
Tet-Trap, a genetic approach to the identification of bacterial RNA thermometers: application to *Pseudomonas aeruginosa*

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ABSTRACT

Modulation of mRNA translatability either by *trans*-acting factors (proteins or sRNAs) or by *cis*-acting riboregulators is widespread in bacteria and controls relevant phenotypic traits. Unfortunately, global identification of post-transcriptionally regulated genes is complicated by poor structural and functional conservation of regulatory elements and by the limitations of proteomic approaches in protein quantification. We devised a genetic system for the identification of post-transcriptionally regulated genes and we applied this system to search for *Pseudomonas aeruginosa* RNA thermometers, a class of regulatory RNA that modulates gene translation in response to temperature changes. As *P. aeruginosa* is able to thrive in a broad range of environmental conditions, genes differentially expressed at 37°C versus lower temperatures may be involved in infection and survival in the human host. We prepared a plasmid vector library with translational fusions of *P. aeruginosa* DNA fragments (PaDNA) inserted upstream of TIP2, a short peptide able to inactivate the Tet repressor (TetR) upon expression. The library was assayed in a streptomycin-resistant merodiploid *rpsL*⁺/*rpsL31* *Escherichia coli* strain in which the dominant *rpsL*⁺ allele, which confers streptomycin sensitivity, was repressed by TetR. PaDNA fragments conferring thermosensitive streptomycin resistance (i.e., expressing PaDNA–TIP2 fusions at 37°C, but not at 28°C) were sequenced. We identified four new putative thermosensors. Two of them were validated with conventional reporter systems in *E. coli* and *P. aeruginosa*. Interestingly, one regulates the expression of *ptxS*, a gene implicated in *P. aeruginosa* pathogenesis.

Keywords: bacterial riboregulators; *Pseudomonas aeruginosa*; *Escherichia coli*; PA5194; *ptxS*; *lpxT*

INTRODUCTION

Bacteria modulate gene expression in response to a variety of chemical and physical signals in order to cope with the challenges posed by a changing environment. Temperature is one of the main physical parameters influencing bacterial growth because it affects both enzymatic reaction rate and macromolecules state. Thus, it is not surprising that bacteria have evolved complex regulatory networks to face sub-lethal temperature changes. In *Escherichia coli*, for instance, both a heat-shock and a cold-shock response involving changes in the expression of tens of genes have been described (Phadtare 2004; Guisbert et al. 2008). On the other hand, a modest temperature increase, within the range of permissive growth temperatures for mesophilic bacteria, is used by some bacterial pathogens as a signal of warm-blooded host invasion to trig-

ger the expression of virulence genes (for review, see Konkel and Tilly 2000).

To either avoid irreversible cell damage or establish a successful host infection, the speediness of the response to a sudden temperature variation is critical, thus posing the problem of fast and precise thermosensing. Bacteria exploit different macromolecules as molecular thermosensors (Klinkert and Narberhaus 2009); in particular, RNA thermometers (RNATs) have been found to control the expression of a variety of heat shock and virulence genes in Gram-negative and Gram-positive bacteria (for review, see Waldminghaus et al. 2005; Kortmann and Narberhaus 2012). An RNAT can be described as a thermolabile secondary structure that sequesters the translation initiation region (TIR) of the

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mRNA at low temperature. Local denaturation due to temperature increase allows ribosome binding and mRNA translation. On this very general theme, which applies to most RNATs described so far, variations of length and localization relative to the AUG of the regions involved in the thermometer structure have been found. For instance, the *E. coli rpoH* thermosensor, for which the definition of RNA thermometer was originally proposed, encompasses RNA regions well within the coding sequence, whereas a widely disseminated class of RNATs like ROSE (Repression Of heat Shock genes Expression) elements form complex secondary structure within the 5' UTR of the mRNA (Narberhaus et al. 1998; Morita et al. 1999a,b; Nocker et al. 2001). Moreover, some RNATs, such as those controlling the expression of bacteriophage λ *cIII* or *E. coli cspA*, rely on the temperature-dependent formation of alternative secondary structures, instead of the simple melting of an unstable one (Altuvia et al. 1989; Giuliadori et al. 2010).

Only two classes of RNATs with common structural themes have been defined so far, ROSE and FourU elements; in both cases, sequence conservation is limited within each class to very short stretches of 4–5 bases in the proximity of the Shine–Dalgarno (SD) region (Waldminghaus et al. 2005, 2007b). Poor sequence conservation hampers the bioinformatic identification of RNATs, which should mainly rely on structural properties (Waldminghaus et al. 2007a); however, the available bioinformatic tools usually overlook non-Watson–Crick interactions that seem to play a relevant role in temperature sensing by some RNATs (Chowdhury et al. 2006). Moreover, unique RNATs, completely unrelated to both ROSE and FourU elements, have been found in different groups of bacteria (Cimdins et al. 2014; for review, see Kortmann and Narberhaus 2012) making it virtually impossible, at the moment, to perform an exhaustive prediction of RNATs by bioinformatic search for conserved structures. Recently, the RNATips (temperature-induced perturbation of structure) web server was presented. It predicts clusters of nucleotides with temperature-sensitive pairing within an RNA sequence (Chursov et al. 2013).

We present here a genetic approach, henceforth called Tet-Trap, to identify RNATs. We have applied Tet-Trap to *Pseudomonas aeruginosa*, for which only a ROSE-like RNAT controlling the expression of the small heat shock protein IbpA has been reported so far (Krajewski et al. 2013). *Pseudomonas aeruginosa* is a Gram-negative, mesophilic bacterium, endowed with a remarkable metabolic versatility reflected by a large genome (Stover et al. 2000). It can infect hosts as diverse as worms, flies, and mammals. In humans, it behaves as an opportunistic pathogen and it is responsible for a variety of serious nosocomial infections (Driscoll et al. 2007). As *P. aeruginosa* is a facultative pathogen, it is conceivable that it can exploit body temperature as a signal for activating the expression of virulence genes specifically required during infection of the warm-blooded host. In fact, a recent transcriptomic survey by RNA-seq of *P. aeruginosa* grown at

28°C and 37°C detected genes preferentially expressed at the body temperature of the mammalian host, among which virulence genes were significantly enriched (Wurtzel et al. 2012). Using Tet-Trap we identified four genes post-transcriptionally regulated by a temperature upshift from 28°C to 37°C. Interestingly, one of them, namely *ptxS*, encodes a protein previously implicated in *P. aeruginosa* virulence (Colmer and Hamood 1998).

RESULTS

The Tet-Trap genetic tool

Tet-Trap is a genetic tool aimed at identifying post-transcriptionally regulated genes, based on the assumption that the *cis*-acting determinant of regulation is located within the 5' UTR and the 5' translated region of the regulated ORF (5'-UTR-TR). In principle, it is made up of three components: a signaling, a sensor, and a reporter system (Fig. 1A). In the signaling system, a sequence encoding a small peptide (TIP2, see below) is fused in frame with the 5'-UTR-TR region of an ORF. Thus, expression of TIP2 as the C-terminus of a fusion protein depends on the ORF translation regulation mechanism. The sensor system exploits the *Tn10 tetA* gene regulatory circuitry to control the expression of the reporter system. In the absence of tetracycline, *tetA* transcription from the *tetAp* promoter is prevented by TetR repressor, which binds to the operator *tetO*; tetracycline acts as the inducer of *tetA* by binding TetR and causing its dissociation from the cognate operator. TIP2 is a gratuitous inducer that mimics the tetracycline effect on TetR, as it leads to repressor dissociation from *tetO*. TIP2 retains this property in end-fused chimeric polypeptides (Goeke et al. 2012). For the reporter system, various reporter genes may be cloned under *tetO*–*tetAp* so that reporter expression depends on and correlates with TIP2 translation.

We constructed two *E. coli* strains with different reporter genes to either select for or counter-select against cells expressing TIP2-tagged polypeptides (Fig. 1B). In these strains, a reporter cassette constituted by the *Tn10 tet* regulatory region (*tetRp*–*tetO*–*tetAp*) (Bertrand et al. 1983) controlling transcription of the reporter gene (under *tetAp* promoter) was inserted in the chromosome. Because the gene for *tetR* is also integrated in the chromosome and constitutively expressed by the *Pcat* promoter in these reporter strains, transcription of the reporter genes is normally switched off. Repression by TetR can be relieved by TIP2, which leads to reporter gene expression. For positive selection of TIP2-expressing cells (strain C-5907), we used the *aadA*:GFP gene as a reporter. It encodes a chimeric protein conferring spectinomycin resistance and green fluorescence (Rizzi et al. 2008). The strain for negative selection against TIP2 expression (C-5920) harbours the streptomycin-resistant *rpsL31* recessive allele and, as reporter cassette, the *rpsL*⁺ allele, which confers streptomycin sensitivity to the otherwise resistant

TABLE 1. Features of the *P. aeruginosa* genomic library

Library size ^a	2.8 × 10 ⁶
<i>p</i> ^b	>99%
Translational fusions ^c	4.8 × 10 ⁴

^aThe total number of clones constituting the genomic library, as estimated by plating an aliquot of the transformants on chloramphenicol. Of note, 2.0 × 10³ CamR clones were obtained in the control ligation (with vector only).

^b*P* is the probability of any DNA sequence of being included in the library according to the equation $N = \ln(1 - P) / \ln(1 - f)$, where *N* is the number of recombinant clones and *f* is the fractional proportion of the genome in a single recombinant, considering an average length of 500 bp and a genome size of 1.3 × 10⁷ bp approximately (the sum of the PAO1 and PA14 genomes that were considered to be unrelated in this calculation).

^cClones growing on spectinomycin and IPTG.

genes translationally coupled to other genes; (3) genes whose translation requires positive regulators absent in *E. coli*; and (4) genes that are positively regulated by small molecules absent in the experimental conditions of the screening. Moreover, constructs encoding toxic fusion proteins would also have been lost.

Application of Tet-Trap to the identification of putative *P. aeruginosa* RNA thermometers

In the *rpsL*⁺ reporter strain (C-5920), the presence of an RNAT in the DNA fragment cloned in frame with ST-TIP2 should result in thermosensitive streptomycin resistance. Thus, no growth should be observed upon transformation of such strain (C-5920) with the translation fusion library on streptomycin plates at 37°C. Conversely, by plating the transformants in the presence of the antibiotic at 28°C, one should expect to select for clones carrying *P. aeruginosa* inserts translationally silent under these conditions and, thus, enrich for putative RNATs. However, upon transformation with the library, we observed a high background of StrR

clones at both 28°C and 37°C, as the plating efficiency on streptomycin was only ~250-fold lower than in the absence of selection. To identify clones carrying putative thermosensors, we screened 1152 StrR transformants grown at 28°C by replica plating on streptomycin and IPTG at 28°C and 37°C. We isolated 16 clones exhibiting thermosensitive, IPTG-dependent streptomycin resistance. The clones carried 12 different *P. aeruginosa* DNA inserts, as determined by sequencing (Table 2 and data not shown). Four clones contained the 5' UTR and 5' end of ORFs in frame with ST-TIP2. In particular, codons 1–53 of *ptxS* (clone #2), 1–49 of PA5194 (#3), and 1–34 of *dsbA* (#4) were fused with ST-TIP2. In clone #1, the cloned ORF is annotated as an intergenic region in the *Pseudomonas* database, but as it is in frame with the downstream PA1031, it could actually represent the 5' end of this gene. Concerning the other fusions, three carried internal fragments of annotated ORFs in frame with ST-TIP2, and in the other five clones the ORFs in frame with ST-TIP2 overlapped with annotated ORFs in a different frame or in antisense orientation (data not shown). It is possible that regulatory sites may actually lie within the internal portion of an ORF. However, as RNATs usually map in the 5' UTR and the first part of the coding region of the genes, we focused on clones carrying such regions fused with ST-TIP2.

We estimated the efficiency of plating (e.o.p.) at 37°C versus 28°C of cultures carrying the constructs 1–4 (Fig. 2B). The *ptxS* fusion showed the strongest effect, with a ≥10⁵ e.o.p. reduction at 37°C on plates supplemented with streptomycin and IPTG; for PA1031 and *dsbA*, the e.o.p. was reduced ~100-fold. Finally, for PA5194, the e.o.p. was reduced ~10-fold and the colonies were very small at 37°C. All strains showed comparable e.o.p. at the two temperatures in the absence of either streptomycin or IPTG. This indicates (1) that the constructs do confer thermosensitive streptomycin resistance and not a generic thermosensitive phenotype, and (2) that the increase in ST-TIP2 expression was not due to temperature-dependent activation of promoters

TABLE 2. *Pseudomonas aeruginosa* regions carrying putative thermosensors

Insert	No. ^a	PAO1 coordinates ^b	Genome annotation ^c	ORF in the construct in frame with ST-TIP2 ^d
1	4	1117570–1117919	PA1030.1 (+; 1)-IR	264 bp at the 5' end of an ORF not annotated in Pseudomonas.com database; the ORF is in frame with the downstream PA1031 (+; 4) ORF
2	1	2487532–2488014	IR- <i>ptxS</i> (+; 1)	Codons 1–53 of <i>ptxS</i>
3	1	5846939–5847277	<i>yrjI</i> (–; 2) -IR-PA5194 (+; 4)	Codons 1–49 of PA5194
4	1	6181479–6181766 ^e	<i>cc4</i> (–; 2)-IR- <i>dsbA</i> (–; 2)	Codons 1–34 of <i>dsbA</i>

^aNumber of sequenced clones carrying the insert.

^bCoordinates refer to GenBank accession number NC_002516.

^cIntergenic regions (IR) and genes (partially) overlapping *Pseudomonas* regions cloned upstream of ST-TIP2 are indicated in the same order in which they are in the corresponding construct (from *Ptac* to ST-TIP2). For the genes, the strand and the confidence rating assigned to the predicted gene function are reported in brackets. 1, genes of known function in *P. aeruginosa*; 2, similarity with well-characterized genes from other bacteria; 4, unknown function (Stover et al. 2000) (www.pseudomonas.com).

^dThe length of the longest predicted ORF in frame with ST-TIP2 is indicated. Whenever an annotated ORF is in frame with ST-TIP2, the region from the initiation codon is reported.

^eRegion cloned upstream of ST-TIP2 in inverted orientation.

in the cloned regions, as it depends on *P_{tac}* induction. This was confirmed by Northern blotting analysis of the transcription pattern of the four constructs at 28°C and 37°C. In all four cases we did not observe any relevant difference in the transcription pattern at the two temperatures (data not shown). On the whole, this analysis confirmed that the expression of the TIP2-tagged peptides 1–4 was post-transcriptionally activated by the temperature upshift.

Validation of the PA5194 and *ptxS* RNA thermosensors in *E. coli* and *P. aeruginosa*

To validate the results of the Tet-Trap, we analyzed translational fusions of two putative thermosensors, i.e., those identified in constructs #2 (*ptxS*) and #3 (PA5194), with conventional reporter genes. For both *ptxS* and PA5194 two constructs each were generated, one carrying the entire *P. aeruginosa* regions originally found in the #2 and #3 ST-TIP2 plasmids, respectively, and the other with the same 3' end of the #2 and #3 inserts, but starting with the first nucleotide transcribed from the endogenous PA5194 or *ptxS* promoters in *P. aeruginosa* (Fig. 3A; Dotsch et al. 2012). As the 5' end of the PA5194 mRNA was not known, we first mapped it by primer extension at 25°C and 37°C and found a single signal of comparable intensity at the two temperatures at position 5847081, 50 nt upstream of the ORF start codon (Fig. 3B).

Two different reporter genes were used in the constructs. In particular, for the *ptxS* fusions we exploited the *bgaB* gene, which encodes a thermostable β -galactosidase (Hirata et al. 1984) and has been already used for the analysis of RNATs (Waldminghaus et al. 2007a; Klinkert et al. 2012). To validate PA5194, which is predicted to encode a membrane protein with a signal peptide at the N-terminus, we cloned the PA5194 fragments in frame with sfGFP, a GFP variant which, unlike β -galactosidase (Oliver and Beckwith 1981), is known to be functional in the periplasm upon translocation across the membrane (Dinh and Bernhardt 2011). β -Galactosidase activity or fluorescence of the chimeric proteins were then monitored at different temperatures. As control, fusions of the 5' UTR and the first nine codons of the *E. coli* *recA* gene with *bgaB* or sfGFP were assayed. All constructs were cloned in the *E. coli*-*P. aeruginosa* shuttle vector pGM931 under the *araBp* promoter. The expression of the reporter genes

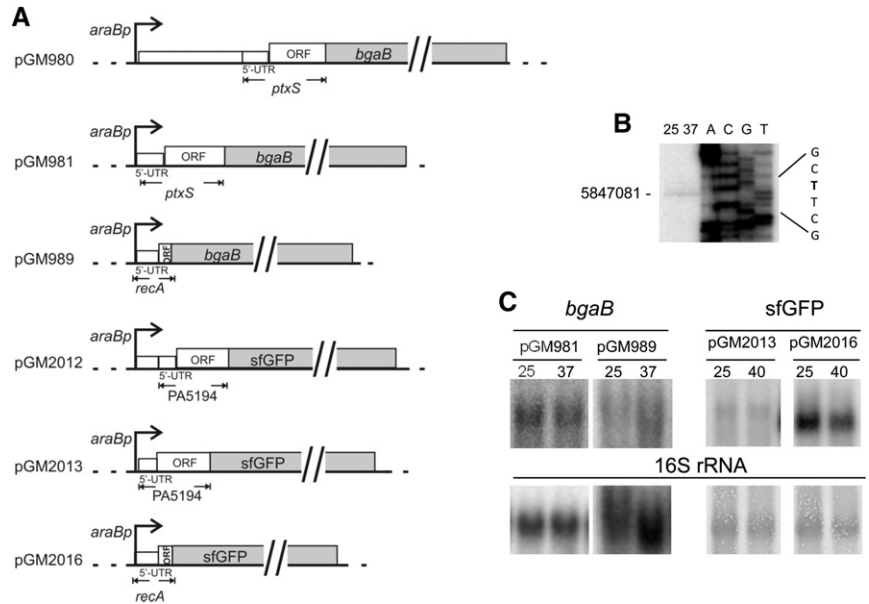


FIGURE 3. Constructs used for validation of putative RNATs and transcriptional analysis. (A) Map of plasmids encoding PtxS-BgaB and PA5194-sfGFP. Control plasmids carrying the 5' end of the *E. coli* *recA* gene in frame with the reporter genes are also represented. Details about plasmid construction and coordinates of the cloned regions are reported in Materials and Methods. *Pseudomonas aeruginosa* and *E. coli* 5' UTRs and ORFs are drawn to scale. (Dotted lines) vector sequences; (bent arrows) *araBp* promoter; (empty boxes) *P. aeruginosa* and *E. coli* DNA; (gray boxes) reporter genes. (B) Mapping the 5' end of the PA5194 mRNA by primer extension. Primer extension was performed with the radiolabeled oligo 3003 on 30 μ g of RNA extracted from exponential cultures of PAO1 grown at 25°C and 37°C. The same oligonucleotide was used for Sanger-sequencing of construct 3. The coordinate of the identified 5' end and the sequence context (in bold the 5' end T) are reported beside the panel. (C) Northern blot analysis of *ptxS*-*bgaB* and PA5194-sfGFP fusions in *P. aeruginosa*. Cultures of the PAO1 strain carrying the plasmids indicated above the lanes were grown at 25°C up to OD₆₀₀ = 0.5. The cultures were induced with 0.1% arabinose, split and further incubated at the temperature indicated above the lanes for 45 min. Northern blotting was performed as described in Materials and Methods after electrophoresis in a 1.5% denaturing agarose gel. The filter was hybridized with oligonucleotides specific for *bgaB* or sfGFP, as indicated on top of the panels. To check gel loading, the filters were either hybridized with the 16S rRNA-specific 1396 oligonucleotide (*bgaB* panels) or stained with methylene blue before the hybridization (sfGFP panels; the bands corresponding to 16S rRNA are shown). The transcript size was roughly estimated by comparison with rRNA migration as follows: pGM981 and pGM989, 2.5 kb; pGM2013, 1.2 kb; pGM2016, 1.1 kb.

was monitored in *E. coli* DH10B cultures grown at 28°C and 42°C upon transcription induction with arabinose (Tables 3 and 4). β -gal expression in the presence of the *ptxS*-*bgaB* fusions was strongly temperature-dependent in *E. coli*, as cultures carrying the long (pGM980) or the short (pGM981) construct showed >20- and 10-fold increases, respectively, in enzymatic activity at 42°C. Conversely, BgaB activity in the *recA* control plasmid (pGM989) increased only twofold. It should be noted that β -galactosidase expression by the long pGM980 construct was much lower at both temperatures than by the short construct pGM981 (Table 3). Reduced reporter expression by the longer fusion could be due to decreased efficiency of translation and/or stability of the mRNA transcribed from *araBp*, which bears at the 5' end a sequence absent in the *P. aeruginosa* *ptxS* mRNA. We then assayed the expression of the *ptxS*-*bgaB* fusion by pGM981 in the *P. aeruginosa* strain PAO1 at 25°C and 37°C. We

TABLE 3. *ptxS* RNAT validation

	<i>E. coli</i> ^a			<i>P. aeruginosa</i> ^a		
	28°	42°	IF ^b	25°	37°	IF ^b
pGM980	3	69	23.3 ± 2.4	nt	nt	na
pGM981	61	827	12.6 ± 1.2	145	787	5.2 ± 0.2
pGM989	855	2115	2.6 ± 0.2	3447	4853	1.4 ± 0.4

^a*bgaB* activity (Miller units) measured as described in Materials and Methods in *E. coli* DH10B and *P. aeruginosa* PAO1 carrying the indicated plasmids. The results of typical experiments are reported. (nt) not tested.

^bInduction Factor of *bgaB* activity at high versus low temperature. The values are the average of two independent determinations. (na) not applicable.

performed the experiments at lower temperatures than in *E. coli* to mimic the temperature upshift due to infection of a mammalian host. We found that *ptxS-bgaB* expression was also clearly thermoregulated in *P. aeruginosa*, as it showed a more than fivefold higher activity at 37°C, whereas *recA-bgaB* expression by the pGM989 control plasmid was not significantly affected by the temperature upshift (Table 3).

For PA5194-sfGFP, we found a sharp increase in the fluorescence of the fusion proteins by both the long and the short constructs at 42°C in *E. coli*, whereas the RecA-sfGFP fluorescence intensity did not increase with temperature (Table 4). In *P. aeruginosa*, a 3.5-fold increase in signal strength was observed at 37°C versus 25°C. An upshift of the temperature to 40°C further increased PA5194-sfGFP expression, whereas RecA-sfGFP expression increased twofold at both 37°C and 40°C (Table 4). Thermoregulated expression of PA5194-sfGFP was confirmed by direct visualization of the cells by fluorescence microscopy (Fig. 4). On the whole, these data show that the *ptxS* and PA5194 regions encoded by the Tet-Trap constructs confer thermoregulated expression to the reporter genes, thus indicating that the determinants of this regulation lie in the cloned portion of the two *P. aeruginosa* genes. Moreover, since the levels of the transcripts of the reporter constructs did not change with temperature, as assessed by Northern blotting (Fig. 3C), translation initiation is the most likely target of this regulation.

To check whether the results obtained with the translational fusions applied also to the full-length *ptxS* and PA5194 proteins, we analyzed the expression in *P. aeruginosa* of variants of the two genes with an HA tag inserted immediately before the stop codon. In agreement with the data obtained with the reporter constructs, the expression of the full-length PtxS and PA5194 proteins was barely detectable at 25°C and strongly induced at higher temperatures (Fig. 5A). Conversely, *ptxS* transcript was expressed at 25°C and increased two- to threefold at temperature ≥37°C, whereas PA5194 mRNA was equally expressed at all the tested temperatures (Fig. 5B). Similar expression profiles were shown also by the endogenous PA5194 and *ptxS* mRNAs (i.e., transcribed from the *P. aeruginosa* chromosome) (Fig. 5C).

Analysis of *ptxS* and PA5194 translation initiation regions' secondary structures

The structure of the first 137 and 126 nt of the *ptxS* and PA5194 mRNAs was probed at different temperatures (25°C–37°C–42°C) by partial digestion with the RNases T1 and A, which cut at the 3' of unpaired guanines and pyrimidines, respectively, and with lead acetate, which cleaves the RNA within single-stranded regions. The results are shown in Figure 6A.

No cuts were observed in the *ptxS* RNA region from nucleotides 30 to 40 at any temperature, whereas the downstream region of the probed RNA was more accessible even at 25°C. Mfold (Zuker 2003) analysis of the transcript predicted several alternative secondary structures with comparable free energy (Fig. 6B and data not shown), among which the structure presented in Figure 6B is in good agreement with the experimental data. The structure consists of two stem-loops (SL1, U18-A40, and SL2, G66-U84) separated by an unstructured region. SL1 is predicted to be stable (ΔG –10.40 kcal/mol), whereas SL2, which involves the GUG start codon, should be relatively unstable (ΔG –5.6 kcal/mol). Moreover, the RNATips program (Chursov et al. 2013) predicted a cluster of nucleotides with thermosensitive pairing in a region overlapping SL2 (Fig. 6C). Indeed, several positions in SL2 (G at positions 66, 67, 73, 74, 78, 84, and 85) were cut by RNase T1 only when the temperature was increased to 37/

TABLE 4. PA5194 RNAT validation

	<i>E. coli</i> ^a			<i>P. aeruginosa</i> ^a				
	28°	42°	IF _{42/28} ^b	25°	37°	IF _{37/25} ^b	40°	IF _{40/25} ^b
pGM2012	290	2603	10.0 ± 1.5	nt	nt	na	nt	na
pGM2013	548	2722	5.6 ± 1.0	419	1366	3.5 ± 0.4	2067	4.9 ± 0.03
pGM2016	128,235	69,913	0.5 ± 0.04	31,083	63,969	2.2 ± 0.1	62,919	2.0 ± 0.1

^asfGFP activity (arbitrary units) determined by Tecan Reader as described in Materials and Methods. The results of a typical experiment with *E. coli* DH10B and *P. aeruginosa* PAO1 carrying the reported plasmids are reported. (nt) not tested.

^bInduction Factor of sfGFP activity at high versus low temperature. The values are the average of at least two independent determinations. (na) not applicable.

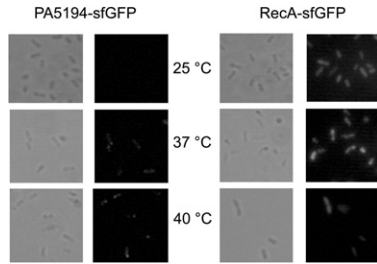


FIGURE 4. Temperature-dependent expression of PA5194-sfGFP by *P. aeruginosa* cells. Images of PAO1 cells carrying plasmids pGM2013 (PA5194-sfGFP) or pGM2016 (RecA-sfGFP) were acquired on a Leica fluorescence microscope. The cells were sampled from cultures grown at the temperatures indicated between the panels as described in Materials and Methods. The same viewfield was captured in contrast phase and fluorescence imaging mode.

42°C (Fig. 6A), suggesting that SL2 can be in equilibrium between an open and a folded conformation and that the temperature upshift may promote unfolding.

For PA5194, we found that the temperature upshift had a modest effect on the accessibility of the RNA to RNases and lead acetate (Fig. 6A). Interestingly, upon degradation with lead acetate and RNase A, increased reactivity at several positions was detected at 42°C, but not at 37°C. This observation is in agreement with in vivo data that showed an effect on PA5194-sfGFP expression by increasing the temperature from 25°C to 37°C and again upon a further increase at 40°C (Table 4). Also for this mRNA, the RNATips analysis suggests that a cluster of residues with thermosensitive pairing overlaps the translation initiation region (Fig. 6C).

The centroid structure calculated at 25°C by RNAfold (Ding et al. 2005) predicts that PA5194 translation initiation region may be involved in the formation of an unstable stem-loop. In particular, three guanidines in the putative Shine-Dalgarno sequence (nucleotides 36–41) should pair with a stretch of uridines (anti-SD) preceding the AUG start codon. The AUG is also predicted to be involved in pairing within the stem (Fig. 6B). Since the results of PA5194 mRNA structural probing were compatible with different predicted secondary structures of the transcript, we decided to test in vivo whether the putative anti-SD could actually play a role in PA5194 regulation. We replaced the three anti-SD uridines (47–49 in Fig. 6B) with either three cytidines (2013CCC mutation) or three adenosines (2013AAA) in the backbone construct pGM2013. The two mutations should either strengthen (2013CCC) or destabilize (2013AAA) the SD-anti-SD pairing and thus have opposite effects on PA5194-sfGFP expression. In particular, the mutated stem-loop in 2013CCC is predicted to have a free energy of -10.38 kcal/mol and could prevent translation also at high temperature (Neupert et al. 2008). In agreement with the bioinformatics prediction, we found that PA5194-sfGFP expression by 2013CCC was repressed in *E. coli* even at 42°C (Fig. 7). Conversely, the 2013AAA mutation led to an increase in fluorescence at all temperatures. In particular, the fluorescence

reached by the wild-type construct at 40°C–42°C was already shown by the mutant at 35°C–37°C. Overall, these data are compatible with a role of the predicted SD-anti-SD interaction in PA5194 regulation.

DISCUSSION

In this work we identified two *P. aeruginosa* genes, namely *ptxS* and PA5194, whose translation is regulated in response to temperature changes. The 5' UTRs of the two genes confer thermoregulated expression to reporter genes in such distantly related bacteria as *E. coli* and *P. aeruginosa*, thus

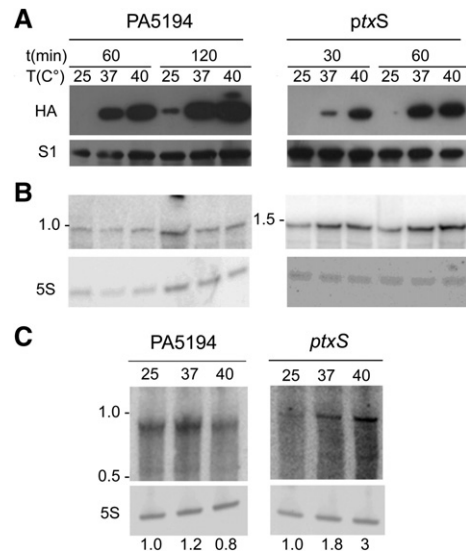


FIGURE 5. Temperature-dependent expression of PA5194 and *ptxS* in *P. aeruginosa*. Proteins (A) and RNA (B) were extracted upon induction with 0.1% arabinose, as detailed in Materials and Methods, from cultures of PAO1 carrying either pPA5194-HA (left) or pPtXS-HA (right). Time and temperature of incubation are indicated above the lanes. (A) Proteins were separated by 10% SDS-PAGE, blotted onto an Hybond ECL filter, and hybridized with HA- or S1-specific antibodies, as indicated beside the panels. No bands were detected in the lanes loaded with not induced samples (data not shown). The PA5194-HA (expected MW, 33.2 kDa) and PtxS-HA (expected MW, 39.9 kDa) migrate in this kind of gels slightly slower than the 28 and 36 kDa bands of the PageRuler Plus Prestained Ladder (ThermoScientific), respectively. (B) The RNA was run on a 5% denaturing urea-polyacrylamide gel, blotted onto a nylon Hybond N membrane and hybridized with either the radiolabeled HA oligonucleotide (PA5194) or the PTXS riboprobe. Migration of the 1.0 and 1.5 kb bands of RiboRuler High Range RNA ladder (ThermoScientific) are reported beside the panels. (C) Northern blot analysis of PA5194 and *ptxS* endogenous mRNAs. Cultures of PAO1 were grown overnight in LD at 37°C and diluted to $OD_{600} = 0.2$ in three flasks containing LD. The flasks were incubated at 25°C–37°C–40°C with agitation up to $OD_{600} = 0.8$. Ten milliliters of samples were taken for RNA extraction. Twenty micrograms of RNA were run on a 5% denaturing urea-polyacrylamide gel, blotted onto a nylon Hybond N membrane, and hybridized with either the radiolabeled PA5194 or the PTXS riboprobes. Migration of the 1.0- and 0.5-kb band of RiboRuler High Range RNA ladder (ThermoScientific) is reported on the left of the panels. The numbers below the panels represent relative expression (RE) of each mRNA with respect to its expression at 25°C. 5S rRNA signals were used for normalization.

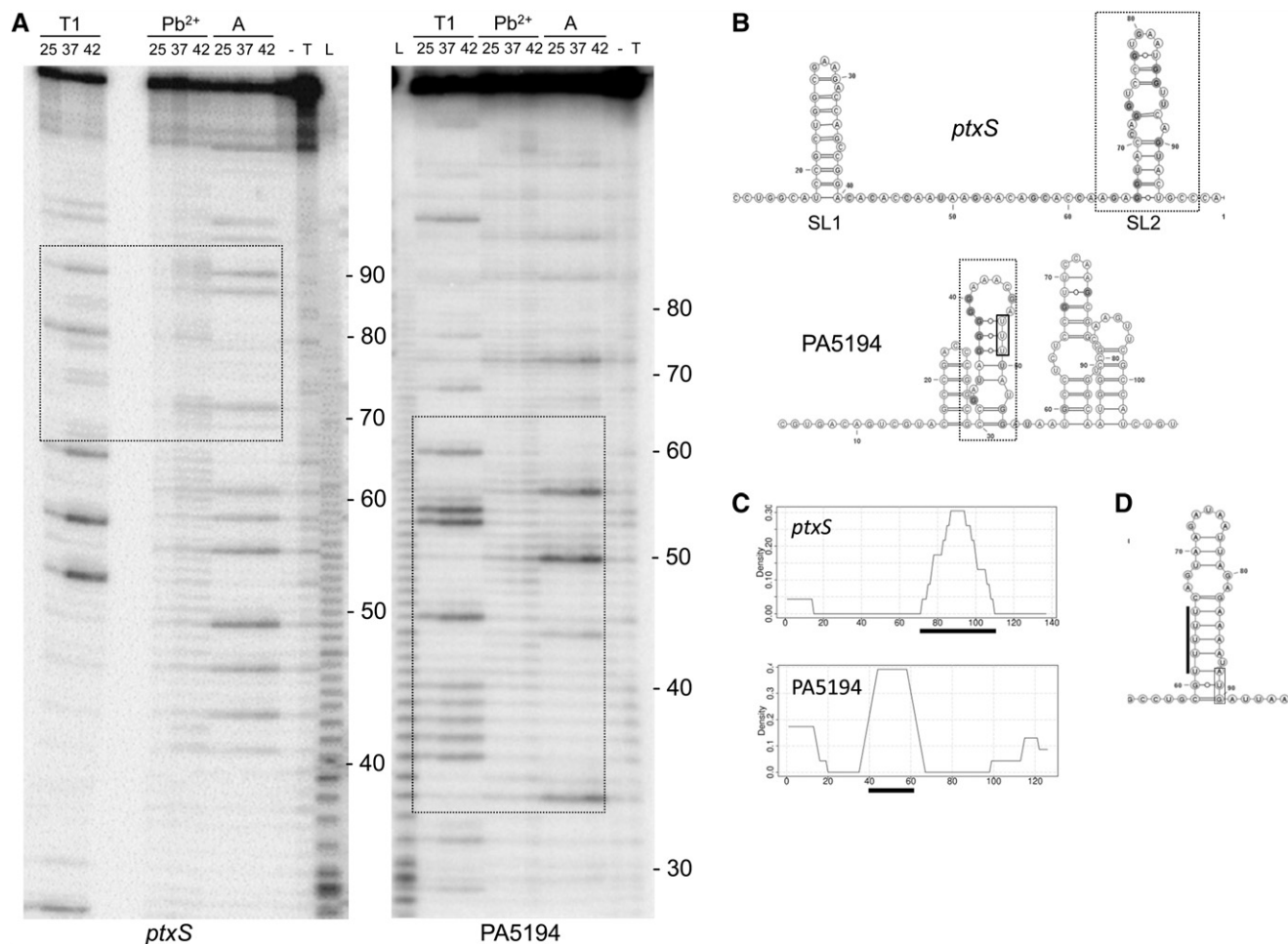


FIGURE 6. In vitro and in silico analysis of the secondary structure of the *ptxS* and PA5194 5' UTRs. (A) Structural analysis of in vitro transcribed *ptxS* (left) and PA5194 (right) RNA with RNase T1 (T1), lead acetate (Pb²⁺) and RNase A (A) at different temperatures (indicated above the lanes in degrees Celsius). (-), *ptxS* or PA5194 RNA incubated at 37°C in the absence of RNases or lead acetate; (T) *ptxS* or PA5194 RNA digested with RNase T1 under denaturing conditions; (L) alkaline digestion of the PA5194 transcript. The dotted boxes encircle regions predicted to fold into secondary structures (shown in panel B) that involve the TIRs of the two RNA molecules. (B) Structure models of *ptxS* and PA5194 with probing results. Cleavage sites are indicated as full gray circles; in particular, positions showing thermo-dependent cleavage are indicated in dark gray. Stem-loops encompassing the SD and start codons of the two genes are enclosed in dotted boxes. The *ptxS* GUG start codon encompasses bases 78–80, the PA5194 AUG start codon bases 51–53. The three U's preceding the AUG in PA5194 that are mutated in pGM2013AAA and pGM2013CC are boxed. (C) Output of the RNATips analysis of the two RNAs showing the density plot of positions along the *ptxS* (upper panel) or PA5194 (lower panel) sequences whose pairing probability is significantly affected by a temperature change from 28°C to 42°C. (D) Structure model of the *E. coli lpxT* TIR. The secondary structure was predicted with the software RNAfold. The start codon of the ORF is boxed; the black bar flanks the five uridine nucleotides involved in the pairing with the TIR. RNA secondary structures in B and D were drawn with the VARNA applet (Darty et al. 2009).

making it unlikely that specific factors acting in trans may be responsible for this regulation. In fact, no ortholog of the *ptxS* gene is present in *E. coli* and no significant sequence similarity exists between the 5' UTRs of PA5194 and its probable orthologous gene *lpxT* (see below). We thus propose that two new RNATs may regulate the translation of *ptxS* and PA5194 in *P. aeruginosa*.

Transcriptional analysis of full-length *ptxS* and PA5194 (Fig. 5) supports this view. In fact, in spite of strong repression of the two proteins at low temperature, their mRNAs are expressed at 25°C and do not (PA5194) or modestly (*ptxS*) increase at higher temperatures. This is unusual for many (most?) transcripts, as efficiently translated mRNAs

are generally more stable than untranslated ones (for review, see Dreyfus 2009), but it has already been reported for other RNATs (Johansson et al. 2002; Kortmann et al. 2011) suggesting that, at low temperature, untranslated mRNAs bearing RNATs may be protected from degradation. This would provide the cells with a pool of translationally silent mRNAs that could be rapidly activated in response to a sudden temperature increase.

No structural similarity of the *ptxS* and PA5194 mRNAs was found with known RNATs after analysis with Mfold (Zuker 2003). However, in vitro probing of the 5' end of the *ptxS* mRNA suggested a secondary structure and a possible mechanism for temperature-dependent translation

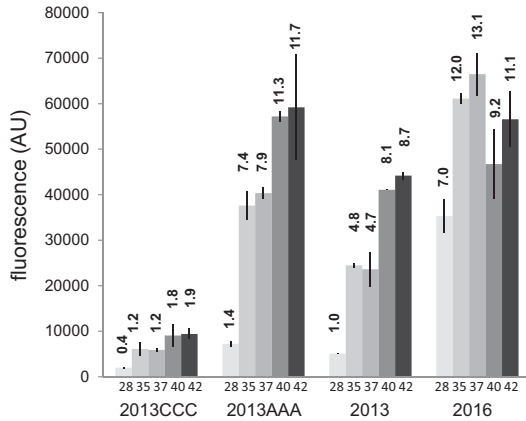


FIGURE 7. Effect of point mutations on PA5194-sfGFP thermoregulation. Cultures of *E. coli* DH10B carrying plasmids pGM2013 (2013) and its mutated derivatives pGM2013CCC (2013CCC) and pGM2013AAA (2013AAA) were grown at the temperatures indicated below the bars and processed as detailed in Materials and Methods. pGM2016 (2016) was analyzed as negative control and showed modest (less than twofold) thermal induction. Fluorescence emission ($F_{485/535}$) was measured in a Packard FluoroCount microplate reader at 485/530 nm. The fluorescence was expressed in arbitrary units (AU) as the ratio $F_{485/535}/OD_{600}$. The average results of two independent measurements are reported with standard deviations. Relative fluorescence with respect to pGM2013 at 28°C is indicated by the numbers on top of the bars.

regulation. In fact, the TIR and the 5' end of the coding sequence may fold into a thermosensitive stem (SL2), which would sequester the TIR at low temperature. As for PA5194, our data support bioinformatic predictions suggesting that the TIR of the gene may fold into an unstable stem-loop with both the SD and the region encompassing the AUG codon involved in base-pairing. However, abolishing the anti-SD-SD pairing in the 2013AAA mutant had only a mild effect on thermal induction. This suggests that this RNAT may primarily act by modulating the start codon accessibility and that the anti-SD-SD interaction could be implicated in the fine tuning of the response. Interestingly, it has recently reported that the cyanobacterial *avashort* RNAT primarily acts by sequestering the AUG start codon (Cimindis et al. 2014).

The physiological role of *ptxS* thermoregulation is not obvious. The gene encodes a repressor of the LacI family that regulates transcription of its own gene and of operons for gluconate transport and degradation (Swanson et al. 1999; Swanson and Hamood 2000). Gluconate transport is the major pathway of glucose uptake when glucose is abundant, upon its oxidation to gluconate (Whiting et al. 1976). The specific effector of PtxS is 2-ketogluconate, whose binding causes dissociation of PtxS from DNA. Recently, it was shown that PtxS interacts with nanomolar affinity with PtxR, whose gene is adjacent to and transcribed divergently from *ptxS*, and that 2-ketogluconate abolishes the interaction (Daddaoua et al. 2012). PtxR is a positive regulator of transcription of *toxA*, which encodes the most toxic virulence factor of *P. aeruginosa* (Hamood et al. 1996; Daddaoua et al.

2012). It has been proposed that in the absence of 2-ketogluconate, PtxS acts as a negative regulator of *toxA* by preventing the interaction of PtxR with the RNA polymerase (Daddaoua et al. 2012). Thus, while high glucose concentrations may induce ToxA expression by inhibiting PtxS interaction with PtxR, PtxS thermal induction may help prevent ToxA expression at high temperature but low glucose concentration. Interestingly, although glucose is far less concentrated in the airways surface liquid (ASL) that lines the respiratory tract than in plasma, in cystic fibrosis patients, which are highly susceptible to *P. aeruginosa* infections, glucose concentration in the ASL is considerably higher than in healthy individuals (Baker et al. 2007; Garnett et al. 2012).

The PA5194 gene is located in a chromosomal region where other functions involved in the response to temperature upshift map. In fact, it is flanked by two divergently transcribed genes, namely *yrfl*, which encodes the chaperone protein HSP33, and PA5195, which is described in the Pseudomonas Genome Database as a probable heat shock protein. The PA5194 gene product is annotated as a membrane protein with unknown function. However, it has an overall 32% identity and 49% similarity with the *E. coli* protein LpxT, and the two genes are predicted to be orthologous by Reciprocal Best-Blast analysis (Montague and Hutchison 2000). Interestingly, the 5' UTR of the *lpxT* mRNA is predicted by RNAfold to form a structure that resembles a FourU RNAT, suggesting that *lpxT* translation may also be thermoregulated (Fig. 6D). LpxT catalyzes the phosphorylation of Lipid A at the 1-position using undecaprenyl pyrophosphate as a phosphate donor, thus increasing the negative charge of the bacterial surface. This modification is relevant not only for LPS stability, but also significantly affects its endotoxicity, as Lipid A lacking one or both phosphate groups has proven to be less toxic in several bacteria (Kong et al. 2011; John et al. 2012; Needham and Trent 2013). The effect of temperature on Lipid A decoration has not been extensively explored so far. However, increased Lipid A phosphorylation in response to a modest temperature upshift (from 37°C to 39°C–41°C) has been observed in *Porphyromonas gingivalis* (Curtis et al. 2011). Moreover, in *P. aeruginosa* the degree of another modification of Lipid A, i.e., acylation, is modulated by temperature (Ernst et al. 2006); it would be interesting to see whether phosphorylation also responds to this stimulus.

Tet-Trap has allowed the identification of two genes post-transcriptionally regulated in response to temperature changes that would have presumably escaped other experimental or bioinformatic approaches. In fact, *ptxS* and PA5194 do not show obvious similarity with known RNATs. This also holds true for *dsbA* and PA1031 5' UTR (data not shown), which were also identified in the Tet-Trap screening. Moreover, *ptxS* and PA5194 are comparably expressed at 25°C and 37°C (Fig. 5C) and, in fact, they were not identified among thermoregulated genes in a recent transcriptomic survey of *P. aeruginosa* (Wurtzel et al. 2012). Finally, as the two genes seem to be inefficiently translated even at high temperature (compare

the PtxS and PA5194 fusions with expression of the RecA control fusion in Tables 3 and 4), the study of their regulation through proteomic approaches would probably have been challenging. We expect that other *P. aeruginosa* RNATs may wait for identification. In fact, we did not fish in our screen the *ibpA* RNAT (Krajewski et al. 2013), which was represented in the *P. aeruginosa* ST-TIP2-fusion library (data not shown). Other rounds of Tet-Trap may be run to identify new *P. aeruginosa* RNATs. Moreover, it would be interesting to apply a Tet-Trap search for RNATs to other Gram-negative bacteria, such as pathogenic strains of *E. coli* or *Salmonella*. As TIP-mediated induction of TetR-controlled gene expression has been proven to be functional also in the Gram-positive bacterium *Staphylococcus aureus* and even in the eukaryote *Saccharomyces cerevisiae* (Gauger et al. 2012; Stoeckle et al. 2012), the Tet-Trap could potentially be adapted to search for RNATs in these systems.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains, plasmids, and oligonucleotides sequences are reported in Supplemental Table S1. *Pseudomonas aeruginosa* strains used here were PAO1 (Stover et al. 2000) and PA14 (Rahme et al. 1995; Lee et al. 2006) (GenBank accession numbers NC_002516 and NC_008463, respectively). *Escherichia coli* coordinates throughout this work refer to GenBank accession number U00096.2, whereas *P. aeruginosa* coordinates refer to PAO1.

Construction of *E. coli* Tet-Trap reporter strains

The SpcR and StrS reporter cassettes were prepared as follows. A cassette constituted by the Tn10 *tet* regulatory region (*tetRp–tetO–tetAp*) (Bertrand et al. 1983) controlling transcription of *kan^R* (under *tetAp* promoter) and *lacZα* (under *tetRp*) was integrated into the C-1a (Sasaki and Bertani 1965) chromosome between the *tonB* and *yciA* loci. The cassette was obtained by three-step-PCR as follows. Three partially overlapping fragments corresponding to the Tn10 *tet* regulatory region, *lacZα* and *kan^R* were synthesized by PCR. Oligonucleotides 2602–2603 on C-5868 genomic DNA (Carzaniga et al. 2009) were used for Tn10 *tet* amplification; 2604–2605 on pUC19 (Yanisch-Perron et al. 1985) for *lacZα*; 2600–2601 on pQE31S1 (Sukhodolets and Garges 2003) for *kan^R*. Then Tn10 *tet* and *lacZα* fragments were used as templates in a PCR reaction with oligonucleotides 2603–2604, obtaining the fragment *lacZα–tet*. Finally, the full-length cassette was obtained by amplification of the *lacZα–tet* and *kan^R* fragments with primers 2604–2606 and was cloned in the SmaI site of pGM742 (Regonesi et al. 2004), giving plasmid pGM932. The insert was excised by digestion with NotI and XmnI and integrated in C-1a/pKD46 between nucleotides 1309870 and 1309872 by λ Red-mediated homologous recombination (Datsenko and Wanner 2000), obtaining C-5898. To replace the *kan^R* gene with the *aadA*:GFP fusion gene, which confers spectinomycin resistance and fluorescence, the *aadA*:GFP ORF was amplified from plasmid pZR80-2 (Rizzi et al. 2008) with the oligonucleotides 2683–2684, harboring 50-nt long tails homologous to the regions flanking the *kan^R* ORF in C-5898. C-5898/pKD46 was transformed with the

above-mentioned PCR fragment; the recombinants were selected on spectinomycin plates and their fluorescence evaluated by Versadoc imaging of the plates. The cassette from a highly fluorescent clone was sequenced and revealed a spontaneous A to G transition within the *tetA* Shine–Dalgarno region (position 916 of Tn10; GenBank accession number AY528506.1). This mutant strain was named C-5899. To replace the *aadA*:GFP gene with *rpsL*, the *rpsL* ORF and the *cat* gene with two flanking FRT sites were amplified by PCR with the oligonucleotides 2713–2714 on C-1a genomic DNA and the oligonucleotides 2636–2712 on pKD3 (Datsenko and Wanner 2000), respectively. These two partially overlapping amplicons were used as DNA templates for a PCR reaction with oligonucleotides 2638–2602. The resulting DNA fragment was used to transform C-5899/pKD46, obtaining C-5912. The reporter construct was then transduced into C-5708, a C-1a derivative carrying the recessive *rpsL31* allele (K42T substitution in S12), which confers streptomycin resistance (Lederberg 1951). The *cat* cassette was excised from the StrS recombinant strain (C-5916) by FLP-mediated recombination, obtaining C-5918. *Pcat–tetR–kan^R* cassettes were amplified (oligonucleotides 2685–2686) from WH1001 derivatives carrying either the *wt* or the weaker -10^{CATTTA} mutant of the *Pcat* promoter upstream of *tetR* (Georgi et al. 2012). Region 806595–808520 of the BW25113 genome was replaced with the two cassettes by λ Red-mediated homologous recombination, obtaining strains KG264 (*Pcat⁺*) and KG265 (*Pcat* -10^{CATTTA}), respectively. We transduced the *Pcat⁺–tetR–kan^R* region from KG264 into C-5899 obtaining C-5901; C-5907 is a C-5901 derivative in which the *kan^R* cassette was removed by FLP-mediated recombination (Datsenko and Wanner 2000). C-5920 was obtained by P1 transduction from KG265 into C-5918.

Tet-Trap plasmids

pGM956 and pGM957 carry a chimeric gene composed of an (SG₄)₅ linker and *trxA* fused upstream of TIP2. The construct was obtained by three-step PCR. (SG₄)₅ was obtained by annealing the partially overlapping oligonucleotides 2689–2690 and extension with *Pfu* polymerase (Stratagene). The *trxA*:TIP2 sequence was amplified by PCR from pWH2354 (Georgi et al. 2012) with the oligonucleotides 2691–2692. The final PCR was performed on the two above-mentioned fragments with oligonucleotides 2692–2693 obtaining the full-length construct (SG₄)₅:*trxA*:TIP2. This was digested with SphI–EcoRI and cloned in pGZ119HE (Lessl et al. 1992) obtaining pGM957. pGM956 is a pGM957 derivative that carries a translation initiation region (TIR) in frame with (SG₄)₅:*trxA*:TIP2. The TIR was obtained by annealing the oligonucleotides 2617–2618 and cloning the fragment in pGM957 between HindIII and SphI sites. Both plasmids were checked by sequencing the relevant regions.

BgaB, *sfGFP*, and *HA* reporter plasmids

To construct the shuttle vector pGM931, the HindIII–PstI pGM362 fragment carrying the transcriptional terminator t_Ω (Briani et al. 2000) was cloned in pBAD24-Δ1 (Carzaniga et al. 2012), obtaining pGM930. The MluI–HindIII pGM930 fragment carrying *araBp*-t_Ω was cloned in pHERD20T (Qiu et al. 2008), obtaining pGM931. All reporter constructs were assembled in pGM931 and checked by sequencing. *bgaB* was amplified by PCR from pBAD2_bgaB (Klinkert et al. 2012) with the primers 2846–2847, digested with NcoI and PstI and cloned in pGM931, obtaining pGM978. pGM978 was digested with NcoI and EcoRI and used as a backbone

for the translation fusions. The following portions of the *ptxS* gene were amplified from the *P. aeruginosa* PAO1 genome and cloned: region 2487531–2488013 (primers 2850–2851; plasmid pGM980); region 2487779–2488013 (primers 2851–2852; plasmid pGM981). The Superfolder GFP (sfGFP) (Pedelacq et al. 2006) gene was amplified from pXG-10SF (Corcoran et al. 2012) with the oligonucleotides 2803–2804, digested with PstI–KpnI and cloned in pGM931, obtaining pGM2011. Translational fusions were obtained by cloning PCR amplified-KpnI-digested PAO1 genomic regions 5846939–5847277 (oligonucleotides 3004–3005) and 5847080–5847277 (oligonucleotides 3004–3006) in KpnI-digested pGM2011, obtaining plasmids pGM2012 and pGM2013, respectively. pGM2013 CCC and pGM2013AAA carry the same region as pGM2013 with the substitution of the three thymidines in position 5847127–5847129 with three cytidines or three adenosines, respectively. The constructs were obtained by three-step PCR on PAO1 DNA with external oligonucleotides 3004 and 3006 and partially overlapping primers 3141–3142 (for pGM2013CCC) or 3141–3143 (for pGM2013AAA), digestion with KpnI of the amplicons and cloning in pGM2011. Control plasmids pGM989 and pGM2016 carry the leader region and the first nine codons of *E. coli recA* in frame with the *bgaB* and sfGFP genes, respectively. The *recA* fragments were amplified from MG1655 genomic DNA with the oligonucleotides 2915–2916 (amplicon Rec1) and 2928–2929 (Rec2). Rec1 was digested with NcoI and EcoRI and cloned in pGM978, obtaining pGM989, whereas Rec2 was digested with KpnI and cloned in pGM2011, obtaining pGM2016. pPA5194-HA was constructed by cloning a KpnI-digested amplicon obtained by PCR with oligonucleotides 3006–3144 on PAO1 DNA in pGM931 digested with KpnI and SmaI. pPtxS-HA was similarly obtained by cloning a NcoI-digested DNA fragment amplified by PCR with oligonucleotides 2852–3145 on PAO1 DNA in pGM931 digested with NcoI and SmaI. All pGM931 derivatives were constructed in *E. coli* and transferred to PAO1 by triparental conjugation (Goldberg and Ohman 1984).

Bacterial cultures were grown in LD (10 g/L Tryptone, 5 g/L Yeast Extract, 5 g/L NaCl) or M9-glucose (0.1% NH₄Cl, 1.6% Na₂HPO₄·12 H₂O, 0.3 KH₂PO₄, 0.5% NaCl, 0.013% MgSO₄, 0.001% CaCl₂ and trace elements, 0.4% glucose). When needed, media were supplemented as follows: 100 µg/mL ampicillin; 30 µg/mL chloramphenicol; 100 µg/mL spectinomycin; 25 µg/mL streptomycin; 0.5 mM IPTG; 0.1%–0.2% arabinose. *Pseudomonas aeruginosa* cultures carrying pGM931 derivatives were grown either in LD supplemented or in M9-glucose supplemented with 300 µg/mL (LD) or 150 (M9-glucose) µg/mL carbenicillin.

Library generation

Genomic DNA was extracted from stationary phase cultures of PAO1 and PA14 with the Puregene Kit. One microgram of PAO1 and PA14 genomic DNA was partially digested with AluI, HaeIII, or the two enzymes for 30 min at 37°C. The digestions were loaded on a 1% agarose gel and bands corresponding to DNA ranging from 300 to 800 nt were cut out from the gel. The digested fragments were purified and pooled. The randomly digested DNA fragments of PAO1 and PA14 were cloned in pGM957 linearized with SmaI, which makes a single cut between *P_{tac}* and the (SG₄)₅-*trxA*-TIP2 chimeric ORF. C-5907 was then transformed with the ligated DNA, and the library was obtained by extracting plasmid DNA from the pool of clones grown in the presence of spectinomycin.

Library sequencing and data analysis

Pseudomonas aeruginosa inserts cloned in the plasmid library described above were amplified by PCR with the oligonucleotides 2739–2740. Ten nanograms of the library DNA were used as template in the amplification reaction. The amplicons were purified by using Agencourt AMPure XP (Beckmann Coulter) in order to remove primer dimers and fragments shorter than 50 bp; the 454 sequencing library was then prepared following the Method Manual for Rapid Library Preparation, GS FLX Titanium (Roche Applied Science). Four hundred fifty-four adaptors with MID indexes were ligated to the ends of the library fragments. The sequencing library was analyzed with Agilent Bioanalyzer High Sensitivity assay and quantified with a NanoDrop fluorimeter by using PicoGreen (Invitrogen, Life Technologies). The library was sequenced in replicate in two lanes corresponding to 1/8 of a GS FLX Titanium Pico Titer Plate (PTP). Genome sequences and annotation files were retrieved from *Pseudomonas* Genome Database (<http://www.pseudomonas.com>). The 52,116 reads generated by pyrosequencing, which were on average 526 bp long, were mapped to the PAO1 and PA14 genomes (GenBank accession numbers NC_002516 and NC_008463, respectively) using Newbler (Roche, 454). The overlap with annotated genes and the coverage were assessed using BEDTools (Quinlan and Hall 2010). In order to define the *P. aeruginosa* core and the PAO1–PA14 strain-specific coding genes, the protein sequences of both strains were blasted against each other (Altschul et al. 1990), 5320 proteins showing sequence similarity ≥90% were defined as “core,” 252 were identified as PAO1-specific and 572 as PA14-specific (Lee et al. 2006; Silby et al. 2011). Three thousand sixty-four core coding genes, 168 PAO1-specific and 194 PA14-specific, for a total of 3426 genes were identified by sequencing. More than 78% of these genes were sequenced with coverage above the detection threshold, which was equal to or >3 reads per gene. The translation initiation region of a gene was considered to be represented in the library if at least one consensus resulting from the mapping covered the start codon of that gene.

β-Galactosidase assays

Escherichia coli DH10B (Grant et al. 1990) cultures were grown at 28°C in LD supplemented with ampicillin up to OD₆₀₀ = 0.5 and induced with 0.1% arabinose. The cultures were split in two and the sub-cultures incubated at 28°C or 42°C. After 30 min, samples were taken to measure OD₆₀₀ and β-galactosidase activity. BgaB activity was measured in permeabilized cells as described by Miller (1972), except that the assay was performed at 55°C. The assay on *P. aeruginosa* was performed by growing PAO1 cultures at 25°C in 40 mL of LD supplemented with carbenicillin (Carb) up to OD₆₀₀ = 0.5. As PAO1 cultures formed macroscopic aggregates under these conditions, the cells were collected by centrifugation and carefully resuspended in 1 mL of LD to eliminate visible aggregates. The cells were then inoculated in 40 mL of LD supplemented with Carb and 0.1% arabinose at 25°C. Twenty milliliters were immediately withdrawn and shifted to 37°C. After 45-min incubation, 15 mL of each sub-culture were centrifuged and resuspended in 0.3 mL of TEDP (0.1 M Tris–HCl, pH 8; 0.001 M EDTA; 0.1 M DTT; 0.1 M PMSF). Crude extracts were obtained by sonication and the protein concentration was evaluated by Bradford assay (Bradford 1976). β-Galactosidase activity at 55°C was assayed by mixing 0.2 mL of crude extract with 0.8 mL of Z buffer (Miller 1972) and 0.2

mL of 4 g/L ONPG in Z buffer. The reaction was stopped by adding 0.5 mL of 1 M Na₂CO₃.

Fluorescence detection

Fluorescence of cells carrying sfGFP fusions was detected either by means of a Tecan Infinity PRO 200 reader or a Packard Fluorocount microplate reader. For Tecan experiments, *P. aeruginosa* PAO1 and *E. coli* DH10B cultures carrying the reporter plasmids were grown 16 h in LD with the appropriate antibiotics and 0.2% arabinose at different temperatures. Cells were collected by centrifugation, washed, and resuspended in PBS at OD₆₀₀ = 0.1–0.5. Two hundred microliter cell samples were transferred in black polystyrene 96-well microplates with clear flat bottom (Corning). OD₅₉₅ and Fluorescence Polarization (FP)_{485/535} were measured. sfGFP activity was expressed in arbitrary units (AU) as the ratio FP_{485/535}/OD₅₉₅. For Packard Fluorocount experiments, DH10B cultures carrying the reporter plasmids were grown and processed as described previously for Tecan experiments, with the difference that the cultures were grown 24 h and the cells were resuspended in PBS at OD₆₀₀ = 0.8–1.0. One hundred microliter cell samples were transferred in black polystyrene 96-well microplates, and fluorescence_{485/535} (F_{485/535}) was measured. The OD₆₀₀ of the culture samples in the wells was assessed by Biophotometer (Eppendorf) reading. The sfGFP activity was expressed in arbitrary units (AU) as the ratio F_{485/535}/OD₆₀₀. *Pseudomonas aeruginosa* cell images were acquired with a Leica fluorescence microscope (Leica AG) equipped with a digital Leica DC150 camera and a 100× oil immersion objective.

Analysis of the expression of HA-tagged *ptxS* and PA5194 variants

Cultures of PAO1 expressing PA5194-HA or PtxS-HA from pGM931 derivatives were grown overnight in LD with carbenicillin at 28°C and diluted to OD₆₀₀ = 0.2 in three flasks containing M9 medium supplemented with carbenicillin, 0.4% glucose, and 0.2% arabinose. The flasks were incubated at 25°C–37°C–40°C. Twenty-five and 3 mL samples were taken after 1 and 2 h for protein and RNA extraction, respectively. RNA was extracted by the RNAsnap procedure (Stead et al. 2012). Protein extraction was performed by resuspending the cell pellet in 2× SDS buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 2% SDS, 0.2% bromophenol blue, and 20% glycerol) and boiling the cells for 15 min. The cells were then centrifuged 10 min at 13,000 rpm, the supernatant was recovered, and an aliquot was analyzed by 10% SDS-PAGE. PageRuler Plus Prestained Protein Ladder weight markers (ThermoScientific) were used as size references. For immunological detection of proteins, the gels were blotted onto a Hybond ECL (GE Healthcare Life Sciences) sheet and incubated with monoclonal anti-HA (12CA5; Roche) or polyclonal anti-S1 (kindly provided by U. Bläsi) antibodies (Sambrook et al. 1989).

Northern blotting and primer extension

Procedures for RNA extraction, Northern blot analysis, in vitro transcription with T7 RNA polymerase and 5' end labeling of oligonucleotides with T4 polynucleotide kinase and [γ -³²P] ATP were previously described (Dehò et al. 1992; Briani et al. 2007). Extraction of RNA from *P. aeruginosa* cultures was performed by phenol–chloroform

treatment of cell lysates (Dehò et al. 1992) or by means of the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. After blotting, the filters were stained by incubating them for 2 min in 0.02% methylene blue and 0.3 M sodium acetate, pH 5.5. Oligonucleotide probes used for Northern blotting were 1396 (16S rRNA); 2865 (*bgaB*); 2871 (sfGFP); HA (Delvillani et al. 2011). The riboprobes PtxS and PA5194 were obtained by in vitro transcription with T7 RNA polymerase and [α -³²P] CTP of DNA fragments obtained by PCR amplification of pPtxS-HA (oligonucleotides 2976–2811) and pPA5194-HA (oligonucleotides 3150–3006), respectively. The 5' end of the PA5194 mRNA was determined by primer extension as previously described (Forti et al. 1995) with the radiolabeled oligonucleotide 3003 on 30 µg of RNA extracted from exponential cultures of PAO1 grown at 25°C and 37°C. The same oligonucleotide was used for Sanger-sequencing of construct 3. Images and densitometric analysis of Northern blots and primer extension gels were obtained by phosphorimaging using ImageQuant software (Molecular Dynamics).

RNA structural probing

The RNAs for structural probing were synthesized by in vitro transcription of proper PCR fragments with T7 RNA polymerase. The DNA templates were obtained by PCR on PAO1 genomic DNA with the oligonucleotides 2909–2910 (*ptxS*) and 3040–3041 (PA5194). The probes were radiolabeled at the 5' end by dephosphorylation with alkaline phosphatase followed by phosphorylation with T4 polynucleotide kinase with [γ -³²P] ATP as a phosphate donor. After labeling, the probes were run on a denaturing polyacrylamide gel and the bands corresponding to the full-length RNAs were gel-eluted. For structural analysis, the probes were denatured by incubation at 95°C for 5 min; 2 × 10⁴ cpm samples were then preincubated in reaction buffer (20 mM Tris-HCl, 2 mM Mg₂Cl, 100 mM NaCl) and 0.66 µg of tRNA mix at 25°C, 37°C, or 42°C for 10 min. One microliter of RNase T1 (final concentration, 66 pg/µL), RNase A (0.04 pg/µL), or lead acetate (0.1 mM) were added to the samples (final volume, 5 µL) and incubation at different temperatures was performed for 5 min before stopping the reactions with 5 µL of RNA loading dye (2 mg/mL xylene cyanol and bromophenol blue, 10 mM EDTA in formamide). A denaturing RNase T1 degradation was performed by incubating each probe (10⁵ cpm) and 0.6 mg/mL tRNA in 1× RNA seq buffer (Ambion) at 50°C for 5 min. RNase T1 (final concentration 0.166 ng/µL) was added and the samples were incubated at 37°C for 5 min before adding 10 µL of RNA loading dye. The RNA ladder was obtained by digesting 2.5 × 10⁵ cpm of probe PA5194 at 95°C for 10 min with 12 µL of Alkaline Buffer (Ambion) and 6 µg of tRNA mix in a final volume of 15 µL. The samples were run on an 8% denaturing polyacrylamide gel. The gel was dried and analyzed by phosphorimaging using ImageQuant software (Molecular Dynamics).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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