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Diversity of small RNAs expressed in *Pseudomonas* species

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Summary

RNA sequencing (RNA-seq) has revealed several hundreds of previously undetected small RNAs (sRNAs) in all bacterial species investigated, including strains of *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Pseudomonas syringae*. Nonetheless, only little is known about the extent of conservation of expressed sRNAs across strains and species. In this study, we have used RNA-seq to identify sRNAs in *P. putida* DOT-T1E and *Pseudomonas extremaustralis* 14-3b. This is the first strain of *P. extremaustralis* and the second strain of *P. putida* to have their transcriptomes analysed for sRNAs, and we identify the presence of around 150 novel sRNAs in each strain. Furthermore, we provide a comparison based on sequence conservation of all the sRNAs detected by RNA-seq in the *Pseudomonas* species investigated so far. Our results show that the extent of sRNA conservation across different species is very limited. In addition, when comparing the sRNAs expressed in different strains of the same species, we observe that numerous sRNAs exhibit a strain-specific expression pattern. These results support the idea that the evolution of most bacterial sRNAs is rapid, which limits the extent of both interspecies and intraspecies conservation.

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Introduction

Bacterial small RNAs (sRNAs) can modulate gene expression and most of them function through base-pairing interactions with mRNA targets that can affect translation initiation, transcription termination or mRNA stability (reviewed in Waters and Storz, 2009). Other sRNAs function by binding to and altering the activity of proteins that regulate gene expression (Waters and Storz, 2009). There are examples of sRNAs involved in transcription reprogramming, carbon metabolism, iron homeostasis, cell envelope homeostasis and coordination of virulence (reviewed in Repoila and Darfeuille, 2009). Until very recently, bacterial sRNAs have been overlooked mainly because there were no high-throughput methods for their detection. Despite the fact that the first bacterial sRNAs have been known for four decades, earlier studies to identify sRNAs have often depended on serendipity, direct detection due to high abundance, protein co-purification and computational predictions based on sequence conservation (reviewed in Vogel and Sharma, 2005). Recent genome-wide studies to identify sRNAs have been based on tiling arrays and, since 2008, primarily on RNA sequencing (RNA-seq), which has enabled the study of the transcriptome at unprecedented depths and in a strand-specific manner.

So far the only *Pseudomonas* species, in which RNA-seq investigations have been carried out to detect sRNAs, are *Pseudomonas aeruginosa* PAO1 and PA14 (Ferrara *et al*., 2012; Gómez-Lozano *et al*., 2012; Wurtzel *et al*., 2012), *Pseudomonas putida* KT2440 (Frank *et al*., 2011) and *Pseudomonas syringae* DC3000 (Filiatrault *et al*., 2010). In this work, we report that RNA-seq has enabled the detection of around 150 novel intergenic sRNAs in *P. putida* DOT-T1E and in *Pseudomonas extremaustralis* 14-3b respectively. Also, we provide a sequence comparison of all intergenic sRNAs validated by RNA-seq in the *Pseudomonas* species investigated so far.

Pseudomonas putida is a ubiquitous bacterium that can be found in soil associated with plant roots as well as in aquatic systems, either in suspension or in biofilms on biotic and abiotic surfaces (Yousef Coronado *et al*., 2008; Rodríguez Herva *et al*., 2010; Jakovleva *et al*., 2012). Survival and proliferation of *P. putida* under such diverse environmental conditions is based on an ample interplay of metabolic activities and the ability of bacteria of this

species to respond to antimicrobial compounds produced by other microorganisms (Daniels and Ramos, 2009). RNA-seq has previously enabled the detection of 36 intergenic sRNAs in *P. putida* KT2440, including RNase P RNA, tmRNA, RgsA/P16, Spot42, RsmZ, RsmY, CrcZ, CrcY and PrrF (Frank *et al*., 2011). In this work, we investigate *P. putida* DOT-T1E, a strain isolated from Granada's wastewater treatment plant that is able to thrive in the presence of high concentrations of organic solvents such as aromatic compounds (Ramos *et al*., 1995). In addition, *P. putida* DOT-T1E has been shown to be resistant to a wide range of toxic compounds such as dyes, heavy metals, bactericidal antibiotics like ampicillin, kanamycin or gentamicin, and bacteriostatic compounds such as chloramphenicol, tetracycline and spectinomycin among others (Ramos *et al*., 1998; Terán *et al*., 2003; Fernández *et al*., 2012). So far 17 sRNAs were annotated in the *P. putida* DOT-T1E, including 6S RNA, 4.5S rRNA, tmRNA, RNase P RNA, PrrF1, PrrF2, RsmY and RsmZ (Winsor *et al*., 2011).

Pseudomonas extremaustralis is a non-pathogenic species isolated from an Antarctic environment whose genome was recently sequenced (López *et al*., 2009; Tribelli *et al*., 2012). This species shows high resistance to cold and oxidative stress and is able to tolerate and degrade hydrocarbons (Tribelli *et al*., 2012). In *P. extremaustralis*, poly-hydroxy butyrate production is associated with high stress resistance such as cold, and genes involved in the biosynthesis of this polymer are located within an adaptive island, which was probably acquired by horizontal gene transfer (Ayub *et al*., 2007; 2009; Tribelli and López, 2011). Additionally, it has been shown that *P. extremaustralis* is able to grow under low oxygen conditions and form well developed biofilms (Tribelli *et al*., 2010; 2013).

Pseudomonas aeruginosa can be found in a wide variety of environmental sources, from water to soil, over a wide range of temperatures. It is an important opportunistic pathogen in humans, where it may cause urinary tract, eye and ear infections, systemic infections in burn patients as well as being a major cause of morbidity and mortality in cystic fibrosis patients due to chronic lung infections (Brennan and Geddes, 2002). Only 44 sRNAs were identified in *P. aeruginosa* until 2012, including 6S RNA, 4.5S rRNA, RNase P RNA, tmRNA, RsmY, RsmZ, CrcZ, PrrF1, PrrF2, and PhrS (reviewed in Sonnleitner *et al*., 2010). The first genome-wide search of sRNAs using RNA-seq in *P. aeruginosa* was reported in 2012 (Gómez-Lozano *et al*., 2012). RNA isolated from exponential and early-stationary phase cultures of *P. aeruginosa* PAO1 was used to prepare three different types of libraries for sequencing. In total, 513 novel intergenic sRNAs were detected in addition to all previously annotated sRNAs except one. The subset of sRNAs detected in each library type was different, showing that transcript identification depends strongly on the specific library preparation strategy used (Gómez-Lozano *et al*., 2012; Gómez-Lozano, Marvig, Molin *et al*., 2014). Later in 2012, two additional independent studies that used RNA-seq to study the transcriptome of *P. aeruginosa* were published. One of the studies investigated strains PAO1 and PA14 at early stationary phase (Ferrara *et al*., 2012). The authors detected the expression of 70 intergenic sRNAs in *P. aeruginosa*, of which 19 were already annotated sRNAs. The other study sequenced *P. aeruginosa* PA14 grown at 28°C and at 37°C (Wurtzel *et al*., 2012). The authors detected the expression of 200 intergenic sRNAs, of which 40 were previously annotated sRNAs (Wurtzel *et al*., 2012).

Pseudomonas syringae pv. *tomato* DC3000 is a model organism for the study of plant–pathogen interactions, which causes bacterial speck on tomato and can infect the model plant *Arabidopsis* (Buell *et al*., 2003). *Pseudomonas syringae* DC3000 has a large repertoire of transporters for the acquisition of nutrients, particularly sugars, as well as genes implicated in attachment to plant surfaces. Comparative genomics analysis confirmed a high degree of similarity with *P. putida* and *P. aeruginosa*, yet revealed 1159 genes unique to *P. syringae* DC3000 (Buell *et al*., 2003). Although 12% of the genes are dedicated to regulation in *P. syringae* DC3000 (Buell *et al*., 2003), little is known about the expression of many predicted gene products and the complex regulatory mechanisms this bacterium uses to monitor and adapt to different environmental cues (Filiatrault *et al*., 2010). RNA-seq has enabled the detection of 25 intergenic sRNAs in *P. syringae* DC3000, including 6S RNA, 4.5S rRNA, RNase P RNA, tmRNA, Spot42, RsmX, RsmY, RsmZ, CrcZ, CrcX, PrrF1 and PrrF2 (Filiatrault *et al*., 2010).

Excluding the highly conserved sRNAs (6S RNA, 4.5S rRNA, RNase P RNA and tmRNA), there is not much knowledge about the extent of sequence conservation of sRNAs in bacteria. This study is the first to investigate to which extent the expression of sRNAs is conserved across different *Pseudomonas* species, including the recently sequenced species *P. extremaustralis* 14-3b.

Results and discussion

Novel sRNAs detected in P. putida *and* P. extremaustralis *14-3b*

Despite the broad use of RNA-seq for transcriptomic studies, this technique has so far only been applied to identify novel sRNAs in three *Pseudomonas* species: *P. aeruginosa* (strains PAO1 and PA14), *P. putida* (strain KT2440) and *P. syringae* (strain DC3000). Here we report the detection of around 150 novel sRNAs in *P. putida*

DOT-T1E and *P. extremaustralis* 14-3b respectively. RNA-seq experiments have been performed using RNA originating from *P. putida* DOT-T1E cultures grown in the presence of eight different antibiotics to get a complete transcriptomic profile and understanding of the mechanisms of resistance of *P. putida* DOT-T1E in the presence of antimicrobial compounds (Appendix S1). These RNA-seq experiments have revealed the existence of 154 intergenic sRNAs, of which 138 are newly detected sRNAs and 16 are already-annotated sRNAs. Here we report the sequences of sRNAs of *P. putida* DOT-T1E conserved in other *Pseudomonas* species (Table S1). Among the novel sRNAs detected, there are sequence homologues to the sRNAs CrcZ, CrcY and Spot42. The complete transcriptomic results including the full list of sRNAs detected will soon be published by Molina-Santiago *et al*. (C. Molina-Santiago, A. Daddaoua, M. Gómez-Lozano, Z. Udaondo, S. Molin and J.L. Ramos, submitted).

In the case of *P. extremaustralis* 14-3b, the RNA was harvested from aerobic cultures at exponential and stationary phases, respectively, and from micro-aerobic cultures at stationary phase (Appendix S1). This is, to the best of our knowledge, the first transcriptomic study performed in this extremophile bacterial species, and it will provide a better understanding of the full coding potential and functional elements of its recently sequenced genome (Tribelli *et al*., unpublished). These experiments have revealed the existence of 156 intergenic sRNAs in the genome of *P. extremaustralis* 14-3b. Here we report the sequences of sRNAs of *P. extremaustralis* 14-3b conserved in other *Pseudomonas* species, which include homologues to 6S RNA, 4.5S rRNA, RNase P RNA, tmRNA, PrrF1/2, RsmZ, RsmY and CrcZ (Table S2). The complete transcriptomic results including the full list of sRNAs detected will be published by Tribelli *et al*.

Overlap of sRNAs between the different Pseudomonas *species*

We have investigated the sequence conservation of sRNAs that are expressed in different *Pseudomonas* species. This has been done by using BLASTN to compare the sequences of the sRNAs that have been detected by RNA-seq so far in *P. aeruginosa* PAO1 and PA14, *P. putida* KT2440 and DOT-T1E, *P. extremaustralis* 14-3b and *P. syringae* DC3000 (Table 1). The extent of sRNAs found to be expressed in two species ranges from 15 sRNAs (*P. extremaustralis* 14-3b versus *P. syringae*) to 28 sRNAs (*P. aeruginosa* versus *P. putida*) (Fig. 1). Tables S1–4 list the sRNA homologues between the *Pseudomonas* species studied and *P. putida* (Table S1), *P. extremaustralis* (Table S2), *P. aeruginosa* (Table S3) and *P. syringae* (Table S4). Only 10 sRNAs are conserved and expressed in all four different species investigated (Fig. 1, Table S6). Four of these are well-known sRNAs: 6S RNA/SsrS, 4.5S rRNA/Ffs/SRP, RNase P RNA and tmRNA/SsrA. The other conserved sRNAs include PrrF1 and PrrF2 (involved in iron metabolism), RsmY and RsmZ (related to virulence in pathogenic strains and to quorum sensing and secondary metabolites in other species), CrcZ [involved in carbon catabolite repression (CCR)] and one sRNA named 2315 in *P. aeruginosa* PAO1. The 2315 sRNA is a homologue of SroG of *Escherichia coli* that was first detected in *P. aeruginosa* by González *et al*. (2008). These sRNAs are formed from the premature termination of transcription in a 5′ untranslated leader mRNA. The particular riboswitch producing SroG is characterized by a conserved RNA structure element termed RFN (Gelfand *et al*., 1999). Flavins such as FMN repress the expression of riboflavin biosynthetic (*rib*) genes by binding to the RFN riboswitch, which causes the premature termination of transcription in the 5′ untranslated *rib* leader mRNAs. In *E. coli* SroG is derived from the leader of *ribB*, which contains an RFN element (Vogel *et al*., 2003). In *P. aeruginosa*, the 2315 sRNA is generated from the *ribC* leader sequence (González *et al*., 2008). In the other sequenced *Pseudomonas*, the homologues of 2315 are located upstream of the T1E_5746 gene in *P. putida* DOT-T1E, the 6,7-dimethyl-8-ribityllumazine synthase gene in *P. extremaustralis* 14-3b and the PSPTO_1840 gene in *P. syringae* DC3000. Homologues of Spot42, t44, RgsA/P16, P26 and TPP are expressed in all species except in *P. extremaustralis* 14-3b, and homologues to CrcY/CrcX are expressed in all species except in *P. aeruginosa* (Tables S1–4).

The evolution of bacterial sRNAs appears to be rapid and, as a result, sequence similarities between sRNAs are limited, even between relatively closely related species (Gottesman and Storz, 2011). This seems to be the case of sRNAs detected in the *Pseudomonas* species, which poses a challenge for the identification and characterization of novel sRNAs based on sequence similarities. The low extent of sequence conservation found in this study might be a consequence of this rapid evolution. The large number of non-conserved sRNAs reported may reflect the metabolic diversification and niche specialization observed in the different *Pseudomonas* species. Indeed most of the highly conserved sRNAs are associated with conserved regulatory networks such as CCR, iron metabolism, quorum-sensing regulation, stationary phase regulation of transcription, ribosome rescue, co- and post-translational transport of proteins, and tRNA processing. From our results, it seems that most of these highly conserved sRNAs are protein-binding RNAs. Seven out of 10 sRNAs conserved across all *Pseudomonas* species

Table 1. Overlap in sRNAs detected in different RNA-seq studies.

	***P. aeruginosa* PAO1** Ferrara *et al.* (2012) 55 sRNAs	***P. aeruginosa* PA14** Ferrara *et al.* (2012) 49 sRNAs	***P. aeruginosa* PA14** Wurtzel *et al.* (2012) 200 sRNAs	***P. syringae* DC3000** Filiatrault *et al.* (2010) 25 sRNAs	***P. putida* DOT-T1E** This study 154 sRNAs	***P. putida* KT2440** Frank *et al.* (2011) 36 sRNAs	***P. extremaustralis* 14-3b** This study 156 sRNAs
***P. aeruginosa* PAO1** Gómez-Lozano *et al.* (2012) 556 sRNAs	38	34	95	22	24	12	21
***P. aeruginosa* PAO1** Ferrara *et al.* (2012) 55 sRNAs	32	17	11	10	8	11	
***P. aeruginosa* PA14** Ferrara *et al.* (2012) 49 sRNAs	16	7	8	4	10		
***P. aeruginosa* PA14** Wurtzel *et al.* (2012) 200 sRNAs	15	20	11	12			
***P. syringae* DC3000** Filiatrault *et al.* (2010) 25 sRNAs	23	14	16				
***P. putida* DOT-T1E** This study 154 sRNAs	15	17					
***P. putida* KT2440** Frank *et al.* (2011) 36 sRNAs	12						

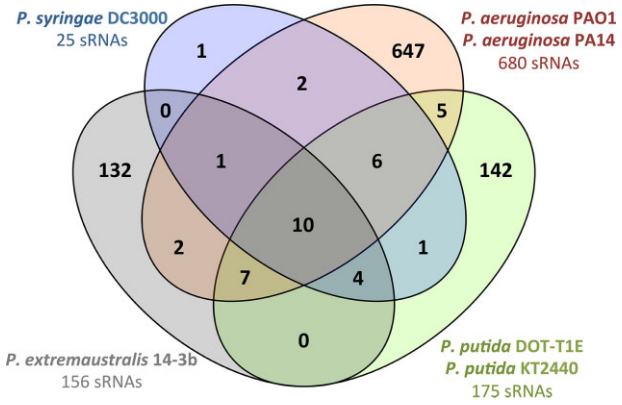

Fig. 1. Venn diagram showing the number of homologues sRNAs in different *Pseudomonas* species.

included in this study regulate the activity of proteins by interacting with them, such as 6S RNA, RNase P RNA, 4.5S RNA, tmRNAs, RsmY, RsmZ and CrcZ. This may be due to the fact that protein-binding sRNAs are more conserved at the structural level than those that act by base pairing, which supports an early evolutionary origin for these regulatory RNAs. However, the structure, mode of action and targets of more sRNAs need to be characterized in order to trace their evolution and identify homologous sRNAs in other species.

One limitation of this study is that the number of sRNAs reported in *P. putida* KT2440 (36 sRNAs) and *P. syringae* DC3000 (25 sRNAs) is most likely an underestimation. The transcriptomic studies of *P. putida* KT2440 and *P. syringae* DC3000 were performed using an older sequencing platform that provided smaller sequencing depths than in later studies. In addition, the RNA extraction methods used in these two studies were not optimal for short RNA species retention (Filiatrault *et al.*, 2010; Frank *et al.*, 2011).

There are several examples of duplicate sRNA genes in bacteria, such as the two Crc-inhibitory sRNAs present in most of the *Pseudomonas* species. Duplicate sRNA genes can allow for increased induction or differential regulation of each the RNA copies, providing more flexibility in their regulatory roles. *Pseudomonas* species lack the CRP-cAMP system, and CCR in these organisms involves the Crc protein, which acts as a translational repressor of genes that encode transport and metabolism functions for less-preferred carbon sources (reviewed in Rojo, 2010). CrcZ is a sRNA crucial for the regulation of Crc activity. CrcZ sequesters Crc by binding to it with high affinity, preventing its repression of translation of mRNAs (Sonnleitner *et al.*, 2009). All sequenced *Pseudomonas* strains encode two Crc-inhibitory sRNAs except *P. aeruginosa*, which encodes only one, CrcZ (Fig. 2). A

Fig. 2. Genomic organization of the sRNAs CrcZ, CrcX and CrcY in different *Pseudomonas* species. Duplicate sRNAs in *P. putida* DOT-T1E. sRNAs are depicted in green, flanking genes are depicted in blue. We have identified homologues of CrcZ and CrcY in both *P. putida* DOT-T1E and *P. extremaustralis* 14-3b. The CrcZ (RNA16) and CrcY (RNA3) homologues in *P. putida* DOT-T1E are encoded in the same intergenic regions as in *P. putida* KT2440, although in a different orientation. In *P. extremaustralis*, 14-3b has a sRNA (sRNA23) encoded antisense to the *crcZ* gene homologue (sRNA22), which resembles the situation in *P. aeruginosa* PAO1 where the sRNA P30 is also encoded antisense to the *crcZ* gene.

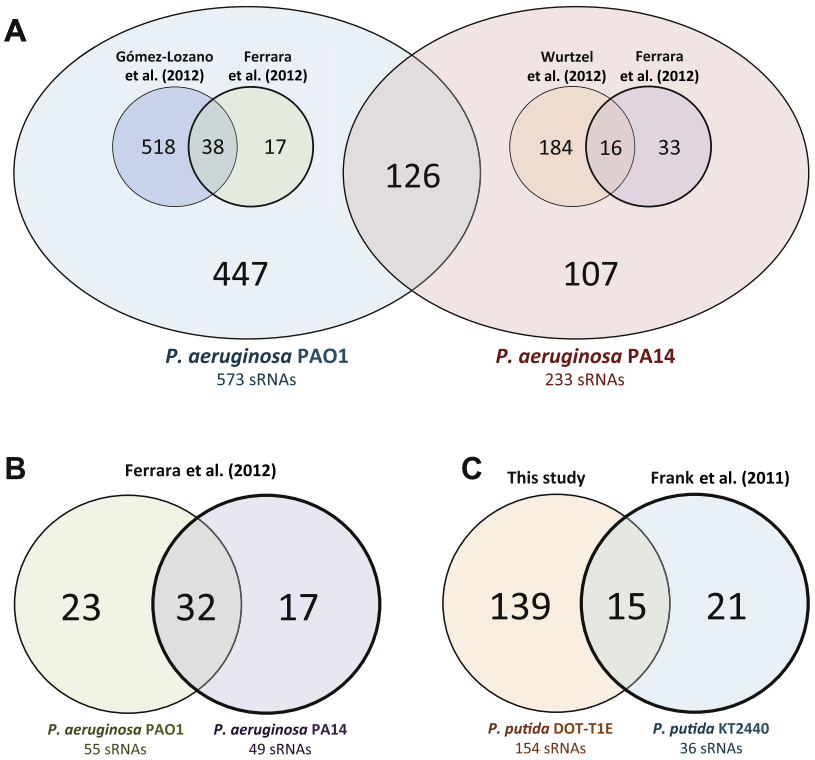

Fig. 3. Venn diagrams showing the number of sRNAs identified in different strains of *P. aeruginosa* and *P. putida*.

similar kind of pattern is observed with the PrrF RNAs, which are encoded as a tandem pair in *P. aeruginosa* (PrrF1–PrrF2), whereas all other sequenced *Pseudomonas* encode the two PrrF RNAs at distant genomic loci. One possible evolutionary explanation is that the original *prrF* gene was duplicated and then moved to a new site, except in the ancestor of *P. aeruginosa* where the duplicated *prrF* gene was lost or did not survive the move, and the original gene then duplicated more recently (Gottesman *et al*., 2007). A similar scenario may explain why *P. aeruginosa* strains only harbor one Crc-binding RNA. *Pseudomonas syringae* DC3000 has a second Crc-regulating sRNA, named CrcX (Filiatrault *et al*., 2013), and *P. putida* KT2440 encodes CrcY, a 368-nt RNA showing 66% identity with CrcZ (Moreno *et al*., 2012). CrcY/CrcX and CrcZ seem to be functionally redundant in these two organisms, as mutation of either gene individually has no effect on CCR, whereas a *crcZcrcY* or a *crcZcrcX* double mutant yields the phenotype expected from loss of Crc inhibition (Moreno *et al*., 2012; Filiatrault *et al*., 2013). We have identified homologues of CrcZ and CrcY in both *P. putida* DOT-T1E and *P. extremaustralis* 14-3b (Fig. 2, Tables S1–2). The CrcZ (RNA16) and CrcY (RNA3) homologues in *P putida* DOT-T1E are encoded in the same intergenic regions as in *P. putida* KT2440, though in a different orientation (Fig. 2). In all *Pseudomonas* species studied so far, all the CrcZ homologues are present in the intergenic region between the *cbrB* and the *pcnB* genes, as reported by Filiatrault *et al*. (2013). There is a sRNA (sRNA23) encoded antisense to the *crcZ* gene homologue (sRNA22) in *P. extremaustralis* 14-3b, which resembles the situation in *P. aeruginosa* PAO1 where the sRNA P30 is also encoded antisense to the *crcZ* gene. Interestingly, there is an additional sRNA encoded downstream of the *crcZ* gene in *P. aeruginosa* strains PAO1 and PA14, reported by Gómez-Lozano *et al*. (2012) and Wurtzel *et al*. (2012) respectively.

Overlap of sRNAs between different strains of the same species

We next investigated the overlap of sRNAs between different strains of the same species (Fig. 3). Taking into account all the studies in *P. aeruginosa*, there are 573 sRNAs known to be expressed in strain PAO1 and 233 sRNAs in strain PA14, of which 126 sRNAs are detected in both strains (Fig. 3A). Table 2 shows the number of sRNAs belonging to the core genome of PAO1 and PA14, representing sRNAs whose sequences are conserved in both strains. Of the 233 sRNAs detected in *P. aeruginosa* PA14, 188 are conserved at the sequence level in *P. aeruginosa* PAO1 (Table 2). Of these 188 sRNAs, 126 (67%) are also expressed in *P. aeruginosa* PAO1. Therefore, there are sRNAs whose sequence is present in both strains but whose expression seems to be strain specific.

Table 2. Number of sRNAs belonging to the core genome of *P. aeruginosa* PAO1 and PA14.

Strain	Study	sRNAs in core genome/total sRNAs detected
P. aeruginosa PAO1	Gómez-Lozano *et al.* (2012) Ferrara *et al.* (2012)	554/573
P. aeruginosa PA14	Ferrara *et al.* (2012) Wurtzel *et al.* (2012)	188/233

When comparing the sRNAs expressed in different *P. putida* strains, we observe the same pattern of strain-specific expression (Fig. 3C), despite that most of the sRNAs detected belong to the core genome of both strains (Table 3). Table S5 contains a list of the sRNAs detected by more than one study in *P. aeruginosa* strains PAO1 and PA14, and the sRNAs detected in both *P. putida* strains KT2440 and DOT-T1E can be found in Table S1. The strain specificity observed in the expression of the core genome sRNAs may be due to the different growth conditions used in the studies that investigated different strains because many sRNAs are only expressed under certain circumstances. However, Ferrara *et al*. (2012) compared the transcriptional response of both strains PAO1 and PA14 under the exact same conditions and found that a substantial number of the detected sRNAs were only expressed in one of the strains (23 intergenic sRNAs were only detected in strain PAO1, and 17 only in strain PA14) (Ferrara *et al*., 2012) (Fig. 3B). Of these intergenic sRNAs, four are in loci unique to PAO1 and seven are in loci unique to PA14. The same extent of strain specificity between *P. aeruginosa* PAO1 and PA14 was also found for the expression of antisense sRNAs (Ferrara *et al*., 2012). The expression of strain-specific sRNAs may also be due to the existence of differences in promoter sequences. However, upon examination of strain-specific antisense sRNAs, it was observed that the predicted antisense promoters were present in both strains even if antisense transcription was only detected in one of them (Gómez-Lozano, Marvig, Tulstrup, *et al*., 2014).

The overlap of intergenic sRNAs between studies focused on the same strain is also surprisingly low. Out of 55 sRNAs detected in strain PAO1 by Ferrara *et al*. (2012), 38 sRNAs were also detected in the same strain in Gómez-Lozano *et al*. (2012) (Fig. 3A). The extent of overlap is lower in the case of the two studies that investigated strain PA14, with only 16 out of 49 sRNAs detected by Ferrara *et al*. (2012) being also detected by Wurtzel *et al*. (2012) (Fig. 3A). This small overlap between the reported sRNAs in the same strain may be caused by the different characteristics of each work, such as the different growth conditions used in each of them, but also the different methods to perform RNA extraction methods and RNA-seq library preparation protocols, as well as differences in sequencing platforms and bioinformatics analyses. Specifically, the library preparation for RNA-seq experiments is known to create considerable bias in the transcripts sequenced. Library preparation requires multiple enzyme-catalysed steps such as sequential oligonucleotide adapter ligations to the 5′and 3 ends of RNAs, reverse transcription (RT) and polymerase chain reaction (PCR). RNA ligase preferences may contribute to the observed bias in sRNA detection (Munafó and Robb, 2010; Hafner *et al*., 2011; Jayaprakash *et al*., 2011; Zhuang *et al*., 2012), as well as the RT reaction and PCR (Taube *et al*., 1998; Sendler *et al*., 2011; Dabney and Meyer, 2012). Recent systematic investigations have revealed method-dependent biases in sRNA quantification, underscoring the importance of library preparation strategy and relative sRNA abundance for successful sRNA detection (Linsen *et al*., 2009; Baker, 2010; Gómez-Lozano *et al*., 2012).

It is important to note that some sRNAs that we have considered to be conserved across different strains of the same species are found as longer or shorter transcripts in one of the strains. One interesting example is the case of the sRNAs pant507 (identified by Gómez-Lozano *et al*., 2012 in *P. aeruginosa* PAO1) and SPA0016 (identified by Ferrara *et al*., 2012 in *P. aeruginosa* PA14). The nucleotide identity between these two sRNAs is higher than 80%, and they are found in the same intergenic region. However, the length of SPA0016 is approximately twice the length of pant507, and it is composed of two tandem sequences very similar to pant507 (Fig. 4).

Conclusion

During the last years, bacterial transcriptomes have been shown to comprise hundreds of previously undetected sRNAs capable of performing diverse regulatory functions. However, despite the broad use of RNA-seq technologies for transcriptome studies, it has only been applied to identify novel sRNAs in three *Pseudomonas* species: *P. aeruginosa, P. putida* and *P. syringae*. In this study, we report the identification of around 150 novel sRNAs in *P. putida* DOT-T1E and *P. extremaustralis*

Table 3. Number of sRNAs belonging to the core genome of *P. putida* KT2440 and DOT-T1E.

Strain	Study	sRNAs in core genome/total sRNAs detected
P. putida KT2440	Frank *et al.* (2011)	30/36
P. putida DOT-T1E	This study	105/154

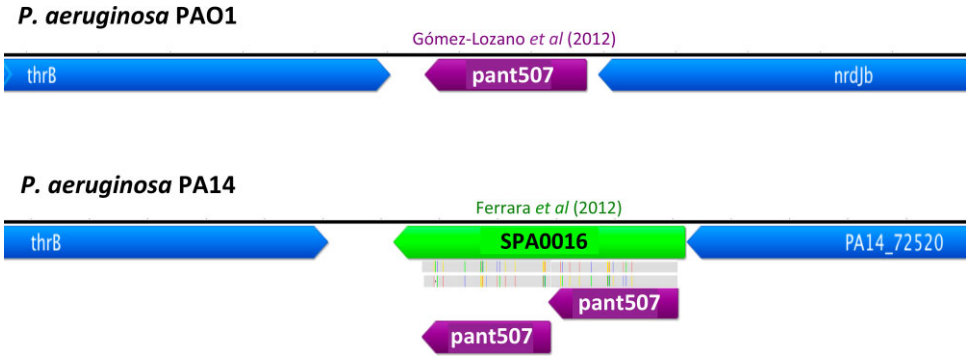

Fig. 4. Homologue sRNAs in *P. aeruginosa* PAO1 and PA14. sRNAs are depicted in purple and green, flanking genes are depicted in blue. Alignment similarities are shown in grey, with mismatches shown in colours.

14-3b respectively. We have also compared all the studies that used RNA-seq to detect expression of sRNAs in *Pseudomonas. Pseudomonas* genomes typically have sizes between 6 and 7 Mbp, and all the *Pseudomonas* genomes included in this study contain around 5500 genes, including a high proportion of regulatory genes. The multitude of sRNAs detected in *Pseudomonas* species suggests the presence of additional layers of regulation, and characterizing their role and their evolution will be highly important for our understanding of these species with such versatile lifestyles and increasing industrial, agricultural, environmental and clinical significance. Bacterial adaptation to new environments involve three levels of regulation: gene regulation that respond to environmental changes, sRNA-associated regulation including evolution of novel sRNA regulatory functions and the acquisition of mutations in regulatory genes which secure fitness for persistent colonization in new niches. Changes in gene regulation in response to new conditions happen in time frames of minutes, while the evolution of new sRNAs and the acquisition of adaptive mutations happen in few to several thousand generations. We here provide information of all sRNAs conserved at the sequence level between the existing studies in *Pseudomonas* species. Our results show that the extent of sequence conservation of sRNAs across different *Pseudomonas* species is very limited, which supports the idea that the evolution of bacterial sRNAs appears to be rapid. In addition, when comparing the sRNAs expressed in different strains of the same species, we observe that many sRNAs exhibit a strain-specific expression, despite the fact that most of these sRNAs belong to the core genome of the strains compared. We anticipate that the results presented here will be important to understand how sRNAs evolve as well as help to characterize the function of the few sRNAs conserved across species based on sequence similarities. However, one limitation of this study is comparing large data sets resulting from different RNA-seq studies because library preparation protocols, sequencing platforms and thresholds for detecting transcripts vary from study to study. A major challenge of transcriptomic studies is how to report reproducibility in order to avoid misleading results when comparing data generated by non-identical methods.

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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:

Table S1. sRNA homologues between *P. putida* and the other *Pseudomonas* species studied.
Table S2. sRNA homologues between *P. extremaustralis* 14-3b and the other *Pseudomonas* species studied.
Table S3. sRNA homologues between *P. aeruginosa* and the other *Pseudomonas* species studied.
Table S4. sRNA homologues between *P. syringae* DC3000 and the other *Pseudomonas* species studied.
Table S5. sRNA homologues between *P. aeruginosa* strains PAO1 and PA14.
Table S6. sRNAs homologues expressed in all four species.
Appendix S1. Experimental procedures.