Multipronged impact of environmental temperature on Staphylococcus aureus infection by phage Kayvirus rodi: Implications for biofilm control

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A B S T R A C T

Environmental cues sometimes have a direct impact on phage particle stability, as well as bacterial physiology and metabolism, having a profound effect on phage infection outcome. Here, we explore the impact of temperature on the interplay between phage *Kayvirus rodi* (phiIPLA-RODI) and its host, *Staphylococcus aureus*. Our results show that phiIPLA-RODI is a more effective predator at room (25 °C) compared to body temperature (37 °C) against planktonic cultures of several strains with varying degrees of phage susceptibility. This result differs from most known examples of temperature-dependent phage infection, in which optimum infection is correlated with the host growth rate. Further characterization of this phenomenon was carried out with strains IPLA15 and IPLA16, whose respective MICs were 7 log units and a 1-log unit higher at 37 °C than at 25 °C. Our results demonstrated that the phage also had a greater impact at room temperature during biofilm development and for the treatment of preformed biofilms. There was no difference in phage adsorption between the two temperatures for strain IPLA16. Conversely, adsorption of phiIPLA-RODI to IPLA15 was reduced at 37 °C compared to 25 °C. Moreover, confocal microscopy analysis indicated that the biofilm matrix of both strains has a greater content of PIA/PNAG at 37 °C than at 25 °C. Regarding infection parameters, we observed longer duration of the lytic cycle at 25 °C for both strains, and infection of IPLA15 by phiIPLA-RODI resulted in a smaller burst size at 37 °C than at 25 °C. Finally, we also found that the rate of phage resistant mutant selection was higher at 37 °C for both strains. Altogether, this information highlights the impact that bacterial responses to environmental factors have on phage-host interactions. Moreover, phage phiIPLA-RODI appears to be a highly effective candidate for biofilm disinfection at room temperature, while its efficacy in biofilm-related infections will require combination with other antimicrobials.

1. Introduction

In order to survive, bacteria have to constantly adapt to their surrounding environment by undergoing physiological and metabolic changes. Such adaptations may also affect their susceptibility to antibiotics or disinfectants, and have an impact on the success of antimicrobial therapies [1,2]. For this reason, it is very important to study the efficacy of different strategies under environmental conditions that mimic potential therapeutic settings. Bacteriophages, viruses that infect and kill bacteria, are no exception. In fact, their own stability and infectivity might be directly affected by variations in certain environmental cues [3,4]. Moreover, given their key role as modulators of bacterial communities, it is also necessary to understand the nature of phage-host interactions in different environments. This might provide new clues regarding differences in the composition of microbial populations observed in distinct niches. Additionally, it will be valuable to predict the potential impact on natural bacterial populations of widespread phage application in human or animal therapy and as decontamination agents.

Temperature is known to play a major part in regulating microbial physiology. For instance, numerous virulence factors of pathogenic bacteria exhibit temperature-dependent expression [5,6]. Indeed, researchers have generally found a tight connection between the production of virulence determinants and infection temperature. In turn, transcription of virulence-related genes was turned off under optimal *in vitro* growth conditions. This phenomenon has not been so well studied regarding antibiotic resistance, although there are some examples in the literature reporting temperature modulation of antimicrobial susceptibility [7,8]. In the case of phages, temperature can influence infection dynamics in multiple ways. On the one hand, stability of the phage particle varies greatly depending on temperature, with higher values generally being more detrimental to phage structure integrity [9]. On the other hand, temperature may affect important infection and multiplication parameters, such as receptor abundance on the cell surface, expression of antiphage defense systems or bacterial growth rate [10–12]. Therefore, the choice of a given phage for a specific

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antimicrobial application should take into account the temperature at which it is more effective. Some phages will then be mainly used in human therapy, while others might be more suitable for biofilm elimination from industrial or clinical surfaces.

Most bacteria in natural and man-made environments are part of complex communities called biofilms, in which a self-produced extracellular matrix protects the individual cells from external challenges. These structures are notoriously resistant to antimicrobials [13]. Furthermore, as the most common lifestyle of bacterial cells, biofilms are frequently involved in bacterial infections, especially chronic infectious diseases [14]. Therefore, it is likely that the majority of phage-bacteria encounters, including those that take place during phage therapy applications, occur in the context of a biofilm. Nonetheless, bacteriophages can penetrate biofilms and infect their target cells, being interesting as potential antibiofilm agents [15]. With this in mind, it is particularly important to assess how changes in environmental factors, like temperature, affect phage infection of biofilm cells. However, the studies available so far had mainly focused on temperature-dependent infection of planktonic cultures.

Kayvirus rodi (phiIPLA-RODI) is a myophage belonging to the *Herelleviridae* family that was isolated from sewage and characterized by Gutiérrez et al. [16], and has the ability to infect staphylococcal biofilms, especially those formed by the human and bovine pathogen *Staphylococcus aureus*. Recent data indicate that biofilm treatment by phiIPLA-RODI is dependent on the environmental pH [17], being more effective at pH values greater than 5.5 In contrast, more acidic environments lead to phage inactivation and promote formation of eDNA-rich biofilms. Until now, all experiments assessing the interaction between this phage and its host had been carried out at 37 °C, a temperature mimicking that found in the human body. However, *S. aureus* biofilms can also grow at room temperature, establishing a reservoir on hospital or industrial surfaces from where it can then start an infection or contaminate foods. Under these conditions, bacterial cells may exhibit different phage susceptibility to that observed at body temperature. With this in mind, the aim of this study was to understand the impact of temperature on the infection of *S. aureus* by phiIPLA-RODI, especially during biofilm formation. This information will help us better define the interactions between this virus and its host in different settings, and will be useful to determine if phiIPLA-RODI is a good candidate for eliminating staphylococcal contamination from surfaces at room temperature and/or in the context of a biofilm-related infection.

2. Materials and methods

2.1. Bacterial strains, bacteriophage and culture conditions

The *S. aureus* strains used in this study are listed in Table 1 and were routinely grown in TSB (Tryptic Soy Broth, Scharlau, Barcelona, Spain) at 37 °C or 25 °C with shaking, or on TSA (TSB supplemented with 2 % (w/v) agar) plates. Bacteriophage phiIPLA-RODI, previously isolated from sewage and characterized by Gutiérrez et al. [16], was propagated on strain IPLA16 as previously described [16].

2.2. Phage susceptibility of planktonic cultures

Phage susceptibility of the different strains was determined as previously described by using a modification of the broth microdilution assay [18]. The minimum inhibitory concentration (MIC) was determined as the lowest starting phage titer that visibly inhibited bacterial growth after 24 h of incubation at 25 °C or 37 °C. The final MIC values for each strain and temperature were determined as the mode of at least three independent experiments.

In order to monitor the evolution of the bacterial population in the presence of increasing phage concentrations, growth of strains IPLA15 and IPLA16 was monitored for 20 h at 25 or 37 °C by measuring the OD_{600} every 15 min using a multiwell plate reader Tecan Infinite M

Table 1
Origin and phage susceptibility of *S. aureus* strains used in this study.

Strain	Description	MIC 25 °C	MIC 37 °C	MLST	Reference
IPLA15	Meat industry surface	10 PFU/ml	10^8 PFU/ml	1	40
IPLA16	Meat industry surface	10 PFU/ml	10^2 PFU/ml	12	40
SA113	Derivative of strain NCTC8325	10^3 PFU/ml	$>10^8$ PFU/ml	8	41
ISP479r	Derivative of strain NCTC8325	10^3 PFU/ml	10^4 PFU/ml	8	42
Newman	Clinical strain	10 PFU/ml	10^6 PFU/ml	254	43
Sa9	Cow mastitis	10 PFU/ml	10^3 PFU/ml	504	44
132	Clinical strain	10^4 PFU/ml	10^8 PFU/ml	8	45
15981	Clinical strain	10 PFU/ml	$>10^8$ PFU/ml	8	46
V329	Cow mastitis	10^4 PFU/ml	10^8 PFU/ml	126	47

Nano (Tecan Trading AG, Seestrasse, Switzerland).

2.3. Isolation of phage resistant mutants

In order to estimate the frequency of selection of phage resistance, overnight cultures of strains IPLA15 and IPLA16 were grown in TSB at 37 °C with shaking. 100 μl from these cultures were mixed with an equal volume taken from a phage stock containing 10^9 PFU/ml and incubated for 10 min at 25 °C or 37 °C. The phage-bacteria mixtures were then added to 5 ml of soft agar (TSB supplemented with agar at 0.7 %) and poured onto TSA plates. Once dry, the plates were incubated at 25 °C or 37 °C for 24 h and, subsequently, the colonies that grew on the agar were counted and used to calculate the frequency of phage resistant mutants by dividing the number of survivors by the inoculum (10^8 CFUs).

Some of the surviving colonies were then picked and analyzed to confirm their lack of phage susceptibility by using the spot assay. Briefly, a 1:10 dilution from an overnight culture of each mutant was prepared in PBS. From this cell suspension, 100 μl were added to 5 ml of soft agar and poured onto a TSA plate. The plates were allowed to air dry for 10 min and then 10 μl of phage stock (10^9 PFU/ml) were spotted on the center of each plate. Following overnight growth at 37 °C, the plates were assessed for the presence of a lytic halo due to the phage. Mutants that did not exhibit halo formation were considered bacteriophage insensitive mutants (BIMs). Additionally, growth of three mutants derived from strain IPLA15 (IPLA15-R1, IPLA15R2 and IPLA15-R3) was monitored in the presence of increasing phage concentrations as described above, and compared to the wild-type strain.

2.4. One-step growth curves

To determine the infection parameters of phage phiIPLA-RODI at each temperature, 10-ml cultures of *S. aureus* IPLA16 and IPLA15 were grown in TSB at 25 °C or 37 °C to an OD_{600} of 0.1 and then pelleted at $4000 \times g$. Each culture was then resuspended in 1 ml of TSB and phage was added at an MOI of 0.1 (10^7 PFU/ml). Adsorption was allowed for 10 min at 25 °C or 37 °C and then the cells in the different samples were pelleted at $12,000 \times g$ in a benchtop centrifuge to eliminate the free phages and resuspended in 10 ml of TSB. The samples were then titrated (time point 0) and then incubated at the same temperature used for the adsorption step. Aliquots were taken every 15 min for titration for a total of 60 or 90 min for experiments carried out at 37 °C or 25 °C, respectively.

2.5. Biofilm treatment assays

S. aureus biofilms were grown as previously described [18]. Briefly, overnight cultures of strains IPLA15 and IPLA16 were diluted in fresh TSBg medium (TSB with 0.25 % glucose) to obtain 10^6 CFU/ml. Two ml from these cell suspensions was used to inoculate each well of a 12-well microtiter plate (Thermo Scientific, NUNC, Madrid, Spain). Biofilms were allowed to develop for 24 h at 25 °C or 37 °C. Next, the planktonic phase was removed and treatment was added to each well (1 ml of TSBg alone or containing 10^9 PFU/ml of phage phiIPLA-RODI). The microtiter plates were incubated again for 6 or 24 h at the same temperature used for biofilm formation. After incubation, the planktonic phase was removed and the number of viable in the adhered phase was determined by serially diluting the samples. In order to harvest the attached phase, the biofilm was washed twice with PBS and subsequently scraped with a sterile pipette tip.

2.6. Efficiency of plating (EOP) and phage adsorption assays

To determine the EOP, a suspension of phage phiIPLA-RODI was titrated on strains IPLA15 and IPLA16 by the overlay agar method. The resulting plates were then incubated at 25 °C or 37 °C for 24 h, after which the number of lytic plaques on each plate was counted. The EOPs were determined by dividing the phage titer obtained under the test conditions by the phage titer estimated under reference conditions (*S. aureus* IPLA16 at 37 °C).

The phage adsorption rate was determined as previously described, with some modifications [19]. First, overnight cultures of *S. aureus* IPLA16 and IPLA15 were diluted 1:100 into fresh TSBg and used to inoculate the wells of a 12-well microtiter plate (2 ml per well). These plates were then incubated at 25 °C or 37 °C for 20–24 h. Afterwards, the planktonic phase was removed and biofilm cells were washed with PBS and scraped and resuspended in 1 ml of PBS and then diluted to an OD_{600} of 1. From these suspensions, 900 μl aliquots (~10^8 CFU/ml) were then mixed with 100 μl of a phage suspension to obtain a final MOI of 0.1. A sample containing only PBS and phage with no bacterial cells was used as a control. Next, phage adsorption was allowed to occur for 5 min at room temperature. Non-adsorbed phage particles were then isolated by centrifuging the samples for 3 min at 10,000×g, and the adsorption rate was calculated as follows:

adsorption rate = [(phage titer in supernatant of control – phage titer in supernatant sample) / (phage titer in supernatant of control)] x 100

2.7. Transcriptional analysis

Total RNA was isolated from *S. aureus* IPLA 16 biofilms grown for 24 h at 25 °C or 37 °C. To harvest the adhered cells, biofilms were washed with PBS upon removal of the planktonic phase, and scraped with a pipette tip in a solution containing 0.5 ml PBS and 1 ml RNA protect (Qiagen). Following 5 min of incubation at room temperature, bacterial cells were pelleted at 5000×g for 10 min and stored at −80 °C until further processing. After thawing the samples, cells were lysed by mechanical disruption in a solution of phenol-chloroform 1:1, glass beads (Sigma) and 80 mM DTT by using a FastPrep®-24. RNA isolation was performed with the Illustra RNA spin Mini kit (GE Healthcare) and the resulting samples were treated with Turbo DNAse (Ambion) to remove traces of genomic DNA. For storage, 1 μl Superase inhibitor (Ambion) was added to each 50-μl sample. RNA concentration and quality were determined by using a microplate spectrophotometer Epoch (Biotek) and agarose gel electrophoresis, respectively.

A total of 10 μg of RNA from each sample were sent to Macrogen Inc. (South Korea) for sequencing using the Illumina HiSeq2000 platform (Illumina, San Diego, CA, USA). FASTQC v. 0.11.3 [20] was used to perform quality control of the reads, and the RNA-seq reads were then mapped to the *S. aureus* IPLA16 genome (GenBank accession number: CP134617.1) with BowTie2 [21]. From the output of this program, only the uniquely mapped reads were kept for the subsequent step. Differential gene expression analysis was carried out using EDGE-pro [22] and the R package DEseq2 [23].

Transcriptional changes in selected genes of strain IPLA15 were analyzed by quantitative reverse transcription-PCR (RT-qPCR). 0.5 μg of purified RNA from 24-h biofilms grown at the two temperatures were converted into cDNA with the iScript™ Reverse Transcription Supermix for RT-qPCR (BioRad) according to the manufacter's instructions. The resulting cDNA was then diluted 1:25 and 2.5 μl were added to each reaction together with Power SYBR Green PCR Master Mix (Applied BioSystems) for qPCR analysis in a 7500 Fast Real-Time PCR system (Applied Biosystems) with the following cycle parameters: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Primers are listed in Table S1. Fold-changes were calculated by the threshold cycle (CT) method [24], using the 16S rRNA gene as a reference.

2.8. Confocal microscopy analysis of biofilms

24-h biofilms of strains IPLA15 and IPLA16 were formed at 25 °C and 37 °C on 2-well μ-slides with a glass bottom (ibidi, USA) by inoculating 2 ml from a cell suspension containing 10^6 CFU/ml. Following removal of the planktonic phase, wells were washed with PBS and subsequently stained with Live/Dead® BacLight™ kit (Invitrogen AG, Basel, Switzerland) and WGA Alexa Fluor® 647 conjugate (Invitrogen, Eugene, Oregon, USA) as indicated by the manufacturers. Briefly, each well was stained for 30 min with 1 ml of PBS containing 1.5 μl of SYTO9, 1.5 μl of propidium iodide and 15 μl of WGA Alexa Fluor® 647 conjugate. The staining solution was then removed and samples were observed with a confocal scanning laser microscope (DMi8, Leica Microsystems) using a 63 × oil objective.

2.9. Biofilm development under phage predation

Overnight cultures of *S. aureus* IPLA16 and IPLA15 were diluted in TSBg to obtain a cell suspension containing 10^6 CFU/ml that was used to inoculate 12-well microtiter plates with 1 ml per well. Afterwards, each well was treated with 1 ml from suspensions containing different phage titers, ranging from 0 (control well) to 10^3 PFU/ml. Biofilms were then allowed to grow for 3, 5, 7, 9 or 24 h at 25 °C. At these time points, the number of viable cells and phage particles was determined for both the planktonic phase and the biofilm. The adhered cells were recovered after washing twice with PBS and scraping with a pipette tip in 1 ml of PBS per well. The resulting suspension was serially diluted, plated on TSA and incubated at 37 °C for 24 h. The next morning, the colonies grown in the different dilutions were counted and used to determine the number of viable cells. The number of active phage particles was determined by titration using the double layer agar technique, using strain IPLA16 as a host and incubation at 37 °C. The pH of the planktonic phase of control (non-infected) biofilms was also monitored with a calibrated pH meter.

The effect of pH on phage-bacteria interaction was further studied by using a modification of the model developed by Fernández et al. [17]. The steps taken to adapt the model parameters for 25 °C are described in Supplementary methods.

2.10. Statistical analysis

Data corresponding to at least three biological replicates was analyzed with Student's t-test. P-values <0.05 were considered significant.

2.11. Genome analysis of mutant strains

Three *S. aureus* IPLA15 mutants with decreased phage susceptibility

were grown overnight on TSA plates at 37 °C. All cells grown on this plate were resuspended in 1 ml PBS to obtain a suspension with approximately 5 x 10⁹ cells/ml. Cells were then pelleted, washed with PBS and resuspended in 0.5 ml of 1 x DNA/RNA Shield buffer (Zymo Research, Irvine, USA). These samples were sent to MicrobesNG (http://www.microbesng.uk) for gDNA isolation and genome sequencing. Purification of gDNA was performed with solid-phase reversible immobilization (SPRI) beads (Beckman Coulter, Brea, USA) after treating the samples with 0.2 mg/ml lysostaphin and 0.1 mg/ml RNase A (ITW Reagents, Spain) for 25 min at 37 °C, and then for 5 min at 65 °C with 0.1 mg/ml proteinase K (VWR Chemicals, Ohio, USA) and 0.5 % v/v SDS (Sigma-Aldrich, Missouri, USA). The genomic libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) as indicated by the manufacturer, and subsequently sequenced on an Illumina HiSeq platform using a 250 bp paired end protocol.

Default parameters were used for all software utilized for genome assembly and analysis unless indicated otherwise. Quality control of the reads was performed with FASTQC v. 0.11.3 [25] and, when necessary, reads were trimmed with Trimmomatic v. 0.39 by using a sliding window quality cutoff of Q15 [26]. Genome assembly was carried out by using SPAdes v. 3.14.1 [27], and quality of the generated assemblies was assessed with QUAST v. 5.0.2 [28]. Sequences shorter than 500 bp were removed prior to genome annotation with the NCBI Prokaryotic Genome Annotation Pipeline v. 5.1 [29]. Comparison of the genomes with that of the wild-type strain (accession numbers CP134618-CP134619) was carried out with the variation analysis webtool on the BV-BRC website (https://www.bv-brc.org/).

3. Results

3.1. Planktonic cells from different S. aureus strains are more susceptible to phiIPLA-RODI at 25 °C than at 37 °C

In order to compare phage susceptibility at ambient and body temperature, we first carried out MIC determination assays with several *S. aureus* strains. The general trend observed for all strains was that phiIPLA-RODI was more successful at inhibiting the bacterial population at 25 °C compared to 37 °C. However, there were two distinct groups of strains. In some cases, the starting phage concentration required for growth inhibition increased by at least 4 log orders (IPLA15, SA113,

Newman, 132, 15981 and V329) at body temperature (Table 1). In contrast, the difference between the two incubation conditions was much smaller in other strains (IPLA16, ISP479r and Sa9) that happened to be the most susceptible at 37 °C (Table 1). We selected IPLA15 and IPLA16 for subsequent experiments as representative of these two groups of strains, (i.e. *S. aureus* IPLA15 with a high difference in phage susceptibility between both temperatures, and *S. aureus* IPLA16 with only one log unit difference between 25 °C and 37 °C).

To observe phage inhibition dynamics more closely, we monitored growth of strains IPLA15 and IPLA16 under different degrees of phage predation at the two temperatures. These experiments confirmed the trends described above and showed that, at 37 °C, strain IPLA15 only started to show some effect of phage infection when the starting concentration of phiIPLA-RODI was 10^3 PFU/ml, with no noticeable impact at lower phage concentrations (Fig. 1A). Also, at higher phage titers ($>10^4$ PFU/ml) we observed delayed growth of the bacterial population, potentially due to the selection of bacteriophage resistant mutants (Fig. 1A). In contrast, growth of IPLA16 at the same temperature was already diminished at 1 and 10 PFU/ml and completely inhibited at higher phage concentrations (Fig. 1B). The results were very different at 25 °C, since growth of both strains was completely inhibited when the starting phage titer was low, specifically 10 PFU/ml and 100 PFU/ml for strains IPLA16 and IPLA15, respectively (Fig. 1C and D).

Next, we compared the efficiency of plating of phage phiIPLA-RODI on liquid overnight cultures of these two strains at 25 °C and 37 °C. The phage titer obtained on IPLA16 grown at 37 °C was used as a reference; as a result, the EOP for this combination was 1. In comparison, titration of IPLA15 at body temperature was significantly lower, with an average EOP value of 0.51 ± 0.24 (p-value = 0.0097). In contrast, the EOPs at room temperature were significant higher for both strains compared to the values obtained at 37 °C, with average values of 1.88 ± 0.52 (p-value = 0.0022) and 1.87 ± 0.37 (p-value = 0.0061) for IPLA15 and IPLA16, respectively.

3.2. Infection by phage phiIPLA-RODI during biofilm development at 25 °C

We had previously characterized biofilm establishment by *S. aureus* IPLA16 in the presence of different starting phage concentrations at 37 °C. Here, we monitored the dynamics between bacterial growth and
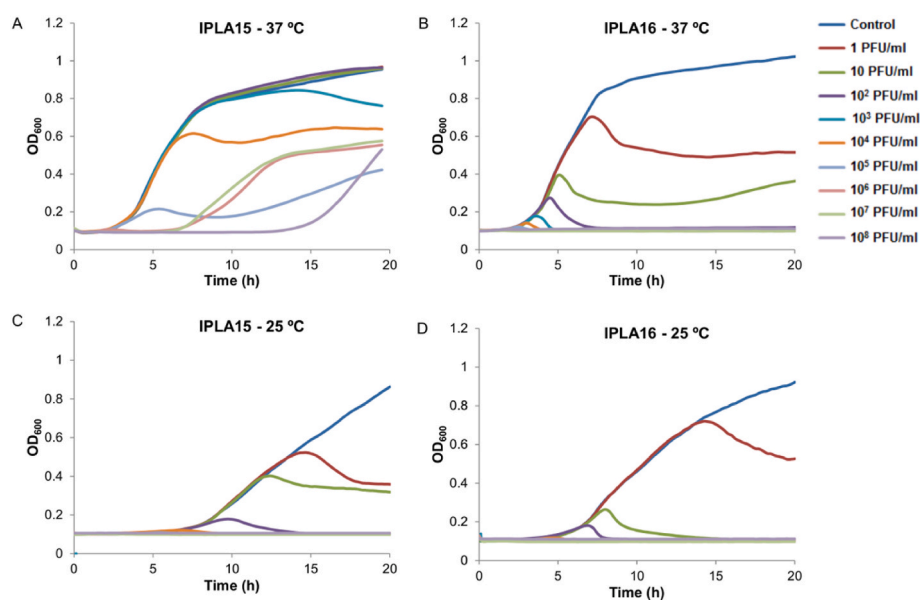

Fig. 1. Growth curves of *S. aureus* strains IPLA15 (A and C) and IPLA16 (B and D) at 37 °C (A and B) and 25 °C (C and D) in the presence of increasing concentrations of phage phiIPLA-RODI ranging from 0 (control) to 10^8 PFU/ml. OD$_{600}$ was monitored for 20 h. Data represent one representative experiment out of three independent repeats showing the same trend.

phage multiplication in biofilms and planktonic cells at 25 °C, and compared it to our previous data obtained at 37 °C [17]. These experiments showed that bacterial growth was slower at room compared to body temperature (Fig. 2A and B). The growth rates of the attached phase during mid exponential and late exponential phase were 2.78 and 2.48 h^{-1}, while the equivalent rates in the planktonic phase were 2.64 and 2.20 h^{-1} (Figs. S1A and S1B). At 37 °C, according to the data obtained by Fernández et al. [17], the growth rates of the biofilm were 3.70 and 2.48 h^{-1}, whereas the values estimated for the planktonic phase were 3.70 and 2.38 h^{-1} (Figs. S1A and S1B).

On the other hand, the phage was more effective at controlling the bacterial population at 25 °C than at 37 °C. Indeed, a starting phage number of 10 PFU/well (MOI = 0.0001) was sufficient to reduce the number of bacteria below the level of detection at 25 °C in both the adhered and the planktonic phases (Fig. 2C and D, respectively), and the same result was observed for a starting phage number of 100 PFU/well (MOI = 0.001) (Fig. 2E and F, respectively). In contrast, 10^3 PFU/well (MOI = 0.01) were required for the same effect on the biofilm population at 37 °C and 10^2 PFU/well (MOI = 0.001) to deplete the planktonic

population below detection levels [18]. However, when comparing the phage propagation rates between the two temperatures, values were generally lower at 25 °C (Figs. S1C and S1D). The only exception to this was phage multiplication in the biofilm during mid logarithmic phase, which occurred at a very similar rate in both cases (11.58 h^{-1} and 12.02 h^{-1} at 25 °C and 37 °C, respectively).

Given the importance of pH for phage-bacteria interactions in the context of biofilm formation at 37 °C, we also monitored the change in pH at 25 °C in control, non-infected cultures during biofilm development (Fig. 2G). After 24 h, the pH value was 5.88, which remained stable even if incubation was prolonged for another 24 h, with an average of 5.80 after 48 h. This value is significantly higher than that obtained in TSBg at 37 °C, which was approximately 4.75 [18], and is above the estimated pH at which phage inactivation starts (about 5.5). Therefore, the lack of phage inactivation may have an impact on the overall outcome of the infection and contribute to the efficacy of the virus.

To study the potential role of pH more in depth, we entered all the above-mentioned parameters into a previously developed simulation model (Supplementary methods). First, we estimated the pH change rate
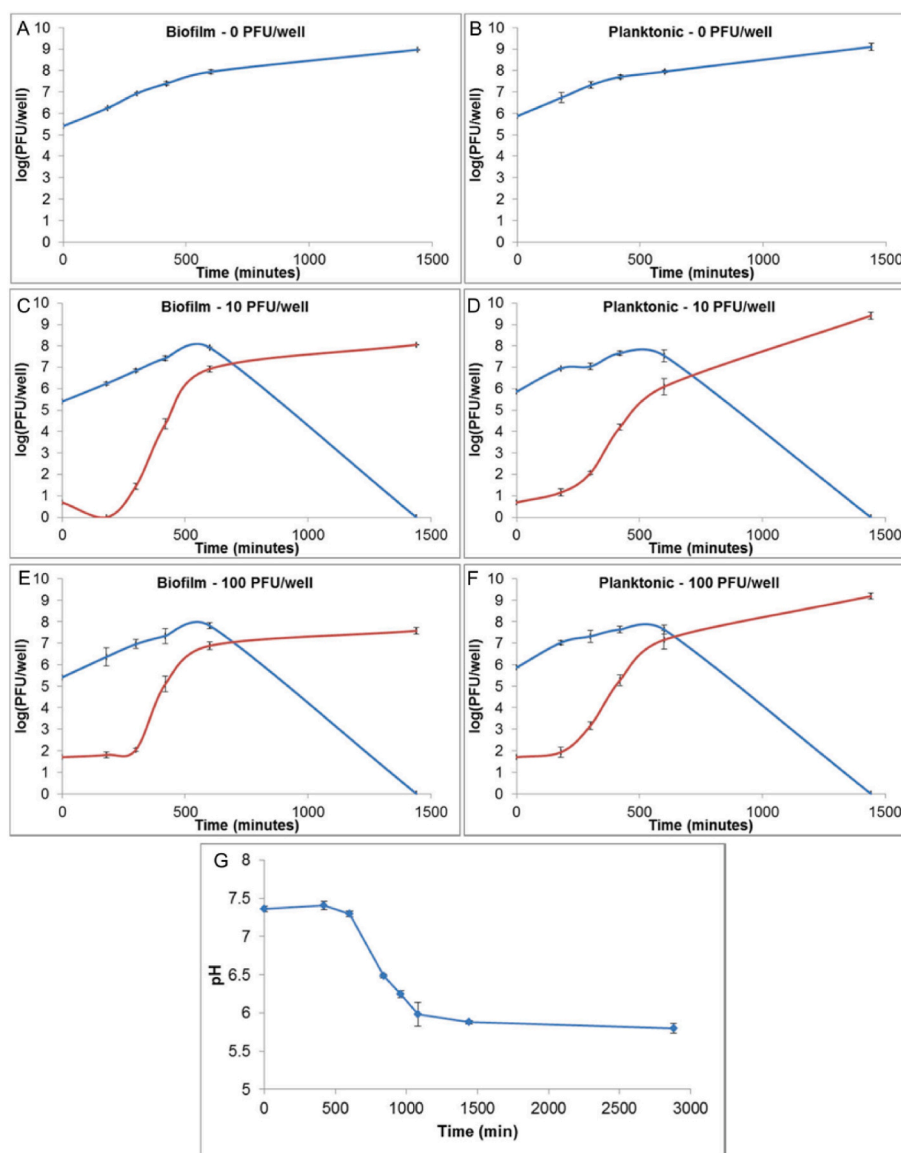

Fig. 2. Development of *S. aureus* IPLA16 biofilms under predation by phage phiIPLA-RODI in TSBg for 24 h at 25 °C. The starting inoculum was 10^6 CFU/well in all cases and the starting phage titer was 0 PFU/well (A and B), 10 PFU/well (C and D) or 100 PFU/well (E and F). In each time point, the number of viable cells (blue line) and phage titer (red line) were determined for the biofilm (A, C and E) and the planktonic phase (B, D and F). The pH of the growth medium was also monitored throughout biofilm development in the control (uninfected) samples (G). Data correspond to the average and standard deviation of three independent repeats.

from the experimental data obtained at 25 °C and entered the resulting values (1 or 0.99, depending on the growth state of the population) in the model and optimized the infection rates (Supplementary methods). When running the model with these parameters, the predicted change in pH throughout growth was quite similar to the experimental values, with a minimum pH of 5.78 reached 20 h after inoculation (Fig. 3A). At 37 °C, the pH decreased by a factor of 0.99 or 0.96 in a 30-min interval depending on the growth stage [17]. This suggests that, in addition to a slower growth, the metabolic pathways active at room temperature might also result in a lesser acidification of the medium compared to those that predominate at 37 °C. The model was then run using these higher rates, but maintaining all the other parameters (bacterial growth rate, phage propagation, etc.) as determined for 25 °C. In this case, the pH did reach 4.75 after approximately 14 h of growth, taking much longer than it does at 37 °C (less than 10 h). Despite the difference in the final pH obtained for these two versions of the model, it must be noted that the predictions regarding the impact of the phage on the bacterial population were exactly the same (Fig. S2). Indeed, even when a low starting MOI of 10 was used, the population was controlled by the phage before the pH went below 6.14, so no phage inactivation was observed (Fig. 3B). Therefore, it appears that the fact that pH was not acidic enough to affect the outcome of phage-bacteria competition at 25 °C under the conditions of the experiment.

With this in mind, we examined whether pH would be decisive for biofilm development under phage predation at room temperature when the starting pH was lower. To do that, we ran the model setting an initial pH of 6 (Fig. 3C–F). Here, we did observe differences in bacterial population outcome depending on the speed of pH decrease. Indeed, when the low pH decrease rate obtained at 25 °C was used in the model, the phage was just as effective as previously seen for a starting pH of 7 (Fig. 3F). In contrast, phage inactivation by pH when using the higher pH change rate obtained at 37 °C prevented elimination of the bacterial population at starting phage concentrations below 100 PFU/well (Fig. 3F).

3.3. Infection of preformed biofilms by phage phiIPLA-RODI is also more effective at 25 °C than at 37 °C

Biofilm cells often exhibit greater resistance against antimicrobial agents than planktonic cells thanks to their complex structure, diversity and specific adaptations. For this reason, the impact of temperature on phage infection might differ between sessile and free-living cells. With this in mind, we assessed the influence of temperature during phage treatment of preformed biofilms. Again, phiIPLA-RODI displayed improved antibacterial activity at 25 °C. After 6 h of treatment we observed an average decrease of 0.85 and 0.51 log units for strains
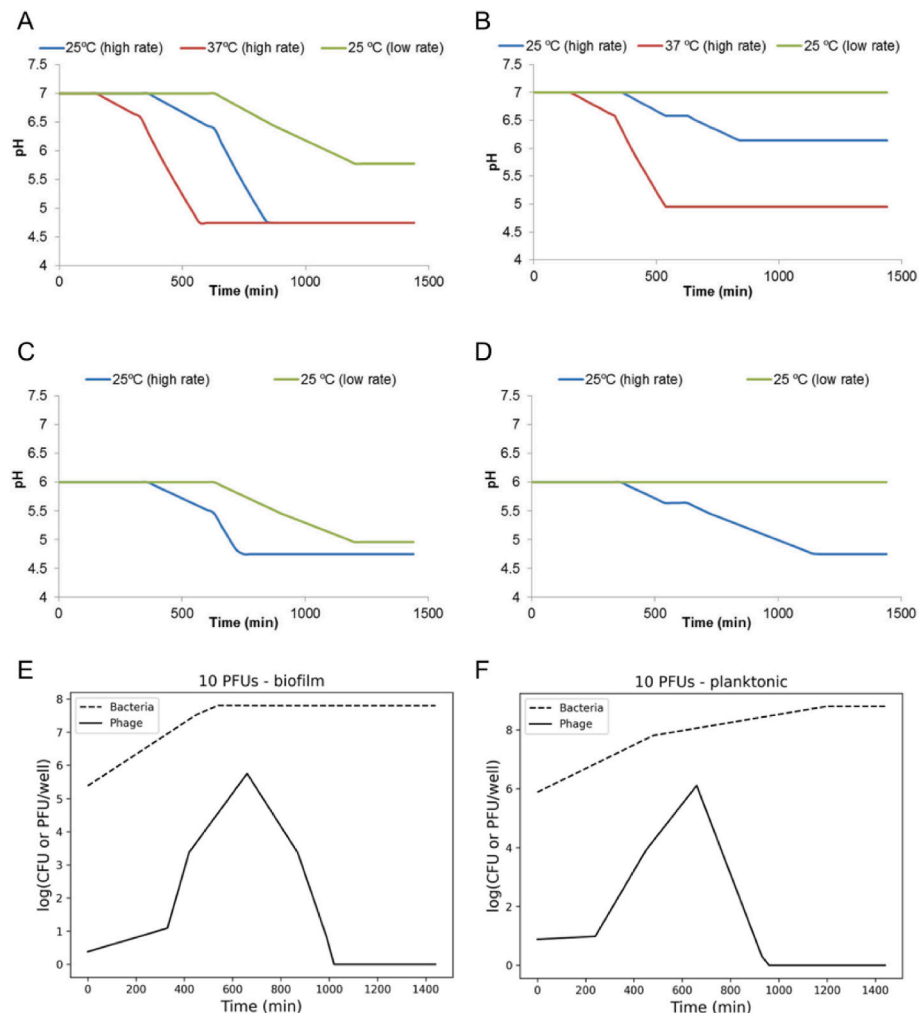

Fig. 3. Impact of pH on temperature-dependent phage infection. Panels A to D represent the changes in the pH of the growth medium during biofilm development of *S. aureus* IPLA16 estimated with a phage-infection model. The starting pH was set at 7 (A and B) or 6 (C and D) and the starting phage titer were 0 PFU/well (A and C) or 10 PFU/well (B and D). The pH changing rate was set at the values obtained at 37 °C (high rate) or at 25 °C (low rate). E and F show the output of the phiIPLA-RODI infection model for biofilms developed at 25 °C for a starting phage concentration of 10 PFU/well in the biofilm (E) and the planktonic phase (F). The lowest possible pH value was set at 4.75, the initial pH was set at 6 and the starting number of cells was always set at 10^6 CFU/well.

IPLA15 and IPLA16, respectively (Fig. 4A). When the treatment duration was 24 h, there was an average 1-log reduction in attached viable cells compared to the untreated control for both strains (Fig. 4B). At 37 °C, however, phage treatment did not result in any difference in cell counts for either strain regardless of treatment duration (Fig. 4A and B).

Observation of untreated biofilms grown at the two temperatures by confocal microscopy showed that the PIA/PNAG content of the matrix

was higher at 37 °C than at 25 °C for both IPLA15 and IPLA16 (Fig. S3), potentially contributing to lesser access of the phage to the cell surface at body temperature.

3.4. Biofilm cells of S. aureus IPLA16 show increased expression of wall teichoic acids (WTA) biosynthesis genes at 25 °C

Next, we examined the transcriptome of *S. aureus* IPLA16 biofilms grown at 25 °C and 37 °C to explore the possible explanation for the difference in phage susceptibility observed between these two temperatures. In total, there were 1381 genes that displayed differential expression between the two assayed conditions (Table S2).

Out of these genes, 40 were involved in processes related to cell wall
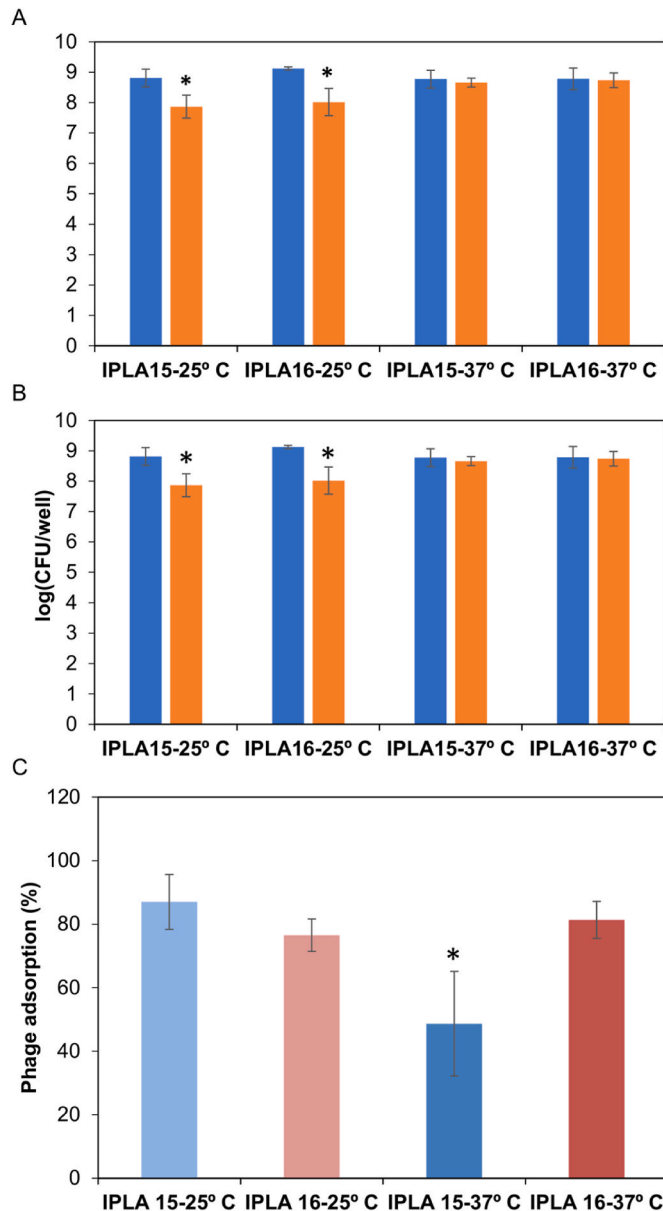

Fig. 4. Biofilm treatment with phage phiIPLA-RODI and phage adsorption to biofilm cells at 25 °C and 37 °C. A and B) 24-h old biofilms of strains IPLA15 and IPLA16 grown at 25 °C or 37 °C and then treated with 10^9 PFU/ml of phage phiIPLA-RODI suspended in TSB (orange bars) or with medium alone (blue bars) and treated for 6 h (A) or 24 h (B) at the same temperature.The number of viable cells in the biofilms was then determined by serial dilutions and plating on TSA plates. Values correspond to the mean and standard deviation of three independent repeats. Statistical analysis was performed by comparing cell counts corresponding to each treatment to its control. C) Sessile cells were harvested from biofilms grown at 25 °C or 37 °C and then incubated with phage phiIPLA-RODI at an MOI of 0.1 for 10 min at room temperature. The number of free, non-adsorbed phages was then determined. Values represent the mean and standard deviation of three independent repeats. Statistical analysis was performed by comparing adsorption rates for each strain at 37 °C to the values estimated at 25 °C. *, p-value <0.05.

Table 2
List of genes related to the cell wall that are dysregulated in biofilms of strain IPLA16 grown at 37 °C compared to those developed at 25 °C according to RNA-seq.

Gene ID	Gene name	Gene product	FC
RL451_08590	tarI'	Ribitol-5-phosphate cytidylyltransferase 2	−6.31
RL451_11505	femA_3	Aminoacyltransferase FemA	−5.94
RL451_06310	mgrA	HTH-type transcriptional regulator MgrA	−5.35
RL451_05990	tagO	putative undecaprenyl-phosphate N-acetylglucosaminyl 1-phosphate transferase	−4.89
RL451_08585	tarJ'	Ribulose-5-phosphate reductase 2	−4.83
RL451_06555	tagG	Teichoic acid translocation permease protein TagG	−4.54
RL451_02975	murG	UDP-N-acetylglucosamine–N-acetylmuramyl- (pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	−4.27
RL451_05525	dltA_1	D-alanine–D-alanyl carrier protein ligase	−4.13
RL451_05515	dltC	D-alanyl carrier protein	−4.12
RL451_08580	tarK	Teichoic acid ribitol-phosphate polymerase TarK	−4.12
RL451_06565	tarA	N-acetylglucosaminyldiphosphoundecaprenol N-acetyl-beta-D-mannosaminyltransferase	−3.77
RL451_06550	tarB	Teichoic acid glycerol-phosphate primase	−3.74
RL451_07585	sle1_2	N-acetylmuramoyl-L-alanine amidase sle1	−3.50
RL451_01200	atl_1	Bifunctional autolysin	−3.46
RL451_08570	tarI	Ribitol-5-phosphate cytidylyltransferase 1	−3.21
RL451_13175	ddl	D-alanine–D-alanine ligase	−3.15
RL451_09095	cap8A_1	Capsular polysaccharide type 8 biosynthesis protein cap8A	−2.97
RL451_02300	pbpH	Penicillin-binding protein H	−2.94
RL451_04600	ftsW	putative peptidoglycan glycosyltransferase FtsW	−2.79
RL451_03200	femA_1	Aminoacyltransferase FemA	−2.78
RL451_06420	sle1_1	N-acetylmuramoyl-L-alanine amidase sle1	−2.72
RL451_05510	dltD	Protein DltD	−2.67
RL451_00535	tagH_1	Teichoic acids export ATP-binding protein TagH	−2.63
RL451_08565	tarJ	Ribulose-5-phosphate reductase 1	−2.53
RL451_01300	murJ	Lipid II flippase MurJ	−2.33
RL451_08575	tarF	Teichoic acid glycerol-phosphate transferase	−2.31
RL451_06540	tarD	Glycerol-3-phosphate cytidylyltransferase	−2.19
RL451_06545	tagX	Putative glycosyltransferase TagX	−2.07
RL451_03910	femA_2	Aminoacyltransferase FemA	2.10
RL451_08885	murQ	N-acetylmuramic acid 6-phosphate etherase	2.22
RL451_06445	graS	Sensor histidine kinase GraS	2.47
RL451_12970	murA2	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2	2.49
RL451_13445	agrA	Accessory gene regulator A	2.67
RL451_03915	lytN	putative cell wall hydrolase LytN	3.20
RL451_09195	wbnH	O-antigen biosynthesis glycosyltransferase WbnH	3.28
RL451_09020	mnaA_1	UDP-N-acetylglucosamine 2-epimerase	3.33
RL451_08950	dltA_2	D-alanine–poly(phosphoribitol) ligase subunit 1	3.34
RL451_12035	atl_3	Bifunctional autolysin	3.36
RL451_02815	ponA	Penicillin-binding protein 1A/1B	3.71
RL451_08455	lytM	Glycyl-glycine endopeptidase LytM	5.48

biosynthesis and turnover and, consequently, their level of expression may have an impact on phage adsorption and infection (Table 2). For instance, multiple genes involved in wall teichoic acids synthesis (*tarI'*, *tagO*, *tarJ'*, *tagG*, *tarK*, *tarA*, *tarB*, *tarI*, *tagH_1*, *tarJ*, *tarF*, *tarD*, and *tagX*) displayed lower expression at 37 °C. Given that WTAs are the receptor of *S. aureus* phages, it is possible that this lesser transcription results in a lower level of adsorption at this temperature compared to 25 °C. We did not observe significant differences in the expression of the gene involved in β-glycosylation of the WTAs (*tarS*). It must be noted that strain IPLA16 does not possess an intact *tarM* gene and, as a result, does not possess a functional α-glycosylase [30]. Genes involved in the D-alanylation of WTAs (*ddl* and *dltD*) also exhibited higher expression at 25 °C although their potential role in affecting phage infection is not clear.

We also found differential expression in four genes involved in phage defense mechanisms, namely RL451_07865, RL451_09500, RL451_09505 and RL451_09525, which were transcribed more at 25 °C (Table S2). However, this does not explain the increased susceptibility at this temperature. If anything, it would suggest the opposite.

Although not directly related to phage infection, we also observed dysregulation of genes involved in biofilm formation. For example, *icaA* and *icaD* (involved in exopolysaccharide biosynthesis), *spa* (coding for protein A), adhesin-encoding gene *fnbA* and serine protease *splB* were all overexpressed at 37 °C (Table S2). In contrast, proteases *aur*, *sspA*, *sspB* and *sspC*, autolysins *atl* and *sle1*, the nuclease *nuc*, as well as the negative regulator of the *ica* operon *icaR* were all expressed more at 25 °C (Table S2). As can be expected, genes involved in the heat shock response, such as intracellular proteases and chaperonins (*clpX*, *clpL*, *clpP_1*, *groS*, *clpC*, *dnaJ*, *groL*, *dnaK* and *clpB*), were overexpressed at 37 °C (Table S2). Genes involved in metabolism were also differentially expressed between the two conditions. For instance, genes involved in acetate and ethanol fermentation (*pflA* and *pflB*), the pyruvate dehydrogenase complex (*pdhA*, *pdhB*, *pdhC* and *pdhD*) and the TCA cycle (*gltA*, *acnA*, *sucD*, *sucC*, *sdhA*, *sdhC* and *mqo1*) displayed higher levels of transcription at 37 °C (Table S2). By contrast, other genes were expressed at a higher rate at 25 °C, such as L-lactate dehydrogenase (*ldh*) and genes involved in nitrate respiration (*narH*, *narG*, *narT* and *narX*) (Table S2).

3.5. Phage phiIPLA-RODI displays increased adsorption to biofilm cells of strain IPLA15 at 25 °C compared to 37 °C

In view of the results obtained with RNA-seq analysis, we tested the adsorption of phage phiIPLA-RODI to biofilm cells of *S. aureus* IPLA16 grown at the two temperatures. However, no significant differences were found between the two conditions (Fig. 4C). In contrast, the phage displayed decreased adsorption to biofilm cells of strain IPLA15 grown at 37 °C compared to the values obtained at 25 °C, with average adsorption rates of 49 % and 87 %, respectively (Fig. 4C).

Based on this result, we examined the expression of genes involved in cell wall biosynthesis in strain IPLA15 at the two temperatures by RT-qPCR (Table S3). Unlike IPLA16, strain IPLA15 did not exhibit differential expression of genes involved in the synthesis of the WTA backbone (*tagO*) or the capsule (*capA*), which were upregulated at 25 °C in IPLA16 (Table 2). However, we did observe a 3-fold higher transcription of *tarM* at 37 °C, a gene involved in α-glycosylation of WTA. This modification might hinder access of the phage to the WTA backbone and, therefore, explain the lower adsorption rate of the phage to IPLA15 at 37 °C. Some genes, *oatA* and *tarS*, did not exhibit any changes in either strain.

3.6. One-step growth curve at 25 °C vs 37 °C

Temperature-dependent differences in infection parameters might also be contributing to the greater susceptibility observed at room temperature in both planktonic cultures and biofilms. This possibility was explored by performing one-step growth curve experiments (Fig. 5). Burst size was significantly lower in strain IPLA15 at 37 °C (9.19 ± 1.67) compared to the same strain at 25 °C (62.64 ± 9.09) (p-value = 0.008) and to strain IPLA16 at 37 °C (57.52 ± 12.97) and 25 °C (64.56 ± 18.36) (p-values = 0.02 and 0.03, respectively). By contrast, there was no significant difference between the burst size values obtained in strain IPLA16 at the two temperatures (p-value = 0.62). The duration of the burst time (latent plus rise period) was longer at 25 °C (75 min) compared to 37 °C (45 min) for both strains.

3.7. Increased selection of phage resistant mutants at 37 °C

In strain IPLA15, growth curve experiments indicated that its greater ability to withstand phage predation at 37 °C compared to 25 °C might be related to the development of a resistant population (Fig. 1A). For this reason, we explored the frequency of phage resistant colony selection at both temperatures and found that, for strains IPLA16 and IPLA15 at 37 °C the values were $2.70 \times 10^{-7} \pm 2.35 \times 10^{-7}$ and $2.93 \times 10^{-6} \pm 6.42 \times 10^{-7}$, respectively, indicating a 1-log increase in the number of bacterial colonies grown in the presence of the phage for the latter
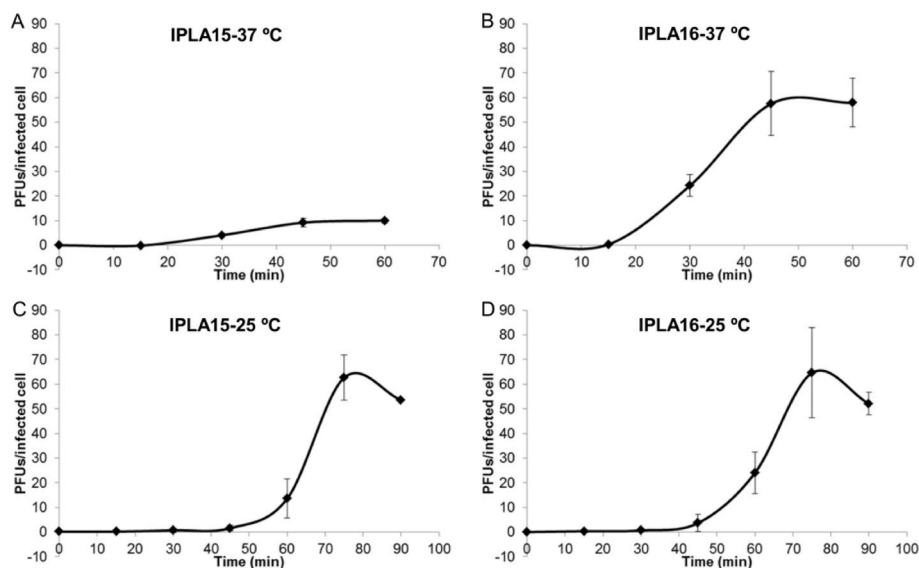

Fig. 5. One-step growth curves of phage phiIPLA-RODI on strains IPLA15 (A and C) and IPLA16 (B and D) at 37 °C (A and B) and 25 °C (C and D). Each data point represents the numbers of PFU per infected cell, and corresponds to the mean and the standard deviation of three independent experiments.

isolate (Fig. 6A). By contrast, the frequency of phage resistance selection at 25 °C was $<10^{-8}$ for both strains.

Regarding the resistant colonies grown at 37 °C, we sought to confirm whether they were bacteriophage insensitive mutants (BIMs). This was the case for all colonies analyzed for strain IPLA16, but IPLA15 resistant colonies were not all BIMs and displayed at least some degree of phage susceptibility in the spot assay (approximately only 20 % of the colonies were BIMs). This prompted us to monitor the growth of three selected mutants in a liquid culture in the presence of a high starting titer of phage phiIPLA-RODI (10^8 PFU/ml) compared to the parental strain. The results of this experiment showed that the mutant strains exhibited an advantage compared to wild type IPLA15 in the presence of the phage, even though they displayed susceptibility in the spot assay (Fig. 6B). The genomes of these three mutants were then sequenced to identify potential mutations that might explain their heightened

resistance to phiIPLA-RODI infection. Interestingly, we identified mutations affecting the Agr quorum-sensing system in all three strains, which has been demonstrated to regulate the expression of *tarM* in stationary phase [31]. Two of them, IPLA15-R1and IPLA15-R2 have frameshift mutations due to the following insertions: 178_179insTTCAACT in *agrA* and 397_398insG in *agrC*, respectively. The third mutant strain, IPLA15-R3, has a non-synonymous mutation in nucleotide 542 (A > G) of *agrA*, leading to the amino acid substitution E181G.

4. Discussion

Understanding the interplay between bacteria and their viruses will provide us with some answers regarding the differences in composition of microbial communities. Adaptation to an environmental niche will not only have an impact on the ability of a microbe to survive, even thrive, in a given set of conditions, but might also affect their susceptibility to some bacteriophages. This would have dramatic consequences regarding bacterial population structure. Moreover, phages are increasingly considered as promising therapeutics against multidrug resistant bacteria and the notoriously persistent biofilms. In this context, it is crucial to know the potential effects that parameters like temperature, pH or nutrient availability may have on the outcome of phage application. This work examined in depth how the dynamics between a virulent phage and its host change depending on temperature.

The staphylococcal phage phiIPLA-RODI is overall a very effective predator, with a broad host range within the *Staphylococcus* genus, being particularly successful against *S. aureus* strains, including clinical MRSA isolates [16,32]. This phage is also a promising biofilm removal agent, although its capabilities are somewhat curtailed in acidic environments [17]. Besides pH, ambient temperature is another key parameter concerning the use of phiIPLA-RODI as an antibiofilm agent. Specifically, we aimed to compare phage infection at body temperature, which would be representative of infection treatment, to predation at room temperature, which would be relevant for surface disinfection in hospitals or the food industry.

Our results show that this phage has better chances of eliminating *S. aureus* contamination at 25 °C than at 37 °C. This same trend was observed for several strains from different origins (clinical, veterinary or food industry) and varying degrees of phage susceptibility. The impact of temperature was particularly noticeable in strains displaying low susceptibility at 37 °C, which turned out to be highly sensitive to the phage at room temperature. Previous studies have described temperature-dependent infection for other bacteriophages infecting *Yersinia enterocolitica*, *Listeria monocytogenes*, *Pseudomonas fluorescens* or lactic acid bacteria [10–12,33,34]. In *S. aureus*, the plaques formed by phage K at 37 °C are smaller than those observed at 30 °C, but only in USA300 strains [35]. Contrary to phiIPLA-RODI, some of these phages thrive under conditions that favor growth of the host, such as the *P. fluorescens* phage ϕS1, which was most effective within a temperature range between 22 °C and 30 °C [36]. Infection at lower temperatures was not as successful and there was no impact on bacterial growth at 37 °C. Similarly, a phage infecting *Y. enterocolitica* was more effective at 25 °C, which is closer to the optimum growth temperature of this bacterium (28 °C), than at 37 °C [10].

A closer look at bacterial growth dynamics in the presence of a high starting MOI revealed that, at least for some strains, development of a phage resistant population was more likely at 37 °C than at 25 °C. This phenomenon would have an effect on bacterial survival under phage predation and contribute to the ability of staphylococcal strains to withstand viral infection at body temperature. This might be due to some extent to the influence of temperature on the mutation rate, established a century ago by Muller [36], and more recently linked to differences in the metabolic rate [37]. Nonetheless, further experiments would be necessary to confirm if other factors are also participating, such as reduced viability at 25 °C. These assays also revealed the
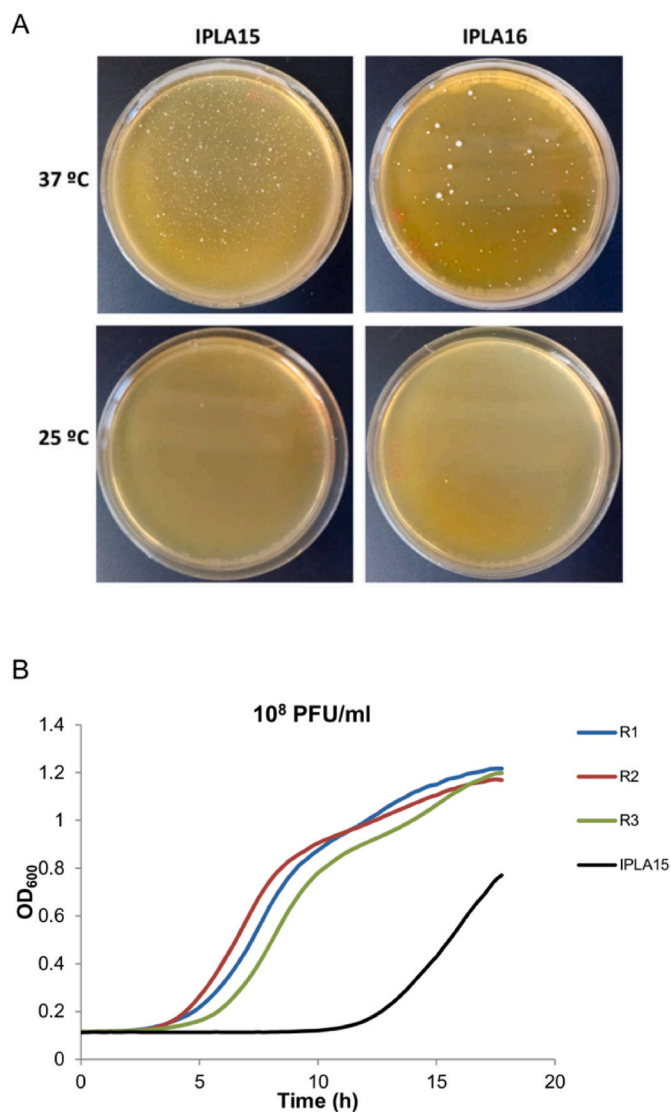
Fig. 6. Phage resistance development at 25 °C and 37 °C in strains IPLA15 and IPLA16. A) Selection of phage resistant mutants of strains IPLA15 and IPLA16 at 25 °C or 37 °C. Each plate was inoculated with 100 μl from an overnight bacterial culture (approximately 10^8 CFUs) and 100 μl from a phage suspension containing 10^8 PFU/ml (10^7 PFUs) mixed with soft agar. These plates were then grown for 24 h at 25 °C or 37 °C. B) Growth curves of parental strain *S. aureus* IPLA15 and 3 phage resistant mutants at 37 °C in the presence of 10^8 PFU/ml of phage phiIPLA-RODI. OD_{600} was monitored for 20 h. Data represent one representative experiment out of three independent repeats showing the same trend.

selection of a high proportion of mutants exhibiting lesser susceptibility but not full resistance to the phage for strain IPLA15 at 37 °C. These cells appear to be partly responsible for the greater ability of this strain to survive when phiIPLA-RODI was added at medium to high MOIs. In turn, given their partial susceptibility to the phage, it is possible that they do support phage propagation, thus favoring phage-host coexistence. Genome analysis of three of these mutants revealed that they harbor mutations affecting the two-component system AgrAC, which is involved in sensing the Agr signal and activating the expression of the quorum-sensing regulon. Yang et al. [31] recently demonstrated that this system controls phage susceptibility in *S. aureus* by decreasing the expression of gene *tarM*, facilitating the infection of some myophages. Lack of a functional AgrAC would, therefore, render the bacterial cells less susceptible to phage attack. In strain IPLA16, such mutants would not have a competitive advantage under phage predation, since this strain does not have a functional TarM protein. Within this context, it makes sense that we did not observe selection of these intermediate resistance mutants in IPLA16.

Phage infection parameters also varied depending on temperature. For instance, the lytic cycle was longer for the two strains tested at 25 °C compared to 37 °C. This is not surprising as phage multiplication requires the bacterial machinery and is, therefore, dependent on the bacterial physiological state [38]. Conversely, burst size only decreased at the 37 °C for strain IPLA15, but did not change for *S. aureus* IPLA16. A study on dairy phages by Zaburlin et al. [12] had already reported longer burst times and a varying impact on burst size at suboptimal growth temperatures. Middelboe [39] also reported an inverse correlation between growth rate and the latent period, but observed a positive correlation with burst size for a marine virus-host system.

Some authors found that temperature sometimes affects phage infection by altering the expression of genes involved in adsorption or phage defense mechanisms. For example, in *Y. enterocolitica*, the *ompF* gene encoding the receptor of phage ϕR1-RT and displays enhanced transcription at 25 °C compared to 37 °C [11]. In turn, *Listeria* exhibited resistance at 25 °C due to higher expression of a restriction-modification system, while being susceptible at 37 °C [10]. In *S. aureus* IPLA16, we observed increased transcription of genes related to WTA biosynthesis and phage defense mechanisms at 25 °C. Evidently, the latter would not explain the better phage efficacy at room temperature. On the other hand, greater production of WTA, the main receptor of bacteriophages in staphylococci, might have explained this phenomenon. However, adsorption of phiIPLA-RODI to strain IPLA16 was similar at the two temperatures, suggesting that the amount of WTA available at 37 °C suffices to obtain maximum adsorption. Unsurprisingly, this strain is amongst the most susceptible at both 37 °C and 25 °C. By contrast, phage adsorption was significantly curtailed at 37 °C in *S. aureus* IPLA15. Transcriptional analysis revealed that this strain did not exhibit differential expression of genes involved in the synthesis of the WTA backbone, but gene *tarM*, involved in WTA glycosylation was expressed more at 37 °C. It is possible that this modification limits access of the phage receptor binding protein (RBP) to the WTA backbone, thereby hindering adsorption to the cell surface. This role of TarM has already been demonstrated for some *S. aureus* phages [31].

Temperature also had an impact on biofilm removal and inhibition by phage phiIPLA-RODI. The better efficacy observed at room temperature is a good sign towards the prospective use of this phage in the development of a surface decontamination product, but would require complementation with other antibacterial or antibiofilm agents for the treatment of biofilm-related infections. The decreased susceptibility of biofilms at 37 °C might also be enhanced by the higher content of PIA/PNAG in the extracellular matrix, as indicated by RNA-seq analysis (*ica* genes were overexpressed at body temperature) and confocal microscopy. We found that the phage was especially effective at preventing biofilm formation even with a low starting phage titer. As would be expected, bacterial growth rates were lower at 25 °C than those estimated by Fernández et al. [17] at 37 °C. Accordingly, phage propagation

rates were also lower in general, with the exception of the biofilm during mid exponential phase. This results in a slower elimination of the bacterial population. However, the phage can actively propagate and kill cells for a longer period at 25 °C due to the delay in the onset of stationary phase at this temperature. This might be perhaps the determining factor behind the slight increase in susceptibility of IPLA16 under these conditions compared to a higher temperature.

Given that pH is a very important parameter in the interplay between phage phiIPLA-RODI and *S. aureus* biofilms, we also studied its potential role at different temperatures. We confirmed that the minimum pH reached at 25 °C during biofilm formation was above that required for phage inactivation. However, further analysis demonstrated that this would not result in improved phage efficacy in our experiments because the phage could eliminate the bacterial population before the time expected for the pH to reach a value of 5.5. However, according to the predictions of a phage infection model, the slower acidification of the growth medium would have an impact at a lower starting pH of 6. It must be noted that our exploration of pH change at the two temperatures suggests that it cannot be explained solely by the slower growth rate, but rather to differences in the prevalent metabolic pathways. Transcriptomic analysis did show differences in the expression of multiple metabolism-related genes, although their overall contribution to medium acidification would require a more in depth study.

In conclusion, infection by phage phiIPLA-RODI of *S. aureus* biofilms and planktonic populations is greatly affected by ambient temperature, with improved efficacy at room compared to body temperature. The mechanisms behind this phenomenon vary in a strain-dependent manner and may include differences in phage resistance development, phage adsorption, infection parameters, prolonged phage action due to delayed onset of the stationary phase, biofilm matrix composition, and metabolic changes leading to a lesser acidification of the surrounding environment. This study reveals that the impact of temperature on phage-host interactions is far more complex than previously thought, with multiple mechanisms playing a role. Moreover, this information has vital repercussions concerning the development of phage-based antibiofilm strategies.

CRediT authorship contribution statement

Lucía Fernández: Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Ana Catarina Duarte:** Writing – review & editing, Visualization, Investigation. **Andrea Jurado:** Investigation, Visualization, Writing – review & editing. **Laura Bueres:** Writing – review & editing, Visualization, Investigation. **Ana Rodríguez:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Pilar García:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Funding

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank R. Muñoz for her help with performing some preliminary experiments, as well as R. Calvo and A. B. Campelo for technical assistance. Strains ISP479r, 132, 15981 and V329 were kindly provided by Dr. A. Toledo-Arana (Instituto de Agrobiotecnología, CSIC-Universidad Pública de Navarra, Spain). We are also grateful to Dr. C. Wolz (University of Tübingen, Germany) and Dr. F. Götz (University of Tübingen, Germany) for providing us with strains Newman and SA113, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2024.100248.

Data availability

RNA-Seq data have been deposited in NCBI's Gene Expression Omnibus (GEO) under the GEO series accession number GSE255751.The whole genome shotgun projects corresponding to the phage-resistant mutant strains IPLA15-R1, IPLA15-R2 and IPLA15-R3 have been deposited at DDBJ/ENA/GenBank under the accession numbers JBJWEZ000000000, JBJWEY000000000 and JBJWEX000000000, respectively.

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