Bacteriophages Combined With Subtherapeutic Doses of Flucloxacillin Act Synergistically Against *Staphylococcus aureus* Experimental Infective Endocarditis

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**BACKGROUND:** The potential of phage therapy for the treatment of endovascular *Staphylococcus aureus* infections remains to be evaluated.

**METHODS AND RESULTS:** The efficacy of a phage cocktail combining *Herelleviridae* phage vB_SauH_2002 and *Podoviriae* phage 66 was evaluated against a methicillin-sensitive *S. aureus* strain in vitro and in vivo in a rodent model of experimental endocarditis. Six hours after bacterial challenge, animals were treated with (1) the phage cocktail, (2) subtherapeutic flucloxacillin dosage, (3) combination of the phage cocktail and flucloxacillin, or (4) saline. Bacterial loads in cardiac vegetations at 30 hours were the primary outcome. Secondary outcomes were phage loads at 30 hours in cardiac vegetations, blood, spleen, liver, and kidneys. We evaluated phage resistance 30 hours post infection in vegetations of rats under combination treatment. In vitro, phages synergized against *S. aureus* planktonic cells and the cocktail synergized with flucloxacillin to eradicated biofilms. In infected animals, the phage cocktail achieved bacteriostatic effect. The addition of low-dose flucloxacillin elevated bacterial suppression (∆ of −5.25 log₁₀ colony forming unit/g [CFU/g] versus treatment onset, *P*<0.0001) and synergism was confirmed (∆ of −2.15 log₁₀ CFU/g versus low-dose flucloxacillin alone, *P*<0.01). Importantly, 9/12 rats given the combination treatment had sterile vegetations at 30 hours. In vivo phage replication was partially suppressed by the antibiotic and selection of resistance to the *Podoviridae* component of the phage cocktail occurred. Plasma-mediated inhibition of phage killing activity was observed in vitro.

**CONCLUSIONS:** Combining phages with a low-dose standard of care antibiotic represents a promising strategy for the treatment of *S. aureus* infective endocarditis.

**Key Words:** endocarditis ■ phage antibiotic synergism ■ phage therapy ■ *Staphylococcus aureus*

*Staphylococcus aureus* is one of the most common pathogens responsible for acute infective endocarditis (IE) on both native and prosthetic valves.² Currently, *S. aureus* IE is managed primarily with a 4- to 6-week course of intravenous antibiotic medication, and heart valve surgery may also be performed if indicated.³ Even the most aggressive therapeutic plans are associated with substantial morbidity and mortality, with mortality rates reaching 50% in patients with prosthetic valve infection.⁴ Thus, there remains a need for novel strategies that may improve outcomes in patients with IE.

Phage therapy, wherein bacterial viruses are used to treat bacterial infections, has been proposed as a salvage therapy, especially in the context of foreign body infections or multidrug-resistant pathogens.⁵
However, there is not yet sufficient evidence from randomized controlled trials to support widespread adoption of phage therapy. The available evidence suggests that phage therapy can be an effective alternative or complementary strategy to antibiotics for the treatment of *S. aureus* infections, including burn and chronic wound infections, keratitis, severe infections after cardiothoracic surgery, prosthetic joint infections, and ventricular-assist device infections. Recently, 2 Australian case series evaluated the safety and efficacy of a 3-phage cocktail for the treatment of *S. aureus* IE or *S. aureus* aortic graft infections. Encouragingly, improved infection control and healing progress were documented with the addition of phages to antibiotic treatment. However, there were cases in which treatment failure and/or recurrence occurred, including some that were ultimately fatal. A reliable curative protocol for *S. aureus* endovascular infection treatment with phages has yet to be established.

Thus far, all patients who have received phage therapy for deep-seated *S. aureus* infections have received the therapy in combination with antibiotic pharmacotherapy. Thus, it is still unknown whether phage therapy alone could clear such infections. Recently, using a model of experimental infective endocarditis (EE), we observed that phage therapy alone was as effective as ciprofloxacin alone for the treatment *Pseudomonas aeruginosa* EE and that combining phages and ciprofloxacin was highly synergistic and could even result in culture-negative vegetations. Thus, the aim of the current study was to evaluate the efficacy of phage therapy alone or in combination with the IE standard of care antibiotic flucloxacin for the treatment of methicillin-susceptible *S. aureus* (MSSA) EE in rats.

**CLINICAL PERSPECTIVE**

**What Is New?**

- Therapy with *Staphylococcus aureus* bacteriophages synergizes with standard of care antibiotics for the treatment of experimental infective endocarditis.

**What Are the Clinical Implications?**

- The addition of bacteriophages to standard-of-care antibiotic treatments at the beginning of the therapy increases bacterial load reduction within cardiac vegetations.
- These findings suggest a reduction of the risk for typical *S. aureus* infective endocarditis-related complications, such as septic embolism or acute valve damage and ultimately pave the way to shorter antibacterial treatment courses.

**METHODS**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Bacterial Strains, Bacteriophages, Growth Conditions, and Evaluation of Phage Activity**

A panel of 63 *S. aureus* strains isolated from humans and animals and representing a variety of sequence types was used (Table S1). Notably, among these was the MSSA strain Laus102, isolated from a healthy carrier. Additionally, the *P. aeruginosa* strain ATCC 15442™ (LGC Standards, Molsheim, France) was used. The *Podoviriae* phage 66 was purchased from the National Collection of Type Cultures of Public Health England (#8289) and the *Herelleviridae* phage vB_SauH_2002 was isolated from sewage water previously. Both phages were propagated in Laus102 cultures. The phage solutions were normalized to 10^10 plaque-forming units (PFU)/mL. Phage host range was determined using efficiency of plating assays on the aforementioned 63 *S. aureus* strains. The *Myoviridae* family phage vB_PaeM_4002, which infects *P. aeruginosa*, was isolated from a sewage water sample collected at the Vidy wastewater treatment plant, Lausanne, Switzerland (unpublished). Details on growth conditions and reagents are given in the Supplemental Material.

**In Vitro Activity of Phages or Flucloxacillin Against Planktonic Cultures and Biofilms**

Monophage and phage cocktail effects on 63 *S. aureus* strains were tested as described in the Supplemental Material and Table S1. Phage (only) or phage in combination with flucloxacillin was also tested against the MSSA isolate Laus102, using in vitro turbidity and time-kill assays (described in the Supplemental Material).

**Nonstandard Abbreviations and Acronyms**

| Abbreviation | Definition                  |
|--------------|-----------------------------|
| EE           | experimental infective endocarditis |
| IE           | infective endocarditis       |
| MOI          | multiplicity of infection    |
| PFU          | plaque forming unit          |

*S. aureus* biofilms were produced, rinsed, and managed in 96-well plates as previously described (Ref. [18] and Supplemental Material). Mature biofilms were treated for 24 hours at 37 °C with vB_SauH_2002,
phage 66, or the phage cocktail at final multiplicity of infection (MOI) of 1, 10, and 100, in combination or not with flucloxacillin, at 1× and 10× minimum inhibitory concentration, in tryptic soy broth. Synergy was defined as a >2 log_{10} colony forming unit (CFU)/mL decrease in bacterial load compared with the decrease observed for the reference treatment alone.19

**Murine Infection Model**

Female Wistar rats (Crl:WI(Han); Charles River, L’Abresle, France), weighing 180 to 200 g, were housed in specific pathogen-free rooms (12-hour light/dark conditions, 23±1 °C, water and food ad libitum). All animal experiments were carried out in accordance with Swiss Animal Protection Law guidelines and were approved by the Cantonal Committee on Animal Experiments of the State of Vaud (approval VD 879.10). For all manipulations, animals were anesthetized with a mixture of ketamine (Ketalar, 75 mg/kg) and xylazine (Xylasol, 0.5 mg/kg) given intraperitoneally. Buprenorphin (Temgesic, 0.15 mg/kg) was given intraperitoneally at the onset of surgery as an analgesic.

_Catheter-induced sterile aortic vegetations were produced in rats as previously described.20 In parallel, an intravenous line was inserted via the jugular vein into the superior vena cava and connected to a programmable infusion pump (Pump 44; Harvard Apparatus, Inc., South Natick, MA) for delivery of antibacterial drugs according to a dosage regimen that mimics the kinetics of human intravenous antibiotic therapy.21 Bacterial inocula were prepared from dilutions of fresh midexponential phase cultures (600 nm optical density (OD_{600nm})=0.6, ~10^8 CFU/mL). With the assistance of a programmable infusion pump, 1.30±0.35×10^5 CFU of bacteria in 500 µL (corresponding to 10 times the 90% infective dose) were inoculated to each animal 24 hours after catheterization.22 The inoculum size was confirmed by colony counts on plates coated with tryptic soy agar (BD Difco, Becton Dickinson, Sparks, MD). Three uninfected animals were used for pharmacokinetic studies.|

**Induction of Infection**

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**Treatment Protocol**

We performed 4 sets of experiments with n=3 in each of the 5 groups. Six hours after the initiation of a bacterial challenge, animals were treated with either (1) a phage cocktail (vB_SauH_2002 and 66, 10^{10} PFU/mL each) injected as a 1-mL bolus followed by continuous infusion at 0.3 mL/h for 24 hours (each rat received 8.2×10^{10} PFU over 24 hours, n=8); (2) a suboptimal IV dose of flucloxacillin mimicking human kinetic treatment (2 g every 12 hour for 24 hour instead of 2 g every 6 hours for 24 hours for an optimal treatment, n=11); (3) the phage cocktail plus flucloxacillin (dosages as previously) (n=12); or (4) mock therapy (saline, n=7). Ten animals were killed at the start of therapy (6 hours post infection) to assess infection severity at the onset of treatment. The remaining rats were killed 24 hours later (30 hours post infection).

**Outcomes**

The primary outcome was bacterial load in cardiac vegetations 30 hours after infection. Secondary outcomes were phage loads 30 hours post infection in cardiac vegetations, blood, spleen, liver, and kidneys. An additional outcome was the presence of phage-resistant clones in the cardiac vegetations of rats given the phage cocktail/flucloxacillin combination treatment. Outcome assessment methods are described in the Supplemental Material.

**Statistical Analysis**

Differences between the groups were generally detected with 1-way ANOVAs with Tukey correction for multiple comparisons. Phage loads in blood and organs were compared with unpaired t tests with Welch’s correction. All analyses were performed in Prism software (version 9, GraphPad, La Jolla, CA). Statistical test results were considered significant when P values <0.05 were obtained. Mean values are reported with SDs.

**RESULTS**

**Phage Cocktail of vB_SauH_2002 and 66 Had Synergistic Activity Against Planktonic S. aureus**

The lytic activity of the *Herelleviridae* phage vB_SauH_2002 (Figure 1A) and the *Podoviridae* phage 66 (Figure 1B) against each of 63 S. aureus strains is summarized in Table S1. The anti-*S. aureus* efficacy ranges of phage vB_SauH_2002 alone, phage 66 alone, or phage vB_SauH_2002 plus phage 66 (equimolar cocktail) covered ~83%, ~59%, and ~92% of the bacterial panel (Table S1). In diluted drop tests (Figure 1C), each of the 2 phages achieved very high titers against the MSSA strain Laus102 (ca. 10^{10} PFU/mL, Figure 1C). With respect to turbidity testing, the 2-phage cocktail achieved more sustained S. aureus-growth inhibition over 24 hours than either vB_SauH_2002 or phage 66 alone at the same MOI of 0.1 (P<0.0001) (Figure 1D through F).

Time-kill assay results are presented in Figure 1G. Notably, a significant loss of bacterial viability (ca. 4 log_{10} CFU/mL) was observed in time-kill assays 2 hours after addition of the phage cocktail at an
MOI of 1 ($P<0.0001$). Bacterial regrowth was observed 24 hours after the phage challenge but could be reduced by the addition of low-dose (1x minimum inhibitory concentration, ie, 0.125 mg/mL) flucloxacillin ($P<0.05$). Interestingly, during the early hours of the time-kill assay experiment, the phage cocktail achieved a greater magnitude of killing (~4 log$_{10}$ CFU/mL at 2 hours) than flucloxacillin (~3 log$_{10}$ CFU/mL at 4 hours) ($P<0.0001$). We did not observe evidence of synergism between the phages and the antibiotic in the time-kill assay experiment (ie, nonsignificant difference between the phages+flucloxacillin and flucloxacillin alone at 24 hours, $P=0.93$).

**Both Phages Synergized With Antibiotics to Clear Biofilms In Vitro**

We further compared the efficacy of each single phage, the phage cocktail, flucloxacillin, and the combination of both for the treatment of MSSA biofilms in vitro (Figure 2). Surprisingly, although phage 66 was
active against planktonic cells and exhibited exopolysaccharide depolymerase activity (evidenced by the formation of halos around PFUs\(^{23}\)) in the diluted drop test assay, phage 66 was ineffective against MSSA biofilms at all MOIs tested (1, 10, and 100) (Figure 2A). In contrast, phage vB_SauH_2002 achieved significant dose-dependent biofilm clearance compared with the control treatment (\(\Delta3.29\pm0.55\log_{10}\text{CFU/mL at MOI=1; } \Delta3.29\pm0.55\log_{10}\text{CFU/mL at MOI=10}\)), with particularly efficacious clearance being achieved at an MOI of 100 (\(\Delta4.51\pm0.55\log_{10}\text{CFU/mL, } P<0.0001\)) (Figure 2A). Moreover, phage vB_SauH_2002 synergized with phage 66 at an MOI of 10 (additional loss of viability of 2.26\(\pm0.55\log_{10}\text{CFU/mL relative to vB_SauH_2002 alone; } P<0.01\) (Figure 2B)). Substantial synergy between the phage cocktail (MOI=1) and low-dose (1x minimum inhibitory concentration) flucloxacillin was observed (2.74\(\pm0.44\log_{10}\text{CFU/mL additional clearance versus the phage cocktail at MOI=1 alone, } P<0.0001\)) (Figure 2B).

**Phages and Flucloxacillin Were Highly Synergistic Against S. aureus Experimental Endocarditis**

At the onset of treatment, which occurred 6 hours after the bacterial challenge (Figure 3A), all rats harbored heavily infected vegetations (7.22\(\pm0.92\log_{10}\text{CFU/g, and mock therapy (saline) allowed the bacterial load to increase to 9.20\(\pm1.05\log_{10}\text{CFU/g 24 hours later (Figure 3B). Bacteriostasis (bacterial load similar to baseline) was observed for 24 hours after administration of the phage cocktail (10^{10} \text{PFU in } 1 \text{mL saline followed by } 8\times10^{10} \text{PFU over 24 hours via continuous intravenous infusion at } 0.3 \text{mL/h). Similarly, bacteriostasis was observed for 24 hours after administration of a low dose of flucloxacillin every 12 hours (simulating antibacterial treatment in human patients) with } 5.35\pm3.16\log_{10}\text{CFU/g versus } 7.22\pm0.92\log_{10}\text{CFU/g at the onset of treatment. In sharp contrast, the combination of both treatments had a highly bactericidal effect (2.62\(\pm1.01\log_{10}\text{CFU/g, ie, } \Delta \text{ of } -5.25\log_{10}\text{CFU/g versus treatment onset, } P<0.0001\) owing to synergistic activity with the phage cocktail (}\Delta \text{ of } -2.15\log_{10}\text{CFU/g versus flucloxacillin alone, } P<0.01)).
Importantly, the vegetations in 9 of 12 rats (75%) treated with the phage cocktail-flucloxacillin combination were culture negative at 24 hours (Figure 3B).

**The Addition of a Subtherapeutic Dose of Flucloxacillin Affected Phage Titers In Vivo**

As shown in Figure 3C, phage titers in blood samples collected from infected animals 24 hours after phage treatment initiation (9.59±0.91 log_{10} PFU/mL) were significantly higher (Δ2.71±0.52 log_{10} PFU/mL, P<0.005) than those in noninfected animals (6.88±0.42 log_{10} PFU/mL). Similarly high phage titers were measured in cardiac vegetations (9.80±0.52 log_{10} PFU/g), spleen (9.20±0.52 log_{10} PFU/g), liver (9.54±0.23 log_{10} PFU/g), and kidneys (9.16±0.61 log_{10} PFU/g) in EE animals. The addition of low-dose flucloxacillin decreased phage titers drastically in all body
compartments (cardiac vegetation $\Delta 1.84 \pm 0.28 \log_{10}$ PFU/mL, $P<0.001$; blood $\Delta 5.06 \pm 0.75 \log_{10}$ PFU/g, $P<0.01$; spleen $\Delta 3.84 \pm 0.27 \log_{10}$ PFU/g, $P<0.0001$; liver $\Delta 3.02 \pm 0.18 \log_{10}$ PFU/g, $P<0.0001$; and kidneys $\Delta 3.92 \pm 0.39 \log_{10}$ PFU/g, $P<0.0001$ versus phage cocktail alone). After rats were on a subtherapeutic dose of flucloxacillin for 24 hours, phage levels in their blood were even lower than those of noninfected animals ($\Delta 2.35 \pm 0.65 \log_{10}$ PFU/mL, $P<0.01$, Figure 3D).

Bacterial Resistance Occurred In Vivo for Phage 66 But Not for vB_SauH_2002

Screening for phage/antibiotic-resistant clones in bacteria recovered from cardiac vegetations treated with the phage and antibiotic in combination resulted in the recovery of $S. aureus$ colonies in 2/12 treated rats (Table S2). Of 36 clones that were recovered, 23 (63%) were susceptible to each single phage and to the phage cocktail (Susceptible, Susceptible, and Susceptible to the phage vB_SauH_2002, phage 66, and the phage cocktail, respectively [SSS] pattern of resistance), whereas 13 were resistant to phage 66 while retaining sensitivity to phage vB_SauH_2002 and the phage cocktail (Susceptible, Resistant, and Susceptible to the phage vB_SauH_2002, phage 66, and the phage cocktail, respectively [SRS] pattern of resistance). No clones harboring resistance to phage vB_SauH_2002 or to the phage cocktail were recovered from any of the animals (Table S2).

Sequencing of the genomes of 6 phage 66-resistant clones showed that all 6 clones harbored single nucleotide polymorphisms in several genes coding for transposases and that 1 clone, namely clone 16C8, harbored 1 additional point mutation in tarS leading to a frameshift (bold and underlined in Table S3). Interestingly, all 13 phage 66-resistant clones were as virulent as the parent strain in a $Galleria mellonella$ infection model (data not shown).

Rat Plasma Impaired the Lytic Activity of $S. aureus$ But Not of $P. aeruginosa$ Phages In Vitro

As shown in Figure 4A, the addition of 10% rat plasma to $S. aureus$ Laus102 cultures 30 min before administration of the phage cocktail at an MOI of 100 inhibited the in vitro bactericidal activity of phages dramatically (7.58±1.36 CFU/mL with plasma versus 2.00±0.00 CFU/mL without plasma, 4 hours after phage treatment, $P<0.0001$). In sharp contrast, phage-induced killing of $P. aeruginosa$ 4 hours after administration of phage vB_PaeM_4002 was not altered significantly by the addition of 10% plasma (2.02±0.07 CFU/mL with plasma and 1.69±0.86 CFU/mL without plasma; Figure 4B, $P=0.33$). Of note, no significant effect on phage killing activity was observed when phages, instead of bacteria, were preincubated for 30 minutes with 10% plasma and washed twice in saline before being added to bacterial cultures in the absence of plasma (data not shown).

DISCUSSION

It is unclear whether phage susceptibility assays performed in vitro predict phage behavior in vivo reliably and to what extent combining phages with standard of care antibiotics represents a potentially promising strategy. In the current study, we designed a 2-phage cocktail for the treatment of EE due to MSSA. We selected the recently isolated and highly lytic Herelleviridae phage vB_SauH_2002 and the Podoviridae phage 66, based on the supposition that its exopolysaccharide depolymerase activity might facilitate antimicrobial activity against bacteria that are embedded in biofilms.

Our standard in vitro phage susceptibility testing results (drop tests, turbidity, and time-kill assays) were encouraging, and further suggested that the 2 phages indeed
produced synergized activity against S. aureus biofilms. Extending these promising in vitro results, we observed a 2.6 log$_{10}$ CFU/g decrease in cardiac vegetations from living rats treated with the phages for 24 hours, compared with mock-therapy controls. However, the phage cocktail alone achieved only a bacteriostatic effect, failing to clear S. aureus EE completely despite effective in vivo phage amplification (also known as phage auto-dosing), which produced a local MOI of ~10$^3$ in cardiac vegetations at the time of necropsy.

The therapeutic failure of phages alone in our S. aureus EE model contrasts with the efficacy of phage therapy alone observed in an S. aureus experimental ventilator-associated pneumonia model. This discrepancy could be related to differing mechanisms of disease. In the pneumonia model, bacterial toxins play a major role in lung tissue destruction and plasma proteins are not expected to interfere much with phage-induced bacterial killing. In contrast, plasma proteins in general and coagulation factors in particular play a major role in S. aureus endovascular infections, wherein plasma fibrinogen and fibronectin attach to the surfaces of circulating S. aureus cells, thereby promoting valve infection indirectly. Hence, inhibition of S. aureus phage activity by blood proteins might limit phage efficacy in the EE model employed in this study. Indeed, we confirmed that preincubating bacterial cells, but not phages, with rat plasma inhibited the bactericidal activity of the phages against S. aureus but not P. aeruginosa. Strong inhibition of S. aureus phage lytic activity by rabbit and human serum was demonstrated in the 1930s, leading researchers at that time to hypothesize that bacteria might be protected by what they called “a colloidal coating of serum,” preventing phage penetration into the bacterial cell surface. Consistent with these almost century-old observations, it was shown recently that S. aureus phage K propagation was impaired in whole blood, plasma, and serum compared with propagation in growth media devoid of blood proteins. The molecular mechanism mediating plasma/serum-mediated phage resistance has yet to be elucidated.

Evidence in support of the therapeutic potential of phage-antibiotic synergisms has been growing. A major potential drawback of the addition of antibiotics is their potential impact on phage pharmacokinetics given that inhibition of bacterial growth also limits the ability of phages to replicate within target bacteria. When a subtherapeutic dosage of flucloxacinil was given concomitantly with our phage cocktail, we detected an altered phage pharmacokinetic profile evidenced by markedly reduced levels of circulating phages compared with levels seen when the phage treatment was administered alone. Notwithstanding, synergy of the 2 treatments emerged in vivo, with 75% of vegetations being found to be culture-negative after only 24 hours of combined treatment.

Regarding resistance selection, all clones recovered from cardiac-vegetation homogenates of rats treated with the phage cocktail/antibiotic combination remained susceptible to flucloxacinil (not shown), the Herelleviridae vB_SauH_2002, and the phage cocktail. Resistance to the Podoviridae phage 66 was observed in one third of the clones. Interestingly, a single phage 66-resistant clone had a mutation leading to a frameshift likely deleterious in tarS. TarM and TarS are involved in α- and β-O-glycosylation of N-acetyl-D-glucosamine residues of the wall teichoic acids, a main phage receptor in S. aureus. TarS-mediated β-O-glycosylation has been shown to be required for S. aureus susceptibility to Podoviridae. Whereas point mutations in tarM have been shown to alter susceptibility to Podoviridae, the present work identified a tarS mutation that may underlie a Podoviridae-resistance mechanism. Of note, the absence of genetic mutations in the remaining phage 66-resistant clones, with the exception of transposase genes likely unrelated to the phage 66-resistance phenotype, suggested the selection in our EE model of an adaptive mechanism of Podoviridae resistance mediated through the differential expression of α- and β-N-acetyl-D-glucosamine, as previously reported in other models of infectious diseases. Finally, resistance to phage 66 did not affect virulence in a Galleria mellonella model of S. aureus infectious disease (not shown), affirming the notion that development of phage resistance is not always associated with an in vivo fitness cost.

CONCLUSIONS

Taken together, the present encouraging results are informative for phage therapy development, particularly for the treatment of S. aureus endovascular infections. For IE applications, S. aureus phages would not be given as monotherapy but rather in combination with antibiotics. Indeed, phages may accelerate bacterial load reduction at infection sites at the start of therapy. This type of intervention may improve infection-related cardiac dysfunction in general, potentially truncating the period of systemic embolization risk, and thus, ultimately, shortening the duration of antibiotic therapy needed. Each of these considerations should be addressed in future translational and clinical trials.

To the best of our knowledge, this study is among very few investigations of synergism between phages and antibiotics in vivo, and the very first to report efficacy of phage-antibiotic combinations for the treatment of S. aureus EE. In a previous study, we investigated P. aeruginosa IE, a relatively uncommon but difficult-to-treat infection, based on the availability of the PhagoBurn phage cocktail. The present model has far greater clinical relevance because S. aureus is the
predominant IE pathogen.\textsuperscript{46} Combination therapy outperformed either phage or antibiotic treatment alone, consistent with recent publications on this topic.\textsuperscript{15,47,48}

Deciphering the mechanisms behind the observed synergy however would require a thorough investigation (eg, transcriptomic analysis\textsuperscript{49}), something that is out of the scope of the present study.

Caution in relation to the use of phage therapy for IE treatment remains warranted. Synergy cannot be assumed and there are potential risks of adverse effects of combining phages with antibiotics.\textsuperscript{50} Moreover, positive results in phage susceptibility testing may or may not translate into positive outcomes in vivo. A systemic understanding of in vivo interactions among bacteria, phages, antibiotics, and host defense mechanisms is needed to better define the role of phage therapy and its modality of prescription for the treatment of endovascular infections in humans.

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Philippe Muhammad, Yazid El-Mastoumy, and Bruno Lucchese

**Abstract**

Infective endocarditis is a serious condition that can be difficult to treat, particularly in cases of methicillin-resistant Staphylococcus aureus (MRSA) or Pseudomonas aeruginosa. In this study, we investigated the effectiveness of phage therapy as an alternative approach to antibiotic treatment.

Methods

We conducted a comprehensive literature review of studies published in the past decade, focusing on the use of bacteriophages to treat MRSA and Pseudomonas aeruginosa infections.

Results

Our analysis revealed that phage therapy offers several advantages over traditional antibiotics, including reduced resistance development and improved efficacy against both MRSA and P. aeruginosa. Additionally, phage therapy appears to have a lower risk of adverse effects compared to conventional antibiotics.

Conclusion

Phage therapy represents a promising alternative treatment strategy for MRSA and Pseudomonas aeruginosa infections, especially in cases where conventional antibiotics are ineffective. Further research is needed to fully understand the potential of phage therapy and to optimize its application in clinical settings.
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Supplemental Material
Data S1. SUPPLEMENTAL METHODS

Bacterial strains, growth conditions, and antibiotic susceptibility testing.

The MSSA strain Laus102, which was isolated from a healthy carrier, and a panel of 62 S. aureus strains that had been previously isolated from humans and cows were used in this study (Supplemental Table 1). All S. aureus strains were stored in TSB (BD Difco™, Becton Dickinson, Sparks, MD) containing 10% (v/v) glycerol at -80 °C and sub-cultured on TSA plates to ensure purity before testing. For liquid cultures, TSB was inoculated with at least five single colonies and incubated for 24 h with agitation (200 rpm) at 37 °C.

The P. aeruginosa strain ATCC® 15442™ (LGC Standards, Molsheim, France) was stored in Lysogeny Broth (LB, BD Difco™, Becton Dickinson, Sparks, MD) containing 10% (v/v) glycerol at -80 °C and sub-cultured on LB agar plates to ensure purity before testing. For liquid cultures, LB was inoculated with at least five single colonies and incubated for 24 h with agitation (200 rpm) at 37 °C.

Flucloxacillin was purchased from OrPha Swiss (Küsnacht, Switzerland). The MICs of flucloxacillin were determined in Muller Hilton Broth (Becton Dickinson, Sparks, MD) using a standard micro-dilution procedure.

Bacteriophages.

The Podoviridae phage 66 and Herelleviridae phage vB_SauH_2002 genomes are publicly available (Genbank accession no. NC_007046 and MW528836, respectively). To produce large quantities of phages, amplification was performed using Laus102 as propagation strain. For each phage preparation, 2 L of TSB was inoculated 1:100 with 20 mL of an overnight culture of Laus102 and incubated at 37 °C under 200rpm until an OD595nm of 0.1 was reached, and then 1 mL of phage stock (10^10 PFU/mL) was added. The culture was further incubated at 37 °C under 200 rpm for 6 h, and then centrifuged twice at 8000 xg for 15 min to remove bacterial debris. The supernatant containing the phages was passed through 0.22-µm filters (vacuum filtration 1000 rapid-filtermax, Techno Plastic Products AG, Trasadingen, Switzerland). The filtrate was further concentrated to 100 mL and buffer exchanged against 3 L of 1× phosphate buffer saline (PBS), pH 7.4 using tangential flow filtration through an mPES/500 KD column (Repligen, Waltham, MA). Phage concentrations in the
purified batches were determined in classical double agar overlay assays (DLAs) \(^{52}\). Briefly, 200 µL of an overnight culture of Laus102 was mixed with 100 µL of serial dilution of the phage preparations and 5 mL of TSB soft agar at 45 °C. This mixture was poured on TSA plates and incubated at 37 °C overnight after the TSB soft-agar layer solidified at room temperature. Concentration of phages was determined by counting PFUs. The equimolar phage cocktail at \(10^{10}\) PFU/mL was assembled after adjusting the concentration of each phage to \(10^{10}\) PFU/mL and by mixing equal volumes of the phages.

Phage vB_PaeM_4002 is a *Myoviridae* previously isolated from a sewage water sample collected at the Vidy wastewater treatment plant in Lausanne, Switzerland (unpublished) using *P. aeruginosa* PAO1 as a host strain. It is similar to the lytic phage vB_Pae_Ps44 (Genbank accession no. NC_028939). vB_PaeM_4002 was purified following the procedure described above, except that the propagation host used was *P. aeruginosa* strain ATCC® 15442™.

**Electron microscopy.**

Four-microliter phage suspension samples were deposited on a lacey carbon copper grid (EMS, Hatfield, PA) previously glow discharged for 30 s at 15 mA. The deposition was conducted in a Vitrobot Mark IV chamber (Thermo Fisher Scientific, Waltham, MA) in 100% humidity. A blotting time of 5 s with a force of -16 was used just before plunge freezing in liquid ethane. The grid was then transferred in an Elsa cryo-transfer holder (Gatan, Pleasanton, CA) and inserted in a 2100 Plus electron microscope (Jeol, Tokyo, Japan). Images (magnification, 120k; pixel size, 0.097 nm; 1-s exposure time) were collected by an XF416 camera (TVIPS GmbH, Gauting, Germany) with SerialEM software at 200 kV (electron dose of 25e\(^{-}/\AA^{2}/s\)) \(^{53}\).

**Determination of phage host range and efficiency of plating.**

Phage host range was determined on various *S. aureus* strains (Supplemental Table S1) using DLA (see above). Efficiency of plating scores were determined by dividing the phage titer in PFU/mL obtained on the tested strain by the phage titer obtained on the amplification strain Laus102 \(^{54}\). All experiments were done in triplicate.

**In vitro turbidity assays.**
One hundred µL of an overnight culture of Laus102 were re-suspended in 10 mL of TSB and incubated at 37 °C under 200 rpm until the OD_{595nm} reached 0.6, corresponding to ~10^8 CFU/mL. Then, 10-μL samples of this bacterial suspension (10^6 CFU) were mixed in 96-well plates (Thermo Scientific, USA) with 280 μL of TSB and 10 μL of various dilutions of the phage solutions to achieve final MOIs of 0.01, 0.1, 1, 10, and 100. The microtiter plates were incubated at 37 °C in an Elx808IU absorbance microplate reader (BioTek®, Sursee, Switzerland) and the OD_{595nm} was recorded every 10 min for 24 h. The microplates were shaken for 3 s before each measurement. All experiments were performed in triplicate.

**Phage time-kill curve assays.**

One hundred-µL samples of an overnight culture of Laus102 were re-suspended in 10 mL of TSB and incubated at 37 °C under 200 rpm until the OD_{595nm} reached 0.6, corresponding to ~10^8 CFU/mL. The culture was diluted 1:100 in 10 mL of fresh TSB supplemented with either the equimolar phage cocktail at a final MOI of 1, flucloxacillin at 1× the MIC, or a combination of both at the same final concentrations and then incubated at 37 °C and 200 rpm. Cell viability was determined 0 h, 2 h, 4 h, and 24 h after inoculation (limit of detection 10^2 CFU/mL). Before plating, samples were diluted in 1× PBS (pH 3) to neutralize the phages. All experiments were performed in triplicate. A similar procedure was used to test vB_Pae_4002 on *P. aeruginosa* strain ATCC® 15442™.

For the experiments in the presence of plasma, 100-µL samples of an overnight culture of Laus102 or *P. aeruginosa* strain ATCC® 15442™ were re-suspended in 10 mL of TSB or LB, respectively, and incubated at 37 °C under 200 rpm until the OD_{595nm} reached 0.6, corresponding to ~10^8 CFU/mL. The culture was diluted 1:100 in 10 mL fresh TSB or LB supplemented 10% rat plasma (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). After a 30-min pre-incubation at room temperature, *S. aureus* phage cocktail or phage vB_Pae_4002 (each at MOI = 100) was added accordingly, and test tubes were placed at 37 °C and 200 rpm. Cell viability was determined 0 h, 2 h, and 4 h after initiation of the phage challenge (limit of detection 10^2 CFU/mL). Before plating, samples were diluted in 1× PBS (pH 3) to neutralize the phages. All experiments were performed in triplicate.

**In vitro S. aureus** mono-species biofilm assay.
Maturation of the biofilm. MSSA Laus102 biofilms were produced in 96-well plates as previously described. Briefly, overnight cultures were diluted 1:100 in TSB, and 100-µL samples of the subsequent solution containing ca. 10⁷ CFU/mL were used to inoculate 96-well polystyrene plates (Greiner Bio-One, Kremsmünster, Austria) (final concentration of bacteria ~10⁶ CFU per well). After a 24-h incubation at 37 °C without shaking, the supernatant was removed from each well, and the remaining adherent biofilm was carefully steam-washed for 45 min using the BiofilmCare™ technology procedure.

Treatment of biofilm. Mature biofilms were treated for 24 h at 37 °C with 10⁸ PFU/mL, 10⁹ PFU/mL, or 10¹⁰ PFU/mL (final MOIs = 1, 10, and 100, respectively) of phage vB_SauH_2002 alone, phage 66 alone, the phage cocktail, or flucloxacillin (1× or 10× MIC). In addition, the phage cocktail at all MOIs was evaluated in combination with both flucloxacillin concentrations.

Evaluation of treatment efficacy. The treated biofilms were rinsed two times with PBS and re-suspended in 100 µl of PBS by scraping the wells with sterile pipette tips. The 96-well microplate was sealed with a plastic film (Dutscher, Brumath, France), put in an ultrasound bath (Bactosonic, Bandelin electronic GmbH & Co.KG, Berlin, Germany) for 10 min at 40 Hz to detach attached bacteria and to remove clusters before determination of viable counts on TSA.

Additional information related to the EE model.

Randomization. Randomization of animals in groups was done using the online tool Research Randomizer (https://www.randomizer.org/).

Flucloxacillin dosing regimen. Rats received a suboptimal IV dose of flucloxacillin mimicking human kinetic treatment (2 g every 12 h for 24 h instead of 2 g every 6 h for 24 h for an optimal treatment). The administration protocol consisted in the infusion of a solution of flucloxacillin (0.3 g/10 mL in saline) according to the following cycle: 2.0mL/h for 15 min., followed by 0.4 mL/h for 1 h 45 min., 0.2 mL/h for 2 h, and 0.005 mL/h for 2 h. After this first 6 h infusion cycle, no treatment was given for 6 h and a second infusion cycle was performed thereafter followed by no treatment for 6 h before euthanasia.

Criteria for euthanasia. Animal welfare was assessed at least two times per day with an in-house welfare score sheet for rodents (see below). Animals were excluded from randomization if we
suspected that the catheter placed into the heart through the carotid artery had potentially damaged the aortic valve or was not properly inserted. Animals were euthanized humanely according to the score and status of the animal as indicated below (termination criteria). The mortality rate after surgery was 10%, and six rats were excluded before infection. Moreover, six rats were further excluded at the end of the experiment because the catheter was not properly inserted.

**Welfare score sheet used in the in vivo experiment of EE rats.**

| Score | 0 | 1 | 2 | 3 |
|-------|---|---|---|---|
| **Haircoat** | | | | |
| Normal | Well groomed | Fur ruffling | General lack of grooming | Hunched up with matted fur |
| **Posture** | | | | |
| Normal | Sporadic hunchback posture | Frequent hunchback posture | Head on cage floor |
| **Activity** | | | | |
| Normal | Decreased activity after slight stimulation | Significant decreased activity after moderate stimulation | Lethargy after moderate stimulation |
| **Breath** | | | | |
| Normal | Shallow | Labored breathing | Breathing noises |
| **Behavior** | | | | |
| Normal | Isolated from cage mates* | | Convulsion |

*This score is not applicable for animals that are isolated in a cage, for instance animals equipped with a “swivel” system.

**Blinding procedure.** The rats receiving saline and phages or saline and antibiotics were connected to the same pumps, rendering the masking of group/treatment assignment challenging and unnecessary since blinding was performed during outcome assignment. Indeed, the technician who performed the experiments to evaluate the bacterial and phage loads in vegetations, and organs was blinded, i.e. she didn’t know from which animal the samples we provided her originated from.

**Bacterial loads in cardiac vegetations.** The presence of macroscopic cardiac valve vegetations was visually validated before being dissected from the heart. After being weight, vegetations were further mechanically homogenized in 1 mL saline. The homogenates were serially diluted and plated in triplicate on TSA plates for bacterial counting. Colonies were counted after an overnight incubation at 37 °C. Remaining vegetation homogenates were stored at -80 °C after the addition of 10% (v/v) glycerol. Phage or flucloxacillin carry over was diluted out through serial dilutions.

**Phage loads in cardiac vegetation, organs, and blood.** After dissection, organs were mechanically homogenized in weight-adapted volumes of saline (1 mL for cardiac vegetations, 2 mL for spleen,
liver, and kidney). Phage loads were determined using a classical DLA (see Materials and Methods). Plates were incubated at 37 °C and plaques were counted the following day.

**Power calculation.** We hypothesized that 100% and 30% of the placebo and phage cocktail/flucloxacillin treated rats would have infected vegetations at 24 h. These estimates, with an \( \alpha = 0.05 \) and a power \((1-\beta) = 0.8\) required a sample size of at least eight animals per group 55.

List of animals in groups.

| Number of animals | Onset of treatment | Saline | Phage cocktail | Flucloxacillin | Phage cocktail + flucloxacillin |
|-------------------|--------------------|--------|----------------|----------------|-------------------------------|
| Considered        | 10                 | 7      | 8              | 11             | 12                           |
| Dead after surgery| 0                  | 3      | 3              | 0              | 0                            |
| With not properly placed catheters | 2      | 2     | 1              | 1              | 0                            |

**Determination of phage-resistance patterns of S. aureus clones recovered in vivo.**

The phage-resistance patterns of the clones recovered in vivo from the rat cardiac vegetations were determined with diluted drop test assays. Cardiac vegetation homogenates (100 μL) were plated on TSA and incubated overnight at 37 °C. Two days later, single colonies were re-suspended in 5 mL fresh TSB and incubated overnight at 37 °C. Overnight bacterial cultures were mixed 1:100 with 15 mL of TSB soft-agar and poured into Petri dishes. The bacterial lawns were then spotted with 5 μL of serial 10-fold dilutions of each phage suspension (vB_SauH_2002, phage 66, and the phage cocktail) and incubated at 37 °C overnight. The results were scored the next day according to the observed lysis phenotypes. Absence and presence of lysis were considered definitive of a resistant phenotype (R) and a susceptible phenotype (S), respectively (Fig. S1).

**Bacterial genome sequencing, assembly, and analysis.**

A bacterial genomic library was prepared with an optimized protocol and standard Illumina adapter sequences, and sequencing was performed with Illumina technology, NovaSeq 6000 (read mode 2 x 150 base pairs). Both processes were performed at Eurofins Genomics Germany GmbH (Ebersberg, Germany). Reads were assembled and contigs annotated using the PATRIC pipeline.
for assembly and annotation, respectively (https://www.patricbrc.org/). Comparative genomics were performed with the PATRIC variation analysis tool set to default parameters.
Table S1. *S. aureus* strains used in this study along with their EOP scores for vB_SauH_2002 and phage 66.

| Human carriage strains from healthy volunteers | EOP score | Covered by the phage cocktail | Reference |
|-----------------------------------------------|-----------|-------------------------------|-----------|
| S. aureus strain                              | Genbank access N° | ST | vB_SauH_2002 | Phage 66 |                                      |           |
| Laus102                                       | JAETX100000000.1  | 8  | 1            | 1        | yes                             | 16        |
| Laus385                                       | CP071350.1      | 8  | 1.5          | 1.25     | yes                             | 16        |
| F60                                           | NA             | 15 | 0.025        | 5.10^{-4} | yes                             |           |
| Human clinical strains                         | EOP score | Covered by the phage cocktail | Reference |
| VRS11b (AID1001123)*†‡                        | AHBV01000000.1  | 5  | 0.35         | 0.225    | yes                             | 57        |
| VRS8 (71080)*††                               | AHBR00000000.1  | 5  | 0            | 0        | no                              | 57        |
| VRS9 (AIS080003)*††                           | AHBS00000000.1  | 5  | 0            | 0.75     | yes                             | 57        |
| VRS10 (AIS100505)*††                          | AHBT00000000.1  | 5  | 0.65         | 0        | yes                             | 57        |
| VRS11a (AIS1001095)*††                        | AHBU00000000.1  | 5  | 0.15         | 0.075    | yes                             | 57        |
| VRS6 (AIS2006032)*††                          | AHBP00000000.1  | 5  | 0.025        | 0.2      | yes                             | 57        |
| VRS7 (AIS2006045)*††                          | AHBQ00000000.1  | 5  | 0.7          | 0        | yes                             | 57        |
| VRS4 (HIP14300)*††                            | AHN00000000.1   | 5  | 0.025        | 0.075    | yes                             | 57        |
| VRS3b (HIP13419)*††                           | AHBM00000000.1  | 5  | 0            | 0.9      | yes                             | 57        |
| VRS2 (HIP11983)*††                            | AHBLO00000000.1 | 5  | 0            | 0        | no                              | 57        |
| VRS3a (HIP13170)*††                           | NBCP00000000.1  | 5  | 0            | 1        | yes                             | 57        |
| VRS1 (HIP11714)*††                            | AHBK00000000.1  | 5  | 0.35         | 0        | yes                             | 57        |
| VRS5 (HIP15178)*††                            | AHBQ00000000.1  | 5  | 0.7          | 0.25     | yes                             | 57        |
| ATCC 29213                                    | LHSU00000000.2  | 5  | 0            | 0        | no                              | 57        |
| I37                                           | CP071352.1      | 8  | 0.85         | 0.25     | yes                             | 16        |
| USA300 FPR3747‡                               | JAFFHX0000000000.1 | 8  | 0            | 0.2      | yes                             | 58        |
| USA300 JE2‡                                   | CP020619.1      | 8  | 0            | 0.8      | yes                             | 58        |
| Yok80                                         | NA             | 8  | 0.025        | 1        | yes                             | This study|
| Yok51                                         | NA             | 22 | 1            | 0.075    | yes                             | This study|
| Yok49                                         | NA             | 30 | 1            | 0.25     | yes                             | This study|
| Yok25                                         | NA             | 45 | 0            | 0        | no                              | This study|
| Yok72‡                                        | NA             | 105| 0.7          | 0.0125   | yes                             | This study|
| Yok53                                         | NA             | 121| 0.75         | 5.10^{-3} | yes                             | This study|
| AW10‡                                        | NA             | 239| 0            | 0.2      | yes                             | This study|
| AW7‡                                         | SRLL00000000.1  | 247| 0.025        | 0.02     | yes                             | 59        |
| COL‡                                         | CP000046.1      | 250| 0.35         | 0        | yes                             | 60        |
| Yok45                                         | NA             | 707| 0            | 0        | no                              | This study|
### Animal strains from bovine mastitis

| Code | GenBank Accession | ST | EOP | efficiency of plating | % coverage |
|------|------------------|----|-----|-----------------------|------------|
| Jn   | CP071362.1       | 8  | 0.025 | 0.03                  | yes        | 61  |
| G04  | CP071369.1       | 8  | 0.5   | 0.125                 | yes        | 61  |
| G36  | CP071366.1       | 8  | 0.8   | 0                     | yes        | 61  |
| G57  | CP071365.1       | 8  | 0.35  | 0                     | yes        | 61  |
| O103 | CP071360.1       | 8  | 0.6   | 0.25                  | yes        | 61  |
| M160 | CP071341.1       | 8  | 0.6   | 0                     | yes        | 16  |
| M283 | CP071337.1       | 8  | 0.45  | 0                     | yes        | 16  |
| M186 | CP071340.1       | 8  | 0.85  | 0                     | yes        | 16  |
| M79  | CP071339.1       | 8  | 0.025 | 0                     | yes        | 16  |
| M385 | CP071333.1       | 8  | 0.45  | 0                     | yes        | 16  |
| M308 | CP071336.1       | 8  | 0.65  | 0                     | yes        | 16  |
| O03  | CP071370.1       | 8  | 0.025 | 0.25                  | yes        | 61  |
| Bc   | CP071374.1       | 8  | 0.025 | 0.2                   | yes        | 61  |
| O100 | CP071361.1       | 8  | 0.025 | 0.3                   | yes        | 61  |
| Je   | CP071363.1       | 8  | 0.025 | 0.075                 | yes        | 61  |
| G34  | CP071367.1       | 8  | 0.025 | 0                     | yes        | 61  |
| M222 | CP071338.1       | 8  | 0.025 | 0                     | yes        | 61  |
| M37  | CP071347.1       | 8  | 0.45  | 0.25                  | yes        | 61  |
| M5   | CP071349.1       | 8  | 0.025 | 0                     | yes        | 61  |
| M20  | CP071348.1       | 8  | 0.45  | 1                     | yes        | 61  |
| M319 | CP071334.1       | 8  | 0.6   | 2                     | yes        | 61  |
| M313 | CP071335.1       | 8  | 0.6   | 0                     | yes        | 61  |
| M124 | CP071343.1       | 8  | 0.025 | 0                     | yes        | 61  |
| M117 | CP071344.1       | 8  | 0.35  | 0                     | yes        | 61  |
| M184 | NA               | 15 | 1     | 0                     | yes        | 61  |
| M356 | NA               | 71 | 0.025 | 0.25                  | yes        | 61  |
| M159 | NA               | 389| 0.025 | 4.10^4                | yes        | 61  |
| M323 | NA               | 389| 0.025 | 0                     | yes        | 61  |
| M3   | NA               | 395| 0.025 | 0.025                 | yes        | 61  |
| M75  | NA               | 504| 0.1   | 0.175                 | yes        | 56  |
| M52  | NA               | 504| 0.025 | 0.9                   | yes        | 61  |
| M86  | CP071346.1       | 1650| 0.6 | 0                     | yes        | 61  |
| M126 | NA               | 1651| 0.025 | 3.5                  | yes        | 61  |

% coverage: 82.54 58.73 92.06

ST, sequence type; EOP, efficiency of plating; NA, not available, *see acknowledgements, †VRSA, ‡MRSA.
Table S2. Phage resistance patterns of clones recovered from the cardiac vegetations of rats treated with the phage cocktail/flucloxacillin combination for 24 h.

| Animal N° | CFU/g vegetations | Number of clones that regrew in TSB | Phage resistance pattern (vB_SauH_2002, phage 66, phage cocktail) |
|------------|-------------------|-------------------------------------|---------------------------------------------------------------|
|            |                   |                                     | SSS               | SRS               |
| 16         | 5.4               | 21                                  | 14                | 6                 |
| 18         | 3.5               | 15                                  | 9                 | 7                 |

S, susceptible; R, resistant.
Table S3. Results of the variant analysis conducted in PATRIC with default parameters between six representative SRS clones recovered from the vegetations of rats treated for 24 h with the phage cocktail/flucloxacillin combination and the Laus1002 wild-type SSS strain.

Clone 16C1

| Contig | Pos   | Score   | Ref_nt | Var_nt | Frameshift | Gene N°* | Function               |
|--------|-------|---------|--------|--------|------------|----------|------------------------|
| 0001   | 525680| 5608.82 | gcc    | gTc    | 496        | Transposase, IS4 family|
| 0001   | 525813| 75.0767 | agt    | Ggt    | 496        | Transposase, IS4 family|
| 0005   | 7195  | 525.68  | ggc    | gTc    | 1625       | Transposase, IS4 family|
| 0005   | 7258  | 844.719 | gat    | Ggt    | 1625       | Transposase, IS4 family|
| 0005   | 7265  | 893.501 | tgt    | Ggt    | 1625       | Transposase, IS4 family|
| 0009   | 87601 | 3107.3  | cag    | Gag    | 2210       | Transposase, IS4 family|
| 0009   | 87676 | 8719.57 | aat    | Gat    | 2210       | Transposase, IS4 family|
| 0009   | 87780 | 13226.1 | aagaga2ta| AAGAA4AGta| yes     | 2211 Transposase, IS4 family|
| 0009   | 87789 | 17524.8 | tgtgtgcag| ttTGttGtgg| 2211 Transposase, IS4 family|
| 0009   | 87812 | 17154.9 | aat    | aAt    | 2211       | Transposase, IS4 family|
| 0009   | 87830 | 10619.7 | gataatttatatatgatgatg | AATATTAATTTTTTATTGATGGT | 2211 Transposase, IS4 family|
| 0009   | 87885 | 8389.01 | ttctat | ttGTGttCAT | 2211 Transposase, IS4 family|
| 0009   | 88220 | 2276.45 | atgacccaa| TTGATCCAaa| 2211 Transposase, IS4 family|
| 0009   | 88235 | 14505.1 | att    | aAt    | 2211       | Transposase, IS4 family|
| 0010   | 42336 | 648.986 | aaa    | Gaa    | 2285       | Hypothetical protein, Lmo2313 homolog [phage A118]|

Synonymous mutations

| Contig | Pos   | Score   | Ref_nt | Var_nt | Frameshift | Gene N°* | Function               |
|--------|-------|---------|--------|--------|------------|----------|------------------------|
| 0001   | 525712| 6093.63 | att    | aAt    | 496        | Transposase, IS4 family|
| 0001   | 525739| 6269.44 | aag    | aaA    | 496        | Transposase, IS4 family|
| 0001   | 525793| 1241.32 | aag    | aaA    | 496        | Transposase, IS4 family|
| 0001   | 525802| 1663.27 | cgt    | cgA    | 496        | Transposase, IS4 family|
| 0005   | 7149  | 190.834 | ttc    | ttT    | 1625       | Transposase, IS4 family|
| 0009   | 87596 | 2442.06 | aat    | aaC    | 2210       | Transposase, IS4 family|
| 0009   | 87749 | 15295.7 | gag    | gaA    | 2210       | Transposase, IS4 family|
| 0009   | 88024 | 23007.5 | cga    | cgT    | 2211       | Transposase, IS4 family|
| 0009   | 88194 | 5005.21 | actctgttt| acTTCTGtt| 2211 Transposase, IS4 family|
| 0010   | 42286 | 6078.02 | act    | acG    | 2285       | Hypothetical protein, Lmo2313 homolog [phage A118]|
## Clone 16C5

### Non-synonymous mutations

| Contig | Pos  | Score  | Ref_nt | Var_nt | Frameshift | Gene_ID | Function                          |
|--------|------|--------|--------|--------|------------|---------|-----------------------------------|
| 0001   | 525680 | 8187.98 | gcc    | gTc    | 496        | Transposase, IS4 family           |
| 0005   | 7258  | 1721.74 | gat    | gGt    | 1625       | Transposase, IS4 family           |
| 0005   | 7265  | 1841.36 | tgt    | Ggt    | 1625       | Transposase, IS4 family           |
| 0009   | 87601 | 4777.89 | cag    | Gag    | 2210       | Transposase, IS4 family           |
| 0009   | 87676 | 11201.7 | aat    | Gat    | 2210       | Transposase, IS4 family           |
| 0009   | 87780 | 15992.4 | aagaga | AAGAAAAGta | yes       | 2211 | Transposase, IS4 family           |
| 0009   | 87789 | 20770.6 | ttggtc | ttTGTGTgg | 2211 | Transposase, IS4 family           |
| 0009   | 87812 | 21642.7 | agt    | aAt    | 2211       | Transposase, IS4 family           |
| 0009   | 87830 | 11586.8 | gataat | AATAATTCAATTTTATGATGGGa | 2211 | Transposase, IS4 family           |
| 0009   | 87885 | 9779.11 | ttctat | ttCCat  | 2211       | Transposase, IS4 family           |
| 0009   | 88220 | 1090.55 | atgaccc | TTGATCCAa | 2211 | Transposase, IS4 family           |
| 0009   | 88235 | 17043.0 | att    | aAt    | 2211       | Transposase, IS4 family           |

### Synonymous mutations

| Contig | Pos  | Score  | Ref_nt | Var_nt | Frameshift | Gene_ID | Function                          |
|--------|------|--------|--------|--------|------------|---------|-----------------------------------|
| 0001   | 525712 | 8026.6 | att    | atC    | 496        | Transposase, IS4 family           |
| 0001   | 525739 | 8395.65 | aag    | aaA    | 496        | Transposase, IS4 family           |
| 0001   | 525793 | 1329.23 | aag    | aaA    | 496        | Transposase, IS4 family           |
| 0001   | 525802 | 1760.09 | cgt    | cgA    | 496        | Transposase, IS4 family           |
| 0009   | 87596 | 3765.39 | aat    | aaC    | 2210       | Transposase, IS4 family           |
| 0009   | 87749 | 17038.1 | gag    | gaA    | 2210       | Transposase, IS4 family           |
| 0009   | 88024 | 21383.9 | cga    | cgT    | 2211       | Transposase, IS4 family           |
| 0009   | 88194 | 4571.23 | acctctgtt | acTTCTGtt | 2211 | Transposase, IS4 family           |
| 0010   | 42286 | 2198.73 | act    | acG    | 2285       | Hypothetical protein, Lmo2313 homolog [phage A118] |
## Clone 16C8

### Non-synonymous mutations

| Contig | Pos  | Score  | Ref_nt | Var_nt   | Frameshift | Gene_ID | Function                                                        |
|--------|------|--------|--------|----------|------------|---------|----------------------------------------------------------------|
| 0001   | 525680 | 5515.24 | gcc    | gTc      | 496        | Transposase, IS4 family |
| 0004   | 150512 | 202.141 | gattttataga | gATTTTATAga | yes        | 1579    | Glycosyl transferase family protein, putative |
| 0005   | 7258  | 4917.17 | gat    | gGt      | 1625       | Transposase, IS4 family |
| 0005   | 7265  | 4599.85 | tgt    | Ggt      | 1625       | Transposase, IS4 family |
| 0009   | 87601 | 6407.54 | cag    | Gag      | 2210       | Transposase, IS4 family |
| 0009   | 87676 | 10902.8 | aat    | Gat      | 2210       | Transposase, IS4 family |
| 0009   | 87780 | 16053.2 | aagaaagt | AAGAAAAGta | yes        | 2211    | Transposase, IS4 family |
| 0009   | 87789 | 19560.9 | tgggtgcgg | tTGTTGGg   | 2211       | Transposase, IS4 family |
| 0009   | 87812 | 20662.2 | agt    | aAt      | 2211       | Transposase, IS4 family |
| 0009   | 87830 | 11884.0 | gataattcttttatgatgt | AATAATTCAATTTTATGATGgt | 2211 | Transposase, IS4 family |
| 0009   | 87885 | 9920.24 | ttctat | ttCCat   | 2211       | Transposase, IS4 family |
| 0009   | 88220 | 2783.96 | atgccaa | TTGATCCAa | 2211       | Transposase, IS4 family |
| 0009   | 88235 | 17981.6 | att    | aAt      | 2211       | Transposase, IS4 family |

### Synonymous mutations

| Contig | Pos  | Score  | Ref_nt | Var_nt | Frameshift | Gene_ID | Function                                               |
|--------|------|--------|--------|--------|------------|---------|--------------------------------------------------------|
| 0001   | 525712 | 7109.39 | att    | atC    | 496        | Transposase, IS4 family |
| 0001   | 525739 | 7076.59 | aag    | aaA    | 496        | Transposase, IS4 family |
| 0001   | 525793 | 2050.16 | aag    | aaA    | 496        | Transposase, IS4 family |
| 0001   | 525802 | 1947.28 | cgt    | cgA    | 496        | Transposase, IS4 family |
| 0009   | 87596  | 4990.44 | aat    | aaC    | 2210       | Transposase, IS4 family |
| 0009   | 87749  | 17628.2 | gag    | gaA    | 2210       | Transposase, IS4 family |
| 0009   | 88024  | 20936.6 | cga    | cgT    | 2211       | Transposase, IS4 family |
| 0009   | 88194  | 5551.04 | acctcttgt | acTTCTGtt | 2211 | Transposase, IS4 family |
| 0010   | 42286  | 3500.84 | act    | acG    | 2285       | Hypothetical protein, Lmo2313 homolog [phage A118] |
Clone 18C1

| Contig | Pos   | Score    | Ref_nt | Var_nt       | Frameshift | Gene_ID | Function                  |
|--------|-------|----------|--------|--------------|------------|---------|---------------------------|
| 0001   | 525680| 8604.29  | gcc    | gTc          | 496        | Transposase, IS4 family   |
| 0005   | 7258  | 262.335  | gat    | gGt          | 1625       | Transposase, IS4 family   |
| 0005   | 7265  | 822.581  | tgt    | Ggt          | 1625       | Transposase, IS4 family   |
| 0009   | 87601 | 5036.85  | cag    | Gag          | 2210       | Transposase, IS4 family   |
| 0009   | 87676 | 11688.4  | aat    | Gat          | 2210       | Transposase, IS4 family   |
| 0009   | 87780 | 15566.7  | aagaga | AAGAAAAGTa   | yes        | 2211 Transposase, IS4 family |
| 0009   | 87789 | 19603.4  | ttggtcgg | ttTGTGTgg     | 2211       | Transposase, IS4 family   |
| 0009   | 87812 | 20831.3  | aat    | aAt          | 2211       | Transposase, IS4 family   |
| 0009   | 87830 | 12640.3  | gataatttaattgatg | AATAATCATTATTCATGATG | 2211 Transposase, IS4 family |
| 0009   | 87885 | 11079.3  | ttctat | ttCCat       | 2211       | Transposase, IS4 family   |
| 0009   | 88235 | 16189.7  | att    | aAt          | 2211       | Transposase, IS4 family   |

| Synonymous mutations |
|----------------------|
| Contig | Pos   | Score    | Ref_nt | Var_nt | Frameshift | Gene_ID | Function                  |
| 0001   | 525712| 9596.15  | att    | atC    | 496        | Transposase, IS4 family   |
| 0001   | 525739| 9276.65  | aag    | aaA    | 496        | Transposase, IS4 family   |
| 0001   | 525793| 1566.82  | aag    | aaA    | 496        | Transposase, IS4 family   |
| 0001   | 525802| 1189.01  | cgt    | cgA    | 496        | Transposase, IS4 family   |
| 0009   | 87596 | 3693.58  | aat    | aaC    | 2210       | Transposase, IS4 family   |
| 0009   | 87749 | 17536.0  | gag    | gaA    | 2210       | Transposase, IS4 family   |
| 0009   | 88024 | 24915.3  | cga    | cgT    | 2211       | Transposase, IS4 family   |
| 0009   | 88194 | 3408.42  | accttctgtt | acTTCTGtt   | 2211 Transposase, IS4 family |
### Clone 18C4

#### Non-synonymous mutations

| Contig | Pos  | Score   | Ref_nt | Var_nt | Frameshift | Gene_ID | Function                  |
|--------|------|---------|--------|--------|------------|---------|---------------------------|
| 0001   | 525680 | 10276.0 | gcc    | gTc    | 496        |         | Transposase, IS4 family   |
| 0005   | 7258  | 1225.1  | gat    | gGt    | 1625       |         | Transposase, IS4 family   |
| 0005   | 7265  | 1314.05 | tgt    | Ggt    | 1625       |         | Transposase, IS4 family   |
| 0009   | 87601 | 3980.07 | cag    | Gag    | 2210       |         | Transposase, IS4 family   |
| 0009   | 87676 | 9695.14 | aat    | Gag    | 2210       |         | Transposase, IS4 family   |
| 0009   | 87780 | 13094.5 | aagaaagta | AAGAAAAGta | yes | 2211 | Transposase, IS4 family |
| 0009   | 87789 | 16499.0 | ttggtgcgg | ttTGTGTgg | 2211 | Transposase, IS4 family |
| 0009   | 87812 | 15960.0 | aat    | aAt    | 2211       |         | Transposase, IS4 family   |
| 0009   | 87830 | 7869.22 | gataattcatttttgtgatgtc | AATAATTCAATTTTTATGATGGT | 2211 | Transposase, IS4 family |
| 0009   | 87885 | 7835.95 | ttatat | tCCat  | 2211       |         | Transposase, IS4 family   |
| 0009   | 88220 | 2190.76 | atgacccca | TTGATCCAa | 2211 | Transposase, IS4 family |
| 0009   | 88235 | 15953.5 | att    | aAt    | 2211       |         | Transposase, IS4 family   |

#### Synonymous mutations

| Contig | Pos  | Score   | Ref_nt | Var_nt | Frameshift | Gene_ID | Function                  |
|--------|------|---------|--------|--------|------------|---------|---------------------------|
| 0001   | 525712 | 10074.7 | aat    | atC    | 496        |         | Transposase, IS4 family   |
| 0001   | 525739 | 8975.51 | aag    | aaA    | 496        |         | Transposase, IS4 family   |
| 0001   | 525793 | 1084.67 | aag    | aaA    | 496        |         | Transposase, IS4 family   |
| 0001   | 525802 | 1072.37 | cgt    | cgA    | 496        |         | Transposase, IS4 family   |
| 0009   | 87596 | 3262.71 | aat    | aaC    | 2210       |         | Transposase, IS4 family   |
| 0009   | 87749 | 14795.8 | gag    | gaA    | 2210       |         | Transposase, IS4 family   |
| 0009   | 88024 | 19495.9 | cga    | cgT    | 2211       |         | Transposase, IS4 family   |
| 0009   | 88194 | 5599.36 | accctgtt | acTCTCTGtt | 2211 | Transposase, IS4 family |
| 0010   | 42286 | 570.972 | act    | acG    | 2285       |         | Hypothetical protein, Lmo2313 homolog [phage A118] |
### Clone 18C10

#### Non-synonymous mutations

| Contig | Pos   | Score | Ref_nt | Var_nt | Frameshift | Gene_ID | Function                      |
|--------|-------|-------|--------|--------|------------|---------|-------------------------------|
| 0001   | 525680| 5608.82| gcc    | gTc    | 496        | Transposase, IS4 family       |
| 0001   | 525813| 75.7067| agt    | Ggt    | 496        | Transposase, IS4 family       |
| 0005   | 7195  | 525.68 | gcc    | gTc    | 1625       | Transposase, IS4 family       |
| 0005   | 7258  | 844.719| gat    | gGt    | 1625       | Transposase, IS4 family       |
| 0005   | 7265  | 893.501| tgt    | Ggt    | 1625       | Transposase, IS4 family       |
| 0009   | 87601 | 3107.3 | cag    | Gag    | 2210       | Transposase, IS4 family       |
| 0009   | 87676 | 8719.57| aat    | Gat    | 2210       | Transposase, IS4 family       |
| 0009   | 87780 | 13226.1| aagaaagta | AAGAAAAGta | yes         | 2211       | Transposase, IS4 family       |
| 0009   | 87889 | 17524.8| ttgtgccg | tTTGTTGgg |            | 2211       | Transposase, IS4 family       |
| 0009   | 87812 | 17154.9| agt    | aAt    | 2211       | Transposase, IS4 family       |
| 0009   | 87830 | 10619.7| gataattcaattttattgatgtg | AATAATTCAATTTTTATTGATGGT | 2211 | Transposase, IS4 family |
| 0009   | 87885 | 8389.01| tctat  | tTCat  | 2211       | Transposase, IS4 family       |
| 0009   | 88220 | 2276.45| atgacccaa | TTGA TCCAa |            | 2211       | Transposase, IS4 family       |
| 0009   | 88235 | 14505.1| att    | aAt    | 2211       | Transposase, IS4 family       |
| 0010   | 42336 | 648.986| aaa    | Gaa    | 2285       | Hypothetical protein, Lmo2313 homolog [phage A118] |

#### Synonymous mutations

| Contig | Pos   | Score | Ref_nt | Var_nt | Frameshift | Gene_ID | Function                      |
|--------|-------|-------|--------|--------|------------|---------|-------------------------------|
| 0001   | 525712| 6093.63| att    | atC    | 496        | Transposase, IS4 family       |
| 0001   | 525739| 6269.44| aag    | aaA    | 496        | Transposase, IS4 family       |
| 0001   | 525793| 1241.32| aag    | aaA    | 496        | Transposase, IS4 family       |
| 0001   | 525802| 1663.27| cgt    | cgA    | 496        | Transposase, IS4 family       |
| 0005   | 7149  | 190.834| ttc    | ttT    | 1625       | Transposase, IS4 family       |
| 0009   | 87596 | 2442.06| aat    | aaC    | 2210       | Transposase, IS4 family       |
| 0009   | 87749 | 15295.7| gag    | gaA    | 2210       | Transposase, IS4 family       |
| 0009   | 88024 | 23007.5| cga    | cgT    | 2211       | Transposase, IS4 family       |
| 0009   | 88194 | 5005.21| acctctgtg | acTTCCTGtt |            | 2211       | Transposase, IS4 family       |
| 0010   | 42286 | 6078.02| act    | acG    | 2285       | Hypothetical protein, Lmo2313 homolog [phage A118] |
Figure S1. Images of representative patterns observed in diluted drop tests for *S. aureus* SSS and SRS clones isolated from the cardiac vegetations of rats treated with the phage cocktail/flucloxacillin combination for 24 h. A. Phage vB_SauH_2002. B. Phage 66. C. Phage cocktail. The SSS pattern observed with the wild-type (WT) strain Laus102 is indicated for comparison in the left panel. S, susceptible; R, resistant.