**ARTICLE**

Impact of temperature-dependent phage expression on *Pseudomonas aeruginosa* biofilm formation

Karishma Bisht, Jessica L. Moore, Richard M. Caprioli, Eric P. Skaar and Catherine A. Wakeman

---

**INTRODUCTION**

Bacteria form highly adaptable multicellular communities called biofilms. A biofilm is a group of microorganisms that adheres to a surface by utilizing extracellular polymeric substances (EPS). The ability of bacteria to form a biofilm varies depending on their environment and bacterial taxonomy. Studies have shown that exposing biofilms to different environmental factors can induce the expression of different sets of genes and ultimately yield distinct biofilm morphologies. Biofilms can be found naturally attached to rocks within streams or rivers where they can beneficially contribute to the ecosystem. However, biofilms are also found growing within industrial settings, such as water pipes, that can cause operational problems and negatively impact industry. Similarly, the biofilms naturally associated with the human body can range from beneficial to pathogenic.

*Pseudomonas aeruginosa* is a ubiquitous opportunistic pathogen associated with hospital-acquired infections. It has been classified by the Centers for Disease Control as a serious threat due to its high level of antibiotic resistance, especially when growing in its clinically relevant biofilm form. Its ability to form vigorous biofilms can contribute to both antibiotic resistance as well as resistance to the human immune system. In general, biofilms account for almost 80% of chronic microbial infections in the human body, and biofilm-forming microbial cells are up to 1000 times more antibiotic resistant than the planktonic counterparts. The EPS matrix surrounding the microbial community consists of proteins, lipids, polysaccharides, and extracellular DNA and helps the bacteria to survive under harsh conditions.

The impact of temperature on bacterial pathogens is well-established. For example, the elevated host temperature triggers the production of *Yersinia* species virulence factors. This phenomenon has also been noticed in bacterial pathogens for plants where virulence genes are repressed at elevated temperatures. These findings highlight the importance of temperature-based regulation of gene expression on host colonization and disease progression with a reverse effect in the case of plant pathogens and human pathogens. Other studies have shown an association between temperature and biofilm formation, regulated by cyclic di-GMP signaling in opportunistic bacterial pathogens, such as *Burkholderia pseudomallei* and *P. aeruginosa*. Due to the wide range of temperatures experienced by *P. aeruginosa* as it transitions from the aquatic or soil environment into the human host, previous studies have already begun to explore thermal regulation in this microbe. One particularly thorough study explored the transcriptomic response in planktonic cells shifting between 22 and 37 °C. Other studies focusing on the impact of temperature shifts on biofilm cells only used short-term exposure to non-physiological heat stresses as a form of biofilm mitigation. Another recent study demonstrated that thermoregulation of biofilm formation in *P. aeruginosa* was strain dependent but consistently more robust at lower temperatures. Our research seeks to expand on these interesting studies by identifying biofilm-specific adaptations of *P. aeruginosa* at both external environmental temperatures and host-relevant temperatures since the biofilm lifestyle of this organism has been intrinsically linked to its ability to colonize both of these niches.

Biofilm formation in *P. aeruginosa* depends on various factors and the genes can be differentially regulated in the presence of varied environmental conditions. Both genetic background and environmental factors can have an effect on the transcriptional profiles and evolutionary trajectory of this pathogen. Temperature shifts can be detected by bacteria utilizing various sensors involving temperature-driven conformational changes in either DNA, RNA, or proteins. For example, RNA thermometers have been reported to be involved in thermoregulation in *P. aeruginosa*. The stress conditions associated with the temperature shifts that *P. aeruginosa* experiences as it transitions from the external environment into the host could contribute to differential protein expression that impacts biofilm formation. By understanding how *P. aeruginosa* regulates these proteins in response to both environmental and host-relevant temperatures, we can potentially identify novel therapeutic strategies to combat the...
biofilm-strengthening proteins with specific relevance to either the host or the industrial environment.

One exciting area of exploration for therapeutic potential is bacteriophages, as these bacteria-targeting viruses can eradicate recalcitrant microbial populations, such as those growing within biofilms and/or associated with certain infections. Conversely, some bacteriophages have recently been found to contribute to the structural integrity of biofilm via incorporation into the EPS matrix, which aids in biofilm adherence to surfaces and tolerance to stress conditions. Pf bacteriophage in particular has been associated with influencing Pseudomonas phenotypes by not only having an effect on biofilm formation and antibiotic resistance but also impacting mammalian immune responses during infection. Differential phage reactivation has been associated with stress responses linked to temperature shifts in other microbes. Similarly, induction and increased release of Pf phages has been linked to temperature shifts in other microbes. The role of temperature fluctuations and temperature-responsive phage proteins on the development of P. aeruginosa biofilms can be beneficial as fluctuations in temperature are a major component of its transition from an environmental lifestyle to a host-associated lifestyle.

RESULTS

Temperature impacts biofilm architecture

Biofilms acquire unique architecture (such as the re-arrangement of the EPS matrix and the cells within) to persist in a wide range of environments, including the host environment. The growth temperature is a striking difference between the external/
consistent with findings by other groups that this environmental microbe and opportunistic pathogen forms slightly more dense biofilms at lower temperatures. However, upon quantifying biofilm-associated colony-forming units (CFUs), we determined that biofilms grown at either temperature have comparable viable cell numbers (Supplementary Fig. 2a).

**Temperature influences protein expression in biofilm**

To investigate the presence of the different proteins expressed at environmental vs. host temperature we used matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS). Pellicle biofilms (biofilms that form at the air-liquid interface) were grown on conductive glass slides for analysis by MALDI IMS (Fig. 2a, b). We saw that the biofilms grown under different temperature conditions exhibit dramatically different protein expression profiles. Some proteins were enriched at host temperature (37 °C), others were enriched at environmental temperature (23 °C), and many proteins were found to be unchanged between these two conditions (Fig. 2c and Supplementary Fig. 3). Overall, these data demonstrate a dramatic shift in biofilm-associated protein production at these two temperatures. As various proteins have been shown to be integral components of EPS in numerous microbes, these findings could be indicative of temperature-specific EPS adaptations.

**Phage Pf1 expression is induced in biofilms at host temperature**

While the MALDI IMS findings supported that the overall biofilm proteome of *P. aeruginosa* is highly impacted by temperature shifts, we sought to determine some of the specific gene expression changes that might contribute to temperature-driven differences in EPS composition. Therefore, we performed transcriptomic analyses of both planktonic and biofilm-associated cells at 23 and 37 °C. Principal component analysis for *P. aeruginosa* PA14 grown in biofilm and planktonic form at 23 and 37 °C revealed a notable clustering effect of the replicates for biofilm as well as the planktonic stage (Fig. 3a). Genes were differentially expressed at the two temperatures in both the planktonic and biofilm state. A total of 175 and 189 genes were differentially regulated in planktonic and biofilm states, respectively, at the two temperatures (Supplementary Fig. 4). At 23 °C, a total of 464 genes were differentially regulated in biofilm vs. planktonic growth state and at 37 °C, a total of 373 genes were differentially regulated in biofilm vs. planktonic growth stage (Supplementary Fig. 4). We used the *P. aeruginosa* Community Annotation Project (Pseudocap) function class assignments (http://pseudomonas.com/pseudocap) to categorize the differentially expressed genes into different functional classes. 94 genes were upregulated in the planktonic state, at environment-relevant temperature while 81 genes were downregulated for this state. In the case of biofilm, 91 genes were upregulated while 98 were downregulated at 23 °C vs. 37 °C (Supplementary Fig. 5a, b). On comparing the gene expression at the same temperature between biofilm and planktonic transcripts, we found a higher number of genes differentially expressed because of this lifestyle switch. At 23 °C, we identified 172 genes upregulated and 292 genes downregulated in biofilm vs. planktonic cells. However, at 37 °C, there were 199 genes upregulated and 176 genes downregulated between biofilm and planktonic cells (Supplementary Fig. 6a, b). We were also able to find genes that were regulated by temperature exclusively in the biofilm state (Fig. 3b) and exclusively in the planktonic state (Supplementary Data 1). There were also genes specifically differentially expressed in biofilm vs. planktonic at the environmental temperature only (Supplementary Data 2) and genes differentially expressed in biofilm vs. planktonic at host temperature only (Supplementary Data 3).

Interestingly, there were 35 genes that were expressed solely dependent on temperature fluctuation and 148 genes which were solely dependent on the planktonic vs. biofilm switch (Supplementary Data 4 and 5). All the raw count data associated with different expression of genes due to thermoregulation in *P. aeruginosa* is also made available (Supplementary Data 6).
When quantifying CFUs in biofilms grown at 37 and 23 °C, we encountered small colony variants (SCVs) specifically in *P. aeruginosa* biofilms grown at 37 °C (Supplementary Fig. 2b, c). The SCVs have previously been reported to be present in the lung adapted *P. aeruginosa* strains exhibiting slow-growing properties, enhanced biofilm formation, and more exopolysaccharide production. In addition, Pf bacteriophage has been previously linked with SCV formation in *P. aeruginosa*, a phenotype promoting persistence at host temperatures. The known association between SCVs and Pf phage directed our focus to some of the phage genes found to be differentially regulated in our transcriptomics data sets. Interestingly certain phages were found to be upregulated at 37 °C while others were expressed higher at 23 °C (Fig. 3c). For example, a classic tailed bacteriophage operon encompassing genes ranging from PA14_08020 to PA14_08280 was more highly expressed at environmentally relevant temperatures (23 °C) whereas the filamentous Pf1 phage proteins ranging from PA14_48880 to PA14_48970 were expressed higher at host-relevant temperatures (37 °C). Of particular interest was PA14_48940, the Pf1 bacteriophage coat B protein (*coaB*), which was the only phage protein that was found to be expressed higher, exclusively in the biofilm state, at the host temperature vs. environmental temperature (Fig. 3c). This protein has been previously reported to be participating in maintaining the structural integrity of the biofilm matrix. Since our transcriptomic analysis showed an increase in the expression of this phage protein at 37 °C, we hypothesized that it could be contributing to the temperature-specific EPS adaptation in *P. aeruginosa* biofilms.

**Phage protein is specifically required for biofilm formation at 37 °C and not 23 °C**

Filamentous phages are abundantly present at sites of chronic infection along with *P. aeruginosa* and CoaB is a major coat protein of this phage. Since our transcriptomic data pointed towards the presence of this phage protein at host temperature, we were particularly interested to investigate the role of CoaB protein of bacteriophage Pf1 in biofilm formation. For our experiments, we used the *coaB* mutant derived from a commercially available transposon mutant library of PA14. The identity of the mutant was confirmed by arbitrary PCR followed by sequencing. We performed the established CV staining protocol to study the biofilm formed by this mutant at the two temperatures. *coaB* mutant strain showed a decrease in biofilm formation relative to...
performed on DNA extracted from bio
of the biological replicates. Unpaired
different days. The mean of each biological replicate was based on three technical replicates. Error bars represent the standard error of mean
48 h. Strains were grown in LB broth at appropriate temperatures. Bars represent the mean of three biological replicates performed on
different days. The mean of each biological replicate was based on three technical replicates. Error bars represent the standard error of mean of the biological replicates. Unpaired t-test (two-tailed) was used to measure statistical significance. *p = 0.038, ns, p = 0.6071. b qPCR was performed on DNA extracted from biofilm grown for 48 h at 23 and 37 °C using primers specific for coaB and the housekeeping gene rplU using an established mechanical dissociation method. Bars represent the mean of three biological replicates performed on different days. The mean of each biological replicate was based on three technical replicates. Error bars represent the standard error of mean of the biological replicates. Unpaired t-test (two-tailed) was used to measure statistical significance. **p = 0.0028. c Congo red binding assay. Extracellular matrix production by the wild type and mutant strain was evaluated on tryptone agar plates containing Congo Red and Coomassie brilliant blue G after incubation at 23 and 37 °C for 72 h. Representative images of the colony morphologies of WT PA14 and the ΔcoaB mutant are shown. Scale bar: 5 mm. d Comparison of the scanning electron microscopy images of Pseudomonas aeruginosa wild-type biofilms and ΔcoaB mutant biofilms grown for 48 h at 23 and 37 °C. Images show ×8000 and ×15,000 magnification and are representative of three independent experiments.

The Pf1 filamentous phage coat B protein specifically contributes to P. aeruginosa PA14 biofilm EPS at human body temperature but not at room temperature. a Crystal violet staining was performed to assess air-liquid biofilm formation in a 96-well microtiter plate at 48 h. Strains were grown in LB broth at appropriate temperatures. Bars represent the mean of three biological replicates performed on different days. The mean of each biological replicate was based on three technical replicates. Error bars represent the standard error of mean of the biological replicates. Unpaired t-test (two-tailed) was used to measure statistical significance. *p = 0.038, ns, p = 0.6071. b qPCR was performed on DNA extracted from biofilm grown for 48 h at 23 and 37 °C using primers specific for coaB and the housekeeping gene rplU using an established mechanical dissociation method. Bars represent the mean of three biological replicates performed on different days. The mean of each biological replicate was based on three technical replicates. Error bars represent the standard error of mean of the biological replicates. Unpaired t-test (two-tailed) was used to measure statistical significance. **p = 0.0028. c Congo red binding assay. Extracellular matrix production by the wild type and mutant strain was evaluated on tryptone agar plates containing Congo Red and Coomassie brilliant blue G after incubation at 23 and 37 °C for 72 h. Representative images of the colony morphologies of WT PA14 and the ΔcoaB mutant are shown. Scale bar: 5 mm. d Comparison of the scanning electron microscopy images of Pseudomonas aeruginosa wild-type biofilms and ΔcoaB mutant biofilms grown for 48 h at 23 and 37 °C. Images show ×8000 and ×15,000 magnification and are representative of three independent experiments.

We first used the Congo red binding assay to evaluate the colony morphology of both the wild type and mutant at both the temperatures. The coaB mutant colony looked similar to the PA14 colony at 23 °C. However, at 37 °C coaB mutant colony looked morphologically different than the wild type with a more wrinkled colony appearance and slight differences in coloration on the Congo Red plates (Fig. 4c). Because the agar colony biofilm in a traditional Congo Red assay is a vastly different growth condition than the pellicle biofilms that were otherwise being analyzed in this study, we also quantified the congo red uptake by pellicle biofilm formed at 23 and 37 °C for both the wild type and mutant. There was no significant difference between the mutant and the wild type at 23 °C, while at 37 °C the coaB mutant had less uptake of the congo red dye when samples were normalized relative to the overall biomass (Supplementary Fig. 8). We next used SEM to see the difference in the biofilm formed by the mutant at both these temperatures. The matrix of the mutant cells looked smoother in comparison to the wild-type matrix which showed the presence of more filament-like structures at 37 °C. No such architectural differences were observed for the mutant matrix at 23 °C (Fig. 4d). Overall, these results show the importance of this phage protein at the host temperature with no impact on biofilm formation at the environmentally relevant temperature of 23 °C.
DISCUSSION
Biofilm-associated infections are particularly recalcitrant to clearance by both antimicrobial therapy and immune function. In addition, the emergence and spread of antibiotic resistance have become a global threat and there is evidence that agricultural and industrial use of antibiotics is a contributor to the aggregation of resistance in the environment. Therefore, studying biofilm adaptations specific to the human host or specific to industrial/environmental (i.e., soil/root associated or aquatic) biofilms will enable the development of antimicrobials targeted for specific use in each case. Such practices will help to prevent the unnecessary spread of microbial resistance to clinically relevant antibiotics. In this study, we wanted to investigate the effect of temperature on biofilm formation in P. aeruginosa, an opportunistic pathogen, which is known to form biofilm in various environments.

We hypothesized that biofilm grown at different temperatures will possess different structural adaptations and have distinct genetic requirements. Our SEM micrographs and CLSM images clearly show a distinct difference in the EPS matrix in the biofilm formed by P. aeruginosa at the environment and host-relevant conditions, supporting our hypothesis. The Congo red binding assay further bolstered our hypothesis by showing differences in EPS dye association with colony biofilms at these temperatures. MALDI IMS, a protein imaging technique that has been used previously to study the protein distribution in biofilms, further demonstrated that pellicle biofilms grown under different temperature conditions exhibit different protein expression profiles, which also supported the possible presence of unique temperature-specific biofilm adaptations.

Next, we analyzed the transcriptomic data for both planktonic and biofilm-associated cells at both 23 and 37°C. We found that the expression of several genes were uniquely impacted by only temperature fluctuation whereas others were unique to only planktonic to biofilm switch. Our observation of SCVs specifically in 37°C biofilms directed our attention to certain phage genes in our transcriptomics data sets that had previously been associated with the SCV phenotype. Indeed, our transcriptomic data supported a temperature-based regulation mechanism for phage proteins like coat b (CoaB) protein of Pf1 prophage, which participates in biofilm function and persistent infection within the host. These data combined with our observation that the coaB mutant was specifically defective in biofilms formation at host-relevant temperatures adds to the growing collection of literature that demonstrates that Pf1 bacteriophage provide structural integrity to P. aeruginosa biofilms and indicates that this phenomenon may be specifically applicable to microbial physiology during infection. This finding may indicate different stressor susceptibilities at specific temperatures. For example, presence of the Pf phage in the host temperature biofilms may yield temperature-specific resistance to aminoglycoside as the incorporation of this phage in EPS has been associated with increased tolerance of this class of antibiotics. Our findings are consistent with previous studies that have found high abundance of these phages at sites of chronic infection as well as literature that demonstrates the contribution of Pf phages to Pseudomonas pathogenesis. Pf phages can not only help this pathogen to tolerate antibiotic exposure but can also play an important role in host colonization and combating mammalian immune system. Other promising roles of Pf bacteriophage include inhibiting the growth of a fungal biofilm, providing structural integrity to Pseudomonas biofilm matrix and its role as an iron chelator in biological systems, thus highlighting its synergistic relationship with P. aeruginosa. The ssDNA (single strand) genome of the Pf phage consists of several genes, of which two encode for coat proteins, CoaA (minor coat protein) and CoaB (major coat protein), and together these genes help this phage to replicate and integrate into the bacterial genome. In our study, we found that CoaB, the major coat protein of Pf1 was not only important for biofilm formation but its expression was thermally regulated. Future studies on understanding how P. aeruginosa regulates its phage proteins in response to both external and host-relevant temperatures can potentially identify novel therapeutic strategies to exploit biofilm-targeting phages while combatting the effects of biofilm-strengthening phage proteins. Because a recent study highlighted the role of temperature in biofilm formation for different P. aeruginosa strains, it will also be important to broaden our understanding of the impact of phage thermoregulation on the biofilm formation in other P. aeruginosa strains in the future.

In addition to studying the strain-to-strain variation in temperature-specific biofilm adaptations, the identification of temperature-specific EPS adaptations may be applicable to numerous other opportunistic pathogens capable of forming biofilms in the environment as well as in the human body. For example, similar MALDI IMS results demonstrating dramatic shifts in the biofilm-associated proteome were observed for Acinetobacter baumannii, another environmental microbe known to opportunistically colonize the human host in different infections (Supplementary Fig. 9). It will be important for future studies to define temperature-specific stressor susceptibilities driven by the different EPS composition produced in different growth environments. Such studies could reveal ideal therapeutic targets for use in the clinic as well as ideal targets to enable the eradication of problematic biofilms in industrial settings.

METHODS
Bacterial strains, media, and growth conditions
P. aeruginosa strain UCBPP-PA14, a highly virulent strain of P. aeruginosa originally isolated from a wound infection, was used in all experiments unless otherwise stated. An isogenic mutant of PA14, the PA14/MrT7::MAR2Tx7 inserted at nucleotide 226 in coab (PA14::coab), was used to examine the importance of the bacteriophage coat protein B in biofilm formation. Strains were routinely grown overnight and maintained at 37°C in Luria-Bertani (LB) broth. Gentamicin was added at 15 μg/mL to maintain the transposon in the PA14::coab mutant. To calculate colony-forming unit for the biofilm cells, bacterial cultures were grown for 48 h at 23 and 37°C in test tubes under static conditions. The planktonic cells were first aspirated out, followed by the addition of 1X PBS and sonication using a Fisher Scientific FB120 Sonic Dismembrator with CL-18 Probe, with a pulse of 20% amplitude for 30 s. Five microliters from the tube was then plated onto Luria Agar plates and left for overnight growth.

MALDI IMS
20 μL of the overnight grown culture of PA14 was added into a fresh 20 mL LB broth, in a 50 mL falcon containing a glass slide. Biofilms were grown on indium-tin oxide-coated glass slides (Delta Technologies, Loveland, CO) for 48 h as described previously. Matrix was applied to sample sections using a TM-Sprayer (HTX Imaging, Carboro, NC). The matrix was sprayed onto the sections at a flow rate of 0.2 ml min⁻¹ using a pushing solvent of 90% acetonitrile. The TM-Sprayer was operated at a speed of 1200 mm min⁻¹ and at a nozzle temperature of 80°C. The spray pattern was set to 2 mm spacing and eight passes of matrix were applied. Imaging mass spectrometry was performed in linear positive ion mode using an Autoflex Speed mass spectrometer (Bruker Daltonics, Billerica, MA) at a 200 μm spatial resolution. Fifty laser shots were acquired per pixel in random-walk mode in ten shot steps. Data were processed using flexImaging version 4.1.

Microtiter biofilm formation assay
To quantify and study biofilm formation at each temperature we used the previously established microtiter biofilm formation assay. Wild-type P. aeruginosa PA14 and the mutant strain were grown overnight in a 96-well round bottom plate in 150 microliters LB broth with shaking at 220 rpm and 37°C overnight. Next day, 5 μL of the overnight grown culture was transferred to a fresh 96-well plate with 145 μL of LB media. This was done for three replicates at three different days. The plates were incubated for 24 h at static conditions and at 37°C.
for 48 h at 23 and 37 °C. An absorbance at 600 nm wavelength was taken after 48 h using Synergy HIi Microplate Reader, Biotek. Planktonic cells were then aspirated out and the remaining biofilm was washed three times with 300 μL of PBS. This step helps remove unattached cells and media components that can be stained in the next step and significantly lowers background staining. Next, 200 μL of 100% ethanol was added to the wells for 15 min. The ethanol was then aspirated out completely and the plates are flipped upside down and left for drying. After the ethanol dried, 200 μL of a 1% solution of CV was added to each well of the microtiter plate. The microtiter plate was then incubated for 15 min at room temperature followed by rinsing it 3–4 times with water by submerging the plate in a tub of water, and blot vigorously on a stack of paper towels to get rid of all the excess water. The microtiter plate was then left to dry for 1–2 h. Finally, 150 μL of 30% acetic acid solution was added to each well of the microtiter plate to solubilize the CV. After an hour of incubation at room temperature, absorbance was taken at 580 nm. Using the biomass baseline, this reading was quantified and analyzed to produce readable data.

**Bacterial RNA extraction**

20 μL of the overnight grown culture of PA14 was added into a fresh 20 mL LB broth, in a 50 mL falcon along with a microscope glass slide. This was left at static conditions at both 23 and 37 °C for 48 h. Three biological replicates were processed for the WT at each temperature. Next, the biofilm from the glass slides was scrapped off using a tip and 700 μL of Qiazol lysis reagent. For disrupting or lysing bacterial cells, the mixture was added onto tubes with beads (Tough Microorganism Lysing Mix (RNase—DNase free, size—2 mL x 0.5 mm Verrel) and lysed in a VWR Bead Mill Homogenizer at 6500 rpm for 1 min (two rounds). For planktonic cells, the 48 h grown culture was centrifuged at 5000 rpm for 10 min, followed by discarding the supernatant and resuspending the pellet in 700 μL of Qiazol lysis reagent. RNA was then extracted using the RNAeasy Mini Kit (Qiagen) according to the manufacturer’s recommendations, and the RNA solution was digested with the RNase-free DNase set (Qiagen), followed by on-column DNase digestion to eliminate any remaining traces of genomic DNA. The purified RNA was quantified using a Take3 plate reader (Synergy HI microplate reader, Biotek). RNA samples with 1.8–2.2 ratio of absorbance at 260/280 nm were kept for further analysis. The samples were then sent to Genewiz for library prep and Illumina HiSeq. Only samples with an RNA integration number greater than 80 were used for cDNA library preparation.

**Analysis of the RNA-seq data**

RNA-seq data were analyzed using Rockhopper software implementing reference-based transcript assembly with UCBPP-PA14 as a reference genome followed by calculating the fold change for the transcripts at each temperature. The full accession number of the UCBPP-PA14 annotation that was used is NC_008463. Each data set was normalized by upper quartile normalization, and then transcript abundance was quantified using reads assigned per the kilobase of target per million mapped reads normalization method. The selection criteria for differential expression required genes to have a fold change of ≥2 and a q value of ≤0.05 to be considered significant. The q value was obtained by adjusting the p value using the Benjamini–Hochberg procedure.

**Congo red binding assay**

To phenotypically assess the different components of the EPS matrix, a colony morphology assay was performed as described previously. Extracellular matrix production by *P. aeruginosa* PA14 and PA14:coaB was evaluated on tryptone agar plates containing Congo Red and Coomassie brilliant blue G after incubation at 23 and 37 °C for 72 h. Five replicates were processed for the WT at each temperature. Next, the biofilm from the glass slides was scrapped off using a tip and 700 μL of Coomassie brilliant blue G after incubation at 23 and 37 °C for 72 h. Images of the colonies were taken daily and resuspended in 1 mL of PBS. OD 600 was taken for all the samples. Forty micrograms per milliliters of Congo red dye was then added to each tube and incubated at 37 °C for 1 h. Samples were then centrifuged at 16,873 × g for 2 min, and supernatants were transferred to a clear 96-well plate for measurement of absorbance at 490 nm using a plate reader (Synergy H1 Microplate Reader, Biotek). PBS + 40 μg/mL Congo red was used as a control. Finally, the exopolysaccharide-bound Congo-red was quantified by subtracting the A490 value of the sample from the A490 value of the control and normalizing the final value to OD 600 reading. All experiments are carried out on three independent days using three replicates each.

**Biofilm imaging**

Biofilms grown on glass slides for 48 h were imaged by confocal laser scanning microscopy (CLSM) (Olympus; FV3000). 20 μL of the overnight grown culture of PA14 was added into a fresh 20 mL LB broth, in a 50 mL falcon along with a microscope glass slide and grown for 48 h. The biofilm was then washed with PBS followed by staining with FilmTracer LIVE/DEAD Biofilm Viability Kit (ThermoFisher). Next, biofilm was incubated with SYTO™ 9 and propidium iodide stains for 20 min in the dark at room temperature as per the manufacturer’s protocol. After that, the biofilm was washed with PBS and the slides were imaged using a OlympusFV3000 microscope with an objective lens of ×60 (oil). Each experiment included three independent biological replicates and three images were taken for each replicate. For each glass slide, five image stacks were taken with a 2-step size of 0.7 μm. ImageJ software was used to calculate the number of live (SYTO9; green) and dead (propidium iodide; red) cells.

**Electron microscopy**

For SEM, static cultures were grown in 50-mL conical tubes with circular cover slips semi-submerged in 2 mL of LB broth. Cover slip samples were handled and processed as described previously. Upon removal of the culture medium, the cover slips were immediately flooded with 25% glutaraldehyde in 0.05 M sodium cacodylate and incubated at room temperature for 1 h. The fixative was removed and replaced immediately with 0.05 M sodium cacodylate to prevent sample dehydration. The cover slips were then incubated in osmium tetroxide for 15 min followed by dehydration with increasing concentrations of ethanol, ranging from 25% to 100%, and CO2 critical point drying. Samples were carbon-coated and visualized with a Hitachi S-4300 scanning electron microscope.

**DNA extraction and quantification of phage by qPCR**

To quantify the phage present in PA14 biofilm we used a protocol that was designed to extract phage from sputum samples and modified it as per our requirement. Briefly, 20 μL of the overnight grown culture of PA14 was added into a fresh 20 mL LB broth, in a 50 mL falcon along with a microscope glass slide and grown for 48 h. The slide was then washed with PBS and biofilm was then scraped off the glass slide and resuspended in 200 μL of PBS. The suspension was then added to a 2 mL tube filled ~1/5 by volume with 1 mm of ceramic beads. For the rest of the protocol QiAamp DNA Minikit (Qiagen) was used. 20 μL of Proteinase K and 200 μL of buffer AL was added to the biofilm cells suspended in 200 μL of PBS. Next, a VWR Bead Mill Homogenizer was used for mechanical disruption, at 6500 rpm for 1 min (two rounds). The homogenized mixture was then used for DNA extraction as per the manufacturer’s protocol. The Pf1 phage ssDNA genome as well as the dsDNA genome of *P. aeruginosa* in biofilm were quantified by qPCR. Two microliters of the DNA extracted from biofilm samples was used as a template in 10-μL qPCR reactions. 5 μL of iQ SYBR Green Supermix (BioRad) and 2 μL primers were used in the final reaction mixture. Cycling conditions were as follows: 95 °C for 2 min, 25 °C for 15 s, 60 °C for 10 s × 40 cycles on a Bioprime Real-Time PCR system. To quantify Pf1 phage, the primers coaB-F (AAGGATCCAGGAAATGCCAGC) and coaB-R (ACGATATAACGGCCGATG) targeting coaB were used. For *P. aeruginosa* quantification, primers targeting the 505 ribosomal subunit gene rplU, rplU-F (CAAGGTGTCATCACTCAAGT) and rplU-R (GCGCCTGACCTTCATGT) were used. All samples were run in triplicate and qPCR was performed on three independent days. The Pf1 phage copy number represent the measured Pf1 phage with the *P. aeruginosa* copy number subtracted to account for the detection of prophage DNA contained in the genome of *P. aeruginosa*.

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). Unpaired t-test (two-tailed) was used to calculate the statistical significance.
REFERENCES

1. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. Science 284, 1318–1322 (1999).
2. Andrews, J. S., Rolfe, S. A., Huang, W. E., Scholes, J. D. & Banwart, S. A. Biofilm formation in environmental bacteria is influenced by different macromolecules depending on genus and species. Environ. Microbiol. 12, 2496–2507 (2010).
3. Hostacka, A., Ciznar, I. & Stefkovicova, M. Temperature and pH affect the production of bacterial biofilm. Folia Microbiol. 55, 75–78 (2010).
4. Barbour, M. el. et al. From the environment to the host: re-wiring of the transcriptome of Pseudomonas aeruginosa from 22 °C to 37 °C. PLoS One 9, e89941 (2014).
5. Hall-Stoodley, L., Costerton, J. W. & Stoodley, P. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. 2, 95 (2004).
6. Galé, S., García-Gutiérrez, C., Miguélez, E. M., Villar, C. J. & Lombó, F. Biofilms in the food industry: health aspects and control methods. Front. Microbiol. 9, 898 (2018).
7. Gutt, B. et al. Beneficial Oral Biofilms as Smart Bioactive Interfaces. Front. Microbiol. 9, 107 (2018).
8. Römling, U. & Balsalobre, C. Biofilm infections, their resilience to therapy and innovative treatment strategies. J. Intern. Med. 272, 541–561 (2012).
9. Chiang, W-C. et al. Extracellular DNA shields against aminoglycosides in Pseudomonas aeruginosa biofilms. Antimicrob. Agents Chemother. 57, 2352–2361 (2013).
10. Goltermann, L. & Tolker-Nielsen, T. Importance of the exopolysaccharide matrix in antimicrobial tolerance of Pseudomonas aeruginosa aggregates. Antimicrob. Agents Chemother. 61, 1–7 (2017).
11. Konkel, M. E. & Tilly, K. Temperature-regulated expression of bacterial virulence genes. Microbes Infect. 2, 157–166 (2000).
12. Jin, S., Song, Y. N., Deng, W. Y., Gordon, M. P. & Nester, E. W. The regulatory VirA gene of Pseudomonas aeruginosa is required for virulence. J. Bacteriol. 175, 6830–6835 (1993).
13. Plumley, B. A. et al. Thermoregulation of Biofilm formation in Burkholderia pseudomallei is disrupted by mutation of a putative dugainline cyclase. J. Bacteriol. 199, e00780–16 (2017).
14. Kim, S., Li, X-H., Hwang, H-J. & Lee, J-I. Thermoregulation of Biofilm formation in Pseudomonas aeruginosa. Appl. Environ. Microbiol. https://aem.asm.org/content/86/22/e01584-20 (2020).
15. O’Toole, A., Ricker, E. B. & Nuxoll, E. Thermal mitigation of Pseudomonas aeruginosa biofilms. Biofouling 31, 665–675 (2015).
16. Schouten, J. D. et al. The impact of quorum sensing and swarming motility on Pseudomonas aeruginosa biofilm formation is nutritionally conditional. Mol. Microbiol. 62, 1264–1277 (2006).
17. Steinmann, R. & Dersch, P. Thermosensing to adjust bacterial virulence in a fluctuating environment. Future Microbiol. 8, 85–105 (2012).
18. Dötsch, A. et al. The Pseudomonas aeruginosa transcriptional landscape is shaped by environmental heterogeneity and genetic variation. mBio 6, e00749–15 (2015).
19. Hurme, R. & Rhen, M. Temperature sensing in bacterial gene regulation — what it all boils down to. Mol. Microbiol. 30, 1–6 (2002).
20. Spiess, C., Bell, A. & Ehrmann, M. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell 97, 339–347 (1999).
21. Johansson, J. RNA thermosensors in bacterial pathogens. Contributions Microbiol. 16, 150–160 (2009).
22. Grosso-Becerra, M. V. et al. Regulation of Pseudomonas aeruginosa virulence factors by two novel RNA thermometers. Proc. Natl Acad. Sci. USA 111, 15562–15567 (2014).
53. Secor, P. R. et al. Biofilm assembly becomes crystal clear - filamentous bacteriophage organize the Pseudomonas aeruginosa biofilm matrix into a liquid crystal. *Microb. Cell* 3, 49–52 (2015).

54. Martínez, E. & Campos-Gómez, J. Pf Filamentous Phage Requires UvrD for Replication in Pseudomonas aeruginosa. *mSphere* 1, e00104–e00115 (2016).

55. Lee, C.-R. et al. Biology of acinetobacter baumannii: pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. Front. Cell. Infect. Microbiol. 7, https://doi.org/10.3389/fcimb.2017.00055 (2017).

56. He, J. et al. The broad host range pathogen Pseudonas aeruginosa strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proc. Natl Acad. Sci. USA* 101, 2530–2535 (2004).

57. Floyd, K. A. et al. Adhesive fiber stratification in uropathogenic escherichia coli biofilms unveils oxygen-mediated control of type 1 Pili. *PLoS Pathog.* 11, e1004697 (2015).

58. McClure, R. et al. Computational analysis of bacterial RNA-Seq data. *Nucleic Acids Res.* 41, e140 (2013).

59. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc.* 57, 289–300 (1995).

60. Okegbe, C. et al. Electron-shuttling antibiotics structure bacterial communities by modulating cellular levels of c-di-GMP. *Proc. Natl Acad. Sci.* 114, E5236–E5245 (2017).

61. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9, 671–675 (2012).

62. Gaddy, J. A., Tomaras, A. P. & Actis, L. A. The Acinetobacter baumannii 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect. Immun.* 77, 3150–3160 (2009).

ACKNOWLEDGEMENTS

We would like to thank Dr. Moamen Elmassry for helping with the use of the UpSetR package, Alex Luecke and other members of the Wakeman Lab for critical reading of this manuscript. Work in the Wakeman lab is supported by NIH/NIGMS (R15GM128072). Work in the Caprioli lab was supported by NIH/NIGMS (P41GM103391-08). Work in the Skaar lab was supported by NIH/NIAID (R01AI138581 and R01AI145992). K.B. was supported by the Doctoral Dissertation Completion Fellowship granted from Texas Tech University Graduate School. K.B. received a publication award from Tech American Society for Microbiology (Tech ASM).

AUTHOR CONTRIBUTIONS

K.B. contributed to the conception, experimental design, data acquisition, interpretation of data, and paper preparation. J.L.M. contributed to the experimental design, data acquisition, and data interpretation of the MALDI IMS/proteomics data as well as paper editing. R.M.C. contributed to data interpretation of the MALDI IMS/proteomics data and paper editing. E.P.S. contributed to data interpretation and paper editing. C.A.W. contributed to the conception, experimental design, data acquisition for biofilms grown for MALDI IMS, interpretation of data, and editing of the paper.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41522-021-00194-8.

Correspondence and requests for materials should be addressed to C.A.W.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021