Many bacteria use the second messenger cyclic diguanylate (c-di-GMP) to control motility, biofilm production and virulence. Here, we identify a thermosensory diguanylate cyclase (TdcA) that modulates temperature-dependent motility, biofilm development and virulence in the opportunistic pathogen *Pseudomonas aeruginosa*. TdcA synthesizes c-di-GMP with catalytic rates that increase more than a hundred-fold over a ten-degree Celsius change. Analyses using protein chimeras indicate that heat-sensing is mediated by a thermosensitive Per-Arnt-SIM (PAS) domain. TdcA homologs are widespread in sequence databases, and a distanty related, heterologously expressed homolog from the Betaproteobacteria order *Gallionellales* also displayed thermosensitive diguanylate cyclase activity. We propose, therefore, that thermodtransduction is a conserved function of c-di-GMP signaling networks, and that thermosensitive catalysis of a second messenger constitutes a mechanism for thermal sensing in bacteria.
Temperature sets physical limits for life on Earth\textsuperscript{1,2}. It affects the physiology and geographic distribution of organisms\textsuperscript{3}, including Eubacteria\textsuperscript{4,5}, across landscapes of all scales. Bacteria express a dynamic range of behaviors following temperature fluctuations, such as cold- or heat-shock responses to thermal extremes\textsuperscript{6,7}, taxis along thermal gradients\textsuperscript{8}, and differential expression of virulence factors at temperatures corresponding to those of endothermic hosts\textsuperscript{9,10}. Unsurprisingly, bacteria have evolved many means for sensing temperature\textsuperscript{11,12}. Known molecular heat-sensing strategies include RNA thermometers\textsuperscript{10}, temperature-sensitive transcription factors\textsuperscript{10}, and transmembrane proteins that initiate signal transduction in response to thermal changes in the thickness of lipid bilayers\textsuperscript{13,14}. Cyclic diguanylate (c-di-GMP) signal transduction is thought to be integral to the control of diverse physiological and social behaviors among bacterial species from nearly every phyllum\textsuperscript{15}. C-di-GMP is enzymatically synthesized by diguanylate cyclases harboring glycine-glycine-aspartate-glutamate-phenylalanine (GGDEF) domains, and degraded by c-di-GMP-specific phosphodiesterases containing glutamate-asparagine-leucine (EAL) or histidine-asparagine-glycine-tyrosine-proline (HD-GYP) domains\textsuperscript{14–17}. Although c-di-GMP signaling has been studied since the 1980s\textsuperscript{18}, one key challenge to understanding it is the simultaneous isolation of smooth and rugose colony morphology variants from biofilms of bacterial species\textsuperscript{14}. Many of these proteins contain putative sensory domains adjacent to their GGDEF, EAL, or HD-GYP domains, yet the vast majority of these sensory domains have no known function.

Here we report the discovery of diguanylate cyclases in which a thermosensitive Per-Arnt-SIM domain is coupled with a GGDEF domain. We show that these proteins display high enzymatic rate–temperature dependencies that are analogous to those described for the cold- and heat-sensing proteins of neurons\textsuperscript{19–21}. We propose that these enzymes constitute molecular thermostatic devices that enable bacteria to calibrate intracellular c-di-GMP levels in response to temperature.

Results

Heat stimulates c-di-GMP biosynthesis. In an effort to identify stimuli of bacterial c-di-GMP signal transduction networks, we exploited the diverse colony phenotypes displayed by different isolates of \textit{Pseudomonas aeruginosa}. The variations in colony morphology of these isolates are often due to differences in their c-di-GMP signaling pathways\textsuperscript{22–26}. A prime example of this phenomenon is the simultaneous isolation of smooth and rugose colony morphology variants from biofilms in laboratory bioreactors and chronic infections\textsuperscript{26–31}. Although there may be exceptions, many smooth colony variants exhibit low intracellular c-di-GMP levels and reduced biofilm production, whereas rugose colony variants have high intracellular c-di-GMP levels and increased biofilm production\textsuperscript{32}. Here, we used a paired collection of smooth and rugose \textit{P. aeruginosa} colony variants previously isolated from the sputum of cystic fibrosis (CF) patients suffering from chronic lung infection\textsuperscript{26,32}. The isolates of each pair were known to be close relatives by pulsed-field gel electrophoresis genotyping\textsuperscript{26,32}. By varying the growth conditions, we found that we could change the colony morphology for a subset of these paired isolates such that one isolate of the pair would switch to the other colony phenotype. This tactic provided a platform for investigating gene–environment interactions affecting intracellular c-di-GMP levels. This approach led to the identification that one strain, \textit{P. aeruginosa} CF39S, displayed temperature-dependent rugose colony morphology, whereas its close genetic relative, strain CF39, did not (Fig. 1a). Consistent with predictions from these colony morphology phenotypes, \textit{P. aeruginosa} CF39 displayed similar biofilm production at all tested temperatures, whereas CF39S had a capacity to produce biofilm that massively increased between 30 and 34 °C (Fig. 1b).

The literature is rife with descriptions that temperature regulates biofilm production in bacteria, yet connections between these observations and c-di-GMP signaling are limited\textsuperscript{33,34}. We posited, therefore, that temperature-dependent increases in \textit{P. aeruginosa} CF39S biofilm production might be correlated with increases in c-di-GMP. To test this hypothesis, nucleotide pools were extracted from cultures of \textit{P. aeruginosa} CF39 and CF39S and c-di-GMP was measured using mass spectrometry. We observed that strain CF39S produced increasing quantities of c-di-GMP with respect to temperature in the physiologically relevant range of 28–41 °C (Fig. 1c). These data suggest that cellular components of \textit{P. aeruginosa} CF39S behave like a molecular thermostat, responding to changes in temperature to fine-tune c-di-GMP synthesis in vivo.

To identify genes linked to the temperature-dependent phenotypes of \textit{P. aeruginosa} CF39S, we carried out a comparative genomic analysis of strains CF39 and CF39S (see “Methods”). This analysis revealed only one high-confidence single nucleotide polymorphism (SNP) between the two strains. This SNP occurred in a gene encoding a putative GGDEF-domain protein that we named the thermosensory diguanylate cyclase A (\textit{tdcA}) (Fig. 1d). \textit{P. aeruginosa} CF39 possesses a base pair deletion in \textit{tdcA} (162ΔG), resulting in a frameshift and truncation of TdcA. The correlation between \textit{tdcA} and temperature-dependent phenotypes of \textit{P. aeruginosa} CF39S was verified using a genetic linkage analysis. Two-step allelic exchange\textsuperscript{35}—in which \textit{tdcA}_{162ΔG} and \textit{tdcA} were replaced with \textit{tdcA} and \textit{tdcA}_{162ΔG} in strains CF39 and CF39S, respectively—reversed the temperature-dependent phenotypes of these isolates (Fig. 1e). Moreover, miniTn7 transposons that were engineered bearing \textit{tdcA} as part of its predicted polycistronic operon, or alternatively, as a fusion between the promoter of that operon and the \textit{tdcA} open reading frame (ORF), were sufficient to confer heat-activated biofilm production to a recipient \textit{P. aeruginosa} PAO1 laboratory strain (Fig. 1e), which naturally lacks \textit{tdcA}. By contrast, \textit{P. aeruginosa} PAO1 acquiring \textit{tdcA}_{162ΔG} did not display heat-activated biofilm production (Fig. 1e). Finally, \textit{P. aeruginosa} PAO1 engineered to express \textit{tdcA} also exhibited thermosensitive synthesis of intracellular c-di-GMP (Supplementary Fig. 1). Taken together, these data demonstrate that \textit{tdcA} is linked to thermal control of biofilm formation and c-di-GMP synthesis in \textit{P. aeruginosa}.

TdcA homologs are conserved among bacteria. Bioinformatics indicate that \textit{tdcA} is encoded on a Tn7-like transposon inserted in the chromosome (Fig. 1d), providing a c-di-GMP signaling module in the accessory genome that gives a gain-of-function to \textit{P. aeruginosa} CF39S. Other genes in this transposon have identity and synteny with the locus of heat resistance (LHR), a horizontally acquired genomic island first identified in \textit{Escherichia coli}\textsuperscript{36}, and later found in many other bacterial species\textsuperscript{37–39}. Genes of the LHR, including orthologous genes of the \textit{P. aeruginosa} CF39S \textit{tdcA} operon, protect cells from heat shock\textsuperscript{39–41}, pressure treatment\textsuperscript{41}, chlorine and oxidizing chemicals\textsuperscript{42} (Fig. 1d). A gene for a small heat shock protein (\textit{shsp20c}) orthologous to one adjacent to the \textit{tdcA} operon is known to confer heat tolerance to \textit{P. aeruginosa}\textsuperscript{43}. However, the best studied LHR from \textit{E. coli} AW1.7 (ref. 44) lacks an ortholog of \textit{tdcA} (Fig. 1d).

The \textit{tdcA} gene is present in <1% of sequenced \textit{P. aeruginosa} genomes in the \textit{Pseudomonas} Genome Database\textsuperscript{45} and is absent from the well-studied laboratory strains PA14 and PAK in addition to PAO1. However, genome-sequenced \textit{P. aeruginosa}...
strains encoding tdcA alleles have been isolated worldwide, originating from both the environment and diverse human infections (Supplementary Fig. 2 and Supplementary Data 1). BLAST searching of the National Center for Biotechnology Information (NCBI) databases using strict search and sequence analysis criteria (see “Methods”) suggests that TdcA homologs are distributed among numerous environmental bacterial species as well as human and veterinary microbiota (Supplementary Fig. 3 and Supplementary Data 1). We observed that a subset of putative TdcA orthologs with >93% identity to *P. aeruginosa* CF39S TdcA (TdcAPs) all occur in the synteny context of the LHR (see Supplementary Data 1 for NCBI accession numbers). By contrast, the remaining putative TdcA homologs, which share 30.2–47.2% identity with TdcAPs, occur in a variety of different genomic contexts. To test the bioinformatic prediction that these latter sequences encode thermosensory diguanylate cyclases, gene synthesis was used to build a construct to express a homolog of TdcAGa in *P. aeruginosa* PAO1 and direct measurements of c-di-GMP in cell lysates by mass spectrometry showed that TdcAGa displays temperature-dependent diguanylate cyclase activity similar to that exhibited by TdcAPs (Supplementary Fig. 3). Taken together, these data suggest that TdcA orthologs and proteins having similarity to it are widespread among bacteria.

**C-di-GMP-dependent thermotransduction.** Thermotransduction is the process by which temperature is perceived by a sensory cell receptor, initiating a signaling cascade that changes cellular physiology. In *P. aeruginosa*, c-di-GMP regulates the expression of genes for synthesis of the biofilm matrix, which includes operons for extracellular polysaccharides (*pel* and *psl*) and an adhesin (*cdr*) (Fig. 2a). We hypothesized, therefore, that c-di-GMP-dependent thermotransduction would upregulate the expression of these gene loci in strains bearing tdcA, driving expression of the biofilm matrix components PEL, PSL, and CdrA in response to changing temperatures. Western blotting using antisera against the proteins necessary for PEL and PSL synthesis revealed a striking upregulation of PelC, PelD, and PslG for *P. aeruginosa* CF39S when incubation temperatures were increased from 30 to 37 °C. A similar trend was observed for PAO1 engineered to express tdcA (Fig. 2b). *P. aeruginosa* strains
bearing tdcA<sub>162AG</sub> as well as wild-type PAO1 did not display these phenotypes (Fig. 2b). By contrast, a P. aeruginosa PAO1 strain containing an activating mutation in the Wsp surface-sensing system (Δ wspF), which has constitutively elevated intracellular c-di-GMP levels (refs. 23, 32), exhibited high abundance of PelC, PeID, and PslG at all temperatures tested (Fig. 2b). Additionally, a bioreporter system, which is comprised of a plasmid-borne fusion between the cdrA promoter (P<sub>cdrA</sub>) and the gene for green fluorescent protein (gfp)<sup>49</sup>, indicated that cdrA transcription in a PAO1 strain bearing tdcA<sup>−</sup> increased linearly 5.2-fold from 31 to 41 °C (Fig. 2c). Taken together, these data indicate that TdcA can differentially regulate a known c-di-GMP-dependent regulon in P. aeruginosa, and that this occurs in response to changing temperatures.

In P. aeruginosa, the c-di-GMP-binding transcription factor FleQ derepresses pel, psl, and cdr operons when c-di-GMP levels are high, and activates transcription of flagellar genes when c-di-GMP levels are low<sup>47-49,50,51</sup>. Unsurprisingly, we observed that strains bearing tdcA exhibited reduced swimming motility as temperatures were increased from 25 to 37 °C (Fig. 2d). We thus hypothesized that TdcA might integrate into the P. aeruginosa c-di-GMP-dependent regulon via FleQ, and reasoned that a ΔfleQ mutation should diminish biofilm production by tdcA<sup>+</sup> strains. Corresponding to this hypothesis, while a P. aeruginosa CF39S ΔfleQ mutant displayed temperature-dependent biofilm formation, the quantity of biofilm it produced was significantly less than it was for wild-type CF39S (Fig. 2e). Similar observations were made for a PAO1 ΔfleQ mutant expressing tdcA (Fig. 2e). Taken together, an interpretation is that TdcA partly integrates into the P. aeruginosa c-di-GMP regulon network, in part, via the c-di-GMP-binding transcription factor, FleQ. Datum points represent 12 technical replicates from each of three biological replicates, and lines and bars represent means and standard deviations, respectively. Temperatures of 25 °C and 37 °C are represented by sky blue and orange lines, respectively. OD<sub>600nm</sub> denotes the optical density measured at a wavelength of 600 nm, and RFU denotes relative fluorescence units. Data represent means and standard deviations of measurements each performed in biological and technical triplicate. The tdcA<sup>−</sup> and tdcA<sup>+</sup> strains are represented by sky blue and orange bars, respectively. OD<sub>600nm</sub> and OD<sub>595nm</sub> denote the optical density measured at wavelengths of 600 nm and 595 nm, respectively. Strains denoted tdcA<sup>−</sup> have the tdcA<sub>162AG</sub> allele.
**P. fluorescens** engineered with *tdcA* and the c-di-GMP bioreporter became luminescent within 30 min of the temperature increase, whereas strains bearing *tdcA*Δ62ΔG or vector controls remained dark (Supplementary Movie 1). These data illustrate that TdcA-dependent thermotransduction drives physiological changes that initiate within minutes and accrue in magnitude over the course of hours.

**Thermal control of *P. aeruginosa* virulence.** Multiple mechanisms of bacterial pathogenesis are regulated by c-di-GMP. Evidence suggests that high c-di-GMP levels reduce expression of the cytotoxic type III secretion system, which, based on prior studies of diguanylate cyclases, is known to contribute to the virulence of *P. aeruginosa* strains in the waxworm *Galleria mellonella*. A key advantage of the *G. mellonella* model is that it is possible to vary external temperatures between 25 and 37 °C without adversely affecting insect survival (Supplementary Fig. 4). We observed that experimental infections with strains bearing *tdcA* and *tdcA*Δ62ΔG alleles produced similar mortality at 25 °C. However, *G. mellonella* infected with the *P. aeruginosa* *tdcA*Δ strain showed significantly lower mortality at 37 °C than those insects infected with *tdcA*Δ62ΔG strain (Supplementary Fig. 4). Attenuation of acute virulence was similarly observed in a *Caenorhabditis elegans* infection model when inoculating bacteria were grown at 37 °C as opposed to 25 °C (Supplementary Fig. 5). Taken together, while we have not yet determined which c-di-GMP-dependent determinants affect lethality in insects and roundworms, these results demonstrate that TdcA is a temperature-dependent regulator of *P. aeruginosa* virulence.

A **thermosensory diguanylate cyclase.** So, what could account for the temperature-dependent synthesis of c-di-GMP in bacterial cells? Sequence analyses predict that TdcA is a 38.7 kDa protein of 342 amino acids that is comprised of an N-terminal Per-Arnt-Sim (thermoPAS) domain, a bacterial protein domain that functions in heat sensing. The thermosensitive Per-Arnt-Sim (thermoPAS) domain shares commonalities that enable the engineering of transient receptor potential (thermoTRP) proteins of the peripheral nervous system in animals—nearly all enzymes display a Q10 value between two and three (Fig. 3b). Our data suggest that similar to the thermoTRP19,21, TdcA is an outlier to this principle (Fig. 3b). Therefore, we assessed the catalytic rate of TdcA-mediated GTP hydrolysis over a range of temperatures, and compared it to the well characterized *P. aeruginosa* diguanylate cyclase, WspR70,75, which is involved in surface sensing.

To begin, temperature-dependent catalytic activity was measured for an equivalent number of enzyme units of WspR and TdcA using the pyrophosphatase assay. One enzyme unit was arbitrarily defined as one micromole of GTP hydrolyzed per minute at 37 °C. WspR displayed canonical temperature-dependent kinetics with a maximal Q10 value of 2.0 between 28 and 37 °C (Fig. 3d, e). By contrast, TdcA activity was undetectable at 25 °C, yet increased with a maximal Q10 value of 10.5 between 28 and 37 °C (Fig. 3d, e). A similar result was obtained for TdcA when c-di-GMP production was directly measured using mass spectrometry (Fig. 3f). However, repeating temperature coefficient calculations with this more sensitive method, which enables the detection of c-di-GMP production in vitro at 25 °C (Fig. 3f), indicated that TdcA has a Q10 = 135 when calculated between 25 and 33 °C (Fig. 3d, see “Methods” and “Discussion”). These results demonstrate that TdcA is not only a bona fide diguanylate cyclase, but also highly thermosensitive.

The **thermosensitive Per-Arnt-SIM (thermoPAS) domain.** We next sought to investigate the putative role for the TdcA PAS3 domain (PAS3TdcA) in temperature sensing. Proteins containing PAS domains share commonalities that enable the engineering of artificial proteins with designer functionalities. For instance, stimulation causes changes in the conserved PAS fold that are transmitted to the PAS-linked effector domain via torque of a linker region69 (Figs. 3a and 4a). We hypothesized, therefore, that PAS3TdcA could be fused to other effectors via its linker to create a chimeric protein with thermosensitive activity.

To test this hypothesis, PAS3TdcA was fused to the GGDEF domain of WspR (GGDEFWspR) to build a synthetic thermosensitive protein (Stp1, Fig. 4a and Supplementary Methods). The activity of Stp1 was assessed in *P. aeruginosa* PAO1 cells engineered to express this protein from an arabinose-inducible expression cassette (araC-PBAD). Here, expression of a constitutively active form of WspR (WspR^{V72D}) (ref. 70) from the
araC-Δrad cassette resulted in intracellular c-di-GMP levels that were similar at both 25 °C and 37 °C (Fig. 3b). By contrast, expression of TdcA resulted in an increase in c-di-GMP levels between these two temperatures, as did expression of Stp1 (Fig. 4b). Although relative levels of c-di-GMP differed between TdcA- and Stp1-overexpressing bacteria, there was a similar 6.5- to 8.1-fold relative increase in intracellular c-di-GMP levels in both strains between 25 and 37 °C (Fig. 4b). We infer from these findings that PAS-TdcA is a thermosensitive Per-Arnt-Sim domain (which we denote “thermoPAS,” after the nomenclature of the thermoTRP), and that it transduces thermal stimuli to regulate the diguanylate cyclase activity of the adjacent GGDEF domain in TdcA.

To corroborate the observation that thermoPAS functions in temperature sensing, we coupled the N-terminus of TdcA, which encompasses the thermoPAS domain and its linker (TdcA1-177), to E. coli LacZ (Fig. 4c). To do this, TdcA1-177 was fused between the LacZ segments that otherwise correspond to the α-peptide (LacZ1-92) and the Ω-fragment (or α-acceptor) of β-galactosidase, resulting in a temperature-sensitive enzyme that we named “thermo-gal.” This chimeric enzyme displayed temperature-sensitive catalytic activity when expressed in E. coli, which is a strain that lacks the entire lac operon as well as the capacity to metabolize L-(-)-arabinose, which is one of the nitrogen sources that E. coli uses to convert the lac operon to a constitutive system.

#### Discussion

Enzymes exhibiting thermosensitive reaction rates that are regulated by a thermoPAS domain exemplify a previously unreported mechanism for cellular temperature sensing. It is possible that the thermoPAS domain may be present in numerous and diverse proteins. PAS domains are characterized by an evolutionary mechanism for cellular temperature sensing. It is possible that differences in activity do not result from changes in protein abundance. Taken together, these data provide evidence that thermoPAS constitutes a molecular thermosensory device that is linked to the temperature-sensing functionality of TdcA.

### References

- [Supplementary Fig. 3](#)
- [Supplementary Fig. 7](#)

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**Fig. 3 TdcA displays thermosensitive catalytic activity.** A Schematic illustrating the predicted domain organization of TdcA. B Substrate-dependent enzyme kinetics of His6-NusA-TdcA. Measurements were performed in triplicate and one independent replicate is shown. C GTP is the substrate for His6-MBP-TdcA. Each bar represents the mean and standard deviation of three independent replicates. ATP, adenosine-5'-triphosphate; GDP, guanosine-5'-diphosphate; GTP, guanosine-5'-triphosphate. D TdcA is an outlier to theory for universal enzymatic rate-temperature dependencies in bacteria. Each datum point represents a Q10 value for an enzyme: Q10 values for WspR and TdcA were calculated in the present work, whereas all other temperature coefficients are derived from published literature values and grouped according to the temperature-dependent activity maximum of the enzyme (psychrophilic, mesophilic, thermophilic). E Temperature-dependent enzyme kinetics of His6-MBP-TdcA. Measurements were performed in technical triplicate and datum points and lines represent individual replicates. F LC-MS/MS measurements of temperature-dependent c-di-GMP production by recombinant His6-MBP-TdcA in vitro. Each datum point represents an independent replicate, and lines and bars represent means and standard deviations, respectively.
TdcA kinetics challenge theory for universal enzymatic rate–temperature dependencies\textsuperscript{72}, yet outliers to this theory are not without precedent or biological relevance. One example includes the thermoTRPs, which couple changes in temperature to ion channel gating to enable the sensation of hot and cold temperatures by sensory neurons\textsuperscript{20,21}. While it is still not clear what part of thermoTRPs sense temperature\textsuperscript{81,82}, the \( Q_{10} \) temperature coefficient has been used to describe the fold-change in electrical current conducted by these proteins per 10 °C change\textsuperscript{20,81,83}. Various thermoTRP isoforms exhibit diverse \( Q_{10} \) values, ranging from ~4 to >200 (ref.19), which bound those of TdcA (\( Q_{10} = 135 \) in the temperature range of 25–33 °C). Because \( Q_{10} \) values depend on the temperature range used to make the calculation\textsuperscript{72}, we acknowledge that this may be a conservative estimate of a physiologically relevant \( Q_{10} \) value for TdcA, which is >2400 in vitro when the calculation is repeated using liquid chromatography tandem mass spectrometry (LC–MS/MS) measurements (Fig. 3f) at 25 and 28 °C (see “Methods”). Using \( Q_{10} \) values as a gauge for thermosensitive biomolecular behavior, we propose, therefore, that thermosensitive catalysis of a second messenger constitutes a rudimentary biochemical mechanism for thermal sensation in bacteria, and furthermore, that c-di-GMP-dependent thermotransduction enables bacteria to regulate diverse aspects of their physiology and social behaviors in response to temperature.

**Methods**

**Strains, physiological buffers, and microbiological media.** Bacterial strains used in this study are listed in Supplementary Table 1. \( P. \) aeruginosa, \( P. \) fluorescens, and \( E. \) coli were routinely grown in lysogeny broth (LB)\textsuperscript{84}, which contained, per liter of milliQ water, 10.0 g tryptone, 5.0 g yeast extract, and 5.0 g NaCl. No-salt lysogeny broth (NSLB) contained, per liter of milliQ water, 10.0 g tryptone and 5.0 g yeast extract, and was supplemented with 15% w/v sucrose when required. Vogel–Bonner Minimal Medium (VBMM)\textsuperscript{85} was prepared as a 10× concentrate, which contained per liter of milliQ water 2.0 g MgSO\textsubscript{4}·7H\textsubscript{2}O, 20 g citric acid, 100 g K\textsubscript{2}HPO\textsubscript{4}, and 35 g Na\textsubscript{2}HPO\textsubscript{4}·4H\textsubscript{2}O, and was adjusted to pH 7.0 and sterilized by filtration. The 10× VBMM solution was diluted as required in sterile milliQ water. A 1 ml aliquot of a 1000× trace metals solution was added per liter of VBMM. The 1000× trace metal solution contained 2 mM FeCl\textsubscript{3}·6H\textsubscript{2}O, 1 mM MnCl\textsubscript{2}·4H\textsubscript{2}O, 0.05 mM Zn(NO\textsubscript{3})\textsubscript{2}·6H\textsubscript{2}O, 1 mM CaCl\textsubscript{2}, 0.1 mM CoSO\textsubscript{4}·7H\textsubscript{2}O, 0.1 mM CuCl\textsubscript{2}·2H\textsubscript{2}O, 0.1 mM Na\textsubscript{2}MoO\textsubscript{4}·2H\textsubscript{2}O, 2 mM

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**Fig. 4 Engineered protein chimeras identify a thermosensitive Per-Arnt-SIM (thermoPAS) domain.** a CLUSTALW sequence alignment of WspR (blue) and TdcA (orange) protein sequences. The TdcA thermoPAS domain was fused to the GGDEF domain of WspR via the region of identity at the N-terminus of the GGDEF domains (black), yielding the chimeric synthetic thermosensory protein 1 (Stp1). b LC–MS/MS measurements of c-di-GMP levels in cells expressing WspR, TdcA, and Stp1 from an arabinose-inducible expression cassette (\( araC\)-\( PBAD\)). Each datum point represents an independent biological replicate, and lines and bars represent means and standard deviations, respectively, for 6–12 independent biological replicates. Temperatures of 25 °C and 37 °C are represented by sky blue and orange bars, respectively. c Internal fusions of the N-terminal portion of TdcA to LacZ (\( β\)-galactosidase) produce an enzyme, thermo-gal, which displays temperature-sensitive catalytic activity when expressed in \( E. \) coli LMG194. Bacteria were grown on LB agar containing X-gal and 1% \( l\)–\( (+)\)-arabinose.
MgSO₄·7H₂O, and 0.2 mM NiCl₂. Semisolid media were prepared by adding 1.5% agar to LB, 1.0% noble agar to VIBMM, respectively. Tryptone agar contained, per liter of milliQ water, 30.0 g tryptone and 1.0% agar. Superbroth contained, per liter of milliQ water, 32.0 g tryptone, 20 g yeast extract, 5 g NaCl, and 5 mL 1 N NaOH. Phosphate-buffered saline (PBS) was purchased at a 20× concentrate and was diluted as needed in sterile milliQ water. Unless noted otherwise, all media, buffers, and water, were sterilized using an autoclave by standard operating procedures. Antibiotics were added as required at the following activity-corrected concentrations: for E. coli, gentamicin (Gm) at 10 µg/mL, carbenicillin (Cb) at 50 µg/mL, tetracycline (Tc) at 10 µg/mL, and kanamycin (Km) at 50 µg/mL; for P. aeruginosa PA01: Cb at 500 µg/mL; Gm at 60 µg/mL for merodiploid and plasmid selection, or 150 µg/mL for miniCTX mutants and Tc at 1000 µg/mL for miniCTX mutants; for P. aeruginosa CF39 and CF39S: Gm at 1000 µg/mL. Where indicated, Congo red was added to media at 40 µg/mL; for P. fluorescens PF-81: Gm at 30 µg/mL for miniTn7 mutants and Km at 500 µg/mL.

Photography of bacterial colonies on agar plates. Overnight cultures of P. aeruginosa were grown in LB at 25°C and 250 r.p.m. These cultures were diluted 1:1000 in LB, and 3 µL of this inoculum was aliquoted onto tryptone agar containing Congo red. These Petri dishes were wrapped in Parafilm® and placed in an incubator set to either 25°C or 37°C and incubated for 18 h. E. coli LMG1914 strains expressing LacZ fusions (pTER132-1, pTER145-1, pEA04-1, or pTER143-1, Supplementary Table 2) were grown on LB + Cb agar containing 158 µM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 1% v/v l-arabinose. These agar plates were inoculated with 3 µL aliquots of E. coli LMG1914 cells that had been collected from LB + Cb agar and suspended in PBS at an optical density at 600 nm (OD600) of 0.3. The plates were then grown at 25°C or 37°C until sufficient color had developed in the colonies (~2–3 days). Images of surface-illuminated colonies were captured on a fluorescence cassette equipped with a digital camera mounted on a Nikon® AZ100 dissection microscope using a 0.5× objective and NIS Elements.-

Transfer of enrichment via increased antibiotic resistance and selection for colonies expressing the fluorescent protein. Dual-marker vectors were built using two different approaches. In the first approach, the alelic exchange vector for ΔpelF allele was built by fusing a constitutively expressed gene for mCherry onto the 3’-end of a ΔpelF deletion allele. To begin, regions upstream and downstream of P. aeruginosa CF39S pelF were cloned using primer pairs oJJH1435/oJJH1436 and oJJH1437/oTER232 (Supplementary Table 3), respectively. A third fragment containing the P. aeruginosa fliC promoter–gene fusion was cloned from CF39S DNA using primer pairs oJJH1438/oJJH1439 and oJJH1440/oJJH1441 and placed in an ed chamber for 8 h.

In the second approach, we incorporated the pDONR-RBSII-GFPMpt3* promoter–gene fusion into the pDONRPEX18Gm backbone, yielding pDONRPEX18Gm-GFPMpt3* (Supplementary Table 2). This produced a dual-marker allelic exchange vector in which deletion alleles could be assembled by LR recombination from only two PCR fragments, one containing the desired fusion and one containing the parental sequence, both cloned into pDONRPEX18Gm using primer pairs oJJH1435/oJJH1436 and oJJH1437/oTER232. This PCR product was gel purified and then co-transformed with pDONRPEX18Gm using BP Clonase II (Invitrogen) according to the manufacturer’s directions. The recombination products were transformed into E. coli. DNA was extracted using electroporation buffer (EB), and transformation reactions were selected on LB + Cm agar. Plasmid DNA was purified and then sequenced using Sanger sequencing using primers M13F and M13R (Supplementary Table 3). These fragments were then gel purified, digested with Smal and then ligated into pEX18Ap that had been similarly digested with restriction enzymes. The ligation mixture was then transformed into E. coli DH5α using electroporation, and transformants were selected on LB + Cb agar. An established protocol for colony PCR of pEX18Ap using M13F and M13R primers35 was used to identify clones with the desired inserts, yielding pLS6 and pLS11, which contained the tdCΔM22 and tdAΔM22 alleles, respectively (Supplementary Table 2). The sequence of the inserts in pLS6 and pLS11 was verified by Sanger sequencing using primers M13F and M13R (Supplementary Table 2).

To facilitate simple genome editing of antibiotic-resistant isolates (such as P. aeruginosa CF39 and CF39S), we created allelic exchange vectors with both an antibiotic resistance marker and an enhanced fluorescent protein encoding a dual-marker vector. One class of these vectors was used to complement ΔpelF mutations in the P. aeruginosa CF39S strains, whereas another class was used to complement ΔtdCΔM22 mutations in P. aeruginosa CF39S. In the first case, the allelic exchange vector for ΔpelF allele was constructed in pEX18Ap (Supplementary Table 2) using established protocols34. Here, primers oLS14 and oLS15, which were tagged with BamHI and PstI restriction sites, respectively, were used to clone tdA and tdCΔM22 from CF39S and CF39 gDNA, respectively. The PCR products were gel purified, digested with BamHI and PstI, and then ligated into pEX18Ap that had been similarly digested with restriction enzymes. The ligation products were transformed into E. coli DH5α using electroporation, and transformants were selected on LB + Cb agar. An established protocol for colony PCR of pEX18Ap using M13F and M13R primers35 was used to identify clones with the desired inserts, yielding pLS6 and pLS11, which contained the tdAΔM22 and tdCΔM22 alleles, respectively (Supplementary Table 2).

A cryogenic stock onto LB agar and grown at 30°C overnight. The next day, bacterial cells were collected from the LB agar with a sterile cotton swab and suspended in LB to an optical density of 600 nm (OD600) of 0.3. This suspension was then diluted 1:10 in LB, and 100 µL aliquots were transferred to the wells of a 96-well microplate. The outer wells of the microplate were filled with 100 µL of milliQ water. These microplates were incubated in a humidified chamber for 8 h. Following incubation, microplate wells were washed three times with milliQ water and air-dried for 10 min. Subsequently, 110 µL of 0.1% crystal violet solution (containing 0.1% w/v crystal violet, 6.3% v/v ethanol, 0.16% v/v phenol, and 0.3% w/v sodium metabisulfite) was added to each well, and the plate was incubated at room temperature and then the crystal violet solution was removed using a multichannel micropipette. Microplates were then washed twice with milliQ water and left to air dry overnight. Biomass-bound crystal violet was solubilized by adding 110 µL of 30% acetic acid to each well. Lastly, 100 µL aliquots of the solubilized crystal violet solution were transferred to a 96-well microplate. Optical density at 550 nm (OD550) or 595 nm (OD595) was measured using an Enspire® microplate reader (Perkin-Elmer).
A similar tactic was used to build ΔfleQ allele. Regions upstream and downstream of P. aeruginosa CF39 floQ were cloned using the primer pairs oTER73/ oTER74 and PTER148/ oTER149. Primers oTER147 and oTER440 were tagged with attB1 and attB2 sequences, respectively (Supplementary Table 3). These fragments were then gel purified and fused via SOE-PCR using primer pair oTER143/ oTER140. The product was then inserted into pDONRPX18Gm–GFPmut3+ using BP Clonase II, and the reaction mixture was transformed into E. coli DH5α using electroporation. Transformed colonies were then selected on LB + Gm agar, identified by colony PCR, and sequence verified using the M13F and M13R primers described above, yielding the plasmid pTER150E (Supplementary Table 2).

**Allelic exchange in P. aeruginosa CF39 and CF39S.** Allelic exchange for pefL, psdL, floQ, and tdac was conducted according to the method of Hmele and colleagues35 with some minor modifications for P. aeruginosa CF39 and CF39S. All suicide vectors were introduced into E. coli DH5α using biparental conjugation with E. coli S17.1 (pRK415). P. aeruginosa CF39 and CF39S plgS6- and plS1-derived merodiploids were selected on VBMM containing 1500 μg/ml Cb. P. aeruginosa pTER124-Δ, pTER130-Δ, and pTER125-derived merodiploids were enriched on VBMM containing 1000 μg/ml Gm, and subsequently selected based on expression of GFPmut3* or mCherry using a Nikon AZ100 stereomicroscope with LED light source and GFP and RFP filters, respectively. All pEX18-derived allelic exchange vectors used in this study encode sacB, and therefore, double-crossover mutations were selected on NSLB + 15% w/v sucrose using established procedures35.

Mutation of a single base pair in the tdcA alleles of strains derived from CF39 and CF39S was identified by sequencing PCR products amplified from E. coli PEX18Gm using BP Clonase II. The reaction mixture was then transformed into E. coli PAO1 gDNA using primer pair oHA11/oHA12. At the same time, the nucleic acid sequences of the native LTGLSNRR sequence. To begin, wpsgR was cloned from P. aeruginosa PAO1 gDNA using primer pair oHA11/oHA12. At the same time, the nucleic acid sequence encoding the TdcA thermosensory protein sequence adjacent to the linker region was assembled from the GGDEF domains of both enzymes and their cognate sensory domains. We designed a chimeric thermosensory enzyme, therefore, by fusing the DNA sequence encoding the TdcA-PA33-linker-LTGLSNRR region to the DNA sequence encoding the WspR GGDEF (wspRgGDEDS) domain, the latter of which was truncated immediately adjacent to and excluded the consensus LTGLSNRR sequence. To begin, wspR was cloned from P. aeruginosa PAO1 gDNA using primer pair oHA11/oHA12. At the same time, the nucleic acid sequence encoding the TdcA thermosensory PA35 domain (tdcAASAS) and its adjacent linker and LTGLSNRR sequence was cloned from P. aeruginosa CF395 gDNA using primer pair oHA13/oHA14. The PCR products were then gel purified and fused via SOE-PCR with primer pair oHA13/oHA14. The oHA12 and oHA13 primers were tagged with Gateway® compatible attR2 and attB1 sites, respectively, and thus the SOE-PCR product was gel purified and recombined with pDONR-PX18Gm using BP Clonase II. The reaction mixture was then transformed into E. coli DH5α, and transformants were selected on LB + Gm agar. A clone with the correct insert was identified by colony PCR sequencing using M13F and M13R primers, yielding the entry vector pHA110, which encoded a fusion between TdcAASAS and WspRgGDEDS that we denoted synthetic thermosensory protein 1 (Stp1, Supplementary Table 2).

**Design and construction of chimeric thermosensory diguanylate cyclase.** A CUSLAI Omega6 alignment and PSI-PRED6,25 analysis of TdcA and WspR primary and secondary sequences revealed a conserved LGTTLNR sequence adjacent to the linker region and the GGDEF domains of both enzymes and their cognate sensory domains. We designed a chimeric thermosensory enzyme, therefore, by fusing the DNA sequence encoding the TdcA-PA33-linker-LTGLSNRR region to the DNA sequence encoding the WspR GGDEF (wspRgGDEDS) domain, the latter of which was truncated immediately adjacent to and excluded the consensus LTGLSNRR sequence. To begin, wspR was cloned from P. aeruginosa PAO1 gDNA using primer pair oHA11/oHA12. At the same time, the nucleic acid sequence encoding the TdcA thermosensory PA35 domain (tdcAASAS) and its adjacent linker and LTGLSNRR sequence was cloned from P. aeruginosa CF395 gDNA using primer pair oHA13/oHA14. The PCR products were then gel purified and fused via SOE-PCR with primer pair oHA13/oHA14. The oHA12 and oHA13 primers were tagged with Gateway® compatible attR2 and attB1 sites, respectively, and thus the SOE-PCR product was gel purified and recombined with pDONR-PX18Gm using BP Clonase II. The reaction mixture was then transformed into E. coli DH5α, and transformants were selected on LB + Gm agar. A clone with the correct insert was identified by colony PCR sequencing using M13F and M13R primers, yielding the entry vector pHA110, which encoded a fusion between TdcAASAS and WspRgGDEDS that we denoted synthetic thermosensory protein 1 (Stp1, Supplementary Table 2).

**Design and construction of thermo-gal and other chimeric β-galactosidase enzymes.** A vector for expression of E. coli β-galactosidase (LacZ) with a C-terminal His6 tag was assembled in vitro from three DNA fragments. The desired construct was then selected on two fragments. These three fragments were then assembled via Gibson assembly97, transformed into E. coli DH5α, and selected on LB + Cb, yielding pTER143 (Supplementary Table 2). This plasmid was then verified by Sanger sequencing using the primers oTER057 and oTER277. Constructs for expressing PAS2PdeO-gal and P-fabPΔ41 were built in an analogous fashion to pTER143 from two PCR products. For PAS2PdeO-gal, the PAS2PdeO fragment was cloned from E. coli DH5α gDNA using primer set oTER143/ oTER144, and the plasmid backbone and lacZ gene were cloned from pTER132 using primer set oTER141/ oTER142. These fragments were then joined via Gibson assembly97, transforming the DNA into DH5α, yielding pEA03 (Supplementary Table 2). This plasmid was then verified by Sanger sequencing using the primers oTER057 and oTER277. A construct for P-fabPΔ41 was built from two PCR products that excluded the nucleotide encoding amino acid residue +15% w/v sucrose using established procedures35.
Molecule Real-Time (SMRT) sequencing
Here, the CF39S genome was sequenced to ~140-fold coverage with four Single E. coli araC-PBAD counts divided by the OD600 measurement for the same strain bearing pMH487.

were standardized to an OD600 of 0.05 in 5 ml VBMM.
The diguanylate cyclase, WspR, requires a phosphorylation signal for activation of cyclase activity; however, the V72D substitution leads to constitutive activation of WspR. A recombinant E. coli vector for producing recombinant WspG<sup>W72D</sup> was built by executing site-directed mutagenesis with primer pair eT2ER269/eT2ER270 on the protein expression vector pETduet: wspR/pPA2133 (ref. 19) to introduce the V72D mutation, generating the plasmid pETduet: wspR<sup>V72D</sup>/pPA2133 (Supplementary Table 3).

To generate a His<sub>6</sub>-MBP-tagged fusion for the expression of recombinant E. coli His<sub>6</sub>-DsbC, the construct was then diluted as required in buffer F (50 mM HEPES, 150 mM NaCl, 1 mM DTT, and 10% glycerol) and stored at −80 °C until use. The concentrated protein was then frozen and stored at −80 °C until cryogenically stored for up to 1 month.

Inhibitor Cocktail (EDTA-free, Roche) and 0.1 mM phenylmethanesulfonyl fluoride (PMSF) were added to induce protein expression. Cells were then frozen and stored at −80 °C until the culture reached an OD<sub>600</sub> of 0.6. The second period, the culture was diluted back to 1:1000 in 55 ml of LB and grown to an OD<sub>600</sub> of 37 °C. Two 10 ml of culture alcohols were taken from the flask and passed through separate filters (0.45 µm, Millipore). One filter was used for determining cell count, while the other was used for nucleotide extractions. Both filters were placed on the same LB agar plate at the desired temperature for 3 h. Following the incubation period, the first filter was washed in 2 ml of PBS and the cells in the PBS were used for viable cell counting. The second filter was processed as follows: microbial metabolites were extracted by transferring the second filter to a Petri dish (60 mm diameter, Fisher Scientific) containing 2 ml of 4 °C 80% HPLC-grade methanol and allowed to incubate for 10 min; methanolic extracts were collected and the filter was washed with 1 ml of 4 °C 80% methanol; the methanolic extract and wash were combined in a 5–ml tube and insoluble debris was pelleted by centrifugation (30 min at 7000 × g); 2 ml of the supernatant was harvested and dried by using a speed vac, and suspended in 200 µl of 50% methanol. The extracted nucleotides were stored at −80 °C until mass spectrometry analysis. After mass spectrometry analysis, the quantified c-di-GMP was normalized using the cell counts obtained for each sample.

Liquid chromatography mass spectrometry (LC-MS) analysis were conducted at the University of California, San Francisco (UCSF) Center for Biotherapy (CMBF) on a Thermo Scientific Q Exactive<sup>®</sup> Mass Spectrometer. Data were acquired in full scan mode (70–1000 m/z) at 140,000 resolving power. Metabolites were ionized via negative mode electrospray using the following source parameters: spray voltage −2000 V, capillary temperature 275 °C, auxiliary gas temperature 325 °C, sheath gas flow 25, auxiliary gas flow 10, sweep gas flow 2. Metabolites were separated using ultra-high performance liquid chromatography (UHPLC) following a reverse phase ion-pairing strategy adapted from Lu and colleagues<sup>10</sup>. Briefly, chromatographic separation of metabolites was achieved using a binary solvent with a commercial metabolite standard c-di-GMP (Sigma-Aldrich), and the assignment was confirmed via MS/MS fragmentations. The solvent gradient was employed: 0–1 min, 0% B; 1–4 min, 0–100% B; 4–5 min, 100% B; 5.5–min, 100% B; 5.5–8 min, 0% B. The c-di-GMP molecular assignment was established by high-resolution MS/MS analysis, co-elution of the microbial metabolite with a commercial metabolite standard c-di-GMP (Sigma-Aldrich) and via MS/MS fragmentations. The c-di-GMP level was quantified using a Lambda 35 UV/VIS Spectrometer with a Peltier device (Perkin-Elmer). Measurements were done using SUPRASIL<sup>®</sup> quartz spectroscopy cells with a light path of 10 mm (Perkin-Elmer).

Nucleotide extractions and c-di-GMP quantification by mass spectrometry. A culture was grown in 5 ml of LB broth at 37 °C at 250 rpm. The following day, the culture was diluted back to 1:1000 in 55 ml of LB and grown to an OD<sub>600</sub> of 0.6 at 37 °C. Two 10 ml of culture aliquots were taken from the flask and passed through separate filters (0.45 µm, Millipore). One filter was used for determining cell count, while the other was used for nucleotide extractions. Both filters were placed on the same LB agar plate at the desired temperature for 3 h. Following the incubation period, the first filter was washed in 2 ml of PBS and the cells in the PBS were used for viable cell counting. The second filter was processed as follows: microbial metabolites were extracted by transferring the second filter to a Petri dish (60 mm diameter, Fisher Scientific) containing 2 ml of 4 °C 80% HPLC-grade methanol and allowed to incubate for 10 min; methanolic extracts were collected and the filter was washed with 1 ml of 4 °C 80% methanol; the methanolic extract and wash were combined in a 5–ml tube and insoluble debris was pelleted by centrifugation (30 min at 7000 × g); 2 ml of the supernatant was harvested and dried by using a speed vac, and suspended in 200 µl of 50% methanol. The extracted nucleotides were stored at −80 °C until mass spectrometry analysis. After mass spectrometry analysis, the quantified c-di-GMP was normalized using the cell counts obtained for each sample.

Liquid chromatography mass spectrometry (LC-MS) analysis were conducted at the California Neuroimaging Research Facility (CMBF) on a Thermo Scientific Q Exactive<sup>®</sup> Mass Spectrometer. Data were acquired in full scan mode (70–1000 m/z) at 140,000 resolving power. Metabolites were ionized via negative mode electrospray using the following source parameters: spray voltage −2000 V, capillary temperature 275 °C, auxiliary gas temperature 325 °C, sheath gas flow 25, auxiliary gas flow 10, sweep gas flow 2. Metabolites were separated using ultra-high performance liquid chromatography (UHPLC) following a reverse phase ion-pairing strategy adapted from Lu and colleagues<sup>10</sup>. Briefly, chromatographic separation of metabolites was achieved using a binary solvent with a commercial metabolite standard c-di-GMP (Sigma-Aldrich), and the assignment was confirmed via MS/MS fragmentations. The solvent gradient was employed: 0–1 min, 0% B; 1–4 min, 0–100% B; 4–5 min, 100% B; 5.5–min, 100% B; 5.5–8 min, 0% B. The c-di-GMP molecular assignment was established by high-resolution MS/MS analysis, co-elution of the microbial metabolite with a commercial metabolite standard c-di-GMP (Sigma-Aldrich) and via MS/MS fragmentations. The c-di-GMP level was quantified using a Lambda 35 UV/VIS Spectrometer with a Peltier device (Perkin-Elmer). Measurements were done using SUPRASIL<sup>®</sup> quartz spectroscopy cells with a light path of 10 mm (Perkin-Elmer).

**UV-Vis spectroscopy of purified proteins.** Purified His<sub>6</sub>-MBP-TdcA and His<sub>6</sub>-MBP-DsbC were diluted in protein buffer B to a concentration of 1 mg/ml for His<sub>6</sub>-MBP-TdcA and 1.15 mg/ml for His<sub>6</sub>-MBP-DsbC, which was an empirically standardization based on the A<sub>280</sub> peak of each sample. The absorbance spectra for His<sub>6</sub>-MBP-TdcA and His<sub>6</sub>-MBP-DsbC were collected in the range of 260–700 nm using a Lambda 35 UV/Vis Spectrometer with a Peltier device with SUPRASIL<sup>®</sup> quartz spectroscopy cells with a light path of 10 mm (Perkin-Elmer). The spectral scan was repeated six times for His<sub>6</sub>-MBP-TdcA and His<sub>6</sub>-MBP-DsbC and each measurement was baseline corrected for buffer absorbance. Absorbance of hemin and flavin mononucleotide (FMN) standards were measured in an identical manner except that these compounds were prepared in ethanol.

**C-di-GMP quantification using pyrophosphate assays.** Diguanylate cyclase activity of purified recombinant TdcA (His<sub>6</sub>-MBP-TdcA or His<sub>6</sub>-NusA-TdcA) and recombinant WspR (His<sub>6</sub>-WspR) was measured using an EnzChek<sup>®</sup> Pyrophosphate Assay (Invitrogen) with modifications<sup>70</sup> also summarized here. High-purity guanosine 5′-triphosphate (GTP) was purchased from Affymetrix. Yeast pyrophosphatase was purchased from Roche. A standard curve was produced for each temperature using 30, 75, 100, and 125 µM pyrophosphate. We used 0.5 mM GTP for all measurements of diguanylate cyclase activity. Data were collected using a Lambda 35 UV/Vis Spectrometer with a Peltier device (Perkin-Elmer). Measurements were done using SUPRASIL<sup>®</sup> quartz spectroscopy cells with a light path of 10 mm (Perkin-Elmer).

**Calculation of temperature coefficient (Q<sub>10</sub>) values.** The temperature coefficient (Q<sub>10</sub>) is the factor by which the rate of reaction increases for every 10 °C (or Kelvin) increase in temperature. The Q<sub>10</sub> value was calculated using the following equation<sup>72</sup>:

\[
Q_{10} = \left( \frac{k_T}{k_{10}} \right)^{10/T_2} \quad (2)
\]
where $k_1$ is the reaction rate measured at temperature $T_1$, and $k_2$ is the reaction rate measured at temperature $T_2$.

Bioinformatic surveys for TdcA homologs and molecular phylogenetics. A search for TdcA protein homologs was executed using a BLASTP search of the NCBI non-redundant nucleotide database implemented within Geneious Prime v2020.1 (ref. 112). Sequences were further analyzed using InterProScan 5 (ref. 113). We set up a series of three filtering criteria to provide high-confidence predictions of other thermosensory diguanylate cyclases: (1) All sequences containing >2 putative domains, as identified using the Conserved Domain Database (CDD), were eliminated from further analysis; (2) Every sequence must possess a putative N-terminal PAS domain and a C-terminal GGDEF domain; and (3) The query TdcA sequence must align to both the PAS and GGDEF domains. This resulted in a list of 300 putative TdcA homologs (Supplementary Data 1).

A phylogenetic tree was built using 39 of these high-confidence TdcA homologs in which genus and species could be unambiguously assigned to the genome. One representative sequence that had the highest identity with the query sequence was included for each species. MUSCLE114 was used to make an alignment of these sequences, and WpRl from *P. aeruginosa* PAO1 (NCBI Accession number AAC07089.1) was used as an outgroup. The phylogenetic tree was built using FastTree2 v2020.1 (ref.112). Sequences were further analyzed using InterProScan 5 (ref.113).

The NCBI non-redundant nucleotide database implemented within Geneious Prime v7.02.®

**Caenorhabditis elegans infection model.** *Caenorhabditis elegans* larvae were used as a model116,117 to assess a change in *P. aeruginosa* virulence with respect to temperature. *C. elegans* larvae were obtained in bulk from The Worm Lady (McGregor, Ontario, CA) and stored at 16 °C until used. Bacterial cultures were grown in LB for 16 h at 37 °C and 275 r.p.m. Cultures were standardized in 0.85% saline using a 0.5 McFarland Standard (DensitChex® Plus, Biomerieux, USA) to obtain a cell density of 1.5 × 10^8 CFU/μl, which was then diluted in PBS to 10 CFU/μl. For infection, the abdomen of each larva was cleaned using a cotton swab and 70% ethanol and placed on an ice pack to minimize their motility. Next, 10 µl of diluted inoculum (100 CFU/larvae) was injected using a Hamilton syringe (Cole-Palmer) in the lower abdominal proleg; 10 µl of sterile PBS was injected as control. A group of ten larvae was used for each strain and condition. Infected larvae were transferred to a clean Petri dish lined with filter paper, incubated at either 25 °C or 37 °C, and scored for survival every 24 h for 72 h. At least four biological replicates were used and survival curves were plotted using Graphpad Prism® v7.02.

**Reporting summary.** Further information on research data availability is in the Nature Research Reporting Summary linked to this article.

**Data availability.**

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**Author contributions**

H.A. and T.E.R. contributed equally to this work. J.J.H. conceived the project. B.Y.G., J.M., and J.J.H. designed the studies. H.A., T.E.R., and J.J.H. wrote the manuscript. H.A., T.E.R., E.A., I.D.R., K.L., L.A.S., and J.J.H. constructed the strains. H.A., T.E.R., E.A., F.L., K.L., N.F., and J.J.H. carried out the motility assays, biofilm assays, and/or photography. H.A., F.L., and N.F. carried out the gene expression measurements. H.A., T.E.R., I.A.S., W.K., G.L.W., F.S.L.B., R.E.B., M.R.P., M.F.H., and J.J.H. performed the genome sequencing and bioinformatic analyses. L.K.J., R.G., and I.A.L. performed the LC-MS/MS measurements. T.E.R., H.A., N.M.G., B.S.T., T.M.L.W., J.G., J.M., and J.J.H. carried out the protein production and/or enzyme assays. Y.L., E.G., M.S., M.S.S., A.K., and A.K.B. performed an analysis of virulence and executed the *Caenorhabditis elegans* and *P. aeruginosa* infection assays. All authors revised and provided feedback on the manuscript.

**Competing interests**

H.A., T.E.R., M.R.P., J.M., and J.J.H. have filed patents for the use of heat-activated gene expression and synthetic proteins in biotechnology. All other authors declare no competing interests.

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