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Temperature-dependent regulation of the *lpxT* gene in *Escherichia coli* and *Pseudomonas aeruginosa*

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Abstract

Bacteria respond to temperature variation through sophisticated regulatory networks that involve different macromolecules and molecular thermosensors. In particular, RNA thermometers (RNATs) are thermolabile secondary structures located within the 5'-UTR of some mRNAs that permit fast response to temperature changes. Typically, the RNATs at low temperature entrap the Translational Initiation Region (TIR) of the cognate mRNA thus inhibiting ribosome binding. When the temperature increases, the RNAT secondary structure becomes unstable and gradually shifts to an open conformation, thus allowing translation. Interestingly, in pathogenic bacteria RNATs have been found to respond to the mammal host temperature (37°C) and control the expression of virulence genes. Therefore, the identification of RNATs in pathogens could provide new information about invasion and pathogenicity strategies.

RNATs are characterized by poor sequence conservation and structural variability that make the bioinformatic analysis hardly applicable to RNATs identification. We developed a new genetic tool, namely the Tet-Trap, aimed to the identification of post-transcriptionally regulated genes. We applied the Tet-Trap to find out new genes regulated by temperature in the opportunistic pathogen *Pseudomonas aeruginosa.* Using this system, we identified four new putative RNATs. Two of them, namely *ptxS* and *lpxT*, were validated with both *in vitro* and *in vivo* approaches. On the whole, our results strongly suggest the presence of new RNATs in the 5'-UTR of both *ptxS* and *lpxT.*

The LpxT protein modifies the outer membrane of Gram negative bacteria by transferring a phosphate group from undecaprenyl-pyrophosphate to

the position 1 of the Lipid A moiety of the lipopolysaccharide (LPS), generating a Lipid A diphosphate specie. One-third of the lipid A found in the *E. coli* outer membrane contains an unsubstituted diphosphate unit at position 1. The high proportion of this modification in Lipid A and its wide distribution among Gram-negative bacteria make relevant the clarification of its regulation and biological role.

We observed that in *E. coli,* the *lpxT* gene 5'-UTR is predicted to fold into an unstable stem-loop entrapping the TIR, thus suggesting a conserved *lpxT* regulatory strategy in *E. coli* and *P. aeruginosa*. This observation prompted us to analyze the expression of the *E. coli lpxT* gene at different temperatures. We found that the *E. coli lpxT* gene expression is temperature-responsive. Moreover, toeprinting and reporter translational fusion experiments indicated that thermoregulation was achieved through translation modulation and that the determinants of thermoregulation were located in the *lpxT* 5'-UTR. Point mutations in the *lpxT* 5'-UTR predicted to change the stability of the stem-loop involving the TIR or enhancing the complementarity of the SD with the 16S rRNA affected thermoregulation, showing that both these elements cooperate in *lpxT* regulation. Overall, our results strongly suggest that in *E. coli*, *lpxT* translation may be modulated in response to temperature variations through a peculiar mechanism based on the combined action of sub-optimal elements.

Sommario

I batteri rispondono alle variazioni di temperatura grazie a sofisticate reti di regolazione che coinvolgono diverse macromolecole e termometri molecolari. In particolare, i termometri a RNA (RNAT) sono strutture secondarie termolabili situate all'interno della 5'-UTR di alcuni mRNA che consentono una rapida risposta alle variazioni di temperatura. Tipicamente, alle basse temperature, gli RNAT sequestrano la regione di inizio della traduzione (TIR) dell'mRNA, inibendo così il legame al ribosoma. Quando la temperatura aumenta, la struttura secondaria dell'RNAT diventa instabile e gradualmente assume una conformazione aperta, consentendo così la traduzione. È interessante notare che in batteri patogeni sono stati trovati RNAT in grado di rispondere alla temperatura dell'ospite mammifero (37°C) e controllare l'espressione di geni di virulenza. Pertanto, l'identificazione di RNAT nei patogeni potrebbe fornire nuove informazioni sulle loro strategie di invasione e patogenicità.

Gli RNAT sono caratterizzati da una scarsa conservazione sia di sequenza che di struttura e ciò rende l'analisi bioinformatica poco applicabile all'identificazione di nuovi RNAT. Nel nostro laboratorio abbiamo sviluppato un nuovo sistema genetico, ovvero il Tet-Trap, finalizzato all'identificazione di geni regolati *in cis* a livello post-trascrizionale, e lo abbiamo applicato alla ricerca di nuovi geni regolati dalla temperatura nel patogeno opportunista *Pseudomonas aeruginosa*. Utilizzando questo sistema, abbiamo identificato quattro nuovi putativi RNAT. Due di essi, ovvero *ptxS* e *lpxT*, sono stati validati sia con approcci *in vitro* che *in vivo*; nel complesso, i nostri risultati

suggeriscono fortemente la presenza di due nuovi RNAT nella 5'-UTR di *ptxS* e *lpxT*.

La proteina LpxT modifica la membrana esterna di molti batteri Gram negativi. Essa catalizza il trasferimento di un gruppo fosfato dall' undecaprenil-pirofosfato alla posizione 1 del Lipide A, la parte lipidica del lipopolisaccaride (LPS), generando così la specie Lipide A pirofosfato. Un terzo del Lipide A presente nella membrana esterna di *E. coli* contiene una unità pirofosfato non sostituita nella posizione 1. L'elevata percentuale di questo tipo di modificazione del Lipide A e la sua ampia distribuzione tra i batteri Gram-negativi rendono rilevanti la comprensione della regolazione e del ruolo biologico di tale modificazione.

La predizione bioinformatica della 5'-UTR del gene *lpxT* di *E. coli* suggerisce che tale sequenza possa formare uno stem-loop, relativamente instabile anche a 28°C, che intrappola la TIR. Ciò suggerisce che sia in *E. coli* che in *P. aeruginosa* il gene *lpxT* possa essere regolato dallo stesso meccanismo molecolare mediato da RNAT. Questa osservazione ci ha spinto ad analizzare l'espressione del gene *lpxT* in *E. coli* (Ec*lpxT*) a diverse temperature, scoprendo che anche la sua espressione è sensibile alla temperatura. Inoltre, esperimenti di toeprinting e saggi di fluorescenza con fusioni traduzionali tra la 5'-UTR di Ec*lpxT* e il gene reporter GFP indicano che la termoregolazione avviene attraverso un meccanismo di modulazione della traduzione e che i determinanti della termoregolazione sono localizzati nella 5'-UTR di Ec*lpxT*. Mutazioni puntiformi nella 5'-UTR di Ec*lpxT* predette conferire un cambiamento nella stabilità dello stem-loop o aumentare la complementarità della SD con l'rRNA 16S interferiscono con la termoregolazione, dimostrando che entrambi questi elementi cooperano nella regolazione di Ec*lpxT.* Complessivamente, i nostri

dati suggeriscono che in *E. coli* la traduzione di *lpxT* possa essere modulata dalla temperatura attraverso un meccanismo peculiare, basato sull'azione combinata di elementi regolativi sub-ottimali.

PART I

State of the Art

1 - Temperature is a critical parameter affecting bacterial growth

A very important physical parameter affecting growth and survival is the temperature. This holds true for all living organisms and especially for unicellular ones, which are particularly exposed to environmental temperature fluctuations. Mesophilic bacteria like *Escherichia coli* and *Pseudomonas aeruginosa*, with an optimal growth temperature (i.e. the temperature at which the growth rate is maximal) of 37°C, can grow, although more slowly, at temperatures ranging from 10°-15° to 42°-45°C. However, even within this permissive window, temperatures approaching to the upper or lower limit are stressful to the cells because of their consequences on cell physiology, macromolecule state and enzymatic reactions, and thus require specific adaptive responses.

The general regulation system activated by an upshift to a sub-lethal temperature (i.e. from 30°C to 42°C) has been named the Heat Shock Response (HSR). In *E. coli,* around 100 genes are induced in the HSR (Arsène *et al.,* 2000; Nonaka *et al*., 2006; Lim and Gross, 2011). More than 50% of the Heat Shock Proteins (HSPs) encoded by such genes are proteases and protein chaperons, including the two major chaperon systems DnaK and GroEL (Georgopoulos *et al.,* 2006; Genevaux *et al*., 2007). Moreover, the HS regulon includes MutL, which is involved in DNA mismatch repair (Tsui *et al.*, 1996), rRNA and tRNA modification systems stabilizing their structure during HS

(Tsui *et al.*, 1996; Bügl *et al*., 2000), RNA polymerase binding proteins, such as TopA, that are probably important for alleviating the topological stress due to active transcription of heat-shock genes (Lim and Gross, 2011; Qi *et al.,* 1996; Cheng *et al.*, 2003), and proteins implicated in ribosome protection and recycling (Korber *et al*, 1999; Korber *et al.*, 2000).

Overall the 25% of the HSPs are involved in the maintenance of the inner membrane integrity, and almost the 8% are transcriptional regulators controlling the expression of membrane proteins, among which membrane-localised transporters (Nonaka *et* al., 2006). This underlines the connection between maintenance of membrane functionality and heat shock response. In fact, in bacteria both high and low temperatures have a strong impact on membrane fluidity. It was show that the composition of membrane fatty acids, and in particular the ratio between Unsaturated and Saturated Fatty Acids (UFA and SFA, respectively; Casadei *et al.*, 2002) changes with the temperature in order to preserve a degree of fluidity compatible with the activity of enzymes and transporters associated with the cell membranes. For example, *Salmonella enterica* exhibits higher UFA content at low temperatures (Álvarez-Ordóñez *et al.,* 2008). The same was reported also for *E. coli* (Casadei *et al.*, 2002), *Lactobacillus acidophilus* (Wang *et al.,* 2005) and *Pediococcus s*pp. (Annous *et al.,* 1999).

Besides the effect on cell membrane, low temperature drastically slows enzymatic reactions and stabilises nucleic acid secondary structures and this, in turn, affects translation of many cellular mRNAs (reviewed by Vigh, 1998; Gualerzi *et al.,* 2003; Lim and Gross., 2011). The Cold Shock (CS) regulon in *E. coli* is induced upon a temperature downshift from 37°C to 10°C and includes 26 cold-shock proteins (CSP) (Goldestein *et al.,* 1990; Gualerzi *et al*.,

2003). Many of these are nucleic acid binding proteins, such as i) proteins involved in resolution of DNA supercoiling, like the Histon-like Nucleoid Structuring proteins (H-NS) (La Teana *et al.,* 1991) and DNA gyrase (Jones *et al.*, 1992); ii) RNA chaperons, such as CspA, i.e. a translational enhancer of cold-shock genes (La Teana *et al.,* 1991; Jones *et al.*, 1992; Goldstein et *et al*., 1990); iii) CspE (Feng *et al.,* 2001) and DeaD RNA helicase (Jones *et al.*, 1996). Cold-shock induces also modifications in the translation apparatus. The translation Initiation Factors IF1, IF2, IF3 are induced at low temperature and IF3 seems to be relevant in favouring translation of CSPs mRNA (Gualerzi *et al.,* 2003).

1.1 - In *E. coli* the major players of heat and cold shock responses are post-transcriptionally regulated through RNA-based mechanisms

The *E. coli* heat-shock response (HSR) is directly regulated by the alternative sigma factor σ^{32} encoded by the *rpoH* gene. RNA polymerase holoenzyme assembled with such sigma factor specifically recognizes the promoters of the HSP genes (Gross, 1996; Yura *et al.,* 2007). σ^{32} expression is tightly regulated to guarantee a rapid response to the temperature upshift. At 30°C, the σ^{32} cellular concentration is very low (about 10-30 copy per cell; Craig and Gross, 1991) because its translation is repressed (see below). Moreover, σ^{32} activity is inhibited by DnaK that directly binds σ^{32} causing its inactivation and targeting it to proteolytic degradation (Tomojasu *et al.,* 1998; Guisbert *et al.,* 2004). Upon a temperature upshift from 30° to 42°C, massive cellular protein unfolding titrates DnaK and abolishes its inhibitory activity on

σ^{32} (Arséne *et al.,* 2000), which can thus associate with the core RNA polymerase and promote transcription of HS genes (including DnaK and GroEL). Once the protein damage has been repaired by the HSPs, DnaK resumes σ^{32} inhibition, and this in turn switches off the HS response (Craig and Gross, 1991; Bukau, 1993).

Temperature modulates not only the activity, but also the expression of σ^{32}. At 42°C, the σ^{32} cellular concentration increases ~17-fold (Straus and Gross, 1987). This change occurs through post-transcriptional mechanisms. In fact, *rpoH* transcription is only moderately induced at high temperatures (Kanemorit *et al.,* 1994), whereas its translation, very low at 30°C, significantly increases at high temperature (Nagai *et al.,* 1991; Nakahigashi *et al.,* 1998).

Studies on *rpoH* regulation led to the discovery of a post-transcriptional mechanism that inhibits mRNA translation at low temperature (Nagai *et al.,* 1991; Nakahigashi *et al.,* 1995; Nakahigashi *et al.,* 1998). In such condition, the *rpoH* mRNA folds into a complex secondary structures that involves the translation initiation region (TIR) and prevents binding of the 30S ribosomal subunit. Upon a temperature upshift, the secondary structure becomes unstable and the AUG start codon and Shine Dalgarno region (SD), namely a purine-rich sequence complementary to the 3'-end of 16S rRNA (Steitz and Jakes, 1975), become accessible. This allows ribosome assembly and mRNA translation. The analysis of the predicted secondary structures of *rpoH* mRNAs expressed by different γ-proteobacteria revealed a high degree of conservation (Nakahigashi *et al.,* 1998), suggesting that the post-transcriptional mechanism that ensures a rapid increase in *E. coli rpoH* expression at high temperature could be conserved in other bacteria. For the *rpoH* riboregulator, the name of RNA thermometer (RNAT) was coined (Storz, 1999).

It should be mentioned that not only high temperature can induce mRNA translation by relieving the inhibition due to a secondary structure, as cold temperature may exert a similar effect. For example, the *E. coli cspA* gene, which encodes the major cold-shock protein induced in the Cold-Shock Response, is transcribed both at 10° and 37°C (Fang *et al.,* 1997), but the mRNA is rapidly degraded at 37°C or higher temperature (Brandi *et al.,* 1996; Goldenberg *et al.,* 1996). Moreover, *cspA* translation is more efficient during the cold-shock. Both these mechanisms cooperate to give a rapid increase of CspA after cold-shock (Goldestein *et al.,* 1990). Differential mRNA stability and translatability are due to a thermolabile secondary structure in the 5'-UTR of *cspA* mRNA that is able to expose the SD region and the AUG at temperatures lower than 20°C (Giuliodori *et al.,* 2010). Thus, two major regulators of HS and CS responses are regulated by thermolabile secondary structures affecting mRNA translation.

1.2 - The secondary structure of the TIR affects mRNA translation

In Bacteria, the first event in the translation of leadered mRNAs (i.e. with a 5'-UTR) is the assembly on the TIR of a pre-initiation complex with the 30S ribosomal subunit and the initiator fMet-tRNA (30S pre-*IC*). The three translation initiation factors IF1, IF2 and IF3 participate in the assembly of the complex and in its conversion to a more stable 30S initiation complex (30S*IC*; recently reviewed by Gualerzi and Pon, 2015).

Different elements and trans-acting factors may impact on the formation of the initiation complex. A pivotal role is played by structural elements of the

mRNA, such as the presence and length of the SD sequence, which contribute to 30S-mRNA complex formation by positioning the 30S in proximity of the start codon and enhancing the thermodynamic affinity of the complex, and secondary (and tertiary) structures involving the TIR (Gualerzi and Pon, 2015). It has been shown that a linear relation exists between the ΔG of formation of the TIR secondary structure (ΔG^0_f) and the fraction of mRNA molecules in the folded state. The lower is the ΔG^0_f, the higher would be the fraction of mRNA molecules in a folded, inaccessible state (de Smith and van Duin, 1990). This is in agreement with the observation that even relatively small changes in the ΔG^0_f of a secondary structure involving the TIR can cause dramatic changes in the translation efficiency (de Smith and van Duin, 1990).

For structured mRNAs, the formation of the 30S*IC* depends on competing equilibria between the formation of either the secondary structure or the 30S-mRNA complex (de Smith and van Duin, 1990; Borujeni and Salis, 2016). It has been estimated that an increase of 1 kcal/mol in free energy of formation of the initiation complex (ΔG_{30S}) compensate for the presence of a secondary structure involving the TIR with a ΔG of formation (ΔG^0_f) of 1 kcal/mol (de Smit and van Duin, 1990). The SD sequence appears to be very relevant for the formation of 30S*IC* on structured mRNA. In fact, strengthening the SD-antiSD interaction may help initiation complex formation by increasing the thermodynamic affinity of the 30S for the TIR. Conversely, unstructured mRNAs are saturated with the 30S even if their TIRs have moderate affinity for the 30S (de Smit and van Duin, 1990).

Regulatory systems that exploit the inhibitory potential of an mRNA secondary structure on translation are widely diffused in bacteria. For instance, RNA riboswitches of Gram negative bacteria usually act as translation

modulators. RNA riboswitches consist of a secondary structure involving the 5' UnTranslated Region (5'-UTR) of the controlled mRNA. The interaction with specific ligands, such as uncharged tRNAs, amino acids or other small molecules, modulates folding of the structure (for a review, see Grundy and Henkin, 2006). The ligands are recognized and bound by the aptamer binding domain and this promotes a structural shift in the expression platform that affects transcription elongation (mainly in Gram positive bacteria) or translation initiation (in Gram negatives; Fig. 1). Typically, riboswitches regulate genes involved in the uptake, biosynthesis, or usage of their ligands (for a review, see, Waters and Storz, 2009; Henkin, 2008; Grundy and Henkin, 2006). A high number of riboswitches have been identified in bacteria and classified in nine classes based on their cognate ligand (for a review, see Winkler and Breaker, 2005). For example, in *Bacillus subtilis* seven classes of riboswitches were found to control the expression of at least the 2% of the bacterial genes (Mandal *et al.,* 2003).

RNA thermometers can be considered as RNA riboswitches responding to a physical signal. In RNA thermometers, the aptamer binding and the expression platform domains are fused in a unique module able to detect intracellular temperature variations, in the order of 1°C, and to regulate accordingly mRNA translation (Chowdhury *et al.,* 2006; Rinnenthal *et al*., 2010). At low temperature the fraction of unstructured RNAT would be very low, thus causing translational inhibition. However, since RNATs have thermodynamically unstable secondary structures, the fraction of folded RNAT would gradually decrease with the temperature, allowing the loading of the 30S on the unfolded mRNA. Thermo-lability of RNATs is determined by features of the 5'-UTR secondary structure, like the presence of bulges, unpaired

nucleotides and non-canonical Watson-Crick base pairs in the double-stranded stems that entrap the TIR (Narberhaus, 2005).

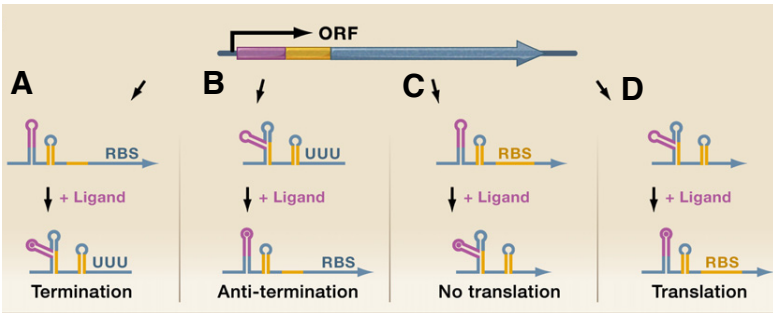

Figure 1: Regulatory function of a metabolite-binding RNA riboswitch.
The 5'-UTR of riboswitches (blue) is composed by an aptamer region (pink) and an expression platform (yellow). In absence of the ligand the expression platform can assume a conformation permissive for transcription elongation (A). When the ligand binds the aptamer domain, a conformational shift causes the formation of a transcriptional terminator. On the other hand, the presence of the ligand can allow the disruption of a transcriptional terminator (B). In Gram negative bacteria, the ligand binding usually causes secondary structure rearrangements leading to entrapment of RBS into an inhibitory stem loop (C), or releasing the RBS and allowing translation (D). Modified from Waters and Storz, 2009.

1.3 - RNA thermometers belong to heterogeneous structural classes

Only two classes of RNATs sharing common structural features have been described so far, ROSE (Repression Of heat Shock gene Expression) and FourU elements; in both cases, sequence conservation is limited to very short sequences of 4-5 bases in the proximity of the Shine-Dalgarno region. On the other hand, many RNATs are unrelated to either classes and can have different degrees of structural complexity. For instance, the RNAT of *rpoH* is complex

and includes a long portion of the ORF, whereas the RNAT found in the *hsp17* gene of the cyanobacterium *Synechocystis* sp. PCC 6803 consists of a single stem-loop 46nt long in which the SD is engaged in a perfect pairing with a UCCU sequence (Fig. 2; Kortmann *et al.,* 2011).

RNATs have been found in the 5'-UTR of genes encoding HSPs, in particular in the 5'-UTR of genes of the class A small heat-shock proteins (sHSPs). These proteins bind to unfolded polypeptides preventing the formation of irreversible aggregates (Lee *et al.,* 1997). sHSPs are particularly abundant in rhizobia species. In *Bradyrhyzobium japonicum* the seven paralogous sHSP genes are organised in five operons. Each of them is controlled by an RNAT belonging to the ROSE class (Narberhaus *et al.,* 1998; Nocker *et al.,* 2001a). ROSE elements regulate sHSPs in various α- and γ-proteobacteria (Waldminghaus *et al.,* 2005), suggesting a specific role of ROSE in sHSPs gene regulation during heat shock.

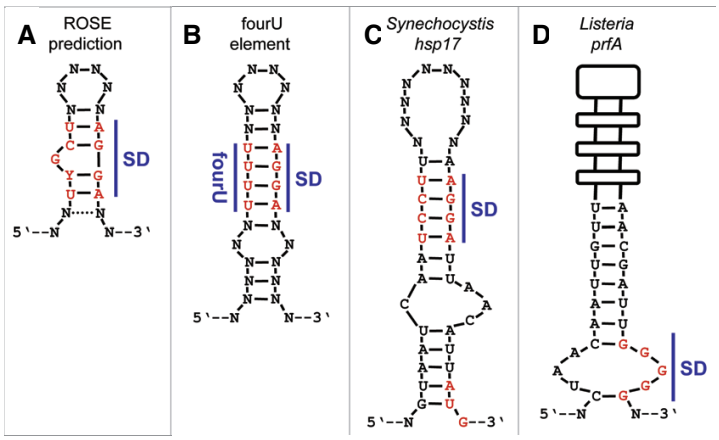

Figure 2: Schematic representation of RNA thermometers.
Schematic representation of A) a ROSE-like element, B) a FourU element. C) Secondary structure of the RNAT controlling expression of the *hsp17* gene. D) Secondary structure of the RNAT controlling expression of *pfrA*. Modified form Narberhaus, 2010.

ROSE elements

ROSE are 60-120 nucleotides long elements characterised by complex secondary structures residing in the 5'-UTR and, in some cases, including few initial codons of the ORF (Narberhaus, 2005). All ROSE elements have predicted secondary structure with three or four stem-loops in which internal loops and bulges are present. Nocker *et al.* (Nocker *et al.,* 2001b) compared fifteen ROSE elements from rhyzobia species. *In silico* analysis revealed high sequence conservation in a stem-loop at the 3'-end of the ROSE, in which the SD and AUG start codon are entrapped (Narberhaus *et al.,* 1998, Nocker *et al.,* 2001b; Fig. 2). In particular, residues involved in base pairing are highly conserved, whereas loop regions are not. Notably, a G residue opposed to the SD sequence forming a bulge is present in all the ROSEs and seems to be at the root of stem instability. Elimination of this residue causes repression of translation at high temperatures. Mutations in the other stem-loops indicate that, albeit not conserved, they are also important for thermoregulation (Nocker *et al.,* 2001b).

A peculiarity of this RNAT class is the transcript lack at low temperature, in the absence of thermally controlled promoters driving ROSE transcription. Mutations destabilising the secondary structure increase the transcript amount at low temperature. It was proposed that the untranslated mRNA at low temperature is targeted by RNases that cause its degradation (Nocker 2001b).

FourU elements

The first FourU RNAT was discovered in *Yersinia pestis* where it regulates the expression of the *lcrF* transcription factor, i.e. the activator of virulence genes (Hoe *et al.,* 1993). However, the name FourU was coined in

2007 by Waldminghaus *et al.* for the RNAT regulating *agsA* gene encoding a sHSP in *Salmonella enterica* (Waldminghaus *et al.,* 2007a).

FourU RNATs are shorter than ROSEs and present a simpler secondary structure with a single hairpin in which the SD sequence pairs to a conserved stretch of four uridines (Fig. 2). The FourU hairpin involves canonical base pairing, with only a mismatch at the base of the stem. Replacing this mismatch with a stable base pair results in complete translation inhibition. Thus, the unpaired nucleotide seems required for melting of the entire structure (Waldminghaus *et al.,* 2007a).

The coordination of Mg^{2+} atoms by the structure has been described for *agsA* RNAT. Since Mg^{2+} affects the melting temperature of the secondary structure *in vitro*, fluctuations in the intracellular concentration of Mg^{2+} may impact on FourU regulation. Indeed, Mg^{2+} intracellular concentration may vary between 1-2mM (Alatossava *et al.,* 1985). A shift between these two concentrations *in vitro* causes a melting temperature increase of ca. 3°C (Rinnenthal *et al.,* 2011).

1.4 - RNA thermometers and virulence

It is well known that some pathogens exploit the host body temperature to activate their virulence genes upon infection (for a review, see Konkel and Tilly, 2000). In the last years, an increasing number of RNATs have been found to be involved in controlling the expression of virulence functions. Interestingly, some master regulators of virulence like LcF, ToxT of *Vibrio cholerae* or PrfA of *Listeria monocytogenes* are all regulated by RNATs that

repress their expression at temperatures below the mammalian body temperature (Böhme *et al.,* 2012 Johansson *et al.,* 2002; Weber *et al.,* 2014). For instance, in *Listeria monocytogenes* it was shown that PfrA, which positively control the transcription of different operons encoding virulence factors, is highly expressed at 37°C, but not at 30°C (Leimeister-Wachter *et al.,* 1992). The 5'-UTR of *pfrA* folds into a secondary structure consisting of a long stem-loop encompassing the SD sequence and the AUG start codon. Curiously, unlike in ROSE, FourU and other RNATs, the SD and AUG are not involved in base paring, but are exposed in a loop (Fig. 2). Point mutations that prevent loop formation cause translational repression of *pfrA* at 37°C, whereas destabilising mutations abolish translational inhibition at 30°C (Johansson *et al.,* 2002). Thus, although the TIR region is not involved in base pair, the overall secondary structure is sufficient to permit post-transcriptional thermoregulation. Conversely, the RNAT modulating the expression of *toxT* transcriptional activator in *V. cholerae* presents the typical FourU features (Weber *et al.,* 2014).

FourU RNATs are also responsible of thermoregulation of genes involved in iron-acquisition in *Shigella dysenteriae* and *Escherichia coli* (Kouse *et al.,* 2013) and in immune evasion and resistance in *Neisseria meningitidis* (Loh *et al.,* 2013).

Since RNATs are involved in virulence genes regulation, the identification of new RNATs in pathogenic bacteria could provide new insights in the comprehension of bacterial strategies implicated in pathogenesis and virulence.

1.5 - Searching new RNATs by *in vitro* and *in vivo* approaches

Poor sequence conservation does not allow the application of conventional bioinformatic tools for homology search to the identification of RNATs. Since the RNAT function depends on the secondary structure, some studies have been implemented to search for regions potentially forming secondary structures immediately upstream of ORFs involved in HSR or virulence (Waldminghaus *et al.,* 2005; Waldminghaus *et al.,* 2007b). Furthermore, bioinformatic tools have been developed to find out temperature-responsive RNA secondary structures on a genome-wide scale (Chursov *et al.,* 2013; Churkin *et al.,* 2014). However, bioinformatic search of conserved features of the 5'-UTRs secondary structure is hampered by the heterogeneity of RNATs and is of limited impact if applied only to genes that are obvious candidate for thermal regulation such as HSP's genes. Moreover, experimental analyses are needed to validate new candidate RNATs found with bioinformatic approaches. *In vitro* and *in vivo* experiments applied to this aim, such as enzymatic or chemical structural probing and reporter analysis expression, are laborious and it is not possible to analyse more than few candidates at a time (Klinkler *et al.,* 2012).

An experimental approach has been recently applied to find new RNATs in *Y. pseudotuberculosis* on a genomic scale (Righetti *et al*., 2016). Such approach stemmed from PARS (Parallel Analysis of RNA Structure), a method previously developed to unveil the "RNA structurome" of *Saccaromyces cerevisiae* grown at 30°C. With PARS the structural profile of more than three thousand mRNAs was obtained and analysed at single nucleotide resolution. Righetti *et al*. applied PARS to the analysis of *Y. pseudotubercolosis* transcriptome folded *in vitro* at 25°, 37° and 42°C. Thermolabile secondary

structures were identified by comparing the RNA structural profile at the three different temperatures. About twenty RNAT candidates were selected for validation, two of which were involved in virulence. *In vivo* validation confirmed thermoregulation for sixteen of the candidates. Interestingly, some of them control the expression of functions previously unrelated to the response to temperature changes (Righetti *et al.,* 2016).

A genetic approach to search new RNATs, i.e. the Tet-Trap, was developed in our laboratory and applied to the research of new RNATs in the opportunistic pathogen *P. aeruginosa* (Delvillani *et al*., 2014). *P. aeruginosa* is a Gram-negative bacterium that can live in a broad range of environments thank to its metabolic versatility (Stover *et al*., 2000). It can cause severe infections in subjects with weakened barriers against bacteria, like burnt, immunocompromised or diabetic patients. In cystic fibrosis patients it elicits chronic lung infections, which are the main cause of the loss of pulmonary function typical of such pathology (Driscoll *et al.,* 2007). A transcriptomic study in *P. aeruginosa* grown at 28°C and 37°C had revealed that virulence genes are preferentially expressed at 37°C, suggesting thermoregulation (Wurtzel *et al.,* 2012). Nevertheless, only a single RNAT was known at the time of our Tet-Trap analysis in *P. aeruginosa* (Krajewski *et al.,* 2013).

The Tet-Trap exploits elements regulating *tetA,* the tetracycline-resistance gene of *Tn10* transposon. This gene is transcriptionally repressed by TetR, which is also encoded in the transposon. In the presence of tetracycline, TetR binds the antibiotic; this causes TetR release from *tetAp* and *tetA* transcription is allowed (Hillen *et al.,* 1994). Besides tetracycline, the TIP2 dodecapeptide is also able to act as allosteric effector of TetR when expressed in chimeric fusions (Goeke *et al.,* 2012). This regulatory elements (i.e. the *tetAp* promoter, TetR

repressor and TIP2 allosteric effector) were combined in the Tet-Trap to obtain reporter strains for either selecting or counter-selecting cells expressing TIP2-tagged polypeptides (Fig. 3). By performing the selection steps at different temperatures, it was possible to identify thermo-regulated genes. Since the Tet-Trap is based on *in vivo* selection of 5'-UTRs conferring thermoregulation to downstream ORFs, relative to bioinformatics or *in vitro* approaches it has the advantage that putative RNAT candidates are selected on a functional basis and in a whole cell context. The development of Tet-Trap and its application to the research of new *P. aeruginosa* RNATs are described in the attached article Delvillani *et al*., 2014 (Part II).

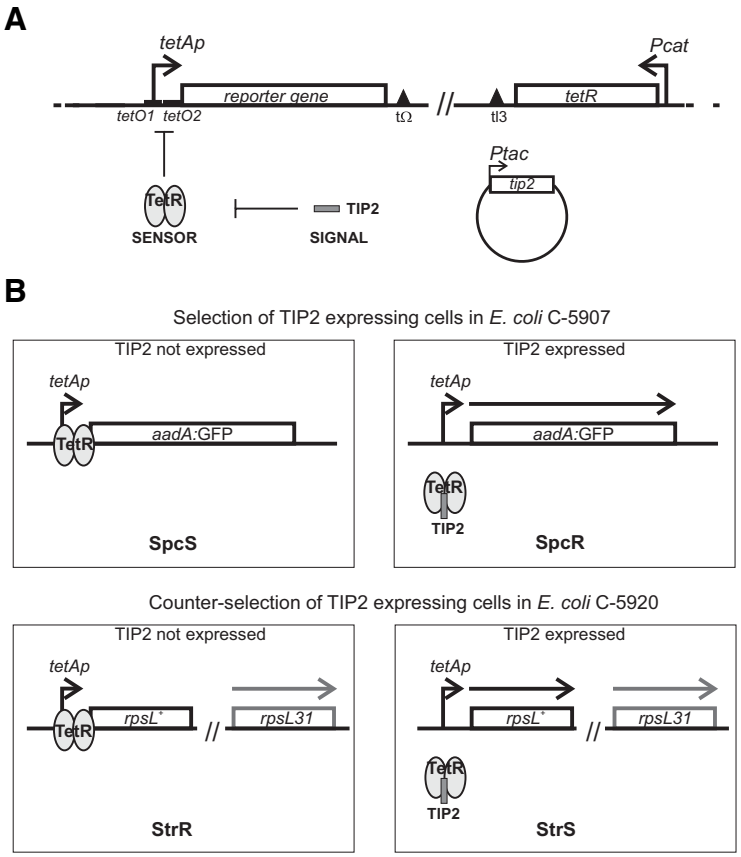

Figure 3: Schematic representation of Tet-Trap system.
A) Schematic representation of Tet-Trap *E. coli* reporter strains. The reporter cassettes (gene *aadA* or *rpsL*), including the *tetAp* promoter, were integrated between *tonB* and *yciA* loci; *tetR* was integrated between *attB* and *bioB* and expressed from the inducible *Pcat* promoter. TIP2 peptide, the allosteric repressor of TetR, was cloned in a vector under the inducible *Ptac* promoter and the *P. aeruginosa* genomic fragments were translationally fused upstream *tip2*. B) Reporter gene expression regulation in the Tet-Trap system. The reporter genes conferring either spectinomycin resistance (*aadA:*GFP gene, upper part; *E. coli* strain C-5907) or streptomycin sensitivity (*rpsL*$^{+}$, lower part; *E. coli* strain C-5920) were cloned under *tetAp* promoter (Delvillani *et al.*, 2014). The C-5920 strain also carries the recessive *rpsL31* gene that confers streptomycin resistance. In both strains, *tetR* is constitutively expressed from *Pcat* promoter. From Delvillani *et al*., 2014.

After a round of Tet-Trap selection, we found four *P. aeruginosa* genes post-transcriptionally regulated by a temperature upshift from 28° to 37°C. We focused our validation analysis on two of them: PA5194, encoding the LpxT protein (see below) and *ptxS*, which codes for a repressor of the LacI family that regulates both operons for gluconate transport and degradation and the expression of toxA gene, encoding the major toxin expressed by *P. aeruginosa* (Swanson *et al.,* 1999; Swanson and Hamood, 2000; Daddaoua *et al.,* 2012). The study of temperature-dependent regulation of *lpxT* in *P. aeruginosa* and *E. coli* has been the object of my research as PhD student.

2 - : The lipopolyshaccaride modification systems

2.1 - The bacterial Lipopolyshaccaride

Gram-negative bacteria, also described as diderms (Gupta, 1998), are surrounded by an essential Outer Membrane (OM) with an important function of selective permeability barrier to both hydrophilic and lipophilic solutes. Protein channels allow the transport of molecules across the OM (Nikaido, 2003). The OM and the cytoplasmic membrane (or Inner Membrane, IM) delimit the periplasm, an aqueous compartment in which the peptidoglycan cell-wall is located (for a review, see Silhavy *et al*., 2010). Despite different OM architectures have been found within diderms, the predominant OM architecture in bacterial world is a bilayer consisting of phospholipids and lipopolysaccharides (LPS) in the inner and outer leaflets, respectively (Nikaido, 2003; Sutcliffe, 2010).

The LPS was first reported as a pyrogenic, not-secreted and heat-labile endotoxin causing fever and disease (reviewed by Caroff and Karibian, 2003). It is an essential molecule in Gram negative bacteria, with the unique known exception of *Neisseria meningitides*, in which it is dispensable (Rittinger *et al.,* 1998). The presence of LPS in the external leaflet of the OM is responsible for the much higher OM impermeability to lipophilic molecules relative to the IM (Nikaido, 2003).

The structure of LPS was finely characterised in enterobacteria like *E. coli* and *Salmonella enterica.* It has a tripartite structure formed by a lipid anchor (the Lipid A) connected to a variable polysaccharide chain (the O-antigen) by an oligosaccharidic core (Raetz *et al.,* 2002). The Lipid A is the lipidic part of LPS and shows less heterogeneity than the O-antigen and oligosaccharidic core. Lipid A biosynthesis is an essential and conserved process in Gram negative bacteria, with the only exception of *Sphingomonas paucimobilis* that produces sphingolipids instead of Lipid A (Kawahara *et al.,* 1991). Indeed, inhibitors of Lipid A biosynthesis are considered potentially good antibiotics (Onishi *et al.,* 1996).

Lipid A is composed by a biphosphorylated β-(1→6)-glucosamine (GlcN) disaccharide backbone substituted with fatty acid chains linked with ester bonds at positions 3 and 3' and amide bonds at positions 2 and 2' (Caroff and Karibian*,* 2003). The length and the number of acyl chains linked to the GlcN backbone is strain-specific and can be modulated by bacteria. The *E. coli* Lipid A is frequently described as an hexa-acylated molecules, although penta- and tetra-acylated species are also present. Indeed, the acylation degree of Lipid A and the number of phosphate groups decorating the GlcN backbone impact on LPS toxicity. For example, *S. enterica* serovar Typhimurium mutants producing a dephosphorylated Lipid A are severely attenuated in a murine model of infection (Caroff and Karibian*,* 2003; Erridge *et al*., 2002; Kong *et al.,* 2012). The oligosaccharide core is divided in two parts. The well conserved inner core is formed by 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) (2→6)-linked to Gln^{II} of Lipid A. One of the two Kdo molecules may be bound to the C-4 position of the first Kdo, which, in *Enterobacteria*, is also linked at its C-5 to three heptose residues (Raetz, 2002; Caroff and Karibian*,* 2003). Phosphate,

pyrophosphate, phosphorylcholine, phosphorylethanolamine groups or other sugars can substitute heptose residues (Nikaido, 2003; Raetz, 2002). The outer core is composed by an oligosaccharide up to six residues long (1→3)-linked to Hep^{II}. It is often branched with glucose, galactose or their derivatives. Its composition varies considerably among bacterial species (Caroff and Karibian, 2003).

The O-antigen is a highly variable region. It is composed by a long oligosaccharidic chain in which more than sixty monosaccharides and thirty non-carbohydrate molecules can be present. Within the same bacterial species different O-antigen molecules are present and define the serological specificity. For example, for *E. coli* 170 different serological types are known (Raetz, 2002). Outer core and O-antigen are not essential. In *E. coli* the minimal LPS portion required for growth is composed by the Lipid A and Kdo. "Deep rough" phenotype is observed in *E. coli* and *Salmonella* mutants lacking the heptose region in the inner core. These mutants show higher sensitivity to hydrophobic molecules, such as hydrophobic antibiotics. On the other hand, some bacterial species, such as *P. aeruginosa*, are not viable without the heptose region (Raetz, 2002).

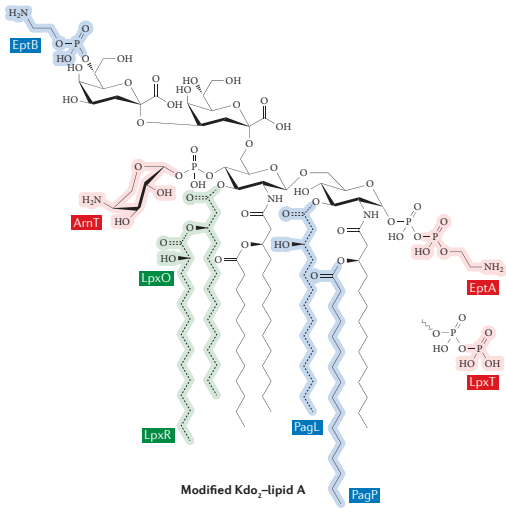

Figure 4: *Salmonella* spp. Lipid A structure and modifications
Modifications highlighted in red are mediated by enzymes (indicated within the boxes) activated by either the Pmr (red), or the Pho TCS (blue). In green modifications controlled by unknown regulators are represented. From Needham and Trent, 2013.

Anionic groups in Lipid A and core region have an important role in membrane stability. Bivalent cations, such as Ca^{2+} and Mg^{2+}, bind to phosphate groups and form a cationic bridge between LPS molecules. This neutralises the phosphate negative repulsion and stabilises the OM (Nikaido, 2003; Raetz, 2002).

2.2 - Lipid A modifications

Lipid A can be covalently modified during its transport to the OM in response to different environmental stimuli. Besides variations of its acylation

and phosphorylation degree, small molecules like phosphoethanolammine or 4-amino-4-deoxy-L-arabinose (Ara4N) can be linked to the glucosamine backbone. Although the physiological meaning of these modifications for free living bacteria is in most cases unclear, it is well known that Lipid A decoration has a deep impact in the interaction between bacteria and the host immune system (reviewed by Raetz *et al*., 2007).

Picomolar levels of Lipid A are detected by Toll-Like Receptor 4 (TLR4) present on the surface of macrophages and endothelial cells of the mammalian immune system. Lipid A bound to TLR4 triggers the biosynthesis of molecules that stimulate inflammation and adaptive immune response, like TNF (Tumor Necrosis Factor) and Interleukin-1β, which are involved in clearance of and tolerance to bacterial LPS (Needham and Trent, 2013). However, prolonged production of these mediators causes cell damage and septic shock (Parrillo, 1993). The *E. coli* Lipid A features that elicit full TLR4 activation are the presence of six fatty acid chains and two phosphate groups in position 1 and 4' of the glucosamine disaccharide backbone. On the other hand, Lipid A modifications can have a strong impact on the recognition by the TLR4 receptor and on the activation of inflammation (Rietschel *et al.,* 1994).

The Two Component System (TCS) PhoPQ is involved in the regulation of Lipid A decoration. This system is present in a large number of bacteria with conserved function and it has been deeply studied in *Salmonella* (for a review, see Prost and Miller, 2008; Groisman, 2001; Groisman *et al.,* 1989). PhoQ is the inner membrane sensor kinase. It responds to low Mg^{2+} and Ca^{2+} concentrations, mild acid pH and presence of antimicrobial peptides in the environment. Interestingly, these conditions are found in macrophage phagosomes (Apulche-Aranda *et al.,* 1992; Bader *et al.,* 2005; Gibbons *et al.,*

2005; Garcia Vescovi *et al.,* 1996; Miller *et al.,* 1989). Antimicrobial peptides (AP) are cationic, amphipathic molecules that promote membrane permeabilisation and bacterial death by binding the negatively charged LPS and forming pores within the membranes. These molecules are important components of the innate immunity and are ubiquitous in nature (Zasloff, 1992).

The PhoP response regulator, upon activation by PhoQ, regulates at least 1% of *Salmonella* genes (Miller *et al.,* 1990). Genes activated by PhoP include the regulon for Mg^{2+} concentration adaptation, genes essential for bacterial virulence and survival within the phagosome and genes involved in Lipid A modification (Fields *et al.,* 1989; Miller *et al.,* 1989; Groisman, 2001; Gunn *et al.,* 1998; Kim *et al.,* 2006; McPhee *et al.,* 2006; Guo *et al.,* 1997; Raetz, 2007; Needham and Trent, 2013). The latest set of genes includes PagP and PagL. PagP catalyses the addiction of palmitate on the acyl chain in position 2 of Lipid A (Guo *et al.,* 1997; Guo *et al.,* 1998; Bishop *et al.,* 2000), a modification that increases antimicrobial peptide resistance (Guo *et al.,* 1998). On the other hand, PagL catalyses the deacylation of *R*-3-hydroxymyristate moiety linked to position 3 of Lipid A (Trent *et al.,* 2001a; Figs. 3 and 4).

Other enzymes involved in acyl chains modifications, but not regulated by the PhoPQ system, are i) the deacylase encoded by *lpxR* gene and active on Lipid A 3' position (Reynolds *et al.,* 2006). It was shown that deacylation of Lipid A helps pathogens to evade innate immune recognition by TLR4 (Kawasaki *et al.,* 2004; Kawano *et al.,* 2010); ii) LpxO, an enzyme that catalyses the hydroxylation of secondary acyl chains in position 3' (Gibbons *et al.,* 2000; Gibbons *et al.,* 2005).

Other Lipid A modifications are directly activated by another TCS, namely PmrAB, which is conserved in several Gram-negative bacteria (Gunn, 2008). PmrB activates its cognate response regulator PmrA by responding to mild acid pH and high concentration of Fe^{3+} and Al^{3+} in the environment (Wösten *et al.,* 2000; Perez *et al.,* 2007; Nishino *et al.,* 2006). In *Salmonella,* PmrA is also activated in a PhoPQ dependent way by PmrD, a small peptide that physically blocks PmrB-dependent PmrA dephosphorilation (Kox *et al.,* 2000; Luo *et al.,* 2013). A recent study demonstrated that *pmrD* can inhibit PmrA dephosphorilation also in *E. coli* (Rubin *et al.,* 2015). PmrA is responsible for the activation of different genetic loci that mediate Lipid A decoration with the two positively charged groups Ara4N (Trent *et al.,* 2001b; Gunn *et al.,* 1998) and phophoetanolammine (pEtN) (Lee *et al.,* 2004). The *pmrE* and *pmrF* loci are necessary for the biosynthesis and addition of Ara4N in position 4' of Lipid A (Trent *et al.,* 2001b; Gunn *et al.,* 1998). On the other hand, pEtN modification is transferred in position 1 by a transferase encoded by the *pmrC* gene (Lee *et al.,* 2004; Fig. 3, Fig. 4).

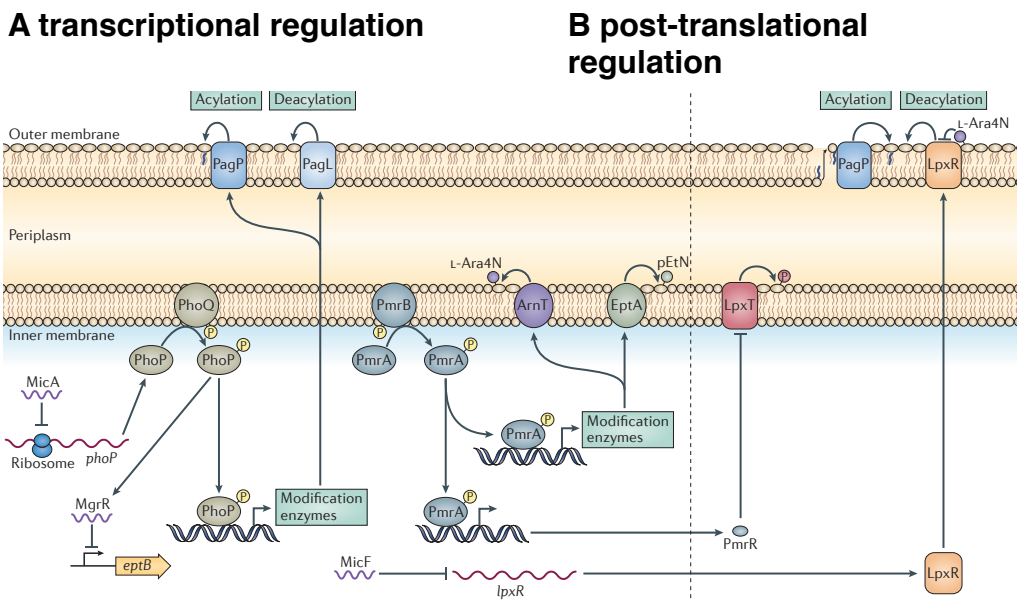

Figure 4: Regulation of Lipid A decorating enzymes
A) Transcriptional regulation by PhoPQ and PmrAB TCSs. Enzymes and sRNAs activated by the TCSs are reported.
B) Post-translational regulation. The small peptide PmrR binds LpxT causing the inhibition of Lipid A 1-PP modification. From Needham and Trent, 2013.

These modifications reduce the negative charge of Lipid A and hence APs and metal ions (such as Fe^{3+} and Al^{3+}) binding on cell surface (Nishino *et al.,* 2006; Wösten *et al.,* 2000). Constitutive activation of PmrAB results in dramatically increased resistance to APs (Roland *et al.,* 1993), whereas *pmrA* mutants show markedly reduced virulence and APs resistance (Gunn *et al.,* 1998; Lee *et al.,* 2004; Gunn *et al.,* 2000; Warner *et al.,* 2013). In agreement with these observations, some Gram-negative pathogenic bacteria intrinsically resistant to the AP polymyxin B show a Lipid A profile completely substituted with Ara4N in position 4' (Hase and Rietschel, 1977; Sidorczyk *et al.,* 1983; Cox and Wilkinson, 1991; Vaara, 1992).

BOX. 1- Two Component Regulatory Systems

Two-Component regulatory Systems (TCSs) are the main bacterial signal transduction systems. Typically, they are able to sense and transduce specific environmental stimuli within the cell, ultimately activating or repressing the expression of specific genes involved in the response to the signal. In their most simple configuration, TCS are composed of two elements: a sensor kinase and a response regulator. The sensor kinase is typically an inner membrane protein that catalyses its auto-phosphorylation on a histidine residue upon receiving the input signal. Then, the phosphate group is transferred to an aspartate residue of the cognate response regulator, which usually acts as transcriptional regulator and controls the output response. TCSs can sense hundreds of different stimuli and have been founded in all known bacteria (for a review, see Laub, 2011).

2.3 - The inner membrane protein LpxT

Another Lipid A decoration widespread in Gram-negative bacteria, including *N. menengitidis, Y. pestis* and *P. aeruginosa* (Cox *et al.,* 2003; Jones *et al.,* 2008; John *et al.,* 2009), is the pyrophosphorylation of Lipid A in position 1 (Lipid A 1-PP). In *E. coli, Salmonella* and *P. aeruginosa* this modification has been attributed to LpxT activity (Touzé *et al.,* 2008; Kato *et al.,* 2012; Nowicki *et al.,* 2014; Fig. 5). However, in *P. aeruginosa* LpxT shows a somehow divergent function, as it can add a phosphate group also in position 4' (Ernst, 1999) and an additional phosphate in position 1, creating a Lipid A 1-PPP species (Nowicki *et al.,* 2014).

LpxT transfers the distal phosphate group of undecaprenyl pyrophosphate (C_{55}-PP) to the Lipid A position 1, thus originating the Lipid A 1-PP species, which represents about 1/3 of Lipid A species in the *E. coli* OM (Raetz *et al*, 2007; Touzé *et al*, 2008; Tatar *et al.,* 2007; Zhou *et al.,* 1999).

The undecaprenyl phosphate (C_{55}-P) is an essential lipid carrier required for the biosynthesis of various bacterial cell wall polymers such as LPS and peptidoglycan (Stevenson *et al.,* 1994; van Heijenoort, 2001). It is synthesised at the cytosolic face of the IM by the UppS synthase. C_{55}-P carries hydrophylic molecules across the IM; after substrate unloading in the periplasm, the carrier is released as C_{55}-PP. In order to be recycled for another round of transport, C_{55}-PP must be dephosphorylated by C_{55}-PP phosphatases (Bouhss *et al.,* 2008). LpxT, together with BacA, YbjG, PgpB, is one of the four *E. coli* C_{55}-PP phosphatases implicated in C_{55}-P recycling (El Ghachi *et al.,* 2005; Tatar *et al.,* 2007). C_{55}-PP dephosphorylation is an essential process in *E. coli* (Bouhss *et al.,* 2008). In fact, although *E. coli* mutants devoid of a single C_{55}-PP phosphatase are viable, mutants lacking all four C_{55}-PP phosphatases, or in which only LpxT is present, are lethal. This suggests that LpxT activity is not sufficient to sustain C_{55}-P recycling. Conversely, LpxT is the only C_{55}-PP phosphatase whose over-expression is toxic for the cells (El Ghachi *et al.,* 2005; our unpublished data). The physiological meaning of double LpxT activity as C_{55}-PP phosphatase and Lipid A kinase, and in particular of Lipid A phosphorylation, is still to be elucidated, but it reveals an unexpected connection between C_{55}-P recycling and Lipid A decoration (Touzé *et al.,* 2008).

Interestingly, an *E. coli lpxT* deletion mutant showed Lipid A modified with pEtN in position 1 also in non-inducing conditions for PmrAB, suggesting

that in wild type cells, LpxT-promoted phosphorylation may compete with pEtN addition (Herrera *et al.,* 2010). In *Salmonella* the small inner membrane peptide PmrR, whose expression is PmrA-dependend, directly binds to LpxT, inhibiting its activity and causing a decrease in Lipid A 1-PP species (Kato *et al.,* 2012; Herrera *et al.,* 2010). LpxT inhibition, together with PmrAB-promoted Lipid A decoration with Ara4N and pEtN, decreases the total negative charge of OM and hence the amount of Fe^{3+} bound on cell surface. This in turn switches off PmrB activation.

This negative feedback loop leads to a continuous adjustment of cell surface properties, and in particular of the total OM charge (Kato *et al.,* 2012). It has been proposed that this may represent a mechanism to modulate the ability of the cell to bind iron, an essential nutrient but also a toxic molecule if in excess, and thus to contribute to iron homeostasis (Wösten *et al*., 2000).

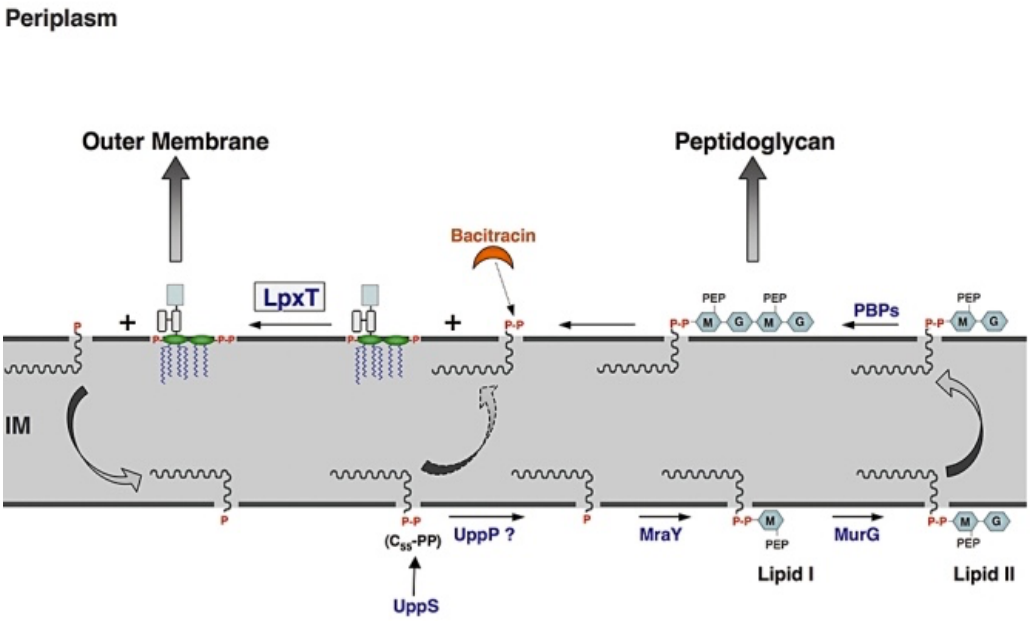

Figure 5: Modification of Lipid A mediated by LpxT and its involvement in C55-P recycling.
Overview of C55-P recycling during the synthesis of peptidoglycan. After the transport across the IM of C55-PP linked to peptidoglycan monomers, the polymerisation of peptidoglycan begins thanks to penicillin-binding proteins (PBPs). This results in generation of free C55-PP. LpxT dephosphorylates C55-PP and transfers the phosphate group to position 1 of Lipid A, generating Lipid A 1-PP and contributing to the recycling of C55-P. From Touzé *et al.,* 2008.

Aim of the Project

As described in the "State of the art" section, we developed a new genetic tool, i.e. the Tet-Trap, to identify *in vivo* new RNATs. We applied this system to the opportunistic pathogen *Pseudomonas aeruginosa,* where only an RNAT was known at the time of our analysis. By using this system, we identified four new putative RNATs and two of these, *ptxS* and *lpxT,* were chosen for validation.

The **first part of my project** was focused on the *in vivo* and *in vitro* validation of the putative RNAT regulating the expression of *lpxT* in *P. aeruginosa.* To this goal, I set up different plasmid reporter systems in *P. aeruginosa* and *E. coli* with the **specific aims** of i) testing whether the expression of LpxT was actually thermoregulated at post-transcriptional level; ii) identifying the determinants of temperature-dependent regulation. I*n vitro* structural probing was also applied in order to define the secondary structure of the *lpxT* 5'-UTR at different temperatures.

On the whole my results confirmed that *P. aeruginosa lpxT* expression is repressed at low temperature (25°-28°C) and that a secondary structure involving the TIR is responsible of thermal regulation. This prompted us to investigate in the **second part of my project** whether thermoregulation acting on *P. aeruginosa lpxT* gene expression was conserved also for the orthologous *E. coli lpxT* gene (Ec*lpxT).*

The **specific aims** of this second part were i) to assess whether the expression of EcLpxT responded to temperature changes; ii) to ascertain whether thermoregulation operated at transcriptional and/or post-transcriptional level; iii) to identify the *in cis* determinants of regulation; iv) to clarify the molecular mechanism of EcLpxT thermal regulation.

Main Results

In this section I will recapitulate the main results presented:

A. in the paper (attached in Part II):

Delvillani, F., **B. Sciandrone**, C. Peano, L. Petiti, C. Berens, C. Georgi, S. Ferrara, G. Bertoni, M. E. Pasini, G. Dehò and F. Briani, 'Tet-Trap, a Genetic Approach to the Identification of Bacterial RNA Thermometers: Application to *Pseudomonas aeruginosa*', *RNA*, 20 (2014), 1963–76; doi.org/10.1261/rna.044354.114

B. in the draft manuscript (attached in Part III):

Sciandrone, B., S. Perego, C. Portugalli, A. Rota, F. Briani, 'Temperature responsive regulation of *Escherichia coli lpxT* is controlled by an RNA thermometer based on an unstable mRNA secondary structure and a sub-optimal Shine-Dalgarno sequence'.

A - Regulation by temperature of the *lpxT* gene in *Pseudomonas aeruginosa*

In the attached paper we describe a genetic tool, i.e. the Tet-Trap, which we developed for the identification of post-transcriptionally regulated genes, and its application to the research of RNATs in the opportunistic pathogen *P. aeruginosa.* The rationale is that genes specifically expressed at 37°C and not at lower temperatures may be involved in the infection of the warm-blooded human host. With our genetic tool, we screened a plasmid library of *P. aeruginosa* 5'-UTRs driving downstream gene expression at 37°C in order to identify those inactive at low temperature (28°C). After a round of Tet-Trap, we identified four new putative RNA thermosensors regulating: *ptxS*, a negative transcriptional regulator of genes of gluconate catabolism involved also in the regulation of virulence genes, such as the gene encoding the exotoxin ToxA (Swasson *et al.,* 1999); *lpxT*, encoded by the PA5491 gene; *dsbA,* a periplasmic protein assisting the folding of secreted proteins, among which also toxins, in many bacterial pathogen including *P. aeruginosa* (Peek and Taylor, 1992; Stenson and Weiss, 2002; Urban *et al.,* 2001; Ha *et al.,* 2003); and PA1031, a gene with unknown function. To validate the results of the Tet-Trap and get insights into the regulatory mechanism controlling temperature response, we further analysed two candidates: *ptxS* and *lpxT.*

My contribution to this work was to analyse the putative thermosensor present in the 5'-UTR of *lpxT*. To do this, I set two constructs, one carrying the whole *P. aeruginosa* region originally found in the *P. aeruginosa* Tet-TRAP

library and the other by cloning a DNA fragment with the same 3'-end but starting with the first nucleotide transcribed from *lpxT* promoter (Dotsch *et al.*, 2012). I mapped it by primer extension 49 nt upstream of the ATG of the ORF. Since LpxT is predicted to be a membrane protein and has a localization signal at the N-term, we used as reporter for translational fusions the sfGFP, a variant of the GFP that should re-fold after translocation through the membrane (Dinh and Bernhardt, 2011) and monitored the expression of the fusions by fluorescence evaluation. A control fusion of the 5'-UTR and first 9 codons of the *E. coli recA* gene with the sfGFP was also assayed. The constructs were cloned in an *E. coli-P. aeruginosa* shuttle vector and were all transcribed from the *araBp* vector promoter. The fluorescence was monitored upon transcription induction in *E. coli* and *P. aeruginosa* at 28° and 42°C (*E. c.*), or 28°, 37° and 40°C (*P. a.*).

In both bacteria, the two *lpxT* constructs conferred thermally inducible fluorescence to the cells. Thermoregulated expression of LpxT-sfGFP was also confirmed by visualizing the cells by fluorescence microscopy. Moreover, since the *lpxT* 5'-UTR region was sufficient to confer thermoregulation to the reporter gene, these results demonstrated that the regulation determinants must lie in such region.

I also analysed the expression in *P. aeruginosa* of the full-length *lpxT* gene with an HA tag inserted immediately before the stop codon, and also in this case I observed that the expression of the LpxT protein was barely detectable at 25°C and strongly induced at 37° and 40°C, whereas the mRNA was equally expressed at all temperatures.

The 5'-UTR of *lpxT* was predicted to fold into a stem-loop involving the Shine-Dalgarno and the AUG of the gene. To validate such prediction, we

analysed *in vitro* the secondary structure of the *lpxT* mRNAs by partial enzymatic and chemical digestion at different temperatures. Since the results of structural probing were compatible with different predicted secondary structures, I mutagenized the putative anti-SD (i.e. the sequence predicted to pair with the SD in the *lpxT* stem) in the GFP reporter plasmid to test its role in *lpxT* regulation. Different mutations were introduced, predicted to either stabilize or destabilize the inhibitory stem, and fluorescence was assayed at different temperatures. On the whole, my data were compatible with regulation of *lpxT* expression by a thermolabile stem-loop sequestering the TIR and suggested that such structure may act by modulating the start codon accessibility.

B - Regulation by temperature of the *lpxT* gene in *Escherichia coli*

In the second part of my PhD training, I focused on *E. coli lpxT* gene regulation. In particular, I investigated whether the *E. coli lpxT* orthologue (hereafter *Ec lpxT)* is also regulated in a temperature-dependent manner. To this aim, I created an *E. coli* recombinant strain whit the *lpxT* chromosomal gene tagged at the C-term with the GFP reporter and I analysed the $LpxT_{GFP}$ protein expression by Western blotting. I found that $LpxT_{GFP}$ expression was strongly induced at high temperature (i.e. 37° and 42°C *vs*. 28°C). Surprisingly, I observed that both the wt *lpxT* and the *lpxT:GFP* mRNA were less abundant and less stable at 42° than at 28°C. This was a surprising observation as translated mRNA are usually more stable than untranslated ones in *E. coli* (Dreyfus, 2009).

By analyzing the sequence immediately upstream of the transcription start site (TSS), we noticed the TAAGGT sequence centered at -10 respect to the TSS. Such sequence is present in a small number of temperature-sensitive σ^D-dependent promoters of *E. coli* (Repoila *et al.,* 2003). By means of transcriptional fusion, we could demonstrate that the 49 pb long region immediately upstream of the *lpxT* TSS indeed contains a temperature sensitive promoter. On the whole, these data ruled out the possibility that thermal induction of LpxT was a consequence of temperature-dependent *lpxT* transcription.

Thermo-responsive LpxT expression was observed also for $LpxT_{HA}$ variant (i.e. LpxT terminally fused with the HA epitope) encoded by a plasmid carrying the *lpxT:HA* gene with the physiological *lpxT* 5'-UTR and under the transcriptional control of the heterologous *araBp* promoter. On the contrary, cells harbouring a control plasmid, in which the 5'-UTR of *lpxT* was replaced with the *recA* 5'-UTR, expressed the LpxT protein at the same level at all the tested temperatures, ruling out post-translational regulation.

Overall, these results indicate that temperature-dependent LpxT expression is achieved through post-transcriptional mechanisms. Bioinformatic prediction of the *lpxT* 5'-UTR secondary structure suggests that it can form an unstable stem-loop involving the SD sequence at low temperatures. Such structure may exert an inhibitory effect on ribosome loading. This was confirmed by the results of toeprinting assays performed at 28° and 42°C, which showed that the assembly of the 30S on the *lpxT* translation initiation region is controlled by temperature in the absence of any additional cellular factor, suggesting that an RNAT may modulate the translation of *lpxT* mRNA.

To assess whether the 5'-UTR was sufficient to confer thermoregulation to heterologous genes, such as the *gfp* reporter, we assayed fluorescence expression by a plasmid construct carrying a translational fusion between the 5'-UTR and first two codons of *lpxT* and the GFP reporter gene, under the control of the heterologous *araBp* promoter. The results of our analysis clearly showed that the *lpxT* 5'-UTR is sufficient to impair the expression of an unrelated gene at low temperature.

The *lpxT* secondary structure has a predicted ΔG of -4.3 kcal/mol at 28°C. It has been claimed that structures with ΔG higher than -5 to -6 kcal/mol should not be able to prevent ribosome binding and thus to reduce translational

efficiency. However, this threshold may not apply to mRNAs with low intrinsic affinity for the 30S. The *lpxT* mRNA belongs to this category as the ORF is preceded by a 3 nt-long, suboptimal SD. We reasoned that because of such feature, even a weak stem-loop like the *lpxT* secondary structure may outcompete the 30S at low temperature and thus affect *lpxT* mRNA translation.

To test this hypothesis, we introduced mutations in *lpxT* 5'-UTR and we cloned these *lpxT* 5'-UTR variants in the same plasmid described above for the fluorescence assay. The mutations were predicted to i) stabilize the *lpxT* secondary structure, ii) destabilize the *lpxT* secondary structure, iii) improve the *lpxT* translation initiation region by increasing the SD complementarity with the 16S anti-SD. Cultures of bacterial strains carrying these plasmids were growth at 28°, 37° and 42°C, and the fluorescence was tested in exponential growth phase. As for stabilizing mutations, we eliminated the bulge in the left arm of the stem and replaced a U·G base pair with a more stable C·G in the stem. As expected, both mutations caused a decrease in fluorescence at all the temperatures. The destabilizing mutations were predicted to increase GFP expression at low temperature and to be silent at high temperature. Two of the three mutants carrying such mutations showed the expected phenotype, whereas the third one had no phenotypic difference respect to the wt. To improve the SD-16S complementarity, we introduced two nucleotide substitutions creating mutants SD1 and SD2. Based on thermodynamic considerations (see Part III), we expected that the mutations may increase fluorescence at both temperatures, but in particular at 28°C. Indeed, a remarkable GFP expression increase was observed at 28°C (around 4- for mutant SD1 and 10-fold for the mutant SD2), whereas GFP activity raised only 2- 3-fold at 42°C. When we combined the

SD2 mutation with one of the destabilizing mutations, thermoregulation was essentially abolished.

These data support the hypothesis that the *lpxT* 5'-UTR secondary structure, albeit unstable, is able to exert an inhibitory effect on *lpxT* translation at low temperature.

Discussion

I draw here some general conclusions about the results that I have presented in this thesis, referring to the Discussions of the article in Part II and the manuscript in Part III for a more accurate treatment of the points arose by my research.

The work presented in this thesis shows that the *lpxT* gene expression is modulated at post-transcriptional level in response to temperature changes both in *E. coli* (Ec) and *P. aeruginosa* (Pa). In spite of the lack of 5'-UTR sequence and secondary structure conservation, such region is the site where regulation occurs in the two systems. In both bacteria, the SD sequence is paired with a stretch of uridines, a typical trait of FourU elements. However, both the Ec and the Pa RNATs are unconventional either for the relative position of regulatory elements or because of their peculiar features. Indeed, in Pa the Us' stretch, which is usually located upstream of the SD, is placed between the SD and the start codon. On the other hand, the Ec RNAT seems to rely on the combination of sub-optimal elements, so that the relative instability of the stem engaging the SD is compensated by poor SD affinity for the 30S ribosomal subunit, leading to translation impairment at low temperature.

For many RNATs, the mRNA expression level and stability are not affected by poor translation at low temperature, probably because of the protection against nucleases provided by the secondary structure of the RNAT itself (Narberhaus, 2002). This seems to apply also to Pa*lpxT* RNAT (Delvillani et al., 2014). Interestingly, the Ec*lpxT* RNAT seems to be unusual also in this respect, as the mRNA is less expressed and less stable at 42°C, when translation is more efficient. This is a puzzling result because translated mRNAs are usually more stable than untranslated ones (Deana and Belasco, 2005). We are currently investigating the molecular bases of this phenomenon that involves

also the 3'-UTR of the *lpxT* gene (see Part III). Whatever the mechanism could be, the interplay between translation and stability of *lpxT* mRNA should limit the extent of LpxT induction both temporally, with the highest expression in the acclimation phase to the temperature upshift, and quantitatively, thus avoiding excessive expression of LpxT, which is toxic when overexpressed (El Ghachi *et al*., 2005; data not shown).

Although the assembly of translation initiation complex on Ec*lpxT* mRNA responds to the temperature also in a purified system, it is possible that in *vivo* *trans*-acting factor may participate in the overall mechanism of temperature–responsive regulation. Our preliminary results point to an involvement of Hfq in the modulation of RNA stability (data not shown).

Regulation by RNATs represents a convenient strategy to achieve fast thermal induction of gene expression. Although the physiological meaning of LpxT activity is still unclear, the observation that, in spite of lack of 5'-UTR sequence conservation, the *lpxT* orthologous genes of *P. aeruginosa* and *E. coli* share this regulatory strategy, suggests that bacterial fitness may benefit from fast modulation of Lipid A phosphorylation degree on the environmental temperature. This consideration is strengthened by the observation of Lipid A phosphorylation in response to a modest temperature upshift (from 37°C to 39-41°C) in *P. gingivalis* (Curtis *et al.,* 2011), a bacterium belonging to the phylum of Bacteroidetes, which is separated by ca. 3 billion years from Proteobacteria (www.timetree.org).

The physiological role of Lipid A pyrophosphorilation is unclear and this makes difficult to rationalize the meaning of temperature-dependent expression of LpxT. Albeit this modification has been mainly studied in bacteria that can thrive in the human host, in the environmental psychotropic bacterium *P.*

syringae the LPS is also phosphorylated both on the Lipid A moiety and on the core oligosaccharide region in response to a temperature upshift (from 0° to 22°C; Ray *et al.,* 1994). This suggests that increasing the degree of LPS phosphorylation may be instrumental in the adaptation to environmental stimuli, not (or at least not only) related with the infection of the mammal host. Negative charges in the LPS should decrease the OM stability because of electrostatic repulsion between flanking LPS molecules. However, enhanced binding of divalent cations such as Mg^{2+} or Ca^{2+} could have the opposite effect, leading to increased rigidity thanks to cationic bridges between LPS molecules (Nikaido *et al.,* 2003). Since high temperature enhances membrane fluidity, inducing LpxT expression in presence of such ions could contribute to the maintenance of membrane stability at high temperature.

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PART II

Content: Published article:

Delvillani, Francesco, Barbara Sciandrone, Clelia Peano, Luca Petiti, Christian Berens, Christiane Georgi, Silvia Ferrara, Giovanni Bertoni, Maria Enrica Pasini, Gianni Dehò and Federica Briani

'Tet-Trap , a Genetic Approach to the Identification of Bacterial RNA Thermometers : Application to Pseudomonas Aeruginosa',

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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 in *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 trigger 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; Giuliodori 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 Tn*10 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

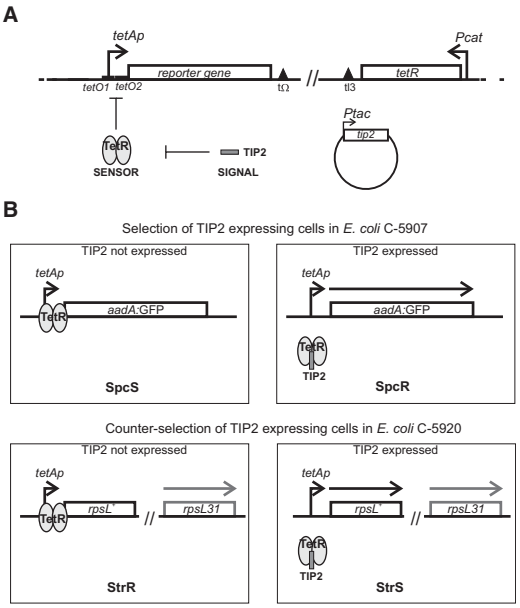

FIGURE 1. *Escherichia coli* reporter strains of Tet-Trap system. (*A*) Outline of Tet-Trap system and regulatory elements. Repression by TetR (the sensor) is relieved by TIP2 expression (the signal) and this triggers the transcription of the reporter gene controlled by *tetAp*. The reporter cassette is integrated between the *tonB* and *yciA* loci (nucleotides 1309870–1309872); the *tetR* cassette between *attB* and *bioB* (806594–808521). Details about strain construction are reported in Materials and Methods. Construct elements are represented by an arbitrary scale. Empty boxes, open reading frames; bent arrows, promoters; triangles, intrinsic bidirectional transcription terminators. (*B*) Reporter gene expression regulation in the Tet-Trap system. The reporter strains carry genes conferring either spectinomycin resistance (*aadA*:GFP gene; *upper* part, strain C-5907) or streptomycin sensitivity ($rpsL^+$; *lower* part, strain C-5920) downstream from the *tetAp* promoter. In C-5920, the endogenous *rpsL* allele carries the *rpsL31* (streptomycin-resistance) recessive mutation. In both strains, the *tetR* gene is constitutively expressed from P_{cat}, and transcription from *tetAp* is repressed unless TIP2 is expressed. (Gray ovals) TetR; (dark gray bar) TIP2 peptide; (thick arrows) mRNAs. Other symbols are as in *A*.

strain (Lederberg 1951). Expression of TIP2-tagged polypeptides from control plasmids renders the strains C-5907 and C-5920 spectinomycin-resistant and streptomycin-sensitive, respectively (data not shown and Fig. 2).

Construction of a *P. aeruginosa* 5′-UTR-enriched genomic library

As first step toward the identification of *P. aeruginosa* RNATs, we constructed a *P. aeruginosa* genomic library enriched for 5′-UTR-TRs, where regions controlling translation initiation usually map in bacteria. Random fragments of PAO1 and PA14 genomic DNA ranging in length between 300 and 800 bp were pooled and cloned in pGM957 (Fig. 2A). This plasmid carries an artificial gene encoding the $(SG_4)_5$:TrxA:TIP2 chimeric polypeptide (hereafter indicated as ST-TIP2) downstream from P_{tac}. *Pseudomonas aeruginosa* DNA fragments were inserted between P_{tac} and ST-TIP2. To select for cells expressing TIP2-tagged peptides, the *aadA:*GFP reporter strain (C-5907) was transformed with the pooled PAO1 and PA14 genomic library, and spectinomycin-resistant transformants were selected in the presence of IPTG. Transformants were also plated in permissive conditions (without spectinomycin or IPTG) to estimate the coverage of the genomic library. As reported in Table 1, the library reached >99% coverage of the combined PAO1 and PA14 genomes. About 1.7% of all transformants (~48,000 clones) were SpcR; these clones were expected to express ST-TIP2 and thus constituted the translational fusion library.

To estimate the coverage of the enriched library, we pooled all SpcR clones, extracted their plasmid DNA, and amplified the genomic inserts by PCR for 454-pyrosequencing. The reads generated by pyrosequencing were mapped on the PAO1 and PA14 genomes. We found that ~60% of the *P. aeruginosa* annotated coding genes (61.5% and 58.1% for PAO1 and PA14, respectively) were represented in the translational fusion library. For 2389 coding genes (69.7% of all genes represented in the library), the cloned region encompassed the 5′ UTR and the beginning of the annotated ORF.

Given the high genomic coverage of the library, we expect that missing genes may belong essentially to the following categories: (1) genes physiologically poorly translated; (2)

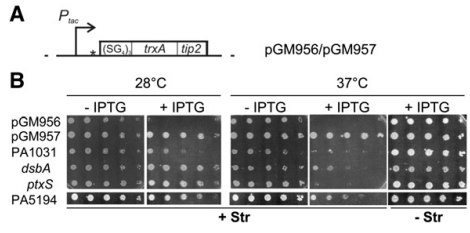

FIGURE 2. Plating efficiency at different temperatures of clones carrying putative RNATs in TIP2 fusions. (*A*) Map of the plasmid constructs encoding ST-TIP2. ST-TIP2 is composed by a flexible $(SG_4)_5$ linker at the N-terminus, followed by an *E. coli* TrxA-Tip2 fusion that effectively induces TetR (Goeke et al. 2012). pGM956 and pGM957 differ by the presence in the former of an in-frame TIR driving translation of ST-TIP2, which is absent in pGM957. Details about plasmid construction and coordinates of the cloned regions are reported in Materials and Methods. (Dotted lines) vector sequence; (bent arrows) IPTG-inducible promoter P_{tac}; (open box) ST-TIP2 ORF; (star) SmaI restriction site exploited for *P. aeruginosa* DNA cloning in pGM957. (*B*) Thermosensitive-streptomycin resistance upon induction of TIP2-tagged protein expression. Serial 10-fold dilutions of C-5920 overnight cultures carrying putative *P. aeruginosa* RNATs cloned upstream of ST-TIP2 in pGM957 were replicated on LD-chloramphenicol plates in the presence or absence of streptomycin (Str) and IPTG and incubated for 16–20 h at the indicated temperatures. C-5920 carrying pGM956 and pGM957 were used as positive and negative controls, respectively, of ST-TIP2 dependent $rpsL^+$ expression.

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

Library size[a]	2.8×10^6
P[b]	>99%
Translational fusions[c]	4.8×10^4

[a]The total number of clones constituting the genomic library, as estimated by plating an aliquot of the transformants on chloramphenicol. Of note, 2.0×10^3 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 1.3×10^7 bp approximately (the sum of the PAO1 and PA14 genomes that were considered to be unrelated in this calculation).
[c]Clones 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 $\geq 10^5$ 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- *ptxS* (+; 1)	Codons 1–53 of *ptxS*
3	1	5846939–5847277	*yrfI* (–; 2) -IR-PA5194 (+; 4)	Codons 1–49 of PA5194
4	1	6181479–6181766[e]	*cc4* (–; 2)-IR-*dsbA* (–; 2)	Codons 1–34 of *dsbA*

[a]Number of sequenced clones carrying the insert.
[b]Coordinates refer to GenBank accession number NC_002516.
[c]Intergenic 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).
[d]The 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.
[e]Region 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).

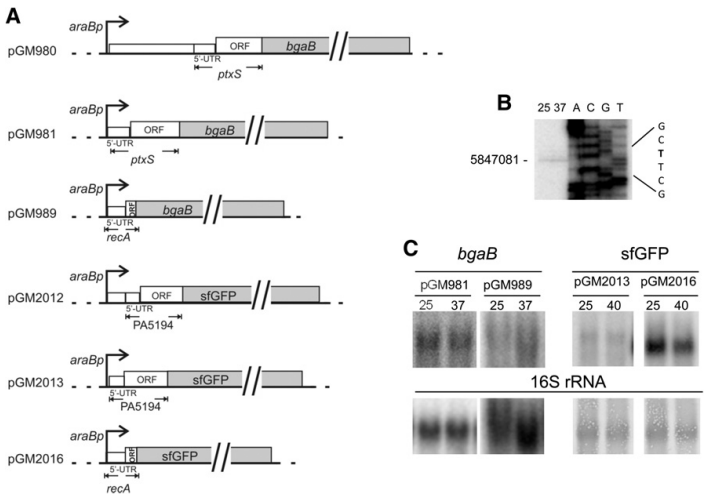

FIGURE 3. Constructs used for validation of putative RNATs and transcriptional analysis. (*A*) Map of plasmids encoding PtxS-BgaB and PA5194-sfGFP. Control plasmids carrying the 5′ end of the *E. coli recA* gene in frame with the reporter genes are also represented. Details about plasmid construction 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 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. 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 PA01 strain carrying the plasmids indicated *above* the lanes were grown at 25°C up to $OD_{600} = 0.5$. 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.

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 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

	E. coli[a]			*P. aeruginosa*[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.
[b]Induction 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

	E. coli[a]			*P. aeruginosa*[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

[a]sfGFP 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.
[b]Induction Factor of sfGFP activity at high versus low temperature. The values are the average of at least two independent determinations. (na) not applicable.

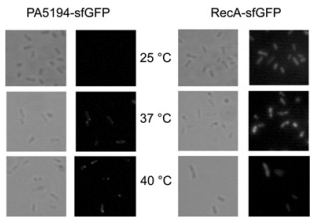

FIGURE 4. Temperature-dependent expression of PA5194-sfGFP by *P. aeruginosa* cells. Images of PAO1 cells carrying plasmids pGM2013 (PA5194-sfGFP) or pGM2016 (RecA-sfGFP) were acquired on a Leica fluorescence microscope. The cells were sampled from cultures grown at the temperatures indicated *between* 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

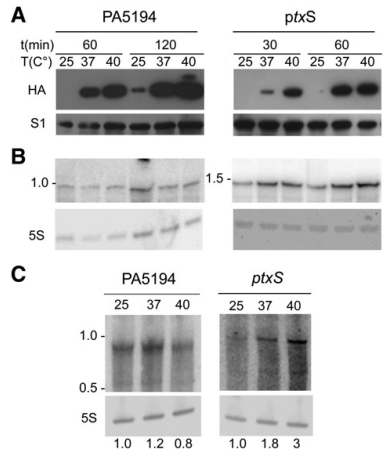

FIGURE 5. Temperature-dependent expression of PA5194 and *ptxS* in *P. aeruginosa*. Proteins (*A*) and RNA (*B*) were extracted upon induction with 0.1% arabinose, as detailed in Materials and Methods, from cultures of PAO1 carrying either pPA5194-HA (*left*) or pPtxS-HA (*right*). Time and temperature 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.

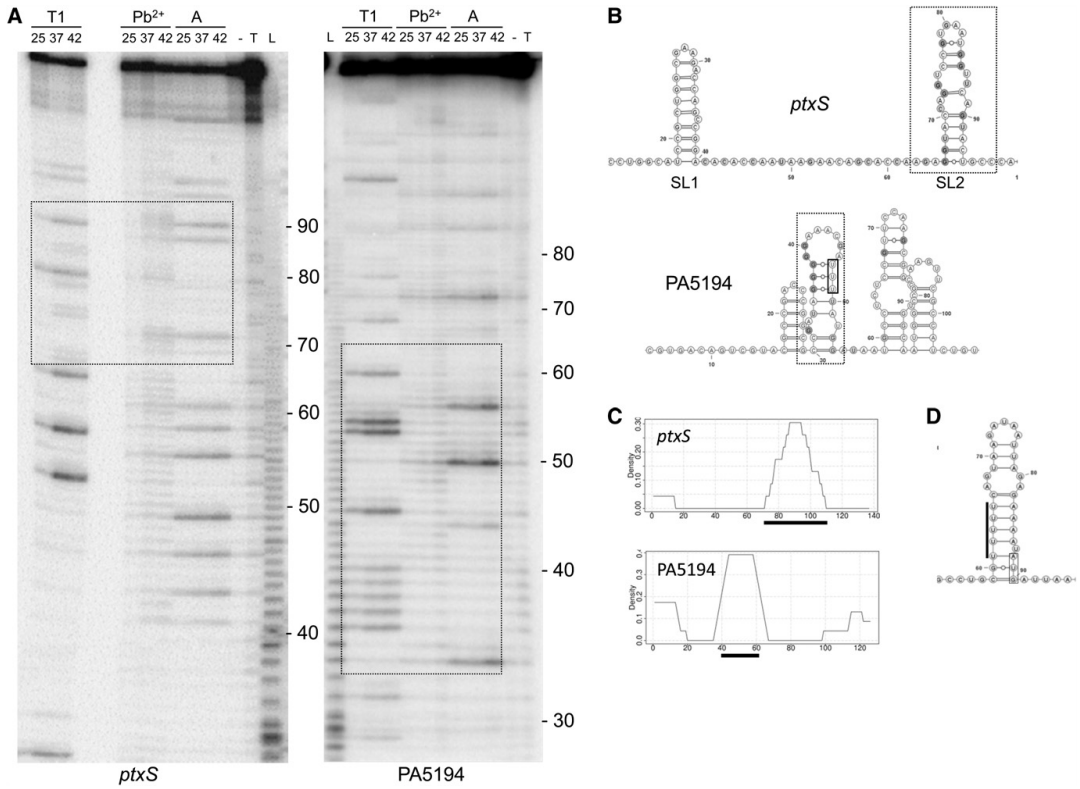

FIGURE 6. In vitro and in silico analysis of the secondary structure of the *ptxS* and PA5194 5′ UTRs. (*A*) Structural analysis of in vitro transcribed *ptxS* (*left*) and PA5194 (*right*) RNA with RNase T1 (T1), lead acetate (Pb^{2+}) and RNase A (A) at different temperatures (indicated *above* the 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

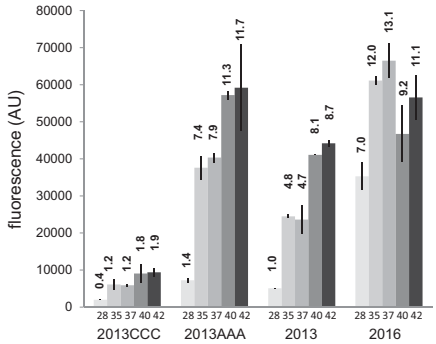

FIGURE 7. Effect of point mutations on PA5194-sfGFP thermoregulation. Cultures of *E. coli* DH10B carrying plasmids pGM2013 (2013) and its mutated derivatives pGM2013CCC (2013CCC) and pGM2013AAA (2013AAA) were grown at the temperatures indicated *below* the bars and processed as detailed in Materials 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 (Cimdins 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 *yrfI*, 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 Tn*10 tet* regulatory region (*tetRp–tetO–tetAp*) (Bertrand et al. 1983) controlling transcription of kan^R (under *tetAp* promoter) and *lacZ*α (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 Tn*10 tet* regulatory region, *lacZ*α and kan^R were synthesized by PCR. Oligonucleotides 2602–2603 on C-5868 genomic DNA (Carzaniga et al. 2009) were used for Tn*10 tet* amplification; 2604–2605 on pUC19 (Yanisch-Perron et al. 1985) for *lacZ*α; 2600–2601 on pQE31S1 (Sukhodolets and Garges 2003) for kan^R. Then Tn*10 tet* and *lacZ*α fragments were used as templates in a PCR reaction with oligonucleotides 2603–2604, obtaining the fragment *lacZ*α-*tet*. Finally, the full-length cassette was obtained by amplification of the *lacZ*α-*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 Tn*10*; 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 $(SG_4)_5$ linker and *trxA* fused upstream of TIP2. The construct was obtained by three-step PCR. $(SG_4)_5$ 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 $(SG_4)_5$:*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 $(SG_4)_5$:*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% NH_4Cl, 1.6% $Na_2HPO_4{\cdot}12\ H_2O$, 0.3 KH_2PO_4, 0.5% NaCl, 0.013% $MgSO_4$, 0.001% $CaCl_2$ 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 $(SG_4)_5$-*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 that 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 Bioanalizer 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 $OD_{600} = 0.5$ 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 OD_{600} 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 $OD_{600} = 0.5$. 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 Na_2CO_3.

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 OD_{600} = 0.1–0.5. Two hundred microliter cell samples were transferred in black polystyrene 96-well microplates with clear flat bottom (Corning). OD_{595} and Fluorescence Polarization $(FP)_{485/535}$ were measured. sfGFP activity was expressed in arbitrary units (AU) as the ratio $FP_{485/535}/OD_{595}$. 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 OD_{600} = 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 OD_{600} 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 $F_{485/535}/OD_{600}$. *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 OD_{600} = 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-^{32}P] 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; 2×10^4 cpm samples were then preincubated in reaction buffer (20 mM Tris–HCl, 2 mM Mg_2Cl, 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 (10^5 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 2.5×10^5 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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Supplementary table S1. Bacterial strains, plasmids, phages and oligonucleotides

Strain	Relevant Genotype	Origin or reference
Pseudomonas aeruginosa		
PAO1		(Stover *et al.*, 2000)
PA14		(Rahme *et al.*, 1995)
Escherichia coli		
C-1a	*E. coli C*, prototrophic	(Sasaki and Bertani, 1965)
C-5708	C-1a *rpsL31*	laboratory collection
C-5868	C-1a Δ*pnp-751* Δ*bcsA*::*cat Tn10*	(Carzaniga *et al.*, 2009)
C-5898	C-1a *tetRp αlacZ tetAp-kanR*	this work
C-5899	C-1a *tetRp-αlacZ tetAp-aadgfp*	this work
C-5901	C-1a *Δbio tetRp- αlacZ tetAp-aadgfp* $Pcat^{+}$*-*$tetR:kan^{R}$	this work
C-5907	C-1a *Δbio tetRp- αlacZ tetAp-aadgfp* $Pcat^{+}$*-tetR*	this work
C-5912	C-1a *tetRp- αlacZ tetAp-*$rpsL^{+}$*:cat*	this work
C-5916	C-5708 *tetRp-αlacZ tetAp-*$rpsL^{+}$*:cat*	this work
C-5918	C-5708 *tetRp- αlacZ tetAp-*$rpsL^{+}$	this work
C-5920	C-5708 *tetRp- αlacZ tetAp-*$rpsL^{+}$ *Pcat-*10^{CATTTA} *-* $tetR:kan^{R}$	this work
BW25113	*E. coli* K-12	(Datsenko and Wanner, 2000)
DH10B		(Grant *et al.*, 1990)
KG264	BW25113 $Pcat^{+}$*-*$tetR:kan^{R}$	this work

KG265	BW25113 *Pcat*-10^{CATTTA} -*tetR:kan*^{R}	this work

Plasmids and phage	**Relevant characteristics**[a]	**Reference**
pBAD2-bgaB	carries *Bacillus stearothermophilus bgaB* gene	(Klinkert *et al.*, 2012)
pBAD24-Δ1	pBAD24 derivative	(Carzaniga *et al.*, 2012)
pGM362	carries pHP45 t_{Ω} terminator	(Briani *et al.*, 2000)
pGM742	CamR; oriVColD	(Regonesi *et al.*, 2004)
pGM930	pBAD24-Δ1 derivative carrying pHP45 t_{Ω} terminator downstream of *araBp*	this work
pGM931	pHERD20T derivative carrying *araBp*- t_{Ω} region of pGM930	this work
pGM932	pGM742 derivative carrying the *lacZ*α- *tetRp-tetO-tetAp- kan*^{R} cassette	this work
pGM956	pGZ119HE derivative. Carries pQE31-S1 Shine-Dalgarno and ATG in frame with ST-TIP2.	this work
pGM957	pGZ119HE derivative. Carries pQE31-S1 Shine-Dalgarno and ATG out of frame with ST-TIP2.	this work
pGM978	pGM931 derivative, contains *bgaB* under pBAD control	this work
pGM980	pGM978 derivative, carries *ptxS* (2487532-2488013) translationally fused to *bgaB* .	this work
pGM981	pGM978 derivative, carries *ptxS* (2487779-2488013) translationally fused to *bgaB*	this work
pGM989	pGM978 derivative, carries *recA* (2334354-2334277) translationally fused to *bgaB*	this work
pGM2011	pGM931 derivative with the insertion of sfGFP	this work
pGM2012	pGM2011 derivative, carries PA5194 (5846939-5847277) translationally fused to sfGFP	this work
pGM2013	pGM2011 derivative, carries PA5194 (5847080-5847277) translationally fused to sfGFP	this work
pGM2013CCC	pGM2013 derivative, carries the substitution of the $TTT_{5847127\ -5847129}$ sequence with three cytidines	this work
pGM2013AAA	pGM2013 derivative, carries the substitution of the $TTT_{5847127\ -5847129}$ sequence with three adenosines	this work

pGM2016	pGM2011 derivative, carries *recA* (2334354-2334277) translationally fused to sfGFP	this work
pGZ119HE	*oriVColD*; CamR ; P_{tac}	(Lessl *et al.*, 1992)
pHERD20T	*P. aeruginosa*-*E. coli* shuttle vector	(Qiu *et al.*, 2008)
pKD46	carries λ RED recombination genes	(Datsenko and Wanner, 2000)
pPA5194-HA	pGM931 derivative, carries PA5194 (5847080-5847931) translationally fused to HA	this work
pPtxS-HA	pGM931 derivative, carries *ptxS* (2487779-2488875) translationally fused to HA	this work
pQE31S1	AmpR; ColE1; *rpsA* under P_{tac} promoter	(Sukhodolets and Garges, 2003)
pUC19	AmpR; ColE1	(Yanisch-Perron *et al.*, 1985)
pWH2354	CamR; p15A; *lacI*^{q}; *trxA*-*TIP2* under P_{tac} promoter	(Georgi *et al.*, 2012)
pXG-10SF	pSC101* replicon; CamR	(Corcoran *et al.*, 2012)
pZR80-2	carries the chimeric *aadA::gfp* gene	(Rizzi *et al.*, 2008)
P1 HTF	High transduction frequency phage P1 derivative	(Wall and Harriman, 1974)

Oligo	**Sequence**[b]
1396	AAGGAGGTGATCCAGCCGCA
2600	CTATCAGTGATAGAGAAAAGTGAAATGATTGAACAAGATGGATTG
2601	TAGTCTCGGTCCCCCATAAAAAAGGGACCTCTAGGGTCCCCAAGTCGGTCA TTTCGAACCCC
2602	CATTAATTCCTAATTTTTGTTGACAC
2603	TTCACTTTTCTCTATCACTGATAG
2604	GAAATTCAGTAAAAGCCTCCGACCGGAGGCTTTTGACTGGCGGGTGTCGGG GCTG
2605	GTGTCAACAAAAATTAGGAATTAATGA TGACCATGATTACGCCAAGC

2606 ATGTCGCGGTTGATCCTGAAGGAAAAC CTC

2617 AGCTTATTAAAGAGGAGAAATTAACTA TGAGAGGCATG

2618 CCTCTCATAGTTAATTTCTCCTCTTTAATA

2636 GGTTCGAAATGACCGACTTGGGGACCCTAGAGGTCCCTTTTTTATGGGGGG TGTAGGCTGGAGCTGCTT

2638 TTGATCCTGAAGGAAAAACCTCGCGCCTTACCTGTTGAGTAATAGTCTCGGT TAAAAAATGCCCTCTTGGGTTA

2683 TGATAGAGTTATTTTACCACTCCCTATCAGTGATAGAGAAAAGTGAAATGG ATCCCGAA GCGGTG

2684 GACCTCTAGGGTCCCCAAGTCGGTCATTTCGAACCCCAGAGTCCCGCTCAT GATGCCTGGAATTAATTCC

2685 AGCCTGCTTTTTTATACTAACTTGAGCGAAACGGGAAGGTAAAAAGACAAC TTCGTCTGTTTCTACTGG

2686 CCATGGGGCTTCTCCAAAACGTGTTTTTTGTTGTTAATTCGGTGTAGACTTT GTGTAGGCTGGAGCTGC

2689 ACCCGGGAGTGGTGGTGGCGGCAGCGGCGGTGGTGGATCCGGTGGCGGTG GCTC

2690 TACCGGTACCGCCGCCACCCGAACCGCCACCGCCAGAGCCACCGCCACCGG A

2691 GGCGGCGGTACCGGTAGCGATAAAATTATTCACCTGACT

2692 CCC**GAATTC**CGTTACCAATGCCACATCCAC AT

2693 CCCTGCAGCATGCAAACCCGGGAGTGGTG GT

2712 TAAAAAATGCCCTCTTGGGTTACATATGAA TATCCTCCTTAGT

2713 TCAGTGATAGAGAAAAGTGAAATGGCAAC AGTTAACCAGC

2714 AAGTCGGTCATTTCGAACCTTACTTAACGGA GAACC

2739 GAATTAAGCTTGCATGCAACC

2740 GCCGCCACCACCACTCC

2803 CTC**GGTACC**AGTAAAGGAGAAGAACTTTTCAC

2804 CTC**CTGCAG**CTATTTGTATAGTTCATCCATGC

2811 GGTTTCAACTCCTGGCATCC

2846 GCG**CCATGG** GGGAGCATATGCGAATCTTC

2847 CCCTGATCTCGACCTGCA

2850 GGG**CCATGG**CTCGCCGATTGATCGCTTTC

2851 CCC**GAATTC**ATGGTCGATGGCGCGCTC

2852 GGG**CCATGG**GGTTTCAACTCCTGGCATCC

2865 TCAACTTAGCATCTTCATACC

2871 GAATTGGGACAACTCCAGTG

2909 *CTAATACGACTCACTATAG*GGTTTCAACTCCTGGCATCC

2910 GGCCTCGGCCACCTGGTT

2915 TCT**CCATGG**CAACAGAACATATTGACTATCC

2916 CAC**GAATTC**TTTCTGTTTGTTTTCGTCGATAG

2928 TCT**GGTACC**CAACAGAACATATTGACTATCC

2929 CAC**GGTACC**TTTCTGTTTGTTTTCGTCGATAG

2976 *CTAATACGACTCACTATAG*GGCCTCGGCCACCTGGTT

3003 GGAACGGAGAGGCATTATCC

3004 GGG**GGTACC**CTTGTCGAAGAGCCAGAAC

3005 GGG**GGTACC**CTGCGCTCGAGATCGAC

3006 GGG**GGTACC**GCTTCGTGACAGTCGTAC

3040 *CTAATACGACTCACTATAG*GGCTTCGTGACAGTCGTAC

3041 GCAGGAGCGCCAGAAGAT

3141	ATGGATAATGCCTCTCCGTT
3142	AACGGAGAGGCATTATCCATAGGGTCGTTTCCCC
3143	AACGGAGAGGCATTATCCATATTTTCGTTTCCCC
3144	TCAAGCGTAGTCTGGGACGTCGTATGGGTAGGCCGAGTCGCGGACCA
3145	TCAAGCGTAGTCTGGGACGTCGTATGGGTAGGCCGAGTCGCGGACCA
3150	*CTAATACGACTCACTATAG*GGCCCCAGAGCCACAGG

[a] *P.aeruginosa* coordinates refer to Genbank accession numbers NC_002516; *E. coli recA* gene coordinates refer to Genbank Accession Number U00096.2.
[b] Boldface characters, restriction sites; italics, T7 promoter

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PART III

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'Temperature responsive regulation of *Escherichia coli lpxT* is controlled by a new RNA thermometer based on an unstable mRNA secondary structure and a sub-optimal Shine-Dalgarno sequence',

Temperature responsive regulation of Escherichia coli lpxT is controlled by a new RNA thermometer based on an unstable mRNA secondary structure and a sub-optimal Shine-Dalgarno sequence

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ABSTRACT

RNA thermometers (RNATs) are thermo-labile mRNA secondary structures that modulate gene translation initiation in response to temperature changes. In a previous work, we found that the expression of *Pseudomonas aeruginosa lpxT*, which encodes a membrane protein phosphorylating the lipid A moiety of the lipopolysaccharide, is induced in response to a temperature upshift and we proposed that an RNAT was responsible for such regulation. Here we show that the expression of the *Escherichia coli* LpxT orthologous protein is also temperature-responsive. Increased LpxT expression at high temperature (i.e. 37°-42° vs. 28°C) depends *in vivo* on post-transcriptional mechanisms. Moreover, *in vitro* assembly of the 30S ribosomal subunit and fMet-tRNA on the *lpxT* transcript is less efficient at 28°C than at higher temperature, suggesting that translation initiation of *lpxT* mRNA is impaired at low temperature. Indeed, the short *lpxT* 5'-UTR (untranslated region) confers thermo-dependent expression to a reporter translational fusion transcribed from a heterologous promoter. The *lpxT* 5'-UTR is predicted to fold into an unstable stem-loop (5'SL) involving the suboptimal Shine-Dalgarno region. Point mutations changing the stability of the 5'SL secondary structure or ameliorating the complementarity of the Shine-Dalgarno with the 16S rRNA affect thermoregulation, showing that both elements cooperate in *lpxT* gene expression regulation. On the whole, our results suggest that *lpxT* is regulated by an unusual RNA thermometer that exploits the combination of sub-optimal elements to confer temperature-responsive translation to the mRNA.

IMPORTANCE

Bacteria have evolved sophisticated regulatory strategies to perceive and respond to temperature changes. A convenient system to quickly adapt gene expression to the temperature is represented by RNA thermometers, which are temperature sensors that modulate the translation of the mRNAs bearing them. Such regulators have been involved in the modulation of genes belonging to different functional categories, among which virulence genes. However, only a small number of RNA thermometers have been functionally characterized so far. Here we report the identification and functional characterization of a new *E. coli* RNAT operating through a peculiar combination of regulatory elements. Our results are important because they provide new information on RNATs' features that may be instrumental in their identification. More in general, our findings reinforce the notion that, as generally acknowledged for transcription, translation initiation efficiency can also be finely tuned to environmental stimuli.

INTRODUCTION

Bacteria have evolved a plethora of regulatory strategies to adapt the cell physiology to the environmental temperature. Basically all steps of the information flux, from DNA transcription to protein stability, can be modulated in response to temperature changes.

In many instances, the molecule acting as the temperature sensor, whether a nucleic acid or a protein, directly regulates gene expression (Klinkert and Narberhaus, 2009). This is the case for RNA thermometers (RNATs), which are RNA-based regulatory systems modulating mRNA translation in response to temperature changes. In simple terms, RNATs can be described as thermolabile mRNA secondary structures involving the translation initiation region (TIR) and preventing ribosome binding at low temperature. Since in RNATs the regions of perfect RNA-RNA pairing are usually short and/or interrupted by bulges and unpaired nucleotides, the formation and stability of the inhibitory structure is deeply affected by small temperature changes, largely within the range of temperatures well tolerated by mesophilic bacteria. Typically, in this type of bacteria, translation of genes controlled by RNATs remains repressed at temperatures up to 28°-30°C and is fully activated at 40°-42°C (Kortmann and Narberhaus, 2012).

Not surprisingly, RNATs have been found upstream of genes implicated in heat stress response such as the *Escherichia coli rpoH* gene and genes encoding chaperone proteins in different bacteria (Cimdins et al., 2014; Krajewski et al., 2013; Morita et al., 1999; Nocker et al., 2001). Moreover, the virulence master regulators of pathogenic bacteria like *Listeria monocytogenes* (Johansson et al., 2002), *Yersinia pestis* (Bohme et al., 2012) and *Vibrio cholerae* (Weber et al.,

2014) are regulated by RNATs calibrated to respond to the mammalian host temperature. Recently, it has been shown that in *Yerinia pseudotuberculosis,* RNATs are exploited to modulate the expression of genes encoding not only chaperons and virulence functions, but also factors implicated in different metabolic pathways (Righetti et al., 2016). Thus RNATs control genes belonging to disparate functional categories, whose quick modulation by temperature is likely to represent an advantage for the bacterium.

We recently developed a genetic approach, Tet-Trap, aimed to find novel RNATs. Its application to *Pseudomonas aeruginosa* led to the identification of several putative RNATs, among which one was demonstrated to confer temperature-dependent regulation to the *lpxT* gene (Delvillani et al., 2014). The LpxT protein modifies the outer membrane by phosphorylating the lipid A moiety of the lipopolysaccharide (LPS) at the 1-position. The phosphate donor is undecaprenyl pyrophosphate, a phosphorylated derivative of the essential transporter of cell wall components undecaprenyl phosphate (Needham and Trent, 2013). Interestingly, in *Porphyromonas gingivalis,* a bacterium evolutionarily very distant from *P. aeruginosa* and lacking an *lpxT* orthologue*,* Lipid A phosphorylation increases upon a small temperature rise (from 37 to 40°C; Curtis et al., 2011). This suggests that bacteria may benefit from tuning the extent of Lipid A phosphorylation to the temperature, irrespective of the enzymatic activity carrying out such modification.

In this study, we investigated whether the *E. coli lpxT* orthologue is also regulated in a temperature-dependent manner. Our results show that temperature-responsive mechanisms operate at both transcriptional and post-transcriptional level in the control of *lpxT* expression. In particular, sub-optimal regulatory elements, namely a short Shine-Dalgarno sequence potentially

sequestered in an unstable inhibitory stem-loop, are combined in a new RNAT that represses *lpxT* expression at low temperature.

RESULTS

The expression of *E. coli* LpxT is induced at high temperature

In *P. aeruginosa lpxT* gene expression is activated in response to a temperature upshift from 25° to 37°-40°C (Delvillani et al., 2014). In order to test whether such a regulatory strategy is conserved also in *E. coli*, we analyzed the expression of the *E. coli* LpxT protein at different temperatures. To this aim, since LpxT-specific antibodies were not available, we C-terminally tagged the *lpxT* open reading frame (ORF) on the chromosome with the GFP reporter. A recombinant $LpxT_{GFP}$ protein with the GFP C-terminally fused to LpxT had been previously shown to be expressed and inserted into the inner membrane (YeiUGFP; Tatar et al., 2007).

Western blotting with GFP-specific antibodies of proteins extracted from the recombinant strain (Fig. 1) showed that $LpxT_{GFP}$ was undetectable or very scarce at 28°C (a faint signal was visible in replicate experiments; see Fig. S1) and sharply and steadily increased at 37° and 42°C. This shows that $LpxT_{GFP}$ (and conceivably LpxT) expression is regulated by temperature.

Temperature-dependent expression of LpxT relies on post-transcriptional mechanisms

Mechanisms operating at different levels in the genetic information flow, from transcription initiation to protein stability control, could be responsible for temperature-responsive regulation.

In order to assess whether transcription modulation was involved, we analyzed the *lpxT* locus transcription profile in the BW25113 parental strain and in its

KG273 derivative by Northern blotting. In exponential cultures of both strains, we detected a single signal compatible with a monocistronic mRNA at all temperatures. The signal was more abundant at 28° than at higher temperature (Fig. 2A and B). This difference was at least in part due to decreased stability at high temperature, as the half-life of *lpxT* mRNA resulted of 3.0±0.8 min at 28° and less than 1 min at 42° (Fig. S2). A similar pattern of expression and decay was observed also for the *lpxT:GFP* allele (Fig. 2B).

A single 5'-end was mapped by primer extension in position 2268826, 28 nt upstream the start codon of the *lpxT* ORF (Fig. 2C). By inspection of the sequence immediately upstream the transcription start site (TSS), we noticed that the hexameric sequence TAAGGT, which is present as -10 in a small number of σ^D-dependent promoters of *E. coli* (Repoila and Gottesman, 2003), is centered exactly at -10 from *lpxT* TSS. Conversely, we could not identify any properly located -35 consensus sequence. Indeed, we found that a DNA fragment spanning the 49 pb region upstream of *lpxT* TSS was able to promote transcription when inserted upstream of a GFP reporter gene in a plasmid construct (Fig. 2D-E). In fact, the GFP mRNA TSS was located at the same position relatively to the putative -10 sequence as the *lpxT* TSS (Fig. 2F), thus confirming that this region contains the *lpxTp* promoter. Interestingly, as observed for *lpxT* mRNA, the GFP mRNA expressed from the plasmid was slightly less abundant at high temperature than at 28°C.

On the whole, these data indicate that *lpxT* is transcribed as a monocistronic mRNA from the *lpxTp* promoter and rule out the possibility that thermal induction of LpxT is a consequence of temperature-dependent *lpxT* transcription.

This notion was further strengthened by the results obtained by analyzing the expression of $LpxT_{HA}$ (i.e. LpxT C-terminally fused with the HA epitope) in

cells hosting the pGM2043 plasmid. In such construct, the *lpxT*$_{HA}$ gene is under the transcriptional control of the heterologous *araBp* promoter. The physiological 5'-UTR of *lpxT* is conserved, whereas the 3'-UTR is replaced by an intrinsic transcription terminator present in the vector. Although the *lpxT*$_{HA}$ transcript expressed by the plasmid is more stable than the *lpxT* mRNA at both 28° and 42°C (Fig. S3), this did not modify the expression pattern of the LpxT protein. In fact, as it had been observed for the expression of the chromosomally encoded LpxT$_{GFP}$ (Fig. 1), the LpxT$_{HA}$ signal tested with HA-specific antibodies was absent at 28°C and increased with the temperature (Fig. 3A). Thus, in spite of being transcribed form different promoters (and carrying different tags), both the chromosomally and the plasmid encoded LpxT variants maintain temperature-responsive expression. This implies that the *in cis* determinants of thermoregulation should be present in the *lpxT*$_{HA}$ construct.

An obvious player in this phenomenon could be the *lpxT* leader sequence, which controls the response to the temperature of the *P. aeruginosa* orthologue (Delvillani et al., 2014). In order to assess whether such region was involved in thermoregulation also in *E. coli*, we replaced the *lpxT* with the *recA* 5'-UTR in pGM2043 plasmid, obtaining pGM2049. As shown in Fig. 3A, we found that LpxT$_{HA}$ was expressed at comparable levels at all tested temperatures in cells carrying pGM2049. Since pGM2043 and pGM2049 differ only for the leader sequence of *lpxT*$_{HA}$ and thus should express identical LpxT$_{HA}$ proteins, these results rule out the possibility that post-translational mechanisms (i.e. LpxT instability at low temperature) may be responsible for poor protein expression at 28°C and point to the 5'-UTR as a pivotal element in *lpxT* thermal regulation. Bioinformatic prediction of the *lpxT* 5'-UTR secondary structure suggests that it can form a stem-loop involving the Shine-Dalgarno (SD) sequence (SL$_{lpxT}$; Fig. 3B), a feature that is a hallmark of RNA thermometers.

The formation of the translation initiation complex on the *lpxT* mRNA is impaired at low temperature

In RNATs, the formation of a secondary structure involving the TIR at low temperature impairs ribosome binding (Narberhaus, 2010). To test whether this occurs also for *lpxT* mRNA, we looked at the assembly of the translation initiation complex by toeprinting assay (Hartz et al., 1988). We used two mRNAs in this assay, the $lpxT_{112}$, which corresponded to the first 112 nt of the transcript from the *lpxTp* promoter, and the *recA*-$lpxT_{112}$ control transcript, in which the first 23 nucleotides of the 28-nt long *lpxT* 5'-UTR were replaced with a portion of the same length of the *recA* leader sequence containing the SD region. The two transcripts were annealed with a complementary oligonucleotide and incubated with the 30S ribosomal subunit and fMet-tRNA at different temperatures. Primer extension was then performed at 37°C. In all reactions we detected two signals, one corresponding to the 5'-end of the transcript (indicated as -29 in Fig. 4) and another one (+15 in Fig. 4) corresponding to the toeprint of the assembled preinitiation complex maps in position +15 relative to the first nucleotide of the ORF. For $lpxT_{112}$ mRNA, the relative intensity of the two signals varied with temperature, as the toeprint signal was respectively stronger at 42° and fainter at 28°C than the 5'-end signal. On the contrary, with the *recA*-$lpxT_{112}$ control transcript, the toeprint signal was stronger than the 5'-end one at both 28° and 42°C. These results demonstrate that the assembly of the 30S on the *lpxT* translation initiation region is controlled by the temperature in the absence of any additional cellular factor, suggesting that an RNAT modulates translation of *lpxT* mRNA.

Both the *lpxT* suboptimal Shine-Dalgarno region and the 5'-UTR secondary structure contribute to temperature-responsive regulation

The data presented above show that the 5'-UTR is necessary for *lpxT* thermoregulation. We wondered whether such sequence was also sufficient to make temperature-responsive the expression of a heterologous gene. In fact, we found that the fluorescence of bacterial cultures carrying a translational fusion between the 5'-UTR and first two codons of *lpxT* and the GFP reporter gene (plasmid pGM2034) was very low at 28°C and increased almost 11- and 36-fold at 37° and 42°, respectively (wt in Fig. 5). Conversely, in the same experimental conditions, a construct with the 5'-UTR and first 9 codons of the *E. coli recA* gene fused with the GFP (plasmid pGM2016; recA-GFP in Fig. 5) was expressed at all temperatures, with around fourfold fluorescence increase at both 37° and 42°C. Thus, the presence of the *lpxT* 5'-UTR is sufficient to confer thermal induction to the expression of an unrelated gene.

The SL_{lpxT} that engages almost entirely the 5'-UTR has a predicted ΔG of -4.3 kcal/mol at 28°C (estimated with mfold; Zuker, 2003). It has been claimed that structures with ΔG higher than -5 to -6 kcal/mol should not be able to prevent ribosome binding and thus to reduce translational efficiency (de Smit and van Duin, 1994; Kubo et al., 1990). However, this threshold may not apply to mRNAs with low intrinsic affinity for the 30S (see Fig. 2 in de Smit and van Duin, 1994). The *lpxT* mRNA belongs to this category as the ORF is preceded by a 3 nt-long, suboptimal SD. Ribosome Binding Site Calculator software (Espah Borujeni et al., 2014) estimates a ΔG for the interaction of the *lpxT* SD with the 16S rRNA of ca. -3.5 kcal/mol at 37°C, whereas for instance the same parameter for the *recA*-GFP expressed by pGM2016 is estimated at about -19 kcal/mol. Indeed, even at 42°C, a condition in which the *lpxT* 5'-UTR should be

unfolded, the GFP expression by pGM2034 is almost three-fold lower than by pGM2016 (Fig.5, 42° C panel). We reasoned that because of such feature, even a weak stem loop like the SL_{lpxT} may outcompete the 30S at low temperature and thus affect *lpxT* mRNA translation.

To assay this hypothesis, i.e. that both the stem-loop and the sub-optimal SD contribute to translation inhibition at low temperature, we introduced in the backbone construct pGM2034 different point mutations and compared the fluorescence of cultures carrying either the pGM2034 plasmid or its mutated derivatives at 28°, 37° and 42°C (Fig. 5). In particular, we assayed mutations predicted to stabilize the SL_{lpxT}. We eliminated the bulge in the left arm of the stem and replaced a U·G base pair with a more stable C·G in the stem (S1 and S2; Figs. 3B and 5). The predicted ΔG of the two mutated SL_{lpxT} was of -9.2 and -7 kcal/mol, respectively. As expected, the two mutations, and in particular S1, caused a sharp decrease in the fluorescence of the cultures at all temperatures. On the contrary, mutations predicted to destabilize the SL_{lpxT} (D1, D2 and D3 in Figs. 3B and 5) were expected to increase the fluorescence at low temperature and to be silent at high temperature. Indeed, such phenotype was exhibited by mutant D3 and, to a minor extent, by D1, whereas the D2 mutation did not significantly affect fluorescence at either temperature. We also tested two mutations that increased the SD complementarity with the 16S anti-SD (SD1 and SD2; Figs. 3B and 5). The two mutations should not prevent the folding of the SL_{lpxT}, as in the mutant SD1, an A·U base pair in the stem is replaced by C·G, which should actually stabilize the stem, whereas in the SD2 a U·A pair is changed into U·G. The two mutants, and in particular the SD2, showed a remarkable fluorescence increase at 28°C (around 4- and 10-fold, respectively), whereas GFP activity raised only two- threefold at 42°C. Interestingly, a double SD2-D1 mutant exhibited an almost twofold increase of the fluorescence with

respect to the single SD2 mutant at 28°C (Fig. 5). For the double mutant, the fluorescence Induction Fold at 37-42° *vs*. 28°C (IF_{37} and IF_{42} reported below the corresponding panels in Fig. 5) was around 4- and 6-fold, respectively, not dissimilar to the IF exhibited by the control *recA*-GFP construct (4.4 fold at both temperatures). Thus, the combination of the two mutations essentially abolished thermoregulation.

On the whole these data support our hypothesis that the SL_{lpxT}, albeit unstable, is nevertheless able to exert an inhibitory effect on *lpxT* translation at low temperature and may thus represent a new RNAT.

DISCUSSION

In this work we report the identification of a new RNAT controlling the expression of the *E. coli lpxT* gene. Different in *vivo* and in *vitro* evidences support this notion. In fact, we demonstrated that a chimeric LpxT-GFP protein expressed from the *lpxT* chromosomal locus is poorly expressed at 28°C and induced at higher temperatures, although its cognate mRNA is expressed at all temperatures and actually more abundant (and more stable) at 28° than at 42°C. These data, together with the observation that thermoregulation is preserved in plasmid fusions expressing LpxT-HA from a heterologous promoter, provided that the *lpxT* leader sequence is present in the construct, discard inefficient transcription initiation as the cause of poor *lpxT* expression at low temperature.

We could not analyse the expression of the untagged LpxT protein. However, the LpxT-GFP fusion is correctly targeted to the membrane when expressed from a plasmid (Tatar et al., 2007; data not shown) and the *lpxT:GFP* and *wt lpxT* mRNAs have similar transcription patterns and, at least at 28°C, comparable stability. Moreover, thermal induction of LpxT is exhibited also by the HA-tagged variant, strongly suggesting that the presence of a C-terminal tag does not affect regulation.

The results of toeprinting experiments suggest that modulation of initiation complex assembly is the mechanism at the root of thermoregulation. *In vitro,* fMettRNA-30S-mRNA complex formation was not completely inhibited at low temperature. This may suggest that in *vivo, trans*-acting factor participate in the mechanism of temperature–responsive regulation and strengthen translation repression. However, it should be noted that, albeit reduced, LpxT expression is

detectable at 28°C (Fig. S1), indicating that also in *vivo* translation is not completely prevented.

Expression data relative to HA and GFP plasmid fusions point to the *lpxT* 5'-UTR as the place where thermoregulation occurs. The *lpxT* 5'-UTR is only 28 nt long and it is predicted to fold into a 25-nt long hairpin with relatively high energy of formation even at 28°C. 5 uridines are predicted to pair with a region encompassing the weak SD of the gene, a trait typical of FourUs, a class of RNATs characterized by the presence of a stretch of 4 uridines paired with the SD (Waldminghaus et al., 2007). However, with respect to other RNATs, the *lpxT* RNAT controls gene expression through a peculiar mechanism based on the combined action of two sub-optimal elements like the thermodynamically unstable SL_{lpxT} and the weak SD region. The SD plays a relevant role in the formation of 30S-mRNA complex on structured mRNA. In fact, for such mRNAs, the formation of the 30S-mRNA initiation complex depends on competing equilibria between the formation of either a secondary structure involving the translation initiation region or the 30S-mRNA complex (de Smit and van Duin, 1990; Borujeni and Salis, 2016). Thus, strengthening the SD-antiSD interaction would increase the affinity of the 30S for the TIR and this in turn would shift the equilibrium between the folded and the unfolded, accessible TIR state towards the second one, ultimately favoring initiation complex formation. Conversely, unstructured mRNAs should be saturated with the 30S even if their TIRs have moderate affinity for the 30S (de Smit and van Duin, 1990). For RNATs, this model predicts that enhanced affinity of the SD for the ribosome should have higher impact on translation efficiency at low temperature, when the secondary structure is folded, than at high temperature. The phenotype of the mutants in the 5'-UTR supports this model, since the GFP relative increase (*vs*. the *wt*) observed in SD mutants is much higher at 28°C

than at 42°C. Thus, for genes with weak SD sequences, a thermodynamically unstable structure may exert an inhibitory effect on translation initiation.

Unexpectedly, we found that at 42°C the *lpxT* transcript is less abundant than at 28°C. This is due to inefficient transcription initiation at high temperature at the *lpxTp*. Moreover, mRNA stability is also decreased at 42°C. This is a puzzling result because translated mRNAs are usually more stable than untranslated ones (Deana and Belasco, 2005). We have not explored the molecular bases of this phenomenon, but the observation that the *lpxT:HA* mRNA expressed by pGM2043 plasmid is more stable than *lpxT* points to the 3'-UTR of the gene, which lacks in the construct, as the site of regulation.

The emerging scenario for *lpxT* thermoregulation is that, as temperature increases, the mRNA may shift from a stable and untranslatable state to an unstable and translatable one. Together with mRNA instability, inefficient transcription initiation also contributes in limiting LpxT expression at high temperature. This would avoid excessive expression of LpxT, which impairs growth when overexpressed (El Ghachi et al., 2005).

Regulation by RNATs represents a convenient strategy to achieve fast thermal induction of gene expression. The observation that, in spite of lack of 5'-UTR sequence conservation, the *lpxT* orthologous genes of *P. aeruginosa* and *E. coli* share this regulatory strategy, suggests that bacterial fitness may benefit from fast modulation of lipid A phosphorylation degree on the environmental temperature. However, since the physiological role of Lipid A pyrophosphorilation is unclear, this makes difficult to understand the physiological role of temperature-dependent expression of LpxT. Albeit this modification has been mainly studied in bacteria that can thrive in the human host, in the environmental psychotropic bacterium *P. syringae* the LPS is also phosphorylated both on the LPS Lipid A and core oligosaccharide in response

to a temperature upshift (from 0 ° to 22°C; Ray et al., 1994). This suggests that increasing the degree of LPS phosphorylation may be instrumental in the adaptation to environmental stimuli, not (or at least not only) related with the infection of the mammal host.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1 and the oligonucleotides in Supplementary Table S1. *E. coli* coordinates throughout this work refer to Genbank Accession Number U00096.2.

Construction of BW25113 lpxT:GFP strain. The GFP reporter cassette was obtained by three step-PCR as follows. In the first step, four DNA fragments were synthesized by PCR, corresponding to i) *lpxT* regions I (2269401-2269564) and II (2269568-2269738). The two fragments were obtained by amplification of MG1655 chromosomal DNA with oligonucleotides 3358-3359 and 3363-3364, respectively; ii) the eGFP coding region, obtained by PCR with primers 3360-3373 on pGM963 (Raneri et al., 2015); iii) the FRT-*kan*^{R}-FRT region, obtained by amplifying plasmid pKD13 (Datsenko and Wanner, 2000) DNA with primers 3374-3362. In the second step, the partially overlapping fragments i) *lpxT* I and eGFP and ii) *kanR* and *lpxT* II were used as templates in two PCR reactions with oligonucleotides 3358-3373 and 3374-3364, respectively. Finally, the full length cassette was obtained by amplification of the two *lpxT* I-eGFP and *lpxT* II-*kanR* fragments with primers 3358-3364 and was integrated in BW25113/pKD46 by λ Red-mediated homologous recombination (Datsenko and Wanner, 2000), obtaining strain BW25113 *lpxT:GFP:kan*. The *kan* cassette was excised from the recombinant strain by FLP-mediated recombination, obtaining strain BW25113 *lpxT:GFP* (hereafter KG273). The LpxT-GFP protein expressed by KG273 carries the Pro

to Gln substitution in position 75 of the eGFP moiety (P42212 entry in www.uniprot.org/uniprot/) of the chimeric protein.

GFP translational fusions. All reporter translational fusions were assembled as pGM2011 (Delvillani et al., 2014) derivatives and checked by sequencing. pGM2032 was obtained by cloning in pGM2011 linearized with *Kpn*I a *Kpn*I-digested PCR fragment encompassing region 2268742- 2268859 and deriving from amplification of MG1655 chromosomal DNA with primers 3210-3211. pGM2034 contains region 2268826-2268859 and was obtained by PCR on pGM2032 with oligonucleotides 3225-3172, digestion with *Nco*I and cloning in pGM2032 digested with *Nco*I. The same procedure with proper oligonucleotides was applied to construct plasmids carrying mutagenized versions of pGM2034 region, namely pGM2044 (oligonucleotides 3284-3172); pGM2045 (3285-3172); pGM2047 (3307-3172); pGM2048 (3308-3172); pGM2055 (3330-3172); pGM2056 (3331-3172); pGM2064 (3348-3172); pGM2065 (3349-3172). Control plasmids pGM2016 carry the leader region and the first 9 codons of *E. coli recA* in frame with sfGFP genes (Delvillani et al., 2014).

GFP transcriptional fusions. Transcriptional fusions were obtained as pGZ119EH derivatives (Lessl et al., 1992). First we cloned the *recA*-sfGFP reporter construct (obtained by PCR amplification of pGM2016 with primers 2804-3274) between the *Eco*RI and *Pst*I sites obtaining pGM2040. The pTAC promoter upstream of the reporter construct was either deleted by *Eco*RI-*Eco*RV digestion, Klenow treatment and ligation, obtaining pGM2054, or replaced with a PCR fragment amplified from *lpxT* locus, namely region 2268778-2268826 (pGM2052; primers 3281-3282).

HA reporter plasmids. The HA coding region was obtained by annealing oligonucleotides 3257-3258 and cloning the double stranded oligonucleotide in pGM930 between *Kpn*I and *Pst*I sites (Delvillani et al., 2014), obtaining plasmid pGM2035. The *lpxT* locus was PCR-amplified with oligonucleotides 3225-3273 (region 2268826-2269564; *lpxT* fragment) or 3286-3273 (regions 1020995-1020975 + 2268846-2269564; *recA-lpxT* fragment) on MG1655 genomic DNA. The two PCR fragments were digested with *Nco*I-*Eco*RI and cloned in pGM2035 cut with the same enzymes, obtaining pGM2043 and pGM2049, respectively. Since *lpxT* expression by multicopy plasmids is toxic (El Ghachi et al., 2005; data not shown), we did all the experiments involving these plasmids with freshly transformed strains.

Bacterial cultures were grown in LD (Briani et al., 2002) or M9-glucose (Raneri et al., 2015). When needed, media were supplemented as follows: 100 µg/ml ampicillin; 30 µg/ml chloramphenicol; 50 µg/ml kanamycin; 0.1-0.2% arabinose.

Western blotting

The culture samples were centrifuged and the cell pellets resuspended in 2× SDS buffer (100 mM Tris– HCl pH 6.8, 200 mM DTT, 2% SDS, 0.2% bromophenol blue, and 20% glycerol) and boiled for 5 min. The samples were then centrifuged 10 min at 13,200 rpm, the supernatants were recovered and an aliquot was analyzed by SDS-PAGE. PageRuler Plus Prestained Protein Ladder weight markers (ThermoScientific) were used as size references. For immunological detection of proteins, the gel was blotted onto a PROTRANTM nitrocellulose membrane (PerkinElmer) and incubated with one of the following antibodies diluted in Blotto (1% dried milk,50 mM Tris-HCl pH=7.5, 150 mM

NaCl, 0.1% Triton X-100): i) polyclonal anti-GFP (A6455; Invitrogen). To minimize unspecific signals, the antibody mix was pre-incubated three hours with a membrane on which crude extracts of BW25113 strain had been blotted; ii) monoclonal anti-HA (12CA5; Roche); iii) polyclonal anti-S1 (kindly provided by U. Bläsi).

Fluorescence expression assay

BW25113 cultures carrying pGM2011 derivatives (i.e. expressing GFP translational fusions) were prepared by inoculating a single colony in LD with 100 µg/ml ampicillin. After overnight incubation at 28°C, the cultures were diluted to OD_{600}= 0.05 in LD with 100 µg/ml ampicillin and 0.1 % arabinose and grown at different temperatures up to OD_{600}= 0.5. For the analysis of transcriptional fusions inserted in pGZ119EH, the cultures were grown up to OD_{600}= 0.4 at 28 and 42°C in LD with 30 µg/ml chloramphenicol. At the stated optical densities, 1 ml samples were taken for fluorescence measurements. Cells were collected by centrifugation, washed, resuspended in PBS at OD_{600}= 0.5-1.0. 100 µl cell samples were transferred in black polystyrene 96 well microplates and both fluorescence$_{485/535}$ ($F_{485/535}$) and OD_{600} were measured by means of an Ensight (PerkinElmer) microplate reader. GFP activity was expressed in arbitrary units (AU) as the ratio $F_{485/535}/OD_{600}$.

Northern Blotting and Primer Extension

Procedures for RNA extraction, *in vitro* transcription with T7 RNA polymerase, Northern blot analysis, staining of the RNA on filters with methylene blue and

5'-end labeling of oligonucleotides with [γ^{32P}]- ATP and T4 polynucleotide kinase were previously described (Delvillani et al., 2014). For mRNA half-life determination, the cultures were grown at 28°C up to mid-exponential phase (OD_{600}= 0.4). Half-cultures were then transferred at 42°C for 10 min before rifampicin addition (0.4 mg/ml final concentration). 2 ml samples were taken immediately before and at successive time points after the addition of the antibiotic for RNA extraction. The half-life of *lpxT* mRNA at 28°C was calculated by analysing the decay curve of the average values, whereas at 42°C this was not possible because *lpxT* mRNA signal was clearly visible only at time 0. Oligonucleotide probes used for Northern blotting were 3172 (GFP); 3258 (HA). The *lpxT*-specific riboprobe was obtained by *in vitro* transcription with T7 RNA polymerase and [α^{32P}] CTP of DNA fragments obtained by PCR amplification of 2268824-2268928 MG1655 genomic region (oligonucleotides 3259-3270). The 5'-end of the *lpxT* mRNA was determined by primer extension as previously described (Forti et al., 1995) with the radiolabelled oligonucleotide 3245 on 10 µg of RNA extracted from cultures of BW25113. The same oligonucleotide was used for Sanger-sequencing of an amplicon obtained by PCR amplification of MG1655 DNA with primers 3210-3245 (region 2372914-2268928). Primer extension on BW25113/pGM2052 was performed on 10 µg with the oligo 3408. The sequencing ladder was obtained by Sanger sequencing of a PCR fragment amplified with primers 2804-3281 on pGM2052 DNA. Images of Northern blots and primer extension gels were obtained by phosphorimaging using ImageQuant software (Molecular Dynamics).

Toeprinting assay

30S ribosomal subunits were purified and toeprinting assay was performed basically as described by Fechter *et al* (2009) (Fechter et al., 2009). Cells from 1 l of an exponential culture at OD_{600}= 1 of *E. coli* MRE600 grown in LD broth at 37°C were collected by centrifugation, washed and resuspended in 2 ml of buffer AE (20 mM $MgCl_2$, 200 mM NH_4Cl, 20 mM Tris-HCl of pH 7.5, 0.1 mM EDTA, 6 mM 2-mercaptoethanol). Cells were mechanically disrupted with a french press (10000 psi) and cell debris removed by ultracentrifugation in TLA100.3 for 30 min, 23500 rpm at 4°C. The supernatant was further ultracentrifuged in TLA100.3 4 h, 52000 rpm at 4°C and the pellet was resuspended in 0.2 ml of AE buffer. The sample was stratified on a 0.8 ml cushion composed by 30% sucrose, 10 mM $MgCl_2$, 500 mM NH_4Cl, 20 mM Tris-HCl of pH 7.5, 0.1 mM EDTA, 6 mM 2-mercaptoethanol and ultracentrifuged in TLA100.3 for 19 h, 41000 rpm at 4°C. The pellet was resuspended in 0.2 ml buffer BE (10 mM $MgCl_2$, 50 mM NH_4Cl, 20 mM Tris-HCl of pH 7.5, 0.1 mM EDTA, 6 mM 2-mercaptoethanol) and extensively dialysed in buffer DE (3 mM $MgCl_2$, 300 mM NH_4Cl, 20 mM Tris-HCl of pH 7.5, 0.15 mM Na_2EDTA, 2 mM DTT) at 4°C. Ribosomal subunits were then separated by sucrose gradient (5-20% w/v in DE buffer) centrifugation in a Beckman SW40.Ti rotor for 2h 20 min, at 35000 rpm and 4°C. The fractions containing the 30S subunits, as estimated by OD_{260} measuring and agarose gel electrophoresis, were collected and dialysed in buffer CE (10 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl of pH 7.5, 0.1 mM EDTA, 1 mM DTT) at 4°C. 30S subunits were concentrated using centrifugal filter units (Millipore) to 10 mg/ml and stored at -80°C. For the toeprinting assay, the $lpxT_{112}$ and the *recA*-$lpxT_{112}$ mRNAs were synthesized by *in vitro* transcription with T7 RNA polymerase

(Promega) of DNA fragments obtained by PCR amplification of MG1655 genomic DNA or pGM2049 DNA with the oligonucleotides 3318-3313 and 3319-3313, respectively. The probes contain the same portions of the *lpxT* coding region (2268854-2268937) and differ for the 5'-UTR. The RNAs (5 pmol) were mixed with 1000000 cpm of primer 3313 radiolabeled with [γ^{32}P]-ATP in 20 µl of 1 mM DTT, heated 1 min at 95 °C and incubated 10 min at 28°C. $MgCl_2$ was added to a final concentration of 10 mM. The mixture was split in three aliquots that were incubated 10 min at 28°, 37° or 42°C. Ternary complex formation was performed by mixing in a final volume of 10 µl 1µl of the renatured RNA-oligonucleotide reaction, 2 µl of 15 mM DTT, 8 mM $MgCl_2$ and 30S ribosomal subunits at a final concentration of 0.2 µM. After 5 min at 28°, 37° or 42°C, the fMet-tRNA (SIGMA; final concentration of 2 µM) was added and incubation protracted for 15 min. cDNA synthesis was performed by adding to each sample 1.5 µl dNTPs 500 µM, 1 µl of buffer TP5X+ (100 mM $MgCl_2$, 100 mM Tris-HCl of pH 7.5, 300 mM NH_4Cl, 5 mM DTT), 0.73 µl of 100 mM $MgCl_2$ and 1.5 µl of H_2O_{DEPC}. After careful mixing, 1.5 µl of AMV reverse transcriptase (15 U; Promega) were added and the reactions incubated 10 min at 37°C. The reactions were stopped by addition of 15 µl of denaturing loading buffer (100mM Tris-HCl of pH 6.8, 200mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol). The samples were loaded onto a 6% polyacrylamide/50% urea sequencing gel and run at 200 W for 2.5 h. The position of the signals was determined by comparison with sequence ladder run simultaneously with the same end-labeled primers and obtained by Sanger sequencing of a PCR fragment amplified with primers 3313-3318 on MG1655 genomic DNA.

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FIGURE LEGENDS

Fig. 1. Expression of LpxT-GFP at different temperatures. Cultures of BW25113 and KG273 were grown overnight in LD medium at 37°C and diluted to OD_{600}= 0.1 in three flasks containing 40 ml of LD. The cultures were grown at 28°, 37° and 42°C up to OD_{600}= 0.8. 4 ml samples were taken for protein extraction. Crude extracts were run in an 8% polyacrylamide- 0.1% SDS gel, blotted onto a nitrocellulose filter and immunodecorated with GFP-specific antibodies. Only the portion of the filter with specific signals is shown (an unspecific signal was visible in all the lanes in the lower portion of the gel). The position where the 55 kDa band of the PageRuler Plus Prestained Protein Ladder (ThermoScientific) weight marker migrated is indicated. The filter was stained with Ponceau before the transfer and the portion of the filter where the LpxT-GFP migrated is shown in the lower panel.

Fig. 2. *lpxT* transcriptional analysis and activity of *lpxTp* promoter. A. *lpxT* transcription pattern. 20 µg samples of RNA extracted from BW25113 cultures at the indicated OD_{600} (OD) and temperatures (below the lanes in °C) were run in a 5% polyacrylamide-urea gel, blotted onto a nylon filter and hybridized with the *lpxT*-specific radiolabeled riboprobe. No other signals were visible. The mRNA was estimated to be ca. 900 nt long by comparison with the migration of the RiboRuler™ High Range RNA ladder (Thermo Scientific; not shown). 5S rRNA stained with ethidium bromide before blotting is shown as loading control. B. Expression and stability of *lpxT* and *lpxT*-GFP mRNAs. Samples for RNA extraction were taken from exponential cultures of BW25113 (*lpxT*) or KG273 (*lpxT*-GFP) grown at 28°C as described in Materials and Methods.

Samples were taken at the time points indicated above the lanes before (0) or after the addition of rifampicin. 20 µg of RNA were run in 1.5% denaturing agarose gels, blotted onto a nylon filter and hybridized with the *lpxT*-specific radiolabeled riboprobe. Signals corresponding to unspecific hybridization with 23S and 16S rRNA were also visible upon long exposition (not shown). 23S stained with methylene blue before transfer is shown as loading control. C. Mapping of the 5'-end of *lpxT* mRNA. Primer extension was performed with the radiolabelled oligo 3245 on 20 µg of RNA extracted from exponential cultures of BW25113 grown at 28°C up to the OD_{600} indicated above the lanes. The same oligonucleotide was used for Sanger-sequencing of a PCR fragment amplified on genomic DNA with the oligonucleotides 3210-3245. The coordinate of the identified 5'-end is reported beside the panel. D, E, F. Activity of *lpxTp*. D. Fluorescence of cultures of BW25113 carrying the plasmids indicated below the bars. The cultures were grown and fluorescence measured as detailed in Materials and Methods. Fluorescence values were normalized by the value of the negative control (BW25113/pGM2054 at 28°C). E, F. 10 µg of RNA extracted from BW25113/pGM2052 cultures grown as described in Materials and Methods up to the OD_{600} indicated above the lanes were either run and blotted as described in A and hybridized with the oligonucleotide 3172 (E), or analysed by primer extension with the oligonucleotide 3408. The sequencing ladder was obtained by Sanger sequencing with the same primer of a PCR fragment amplified with oligonucleotides 2804-3281 on pGM2052 DNA.

Fig. 3. 5'-UTR role in *lpxT* regulation and secondary structure prediction. A. Exponential cultures of BW25113 carrying either pGM2043 (left panels) or pGM2049 (right panels) were diluted to OD_{600}= 0.2 in three flasks containing

M9 medium supplemented with ampicillin, 0.4% glycerol and 0.2% arabinose. The flasks were incubated 60 min at 28°-37°-42°C. 10 ml samples were taken for protein extraction. Crude extracts prepared as described in Materials and Methods were run on 12% polyacrylamide gels. Proteins were immunodecorated with the HA-specific or the S1-specific (as loading control) antibodies. B. Structure with the MFE (minimum free energy) at 28°C was calculated by mFold version 2.3 (Zuker, 2003) and drawn with the VARNA applet (Darty *et al.,* 2009). The SD is indicated; the start codon is in lower case letters. Mutations obtained by site directed mutagenesis are indicated.

Fig. 4. Toeprinting analysis of translation initiation complex assembly on *lpxT* mRNA. Toeprinting analysis was performed as detailed in Materials and Methods on *lpxT* or *recA*-*lpxT* transcripts. The two RNAs have the same 5'-UTR of identical length. Incubation of the RNA with the 30S ribosomal subunit and fMet-tRNA was performed at the temperature indicated above the lanes. Primer extension was performed at 37°C with the radiolabeled primer 3313. The relative position of the signals corresponding to the initiation complex (+15) or to the unbound RNA (-29) respect to the first nt of the ORF (+1) is indicated. The sequencing ladder was obtained by Sanger sequencing with the same primer of a PCR fragment amplified with oligonucleotides 3318-3313 on BW25113 genomic DNA.

Fig. 5. Expression of translational fusions between the *lpxT* 5'-UTR and the GFP and effect of mutation in the 5'-UTR. Fluorescence was estimated at the indicated temperatures as described in Materials and Methods on exponential cultures of BW25113 carrying pGM2016 (*recA*-GFP), pGM2034 (wt) or its mutated derivatives pGM2045 (S1), pGM2047 (S2), pGM2048 (D1),

pGM2055 (D2), pGM2044 (D3), pGM2056 (SD1), pGM2064 (SD2) and pGM2065 (SD2-D1). Mutations are named as in Fig. 3B. At least 9 independent cultures (i.e. deriving from independent clones) of each strain were analysed in duplicate experiments. To compare results obtained in experiments done in different days, fluorescence was normalized for the highest value obtained in each experiment for pGM2016 at 42°C. The median (line) and average (x) are reported inside the boxes. The whiskers represent the minimum and maximum values observed. Statistical significance of difference relative to the wt at each assayed temperature was evaluated with t-test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. The numbers above the boxes are relative expression *vs*. the wt at the various temperature (calculated by dividing each average value for the wt average). The numbers below 37° and 42°C panels represent Induction Fold respect to 28°C (calculated by dividing the average value at 37°C or 42°C for the average 28°C value).

TABLE 1. Bacterial strains and plasmids

Strain	Relevant Genotype	Origin or reference
BW25113	*E. coli* K-12	(Datsenko and Wanner, 2000)
DH10B		(Grant *et al.,* 1990)
KG273	BW25113 *lpxT:*GFP	this work
MG1655	F^- lambda$^-$ *ilvG*$^-$ *rfb*-50 *rph*-1	(Blattner *et al.,* 1997)
MRE600	*rna*$^-$	(Wade *et al.,* 1966)

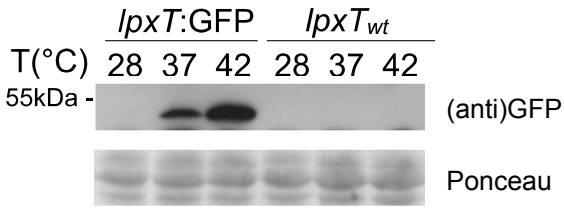
Plasmids and phage	Relevant characteristics[a]	Reference
pGM930	pBAD24-Δ1 (Carzaniga *et al.,* 2012) derivative with the insertion of t_Ω region	(Delvillani *et al.,* 2014)
pGM931	pHERD20T derivative carrying *araBp*-t_Ω region of pGM930	(Delvillani *et al.,* 2014)
pGM963	pGM931 (Delvillani *et al.,* 2014) derivative with the insertion of eGFP gene lacking the AUG start codon	(Raneri *et al.,* 2015)
pGM2011	pGM931 derivative with the insertion of sfGFP	(Delvillani *et al.,* 2014)
pGM2016	pGM2011 derivative, carries *recA* (2334354-2334277) translationally fused to sfGFP	(Delvillani *et al.,* 2014)

pGM2032	pGM2011 derivative, carries *lpxT* (2268742-2268859) translationally fused to sfGFP	This work
pGM2034	pGM2011 derivative, carries *lpxT* (2268826-2268859) translationally fused to sfGFP	This work
pGM2035	pGM930 derivative, carries the HA epitope coding region downstream of the *araBp* promoter	This work
pGM2040	pGZ119EH derivative, carries the *recA:sfGFP* reporter gene between *Eco*RI and *Pst*I sites	This work
pGM2043	pGM2035 derivative, carries *lpxT* (2268826-2269564) translationally fused to the HA region	This work
pGM2044	substitution TTTTT$_{-23/-27}$-GGGGG in the *lpxT* 5'-UTR of pGM2034	This work
pGM2045	deletion $\Delta_{-20/-21}$ in the *lpxT* 5'-UTR of pGM2034	This work
pGM2047	substitution T$_{-5}$-C in the *lpxT* 5'-UTR of pGM2034	This work
pGM2048	substitution CT$_{-22/-23}$-AA in the *lpxT* 5'-UTR of pGM2034	This work
pGM2049	pGM2035 derivative, carries recA-*lpxT* (2268826-2269564) translationally fused to the HA region	This work
pGM2052	pGM2040 derivative, carries the region 2268778-228826 between *Eco*RV and *Eco*RI restriction sites	This work

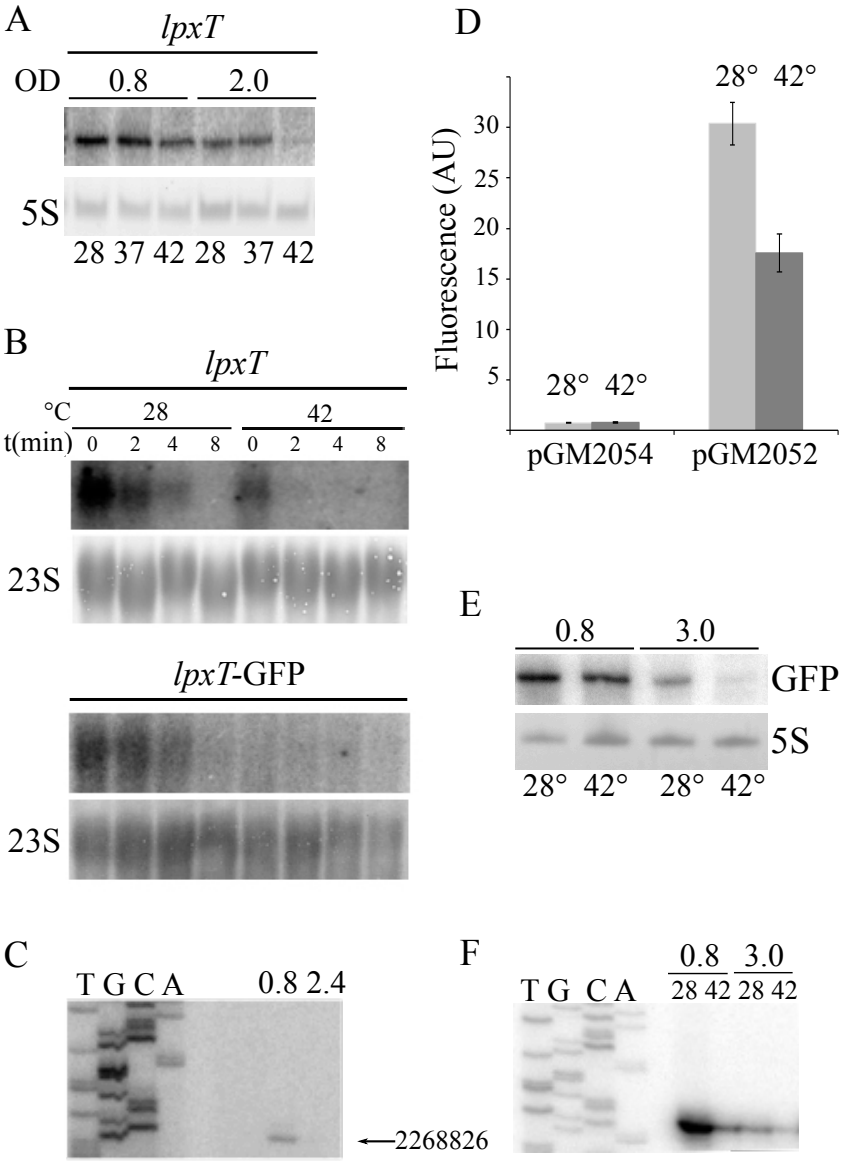
pGM2054	pGM2040 derivative, deletion of the region between *EcoR*V and *EcoR*I	This work
pGM2055	substitution A_{-18}-C in the *lpxT* 5'-UTR of pGM2034	This work
pGM2056	substitution T_{-10}-G in the *lpxT* 5'-UTR of pGM2055	This work
pGM2064	substitution A_{-9}-G in the *lpxT* 5'-UTR of pGM2034	This work
pGM2065	substitution A_{-9}-G in the *lpxT* 5'-UTR of pGM2048	This work
pGZ119HE	*oriVColD*; CamR ; P_{tac}	(Lessl *et al.,* 1992)
pKD46	carries λ RED recombination genes	(Datsenko and Wanner, 2000)
pKD13		(Datsenko and Wanner, 2000)

[a] *E. coli* coordinates refer to Genbank Accession Number U00096.2.

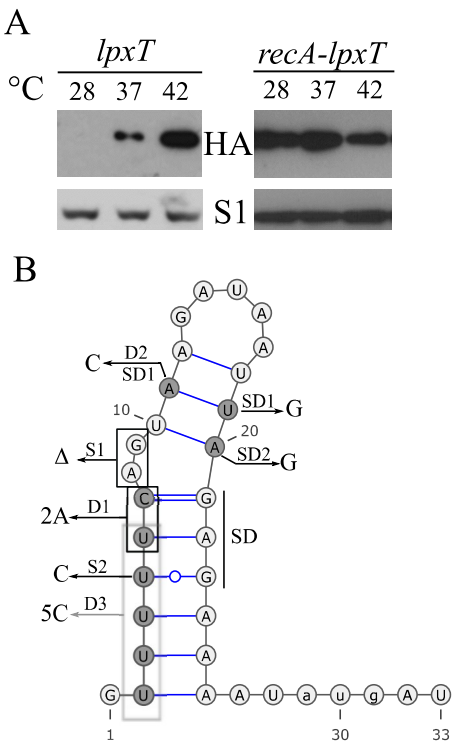
FIGURE



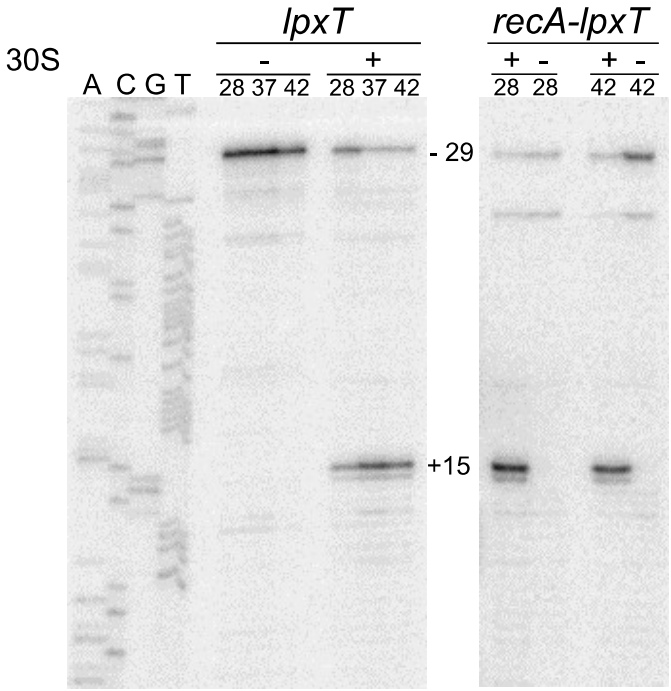
Sciandrone et al., Fig. 1



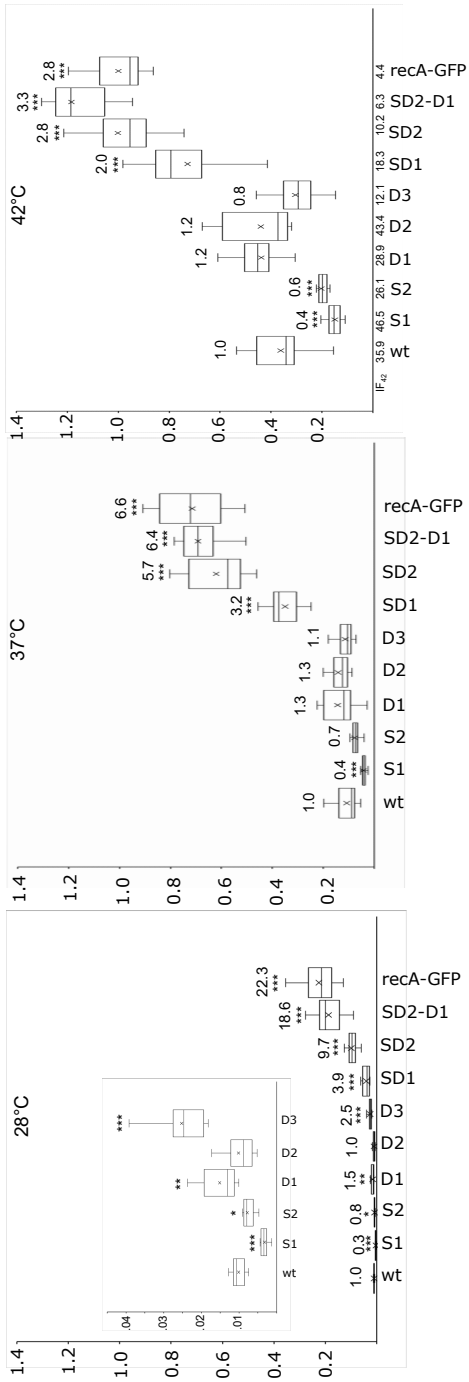
Sciandrone et al., Fig. 2



Sciandrone et al., Fig. 3



Sciandrone et al., Fig. 4



Sciandrone et al., Fig. 5

SUPPLEMENTARY TABLE S1. Oligonucleotides

Name	Sequence[a]
2804	CTCCTGCAGCTATTTGTATAGTTCATCCATGC
3172	GACAAGTGTTGGCCATGGAAC
3210	GGGGTACCTTTCCAGCAGCGAAGCTG
3211	CCGGTACCAATCATATTTTCTCTAATTATCTTACT
3225	GGGCCATGGTTTTTCAGTAAGATAATTAGAG
3245	GGGGATATACCAGGAAAGAAAC
3257	CGAATTCTACCCATACGATGTTCCAGATTACGCTTGACTGCA
3258	GTCAAGCGTAATCTGGAACATCGTATGGGTAGAATTCGGTAC
3259	GCGTTTTTCAGTAAGATAATTAGAG
3270	**TAATACGACTCACTATGGG**GGGGATATACCAGGAAAGAAAC
3273	GGAATTCTTTGTTTTGGAAATGTTTGTTTTTTC
3274	GGAATTCCAACAGAACATATTGACTATCC
3281	ATCGCGCGCTGTTGAAGCTTC
3282	GGAATTCGCAGGCAACCTTACGCG

3284 GGGCCATGGCCCCCCAGTAAGATAATTAGAGAAAATATGAT
T

3285 GGGCCATGGTTTTTCTAAGATAATTAGAGAAAATATGATT

3286 GGGCCATGGTATTACCCGGCATGACAGGAGAAAATATGATT
AAAAATTTGCCGC

3307 GGGCCATGGTTTCTCAGTAAGATAATTAGAGAAAATATGAT
T

3308 GGGCCATGGTTTTAAAGTAAGATAATTAGAGAAAATATGAT
T

3313 ATGATTAACGGGGATATACCAGG

3318 **CTAATACGACTCACTATAG**GTTTTTCAGTAAGATAATTAG
AGAAAATA

3319 **CTAATACGACTCACTATAG**GGTATTACCCGGCATGACAGG

3330 GGGCCATGGTTTTTCAGTCAGATAATTAGAGAAAATATGAT
T

3331 GGGCCATGGTTTTTCAGTCAGATAATGAGAGAAAATATGAT
T

3348 GGGCCATGGTTTTTCAGTAAGATAATTGGAGAAAATATGAT
T

3349 GGGCCATGGTTTTAAAGTAAGATAATTGGAGAAAATATGAT
T

3358 CCAGAGTAATGATTGGCGCACAC

3359 TTTGTTTTGGAAATGTTTGTTTTTTCCTGG

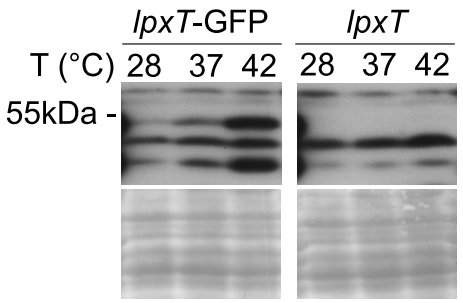
3360 GGAAAAAACAAACATTTCCAAAACAAAAGTAAAGGAGAA
GAACTTTTCAC

3362 CCCTGATGATGTTAATTACTGTGAGCTGTCAAACATGAGAA
TTAATTCCG

3363	CTCACAGTAATTAACATCATCAGGG
3364	CAGAAAGTTAATAAGCGGGGTTGG
3373	GAAGCAGCTCCAGCCTACACCTGCAGCTATTTGTATAGTTCAT
3374	ATGAACTATACAAATAGCTGCAGGTGTAGGCTGGAGCTGCTTC
3408	CCAGTGAAAAGTTCTTCTCCTTTAC

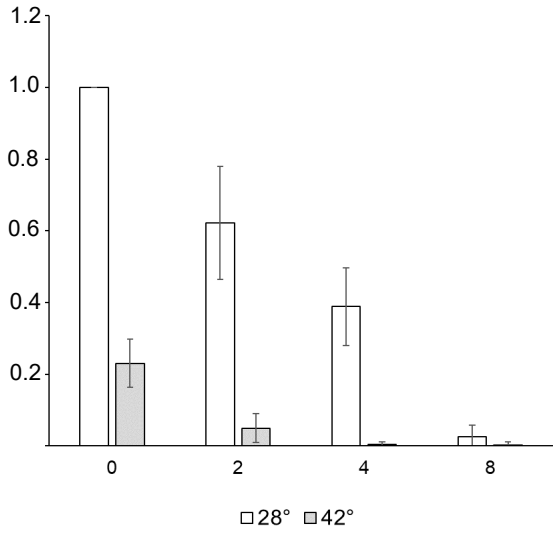
[a] Boldface characters, T7 promoter; underlined, restriction sites

SUPPLEMENTARY FIGURES



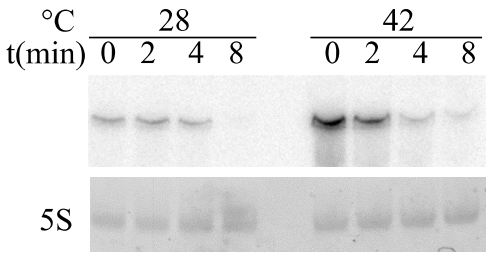
Sciandrone et al., Fig. S1

Fig. S1. Expression of LpxT-GFP at different temperatures. Crude extracts obtained from BW25113 (not shown) and KG273 exponential cultures grown at the temperatures indicated above the lanes were run in a 10% polyacrylamide-0.1% SDS gel, blotted onto a nitrocellulose filter and immunodecorated with GFP-specific antibodies. The position where the 55 kDa band of the PageRuler Plus Prestained Protein Ladder (ThermoScientific) weight marker migrated is indicated. The filter was stained with Ponceau before the transfer and the portion of the filter where the LpxT-GFP migrated is shown in the lower panel. In this experiment the GFP antibody was not pretreated to minimize unspecific hybridization (see Materials and Methods). As a consequence, several additional signals were visible. Specific LpxT-GFP signal was individuated by estimated MW and by comparison between the KG273 and the BW25113 band patterns.



Sciandrone et al., Fig. S2

Fig. S2. Half-life of *lpxT* mRNA at different temperatures. Northern blotting signals corresponding to *lpxT* mRNA extracted before (0) and at different time points after adding rifampicin, as described in Materials and Methods and in Fig. 2B legend, were quantified and normalized on the value of the sample collected at 28°C, time 0. The graph represents the average and standard deviation (vertical bars) of four independent experiments.



Sciandrone et al., Fig. S3

Fig. S3. Expression of LpxT-HA at different temperatures. Expression and stability of *lpxT-HA* mRNA. Samples for RNA extraction were taken from exponential cultures of BW25113/pGM2043 grown at 28°C. Half cultures were shifted at 42°C 10 min before first sampling (t=0). Rifampicin (0.4 mg/ml) was immediately added and samples were taken at the time points indicated above the lanes. 15 μg of RNA were run in a 5% polyacrylamide-urea gel, blotted onto a nylon filter and hybridized with the HA-specific oligonucleotide. No other signals were visible.