LasR regulates protease IV expression at suboptimal growth temperatures in *Pseudomonas aeruginosa*

Running title: LasR regulates protease IV expression

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ABSTRACT

The opportunistic pathogen *Pseudomonas aeruginosa* causes debilitating lung infections in people with cystic fibrosis, as well as eye, burn, and wound infections in otherwise immunocompetent individuals. Many of *P. aeruginosa*'s virulence factors are regulated by environmental changes associated with human infection, such as a change in temperature from ambient to human body temperature. One such virulence factor is protease IV (PIV). Interestingly, *piv* expression is higher at ambient temperatures (22-28°C) compared to human body temperature (37°C). We found that *piv* expression was thermoregulated at stationary phase, but not exponential phase, and that *piv* is thermoregulated at the level of transcription. Protein levels of known transcriptional regulators of *piv*, the quorum sensing regulator LasR and the gene-silencing histone nucleoid silencing proteins MvaT/MvaU, were not thermoregulated. Using a transcriptional reporter for *piv*, we show that LasR activates *piv* expression at stationary phase at 25°C but not 37°C, while MvaT/MvaU are not required for *piv* thermoregulation. We also identified a *las* box in the *piv* promoter, which is important for *piv* thermoregulation. We propose that LasR directly regulates *piv* at stationary phase at 25°C but has a negligible impact at 37°C. Here, we show that *piv* is uniquely regulated by LasR in a temperature-dependent manner. Our findings suggest that the LasRI quorum sensing regulon of *P. aeruginosa* may not be fully characterized and that growth at non-standard laboratory conditions such as lower temperatures could reveal previously unrecognized quorum sensing regulated genes.

IMPORTANCE

Pseudomonas aeruginosa is a versatile opportunistic pathogen capable of causing many different types of infections that are often difficult to treat, such as lung infections in people with cystic fibrosis. Temperature regulates the expression of many virulence factors that contribute to *P. aeruginosa*'s ability to cause infection, yet our mechanistic understanding of virulence factor

thermoregulation is poor. In this study, we show that the virulence factor protease IV is thermoregulated at the level of transcription via temperature-dependent upregulation by the quorum sensing regulator, LasR. Mechanistic studies of virulence factor thermoregulation will expand our understanding of how *P. aeruginosa* experiences different environments, including the mammalian host. Our work also highlights the importance of growth conditions in studying gene regulation, as it elucidates the regulation of protease IV by LasR, which was previously not well understood.

INTRODUCTION

Pseudomonas aeruginosa is a highly adaptable bacterial pathogen that causes lung, burn, wound, and corneal infections. To survive in the diverse niches of the human body, *P. aeruginosa* adapts to its environment and regulates the expression of many virulence factors that assist in nutrient acquisition and immune evasion. A major environmental cue that regulates virulence in many pathogenic bacteria, including *P. aeruginosa*, is the change in temperature from ambient or room temperature to that encountered in or on the human body (1, 2). Human body temperature is generally accepted to be 37°C but can be lower at body sites exposed to the environment, such as the skin and eyes.

Many virulence factors in *P. aeruginosa* are regulated by temperature and mechanisms of thermoregulation have been studied at the post-transcriptional (2) and post-translational levels (3–5). Thermoregulation at the level of transcription has not been as extensively studied, despite the finding that expression of 6.4% of the genome is regulated by temperature, with many virulence factors upregulated at 37°C compared to ambient temperatures (6, 7). Interestingly, expression of the gene encoding the virulence factor protease IV (PIV) is greatly upregulated at ambient temperatures compared to 37°C in both of the laboratory strains PAO1 and PA14 (6, 7). PIV is a serine protease that degrades a broad range of immune molecules, including the iron sequestering lactoferrin and transferrin, complement C3 found in blood, as well as alveolar surfactant proteins and IL-22 that provide protection in the lungs (8–11). PIV has been extensively characterized as a key virulence factor for *P. aeruginosa* eye infections and strains lacking PIV are rendered avirulent in corneal infections (12–15). Corneal infections can be difficult to treat with severe patient outcomes, as seen in a recent carbapenem-resistant *P. aeruginosa* outbreak caused by contaminated artificial tears that resulted in multiple deaths, instances of moderate to complete loss of vision, and enucleation due to the infection (16).

PIV is initially translated as a ~48 kD pre-proenzyme that contains an N-terminal signal sequence, an inactivating propeptide, and the protease domain (17). The signal sequence is cleaved during secretion and the remaining propeptide renders the 45 kD proenzyme catalytically inactive; release of the propeptide is required to yield the 26 kD, enzymatically active ("mature"), protease. Cleavage of the propeptide has been reported to occur autocatalytically and by the *P. aeruginosa* elastase LasB in bacterial supernatants (9, 18, 19).

Transcriptional regulation of *piv* is also complex and not fully understood. *piv* expression is directly upregulated by the sigma factor PvdS under iron starvation conditions, leading to the gene's alternative name *prpL* (PvdS-regulated endoprotease, lysyl class) (20). *piv* expression is directly repressed by the histone nucleoid structuring (H-NS) family members MvaT and MvaU, which act together to negatively regulate the same global regulon (21, 22). *piv* is also regulated by the LasRI quorum sensing system (23–25). The LasRI quorum sensing system is a mechanism for *P. aeruginosa* to coordinate expression of certain genes at high cell densities through the audoinducer synthase LasI synthesizing the freely diffusible homoserine lactone (HSL) autoinducer 3-oxo-C12-HSL (3O-C12-HSL), which complexes with transcription factor LasR to activate its function as a positive regulator of hundreds of genes (23–26). LasR has not been found to directly bind to the *piv* promoter and the mechanism(s) by which it regulates *piv* is not clear (27). Additionally, how *piv* expression is regulated by temperature is not understood and is important for our understanding of how *P. aeruginosa* adapts to body sites which can experience lower temperatures due to exposure to the environment.

We found that *piv* thermoregulation occurs at the level of transcription due to significantly higher promoter activity at room temperature (25°C) compared to human body temperature (37°C). Higher *piv* expression at ambient temperatures was reflected in a higher level of mature PIV protein, suggesting that temperature regulation could affect the amount of this virulence factor produced by *P. aeruginosa* in infection sites experiencing different temperatures. Our

study outlines the role of LasR as an activator of *piv* expression at 25°C but not 37°C and provides evidence for direct LasR regulation of *piv*. This suggests that LasRI quorum sensing may regulate certain genes disparately under conditions different from those standardly used in the laboratory.

RESULTS

Thermoregulation of *piv* depends on growth phase.

A previous transcriptomic study from our group showed by microarray analysis that *piv* was more highly expressed at 22°C compared to 37°C in *P. aeruginosa* PAO1 (6). Similar results were found in an RNA-seq analysis of *P. aeruginosa* PA14 grown at 28°C compared to 37°C (7). However, both transcriptomes were determined for cells at stationary phase and only examined two temperatures: a “low” or ambient temperature and human body temperature, 37°C. We wanted to examine *piv* expression at additional temperatures to characterize if *piv* thermoregulation is binary with a ‘high’ and ‘low’ expression state or continuous with expression changing gradually as temperature changes. We also wanted to determine if growth phase affected *piv* thermoregulation. Wild-type *P. aeruginosa* PAO1 was grown in parallel at 25°C, 30°C, 37°C, and 42°C and RNA extracted from cultures at each temperature, first at exponential phase and then from the same cultures at early stationary phase (Fig. 1A). *piv* expression at each temperature was determined relative to 37°C (a standard laboratory condition) at each growth phase using RT-qPCR. Interestingly, we found that *piv* thermoregulation depended on growth phase. At exponential phase, *piv* expression was not regulated by temperature (Fig. 1B). However, at stationary phase, *piv* expression at 25°C and 30°C was ~16-fold and ~10-fold higher, respectively, than at 37°C and ~6-fold lower at 42°C than 37°C. These results support *piv* thermoregulation as continuous within the range of tested temperatures, with highest

126 expression observed at 25°C and expression decreasing gradually as temperature increases to
127 42°C.

128 We then determined whether *piv* transcript levels were reflected in levels of PIV protein
129 in cells grown at different temperatures. To test this, we used PAO1 PIV VSV-G, in which the
130 native *piv* locus was tagged at the C-terminus with the vesicular stomatitis virus G (VSV-G)
131 epitope. The C-terminal placement of the VSV-G tag would allow for detecting the 48 kD pre-
132 proenzyme, the 45 kD proenzyme, and the 26 kD mature enzymatic forms of PIV, as processing
133 of the full-length gene product involves proteolysis from the N-terminus (9, 17). PAO1 PIV VSV-
134 G was grown at 25°C, 30°C, 37°C, and 42°C to early stationary phase and cell lysates were
135 subjected to quantitative western blotting with VSV-G antibodies (αVSV-G) as well as with RNA
136 polymerase α subunit antibodies (αRpoA) as a temperature independent control (Fig 2A,B). We
137 found that the abundance of the 26 kD mature PIV VSV-G domain was significantly higher at
138 25°C and 30°C than at 37°C. Mature PIV VSV-G was hardly detected at 42°C. An unprocessed
139 form of PIV could be detected at approximately 50 kD at 25°C and 30°C but not at 37°C or
140 42°C; however, we were not able to distinguish between the 48 kD pre-proenzyme and 45 kD
141 proenzyme forms of PIV. The abundance of mature PIV VSV-G at each temperature agrees well
142 with *piv* expression data, suggesting there is likely not temperature-dependent post-
143 transcriptional modification of *piv*. Together, these results show that *piv* thermoregulation
144 depends on growth phase and thermoregulation of *piv* gene expression is reflected in the
145 abundance of PIV protein.

146 **Transcriptional regulators of *piv* are not thermoregulated.**

147 Previous studies have shown that LasRI quorum sensing positively regulates *piv* (23–25) and
148 the H-NS family members MvaT and MvaU directly repress *piv* expression (21, 22). One
149 possible mechanism for the thermoregulation of *piv* expression could be thermoregulation of
150 transcriptional regulators of *piv*. We asked if the quorum sensing regulator LasR was more

abundant at 25°C than 37°C, and/or if the repressors MvaT and MvaU were more abundant at 37°C than 25°C, as a possible mechanism for *piv* thermoregulation. We first examined gene expression of *lasR*, *mvaT*, and *mvaU* in PAO1 grown at 25°C and 37°C at both exponential and stationary phases (Fig. 3A). The expression of each transcriptional regulator was not significantly regulated by temperature, even at stationary phase, when *piv* is most thermoregulated. This suggested that LasR, MvaT, and MvaU are not themselves thermoregulated, which is consistent with other studies that did not identify these genes as thermoregulated (6, 7). To confirm that the protein abundance of LasR, MvaT, and MvaU was not regulated by temperature, we assessed the level of each protein in cells grown at 25°C and 37°C to stationary phase by quantitative western blotting. Blotting in PAO1 with antibodies raised to LasR in rabbits (25) confirmed that the level of LasR protein was not significantly different at 25°C compared 37°C (Fig. 3B). Blotting with αVSV-G antibodies in the PAO1 MvaT VSV-G (Fig. 3C) and PAO1 MvaU VSV-G strains (Fig. 3D) with C-terminal VSV-G epitope tagged *mvaT* and *mvaU* at the native loci also showed that levels of MvaT VSV-G and MvaU VSV-G were also not significantly different at 25°C compared to 37°C. These results indicate that transcriptional regulators LasR, MvaT, and MvaU are not themselves thermoregulated.

piv expression is regulated by temperature at the level of transcription.

Another possible mechanism for the thermoregulation of *piv* expression could be temperature dependent *piv* promoter activity. To test this, we made a transcriptional reporter by fusing the 200 bp upstream of the *piv* coding sequence to an unstable green fluorescent protein variant, *gfp*(ASV) (28) that has been previously used for transcriptional reporters (29). PAO1 carrying the P_{piv}-*gfp*(ASV) reporter plasmid (pRD91) was grown at 25°C and 37°C and assayed for fluorescence at exponential phase (Fig. 4A) and then again at stationary phase (Fig. 4B). PAO1 carrying a no-promoter *gfp*(ASV) reporter plasmid (pRD87) was used to assess background fluorescence, which was overall low at both temperatures and growth phases (Fig. S1). At both

exponential and stationary phases, *piv* promoter activity in PAO1 was higher at 25°C than 37°C, with promoter activity at 37°C not above the background (Fig. 4A,B). Promoter activity at 25°C was also higher at stationary phase than exponential phase. The finding that promoter activity was thermoregulated and affected by growth phase suggests that the growth phase dependent thermoregulation of *piv* observed in Fig. 1 is due to differences in *piv* promoter activity.

Although the abundance of transcriptional regulators LasR, MvaT, and MvaU was not thermoregulated, we wondered if their regulation of *piv* was affected by temperature since the *piv* promoter is sufficient to drive thermoregulation. To test if LasR was required for *piv* thermoregulation, we assayed a Δ*lasR* mutant carrying the P_{piv}-*gfp*(ASV) reporter for fluorescence at 25°C and 37°C as described; there was no promoter activity above background levels at either 25°C or 37°C, at both exponential and stationary phases (Fig. 4A, B). Expressing LasR in single copy from the ectopic Tn*7* site complemented the Δ*lasR* mutation, restoring wild-type *piv* promoter thermoregulation. This shows that LasR is required for *piv* promoter activity at both 25°C and 37°C. To further confirm that *piv* was regulated by LasRI quorum sensing, we deleted the gene encoding the LasI autoinducer synthase and assayed the Δ*lasI* mutant for activity from the *piv* transcriptional reporter (Fig. 4A,B). Promoter activity in the Δ*lasI* mutant phenocopied that of the Δ*lasR* mutant, supporting *piv* being regulated by LasRI quorum sensing.

In the Δ*mvaT*Δ*mvaU* mutant, promoter activity at exponential phase was overall higher at both temperatures than in PAO1 and was slightly thermoregulated; however, at stationary phase promoter activity was highly thermoregulated (Fig. 4). Expressing either MvaT or MvaU in single copy from the ectopic Tn*7* site complemented the Δ*mvaT*Δ*mvaU* mutation. Since promoter activity is thermoregulated at stationary phase in the Δ*mvaT*Δ*mvaU* mutant, this result indicates that MvaT and MvaU are not required for *piv* thermoregulation. To test if LasR was responsible for thermoregulation in the Δ*mvaT*Δ*mvaU* mutant and to assess the role of LasR in *piv* thermoregulation in the absence of strong repressors MvaT and MvaU, we deleted *lasR*

from the Δ*mvaT*Δ*mvaU* strain. At exponential phase, promoter activity in Δ*mvaT*Δ*mvaU*Δ*lasR* was overall derepressed and slightly thermoregulated, phenocopying Δ*mvaT*Δ*mvaU* (Fig. 4A,B). However, at stationary phase, promoter activity in Δ*mvaT*Δ*mvaU*Δ*lasR* was significantly less thermoregulated due to a decrease in *piv* promoter activity at 25°C with no change at 37°C. Thus, deleting *lasR* from Δ*mvaT*Δ*mvaU* revealed that LasR only increases promoter activity at stationary phase at 25°C and that LasR does not seem to regulate *piv* at 37°C.

Collectively, these results reveal that *piv* thermoregulation is occurring due to temperature dependent promoter activity which requires the transcriptional regulator LasR but not MvaT nor MvaU.

LasR directly regulates *piv*.

To determine how *piv* expression was being thermoregulated by LasR, we first analyzed the *piv* promoter region more closely. We had found that 200 nucleotides upstream of the *piv* coding region (Fig. 5A) was sufficient for thermoregulation of its promoter activity. The *piv* upstream region was recently identified as containing a σ^{70}-dependent promoter (22). Using SAPPHIRE (30), a predictor for σ^{70} promoters in *Pseudomonas* spp., we identified the putative -35 and -10 elements for the sigma factor RpoD (Fig. 5A, underlined regions). Putative transcription start sites (TSSs) for *piv* were mapped using 5' rapid amplification of cDNA ends (5' RACE) in PAO1 (Fig. 5A, highlighted bases). We found that transcription began at positions 151C or 152G of the promoter region sequence. The predicted -35 and -10 elements are appropriately positioned upstream of our TSSs such that RpoD could be directing transcription of *piv* beginning at 151C or 152G of the promoter.

We then analyzed the region upstream of the TSSs and predicted σ^{70} -35 and -10 elements and identified a 16 bp sequence (Fig. 5A, boxed region) that resembles a canonical *las* box, particularly the one identified for non-cooperative LasR binding (27). We hypothesized that LasR could be directly regulating *piv* by binding to this region. To test this, we introduced

point mutations to the putative *las* box in the P_{piv}-*gfp*(ASV) reporter plasmid (pRD91) at highly conserved positions that we predict would either disrupt (Fig. 5B, pink base changes) or improve (Fig. 5B, green base changes) potential LasR binding. The predicted deleterious mutations were among ones that have been previously shown to abolish LasR binding to and greatly reduce activity of the *pqsR* promoter (31, 32). We then assayed the effects of these mutations on *piv* promoter activity at 25°C and 37°C in PAO1 (Fig. 5C). The predicted deleterious 1C→A and 14T→A mutations of the putative *las* box reduced *piv* promoter activity slightly at exponential phase and greatly at stationary phase at 25°C only. Since these mutations greatly reduced the effect of growth phase on *piv* promoter activity only at 25°C, these results are consistent with the role of LasR as a quorum sensing regulator that is more active at stationary phase. The 16T→G mutation, predicted to improve LasR binding, increased promoter activity at both 25°C and 37°C; however, promoter activity was still highly thermoregulated. These results are consistent with our prediction for the location of a *las* box in the *piv* promoter.

DISCUSSION

Here, we found that *piv* thermoregulation occurs at the level of transcriptional regulation and requires the quorum sensing regulator LasR but not the H-NS family members MvaT/MvaU. By leveraging a Δ*mvaT*Δ*mvaU* mutant strain in which *piv* expression was depressed, we show that LasR acts as a positive regulator of *piv* at 25°C with little to no effect on *piv* expression at 37°C. This is consistent with our findings that *piv* expression is significantly thermoregulated at stationary phase but not at exponential phase, as LasR abundance and activity is higher at stationary phase (25). We also identify a putative *las* box in the *piv* promoter and provide evidence that LasR directly regulates *piv*.

The role of *piv* and its thermoregulation in *P. aeruginosa* virulence

Thermoregulation of *piv* may underpin the importance of this protease for infections of surface exposed body sites, such as the eyes or skin. Corneal infections by *P. aeruginosa* can lead to permanent loss of vision and death as seen in a recent outbreak caused by contamination of artificial tear drops (16). PIV causes epithelial erosion in the cornea and contributes to bacterial virulence, and *P. aeruginosa* lacking PIV is practically avirulent in corneal models of infection (12–15). PIV also severely impaired wound healing and contributed to elevated levels of inflammation in a wound model of infection in mice (33, 34). At temperatures lower than 37°C, which could be experienced by the bacteria in surface exposed infections, many virulence factors such as LasB and type III secretion are downregulated, while PIV is upregulated (6, 7). The unique thermoregulation of PIV may in part contribute to the severity of corneal infections and to the success of *P. aeruginosa* as an ocular pathogen, as well as the ability of *P. aeruginosa* to cause chronic infections of wounds.

Thermoregulation of *piv* has impacted its study.

Many studies of PIV processing, secretion, and post-secretion activation have utilized *in vitro* experiments or expressed PIV in heterologous hosts, which has led to some inconsistencies in the model for PIV post-secretion activation. One study in PAO1 showed that the propeptide could only be degraded by protease LasB in *in vitro* assays (although the ability of PIV to degrade its own propeptide was not tested) and proposed that the PIV proenzyme is secreted into supernatant and then processed into the mature form by LasB (18). However, earlier studies found that PIV purified from the supernatant of PA103-29, a derivative of PA103 that lacks LasB, was enzymatically active, as was PIV when expressed in *Pseudomonas putida* and *Escherichia coli* (14, 17, 35). In both heterologous hosts, mature PIV was found in cell supernatants, indicating that LasB is not required for the removal of the propeptide from the proenzyme and suggesting that this maturation step may occur autocatalytically. Mutants of PIV that lacked catalytic activity could not be processed from the proenzyme into the mature protease, further supporting an autocatalytic cleavage mechanism of the propeptide (9). We

277 found significant amounts of mature PIV inside cells of PAO1 at 25°C and 30°C (Fig. 2), as well
278 as low levels of precursor forms, which supports a path for the maturation of PIV inside cells
279 and not just in the supernatant. As LasB is inactive in the cytoplasm of *P. aeruginosa*, we
280 believe our results show that PIV can mature autocatalytically in PAO1.

281 Prior studies on the secretion and protease activity of PIV have been hampered due to
282 the low amount of PIV produced from the native chromosomal locus (8, 17). We postulate that
283 the low level of PIV observed by others was due to thermoregulation of *piv* expression, as the
284 studies were all conducted at 37°C. Consistent with this, we detected abundant PIV in both
285 mature and precursor(s) forms at 25°C and 30°C but very little mature and no precursor PIV at
286 37°C or higher (Fig. 2). Our findings demonstrate that PIV produced from its native locus can be
287 easily detected in cells grown at 25°C and propose this as a viable alternative to yield high *piv*
288 expression and/or high PIV protein levels for further studies of PIV secretion and maturation.

289 **A model for thermoregulation of *piv* by LasR**

290 We propose a model for *piv* thermoregulation in which LasR activates *piv* transcription at
291 stationary phase by interacting with a newly identified *las* box in the *piv* promoter at 25°C but not
292 at all, or very lowly, at 37°C and higher (Fig. 6). In wild-type cells growing at 25°C, LasR
293 increases *piv* promoter activity while MvaT and MvaU appear to have no regulatory effect. Given
294 that the loss of *lasR* results in no detectable promoter activity at 25°C and that further deletion of
295 *mvaT* and *mvaU* from Δ*lasR* restores promoter activity (Fig. 4), we suspect that MvaT/MvaU do
296 not repress at 25°C due to LasR interfering with MvaT/MvaU interacting with the *piv* promoter. In
297 a wild-type cell growing at 37°C, *piv* promoter activity is barely detectable and LasR appears to
298 have no regulatory effect on *piv* (Fig. 4). MvaT/MvaU repress *piv* at 37°C, which we suggest is
299 due to the lack of LasR interference at the promoter. And while PvdS is a known regulator of *piv*
300 in low iron conditions, since our experiments were all conducted in iron replete LB media in
301 which PvdS is inactive, *piv* thermoregulation must not require PvdS.

It remains unclear how temperature is modulating LasR regulation of *piv*. In agreement with prior studies (36), we determined that LasR expression and protein levels are not temperature dependent (Fig. 3) and thus the amount of LasR is not driving higher *piv* expression at lower temperatures. Additionally, although translation of LasI is slightly higher at 37°C than 30°C due to an RNA thermometer, this has been shown to only result in a small increase of 3O-C12-HSL levels that had no effect on expression of the LasR-regulated *lasB* or the amount of the gene product elastase (36). Given this and that *piv* expression is higher at ambient temperatures of 25°C-30°C than 37°C (the opposite of how LasI is thermoregulated), we do not believe that the general activity of the LasRI quorum system itself is thermoregulated nor responsible for thermoregulating *piv*. A second quorum sensing system in *P. aeruginosa*, the RhlRI system, is thermoregulated due to an RNA thermometer that results in higher RhlR levels at 37°C. The thermoregulation of the transcriptional activator RhlR does result in higher levels of RhlR-regulated virulence factors, like rhamnolipids, at 37°C (36); however, *piv* is not regulated by the RhlRI quorum sensing system, and furthermore is upregulated at temperatures lower than 37°C, indicating that RhlRI is not responsible for *piv* thermoregulation (25).

We postulate that thermoregulation of *piv* by LasR involves an interaction between LasR and specifically the *piv* promoter that is temperature sensitive. Lower temperatures such as 25°C may favor LasR interacting with the *piv* promoter at the identified *las* box to mediate *piv* thermoregulation. A previous study that identified promoters directly bound by LasR in PAO1 failed to identify the *piv* promoter as one, despite identifying several known and novel LasR targets (27). In retrospect, this was likely because the experiment in this previous study was conducted at 37°C (27), a temperature at which our findings show that LasR does not regulate *piv*. We find a mutation designed to ‘improve’ the *las* box sequence within the *piv* promoter increases promoter activity at 37°C to detectable, albeit still low, levels (Fig. 5). This further supports that LasR directly regulates *piv*, but only under certain temperature conditions, which

could explain why prior experiments conducted at 37°C failed to identify *piv* as a directly LasR-regulated gene. To the best of our knowledge, this is the only gene known to be directly regulated by LasR in a temperature-dependent manner.

It is interesting that mutating the *las* box sequence toward consensus at highly conserved positions did not result in equal promoter activity at 25°C and 37°C (Fig 5). Thermoregulation of *piv* by LasR must not be solely due to the sequence of the *las* box. It is notable that the position of the *las* box is farther upstream of the *piv* TSSs we identified compared to the position of the *las* box in some other LasR bound promoters (7). The *piv* promoter is also unusually AT rich at ~59% compared to the *P. aeruginosa* PAO1 genomic average of ~33%. Genomic DNA that is high in AT content is more flexible and has been documented to adopt temperature sensitive secondary structures as "DNA thermometers" that can mediate temperature-dependent transcription factor binding and subsequent gene expression (1). The position of the putative *las* box within the *piv* promoter could be accessible to LasR at 25°C, but not 37°C, due to temperature-dependent structural changes of the promoter itself.

As previously discussed, LasR regulated genes have been extensively studied as part of the quorum sensing regulon in *P. aeruginosa* (23–25, 37). However, *piv* had not been identified as directly regulated by LasR prior to this study, likely due to low or nonexistent direct LasR regulation of *piv* under the standard laboratory growth temperature of 37°C. This raises the possibility that LasR activates transcription of other unknown genes only at specific conditions, included but not limited to non-optimal growth temperatures. Studying gene expression under non-standard laboratory conditions that mimic various other infection environments may expand our understanding of virulence factor regulation and quorum sensing in the diverse environments that *P. aeruginosa* can survive in.

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MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains, plasmids, and oligonucleotides are listed in the Supplemental Material, Tables S1-S3. Standard molecular biology practices were used to construct plasmids and all constructed plasmids were confirmed by sequencing. Mutant strains were confirmed by PCR and sequencing. Full plasmid and strain construction details are available in the Supplemental Material.

Culture conditions

Overnight cultures of *P. aeruginosa* were routinely grown in 3 mL of lysogeny broth (LB) at 37°C in a rolling drum. As appropriate, *E. coli* growth media was supplemented with gentamicin (15 μg/mL), carbenicillin (100 μg/mL), or tetracycline (10 μg/mL) and *P. aeruginosa* media with gentamicin (60 μg/mL), carbenicillin (300 μg/mL), or tetracycline (100 μg/mL), unless otherwise noted.

RNA extraction and DNase treatment

To measure transcript levels at different growth temperatures, biological triplicates of PAO1 grown overnight at 37°C were subcultured to an initial OD_{600} of 0.05 in 25 mL LB and incubated shaking at 200 rpm at the indicated temperature. At exponential phase (OD_{600} ~0.5), 10^9 cells were centrifuged and resuspended in 1 mL Tri-Reagent (Millipore Sigma) and RNA extracted according to the manufacturer's instructions, using chloroform. The cultures were allowed to continue growing until early stationary phase (OD_{600} ~2.0) and RNA extracted again as described. 5 μg of extracted RNA was DNase treated with TURBO DNase (ThermoFisher) according to the manufacturer's instructions for rigorous DNase treatment.

Real time quantitative PCR (RT-qPCR)

One-step RT-qPCR was performed on DNase treated RNA using Power SYBR™ Green RNA-to-CT™ 1-Step Kit (Applied Biosystems) on a LightCycler 96 (Roche) using LightCycler software v1.1.0.1320. Reactions were conducted in technical triplicate per each biological sample and the C_q values were averaged for gene expression analysis using the temperature insensitive *omlA* (6) as an internal control gene. The relative expression of a gene at a given temperature *X*°C compared to 37°C was calculated and plotted as $\log_2(2^{-\Delta\Delta Ct})$, where $\Delta\Delta Ct$ = (C_q gene at *X*°C - C_q *omlA* at *X*°C) – (C_q gene at 37°C – C_q *omlA* at 37°C).

Immunoblotting

Overnight biological triplicates were subcultured to an initial OD_{600} of 0.05 in 25 mL LB and incubated shaking at 200 rpm at the indicated temperature. At early stationary (OD_{600} ~2.0) phase, 0.5 mL was collected and resuspended in 475 μL Laemlli buffer with 25 μL β-mercaptoethanol and boiled for 15 minutes. 10 μL of prepared cell lysate sample was electrophoresed, transferred to a low-fluorescence PVDF membrane (Bio-Rad), and blocked for 1 hour in Intercept Blocking Buffer (Li-Cor). Primary antibodies against the VSV-G epitope tag (Sigma, batch # 0000143676) were used at 1:5,000 overnight at 4°C and primary antibodies against LasR (25) used at 1:1,000 overnight at 4°C with fluorophore conjugated secondary

antibodies against rabbits used at 1:10,000 (Li-Cor, Lot No. D20803-09). Primary antibodies against the α-subunit of RNA polymerase were used at 1:5,000 (BioLegend, Lot No. B376827) with fluorophore conjugated antibodies against mice (Li-Cor, Lot No. D20601-01) used at 1:10,000. Membranes were imaged on a BioRad ChemiDoc Imager. Primary antibodies against LasR were validated by immunoblotting cell lysate from PAO1 and PAO1 Δ*lasR* (Fig. S2). Densitometry analysis was conducted on raw image files using ImageJ.

Transcriptional reporter assays

Biological triplicates of strains with a transcriptional reporter plasmid were grown overnight at 37°C and subcultured to an initial OD_{600} of 0.1 in 25 mL LB supplemented with gentamicin 30 μg/mL and incubated shaking at 200 rpm at each 25°C and 37°C. At exponential phase (OD_{600} ~0.5), 10^9 cells were centrifuged at 10,000 rpm for 2 minutes, supernatant removed, and the pellet resuspended in 1 mL PBS. 200 μL of each sample was then added to a black sided 96 well plate in technical triplicate and the fluorescence measured (excitation 485 nm, emission 515 nm) on a Synergy H1 plate reader (BioTek). At stationary phase (OD_{600} ~2.0), 10^9 cells were sampled from the same cultures and fluorescence measured as described. The RFU of 200 μL PBS on each plate was subtracted from technical replicates, which were then averaged per biological replicate. Background was determined by averaging all values from a transcriptional reporter assay of PAO1 carrying pRD87, a no-promoter control of the reporter plasmid.

5' rapid amplification of cDNA ends (5' RACE)

PAO1 was grown at 25°C in LB to late log phase (OD_{600} ~0.8), RNA extracted using MasterPure RNA Purification Kit (Epicentre), and treated with TURBO DNase as described. 5' RACE was conducted using the 5' RACE System for Rapid Amplification of cDNA Ends (ThermoFisher) according to manufacturer's instructions with modifications: ThermoScript Reverse Transcriptase (Invitrogen) was used in place of the kit's reverse transcriptase with betaine added to 0.5 M for the reverse transcriptase step.

Statistical analyses

All statistical tests were conducted as described using Prism version 10.

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550

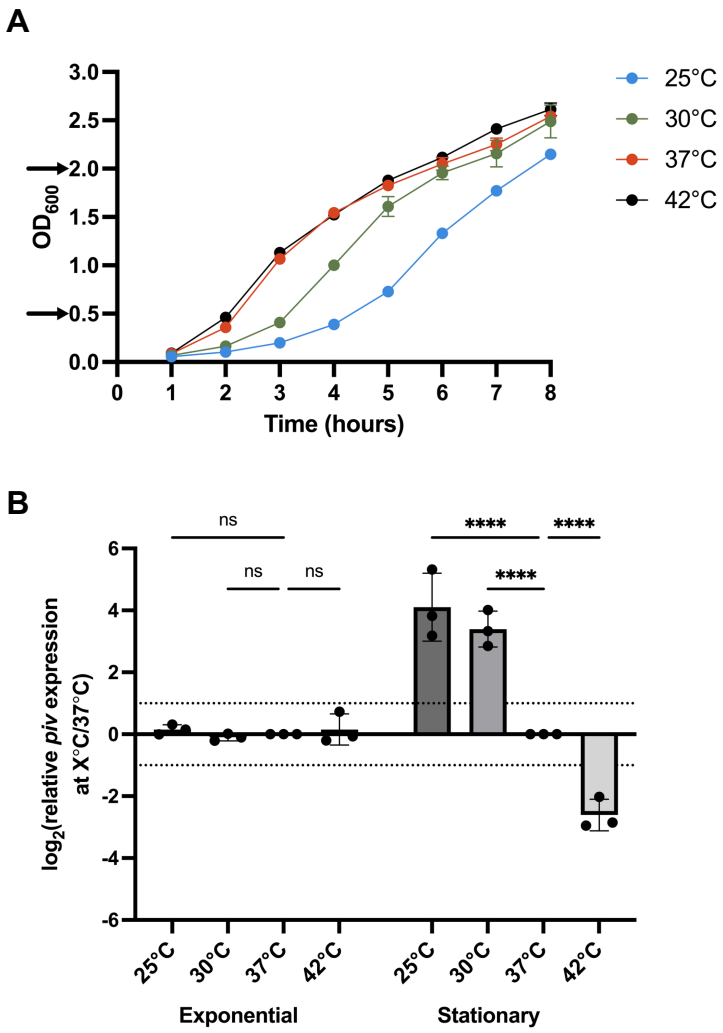

Figure 1. Temperature regulation of *piv* depends on growth phase. A) PAO1 was grown at 25°C, 30°C, 37°C, and 42°C. RNA was extracted from an equal number of cells first at exponential phase and then from the same cultures at early stationary phase, at points during bacterial growth indicated by arrows on the y-axis. The average growth of three biological replicates is shown with error bars representing standard deviation. B) Expression of *piv* at each temperature was calculated relative to 37°C using *omlA* as a temperature insensitive, internal control gene. RT-qPCR was conducted in technical triplicate and the mean of three biological replicates is shown with error bars representing standard deviation. Statistical significance was determined by two-way ANOVA with Dunnett's multiple comparisons: * $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.0002$, **** $p < 0.0001$, ns – not significant. Values between the dotted lines were not considered biologically significant.

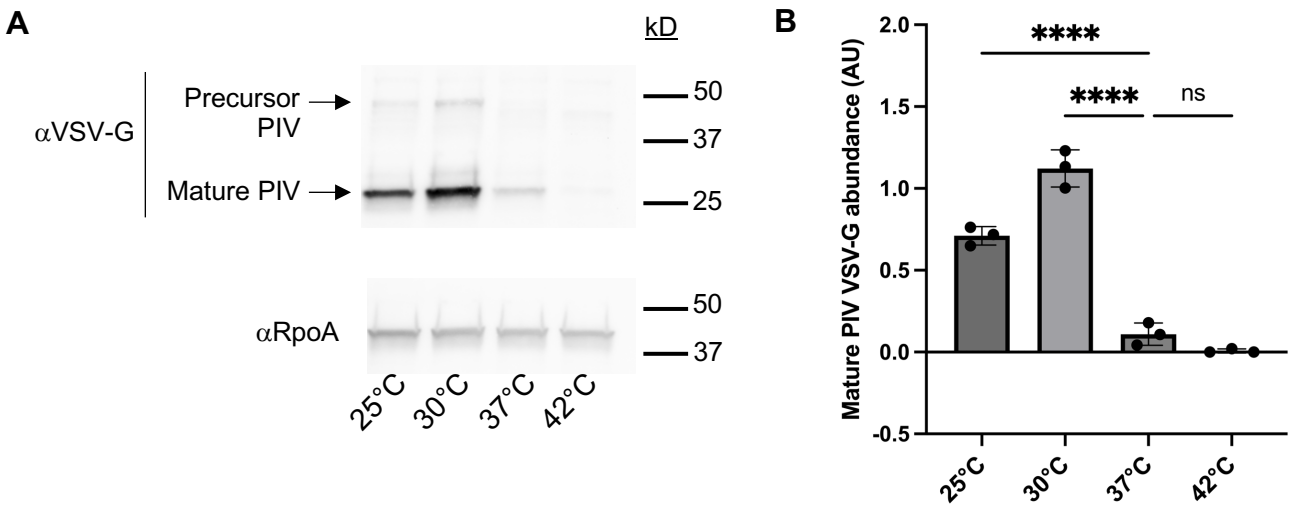

Figure 2. Temperature regulation of *piv* expression is reflected by levels of PIV protein. A) Cell lysates of PAO1 PIV VSV-G grown to early stationary phase at 25°C, 30°C, 37°C, and 42°C were probed with antibodies against the VSV-G tag (αVSV-G) and the RNA polymerase α subunit (αRpoA). A representative image of three biological replicates is shown. B) The amount of mature PIV VSV-G protein at each temperature was determined relative to the amount of RpoA using ImageJ. The mean of three biological replicates is shown with standard deviation. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparisons: * $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.0002$, **** $p < 0.0001$, ns – not significant.

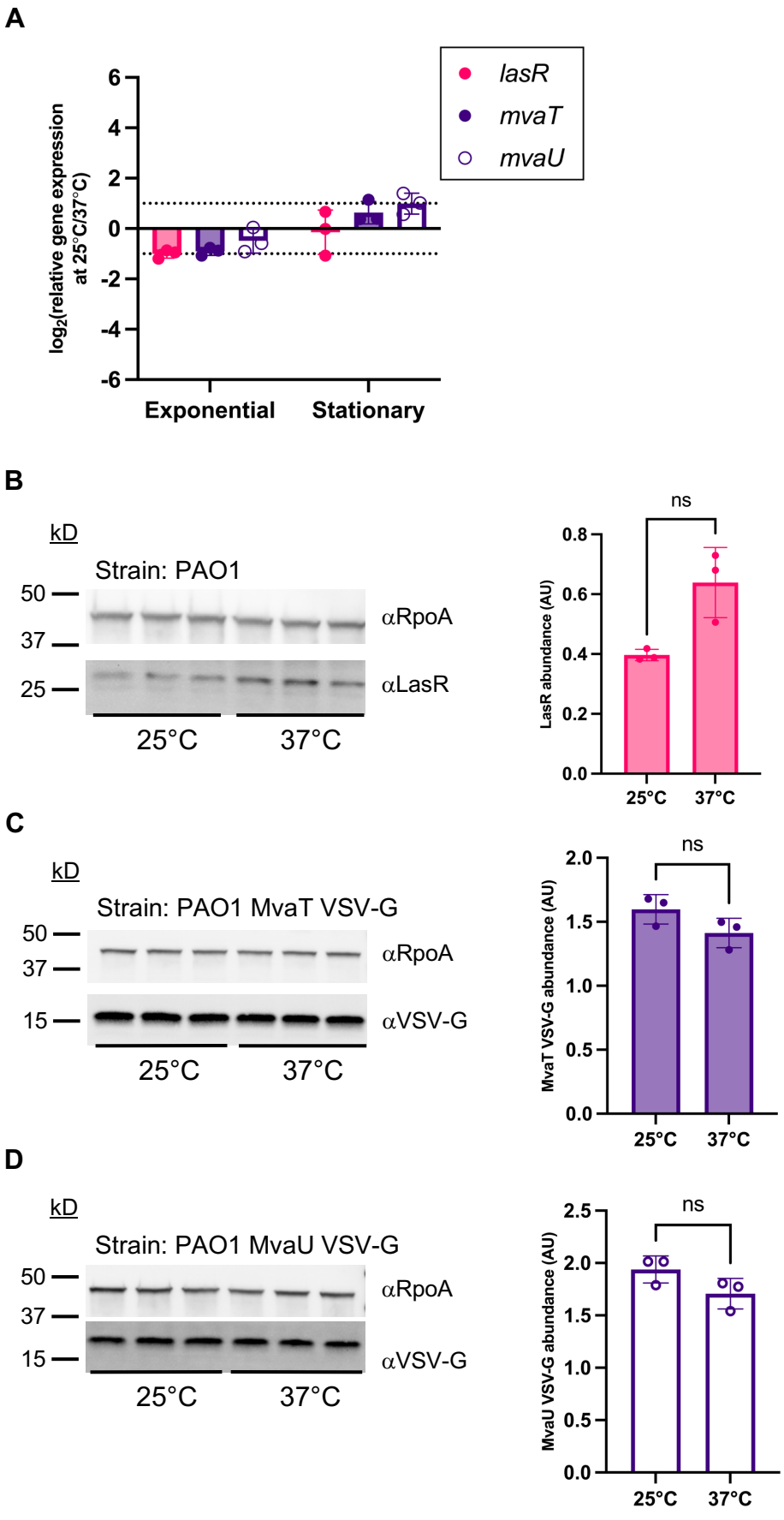

Figure 3. Transcriptional regulators of *piv* are not thermoregulated. A) PAO1 was grown at 25°C and 37°C and RNA extracted at exponential and stationary phases as previously described. Expression of genes was determined by RT-qPCR using *omlA* as an internal control gene and calculated for 25°C relative to 37°C. RT-qPCR was conducted in technical triplicate. The mean of three biological replicates analyzed is shown with error bars representing standard deviation. Values between the dotted lines were not considered biologically significant. B,C,D) Western blots (left) and subsequent quantification (right) of indicated transcription factors in cells grown at 25°C and 37°C to stationary phase. B) PAO1 was probed with αLasR antibodies. C) PAO1 MvaT VSV-G was probed with αVSV-G antibodies. D) PAO1 MvaU VSV-G was probed with αVSV-G antibodies. All samples were probed with αRpoA as a loading control. Biological triplicates for each protein are shown. For densitometry analysis, the amount of LasR (B), MvaT VSV-G (C), and MvaU VSV-G (D) protein was determined relative to the amount of RpoA in the same sample using ImageJ. The mean of three biological replicates is shown with standard deviation. Statistical significance was determined by two-tailed t test, ns – not significant.

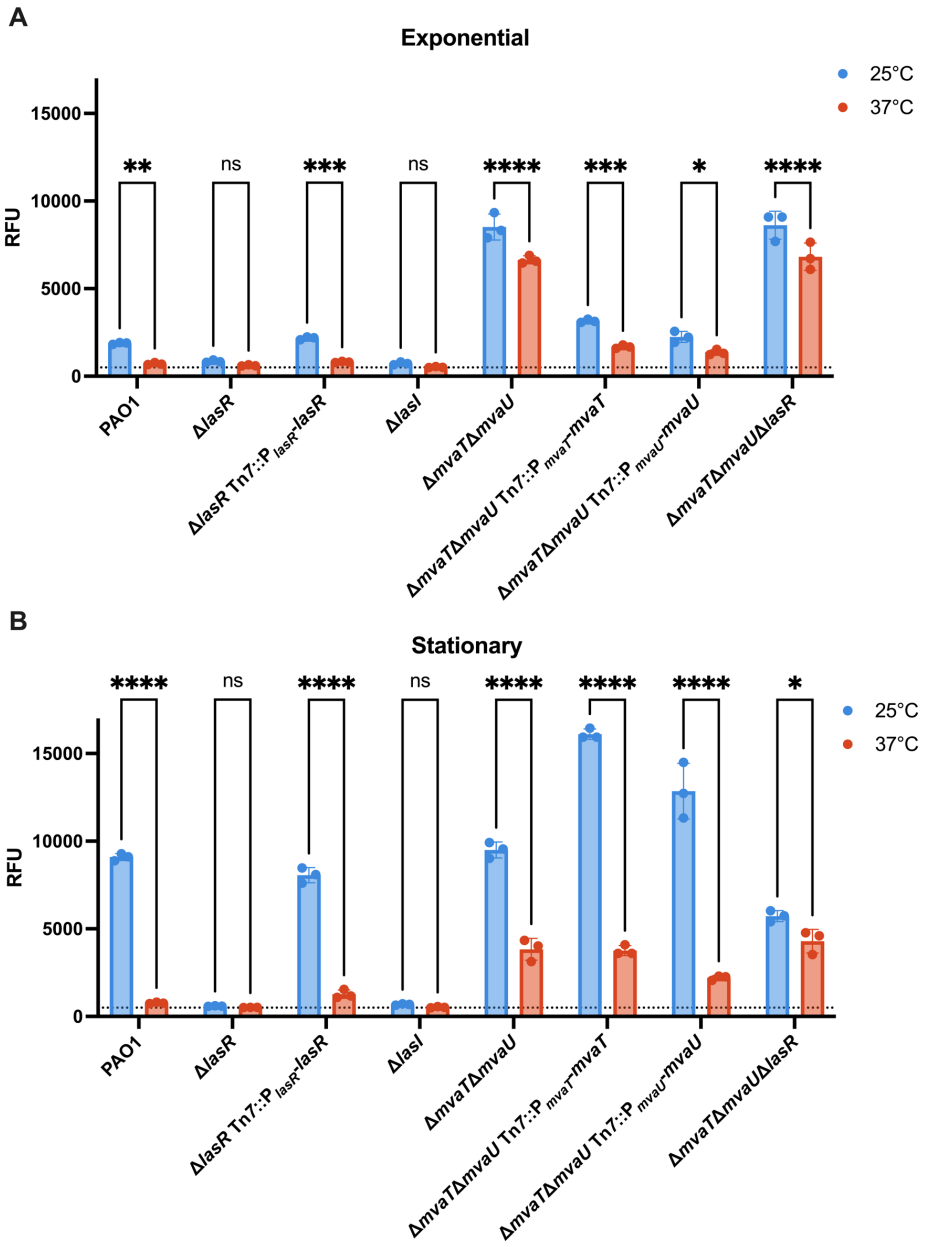

Figure 4. Temperature alters activity of the *piv* promoter through transcriptional regulator LasR, but not MvaT/MvaU. The indicated strains carrying the P_{piv}-*gfp*(ASV) transcriptional reporter (pRD91) were grown at 25°C and 37°C. An equal number of cells were sampled for fluorescence (RFU) measurements (excitation 485 nm, emission 515 nm) first at exponential phase (A) and then at early stationary phase (B). The dotted line indicates background fluorescence of PAO1 carrying a no-promoter *gfp*(ASV) reporter plasmid (pRD87). Three biological replicates were performed, with RFU measurements conducted in technical triplicate per each. The mean of three biological replicates is shown with error bars representing standard deviation. Statistical significance was determined by two-way ANOVA with Šídák multiple comparisons: * $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.0002$, **** $p < 0.0001$, ns – not significant. Statistical comparisons shown are for a given strain grown at 25°C versus 37°C.

A

```
1
TGGTAGAGAGAGCAATCCAACATCAATGGCAAGTGAAGGA

41
AATAGCTATCTATTCTGCTATCCACAAACGCTTCTTCATA
      las box

81
TTTAATAGCTAGCAGAAACGAATATAAAAAATATTTGAAC
                                  σ70 −35

                                      TSS
121
GACTCCGTCACACCTGCATATCTTTCGCCACGCGAACGAT
                  σ70 −10

161
TGAGGAAGTTGCCCTCCCAAAAAACTGAAGGAGTCGATTC
```

B

```
consensus las box: CTAnnnnnnnnnnTAG
      piv las box: CTATCTATTCTGCTAT
          1C→A:    ATATCTATTCTGCTAT
         14T→A:    CTATCTATTCTGCAAT
         16T→G:    CTATCTATTCTGCTAG
```

C

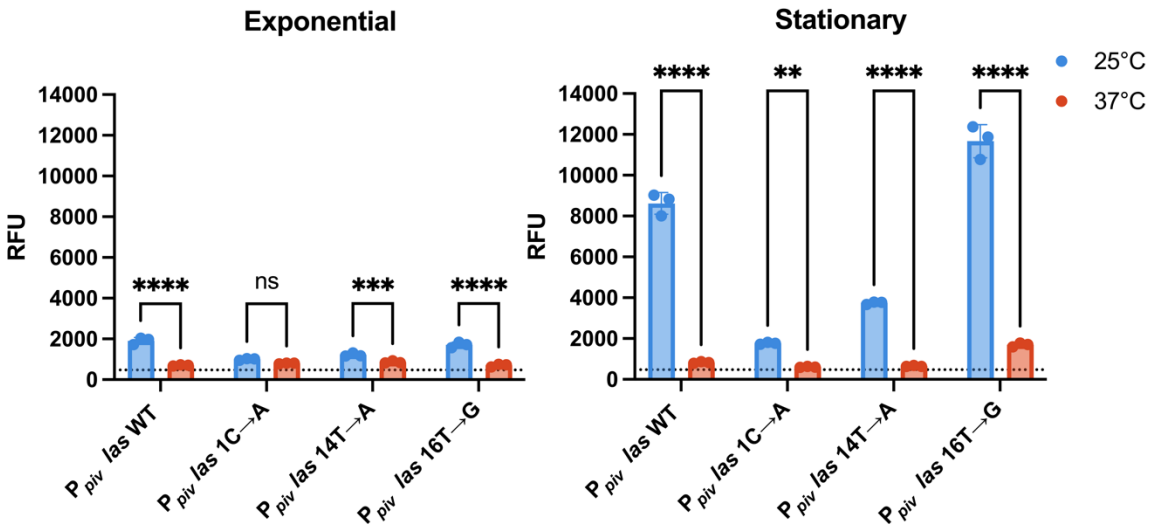

Figure 5. Mutations to a putative *las* box in the *piv* promoter region alter promoter activity. A) Sequence of the 200 nucleotides upstream of the *piv* start codon that are sufficient for temperature and growth phase regulation. Putative σ^{70} -35 and -10 elements identified with

SAPPHIRE (30) are underlined. Nearby transcription start sites (TSSs) were identified by 5' RACE are highlighted. A DNA motif with similarity to canonical *las* boxes (27) was identified upstream of transcription initiation regions and is boxed. B) Mutations made to the *las* box within the *piv* promoter of the P_{piv}-*gfp*(ASV) transcriptional reporter plasmid (pRD91). Mutations are predicted to disrupt (in pink) or improve (in green) LasR binding based on comparison to the consensus sequence. C) PAO1 carrying *las* box mutants of the P_{piv}-*gfp*(ASV) transcriptional reporter shown in B) were grown at 25°C and 37°C and fluorescence measurements taken at exponential (left) and stationary (right) phases as previously described. The mean of three biological replicates analyzed with standard deviation is shown. Statistical significance was determined two-way ANOVA with Šídák multiple comparisons: * $p < 0.0332$, ** $p < 0.0021$, *** $p < 0.0002$, **** $p < 0.0001$.

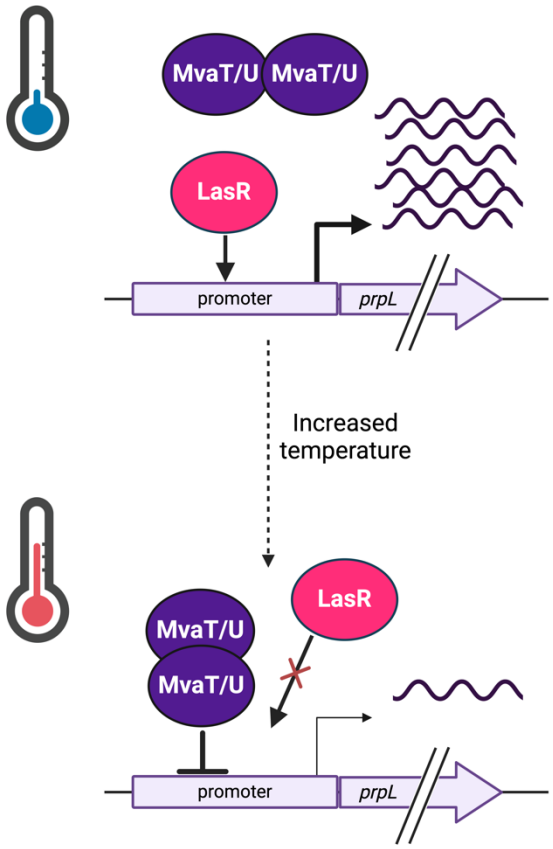

Figure 6. The proposed model for the transcriptional thermoregulation of *piv* by the quorum sensing regulator LasR. At 25°C (blue thermometer), LasR drives high expression of *piv* at stationary phase by directly interacting with the *piv* promoter while MvaT/MvaU do not repress expression. At 37°C (red thermometer), LasR no longer directly regulates *piv*, which allows MvaT/MvaU to repress *piv* expression.