-	5152482	5152317	166	-	PP_4535	PP_4536	< < >	
297	Pit320					5154447	5154381	67	-	PP_4537	PP_4538	< < <	3'UTR/5'UTR
298	Pit234	5161120	5161207	88	+	5161125	5161200	76	+	PP_4543	PP_4545	< > >	5'UTR/actuaton
299	Pit153	5219073	5218904	170	-	5219035	5218904	132	-	PP_4598	PP_4599	> > >	
300	Pit154	5222784	5222625	160	-	5222782	5222624	159	-	PP_4602	PP_4603	< < <	
301	Pit155	5224318	5224503	186	+	5224323	5224487	165	+	PP_4603	PP_4604	< > <	
302	Pit156	5237100	5237532	433	+	5237092	5237538	447	+	PP_4613	PP_4614	> > >	5'UTR/actuaton
303	Pit235	5248771	5248836	66	+	5248740	5248877	138	+	PP_4623	PP_4624	< > <	
304	Pit236	5291965	5291896	70	-	5291964	5291876	89	-	PP_4665	PP_4666	> < <	3'UTR
305	PrrF1	5325410	5325493	84	+	5325413	5325488	76	+	PP_4685	PP_4686	> > <	
306	CrcZ	5338261	5338650	390	+	5338126	5338653	528	+	PP_4696	PP_4697	> > >	
307	P30	5338614	5338287	328	-					PP_4696	PP_4697	> < >	
308	Ribosomal S15 leader	5354579	5354741	163	+					PP_4709	PP_4710	< < <	
309	P32	5373351	5373255	97	-	5373295	5373226	70	-	PP_4724	PP_4725	< < <	
310	SsrA tmRNA	5389987	5390417	431	+	5389996	5390416	421	+	PP_4738	PP_4739	> > >	
311	Pit157	5390140	5390072	69	-					PP_4738	PP_4739	> < >	
312	Pit158	5390374	5390212	163	-					PP_4738	PP_4739	> < >	
313	C4_AS_RNA_5	5390629	5390766	138	+	5390559	5390631	73	+	PP_4738	PP_4739	> > >	
314	Pit237/IGR 4740	5391559	5391367	193	-					PP_4739	PP_4740	> < >	
315	Pit159	5392106	5392005	102	-	5392199	5391937	263	-	PP_4739	PP_4740	> < >	
316	Pit321					5392702	5392626	77	-	PP_4739	PP_4740	> < >	
317	Pit160	5401042	5400921	122	-	5401043	5400968	76	-	PP_4743	PP_4744	> < <	
318	Pit238	5414418	5414335	84	-	5414413	5414325	89	-	PP_4755	PP_4756	< < <	3'UTR
319	Pit322					5432075	5432151	77	+	PP_4771	PP_4472	> > <	3'UTR
320	P24	5437810	5437675	136	-					PP_4775	PP_4776	< < >	
321	Pit161	5453316	5453130	187	-	5453314	5453147	168	-	PP_4790	PP_4791	> < >	
322	Pit323					5525820	5525749	72	-	PP_4858	PP_4859	> < >	
323	Pit162	5545522	5545297	226	-	5545520	5545260	261	-	PP_4877	PP_4878	< < <	
324	TPP_RS_2	5596335	5596174	162	-	5596332	5596257	76	-	PP_4922	PP_4923	< < >	
325	Pit324					5630415	5630379	37	-	PP_4946	PP_4947	< < >	
326	SAH RS	5667848	5667999	152	+	5667829	5667904	76	+	PP_4975	PP_4976	< > >	
327	Pit239	5671617	5671675	59	+					PP_4978	PP_4979	> > >	3'UTR/5'UTR
328	Pit325					5675963	5676118	156	+	PP_4982	PP_4983	< > <	
329	Pit240	5696566	5696596	31	+					PP_4999	PP_5000	> > >	3'UTR
330	Pit326					5724373	5724298	76	-	PP_5024	PP_5025	< < <	3'UTR
331	Pit163	5756976	5756808	169	-	5756973	5756827	147	-	PP_5049	PP_5050	> < >	
332	Pit327					5773052	5773133	82	+	PP_5061	PP_5062	< > <	
333	Pit241	5879122	5879080	43	-	5879163	5879088	76	-	PP_5152	PP_5153	< < <	3'UTR
334	Pit242	5883991	5883928	64	-	5884019	5883944	76	-	PP_5157	PP_5158	> < <	3'UTR
335	Pit243	5914177	5914212	36	+					PP_5184	PP_5185	> > <	
336	Pit244	5927985	5927944	42	-	5927985	5927910	76	-	PP_5195	PP_5196	< < <	3'UTR
337	6S/SsrS	5934661	5934846	186	+	5934666	5934789	124	+	PP_5202	PP_5203	> > >	
338	Pit164	5934845	5934662	184	-					PP_5202	PP_5203	> < >	
339	Pit165	5941437	5941310	128	-	5941435	5941359	77	-	PP_5208	PP_5209	> < <	
340	Pseudomon-Rho	5948619	5948465	155	-	5948619	5948465	155	-	PP_5214	PP_5215	< < <	
341	Pit166	5971907	5971848	60	-					PP_5237	PP_5238	> < >	
342	Pit328					5974561	5974634	74	+	PP_5238	PP_5239	> > >	
343	Pit167	5988840	5988892	53	-					PP_5246	PP_5247	> > >	
344	Pit168	5989914	5989780	135	-	5989914	5989768	147	-	PP_5247	PP_5248	> < <	
345	Pit169	6038999	6039220	222	+	6039000	6039151	152	+	PP_5290	PP_5291	< > <	
346	Pit245	6086333	6086381	49	+	6086333	6086417	85	+	PP_5338	PP_5339	> > >	3'UTR
347	Pit170	6128020	6128117	98	+	6127981	6128102	122	+	PP_5375	PP_5376	< > >	
348	Pit171	6128647	6128550	98	-	6128647	6128572	76	-	PP_5375	PP_5376	< < >	
349	Pit172	6137098	6137180	83	+	6137092	6137322	231	+	PP_5384	PP_5385	> > >	3'UTR
350	Pit246	6152374	6152488	115	+	6152371	6152502	132	+	PP_5395	PP_5396	> > <	
351	Pit174	6158691	6158385	307	-	6158679	6158305	375	-	PP_5401	PP_5402	> < <	
352	Pit175	6158908	6158983	76	+	6158912	6159157	246	+	PP_5401	PP_5402	> > <	
353	Pit176	6159167	6158772	396	-	6159049	6158973	77	-	PP_5401	PP_5402	> < <	
354	Pit177	6166421	6166467	47	+	6166422	6166598	177	+	PP_5406	PP_5407	< > >	
355	Pit178	6166629	6166470	160	-	6166637	6166449	189	-	PP_5406	PP_5407	< < >	

Table S3: Antisense sRNAs detected in this study.

		KB1				KB4				
Nr.	Name	Start	Stop	Length	Strand	Start	Stop	Length	Strand	Antisense to
1	Pat001	19921	19760	162	-	19918	19842	77	-	antisense: PP_0015
2	Pat219	25457	25382	76	-	25457	25382	76	-	antisense: PP_0020
3	Pat002	25968	25814	155	-	25962	25888	75	-	antisense: PP_0021
4	Pat003					27002	27079	78	+	antisense: PP_0022
5	Pat220	41464	41321	144	-	41464	41389	76	-	antisense: oprP
6	Pat221	49586	49655	70	+	49586	49655	70	+	antisense: PP_0044
7	Pat005	53466	53341	126	-					antisense: PP_0046
8	Pat222					66811	66738	74	-	antisense: PP_0057
9	Pat223	95041	95181	141	+	95047	95122	76	+	antisense: PP_0085
10	Pat006	107948	107822	127	-	107921	107822	100	-	antisense: PP_0102
11	Pat224	108184	108126	59	-	108184	108126	59	-	antisense: PP_0102
12	Pat225	108471	108388	84	-	108471	108388	84	-	antisense: PP_0102
13	Pat226	113274	113123	152	-	113303	113123	181	-	antisense: PP_0107
14	Pat009					122729	122828	100	+	antisense: PP_0116
15	Pat011	131069	131319	251	+	131063	131138	76	+	antisense: engB
16	Pat227	131522	131444	79	-	131519	131444	76	-	antisense: PP_0125
17	Pat228					164809	164883	75	+	antisense: pntB
18	Pat229	167291	167427	137	+	167291	167427	137	+	antisense: PP_0157
19	Pat030	188141	188054	88	-					antisense: PP_0165
20	Pat031	195204	195101	104	-	195204	195129	76	-	antisense: PP_0168
21	Pat230	233702	233665	38	-	233702	233633	70	-	antisense: PP_0180
22	Pat231					243638	243712	75	+	antisense: fkl
23	Pat033	305642	305955	314	+	305642	305955	314	+	antisense: PP_0251
24	Pat232	322154	322204	51	+	322202	322284	83	+	antisense: PP_0267
25	Pat034	335034	335124	91	+	335039	335114	76	+	antisense: PP_t02 PP_0277
26	Pat233	349198	349035	164	-	349292	349120	173	-	antisense: PP_0288
27	Pat234					353063	352988	76	-	antisense: hisF
28	Pat235					355495	355570	76	+	antisense: PP_0296
29	Pat236	360179	360264	86	+	360177	360292	116	+	antisense: PP_0299
30	Pat035	472837	472999	163	+	472837	472999	163	+	antisense: rpoD
31	Pat036	479370	479480	111	+	479379	479450	72	+	antisense: cca
32	Pat237	519207	519109	99	-	519207	519134	74	-	antisense: argC
33	Pat044	532298	532225	74	-					antisense: PP_0439
34	Pat045	532422	532382	41	-	532422	532382	41	-	antisense: PP_t05
35	Pat046	532667	532601	67	-	532667	532601	67	-	antisense: PP_t07
36	Pat047	534147	534049	99	-	534191	534034	158	-	antisense: secE PP_t08
37	Pat238					536145	536102	44	-	antisense: rplA
38	Pat048	536549	536474	76	-	536549	536474	76	-	antisense: rplJ
39	Pat239					552421	552346	76	-	antisense: rplW
40	Pat240	558812	558712	101	-	558812	558712	101	-	antisense: rplR rpsE
41	Pat241	594252	594326	75	+	594252	594326	75	+	antisense: PP_0505
42	Pat051	609655	609736	82	+	609661	609736	76	+	antisense: PP_0525
43	Pat052	612235	612442	208	+	612227	612499	273	+	antisense: dxs
44	Pat242	638815	638891	77	+	638815	638891	77	+	antisense: PP_0551
45	Pat243	650140	650229	90	+	650140	650229	90	+	antisense: accB
46	Pat062/IGR 0601					707163	707395	233	+	antisense: rpsT
47	Pat244					733803	733727	77	-	antisense: PP_t15 PP_t14
48	Pat245					737454	737339	116	-	antisense: PP_0629
49	Pat064	744829	745064	236	+	744829	745064	236	+	antisense: PP_0637
50	Pat246	755039	755211	173	+	755039	755211	173	+	antisense: PP_0645
51	Pat247	815245	815170	76	-	815245	815170	76	-	antisense: PP_0701
52	Pat065					839743	839818	76	+	antisense: ipk PP_t18
53	Pat248					843047	843121	75	+	antisense: PP_0725
54	Pat249	843953	844031	79	+	843953	844031	79	+	antisense: PP_0726
55	Pat250	870447	870372	76	-	870447	870372	76	-	antisense: PP_0752
56	Pat251	951189	951118	72	-	951189	951118	72	-	antisense: cyoA cyoB
57	Pat252	980226	980154	73	-	980226	980154	73	-	antisense: iscA iscU
58	Pat253					983604	983530	75	-	antisense: PP_0848
59	Pat074	1086738	1086836	99	+					antisense: PP_0941
60	Pat075	1101445	1101393	53	-	1101531	1101329	203	-	antisense: ttg2C ttg2B
61	Pat254	1120241	1120320	80	+	1120264	1120320	57	+	antisense: PP_0981
62	Pat076	1137380	1137511	132	+	1137364	1137440	77	+	antisense: PP_0998
63	Pat079	1175786	1175596	191	-	1175786	1175596	191	-	antisense: guaB
64	Pat255					1179358	1179434	77	+	antisense: PP_1033
65	Pat256	1180261	1180327	67	+	1180261	1180327	67	+	antisense: PP_1033
66	Pat257					1271263	1271188	76	-	antisense: PP_1112
67	Pat080	1282153	1282278	126	+	1282153	1282228	76	+	antisense: PP_1118

68	Pat081	1292418	1292348	71	-	1292418	1292360	59	-	antisense: estC PP_1128
69	Pat258	1410934	1411008	75	+	1410934	1411008	75	+	antisense: PP_1232
70	Pat259					1466653	1466727	75	+	antisense: PP_1283
71	Pat260	1507002	1506939	64	-	1506999	1506924	76	-	antisense: sspA
72	Pat261	1531772	1531806	35	+					antisense: PP_1344
73	Pat107	1583401	1583294	108	-	1583401	1583294	108	-	antisense: ttgR
74	Pat262	1602163	1602220	58	+	1602163	1602220	58	+	antisense: PP_1405
75	Pat108	1606529	1606700	172	+	1606529	1606606	78	+	antisense: PP_t25 phaG
76	Pat110	1718854	1718779	76	-	1718921	1718779	143	-	antisense: PP_1514
77	Pat263	1742465	1742540	76	+	1742465	1742540	76	+	antisense: PP_1539
78	Pat111	1745815	1746239	425	+	1745815	1746239	425	+	antisense: PP_1544
79	Pat112	1748033	1747838	196	-	1748033	1747916	118	-	antisense: PP_1547
80	Pat264					1750500	1750425	76	-	antisense: PP_1550
81	Pat113	1751034	1750827	208	-	1751034	1750959	76	-	antisense: PP_1551
82	Pat265					1809775	1809702	74	-	antisense: eno
83	Pat266	1833639	1833900	262	+	1833639	1833900	262	+	antisense: PP_1637
84	Pat114	1848631	1848538	94	-	1848654	1848474	181	-	antisense: cysM
85	Pat115	1912172	1912298	127	+	1912172	1912298	127	+	antisense: PP_1712
86	Pat267	1990208	1990136	73	-	1990202	1990136	67	-	antisense: PP_1780
87	Pat268	2008260	2008308	49	+					antisense: PP_1791
88	Pat269	2015860	2015916	57	+	2015860	2015916	57	+	antisense: PP_1795
89	Pat270					2090712	2090638	75	-	antisense: PP_1868
90	Pat271	2099746	2099822	77	+	2099746	2099822	77	+	antisense: PP_1875
91	Pat122					2103029	2102956	74	-	antisense: PP_t34
92	Pat125	2162776	2162557	220	-	2162776	2162557	220	-	antisense: PP_1919
93	Pat272					2194359	2194434	76	+	antisense: PP_1940
94	Pat129					2242165	2242090	76	-	antisense: PP_t41
95	Pat273	2295883	2295929	47	+	2295883	2295929	47	+	antisense: PP_2023
96	Pat274	2388227	2388152	76	-	2388227	2388152	76	-	antisense: pyrD
97	Pat275	2414874	2414799	76	-	2414874	2414799	76	-	antisense: PP_2115
98	Pat276	2543095	2543170	76	+	2543095	2543170	76	+	antisense: PP_2236
99	Pat277	2597871	2597797	75	-	2597871	2597797	75	-	antisense: PP_2276
100	Pat278	2660081	2660156	76	+	2660081	2660156	76	+	antisense: PP_2331
101	Pat279					2684909	2684828	82	-	antisense: PP_2353
102	Pat280	2711455	2711530	76	+	2711455	2711530	76	+	antisense: metH
103	Pat281					2715026	2715180	155	+	antisense: metH
104	Pat282					2770824	2770899	76	+	antisense: PP_2423
105	Pat144	2809277	2809202	76	-	2809277	2809202	76	-	antisense: PP_2464
106	Pat146	2820008	2819853	156	-					antisense: PP_2473
107	Pat283	2826900	2826819	82	-	2826912	2826837	76	-	antisense: PP_2479
108	Pat147	2837590	2837724	135	+	2837546	2837621	76	+	antisense: PP_2489
109	Pat284					2947881	2947806	76	-	antisense: PP_2580
110	Pat285	2964325	2964403	79	+	2964325	2964403	79	+	antisense: PP_2594
111	Pat286	3006537	3006438	100	-	3006513	3006438	76	-	antisense: PP_2627
112	Pat287	3107884	3107959	76	+	3107884	3107959	76	+	antisense: PP_2719
113	Pat288	3138003	3137844	160	-	3138003	3137935	69	-	antisense: PP_2754
114	Pat289	3204208	3203981	228	-	3204208	3204133	76	-	antisense: PP_2810
115	Pat290	3365864	3365804	61	-	3365942	3365867	76	-	antisense: PP_2962
116	Pat291					3383603	3383673	71	+	antisense: PP_2985
117	Pat292	3409940	3409788	153	-	3409940	3409865	76	-	antisense: PP_3023
118	Pat293	3506876	3506770	107	-	3506876	3506803	74	-	antisense: PP_3104
119	Pat294					3531837	3531910	74	+	antisense: PP_3120
120	Pat295					3566458	3566362	97	-	antisense: PP_3149
121	Pat152	3730017	3729933	85	-	3730045	3729907	139	-	antisense: PP_3296
122	Pat153	3732154	3732351	198	+	3732154	3732351	198	+	antisense: PP_3299
123	Pat296	3800611	3800543	69	-	3800611	3800543	69	-	antisense: PP_3360
124	Pat155	3865516	3865591	76	+	3865516	3865591	76	+	antisense: PP_3414
125	Pat297	3899536	3899700	165	+	3899536	3899751	216	+	antisense: PP_3442
126	Pat298	3926495	3926355	141	-	3926495	3926378	118	-	antisense: PP_3462
127	Pat299					4007135	4007210	76	+	antisense: ggt-1
128	Pat159	4070962	4071052	91	+	4070962	4071037	76	+	antisense: PP_3584
129	Pat300	4152549	4152812	264	+	4152549	4152812	264	+	antisense: PP_3656
130	Pat301	4154046	4154121	76	+	4154046	4154121	76	+	antisense: PP_3657
131	Pat161	4179650	4179371	280	-	4179650	4179371	280	-	antisense: PP_3677
132	Pat302	4188168	4187985	184	-	4188168	4187985	184	-	antisense: PP_3684
133	Pat303					4191800	4191875	76	+	antisense: PP_3686
134	Pat304					4208858	4208785	74	-	antisense: PP_3692
135	Pat305	4211100	4211280	181	+	4211100	4211280	181	+	antisense: PP_3693
136	Pat306	4212757	4212832	76	+	4212757	4212832	76	+	antisense: PP_3695
137	Pat162	4220579	4220290	290	-	4220579	4220290	290	-	antisense: PP_3699
138	Pat307	4235010	4235083	74	+	4235010	4235083	74	+	antisense: PP_3711

139	Pat163	4278924	4278837	88	-					antisense: PP_3750
140	Pat308	4303911	4303986	76	+	4303911	4303986	76	+	antisense: PP_3775
141	Pat309					4306053	4306128	76	+	antisense: proC-1
142	Pat310	4325660	4325585	76	-	4325660	4325585	76	-	antisense: PP_3796
143	Pat311	4351780	4351870	91	+	4351780	4351870	91	+	antisense: PP_3824
144	Pat312					4362494	4362409	86	-	antisense: PP_3838
145	Pat313	4364756	4364883	128	+	4364756	4364883	128	+	antisense: PP_3841
146	Pat166	4367195	4367492	298	+					antisense: PP_3844
147	Pat167	4369700	4369775	76	+	4369700	4369775	76	+	antisense: PP_3846
148	Pat168	4373555	4373441	115	-	4373555	4373326	230	-	antisense: PP_3849
149	Pat314					4377290	4377365	76	+	antisense: PP_3852
150	Pat315					4401508	4401583	76	+	antisense: PP_3881
151	Pat170	4410811	4410886	76	+	4410752	4410911	160	+	antisense: PP_3894
152	Pat316					4416188	4416271	84	+	antisense: PP_3902
153	Pat317					4418557	4418485	73	-	antisense: PP_3908
154	Pat318	4440455	4440360	96	-	4440454	4440321	134	-	antisense: PP_3936 PP_3935
155	Pat319	4465308	4465377	70	+	4465308	4465377	70	+	antisense: PP_3957
156	Pat320	4552983	4553057	75	+	4552983	4553057	75	+	antisense: PP_4040
157	Pat173	4636333	4636446	114	+	4636333	4636446	114	+	antisense: PP_4101
158	Pat321					4644939	4644974	36	+	antisense: PP_4110
159	Pat322					4681506	4681427	80	-	antisense: gloB
160	Pat174	4724673	4725037	365	+	4724691	4724961	271	+	antisense: PP_4182
161	Pat175	4735175	4735250	76	+	4735175	4735250	76	+	antisense: sucA
162	Pat176	4736516	4736591	76	+	4736498	4736573	76	+	antisense: sdhB
163	Pat177	4743871	4743975	105	+					antisense: PP_4197
164	Pat323	4765296	4765224	73	-	4765296	4765224	73	-	antisense: pvdE
165	Pat178	4767604	4767353	252	-	4767604	4767353	252	-	antisense: fpvA
166	Pat180	4909745	4909605	141	-	4909745	4909605	141	-	antisense: PP_4318
167	Pat324	4942107	4941808	300	-	4942107	4942032	76	-	antisense: PP_4349
168	Pat182	4965153	4965486	334	+	4965153	4965486	334	+	antisense: fleQ
169	Pat184	4967073	4967202	130	+	4967042	4967202	161	+	antisense: fliD
170	Pat187	5027696	5027553	144	-	5027696	5027621	76	-	antisense: PP_4431
171	Pat188	5032227	5031991	237	-					antisense: dadA-1
172	Pat325					5045308	5045505	198	+	antisense: PP_4449
173	Pat326	5070060	5070135	76	+	5070060	5070135	76	+	antisense: PP_4466
174	Pat327	5075624	5075843	220	+	5075624	5075843	220	+	antisense: PP_t63 PP_t62
175	Pat190					5079837	5079912	76	+	antisense: PP_t68 csrA
176	Pat191	5172823	5173081	259	+	5172823	5173088	266	+	antisense: fadD
177	Pat328					5202132	5202057	76	-	antisense: PP_4583
178	Pat329	5205213	5205286	74	+	5205213	5205286	74	+	antisense: PP_4586
179	Pat192	5208453	5208715	263	+					antisense: PP_4589 PP_4590
180	Pat330	5241222	5241185	38	-					antisense: PP_4618
181	Pat331	5266118	5266206	89	+	5266098	5266172	75	+	antisense: cstA
182	Pat332	5274011	5273948	64	-					antisense: PP_4648
183	Pat333					5325460	5325333	128	-	antisense: PP_4685
184	Pat334	5329706	5329629	78	-	5329706	5329629	78	-	antisense: PP_4690
185	Pat335					5380358	5380414	57	+	antisense: fur
186	Pat204	5477127	5477220	94	+	5477178	5477242	65	+	antisense: PP_4814
187	Pat336	5490132	5490008	125	-	5490132	5490057	76	-	antisense: PP_4825
188	Pat206	5529009	5529276	268	+	5529013	5529383	371	+	antisense: PP_4863
189	Pat207	5549456	5549352	105	-	5549456	5549309	148	-	antisense: PP_t73
190	Pat337	5564551	5564626	76	+	5564551	5564626	76	+	antisense: miaA
191	Pat338					5680664	5680731	68	+	antisense: PP_4986
192	Pat213	5723350	5723478	129	+	5723350	5723425	76	+	antisense: PP_5024
193	Pat214	5744150	5744434	285	+	5744150	5744377	228	+	antisense: glgP
194	Pat339					5780452	5780377	76	-	antisense: PP_5066
195	Pat215	5954524	5954598	75	+	5954415	5954621	207	+	antisense: PP_5219
196	Pat216	5977354	5977135	220	-					antisense: PP_5239
197	Pat340	5980495	5980409	87	-	5980467	5980418	50	-	antisense: PP_5242
198	Pat217	6011765	6011699	67	-	6011839	6011764	76	-	antisense: rep
199	Pat341	6097191	6096907	285	-	6097200	6097125	76	-	antisense: PP_5348
200	Pat342	6132149	6132382	234	+	6132124	6132382	259	+	antisense: copA
201	Pat343	6139149	6139025	125	-	6139149	6138988	162	-	antisense: PP_5386
202	Pat344	6143379	6143251	129	-	6143379	6143304	76	-	antisense: PP_5387

Table S4: Differentially expressed sRNAs in wild-type and Δ*hfq* mutant strains (KB1 dataset).

4-1. Wt Ex compared Wt Tr and Wt St			
Nr.	sRNA	Wt Ex:Wt Tr	Wt Ex:Wt St
1	Pit138	2	2.9
2	Pit174	2.1	2.3
3	Pit094	2.2	3.4
4	Pit146	2.2	6.2
5	Pit168	2.2	18.4
6	Pit043	2.3	10.4
7	Pit209	2.3	3.4
8	TPP_RS_1	2.3	4.4
9	TPP_RS_2	2.3	4.2
10	Pit206	2.4	2.3
11	Pit088	2.5	3.1
12	Pit177	2.5	3.3
13	Pit205	2.6	6.3
14	Pit187	2.7	3.1
15	Pit130	2.8	2.2
16	RsmY	2.8	3
17	Pit165	2.9	12
18	Pit167	2.9	3.5
19	Pit061	3	3.6
20	Pit140/IGR 3917	3.2	3.6
21	Cobalamin_RS_3	3.3	3.8
22	P15	3.3	3.2
23	Pat318	3.3	34.2
24	Pat012	3.4	4.1
25	P26	3.5	6.3
26	Pit001	3.8	9.8
27	Pit149	3.8	2.2
28	Pat182	3.9	2.4
29	Pat033	4.3	9
30	sucA-II_RNA	4.4	3.9
31	P32	4.5	4.3
32	Pit097	4.5	5
33	Pit235	4.5	42.1
34	Pit077	4.7	13.9
35	Pat183	4.9	4.2
36	Pit068	5	18.3
37	Pit041	5.1	12
38	Pit178	5.2	10.9
39	Pit197	5.7	10.3
40	Pit144	6.2	14.3
41	Cobalamin_RS_1	6.5	6.8
42	Pit207	7.4	3.6
43	Pit005	7.8	18.8
44	Pit002	7.9	47.2
45	Pseudomon-Rho	8.4	7.3
46	gyrA	8.7	11
47	Pit190	8.7	16.5
48	groES	9.4	22
49	Pat107	9.4	3
50	Pit139	9.4	9
51	RNA7	9.9	2.2
52	SAH_RS	10.5	5.3
53	Pat185	11.2	5.1
54	Pit065	13.4	12.4
55	YybP-YkoY	13.6	9.7
56	Alpha_RBS	15.5	16.7
57	Pat099	15.6	20.3
58	Pat175	15.7	12.5
59	rpsL_leader	17.4	20.3
60	P24	21.6	21.6
61	Pat118	21.6	9.4
62	Pit025	22.2	22.6
63	t44	23.6	11.9
64	Pit195	25.5	32.7
65	Cobalamin_RS_2	32	32
66	Pit211	37	3.9
67	Pit245	39.8	31.8
68	Pat102	7.6	
69	Pat112	3.2	
70	Pit008	2.6	
71	Pit074	2.2	
72	Pit194	2.5	
73	Pit224	6.8	
74	Pit227	3.3	
75	PrrF1	2.9	
76	Pat297		3.2
77	Pat002		4
78	Pat009		21.6
79	Pat077		26.8
80	Pat078		26.8
81	Pat108		2.1
82	Pat168		6.5
83	Pat174		6.4
84	Pat184		3.1
85	Pat187		2.9
86	Pat188		4.3
87	Pat189		11.3
88	Pit003		4.5
89	Pit006		5.7
90	Pit007		2.2
91	Pit009		2.8
92	Pit015		5.5
93	Pit053		3.1
94	Pit054		2.6
95	Pit071		3.5
96	Pit083		3.2
97	Pit087		2.6
98	Pit108		2
99	Pit115		4.9
100	Pit143		5.9
101	Pit148/IGR 4451		3
102	Pit151		2.5
103	Pit152		2.1
104	Pit166		8.6
105	Pit175		7
106	Pit176		4.5
107	Pit179		2.4
108	Pit182		3.4
109	Pit193/IGR 0886		2.6
110	Pit201		2.1
111	Pit220		3.5
112	Pit223/IGR 4095		3.7
113	Pit231		9.6
114	Pit243		2

4-2. Wt Tr compaerd to Wt Ex		
Nr.	sRNA	Wt Tr:Wt Ex
1	Pat047	317.1
2	Pat236	127.2
3	Pit184	104
4	Pit185	82
5	Pat081	63.9
6	RNA2	60.2
7	Pit183	47.3
8	Pit199	45.2
9	RNA1	36.2
10	Pit230	31.2
11	rmf	31.1
12	Pat101	30.6
13	Pit238	27.7
14	Pat330	26.8
15	Pat173	25.1
16	Pat190	23.2
17	Pat044	21.4
18	Pit156	20.9
19	Pat163	19.5
20	Pat064	19.5
21	Pat208	19.5
22	Pat011	17.9
23	Pit216	11.5
24	Pit171	11.3
25	Pit135	10.7
26	Pat223	10.1
27	Pit236	9.3
28	Pit113	8.9
29	Pit217	8.8
30	Pit040	8.8
31	Pat260	8.1
32	Pit214	6.9
33	Pit192	6.6
34	Pit246	5.8
35	Pat051	5.8
36	Pit134	5.3
37	Pit119	5.3
38	Pit089	5.3
39	Pit203	5.2
40	Pit181	5
41	Pat079	4.9
42	Pit208	4.8
43	Pat111	4.5
44	Pat006	4.3
45	Pit233	4.3
46	Pat048	4.2
47	Pit026	4.2
48	Pit180	4.1
49	Pit059	3.7
50	Pit109	3.4
51	Pit036	3.2
52	Pit147	3.1
53	Pit027	3
54	Pit193/IGR 0886	2.9
55	RgsA/P16	2.8
56	Pit215	2.8
57	Pit038	2.8
58	Pat170	2.8
59	Pat297	2.7
60	Pit240	2.6
61	Psr2/CrcY	2.6
62	Pit231	2.5
63	Pat332	2.5
64	Pit202	2.5
65	Pit019	2.4
66	Pat192	2.4
67	Pit030	2.4
68	Pit212	2.2
69	Pit009	2.1
70	Pit138	-2
71	Pit174	-2.1
72	Pit094	-2.2
73	Pit146	-2.2
74	Pit168	-2.2
75	Pit074	-2.2
76	Pit209	-2.3
77	Pit043	-2.3
78	TPP_RS_1	-2.3
79	TPP_RS_2	-2.3
80	Pit206	-2.4
81	Pit194	-2.5
82	Pit088	-2.5
83	Pit177	-2.5
84	Pit008	-2.6
85	Pit205	-2.6
86	Pit187	-2.7
87	RsmY	-2.8
88	Pit130	-2.8
89	Pit167	-2.9
90	Pit165	-2.9
91	PrrF1	-2.9
92	Pit061	-3
93	Pit140/IGR 3917	-3.2
94	Pat112	-3.2
95	P15	-3.3
96	Pit227	-3.3
97	Pat318	-3.3
98	Cobalamin_RS_3	-3.3
99	Pat012	-3.4
100	P26	-3.5
101	Pit001	-3.8
102	Pit149	-3.8
103	Pat182	-3.9
104	Pat033	-4.3
105	sucA-II_RNA	-4.4
106	Pit235	-4.5
107	Pit097	-4.5
108	P32	-4.5
109	Pit077	-4.7
110	Pat183	-4.9
111	Pit068	-5
112	Pit041	-5.1
113	Pit178	-5.2
114	Pit197	-5.7

4-3. Wt St compared to Wt Ex		
Nr.	sRNA	Wt St:Wt Ex
1	Pat047	159.8
2	RNA2	82.1
3	Pit217	47.5
4	Pat173	41.6
5	Pat223	40.9
6	RNA1	38.9
7	Pat150	38.9
8	Pit215	37.2
9	Pit218	36
10	Pat101	32.4
11	Pit230	31.1
12	RsmZ	30
13	Pat208	28.5
14	Pat081	28.5
15	Pit236	28.1
16	Pat144	27.2
17	Pit110	27
18	rmf	26.2
19	Pat011	24.4
20	Pit040	23.6
21	Pat153	19.3
22	Pat330	18.8
23	Pit213	18.3
24	Pit210	16.9
25	Pat207	16.7
26	Pat260	12.3
27	Pat332	12.1
28	Pit183	11.9
29	Pit239	11.5
30	RNA10	10.6
31	Pit208	9.9
32	Pit234	9.8
33	RgsA/P16	9.3
34	Pit216	8.1
35	Pat147	7.8
36	Pit199	7.5
37	Pat006	7
38	Pit224	6.8
39	Pat261	6.7
40	Pat290	6.6
41	Pat030	6.5
42	PrrF2	6.5
43	Pit171	6.1
44	Pat155	6
45	Pit051	5.7
46	Pit119	5.5
47	Pit192	5.4
48	Pit019	5.1
49	Pit189	5.1
50	Pit052	4.7
51	Pit017	4.6
52	Pit126	4.6
53	Pit202	4.4
54	Pit059	4.4
55	Pit225	4.3
56	Pit181	3.8
57	Pit056	3.7
58	Pit154	3.7
59	Pit105	3.7
60	Pit124	3.7
61	Pit049	3.7
62	Pit132/IGR 3586	3.7
63	PrrF1	3.4
64	Pit162	3.4
65	Pit226	3.3
66	Pit109	3.3
67	Pat192	3.3
68	Pit203	3.1
69	Pit137	3
70	Pit196	2.7
71	Pit170	2.7
72	Pit212	2.6
73	Pat268	2.5
74	Pat111	2.5
75	C4_AS_RNA_4/IGR 4535	2.4
76	Pit089	2.4
77	Pit227	2.4
78	Pit242	2.3
79	Pit023	2.2
80	Pit008	2.2
81	Pat112	2.1
82	Pit229	2.1
83	Pit108	-2
84	Pit243	-2
85	Pit152	-2.1
86	Pat108	-2.1
87	Pit201	-2.1
88	Pit244	-2.2
89	RNA7	-2.2
90	Pit149	-2.2
91	Pit130	-2.2
92	Pit007	-2.2
93	Pit174	-2.3
94	Pit206	-2.3
95	Pit179	-2.4
96	Pat182	-2.4
97	Pit151	-2.5
98	Pit054	-2.6
99	Pit193/IGR 0886	-2.6
100	Pit087	-2.6
101	6S/SsrS	-2.7
102	Pit009	-2.8
103	Pit138	-2.9
104	Pat187	-2.9
105	Pit148/IGR 4451	-3
106	RsmY	-3
107	Pat107	-3
108	Pit187	-3.1
109	Pit088	-3.1
110	Pit053	-3.1
111	Pat184	-3.1
112	Pit083	-3.2
113	Pat297	-3.2
114	P15	-3.2

4-6. Δ*hfq* St compared to Wt St		
Nr.	sRNA	Δ*hfq St*:Wt St
1	Pat175	14.4
2	Pit211	10.2
3	t44	9.9
4	Cobalamin_RS_2	8.9
5	P30	8.9
6	Pat186	8.9
7	SAH_RS	8.8
8	Pat007	8.8
9	Pit172	8.6
10	Pat188	7.4
11	Pit026	7.1
12	Pit118	7
13	gyrA	6.8
14	Pit077	6.8
15	Cobalamin_RS_1	6.3
16	Pat161	6.2
17	Pit197	6.1
18	Pit005	5.8
19	Pat183	5.8
20	Pit103	5.7
21	YybP-YkoY	5.6
22	Pit117	5.3
23	Pit120	5.1
24	Pat102	4.7
25	Pit021_Pit022	4.6
26	rpsL_leader	4.5
27	Pit223/IGR 4095	4.5
28	Pit234	4.5
29	Pseudomon-Rho	4.5
30	Pit188	4.4
31	Pit192	4.4
32	Alpha_RBS	4.3
33	Pat164	4.3
34	Pit130	4.2
35	Pit100	4
36	Pit207	4
37	Pat146	3.8
38	Pit237/IGR 4740	3.7
39	Pit071	3.7
40	Pit236	3.7
41	Pit065	3.7
42	TPP_RS_2	3.7
43	Pat177	3.6
44	Pit201	3.6
45	Pat108	3.5
46	Pit235	3.5
47	Pat180	3.4
48	Pat206	3.3
49	Pit115	3.3
50	Pat162	3.3
51	Pit015	3.2
52	Pat182	3.2
53	Pit215	3.1
54	Pit116	3.1
55	sucA-II_RNA	3
56	Pit097	3
57	Pat268	3
58	Pit200	2.9
59	Pit184	2.9
60	Pat006	2.8
61	Pat213	2.8
62	Pit002	2.7
63	Pit137	2.7
64	Cobalamin_RS_3	2.7
65	Pit025	2.7
66	Pit091	2.7
67	Pit132/IGR 3586	2.6
68	Pit056	2.6
69	Pit154	2.6
70	Pit124	2.6
71	Pit049	2.6
72	Pit105	2.6
73	Pit040	2.6
74	Pit216	2.6
75	Pit162	2.6
76	Pit043	2.6
77	Pat332	2.5
78	Pat190	2.5
79	Pit111	2.5
80	Pat100	2.5
81	Pat160	2.5
82	Pit094	2.5
83	Pit054	2.4
84	Pit076	2.4
85	Pit087	2.4
86	Pat193	2.3
87	Pat154	2.3
88	Pat083	2.3
89	Pit061	2.3
90	Pit004	2.3
91	Pit218	2.3
92	TPP_RS_1	2.3
93	Pit165	2.2
94	Pit083	2.2
95	Pit138	2.2
96	Pit214	2.2
97	Pit019	2.1
98	Pit006	2.1
99	Pit143	2.1
100	Pit114	2.1
101	Pit003	2.1
102	Pat052	2
103	RNA2	-2
104	RgsA/P16	-2.1
105	Pit199	-2.2
106	Pat111	-2.2
107	Pit195	-2.3
108	Pit052	-2.3
109	Pit246	-2.3
110	Pit227	-2.4
111	Pit239	-2.6
112	RsmY	-2.6
113	Pit208	-2.8
114	Pit213	-3.5

115	Pit244		2.2
116	Pat006	-4.3	-7
117	Pat011	-17.9	-24.4
118	Pat047	-317.1	-159.8
119	Pat081	-63.9	-28.5
120	Pat101	-30.6	-32.4
121	Pat111	-4.5	-2.5
122	Pat173	-25.1	-41.6
123	Pat192	-2.4	-3.3
124	Pat208	-19.5	-28.5
125	Pat223	-10.1	-40.9
126	Pat260	-8.1	-12.3
127	Pat330	-26.8	-18.8
128	Pat332	-2.5	-12.1
129	Pit019	-2.4	-5.1
130	Pit040	-8.8	-23.6
131	Pit059	-3.7	-4.4
132	Pit089	-5.3	-2.4
133	Pit109	-3.4	-3.3
134	Pit119	-5.3	-5.5
135	Pit171	-11.3	-6.1
136	Pit181	-5	-3.8
137	Pit183	-47.3	-11.9
138	Pit192	-6.6	-5.4
139	Pit199	-45.2	-7.5
140	Pit202	-2.5	-4.4
141	Pit203	-5.2	-3.1
142	Pit208	-4.8	-9.9
143	Pit212	-2.2	-2.6
144	Pit215	-2.8	-37.2
145	Pit216	-11.5	-8.1
146	Pit217	-8.8	-47.5
147	Pit230	-31.2	-31.1
148	Pit236	-9.3	-28.1
149	RgsA/P16	-2.8	-9.3
150	rmf	-31.1	-26.2
151	RNA1	-36.2	-38.9
152	RNA2	-60.2	-82.1
153	Pat236	-127.2	
154	Pat044	-21.4	
155	Pat048	-4.2	
156	Pat051	-5.8	
157	Pat064	-19.5	
158	Pat079	-4.9	
159	Pat163	-19.5	
160	Pat170	-2.8	
161	Pat190	-23.2	
162	Pat297	-2.7	
163	Pit009	-2.1	
164	Pit026	-4.2	
165	Pit027	-3	
166	Pit030	-2.4	
167	Pit036	-3.2	
168	Pit038	-2.8	
169	Pit113	-8.9	
170	Pit134	-5.3	
171	Pit135	-10.7	
172	Pit147	-3.1	
173	Pit156	-20.9	
174	Pit180	-4.1	
175	Pit184	-104	
176	Pit185	-82	
177	Pit193/IGR 0886	-2.9	
178	Pit214	-6.9	
179	Pit231	-2.5	
180	Pit233	-4.3	
181	Pit238	-27.7	
182	Pit240	-2.6	
183	Pit246	-5.8	
184	Psr2/CrcY	-2.6	
185	Pat261		-6.7
186	Pat268		-2.5
187	Pat290		-6.6
188	C4_AS_RNA_4/IGR 4535		-2.4
189	Pat030		-6.5
190	Pat112		-2.1
191	Pat144		-27.2
192	Pat147		-7.8
193	Pat150		-38.9
194	Pat153		-19.3
195	Pat155		-6
196	Pat207		-16.7
197	Pit008		-2.2
198	Pit017		-4.6
199	Pit023		-2.2
200	Pit049		-3.7
201	Pit051		-5.7
202	Pit052		-4.7
203	Pit056		-3.7
204	Pit105		-3.7
205	Pit110		-27
206	Pit124		-3.7
207	Pit126		-4.6
208	Pit132/IGR 3586		-3.7
209	Pit137		-3
210	Pit154		-3.7
211	Pit162		-3.4
212	Pit170		-2.7
213	Pit189		-5.1
214	Pit196		-2.7
215	Pit210		-16.9
216	Pit213		-18.3
217	Pit218		-36
218	Pit224		-6.8
219	Pit225		-4.3
220	Pit226		-3.3
221	Pit227		-2.4
222	Pit229		-2.1
223	Pit234		-9.8
224	Pit239		-11.5
225	Pit242		-2.3
226	PrrF1		-3.4
227	PrrF2		-6.5
228	RNA10		-10.6
229	RsmZ		-30

115	Pit144	-6.2
116	Cobalamin_RS_1	-6.5
117	Pit224	-6.8
118	Pit207	-7.4
119	Pat102	-7.6
120	Pit005	-7.8
121	Pit002	-7.9
122	Pseudomon-Rho	-8.4
123	Pit190	-8.7
124	gyrA	-8.7
125	Pit139	-9.4
126	groES	-9.4
127	Pat107	-9.4
128	RNA7	-9.9
129	SAH_RS	-10.5
130	Pat185	-11.2
131	Pit065	-13.4
132	YybP-YkoY	-13.6
133	Alpha_RBS	-15.5
134	Pat099	-15.6
135	Pat175	-15.7
136	rpsL_leader	-17.4
137	P24	-21.6
138	Pat118	-21.6
139	Pit025	-22.2
140	t44	-23.6
141	Pit195	-25.5
142	Cobalamin_RS_2	-32
143	Pit211	-37
144	Pit245	-39.8

115	Pit177	-3.3
116	Pit094	-3.4
117	Pit209	-3.4
118	Pit182	-3.4
119	Pit220	-3.5
120	Pit167	-3.5
121	Pit071	-3.5
122	Pit140/IGR 3917	-3.6
123	Pit061	-3.6
124	Pit207	-3.6
125	Pit223/IGR 4095	-3.7
126	Cobalamin_RS_3	-3.8
127	Pit211	-3.9
128	sucA-II_RNA	-3.9
129	Pat002	-4
130	Pat012	-4.1
131	TPP_RS_2	-4.2
132	Pat183	-4.2
133	Pat188	-4.3
134	P32	-4.3
135	TPP_RS_1	-4.4
136	Pit003	-4.5
137	Pit176	-4.5
138	Pit115	-4.9
139	Pit097	-5
140	Pat185	-5.1
141	SAH_RS	-5.3
142	Pit015	-5.5
143	Pit006	-5.7
144	Pit143	-5.9
145	Pit146	-6.2
146	Pit205	-6.3
147	P26	-6.3
148	Pat174	-6.4
149	Pat168	-6.5
150	Cobalamin_RS_1	-6.8
151	Pit175	-7
152	Pseudomon-Rho	-7.3
153	Pit166	-8.6
154	Pit139	-9
155	Pat033	-9
156	Pat118	-9.4
157	Pit231	-9.6
158	YybP-YkoY	-9.7
159	Pit001	-9.8
160	Pit197	-10.3
161	Pit043	-10.4
162	Pit178	-10.9
163	gyrA	-11
164	Pat189	-11.3
165	t44	-11.9
166	Pit165	-12
167	Pit041	-12
168	Pit065	-12.4
169	Pat175	-12.5
170	Pit077	-13.9
171	Pit144	-14.3
172	Pit190	-16.5
173	Alpha_RBS	-16.7
174	Pit068	-18.3
175	Pit168	-18.4
176	Pit005	-18.8
177	Pat099	-20.3
178	rpsL_leader	-20.3
179	P24	-21.6
180	Pat009	-21.6
181	groES	-22
182	Pit025	-22.6
183	Pat078	-26.8
184	Pat077	-26.8
185	Pit245	-31.8
186	Cobalamin_RS_2	-32
187	Pit195	-32.7
188	Pat318	-34.2
189	Pit235	-42.1
190	Pit002	-47.2

115	PrrF2	-3.9
116	Pit224	-4
117	Pit059	-5.1
118	Pit180	-11.4
119	Pat169	-11.5
120	Pit230	-20.2
121	Pit140/IGR 3917	-20.6
122	Pit245	-25.5
123	Pat330	-26.2
124	Pit139	-29.8
125	Pat170	-67.7
126	Psr2/CrcY	-146.3
127	Pit222	-307
128	CrcZ	-358.2
129	Pit221	-3826.2

Table S4: Differentially expressed sRNAs in wild-type and Δhfq mutant strains (KB1 dataset).

	4-4. Δ*hfq* Ex compared to Wt Ex		
	Nr.	sRNA	Δ*hfq* Ex:Wt Ex
	1	Pit192	48.6
	2	Pat330	11.2
	3	Pit216	10.6
	4	Pit118	8.8
	5	Pit038	5.5
	6	Pit207	5.5
	7	Pat182	5.2
	8	Pit102	4.7
	9	Pit021_Pit022	4.5
	10	Pit223/IGR 4095	4.3
	11	Pit218	3.5
	12	Pit213	3.5
	13	Pit032	3.2
	14	Pit100	2.3
	15	Pit085	2.2
	16	Pit154	2
	17	Pit056	2
	18	Pit124	2
	19	Pit105	2
	20	Pit049	2
	21	Pit132/IGR 3586	2
	22	Pit195	-2.1
	23	Pat174	-2.1
	24	Pit007	-2.2
	25	P26	-2.2
	26	Pit025	-2.3
	27	Pit175	-2.4
	28	Pit002	-2.5
	29	Pit225	-2.7
	30	Pit001	-2.7
	31	Pit190	-2.8
	32	PhrS	-3
	33	Pit198	-3.2
	34	Spot42-like/spf/ErsA	-3.6
	35	PrrF1	-3.6
	36	Pit149	-5.5
	37	PrrF2	-6.3
	38	Pit245	-6.3
	39	Pat318	-12.3
	40	Pit224	-12.3
	41	Psr2/CrcY	-63.2
	42	Pat170	-68.1
	43	Pit140/IGR 3917	-73.3
	44	CrcZ	-98.6
	45	Pit139	-269.4

4-5. Δ*hfq* Tr compared to Wt Tr		
Nr.	sRNA	Δ*hfq Tr*:Wt Tr
1	Pit121	26.4
2	Pit218	23.4
3	Pit118	13.1
4	Pit211	11.2
5	Pat175	9.5
6	Pit192	9.5
7	groES	9.4
8	Pit207	8.1
9	Pit117	8
10	t44	7.9
11	Pat182	6.2
12	YybP-YkoY	6.2
13	Pat099	5.6
14	Pat033	5.4
15	Pit120	4.7
16	Pat180	4.6
17	RNA7	4.6
18	SAH_RS	4.4
19	TPP_RS_1	4
20	gyrA	3.6
21	Pseudomon-Rho	3.3
22	Pit032	3.3
23	rpsL_leader	3.3
24	sucA-II_RNA	3.3
25	Pat112	3.2
26	RsmZ	3.1
27	Alpha_RBS	3
28	Pit102	3
29	Pit038	3
30	Pit065	2.6
31	Pit215	2.6
32	Pat332	2.6
33	PhrS	2.5
34	Pat174	2.4
35	TPP_RS_2	2.4
36	Pit245	2.4
37	Pit200	2.3
38	Pit077	2.3
39	Pit019	2.3
40	Pit132/IGR 3586	2.2
41	Pit105	2.2
42	Pit154	2.2
43	Pit124	2.2
44	Pit056	2.2
45	Pit049	2.2

	46	Pit222	-547.2
	47	Pit221	-2226.7

46	Pit162	2.1
47	Pit007	-2.1
48	Pit134	-2.2
49	Pit147	-2.4
50	Pit231	-2.5
51	Pit213	-2.9
52	Pit246	-2.9
53	Pit135	-3
54	PrrF1	-3
55	Pit208	-3.4
56	Pit214	-3.6
57	Pit212	-3.6
58	Pit027	-3.7
59	Pit240	-3.9
60	Pit230	-4.8
61	rmf	-4.8
62	PrrF2	-5.3
63	Pit015	-6
64	Pat047	-6.1
65	Pit199	-6.1
66	Pit238	-9.9
67	Pit156	-11.6
68	Pat318	-14.4
69	Pit183	-17.1
70	Pat297	-20
71	Pat044	-21.4
72	Pit140/IGR 3917	-23.2
73	Pit050	-23.2
74	Pit180	-25.2
75	Pit139	-28.8
76	Pat169	-28.8
77	Pat170	-191.6
78	Psr2/CrcY	-194.5
79	Pit221	-220.6
80	CrcZ	-297.4
81	Pit222	-564.3
82	Pit185	-587.6
83	Pit184	-1168.2
84	Pat236	-1425.1

Table S5: Fold enrichment of sRNA binding in the co-immunoprecipitation with Hfq protein (KB4 dataset).

5-1. coIP Ex:C Ex		
Nr.	sRNA	Fold
1	Pit320	1254.9
2	Pit046	265
3	Pat314	260.8
4	Pat339	253.5
5	Pit247	242.3
6	Pit322	157.7
7	Pat259	93.5
8	Pat295	88.9
9	Pit298	82.4
10	Pat246	79.2
11	Pit310	62.9
12	Pit144	57.2
13	Pit265/IGR0752	52.9
14	Pat277	46.6
15	Pat240	45.5
16	Pit318	40.2
17	Pat270	38.6
18	Pat265	35.6
19	Pit297	35.6
20	Pit248	34.9
21	Pit329	33.3
22	PhrS	33.2
23	Pit252	30.8
24	Pit312	30.2
25	RNA6	30
26	Pit193/IGR0886	29.4
27	Pit272	28.3
28	Pit269	28.2
29	Pat257	26.1
30	Pit313	25.9
31	Pit143	24.8
32	Pit165	23.4
33	Pat249	23.3
34	Pit121	23.1
35	Pit256	23.1
36	Pit299	22.5
37	Pit085	21.7
38	Pat333	20.3
39	Pit001	20.2
40	Pit300	18.9
41	RgsA/P16	18.8
42	Pat045	18.4
43	Pit050	17.9
44	Pit304	17.9
45	Pit213	17.4
46	Pit195	16.9
47	Pat222	16.5
48	Pat298	16.2
49	Pat236	16
50	Pit229	15.6
51	Pit245	14.1
52	Pit316	14
53	Pit154	13.7
54	Pit214	13.6
55	Pit056	13.5
56	Pit019	13.3
57	PrrF1	13.1
58	Pat247	13
59	Pit182	12.7
60	Pit132/IGR3586	12.6
61	Pit216	12.5
62	Pat255	12.4
63	Pit139	12
64	Psr2/CrcY	12
65	Pat243	11.9
66	Pit061	11.8
67	Pat281	11.7
68	Pit049	11.5
69	Pit124	11.5
70	Pit302	11.2
71	Pat256	11.2
72	Pit286	11.2
73	Pit059	10.8
74	Pit328	10.7
75	Pit137	10.6
76	Pit105	10.6
77	Pat245	9.9
78	Pat180	9.8
79	Pat034	9.8
80	Pit130	9.7
81	Pit230	9.7
82	Pat215	9.6
83	CrcZ	9.6
84	Pit185	9.5
85	Pit253	9.5
86	Pat115	9
87	Pit315	8.8
88	Pat338	8.7
89	Pit079	8.7
90	Pit116	8.5
91	Pit089	8.3
92	Pat313	8.1
93	Pit288	7.9

5-2. coIP Tr:C Tr		
Nr.	sRNA	Fold
1	Pit320	2618.3
2	Pat314	321.1
3	Pat259	225.4
4	Pit329	211.1
5	Pit247	107
6	Pit213	66
7	Pit298	64.7
8	Pat222	61.9
9	Pit272	59.1
10	Pit046	43.5
11	PhrS	40.8
12	Pat295	39.4
13	Pat277	38.5
14	Pat240	38.3
15	Pat339	37.2
16	Pit245	36.3
17	Pit322	35.3
18	Pit143	33.2
19	Pit144	33
20	Pit265/IGR0752	32
21	Pit193/IGR0886	30.6
22	Pit269	30
23	Pit229	25.9
24	Pat281	25.4
25	Pat333	24.6
26	Pat257	23.1
27	Pit310	23
28	Psr2/CrcY	22
29	Pit286	21.8
30	RNA6	21.5
31	Pat247	21.3
32	Pit318	21.2
33	Pit001	21.1
34	Pit299	20.7
35	Pit085	20.2
36	Pat243	20
37	Pit297	18.8
38	Pit300	18.1
39	Pit302	17.8
40	Pat246	17.3
41	Pit259	17.2
42	Pit234	17.1
43	Pat255	17
44	Pat249	16.4
45	Pat270	15.9
46	Pat298	15.4
47	Pit185	14.6
48	Pit252	14.3
49	Pit089	13.2
50	Pit025	12.3
51	Pat236	12.2
52	CrcZ	12.1
53	Pit165	11.3
54	Pat317	10.8
55	Pit313	10.8
56	Pit256	10.2
57	Pit316	9.8
58	Pit248	9.8
59	RgsA/P16	9.8
60	Pit014	9.4
61	Pat115	9.4
62	Pit061	9
63	Pit285	8.7
64	Pit304	8.6
65	Pit317	8.2
66	Pit325	7.6
67	Pat265	7.5
68	Pit037	7.3
69	Pat227	7.2
70	Pat034	7
71	PrrF1	7
72	Pit301	6.8
73	Pit262	6.7
74	Pit312	6.7
75	P6	6.3
76	Pit019	6.2
77	Pit282	6.2
78	Pit255	6.2
79	Pit253	6.1
80	Pat062/IGR_0601	6.1
81	Pit154	5.9
82	Pat313	5.8
83	P26	5.8
84	Pit130	5.8
85	Pit315	5.6
86	Pat245	5.6
87	Pit137	5.5
88	Pit271	5.3
89	Pat253	5.3
90	Pit056	5.3
91	Pat152	5.2
92	Pit326	5.1
93	Pit105	5.1

5-3. coIP St:C St		
Nr.	sRNA	Fold
1	Pit320	529.8
2	Pit329	428.3
3	Pit247	149.6
4	Pat314	107.6
5	Pat240	64.8
6	Pit213	57.3
7	Pat246	50.7
8	Pit322	48.6
9	Pat333	37.7
10	Pat270	36.3
11	PhrS	35.9
12	Pit248	33.1
13	Pit298	29.3
14	Pit253	29.2
15	Pit310	24.3
16	Pat281	22.3
17	Pit272	22
18	Pat236	21.8
19	Pat298	21.3
20	Pat317	20.4
21	Pit229	20.2
22	Pit316	20.1
23	Pat295	19.9
24	Pit245	19.9
25	Pit252	19.4
26	Pit312	18.7
27	Pit269	18.4
28	Pit313	17.9
29	Pit297	17.9
30	Pit265/IGR0752	17.7
31	Pit300	17.5
32	Pit216	16.8
33	Pit304	16.5
34	Pit144	15.6
35	Pit046	15.5
36	Pit214	15.5
37	Pat338	15.4
38	Pit121	15
39	Pat339	14.6
40	Pat243	14.4
41	Pit083	13.9
42	Pit193/IGR0886	13.8
43	Pat247	13.1
44	Pit302	12.4
45	Pat256	11.2
46	Pat249	10.9
47	Pit286	10.9
48	Pit185	10.9
49	Pit195	10.8
50	P6	10.6
51	Pit282	10.4
52	Pit061	10.4
53	Pit230	10.4
54	Pit262	10.3
55	Pit139	10.3
56	CrcZ	10
57	Pit318	9.8
58	Pit116	9.8
59	Psr2/CrcY	8.6
60	Pit001	8.5
61	Pat215	8.1
62	Pat265	8
63	groES	7.8
64	PrrF1	7.7
65	Pit236	7.6
66	RNA6	7.6
67	Pat184	7.5
68	Pat227	7.2
69	Pit259	7.2
70	Pat033	6.9
71	Pit319	6.8
72	Pit165	6.8
73	Pit143	6.8
74	C4_AS_RNA_5	6.6
75	Prrf2	6.6
76	Pat245	6.6
77	Pit019	6.5
78	Pat277	6.4
79	Pit285	6.4
80	Pit328	6.3
81	Pat257	6.2
82	Pit299	6
83	Pit076	5.9
84	Pit288	5.9
85	Pit036	5.8
86	Pat152	5.8
87	Pat313	5.7
88	Pit059	5.7
89	Pit113	5.6
90	Pit182	5.5
91	Pit045	5.3
92	Pit050	5.3
93	sucA-II_RNA	5.2

5-4. sRNAsin common in all three conditions				
Nr.	sRNA	coIP Ex:C Ex	coIP Tr:C Tr	coIP St:C St
1	Pit320	1254.9	2618.3	529.8
2	Pit046	265	43.5	15.5
3	Pat314	260.8	321.1	107.6
4	Pat339	253.5	37.2	14.6
5	Pit247	242.3	107	149.6
6	Pit322	157.7	35.3	48.6
7	Pat259	93.5	225.4	4.1
8	Pat295	88.9	39.4	19.9
9	Pit298	82.4	64.7	29.3
10	Pat246	79.2	17.3	50.7
11	Pit310	62.9	23	24.3
12	Pit144	57.2	33	15.6
13	Pit265/IGR0752	52.9	32	17.7
14	Pat277	46.6	38.5	6.4
15	Pat240	45.5	38.3	64.8
16	Pit318	40.2	21.2	9.8
17	Pat270	38.6	15.9	36.3
18	Pit297	35.6	18.8	17.9
19	Pat265	35.6	7.5	8
20	Pit248	34.9	9.8	33.1
21	Pit329	33.3	211.1	428.3
22	PhrS	33.2	40.8	35.9
23	Pit252	30.8	14.3	19.4
24	Pit312	30.2	6.7	18.7
25	RNA6	30	21.5	7.6
26	Pit193/IGR0886	29.4	30.6	13.8
27	Pit272	28.3	59.1	22
28	Pit269	28.2	30	18.4
29	Pat257	26.1	23.1	6.2
30	Pit313	25.9	10.8	17.9
31	Pit143	24.8	33.2	6.8
32	Pit165	23.4	11.3	6.8
33	Pat249	23.3	16.4	10.9
34	Pit121	23.1	3	15
35	Pit256	23.1	10.2	4.4
36	Pit299	22.5	20.7	6
37	Pit085	21.7	20.2	4.9
38	Pat333	20.3	24.6	37.7
39	Pit001	20.2	21.1	8.5
40	Pit300	18.9	18.1	17.5
41	RgsA/P16	18.8	9.8	2.4
42	Pit304	17.9	8.6	16.5
43	Pit050	17.9	4.8	5.3
44	Pit213	17.4	66	57.3
45	Pit195	16.9	2.7	10.8
46	Pat298	16.2	15.4	21.3
47	Pat236	16	12.2	21.8
48	Pit229	15.6	25.9	20.2
49	Pit245	14.1	36.3	19.9
50	Pit316	14	9.8	20.1
51	Pit214	13.6	3.1	15.5
52	Pit019	13.3	6.2	6.5
53	PrrF1	13.1	7	7.7
54	Pat247	13	21.3	13.1
55	Pit182	12.7	2.8	5.5
56	Pit216	12.5	2.5	16.8
57	Pat255	12.4	17	4.8
58	Pit139	12	5	10.3
59	Psr2/CrcY	12	22	8.6
60	Pat243	11.9	20	14.4
61	Pit061	11.8	9	10.4
62	Pat281	11.7	25.4	22.3
63	Pit302	11.2	17.8	12.4
64	Pat256	11.2	4.9	11.2
65	Pit286	11.2	21.8	10.9
66	Pit328	10.7	4.5	6.3
67	Pit137	10.6	5.5	2
68	Pat245	9.9	5.6	6.6
69	Pat034	9.8	7	5.1
70	Pat180	9.8	2.5	3.1
71	Pit230	9.7	4.2	10.4
72	Pit130	9.7	5.8	4.5
73	CrcZ	9.6	12.1	10
74	Pat215	9.6	2.1	8.1
75	Pit253	9.5	6.1	29.2
76	Pit185	9.5	14.6	10.9
77	Pat115	9	9.4	3.2
78	Pit315	8.8	5.6	2.5
79	Pat338	8.7	4.5	15.4
80	Pit116	8.5	4	9.8
81	Pit089	8.3	13.2	4.8
82	Pat313	8.1	5.8	5.7
83	Pit288	7.9	3.4	5.9
84	Pit285	7.8	8.7	6.4
85	Pit036	7.8	4.9	5.8
86	Pit037	7.6	7.3	2.4
87	Pit083	7.1	3.4	13.9
88	C4_AS_RNA_5	7	2.1	6.6
89	Pit235	6.9	3.3	4
90	Pit113	6.8	3.4	5.6
91	Pat317	6.6	10.8	20.4
92	Pit262	6.6	6.7	10.3
93	Pit326	6.6	5.1	4.5

94	Pit036	7.8
95	Pit285	7.8
96	Pit037	7.6
97	Pit083	7.1
98	C4_AS_RNA_5	7
99	Pit235	6.9
100	Pit187	6.8
101	Pit113	6.8
102	Pit262	6.6
103	Pat317	6.6
104	Pit326	6.6
105	Pat129	6.5
106	Pat227	6.5
107	Pit296	6.4
108	Pit025	6.2
109	Pit045	5.9
110	Pat253	5.8
111	Pit323	5.7
112	Pit282	5.7
113	Pit159	5.7
114	Pit259	5.4
115	Pat152	5.4
116	Pit277	5.4
117	Pit276	5.4
118	Pat271	5.2
119	Pit319	5.1
120	Pit309	5.1
121	Pat006	5
122	Pit317	5
123	Pit162	5
124	Pit219	4.9
125	Pit305	4.8
126	Pat223	4.7
127	Pat062/IGR_0601	4.5
128	Pit260	4.4
129	Pat288	4.3
130	Pit118	4.2
131	Pit325	4.2
132	Pit324	4.1
133	Pit236	4.1
134	P6	4.1
135	Pit301	4
136	Spot42-like/spf/ErsA	4
137	Pat282	3.8
138	Pit295	3.6
139	YybP-YkoY	3.6
140	Pit014	3.6
141	Pit250	3.5
142	Pit255	3.4
143	Pat206	3.4
144	Pit076	3.3
145	Pit052	3.3
146	P26	3.2
147	Pat329	3.2
148	Pat344	3
149	Pit160	2.9
150	Pat113	2.9
151	Pat337	2.8
152	Pat033	2.7
153	Pit068	2.7
154	Pit274	2.7
155	Pit077	2.6
156	Pit038	2.6
157	Pit231	2.5
158	Pat280	2.5
159	Pit261	2.5
160	TPP_RS_2	2.4
161	Pat242	2.4
162	Prrf2	2.3
163	Pit054	2.3
164	Pit136	2.2
165	Pit009	2.2
166	gyrA	2.1
167	Pat233	2.1
168	Alpha_RBS	2.1
169	Pit254	2
170	Pit044	2
171	SAH_RS	-2
172	Pit047	-2
173	RNA2	-2
174	Pat076	-2.1
175	RNA1	-2.1
176	Pat316	-2.1
177	Pit228	-2.1
178	Pat312	-2.1
179	Pat274	-2.1
180	Pit275	-2.1
181	Pit027	-2.1
182	Pat047	-2.1
183	Pat213	-2.2
184	Pat310	-2.2
185	Pit205	-2.2
186	Pat324	-2.2
187	Pit294	-2.3
188	Pit100	-2.3
189	Pit307	-2.3
190	Pat328	-2.3

94	Pit319	5
95	Pit049	5
96	Pit139	5
97	Pit132/IGR3586	4.9
98	Pat256	4.9
99	Pit036	4.9
100	Pit045	4.9
101	Pit124	4.9
102	Pit050	4.8
103	Pit076	4.6
104	Pit328	4.5
105	Pat338	4.5
106	Pit159	4.5
107	Pit170	4.4
108	Pit296	4.3
109	Pit230	4.2
110	Pit277	4.2
111	Pat009	4
112	Pit116	4
113	Pit309	4
114	Pat129	3.7
115	Pat237	3.6
116	Pit231	3.6
117	Pat113	3.5
118	Pit288	3.4
119	Pat110	3.4
120	Pit083	3.4
121	Pit113	3.4
122	Pit274	3.4
123	Pit235	3.3
124	Pit162	3.3
125	Pit284	3.3
126	Pit251	3.2
127	Pit009	3.1
128	Pit214	3.1
129	Pit187	3.1
130	Pat065	3
131	Pat242	3
132	Pit121	3
133	Pat344	3
134	Pit305	2.9
135	Pat280	2.9
136	Pat337	2.9
137	Pat282	2.9
138	Pit261	2.8
139	Pit182	2.8
140	YybP-YkoY	2.8
141	Pat241	2.8
142	Pit219	2.8
143	Pat233	2.8
144	Pit052	2.8
145	Pit195	2.7
146	sucA-II_RNA	2.7
147	Pit295	2.6
148	Pat011	2.6
149	Pat288	2.6
150	Pit294	2.6
151	Pit276	2.5
152	Pit216	2.5
153	Pat180	2.5
154	Pat220	2.5
155	Pat036	2.4
156	Pat213	2.4
157	Pit236	2.3
158	Pit066	2.3
159	Pit068	2.3
160	Pat223	2.3
161	Pit160	2.3
162	Pit051	2.2
163	Pat184	2.1
164	rmf	2.1
165	Pat206	2.1
166	C4_AS_RNA_5	2.1
167	Pit263	2.1
168	Pat215	2.1
169	Spot42-likespfErsA	2.1
170	Pit093	2
171	Pat320	-2
172	Pit283	-2.1
173	Pat052	-2.1
174	RnpBP28	-2.1
175	Pit241	-2.1
176	Pat343	-2.1
177	Pat305	-2.1
178	Pit176	-2.2
179	Pat341	-2.2
180	Pat162	-2.2
181	Pat187	-2.2
182	Pit032	-2.2
183	Pit293	-2.2
184	Pit017	-2.2
185	Pat003	-2.2
186	Pit062	-2.3
187	Pat235	-2.3
188	Pat111	-2.3
189	Pit249	-2.4
190	Pat290	-2.4

94	Pat034	5.1
95	Pit085	4.9
96	Pat255	4.8
97	Pit089	4.8
98	YybP-YkoY	4.8
99	Pit068	4.8
100	Pit326	4.5
101	Pit130	4.5
102	Pat329	4.5
103	Pat286	4.4
104	Pit271	4.4
105	Pit256	4.4
106	Pit263	4.3
107	Pit009	4.3
108	Pat253	4.2
109	Pit025	4.2
110	Pit054	4.1
111	Pit118	4.1
112	Pat259	4.1
113	Pit234	4
114	Pit235	4
115	Pit295	3.9
116	Pit260	3.8
117	Pit277	3.5
118	Spot42-likespfErsA	3.4
119	Pit040	3.3
120	Pat223	3.3
121	Pit317	3.2
122	Pat115	3.2
123	Pit160	3.2
124	Pit309	3.2
125	Pit305	3.1
126	Pat180	3.1
127	Pit301	3
128	Pit159	3
129	Pat113	2.9
130	Pat237	2.9
131	Pit219	2.8
132	Pit276	2.8
133	Pit323	2.7
134	Pat062/IGR_0601	2.7
135	Pat065	2.7
136	Pit325	2.7
137	Pit014	2.6
138	t44	2.6
139	Pit315	2.5
140	Pat280	2.5
141	Pat344	2.5
142	Pat288	2.5
143	Pit296	2.4
144	Pat206	2.4
145	Pit037	2.4
146	Pit324	2.4
147	Alpha_RBS	2.4
148	Pit261	2.4
149	RgsA/P16	2.4
150	Pat337	2.3
151	Pit303	2.3
152	Pat318	2.2
153	Pit052	2.2
154	Pit005	2.1
155	Pat241	2.1
156	Pit044	2.1
157	Pit251	2.1
158	Pit274	2
159	Pit137	2
160	Pat242	2
161	Pit079	2
162	Pit203	-2
163	Pat341	-2
164	Pat300	-2
165	Pat343	-2
166	Pat269	-2
167	Pit291	-2
168	Pat238	-2.1
169	Pit027	-2.1
170	Pit233	-2.1
171	Pat263	-2.1
172	Pat289	-2.1
173	Pit097	-2.1
174	Pit088	-2.2
175	Pit119	-2.2
176	Pit249	-2.2
177	Pat252	-2.2
178	Pit015	-2.3
179	Pat254	-2.3
180	Pat204	-2.4
181	Pit032	-2.4
182	Pit091	-2.4
183	Pat009	-2.5
184	Pit201	-2.5
185	Pat108	-2.5
186	Pat319	-2.5
187	Pit060	-2.5
188	Pat321	-2.5
189	Pat308	-2.5
190	Pat310	-2.5

94	Pat227	6.5	7.2	7.2
95	Pit296	6.4	4.3	2.4
96	Pit025	6.2	12.3	4.2
97	Pit045	5.9	4.9	5.3
98	Pat253	5.8	5.3	4.2
99	Pit282	5.7	6.2	10.4
100	Pit159	5.7	4.5	3
101	Pit259	5.4	17.2	7.2
102	Pat152	5.4	5.2	5.8
103	Pit277	5.4	4.2	3.5
104	Pit276	5.4	2.5	2.8
105	Pit319	5.1	5	6.8
106	Pit309	5.1	4	3.2
107	Pit317	5	8.2	3.2
108	Pit219	4.9	2.8	2.8
109	Pit305	4.8	2.9	3.1
110	Pat223	4.7	2.3	3.3
111	Pat062/IGR_0601	4.5	6.1	2.7
112	Pat288	4.3	2.6	2.5
113	Pit325	4.2	7.6	2.7
114	P6	4.1	6.3	10.6
115	Pit236	4.1	2.3	7.6
116	Spot42-likespfErsA	4	2.1	3.4
117	Pit301	4	6.8	3
118	YybP-YkoY	3.6	2.8	4.8
119	Pit295	3.6	2.6	3.9
120	Pit014	3.6	9.4	2.6
121	Pat206	3.4	2.1	2.4
122	Pit076	3.3	4.6	5.9
123	Pit052	3.3	2.8	2.2
124	Pat344	3	3	2.5
125	Pit160	2.9	2.3	3.2
126	Pat113	2.9	3.5	2.9
127	Pat337	2.8	2.9	2.3
128	Pit068	2.7	2.3	4.8
129	Pit274	2.7	3.4	2
130	Pat280	2.5	2.9	2.5
131	Pit261	2.5	2.8	2.4
132	Pat242	2.4	3	2
133	Pit009	2.2	3.1	4.3
134	SAH_RS	-2	-8.1	-2.6
135	Pat316	-2.1	-2.5	-4.1
136	Pit205	-2.2	-4.2	-3.9
137	Pat213	-2.2	2.4	-4
138	Pat328	-2.3	-4.7	-2.8
139	Pit100	-2.3	-2.5	-3.7
140	Pat292	-2.4	-3.6	-2.8
141	Pit293	-2.5	-2.2	-3.2
142	Pit032	-2.6	-2.2	-2.4
143	Pat294	-2.6	-2.8	-3.2
144	Pat226	-2.6	-3.6	-3.9
145	Pit238	-2.7	-5.1	-2.7
146	Pat340	-2.7	-5.3	-3.6
147	Pat225	-2.7	-4.4	-4.8
148	Pit060	-2.8	-3.7	-2.5
149	Pit098	-2.9	-5.4	-4
150	SsrA/tmRNA	-2.9	-4.2	-6.4
151	Pat144	-2.9	-12.5	-7.6
152	RsmZ	-2.9	-23.7	-19.7
153	Pat308	-3	-4.1	-2.5
154	Pat080	-3	-2.5	-3.6
155	Pit120	-3	-11.6	-4.5
156	Pat204	-3.1	-3.7	-2.4
157	Pat300	-3.2	-3.2	-2
158	Pat273	-3.2	-2.9	-6.2
159	RNA7	-3.2	-4.2	-7.1
160	Pit176	-3.3	-2.2	-4.6
161	Pit268	-3.4	-4	-4
162	Pit189	-3.5	-5.6	-2.8
163	Pat114	-3.6	-2.5	-3.1
164	Pit241	-3.7	-2.1	-2.6
165	Pat307	-3.7	-4.3	-3.4
166	Pat290	-3.8	-2.4	-2.6
167	Pat291	-3.8	-3.3	-5.9
168	Pit031	-3.9	-3.1	-4.6
169	Pit035	-4	-6.8	-2.6
170	RNA9	-4.1	-5.1	-3.8
171	Pat238	-4.3	-2.5	-2.1
172	Pat343	-4.4	-2.1	-2
173	Pat341	-4.4	-2.2	-2
174	Pat302	-4.5	-3.4	-3.5
175	Pat235	-4.6	-2.3	-3.3
176	Pit161	-4.7	-7.1	-7.1
177	Pit133	-4.7	-9	-7.1
178	Pit048	-5	-8.1	-6.9
179	Pat276	-5.2	-3.6	-3.9
180	Pit125	-5.2	-7.3	-7.5
181	Pit055	-5.2	-7.6	-7.6
182	Pat309	-5.3	-2.6	-3.8
183	Pit149	-5.3	-2.9	-4.3
184	Pat335	-5.3	-9.5	-16.8
185	Pit107	-5.4	-7.4	-7.7
186	Pit155	-5.7	-7.4	-7
187	Pat299	-5.8	-6.3	-7.1
188	Pit217	-6	-4.7	-6.7
189	Pat321	-6.2	-38.5	-2.5
190	Pit134	-6.6	-7.4	-5

191	Pit266	-2.4
192	Pit015	-2.4
193	Pat292	-2.4
194	Pat305	-2.5
195	Pat219	-2.5
196	Pat325	-2.5
197	C4_AS_RNA_2	-2.5
198	Pit293	-2.5
199	Pit070	-2.5
200	Pat231	-2.6
201	Pat294	-2.6
202	Pit032	-2.6
203	Pseudomon-Rho	-2.6
204	Pat226	-2.6
205	Pit238	-2.7
206	Pat225	-2.7
207	Pat340	-2.7
208	Pit203	-2.7
209	Pat075	-2.8
210	Pit060	-2.8
211	Pit227	-2.8
212	RsmZ	-2.9
213	Pat144	-2.9
214	Pit098	-2.9
215	SsrA/tmRNA	-2.9
216	Pit109	-3
217	Pit120	-3
218	Pat080	-3
219	Pat308	-3
220	Pat315	-3.1
221	Pat178	-3.1
222	Pit289	-3.1
223	Pat204	-3.1
224	Cobalamin_RS_1	-3.2
225	Pat273	-3.2
226	Pat300	-3.2
227	RNA7	-3.2
228	Pit002	-3.3
229	Pit200	-3.3
230	Pit176	-3.3
231	Pat336	-3.3
232	C4_AS_RNA_3	-3.3
233	Pit268	-3.4
234	Pit006	-3.5
235	Pit189	-3.5
236	Pit101	-3.6
237	Pat320	-3.6
238	Pat114	-3.6
239	Pit241	-3.7
240	Pat170	-3.7
241	Pat307	-3.7
242	Pat290	-3.8
243	Pat291	-3.8
244	Pit031	-3.9
245	Pit258	-4
246	Pit035	-4
247	RNA9	-4.1
248	Pit026	-4.3
249	Pat001	-4.3
250	Pat238	-4.3
251	Pat343	-4.4
252	Pat341	-4.4
253	Pat168	-4.5
254	Pat302	-4.5
255	Pat258	-4.5
256	Pat235	-4.6
257	Pit161	-4.7
258	Pat217	-4.7
259	Pit133	-4.7
260	Pit081	-4.9
261	Pit048	-5
262	Pit125	-5.2
263	Pat276	-5.2
264	Pit055	-5.2
265	Pat335	-5.3
266	Pat309	-5.3
267	Pit149	-5.3
268	Pit107	-5.4
269	Pit186	-5.5
270	Pit155	-5.7
271	Pit141	-5.7
272	Pat299	-5.8
273	Pit217	-6
274	Pat321	-6.2
275	Pat228	-6.6
276	Pit134	-6.6
277	Pit224	-6.9
278	Pit008	-6.9
279	Pit267	-7.5
280	Pit327	-7.5
281	Pit206	-7.8
282	Pit233	-7.8
283	Pit088	-8.1
284	Pat221	-8.1
285	Pat319	-8.3
286	Pat303	-8.4
287	Pat248	-8.4

191	Pat108	-2.5
192	Pit201	-2.5
193	Pat316	-2.5
194	Pat174	-2.5
195	Pat080	-2.5
196	Pit178	-2.5
197	Pit211	-2.5
198	Pit100	-2.5
199	Pit194	-2.5
200	Pit327	-2.5
201	Pit070	-2.5
202	Pat238	-2.5
203	Pat114	-2.5
204	Pit177	-2.6
205	Pit099	-2.6
206	Pat309	-2.6
207	Pit233	-2.6
208	Pit094	-2.7
209	Pit142	-2.7
210	Pit206	-2.7
211	Pat294	-2.8
212	Pit149	-2.9
213	Pit101	-2.9
214	Pat273	-2.9
215	Pat274	-2.9
216	Pat228	-2.9
217	Pit088	-3
218	groES	-3.1
219	Pit031	-3.1
220	Pat190	-3.1
221	Pat076	-3.2
222	Pat300	-3.2
223	Pat291	-3.3
224	Pat302	-3.4
225	Pat224	-3.4
226	Pit289	-3.4
227	Pit270	-3.5
228	Pat175	-3.5
229	Pat292	-3.6
230	Pat153	-3.6
231	Pat226	-3.6
232	Pat276	-3.6
233	Pit002	-3.7
234	Pit060	-3.7
235	Pat204	-3.7
236	Pat248	-3.8
237	Pit026	-3.8
238	Pit081	-3.9
239	Pit258	-3.9
240	Pseudomon-Rho	-4
241	Pit268	-4
242	Pat308	-4.1
243	Pit246	-4.1
244	Pat221	-4.1
245	SsrA/tmRNA	-4.2
246	RNA7	-4.2
247	Pit205	-4.2
248	Pit097	-4.2
249	Pat307	-4.3
250	Pit034	-4.3
251	Pit224	-4.4
252	Pat225	-4.4
253	Pat304	-4.4
254	Pat328	-4.7
255	Pit275	-4.7
256	Pit217	-4.7
257	Pit200	-4.7
258	Pat079	-4.7
259	rpsL	-4.9
260	Pat342	-5.1
261	RNA9	-5.1
262	Pit238	-5.1
263	Pat340	-5.3
264	Pit098	-5.4
265	Pat311	-5.6
266	Pit008	-5.6
267	Pit186	-5.6
268	Pit189	-5.6
269	Pit184	-6.1
270	Pat299	-6.3
271	Pit311	-6.3
272	Pat182	-6.6
273	Pit035	-6.8
274	Pit117	-7
275	Pit161	-7.1
276	Cobalamin_RS_1	-7.3
277	Pit125	-7.3
278	Pit107	-7.4
279	Pit155	-7.4
280	Pit134	-7.4
281	Pit055	-7.6
282	Pat283	-7.7
283	Pat234	-7.8
284	Pat272	-8
285	SAH_RS	-8.1
286	Pit048	-8.1
287	Pit133	-9

191	Pit017	-2.5
192	Pat258	-2.5
193	Pat287	-2.5
194	Pat244	-2.5
195	Pat173	-2.5
196	Pat290	-2.6
197	Pit035	-2.6
198	Pit126	-2.6
199	SAH_RS	-2.6
200	Pit241	-2.6
201	Pit284	-2.7
202	Pat153	-2.7
203	Pit307	-2.7
204	Pat336	-2.7
205	Pat278	-2.7
206	Pat001	-2.7
207	Pit238	-2.7
208	Pat111	-2.7
209	Pit123	-2.7
210	Pat292	-2.8
211	Pit266	-2.8
212	Pit189	-2.8
213	Pat187	-2.8
214	Pat328	-2.8
215	Pat081	-2.8
216	Pit006	-2.8
217	Pit177	-2.9
218	Pat250	-2.9
219	Pit065	-2.9
220	Pat052	-3.1
221	Pit109	-3.1
222	Pit114	-3.1
223	rmf	-3.2
224	Pit293	-3.2
225	Pat294	-3.2
226	Pit102	-3.3
227	Pat235	-3.3
228	Pat307	-3.4
229	Pat047	-3.4
230	Pat147	-3.4
231	Pit311	-3.5
232	Pat302	-3.5
233	Pat175	-3.5
234	Pat191	-3.5
235	Pit175	-3.6
236	Pat340	-3.6
237	Pit142	-3.6
238	RnpBP28	-3.6
239	Pat080	-3.6
240	Pat155	-3.7
241	Pit100	-3.7
242	RNA9	-3.8
243	Pat309	-3.8
244	Pit308	-3.8
245	Pit212	-3.8
246	Pit205	-3.9
247	Pit210	-3.9
248	Pat226	-3.9
249	Pat276	-3.9
250	Pit268	-4
251	Pit098	-4
252	Pat213	-4
253	Pat316	-4.1
254	Pit283	-4.1
255	Pat304	-4.2
256	Pit149	-4.3
257	Pat232	-4.4
258	Pit120	-4.5
259	Pit176	-4.6
260	Pit031	-4.6
261	Pit267	-4.7
262	Pat303	-4.8
263	Pat225	-4.8
264	Pit134	-5
265	Pat264	-5.3
266	Pat239	-5.5
267	Pat228	-5.5
268	Pat112	-5.8
269	Pat291	-5.9
270	Pat234	-6
271	Pat273	-6.2
272	Pat315	-6.3
273	SsrA/tmRNA	-6.4
274	Pit217	-6.7
275	Pit048	-6.9
276	Pit155	-7
277	RNA7	-7.1
278	Pit161	-7.1
279	Pit133	-7.1
280	Pat299	-7.1
281	Pat248	-7.5
282	Pit125	-7.5
283	Pit055	-7.6
284	Pat144	-7.6
285	Pit314	-7.6
286	Pit107	-7.7
287	Pit327	-7.7

191	Pat228	-6.6	-2.9	-5.5
192	Pit008	-6.9	-5.6	-13.4
193	Pit267	-7.5	-17.3	-4.7
194	Pit327	-7.5	-2.5	-7.7
195	Pit233	-7.8	-2.6	-2.1
196	Pit088	-8.1	-3	-2.2
197	Pat221	-8.1	-4.1	-9.1
198	Pat319	-8.3	-20.5	-2.5
199	Pat248	-8.4	-3.8	-7.5
200	Pat342	-8.5	-5.1	-11.9
201	Pit308	-8.8	-11.4	-3.8
202	Pat264	-9.2	-15.4	-5.3
203	Pat279	-9.8	-47.2	-14.2
204	6S/SsrS	-9.8	-11.5	-16.5
205	Pat304	-10.2	-4.4	-4.2
206	SRP	-10.2	-11.3	-21.1
207	Pat311	-10.7	-5.6	-8.5
208	Pat079	-11.2	-4.7	-9.7
209	Pit080	-11.6	-63.6	-12.2
210	Pat272	-12.8	-8	-9.3
211	Pit246	-14.8	-4.1	-12.6
212	P32	-15.9	-69.7	-10.3
213	Pit321	-18.2	-13.7	-14.5
214	Pit314	-19.2	-26.4	-7.6
215	Pat322	-27.2	-15.6	-24.4
216	Pat283	-43.9	-7.7	-30
217	Pat275	-83.8	-15.5	-28.6
218	Pit273	-93.2	-61.8	-74.1

288	Pat342	-8.5
289	Pit308	-8.8
290	Pat264	-9.2
291	Pat279	-9.8
292	6S/SsrS	-9.8
293	Pat239	-9.9
294	Pat173	-10
295	Pat304	-10.2
296	SRP	-10.2
297	Pat311	-10.7
298	Pat079	-11.2
299	Pit080	-11.6
300	Pat272	-12.8
301	Pit246	-14.8
302	P32	-15.9
303	Pat161	-16
304	Pit321	-18.2
305	Pit314	-19.2
306	Pat322	-27.2
307	Pit184	-40.3
308	Pat283	-43.9
309	Pat275	-83.8
310	Pit273	-93.2

288	Pat168	-9
289	Pit227	-9.3
290	Pat335	-9.5
291	Pat112	-9.6
292	C4_AS_RNA_3	-10.4
293	Pit123	-10.8
294	SRP	-11.3
295	Pit308	-11.4
296	6S/SsrS	-11.5
297	Pit120	-11.6
298	Pat144	-12.5
299	Pit321	-13.7
300	Pat264	-15.4
301	Pat275	-15.5
302	Pat322	-15.6
303	Pat284	-16.3
304	Pit267	-17.3
305	Pat319	-20.5
306	RsmZ	-23.7
307	Pit314	-26.4
308	Pat321	-38.5
309	Pat279	-47.2
310	Pit273	-61.8
311	Pit080	-63.6
312	P32	-69.7
313	Pat161	-167.3

288	Pat217	-8
289	Pat311	-8.5
290	Pat221	-9.1
291	Pat272	-9.3
292	Pat079	-9.7
293	P32	-10.3
294	Pat342	-11.9
295	Pit080	-12.2
296	Pit246	-12.6
297	Pit008	-13.4
298	Pat279	-14.2
299	Pit321	-14.5
300	6S/SsrS	-16.5
301	Pat335	-16.8
302	RsmZ	-19.7
303	SRP	-21.1
304	Pat322	-24.4
305	Pat275	-28.6
306	Pat283	-30
307	Pit273	-74.1

Table S7: Strains, plasmids and oligonucleotides used in this study.

underlined: FLAG sequence

Plasmids

Plasmid	Genotype	Source
pSIJ218	*oriV*(R6K), *lacZα* fragment with I-SceI sites; KmR; T7 polymerase flanked by homologous regions for insertion after *glmS* gene	Calero et al., 2016
pSWI-1	*oriV*(RK2), *xylS-Pm→I-SceI*; Ap^{R}	Martinez-Garcia et al., 2011
pKB1	pSIJ218 derived *hfq* 3FLAG	This study

Strains

Strain	Genotype	Source
P. putida KT2440	Prototrophic, wild-type strain derived from *P. putida* mt-2 deprived of the pWW0 TOL plasmid; Mt-2 *hsdR*1 (r^{-} m^{+})	Bagdasarian et al., 1981
P. putida Δ*hfq*	KT2440 derivative with a full deletion of *hfq*	Arce-Rodríguez et al., 2015
KL3	*P. putida* KT2440 *hfq*::3xFLAG	
E. coli DH5α	φ80dlacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rk- mk+) supE44 thi1 gyrA relA1	Lab collection
DH5αλpir	λpir phage lysogen of DH5α	Lab collection

Oligonuclotides

Oligonucleotide	Sequence (5'-3')	Purpose
KB1_pEMG_rv	ATTACCCUGTTATCCCTATACTGGCCGT	Amplification of the pEMG backbone
KB2_pEMG_Hfq_fw	ACAAGTUCTAGGGATAACAGGGTAATCCGGCGTA	Amplification of the pEMG backbone
KB3_pEMG_Hfq_fw	AGGGTAAUCAGCATGACAGCTCATCGTCG	Amplification upstream of *hfq* gene
KB4_Hfq_FLAG_rv	ATGTCGUGGTCCTTGTAGTCACCGTCGTGGTCCTTGTAGTCGGCGTTGCCTGGCTCGCT	Amplification upstream of *hfq* gene, introducing FLAG sequence at the end of the *hfq* gene
KB5_Hfq_FLAG_fw	ACGACAUCGACTACAAGGACGACGACGACAAGTGATAG GAG CCT GCA TTG TTC TTT GAG	Amplification downstream of *hfq* gene, introducing FLAG sequence at the end of the *hfq* gene
KB6_pEMG_Hfq_rv	AACTTGUGCGGCAGGTGGCGAATGAA	Amplification downstream of *hfq* gene
KB7_pEMG_ck_fw	ATGTGCTGCAAGGCGATTAAGTTGGGT	Colony PCR check
KB8_pEMG_ck_rv	GGCTCGTATGTTGTGTGGAATTGTG	Colony PCR check
KB9_Hfq_ck_fw	TGC GAA GGG GCG GCC TAA TTT AT	Colony PCR check
KB10_wt_fw	ACC GCC AGT TGG CCG AAG TCG A	Colony PCR check
KB11_wt_rv	AGTTGCTCGACTCTTCGAGCGTAGC	Colony PCR check
KB12_FLAG_fw	GAC TAC AAG GAC GAC GAC GAC AA	Colony PCR check
KB13_FLAG_rv	TTGTCGTCGTCGTCCTTGTAGTC	Colony PCR check
KB_NB_5S	ATG GGA TCA GGT GGT TCC AAT GCT	Northern blot
KB_NB_Pit003	GCG CTT AGC GAA GCA GAC TTG	Northern blot
KB_NB_Pit017	TGT TCG AAG AAG CCA ACG GCA GCG AGG GCG AGA CCA T	Northern blot
KB_NB_Pit023	AGT CGA ACC TGC CCG GGA ACG G	Northern blot
KB_NB_Pit032	GCT GTA ACC GAG CAT TCT GC	Northern blot
KB_NB_Pit051	GTA CGG CAC CAA CGA GGA TG	Northern blot
KB_NB_Pit052	TGC CAG TCT ACG TGG TCG AG	Northern blot
KB_NB_Pit142	GAG ATT CAA ACC CTT ATC CTA GGC TC	Northern blot
KB_NB_Pit165	GCT CTG TAA TTG CTG GTC GAA C	Northern blot
KB_NB_Pit192	TG TCC GAA CCT GTC GAG ATC CTG TG	Northern blot
KB_NB_Pit200	TGG GGA CTT CTA CGG TGG GTA	Northern blot
KB_NB_Pit211	GAT TTG CAC CAC CTG ATT TTG AG	Northern blot
KB_NB_Pit217	CAG ACG CAG TGA ACA CTC CCT G	Northern blot
KB_NB_Pit221	GTC TCC AGG GCT TCT AGC TAG	Northern blot
KB_NB_Pit229	TGT GAG CTC TGA AGC GGC CG	Northern blot
KB_NB_Pit235	CGA GAC GTC AGA TCA AGG AGC T	Northern blot
KB_NB_Pit245	GAA AGG TTG AGA GGT GTC TAG TC	Northern blot
KB_NB_Pat223	AGT GCT CTC CAT GGT TGA CAG TGA	Northern blot

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PAPER 3

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Genome-wide mapping of transcription start sites yields novel insights into the primary transcriptome of *Pseudomonas putida*

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Summary

The environmental bacterium *Pseudomonas putida* is an organism endowed with a versatile metabolism and stress tolerance traits that are desirable in an efficient production organism. In this work, differential RNA sequencing was used to investigate the primary transcriptome and RNA regulatory elements of *P. putida* strain KT2440. A total of 7937 putative transcription start sites (TSSs) were identified, where over two-thirds were located either on the opposite strand or internal to annotated genes. For TSSs associated with mRNAs, sequence analysis revealed a clear Shine–Dalgarno sequence but a lack of conserved over-represented promoter motifs. These TSSs defined approximately 50 leaderless transcripts and an abundance of mRNAs with long leader regions of which 18 contain RNA regulatory elements from the Rfam database. The thiamine pyrophosphate riboswitch upstream of the *thiC* gene was examined using an *in vivo* assay with GFP-fusion vectors and shown to function via a translational repression mechanism. Furthermore, 56 novel intergenic small RNAs and 8 putative actuaton transcripts were detected, as well as 8 novel open reading frames (ORFs). This study illustrates how global mapping of TSSs can yield novel insights into the transcriptional features and RNA output of bacterial genomes.

Introduction

Pseudomonas putida is a ubiquitous Gram-negative rod-shaped bacterium that has been used as a laboratory model for environmental bacteria and intensively studied regarding potential applications in industrial biotechnology (Poblete-Castro *et al*., 2012). Its simple nutritional requirements enable it to thrive in a wide variety of water and soil environments, including strains that colonize the rhizosphere and sites contaminated with chemical waste. Although features such as a versatile intrinsic metabolism, general robustness towards stress, and the ability to synthesize bioactive secondary metabolites are shared with other pseudomonads, *P. putida* is non-pathogenic and lacks the virulence factors harbored by other members of the genus that are human and plant pathogens (Nikel *et al*., 2014). A notable trait of *P. putida* is a superior tolerance to organic solvents (Ramos *et al*., 2015), as well as the ability of some strains to metabolize xenobiotic compounds. These characteristics combined with the availability of tools for genetic manipulation make *P. putida* an attractive host for heterologous gene expression and cell factory for the recombinant biosynthesis of natural products (Loeschcke and Thies, 2015).

One of the best characterized *P. putida* strains is KT2440 (Regenhardt *et al*., 2002), a plasmid-free derivative of the toluene-degrading strain mt-2 (Nakazawa, 2002). It is the preferred host for genetic manipulation and has been certified as a biosafety strain (Federal Register, 1982), a status that allows for industrial-scale production. The 6.2 Mb genome sequence confirmed the avirulence of the strain, and enabled a greater understanding of its physiology and metabolic repertoire (Nelson *et al*., 2002; Belda *et al*., 2016). Several genome-scale metabolic models have been developed and used to investigate the potential of the strain for the production of biochemicals (Nogales *et al*., 2008; Puchalka *et al*., 2008; Sohn *et al*., 2010; Oberhardt *et al*., 2011; Belda *et al*., 2016).

RNA sequencing (RNA-seq) technology has emerged in recent years as the method of choice for transcriptome analysis and was used in an earlier study of *P. putida* KT2440 (Frank *et al*., 2011). The method of differential RNA sequencing (dRNA-seq) (Sharma and Vogel, 2014)

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distinguishes primary and processed transcripts, enabling the global determination of transcription start sites (TSSs). As these define 5′ untranslated regions (5′UTRs) of mRNA transcripts, dRNA-seq facilitates the mapping and annotation of RNA regulatory elements in leader regions, including *cis*-acting metabolite-binding riboswitches. To date, over twenty families of riboswitches with known cognate ligands have been identified (Lunse *et al*., 2014; Ramesh, 2015), as well as several orphan riboswitches with unknown ligands (Breaker, 2011). Comparative genomics approaches have been used to reveal a number of conserved RNA motifs in bacteria and archaea. Although RNA elements have been also predicted in *Pseudomonas* spp. (Weinberg *et al*., 2007, 2010; Naville and Gautheret, 2010) and *P. putida* KT2440 (Frank *et al*., 2011; Sun *et al*., 2013), no studies with detailed characterization have been reported thus far.

In this work, a dRNA-seq approach was employed to gain insights into the *P. putida* KT2440 transcriptome, including *cis*-regulatory elements in 5′UTRs. Nearly 8000 TSSs were identified in four different growth conditions, where the majority were located either opposite of or internal to annotated genes. For the TSSs associated with mRNAs, sequence analysis showed a conserved Shine–Dalgarno sequence in leader regions but a lack of overrepresented sequence motifs in promoter regions. The study documents the discovery of roughly 50 leaderless mRNAs and hundreds of mRNAs with long leader regions, where the latter include 18 conserved RNA regulatory elements. Using plasmid reporter fusions, a thiamine pyrophosphate (TPP) riboswitch element is demonstrated to function via a translational repression mechanism. This is, to our knowledge, the first *in vivo* riboswitch characterization in *P. putida*.

Results and discussion

Experimental approach

The dRNA-seq approach was used to map and investigate TSSs in *P. putida* strain KT2440. The method, described previously by Sharma *et al*. (2010), is based on the use of exonuclease enzyme that specifically degrades 5′monophosphorylated RNAs but not 5′triphosphorylated RNAs from the total RNA sample. This leads to a selective depletion of the processed RNAs and enrichment of primary RNA transcripts in the exonuclease-treated sample relative to the untreated total RNA sample. A comparison of sequencing reads between the treated and untreated samples allows for genome-wide TSS identification and improvement of the genome annotation.

Cells were grown in M9 minimal medium with either glucose or citrate as sole carbon sources and harvested in exponential and stationary phases (Supporting Information Fig. S1). Citrate was chosen as *P. putida* uses organic acids and amino acids, abundant in plant root exudates, as preferred carbon sources (Vilchez *et al*., 2000; Lugtenberg *et al*., 2001; Revelles *et al*., 2005), and the capability to grow on citrate is a feature distinct from *Escherichia coli*, which cannot normally use citrate as an energy source under oxic conditions (Blount *et al*., 2008). Unlike the model bacteria *E. coli* and *Bacillus subtilis*, glucose is not the favorite carbon source for pseudomonads (Rojo, 2010). In *P. putida*, succinate is consumed faster than glucose when the two substrates are provided simultaneously, although glucose is still well assimilated compared with other carbon sources (La Rosa *et al*., 2015a,b). Furthermore, the two chosen substrates, glucose and citrate, activate either glycolytic or gluconeogenic physiological regimes (Munoz-Elias and McKinney, 2006; Chavarria *et al*., 2013), respectively, leading to the expression of distinct metabolic genes (Kim *et al*., 2013).

Total RNA was extracted and split into two equal parts, where one was treated with exonuclease (Supporting Information Fig. S2). The untreated and treated RNA samples were used for preparation of strand-specific cDNA libraries for sequencing on the Illumina HiSeq platform. Strand-specific sequencing resulted in 2.5–14.6 million reads per sample, where on average 97% of the reads mapped to the reference chromosome (Supporting Information Table S1). Although the majority of reads mapped to rRNA, comparison of the percentage of rRNA reads between respective untreated and treated samples indicated a reduction in processed transcripts in the exonuclease-treated samples (Supporting Information Table S1). Moreover, despite the presence of ribosomal RNA, a sufficient number of reads were generated for transcriptional mapping in each condition (Creecy and Conway, 2015).

Identification of transcription start sites: the majority are antisense or internal relative to annotated genes

Transcription start sites were identified using TSSpredator software (Dugar *et al*., 2013), which normalizes the expression data and detects TSSs at genomic positions where a significant number of reads show major enrichment in the treated compared to the untreated samples. A total of 7937 putative TSSs were predicted in the *P. putida* KT2440 genome (Supporting Information Table S2), of which 762 (10%) were unique in exponential phase and 635 (8%) unique in stationary phase samples for cells grown in citrate (Fig. 1A). For cells grown in the presence of glucose, 606 (8%) and 1061 (13%) unique putative TSSs were predicted in exponential and stationary phase samples respectively. Nearly half of the TSSs (48%) were identified in the same growth phase, including 2238 TSSs (28%) in stationary phase and 1613 (20%) in exponential phase for both carbon sources. However, 42% of TSSs were clearly associated with growth in either glucose or citrate as sole carbon sources. Surprisingly, only 47 TSS

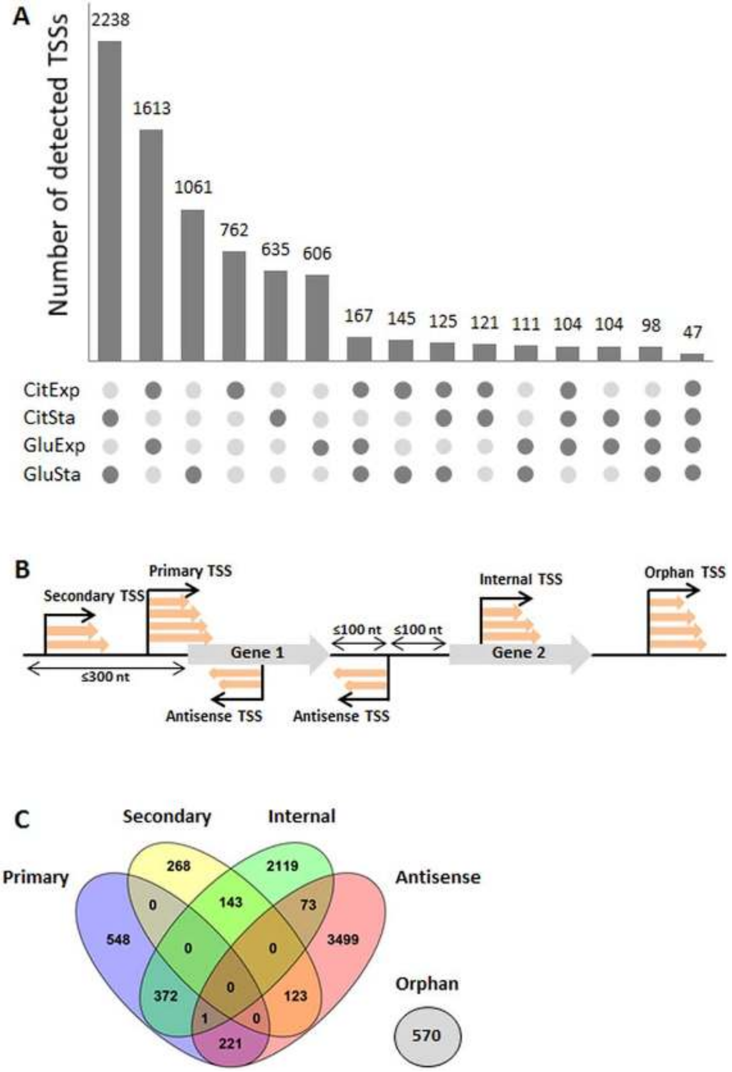

Fig. 1. Genome-wide identification of transcription start sites.
A. The numbers of identified transcription start sites (TSSs) in the four conditions with either glucose (Glu) or citrate (Cit) as sole carbon sources in exponential (Exp) or stationary (Sta) growth phases are shown. Dark and light dots indicate the presence or absence of TSSs in a growth condition respectively.
B. Schematic illustration of categories used for TSS classification (including primary, secondary, internal, antisense and orphan TSS groups) based on their genomic context relative to annotated genes.
C. Venn diagram showing the distribution of identified TSSs into the categories depicted in panel B. A TSS can be associated with more than one group.

(0.6%) were detected in all four conditions (Fig. 1A). These data show a similarity in the positions of transcription initiation between the two carbon sources when the same growth phase is considered. The biggest difference was observed between the exponential and stationary phase growth conditions, underscoring the vast changes in gene expression to respond to the different physiological status of the cell in the two growth phases. Therefore, the two growth phases introduce more variability than the two carbon sources. Additionally, concerning the TSSs found in only one condition, approximately 7% of the 3064 unique TSSs corresponded to TSSs with positions varying by 10 or less nucleotides (nt) in different conditions. The rest were composed of TSSs corresponding to specific expression in one condition, or TSSs with major coordinate differences in different conditions.

The identified TSSs were classified based on their putative origin and genomic context into primary, secondary, internal, antisense and/or orphan TSSs categories (Fig. 1B) (Dugar *et al.*, 2013). TSSs located within 300 nt upstream of an annotated gene were designated as primary and secondary, with the former exhibiting the most cDNAs and strongest expression relative to the latter. TSSs located within and on the same strand of annotated genes were defined as internal, whereas antisense TSSs were positioned either inside or at a maximal distance of 100 nt relative to annotated genes on the opposite strand. Orphan TSSs were not in close proximity to annotated genes and belonged to none of the aforementioned categories (Fig. 1B and C). Surprisingly, the numbers of primary and secondary TSSs were considerably lower than the numbers of internal and antisense TSSs. One factor contributing to the low number of primary TSSs is that these could be placed in other TSS categories such as internal and orphan in the case of long leaders with lengths greater than 300 nt. This suggests that a threshold length of 300 nt upstream of start codons for definition of primary and secondary TSSs, although sufficient in the organisms studied previously (Irnov *et al.*, 2010; Sharma *et al.*, 2010; Filiatrault *et al.*, 2011; Kroger *et al.*, 2012; Sahr *et al.*, 2012; Schmidtke *et al.*, 2012; Wurtzel *et al.*, 2012; Dugar *et al.*, 2013; Wiegand *et al.*, 2013; Kopf and Hess, 2015; Nuss *et al.*, 2015; Papenfort *et al.*, 2015), is not optimal for all bacteria and that longer leader regions may be more prevalent in *P. putida*.

Nearly half of the identified TSSs were classified as antisense, indicating a high level of transcription initiation on the antisense strand. Antisense transcription is now highly reported in transcriptome analysis, and several studies have revealed the presence of a large number of transcripts antisense to annotated genes and the 5′ or 3′ ends of mRNAs in different organisms (Georg *et al.*, 2009; Liu *et al.*, 2009; Toledo-Arana *et al.*, 2009; Dornenburg *et al.*, 2010; Filiatrault *et al.*, 2010). Acting via extensive base pairing, antisense RNAs regulate the expression of the gene on the opposite strand by modulating the transcription, stability or translation of the specific target. In some cases, antisense RNAs can play a dual role by also functioning as an mRNA encoding a small protein (Silby and Levy, 2008), or regulating several genes other than the target on the opposite strand with the RNA chaperone Hfq (Opdyke *et al.*, 2004; Mandin *et al.*, 2007; Arnvig and Young, 2009). Despite the high number of antisense transcripts identified, only a few have been functionally characterized. Besides antisense transcripts acting as RNA regulators, antisense transcription may also represent noise due to nonspecific transcription in bacteria (Georg and Hess, 2011; Thomason *et al.*, 2015). At least one antisense TSS was identified for 1991 (36%) of *P. putida* KT2440 genes. Previous studies identified putative antisense RNAs for 12% of all genes in *Mycoplasma pneumoniae* (Guell *et al.*, 2009), 2% in *Sinorhyzobium meliloti* (Schluter *et al.*, 2010), less than 1% in *Bacillus subtilis* (Irnov *et al.*, 2010), and 22% in *E. coli* (Dornenburg *et al.*, 2010), without any further analysis to validate the presence of the antisense RNAs. Similar to *P. putida*, *Helicobacter pylori* was reported to have antisense RNAs on 46% of all genes, of which 21 RNA transcripts were supported with additional experiments (Sharma *et al.*, 2010).

A direct comparison with other TSS identification studies is not straightforward due to different experimental approaches and TSS classification schemes. However, three previous studies used the same experimental approach (dRNA-seq methodology followed by Illumina sequencing and TSSpredator analysis) in different bacteria and growth conditions (Dugar *et al.*, 2013; Bischler *et al.*, 2015; Thomason *et al.*, 2015). *Pseudomonas putida* has the highest percentage of orphan TSSs (6%) compared to *E. coli* (3%), *Campylobacter jejuni* (2%) and *H. pylori* (2%), suggesting the presence of a large number of sRNA candidates and unannotated ORFs (Supporting Information Fig. S3). Even though a direct comparison with other TSS identification studies is not possible, it is worth mentioning that similar to *P. putida*, high percentages of orphan TSSs were previously observed in cyanobacteria (Kopf and Hess, 2015). Transcriptomic analysis of seven cyanobacteria by dRNA-seq revealed high levels of transcription in non-coding regions among all the TSSs identified, where the percentage of orphan TSSs varied between 5.1% and 26.7% depending on the organism (Mitschke *et al.*, 2011; Voss *et al.*, 2013; Kopf *et al.*, 2014, 2015; Pfreundt *et al.*, 2014; Voigt *et al.*, 2014).

In order to confirm the TSS predictions, the full-length sequences of three selected gene transcripts were determined by 5′RACE (rapid amplification of cDNA ends). The TSS predictions were validated in the following genes with high expression levels or differential expression between growth on glucose and citrate: PP0147, a citrate transporter; PP4010, cold-shock protein D (*cspD*); and PP1623, the RNA polymerase sigma factor (*rpoS*). In all cases there was good agreement between RACE determined and predicted TSS positions, with a maximum divergence of 9 nucleotides (Supporting Information Table S3). This lends reliability to the TSS predictions and the potential of TSSpredator as a valid automated TSS prediction tool. Nevertheless, the accuracy of the TSSs coordinates can be improved by modifying the library preparation protocol in order to reduce the relative amount of rRNA and by increasing the sequencing depth. Another possibility is to increase sensitivity in TSSpredator parameters, leading to a higher number of identified TSSs but also an increase in false positives. Therefore, the TSSpredator parameters (see Experimental procedures) chosen for this study were an optimal compromise between the number and accuracy of TSSs coordinates identified. Moreover, the 5′RACE result for the *rpoS* transcript revealed a primary TSS located 369 nucleotides upstream of the ATG start codon (Supporting Information Fig. S4). This TSS was positioned inside the upstream PP1622 gene and therefore classified as an internal TSS. Thus, not all primary TSSs are within 300 nucleotides of the start codon and some internal TSSs may function as the primary TSS of the downstream mRNA. This finding can account in part for the high number of internal TSSs relative to primary and secondary TSSs identified in *P. putida*.

A common feature of bacterial genomes is the polycistronic organization of several genes in an operon, where sets of co-regulated and co-transcribed genes are transcribed as a single mRNA, allowing rapid adaptation to environmental changes (Lawrence, 2002). A total of 1076 multi-gene operons were predicted here in *P. putida* (Supporting Information Table S4) that were mostly composed of two (59%) or three (20%) genes and seven operons included more than ten genes (Supporting Information Fig. S5). Considering that *P. putida* KT2440 has 5350 coding sequences and 3120 (58%) were predicted to be organized in multi-gene operons, the remaining 2230 (42%) could be single-gene operons or not expressed under the studied conditions and therefore not categorized. Although information on the positions of 3′ ends are also required for a precise mapping of operons, the above estimation is based on the pattern of expressed genes under the studied conditions and is similar to the single- and multi-gene operon composition of *E. coli* (Conway *et al*., 2014).

Therefore, there are several possible explanations for the absence of identified primary TSSs for some annotated genes and the relative low number of TSSs in this class including: (i) the gene is not expressed under the tested conditions, (ii) the TSS is longer than 300 nt and thus it is not classified as a primary TSS but either an orphan or internal TSS, (iii) the gene is part of an operon so it is co-transcribed and shares the TSS with the upstream gene and (iv) the gene is part of an operon with an internal promoter and terminator and therefore the TSS is classified as internal.

Investigation of 5′ untranslated regions reveals an abundance of mRNAs with long leaders

A total of 1676 primary and secondary TSSs were identified, of which 1599 were associated with mRNAs defining the 5′UTR regions of protein-coding genes, and 77 were related to RNAs transcripts (rRNA and tRNA). The 5′UTRs or leader regions of mRNA, defined by the transcription start site and the nucleotide just before the start codon were examined in *P. putida*. Leaderless mRNAs in bacteria with an mRNA starting at the first codon or up to ten nucleotides upstream were once considered rare (Moll *et al*., 2002; Laursen *et al*., 2005), but recent studies have shown that they are much more common (Brock *et al*., 2008; Sharma *et al*., 2010; Sahr *et al*., 2012; Schmidtke *et al*., 2012; Cortes *et al*., 2013; Schluter *et al*., 2013; Kopf *et al*., 2015; Shell *et al*., 2015). Out of the 1599 TSSs associated with mRNAs, 88 defined mRNAs with a 5′UTR of ten nucleotides or less. Of these, 51 mRNAs were leaderless with no other longer TSS identified (Supporting Information Table S5) and the other 37 mRNAs had both leadered and leaderless variants. A significant fraction of the leaderless mRNAs encode gene products with functions related to nucleic acids (DNA binding proteins, DNA/RNA modification and nucleotide synthesis enzymes) or of unknown function. This result indicates that leaderless transcripts may be more frequent in certain gene function categories than others; for instance information storage and processing categories tend to have a higher fraction of leaderless mRNAs than metabolic genes (Nakagawa *et al*., 2010; Zheng *et al*., 2011). A previous study on the *P. putida* KT2440 transcriptome identified eight leaderless mRNAs out of 170 highly expressed 5′UTRs (Frank *et al*., 2011). In this study, leader regions longer than 100 nucleotides are reported for six of these transcripts. The larger number of leaderless mRNAs identified here is likely due to the different conditions examined and the dRNA-seq approach that specifically reveals features of 5′UTRs (Sharma and Vogel, 2014).

The 5′UTR length distribution of the TSSs associated with mRNA is shown in Fig. 2A. There is a median 5′UTR length of 136 nt and a high number of 5′UTRs with lengths between 100 and 300 nt. Nearly 1000 leaders are longer than 100 nt, and about half of these are longer than 200 nt. This is an underestimate as there are likely to be a significant number of mRNAs with leaders longer than 300 nucleotides that are not taken into account here. This differs from previously reported distributions in other organisms, which have median 5′UTR lengths between 33 and 54 nt and the highest 5′UTR frequencies in the 20–40 nt range using a variety of methods (Irnov *et al*., 2010; Sharma *et al*., 2010; Filiatrault *et al*., 2011; Kroger *et al*., 2012; Sahr *et al*., 2012; Schmidtke *et al*., 2012; Wurtzel *et al*., 2012; Dugar *et al*., 2013; Wiegand *et al*., 2013; Kopf and Hess, 2015; Nuss *et al*., 2015; Papenfort *et al*., 2015). Our result shows that *P. putida* KT2440 has many potential mRNAs with long 5′UTRs compared with other bacteria examined to date. These long leader regions may mediate regulation on downstream genes via specific RNA

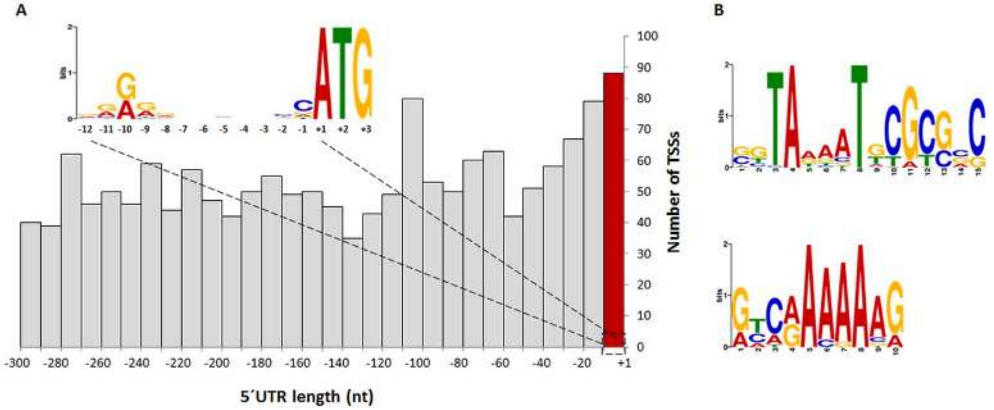

Fig. 2. Leader regions and analysis of promoter motifs.
A. Plot showing the distribution of 5UTR lengths based on 1599 primary and secondary TSS of mRNAs. 5UTRs with lengths of 10 nt or less are shown in red. The insert shows the overrepresented motif for the 5UTRs, and consists of the Shine–Dalgarno sequence and the start codon.
B. Two overrepresented motifs found in promoter regions, including the −10 box (top) and the A_5 sequence (bottom).

secondary structures (Winkler and Breaker, 2003; Araujo *et al*., 2012), such as *cis*-acting riboswitches (Coppins *et al*., 2007) and be targeted via base-pairing interactions with *trans*-acting sRNA regulators (Waters and Storz, 2009). Therefore, extended 5′UTR regions in *P. putida* suggest a high potential for mRNA regulation and the presence of *cis*-regulatory elements.

The sequences adjacent to the TSSs of mRNAs were investigated for the presence of Shine-Dalgarno sequences by using the Multiple EM for Motif Elicitation (MEME) tool for motif discovery (Bailey *et al*., 2009). The Shine–Dalgarno sequence was searched for in the regions surrounding the start codons using genomic sequences corresponding to 40 nucleotides upstream and downstream of the start codon as input. The Shine–Dalgarno sequence was clearly identified within the 12 nucleotides upstream of the ATG start codon (Fig. 2A).

The promoter regions were also investigated by MEME using the sequences 50 nucleotides upstream of the transcription start site. For this search, TSSs of tRNA and rRNA genes were also included (1676 total input sequences). This yielded two motifs with significant *E*-values ($< 10^{-30}$) including a possible −10 box and an A_5 sequence (Fig. 2B). Interestingly, the motifs were found in surprisingly few input sequences, 77 and 63, for the −10 box and A_5 motifs respectively. Moreover, the motifs did not have a specific nucleotide position, as their locations varied relative to the TSS between the different sequences. A previous transcriptomic study found the pentameric polyA motif but neither −10 nor −35 region motifs (Frank *et al*., 2011). These results emphasize the relative lack of overrepresented promoter motifs with a clear position for *P. putida* transcripts. It has been noted previously that TSS neighborhoods can be highly heterogeneous with different promoter architectures affecting the position of transcription initiation depending on the growth phase and the environment (Narlikar, 2014). Therefore the difficulty in finding conserved overrepresented motifs in specific locations in *P. putida* promoter regions suggests the presence of a different promoter architecture and high level of heterogeneity surrounding the TSS.

Cis-regulatory RNA elements in 5′UTR regions

As 5′UTRs contain elements that exert *cis*-regulation on downstream genes, the identification and characterization of these elements can contribute to the understanding of bacterial adaptation under different conditions. The high number of mRNAs with long leader regions in *P. putida* KT2440 prompted the investigation of these 5′UTRs for possible regulatory functions. In this respect much is still unknown for *P. putida*, where the *cis*-regulatory elements known to date have been based on sequence comparison of transcriptomic data (Frank *et al*., 2011) and comparative genomic analysis (Weinberg *et al*., 2007; 2010; Sun *et al*., 2013), but further characterization is lacking.

Therefore, 5′UTRs defined by primary and secondary TSSs with lengths of 80–300 nucleotides were investigated for possible *cis*-regulatory RNA structures by searching for homologies with annotated sequences in Rfam databases (Nawrocki *et al*., 2015). The input sequences included those from 100 nucleotides upstream of the identified TSS

Table 1. *Cis*-RNA regulatory elements in 5′UTRs.

Number	Rfam motif	Predicted TSS position[a]	Strand	Downstream gene number and annotation		Reference[d]
1	*gabT*	85	+	PP0214	*gabT*: 4-aminobutyrate aminotransferase	Weinberg *et al.* (2010) (*Pseudomonas*)
2	*rpsL* pseudo	95	+	PP0449	*rpsL*: 30S ribosomal protein S12	Naville and Gautheret. (2010) (Pseudomonadaceae)
3	Alpha RBS	238	+	PP0476	*rpsM*: 30S ribosomal protein S13	Schlax *et al.* (2001) (*E. coli*)
4	FMN	186; 196	-	PP0530	*ribB*: 3,4-dihydroxy-2-butanone 4-phosphate synthase	Frank *et al.* (2011) (*P. putida* KT2440)
5	YybP-YkoY leader[b]	178	-	PP0760	hypothetical protein	Frank *et al.* (2011) (*P. putida* KT2440)
6	2 group II (D1D4-3 and D1D4-1)[c]	279	+	PP1250	group II intron-encoding maturase	Lehmann and Schmidt (2003)
7	Pseudomon-*groES* RNA	111	+	PP1360	*groES*: co-chaperonin *GroES*	Weinberg *et al.* (2010) (*Pseudomonas*)
8	Cobalamin[b]	246	+	PP1672	*cobO*: cob(I)yrinic acid a,c-diamide adenosyltransferase	Frank *et al.* (2011) (*P. putida* KT2440)
9	*gyrA* RNA	122; 148	+	PP1767	*gyrA*: DNA gyrase subunit A	Weinberg *et al.* (2010) (*Pseudomonas*)
10	2 group II (D1D4-3and D1D4-1)[c]	280	+	PP1846	group II intron-encoding maturase	Lehmann and Schmidt (2003)
11	Cobalamin[b]	197	-	PP2418	hypothetical protein	Sun *et al.* (2013) (*P. putida* KT2440)
12	TPP	135	+	PP3185	*pet18*: TenA family transcriptional activator	Sun *et al.* (2013) (*P. putida* KT2440)
13	Cobalamin	217; 248	-	PP3508	*cobW*: cobalamin biosynthesis protein *CobW*	Sun *et al.* (2013) (*P. putida* KT2440)
14	*sucA-II* RNA	110; 235	-	PP4189	*sucA*: 2-oxoglutarate dehydrogenase E1	Weinberg *et al.* (2010) (Pseudomonadales)
15	Ribosomal S15 leader[b]	107	-	PP4709	30S ribosomal protein S15	Sun *et al.* (2013) (*P. putida* KT2440)
16	TPP	246; 252	-	PP4922	*thiC*: thiamine biosynthesis protein *ThiC*	Frank *et al.* (2011) (*P. putida* KT2440)
17	SAH	152	+	PP4976	*ahcY*	Weinberg *et al.* (2007) (Proteobacteria)
18	Pseudomon-*Rho*	128; 136	-	PP5214	*rho*: transcription termination factor *Rho*	Weinberg *et al.* (2010) (*Pseudomonas*)

a. The TSS position is reported as the leader length or number of nucleotides upstream of the downstream gene.
b. Regulatory element found by visual inspection on IGV profiles.
c. Ribozyme.
d. The organisms described in the cited references are indicated in parentheses.

to 50 nucleotides downstream of the first codon. In addition to the *in silico* search, manual inspection of TSS read profiles was performed with Integrative Genomics Viewer (IGV) (Robinson *et al.*, 2011; Thorvaldsdottir *et al.*, 2013). A total of 18 *cis*-RNA regulatory elements were found with homology to known RNA motifs using the Rfam database (Table 1). The riboswitch elements predicted previously in *P. putida* KT2440 by transcriptomics (Frank *et al.*, 2011) and comparative genomics (Sun *et al.*, 2013) were confirmed here. Moreover, other relevant motifs were also found, including those related to the genus *Pseudomonas* (Weinberg *et al.*, 2010) and phylum Proteobacteria (Weinberg *et al.*, 2007; Naville and Gautheret, 2010), and two group II catalytic RNA (ribozymes) elements that occur in all domains of life (Lehmann and Schmidt, 2003). Finally, a RNA element previously described in E. coli associated with repression of the ribosomal protein S13 operon by the ribosomal protein S4 (Schlax *et al.*, 2001) was identified in *P. putida* KT2440.

The TPP riboswitch upstream of the thiC gene acts via a translational inhibition mechanism

The TPP riboswitch upstream of the *thiC* gene was chosen from the list of predicted *cis*-regulatory RNA elements (Table 1) as it has been characterized in other bacteria and suitable for further investigation of the ligand-dependent regulatory mechanism in *P. putida*. The identified TPP

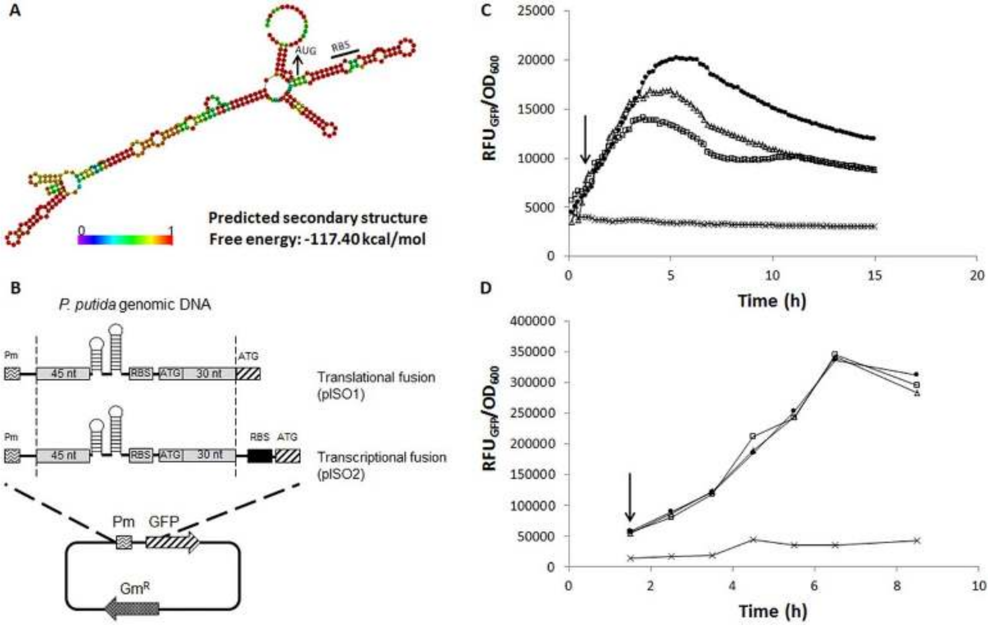

Fig. 3. Characterization of the ligand-dependent regulatory mechanism of the TPP riboswitch upstream of the *thiC* gene by GFP reporter fusion systems.
A. The secondary structure of the TPP riboswitch predicted by RNAfold WebServer (Gruber *et al.*, 2008). The base coloring represents the base-pairing probability. The free energy of the conformation is reported.
B. Representation of the two reporter fusion systems: the translational (pISO1) and the transcriptional (pISO2) fusion plasmids.
C, D. Fluorescence levels in the absence and presence of the ligands (TPP and thiamine) in the translational fusion (C) and transcriptional fusion (D) plasmids. There is a repression of fluorescence in the translational fusion when either TPP or thiamine are added. However, fluorescence levels in the transcriptional fusion are unchanged by ligand addition. Arrows indicate the points of ligand addition. RFU/OD$_{600}$ graphs are showed: curves with no induction and no ligand (×), curves with induction but absence of ligand (•), and curves with 0.5 mM TPP (Δ) and 5 μM thiamine (□) after induction.

sequence folded in a stem-loop structure stabilized by a negative free energy value ($\Delta G = -117.40$ kcal/mol), predicted by RNAfold WebServer (Gruber *et al.*, 2008) (Fig. 3A). The TPP riboswitch (or Thi-box) binds directly to its natural ligand TPP, the active form of thiamine (vitamin B1), and represses the expression of thiamine-related genes (Miranda-Rios, 2007). The regulatory mechanisms of the TPP riboswitches upstream of the *thiC* and *thiM* genes in *E. coli* (Winkler *et al.*, 2002; Ontiveros-Palacios *et al.*, 2008; Caron *et al.*, 2012), and the *thi* operon (*tenA*) in *B. subtilis* (Mironov *et al.*, 2002) have been studied in the presence of TPP and thiamine. The involved Thi-box elements in *E. coli* and *B. subtilis* differ in their mechanism of action, where they function via translational repression (Winkler *et al.*, 2002) and transcription termination (Mironov *et al.*, 2002) mechanisms respectively. This has led to the suggestion that the TPP riboswitch induces transcription termination in Gram-positive bacteria and inhibits translation initiation in Gram-negative bacteria (Nudler and Mironov, 2004). Thus, it was of interest to test this hypothesis by investigating the regulatory function of the specific TPP riboswitch sequence predicted in *P. putida*.

The regulatory mechanism of the TPP riboswitch upstream of the *thiC* gene was tested by using a translational fusion with a GFP reporter in a plasmid construct. The resulting plasmid (pISO1) contained the inducible promoter Pm (Marques *et al.*, 1998; Miura *et al.*, 1998; Winther-Larsen *et al.*, 2000), the natural genome sequence of *P. putida* KT2440 (including the putative riboswitch domain, the natural ribosome binding site (RBS) and 30 nt of the natural downstream gene *thiC*), and the GFP gene (Fig. 3B). The KT2440 (pISO1) strain was grown in a microtiter plate, induced with 3-methylbenzoate for GFP expression, followed by monitoring the level of fluorescence with and without TPP and thiamine ligands. The plasmid-transformed strain showed a reduction of relative fluorescence units (RFU) when TPP or thiamine were added to the media compared to the absence of ligand

(Fig. 3C), while the RFU levels in the strain harboring the original plasmid without riboswitch sequence were not affected. This confirmed the regulatory mechanism of the riboswitch sequence, which repressed the expression of the downstream GFP gene in the presence of either ligand. Different ligand concentrations between 10 nM and 1.5 mM were tested, and addition of 0.5 mM TPP and 5 μM thiamine led to RFU reductions of 21% and 35% for TPP and thiamine respectively. These ligand concentrations yielded the maximum extent of repression, as larger effects were not observed with higher ligand concentrations.

In vitro studies have demonstrated a stronger binding of the TPP ligand to the *thiC* Thi-box structure compared with the precursor thiamine, with the riboswitch exhibiting more than 1000-fold discrimination between the two ligands (Winkler *et al*., 2002; Yamauchi *et al*., 2005; Edwards and Ferre-D'Amare, 2006; Lang *et al*., 2007; Ontiveros-Palacios *et al*., 2008; Haller *et al*., 2013). Therefore the 100-fold lower thiamine concentration relative to TPP concentration required for repression of GFP expression observed in this study may be a consequence of differences in ligand uptake into the cell. Thiamine is synthesized by most prokaryotes (Begley *et al*., 1999; Jurgenson *et al*., 2009), and can alternatively be taken up from the environment (Webb *et al*., 1998), but the responsible transporters and cellular uptake mechanisms remain unclear in many organisms, including *P. putida* (Webb *et al*., 1998; Jurgenson *et al*., 2009; Rodionov *et al*., 2009; Jeanguenin *et al*., 2012; Rodionova *et al*., 2015). Therefore, the difference in the active concentrations of TPP and thiamine could be related to the specificity of the transporter. Specifically, the transporter may have higher affinity for thiamine that facilitates its entry, while TPP transport may be less efficient and require higher concentrations for activity.

To better understand the regulatory mechanism and confirm the expected translational inhibition of the TPP riboswitch, a plasmid vector was constructed with a transcriptional fusion of the TPP motif and the GFP gene (pISO2). For this fusion, in addition to the natural RBS introduced with the riboswitch sequence from the *P. putida* genome, a second RBS was introduced just upstream of the reporter gene (Fig. 3B). In this construct, the translation repression activity of the riboswitch should only sequester the natural RBS but not the second RBS, and thus allow GFP expression in the presence or absence of ligand.

The RFU levels of *P. putida* KT2440 (pISO2) with and without ligands, were monitored during growth, and dilution factors were applied to avoid overflow measurement of fluorescence due to the two RBS sequences. The transcriptional fusion plasmid allowed a continuous expression of GFP and no repression of fluorescence was observed upon ligand addition (Fig. 3D). In this model, the reporter expression was dependent on the level of the mRNA and its translation regulated by the second RBS, which was not sequestered by the riboswitch structure. This confirms the translational repressor activity of the TPP riboswitch upstream of the *thiC* gene in *P. putida* KT2440. In the case of a mechanism involving transcription termination in the presence of the ligands, there would be no transcribed mRNA and consequently no expression of GFP. Our data supporting the translational repression mechanism of the TPP riboswitch in *P. putida* KT2440 is consistent with the hypothesis that in Gram-negative bacteria, Thi-box elements act by interfering with RBS-ribosome recognition instead of transcription termination. This work represents the first *in vivo* riboswitch characterization in *P. putida*.

Identification of small RNA candidates derived from intergenic regions and 5′ UTRs

In addition to *cis*-regulatory RNA elements, 80 putative intergenic small RNA transcripts were identified based on computational prediction and visual inspection of expression profiles (Supporting Information Table S6) (Supporting Information Fig. S6B). Twenty-four transcripts were annotated previously or found to have homology to known sRNAs or RNA motifs in the Rfam database (Nawrocki *et al*., 2015). The other 56 sRNAs (named Pit for *P. putida* intergenic transcript) were novel sRNAs candidates, and a subset of a complete list of putative sRNAs identified in another study with a deeper sequencing depth (Bojanovič *et al*., manuscript in preparation). Moreover, three additional sRNAs (RNA1, RNA2, RNA3) were detected here but not in Bojanovič *et al.* (manuscript in preparation), likely due to the different library preparation strategies and the dRNA-seq approach used here. A previous study identified 36 sRNAs, of which 14 were novel (Frank *et al*., 2011). In this study, 6 of the latter 14 were detected (named as IGR in Supporting Information Table S6). The reason the other 8 were not detected may be attributed to the expression of sRNAs only in specific growth conditions and differences in experimental protocols for RNA isolation and library construction as documented previously (Gomez-Lozano *et al*., 2012).

Further analysis with IGV revealed an additional 8 transcripts (Supporting Information Table S7) with read profiles consistent with actuatons (Supporting Information Fig. S6C). Actuatons are a class of sRNAs characterized by a high number of reads in the 5′UTR and the presence of a terminator in the proximity of the downstream gene. The downstream mRNA lacks its own TSS and originates from terminator read-through, such that these sRNAs and their downstream mRNAs are joined in a unique transcriptional unit (Kopf and Hess, 2015). This group of sRNAs is expected to function as possible regulators.

Identification of novel ORFs

The TSS prediction revealed 570 orphan TSSs, which were used for the identification of putative novel ORFs in *P. putida* KT2440. The DNA sequences between the predicted orphan TSSs and the downstream annotated genes were collected and analysed by the *in silico* gene finders GLIMMER (Salzberg *et al.*, 1998; Delcher *et al.*, 2007) and GeneMark (Lukashin and Borodovsky, 1998; Besemer and Borodovsky, 2005). The RNA-seq data were then used to confirm the transcription of the ORFs predicted by both GLIMMER and GeneMark. Twenty-one putative ORFs were identified (Supporting Information Table S8) and classified into two categories. In the first, the sequences from the two gene finders were completely overlapping, having the same translational start and stop positions. In the second, the sequences had different start sites predicted by GLIMMER and GeneMark but the same stop position. Of the 21 predicted ORFs, 12 belonged to the first and 9 to the second category. The functions of the putative ORFs were evaluated by sequence homology in protein Blast (Johnson *et al.*, 2008). Five ORFs were homologous to functional proteins or specific domains in *Pseudomonas* and other organisms (PP3108.2 and PP3108.4: rhs family protein, PP1810.1: DUF 3077 superfamily, PP1935.4: resolvase, PP2509.1: diadenosine tetraphosphate hydrolase), while the 16 remaining ORFs were hypothetical proteins (Supporting Information Table S8).

From the 21 putative ORFs identified here, 8 were also detected previously in Frank *et al.* (2011) with exactly the same coordinates, and 5 with a different start position. The remaining 8 are novel ORFs that have not been described previously (Supporting Information Table S8). The different numbers of putative ORFs identified in the two studies can be due in part to different patterns of gene expression in the investigated conditions and also to the fact that the analysis performed here is limited to the orphan TSSs.

Concluding remarks

This study is the first genome-wide TSS analysis in *P. putida* under different growth conditions, and provides a deeper understanding of its metabolic versatility and ability to adapt to different environments. The novel genomic features uncovered here prompt intriguing questions regarding promoter selection and variability in *P. putida* under different conditions, as well as the number of antisense transcripts and whether these are located opposite genes in specific functional categories. The hundreds of mRNAs with long leader regions highlight the issue of their biological function and the wider role of *cis*-regulation in *P. putida*. The structures of these leaders would be interesting to study with next-generation sequencing approaches to probe RNA structure on a global scale or the RNA structurome with the aim of identifying RNA thermometers (Righetti and Narberhaus, 2014) and other *cis*-regulatory elements such as riboswitches. The work underscores the complexity and diversity of bacterial transcriptomes and as the interest in *P. putida* as biotechnological tool is increasing, the genomic features identified here are a benchmark for future studies of its gene expression and metabolic engineering.

Experimental procedures

Bacterial strains and growth conditions

Pseudomonas putida strain KT2440 was used in all experiments. *Pseudomonas putida* was cultivated in M9 minimal medium (per liter: $Na_2HPO_4 \cdot 12H_2O$, 70 g; KH_2PO_4, 30 g; NH_4Cl, 10 g; NaCl, 5 g) supplemented with ammonium iron citrate, magnesium sulfate and trace metals (per liter: H_3BO_3, 300 mg; $ZnCl_2$, 50 mg; $MnCl_2 \cdot 4H_2O$, 30 mg; $CoCl_2$, 200 mg; $CuCl_2 \cdot 2H_2O$, 10 mg; $NiCl_2 \cdot 6H_2O$, 20 mg; and $NaMoO_4 \cdot 2H_2O$, 30 mg) (Abril *et al.*, 1989). The medium included either sodium citrate (10 mM) or glucose (0.5% (w/v)) as sole carbon sources. All liquid cultures were grown at 30°C with vigorous shaking at 250 rpm. The cultures used for RNA isolation were grown from single colonies isolated from LB agar plates containing 25 μg/mL chloramphenicol grown overnight at 30°C. These were used to inoculate 5 mL M9 medium supplemented with chloramphenicol (25 μg/mL). The overnight cultures were diluted to a starting OD_{600} of 0.05 in 100 mL M9 medium in 250 mL Erlenmeyer flasks.

Escherichia coli strain NEB5α (New England Biolabs) was used for cloning and propagation of plasmids (Supporting Information Table S9). Chemically competent cells of NEB5α were prepared as described elsewhere (Inoue *et al.*, 1990) and had an estimated transformation efficiency of 2.6–3.3 × 10^7 CFU/μg DNA. *Escherichia coli* was propagated at 37°C in LB supplemented with gentamicin (10 μg/mL) when required.

Cell harvest and RNA isolation

Pseudomonas putida KT2440 cells were harvested in mid-exponential phase ($OD_{600} \sim 0.5$ and 1 for citrate and glucose cultures, respectively) and in early-stationary phase ($OD_{600} \sim 1.5$ and 4.9 for citrate and glucose cultures, respectively). Cells were harvested by transferring 20 mL of each culture into 50-mL Falcon tubes containing 4 mL of stop solution (5% phenol in 95% ethanol, 4°C), vortexed for 15 s and kept on ice for 5 min. Following centrifugation (8000 rpm, 2 min, 4°C) in a Multifuge X3 Fr centrifuge (Thermo Scientific), cells were resuspended in 2 mL of supernatant by pipetting and split into two RNase-free 1.5 mL tubes. After centrifugation (7000 x g, 5 min, 4°C) and removal of supernatant, the pellet was dissolved in 1 mL of TRIzol Reagent (Invitrogen, Life Technologies), incubated 5 min at room temperature and stored at −80°C. Total RNA extraction and DNA removal by treatment with DNaseI were performed as previously described (Gomez-Lozano *et al.*, 2012). The integrity of total RNA, the presence of rRNAs and tRNAs, as well as DNA contamination were assessed with a RNA 6000 Nano chip on Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was extracted from two biological replicate cultures for each condition.

Exonuclease treatment

The sample preparation for dRNAseq was accomplished essentially as described previously (Sharma *et al.*, 2010). Briefly, total RNA was divided in two equal portions, where one was incubated with Terminator TM 5′phosphate-dependent exonuclease (TEX) (Epicentre Illumina TER51020) to generate the primary transcript enriched library and the other left untreated. The exonuclease reaction was performed with 5 μg of total RNA sample, using 2 units of TEX for 1 h at 30°C. RiboLock RNase Inhibitor (Thermo Scientific E00381) was added to the reaction mixture (20 U) to preserve RNA integrity. The reaction was stopped with 1 μL of 100 mM EDTA. The RNA integrity and abundance of 16S and 23S rRNAs were assessed with a RNA 6000 Nano chip on the Agilent 2100 Bioanalyzer (Agilent Technologies) (Supporting Information Fig. S2).

TEX treatment was followed by phenol extraction and ethanol precipitation of mRNA, as described by the manufacture (Epicentre Illumina TER51020). RNase-free water was added to the reaction for a final volume of 200 μL. The extraction was performed once with an equal volume of buffer-saturated phenol, followed by vortexing and centrifugation at (14500 x g, 2 min, 4°C). The aqueous phase was transferred to a new RNase-free tube, followed by addition of 1 mL precipitation mix (0.1 volume of 3 M sodium acetate pH 5.5, 2.5 volumes of cold ethanol 100%, 0.02 volume of glycogen 20 mg/μl (Thermo Scientific R0551)). After mixing thoroughly, the reaction was kept at −20°C for 30 min. The RNA was pelleted by centrifugation (14500 x g, 30 min, 4°C), and the supernatant discarded. The RNA pellet was washed with 500 μL of 70% ethanol and precipitated by centrifugation (14500 x g, 5 min, 4°C). The supernatant was discarded and the RNA pellet resuspended in 20 μL of RNase-free water. The final RNA samples were quantified using a NanoDrop 8000 (Thermo Scientific).

Library preparation and RNA sequencing

Sequencing libraries were constructed using the Illumina® TruSeq® Stranded mRNA Sample Preparation kit (Sultan *et al.*, 2012). Each final library was validated with a DNA 1000 chip on the Agilent 2100 Bioanalyzer and concentration measured using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies). The concentration of each library was normalized to 10 nM using 10 mM Tris-Cl, pH 8.5, 0.1% Tween 20. Then, 10 μL of each normalized library were pooled together. The final pooled library sample was validated with the DNA High Sensitivity Assay on Agilent 2100 Bioanalyzer (Agilent Technologies) and the concentration confirmed on a Qubit 2.0 Fluorometer. The libraries were sequenced using the Illumina HiSeq2000 platform (Beckman Coulter Genomics).

Data analysis

The sequencing reads were initially checked for quality by evaluation of average quality per reads Phred score and mapped onto the *P. putida* KT2440 genome (RefSeq Accession No. NC_002947.3) with Bowtie2 (Langmead and Salzberg, 2012). Mapping output files were sorted and indexed with SAMtools (Li *et al.*, 2009) and then converted to .wig files. The transcription start sites were identified by TSSpredator (Dugar *et al.*, 2013) by processing the reads from biological replicate samples together and using the 'more sensitivity' parameter settings, which determine TSSs by *step height* and *processing site factor* values of 0.2 and 2 respectively. The assignment of primary and secondary TSSs was performed using the default value of a 300 nt maximal upstream distance from the start codon.

Statistical and data analysis were handled by R Bioconductor and Microsoft Excel. Promoter analysis was conducted by MEME Suite (Bailey *et al.*, 2009) and *cis*-RNA secondary structures in 5′UTR regions were searched for homologies against the Rfam databases (Nawrocki *et al.*, 2015). Operon and sRNA prediction were performed by Rockhopper (McClure *et al.*, 2013; Tjaden, 2015). Visual inspection of identified putative sRNAs was done by the Integrative Genomics Viewer (IGV) (Robinson *et al.*, 2011; Thorvaldsdottir *et al.*, 2013). Novel ORFs were predicted by the *in silico* gene finders GLIMMER (Salzberg *et al.*, 1998; Delcher *et al.*, 2007) and GeneMark (Lukashin and Borodovsky, 1998; Besemer and Borodovsky, 2005) and transcription confirmed with RNA-seq data. ORFs functions were searched by sequence homology in protein Blast (Johnson *et al.*, 2008).

5′ RACE

The 5′ends of mRNA transcripts were confirmed by RACE procedures published previously (Vogel and Wagner, 2005; Gomez-Lozano *et al.*, 2012) with modifications. In our approach, the Tobacco Acid Pyrophosphatase treatment step of the DNase-treated total RNA was replaced by the TEX treatment (described above) followed by RNA 5′polyphosphatase (Epicentre Illumina RP8092H), which removes the γ and β phosphates from 5′triphosphorylated RNAs and has no activity on 5′monophosphorylated ends. Briefly, the untreated and treated TEX samples were incubated at 37°C with RNA 5′Polyphosphatase (20 Units) for 30 min. Following RNA purification, an RNA adapter was ligated to the 5′ends using T4 RNA ligase (Thermo Scientific EL0021). The adapter-RNA complex was reverse transcribed by Thermoscript RT-PCR System (Invitrogen 11146) using a gene specific primer (GSP1) for the mRNA. The resulting cDNA was amplified by PCR reaction with Phusion HotStart II High-Fidelity DNA polymerase (Thermo Scientific F-548S/L) using a second gene specific primer (GSP2) and an adapter-specific primer. The PCR products were checked on an agarose gel and sequenced with the PCR amplification primers at Eurofins Genomics (Denmark). The oligonucleotides used in this study are listed in Supporting Information Table S9.

Plasmid construction and uracil excision cloning

PCR products were obtained using proof-reading PfuX7 polymerase (Nørholm, 2010) in Phusion HF Buffer (Thermo Scientific). DNA fragments were amplified with 20 cycles in a 50 μL reaction volume using a C1000 Touch^{TM} Thermal Cycler (BioRad). Amplicons were purified with PureLink^{TM} Quick Gel Extraction and PCR Purification Combo Kit (Life Technlogies) and quantified using NanoDrop 8000 (Thermo Scientific). Translational (pISO1) and transcriptional (pISO2) fusions of the TPP riboswitch and the reporter GFP in plasmid constructs were obtained via uracil excision cloning as described elsewhere (Cavaleiro *et al*., 2015a,b). Oligonucleotides and plasmids used are listed in Supporting Information Table S9. Transformants were checked by colony PCR. Positive plasmids were isolated using the NucleoSpin R plasmid QuickPure Kit (Macherey-Nagel), sequenced at Eurofins Genomics (Denmark) and transformed into *P. putida* KT2440 (Martinez-Garcia and de Lorenzo, 2011).

Riboswitch characterization

Kinetic and manual assays for GFP fluorescence expression were performed using a SynergyMx 96-microtiter plate reader (BioTeck). Overnight cultures of bacteria grown in M9 medium with glucose (0.5% (w/v)) and gentamicin (10 μg/mL) were diluted to a starting OD_{600} of 0.2 with fresh media and transferred to a 96 well microtiter plate. The plate was incubated at 30°C for 2 h with shaking until an OD_{600} of 0.2–0.3 was reached, followed by addition of the inducer 3-methylbenzoate at a final concentration of 0.5 mM. Kinetic assays were performed in the plate reader; where both cell density (OD_{600}) and fluorescence (RFU (485, 528)) were measured every 10 min for *P. putida* KT2440 (pISO1) after induction, while manual measurements were performed every hour for *P. putida* KT2440 (pISO2). At 1.5 h after induction, ligands were added to the wells in different concentrations (10 nM, 100 nM, 1 μM, 5 μM, 10 μM, 100 μM, 0.5 mM, 1 mM and 1.5 mM). Kinetic curves were monitored for 15 h until the entry into stationary phase.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Growth curves of *P. putida* KT2440. Cells were grown in M9 minimal medium in the presence of glucose (0.5% (w/v)) or citrate (10 mM) as sole carbon sources. Cell growth was carried out at 30° C with shaking at 250 rpm, and monitored by measuring optical density at 600 nm. Growth rate in glucose μ_{MAX} (h^{-1}) 0.55 ± 0.01, growth rate in citrate μ_{MAX} (h^{-1}) 0.40 ± 0.05. Arrows indicate the cell harvest points. Error bars represent the standard deviations of three biological replicates.

Fig. S2. Size profiles of total and exonuclease-treated RNA samples. RNA extracted from *P. putida* KT2440 cells was analysed with the RNA 6000 Nano chip on the Bioanalyzer (Agilent). Example profiles of total RNA (A) and exonuclease-treated RNA (B) samples. The peaks corresponding to 16S and 23S rRNAs are labeled (16S, 23S). The peaks marked with asterisks (*) correspond to 5S rRNA, tRNAs and RNA transcripts shorter than 120 nt. The absence of 16S and 23S rRNA peaks in (B) shows the effect of the exonuclease enzyme on processed RNAs.

Fig. S3. Comparison of TSS classification between differential RNA-seq studies in other bacteria. The *P. putida* KT2440 TSS classification in this study is compared with that of previous studies in *Campylobacter jejuni* (Dugar et al., 2013), *Escherichia coli* (Thomason et al., 2015) and *Helicobacter pylori* (Bischler et al., 2015). All studies used exonuclease treatment, the software TSSpredator for TSS identification and the same TSS class definitions.

Fig. S4. 5′RACE experiment to confirm predicted TSSs. A 5′RACE experiment was performed on the *rpoS* transcript (PP1623) in all growth conditions (GluExp, CitExp, GluSta, CitSta). Total RNA samples were divided into two equal parts where one was treated with exonuclease enzyme (+) and the other left untreated (-). Both samples were treated with RNA polyphosphatase, which removes the two phosphate groups at the 5′ends of primary RNA transcripts. Then the RNA adapter was ligated to the 5′ends, followed by reverse transcription performed with the gene specific primer that is specific for the *rpoS* transcripts. The cDNA was amplified by PCR with a second specific primer. A. The 2% agarose gel shows the primary bands (*) after RACE that were sequenced. An enriched band corresponding to the primary transcript is observed for the '+' samples, whereas the '-' samples contain both primary and shorter processed transcripts. B. Schematic representation of the read profile surrounding the *rpoS* gene in the glucose exponential growth condition. The picture shows the mapped reads to the *rpoS* and the upstream *nlpD* genes (PP1622) in both untreated (top) and treated (bottom) samples. The treated sample shows a higher number of reads corresponding to the primary transcript comparing with the untreated sample. The TSSpredator identified TSS coordinates (solid line) internal to *nlpD* gene. The 5′RACE results (dashed line) show the point where the TSS has been determined by the 5′RACE experiment. There is a difference of only 4 nt between the predicted and RACE determined TSS positions.

Fig. S5. Overview of multi-gene operons identified in *P. putida* KT2440. A total of 1076 multi-gene operons were found in *P. putida* KT2440. The graph summarizes the operons based on the number of genes they contain.

Fig. S6. Profiles of transcript categories. The three different profiles associated with particular transcript categories detected in this study are illustrated, where arrows denote TSSs. A. Read profile of the TSS associated with an expressed mRNA, where there is a high intensity of reads mapping to the 5′UTR and expression that continues through the gene. B. Typical sRNA profile with high expression in an intergenic region. C. Actuaton profile with a high number of reads just downstream of the TSS in the 5′UTR, followed by low or no expression extending through the downstream gene.

Table S1. Mapping statistics.

Table S2. Transcription start sites predicted by TSSpredator.

Table S3. Summary of 5′RACE results.

Table S4. Multi-gene operons.

Table S5. Leaderless mRNAs.

Table S6. Intergenic small RNA transcripts.

Table S7. Putative actuatons.

Table S8. Putative ORFs.

Table S9. Strains, plasmids and oligonucleotides used in this work.

Table S1. Mapping statistics.

The total number of reads per sample obtained by dRNA-seq and their mapping on the *Pseudomonas putida* KT2440 genome. The number of reads mapping to rRNA and unique locations are also reported.

Sample	CitExp1	ECitExp1	CitExp2	CitSta1	ECitSta1	CitSta2	ECitSta2
Total reads	**14606687**	**7319772**	**2467876**	**10773486**	**13792053**	**2529327**	**4425463**
Mapped reads (%*)	**13990359 (96%)**	**7216017 (99%)**	**2239434 (91%)**	**10493349 (97%)**	**13557678 (98%)**	**2371249 (94%)**	**4346004 (98%)**
rRNA reads (%)**	13570648 (97%)	5772814 (80%)	2172251 (97%)	10283482 (98%)	11930757 (88%)	2323824 (98%)	3433343 (79%)
Unique reads (%)**	419711 (3%)	1371043 (19%)	89577 (4%)	314800 (3%)	1626921 (12%)	47425 (2%)	912661 (21%)

Sample	GluExp1	EGluExp1	GluExp2	EGluExp2	GluSta1	EGluSta1	GluSta2	EGluSta2
Total reads	**4979158**	**8498168**	**7357738**	**6214340**	**5662585**	**9756498**	**10872646**	**7362417**
Mapped reads (%*)	**4618726 (93%)**	**8273117 (97%)**	**7112066 (97%)**	**6087137 (98%)**	**5466349 (97%)**	**9466666 (97%)**	**10620757 (98%)**	**7199819 (98%)**
rRNA reads (%)**	4341602 (94%)	6783956 (82%)	6756463 (95%)	4747967 (78%)	5302359 (97%)	8803999 (93%)	10408342 (98%)	6047848 (84%)
Unique reads (%)**	277124 (6%)	1489161 (18%)	355603 (5%)	1217427 (20%)	163990 (3%)	662667 (7%)	212415 (2%)	1079973 (15%)

* The % of mapped reads relative to the total number of reads.

** The % of reads relative to the total number of mapped reads.

Abbreviations: Cit, citrate; Glu, glucose, Exp, exponential phase; Sta, stationary phase; E, exonuclease-treated sample.

Table S3. Summary of 5´RACE results.

RACE was performed on three genes in both treated and untreated contidion.

The PP designations of the genes, strand location, tested conditions, as well as the TSS coordinates predicted by TSS predator, detrmined by 5´RACE, and the difference between them are given.

Sample number	**Gene number**	**Gene designation**	**Strand**	**Condition tested**	**Predicted TSS**	**5' RACE TSS**	**Difference (nt)**
1	PP0147	citrate transporter	-	CitExp	157929	157923	6
2	PP4010	*cspD*	+	CitSta	4520037	4520028	9
3	PP4010	*cspD*	+	GluSta	4520037	4520028	9
4	PP1623	*rpoS*	+	CitExp	1818387	1818383	4
5	PP1623	*rpoS*	+	GluExp	1818387	1818383	4
6	PP1623	*rpoS*	+	CitSta	1818387	1818383	4
7	PP1623	*rpoS*	+	GluSta	1818387	1818383	4

Abbreviations: Cit, citrate; Glu, glucose, Exp, exponential phase; Sta, stationary phase.

Table S5. Leaderless mRNAs.

A total of 51 leaderless mRNAs have been identified.

For each leaderless mRNA, the genomic coordinate of start position, strand, locus information (locus designation, gene product, gene length), and UTR length are reported.

Position	Strand	Locus tag	Product	Gene length
72853	-	PP0061	glycyl-tRNA synthetase subunit alpha	948
475702	+	PP0390	DNA-binding/iron metalloprotein/AP endonuclease	1026
605646	+	PP0520	phosphatidylglycerophosphatase A	504
740838	+	PP0634	fimbrial protein pilin	411
874415	+	PP0759	hypothetical protein	840
1242472	-	PP1082	bacterioferritin	474
1242889	-	PP1083	BFD (2Fe-2S)-binding domain-containing protein	219
1257452	+	PP1100	deoxycytidine triphosphate deaminase	567
1311534	-	PP1144	diguanylate cyclase	2265
1390189	+	PP1213	aspartyl-tRNA synthetase	1776
1417530	+	PP1240	phosphoribosylaminoimidazolesuccinocarboxamide synthase	711
1733866	-	PP1526	beta-(1-3)-glucosyl transferase	2592
1778150	-	PP1586	killer protein	279
1799198	+	PP1605	ribonuclease HII	624
1848550	+	PP1654	cysteine synthase B	900
2080503	+	PP1858	elongation factor P	570
2227402	-	PP1964	deoxynucleotide monophosphate kinase	804
2380327	+	PP2089	OmpF family protein	1035
2478293	+	PP2172	hypothetical protein	444
2624401	+	PP2296	hypothetical protein	357
2882011	-	PP2536	glutathione S-transferase	624
3282918	-	PP2884	XRE family transcriptional regulator	546
3328446	+	PP2927	hypothetical protein	1047
3727917	-	PP3293	hypothetical protein	426
4127622	+	PP3631	hypothetical protein	651
4148759	-	PP3652	amino acid transporter LysE	633
4257617	-	PP3730	transcriptional regulator	717
4266462	-	PP3738	GntR family transcriptional regulator	714
4361547	-	PP3836	hypothetical protein	336
4460531	+	PP3954	hypothetical protein	951
4519523	-	PP4008	ATP-dependent Clp protease ATP-binding subunit ClpA	2271
4547348	-	PP4035	NCS1 nucleoside transporter	1491
4676114	+	PP4139	hypothetical protein	219
4873618	-	PP4282	aquaporin Z	693
4936996	+	PP4345	GntR family transcriptional regulator	693
5081537	-	PP4473	aspartate kinase	1236
5120350	+	PP4507	TrkH family potassium uptake protein	1455
5143783	-	PP4527	hypothetical protein	927
5210142	-	PP4591	ribonuclease D	1134
5270336	-	PP4644	DNA repair protein RadA	1371
5431242	-	PP4770	periplasmic ligand-binding sensor protein	759
5451465	+	PP4790	apolipoprotein N-acyltransferase	1518
5741325	-	PP5038	hypothetical protein	255
5745862	-	PP5041	glycogen/starch/alpha-glucan phosphorylase	2451
5904741	-	PP5177	ornithine carbamoyltransferase	894
5936466	+	PP5206	secretion protein HlyD family protein	960
6033659	+	PP5285	bifunctional phosphopantothenoylcysteine decarboxylase/phosphopantothenate synthase	1212
6104119	+	PP5354	hypothetical protein	408
6108146	-	PP5357	pyridoxamine kinase	873
6147816	-	PP5393	heavy metal transport/detoxification protein	201
6175174	-	PP5412	ATP synthase F0F1 subunit epsilon	420

Table S6. Intergenic small RNA transcripts.

A total of 80 small RNAs candidates have been identified and are listed according to their genomic coordinates.
For each transcript the name, start and stop coordinates, length, strand, 5´ and 3´ flanking genes, and orientation relative to the flanking genes are indicated.

Number	Name	Start	Stop	Length	Strand	5´Flanking gene	3´Flanking gene	Orientation
1	Pit003	16329	16281	49	-	PP0013	PP0014	> < >
2	Pit006	58407	58555	149	+	PP0049	PP0050	< > <
3	Spot42-like/spf/ErsA*	130367	130539	173	+	PP0123	PP0124	> > <
4	C4 AS RNA 1*	335696	335870	175	+	PP0277	PP0278	< > >
5	RsmY*	450782	450934	153	+	PP0370	PP0371	> > <
6	Pit020	450917	450814	104	-	PP0370	PP0371	> < <
7	P27*	536446	536303	144	-	PP0444	PP0445	> < >
8	P26*	537405	537502	98	+	PP0446	PP0447	> > >
9	Pit024	611076	610907	170	-	PP0525	PP0526	< < >
10	Pit025	624099	624004	96	-	PP0536	PP0537	< < <
11	Pit031	751928	752255	328	+	PP0640	PP0641	< > <
12	C4 AS RNA 2*	759558	759654	97	-	PP0651	PP0652	> > <
13	Pit048	1296778	1296615	164	-	PP1132	PP1133	< < >
14	Pit049	1298345	1298507	163	+	PP1132	PP1133	> > <
15	PhrS*	1316300	1316400	101	+	PP1148	PP1150	> > >
16	Pit051	1349036	1349140	105	+	PP1173	PP1174	< > <
17	Pit052	1349617	1349735	119	+	PP1174	PP1175	< > <
18	Pit054	1388590	1388487	104	-	PP1209	PP1210	< < >
19	Pit055	1440293	1440130	164	-	PP1259	PP1260	< < >
20	Pit056	1441860	1442022	163	+	PP1259	PP1260	> > <
21	RnpB/P28*	1512685	1513069	385	+	PP1326	PP1328	> > >
22	Pit058	1626951	1627100	150	+	PP1426	PP1427	< > >
23	T44*	1785122	1785242	121	+	PP1590	PP1591	< > >
24	RsmZ*	1822011	1822190	180	+	PP1624	PP1625	> > <
25	Pit064	1847250	1847088	163	-	PP1652	PP1653	> < >
26	RNA1**	1995669	1995866	198	+	PP1781	PP1782	< > <
27	Pit077	2151206	2150992	215	-	PP1905	PP1906	< < >
28	RgsA/P16*	2229834	2229726	109	-	PP1967	PP1968	> < >
29	C4 AS RNA 3*	2302915	2302823	93	-	PP2027	PP2026	< < <
30	Rmf*	2388735	2388345	391	-	PP2095	PP2096	> < >
31	Pit092	2435418	2435212	207	-	PP2133	PP2134	> < >
32	RNA2**	2608031	2608171	141	-	PP2284	PP2285	> < >
33	Pit094	2622634	2623131	498	+	PP2294	PP2295	> > >
34	Pit097	2672559	2672471	89	-	PP2339	PP2340	< < >
35	Pit098	2674735	2674968	234	+	PP2343	PP2344	> > <
36	RNA3**	2710973	2710798	176	-	PP2373	PP2374	> < <
37	C4 AS RNA 6*	2855850	2855745	106	-	PP2507	PP2508	> < >
38	Pit105	2925591	2925749	159	+	PP2563	PP2564	< > <
39	Pit107	2939084	2939246	163	+	PP2570	PP2571	< > <
40	Pit109	3261547	3261423	125	-	PP2859	PP2858	< < <
41	Pit110	3275580	3275861	282	+	PP2873	PP2874	> > >
42	Pit114	3450217	3450305	89	+	PP3067	PP3068	> > >
43	P15*	3466266	3466060	207	-	PP3080	PP3081	< < <
44	Pit124	3826437	3826208	230	-	PP3381	PP3380	< < <
45	Pit125	3828006	3828169	164	+	PP3381	PP3382	< > <
46	Pit126	3967909	3967809	101	-	PP3497	PP3498	> > >
47	Pit127	3971957	3971765	193	-	PP3501	PP3502	> < >
48	Psr2*	4013251	4013565	315	+	PP3540	PP3541	> > <
49	Pit130	4022619	4022473	147	-	PP3548	PP3547	< < <
50	Pit132/IGR 3586	4073874	4073623	252	-	PP3586	PP3585	< < <
51	Pit133	4075444	4075605	162	+	PP3587	PP3586	< > <
52	Pit136	4224280	4224507	228	+	PP3703	PP3704	< > <
53	Pit137	4302495	4302290	206	-	PP3774	PP3775	> < <
54	Pit140/IGR 3917	4425377	4425164	214	-	PP3916	PP3917	> < >
55	PrrF2*	4595167	4595310	144	+	PP4069	PP4070	> > >
56	Pit144	4595281	4595233	49	-	PP4069	PP4070	> < >
57	IGR 4095	4630733	4630507	227	-	PP4094	PP4095	> < >
58	Bacteria small SRP*	4858503	4858392	112	-	PP4273	PP4274	> < <
59	Pit148/IGR 4451	5047215	5047412	198	+	PP4450	PP4451	> > <
60	Pit149	5103279	5103410	132	+	PP4491	PP4492	> > >
61	Pit151	5140624	5140398	227	-	PP4524	PP4525	> < <
62	C4 AS RNA 4/IGR 4535*	5148997	5148876	122	-	PP4535	PP4534	< < <
63	Pit153	5219064	5218924	141	-	PP4598	PP4599	> > >
64	Pit154	5222758	5222598	161	-	PP4603	PP4602	< < <
65	Pit155	5224327	5224489	163	+	PP4603	PP4604	< > <
66	PrrF1*	5325394	5325485	92	+	PP4685	PP4686	> > <
67	CrcZ*	5338284	5338625	342	+	PP4696	PP4697	> > >
68	P31*	5373151	5373213	63	+	PP4724	PP4725	< > <
69	P32*	5373351	5373255	97	-	PP4724	PP4725	< < <
70	SsrA tmRNA*	5389989	5390412	424	+	PP4738	PP4739	> > >
71	IGR 4740	5391608	5391314	295	-	PP4739	PP4740	> < >
72	P24*	5437800	5437675	126	-	PP4775	PP4776	< < >
73	Pit161	5453307	5453144	164	-	PP4790	PP4791	> < >
74	Pit162	5545496	5545288	209	-	PP4879	PP4878	< < <
75	Pit163	5756969	5756716	254	-	PP5049	PP5050	< < >
76	6S RNA/SsrS*	5934661	5934846	186	+	PP5202	PP5203	> > >
77	Pit168	5989892	5989792	101	-	PP5247	PP5248	> < <
78	Pit169	6039010	6039211	202	+	PP5290	PP5291	< > <
79	Pit172	6137172	6137302	131	+	PP5384	PP5385	> > >
80	Pit176	6159099	6158994	106	-	PP5401	PP5402	> < <

* Annotated sRNA.
** Additional sRNAs not identified in Bojanovič *et al.*, manuscript in preparation.

Table S7. Putative actuatons.

For each transcript the name, start and stop coordinates, length, strand, 5´and 3´flanking genes and orientation relative to the flanking genes are reported.

Number	Name	Start	Stop	Length	Strand	5´Flanking gene	3´Flanking gene	Orientation
1	RNA4	611063	610868	195	-	PP0525	PP0526	< < >
2	RNA5	1532000	1531903	97	-	PP1344	PP1345	< < >
3	RNA6	1607721	1607616	105	-	PP1409	PP1408	< < <
4	RNA7	1748828	1748728	100	-	PP1549	PP1548	< < <
5	RNA8	2532043	2532142	99	+	PP2222	PP2223	> > >
6	Pit108	3023086	3023256	170	+	PP2638	PP2639	> > >
7	RNA09	4170053	4170158	105	+	PP3668	PP3669	< > >
8	RNA10	4564500	4564595	95	+	PP4049	PP4050	< > >

Table S8. Putative ORFs.

Putative ORFs are identified by the gene finders GLIMMER and GeneMark.

Table A. lists the ORFs that have been found in both gene finders and have the same translational coordinates.

Table B. lists the ORFs that show a different translational start site between the two gene finders but same stop site.

For each putative ORF, coordinates, strand, length, predicted TSS, flanking genes, orientation and Blastp result are reported.

A. Novel ORFs with same translational coordinates

ORF[a]	Coordinates	Strand	Length (bp)	Predicted TSS	5´Flanking gene	3´Flanking gene	Orientation	Blastp
PP0284.1	343442-342999	-	444	343871	PP0284	PPt03	> < >	hypothetical protein
PP0636.1	744476-744916*	+	441	743427	PP0636	PP0637	< > <	hypothetical protein (p)
PP0651.2	759860-760087*	+	228	759513	PP0651	PP0652	> > <	hypothetical protein
PP0651.3	758879-758760	-	120	759671	PP0651	PP0651	> <[b] >	hypothetical protein
PP1115.1	1275412-1275113**	-	300	1275760	PP1115	PP1116	< < <	hypothetical protein
PP1810.1	2037877-2037602	-	276	2038030	PP1810	PP1811	> < >	DUF 3077 superfamily
PP1935.1	2182187-2182579*	+	393	2181461	PP1935	PP1936	< > <	hypothetical protein
PP2874.1	3275654-3275842	+	189	3275596	PP2874	PP2875	> > <	hypothetical protein
PP3108.2	3516293-3516916**	+	624	3515633	PP3108	PP3109	> > >	rhs family protein
PP3108.4	3516127-3516246	+	120	3515633	PP3108	PP3109	> > >	type IV secretion protein rhs-like protein
PP3688.1	4197678-4197145*	-	534	4198048	PP3688	PP3689	< < >	hypothetical protein **(P)**
PP4535.2	5152241-5151921*	-	321	5152397	PP4535	PP4536	< < >	hypothetical protein **(P)**

B. Novel ORFs with different start codon position

ORF[a]	Start site Glimmer/GeneMark	Stop site	Strand	Length Glimmer/GeneMark (bp)	Predicted TSS	5´Flanking gene	3´Flanking gene	Orientation	Blastp
PP1115.2	1276318/1276015	1276452	+	135/438	1274407	PP1115	PP1116	< > <	hypothetical protein
PP1919.1	2163137*/2163278	2164150	+	1014/873	2162727	PP1919	PP1920	> > <	hypothetical protein (p)
PP1935.3	2183476/2183269**	2183766	+	291/498	2181461	PP1935	PP1936	< > <	hypothetical protein
PP1935.4	2184249/2183940*	2184506	+	258/567	2181461	PP1935	PP1936	< > <	ser recombinase superfamily, HTH hin like superfamily **(P)**
PP1936.1	2188357/2188522*	2187608	-	750/915	2188873	PP1936	PP1937	< < >	hypothetical protein **(P)**
PP2294.1	2623027/2622829**	2623161	+	135/333	2621650	PP2294	PP2295	> > >	hypothetical protein
PP2509.1	2857541/2857313**	2857699	+	159/387	2856899	PP2509	PP2510	< > <	diadenosine tetraphosphate hydrolase **(P)**
PP3066.3	3448342/3448378	3447872	-	471/507	3450644	PP3066	PP3067	> < >	hypothetical protein **(P)**
PP5237.1	5971944/5971962	5971831	-	114/132	5975951	PP5238	PP5237	> < >	hypothetical protein (p)

[a] ORF name is assigned based on the 5´flanking gene and consecutive number from Frank *et al.*.

[b] Internal and on the opposite strand of the gene.

* Found also in Frank *et al.* with the same coordinates.

** Found also in Frank *et al.* with a different start position.

(p) Highly conserved in *Pseudomonas putida.*

(P) Highly conserved in *Pseudomonas spp.*

Table S9. Strains, plasmids and oligonucleotides used in this work.

Strain	Genotype	Ref.
P. putida KT2440	*rmo- mod+*	DSMZ
E. coli NEB5α	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	NEB

Plasmid	Genotype	Ref.
pPCV31	*xylS*, *gfp* expressed fom Pm promoter in pSEVA, pBBR1 origin of replication, Gm^R	Unpublished
pISO1	pPCV31 with TPP riboswitch upstream of *gfp*	This work
pISO2	pISO1 with RBS between TPP riboswitch and *gfp*	This work

Oligonucleotide	Sequence	Application
GSP1 PP0147	GGCGGCGCAGCAGATCATGT	5´RACE of citrate transporter
GSP2 PP0147	GCGGTGCCGACCGAGACTTTCA	5´RACE of citrate transporter
GSP1 PP4010	GCGGCTCGCCCAAGGCTCTG	5´RACE of *cspD*
GSP2 PP4010	GCAGCGGCCGCGGCATCTTT	5´RACE of *cspD*
GSP1 PP1623	CGGCCGGCAGGGTCACCCTT	5´RACE of *rpoS*
GSP2 PP1623	CCGCTTTCGTCGCCGCTCTT	5´RACE of *rpoS*
RNA_adapter	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA	5´RACE RNA adapter
Adapter_primer	GCTGATGGCGATGAATGAACACTGC	5´RACE adapter primer
ITD1	AGCTTGUCCAGCAGGGTTGTCCAC	USER cloning: construction of pISO1
ITD2	ACAAGCUGATGGACAGGCTGCG	USER cloning: construction of pISO1
ITD3	ATGGTCAUGACTCCATTATTATTGTTTCTGTTGC	USER cloning: construction of pISO1
ITD4	ATGACCAUGCCTAGGCCGCGGCCGCGCGCATTTACCTGCTTGGCTTTGCTGACC	USER cloning: construction of pISO1
ITD5	ATCGCTUTTTCTTGTTTGGTCATCACAGG	USER cloning: construction of pISO1
ITD6	AAGCGAUCAACCTCAGCATGAGTAAAGGAGAAGAACTTTTCACTGGAG	USER cloning: construction of pISO1
ITD32	ATCAACCUCAGCGCTGAGGCGATAGGAGGAATATACCATGAGTAAAGGAGAAGAACTTTTCACTGGAG	USER cloning: construction of pISO2
ITD33	AGGTTGAUCGCTTTTTCTTGTTTGGTCATC	USER cloning: construction of pISO2
ITD17	GCGGAGCTATCCAACGGCGG	plasmid sequencing
ITD18	GGACAGGGCCATCGCCAATTGG	plasmid sequencing
ITD19	GCTCGCGGCCATCGTCCACA	plasmid sequencing
ITD20	CCGCCAATTCGTCGCCCCATG	plasmid sequencing
ITD21	CAGTGGAGAGGGTGAAGGTGATGC	plasmid sequencing
ITD22	GGCGACTGCCCTGCTGCGTA	plasmid sequencing