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ORIGINAL RESEARCH

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 (Δ of -5.25 $\log_{10}$ colony forming unit/g [CFU/g] versus treatment onset, $P<0.0001$) and synergism was confirmed (Δ of -2.15 $\log_{10}$ 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[1] 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]

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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.

Nonstandard Abbreviations and Acronyms

EE	experimental infective endocarditis
IE	infective endocarditis
MOI	multiplicity of infection
PFU	plaque forming unit

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,[6,7] keratitis,[8] severe infections after cardiothoracic surgery,[9] prosthetic joint infections,[10,11] and ventricular-assist device infections.[12]

Recently, 2 Australian case series evaluated the safety and efficacy of a 3-phage cocktail for the treatment of patients with *S. aureus* IE or *S. aureus* aortic graft infections.[13,14] Encouragingly, improved infection control and healing progress were documented with the adjunction 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.[15] 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 flucloxacillin for the treatment of methicillin-susceptible *S. aureus* (MSSA) EE in rats.

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.[16] 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.[17] 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).

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.

Induction of Infection

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[21]) 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.

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

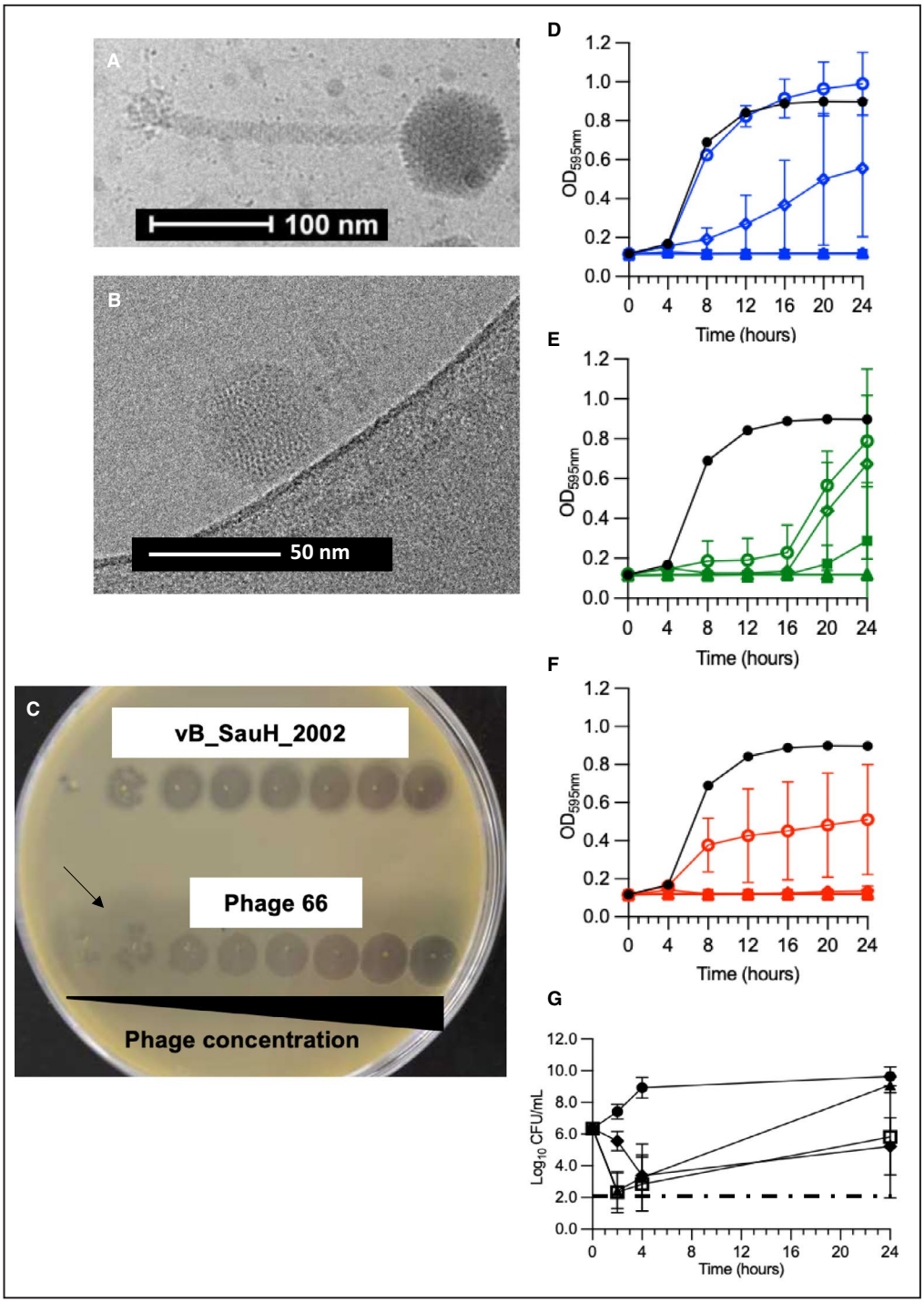
Differences between the groups were generally detected with 1-way ANOVAs with Tukey correction for multiple comparisons. Phage loads in blood and organs were compared with unpaired *t* tests with Welch's correction. All analyses were performed in Prism software (version 9, GraphPad, La Jolla, CA). Statistical 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 (1× 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

Figure 1. In vitro activity against MSSA Laus102 of *Myoviridae* phage vB_HSa_2002, *Podoviridae* phage 66, or both in a 2-phage cocktail. Electron micrograph of (A) vB_SauH_2002 and (B) phage 66.
Antibacterial activity was tested through (**C**) diluted drop tests, (**D** through **F**) turbidity assays, and (**G**) time-kill assays. **C**, The black arrow indicates the halo surrounding phage 66 lysis zones. Both phages were serially 10-time diluted from right to left (starting concentration was 10^9 PFU/mL for both phages). **D**, Phage vB_SauH_2002 (blue). **E**, Phage 66 (green), and (**F**) phage cocktail (red). (**D** through **F**) Control without phages (closed black circles); phages at MOI=0.01 (open circles); phages at MOI=0.1 (open diamonds), MOI=1 (open triangles), MOI=10 (closed squares), and MOI=100 (closed triangles). **G**, Time-kill assays were performed by challenging MSSA Laus102 with saline (closed circles); phage cocktail at MOI=1 (closed triangles), flucloxacillin at 1× MIC (closed diamonds) or a combination of both treatments (open squares). Means (±SDs) of 3 independent experiments performed in triplicate are shown in panels (**D**–**G**). One-way ANOVA with Tukey correction for multiple comparison statistical tests was performed to compare either areas under the curves of curves obtained at MOI=0.1 (Figures 1D through 1F) or 24-hour time points (Figure 1G). CFU indicates colony forming unit; MIC, minimum inhibitory concentration; MOI, multiplicity of infection; MSSA, methicillin-susceptible *Staphylococcus aureus*; and PFU, plaque forming unit.

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 (Δ3.22±0.55 $\log_{10}$ CFU/mL at MOI=1; Δ3.29±0.55 $\log_{10}$ CFU/mL at MOI=10), with particularly efficacious clearance being achieved at an MOI of 100 (Δ4.51±0.55 $\log_{10}$ 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±0.55 $\log_{10}$ CFU/mL relative to vB_SauH_2002 alone; $P<0.01$) (Figure 2B). Substantial synergy between the phage cocktail (MOI=1) and low-dose (1× minimum inhibitory concentration) flucloxacillin was observed (2.74±0.44 $\log_{10}$ CFU/mL additional clearance versus the phage cocktail at MOI=1 alone, $P<0.0001$) (Figure 2B).

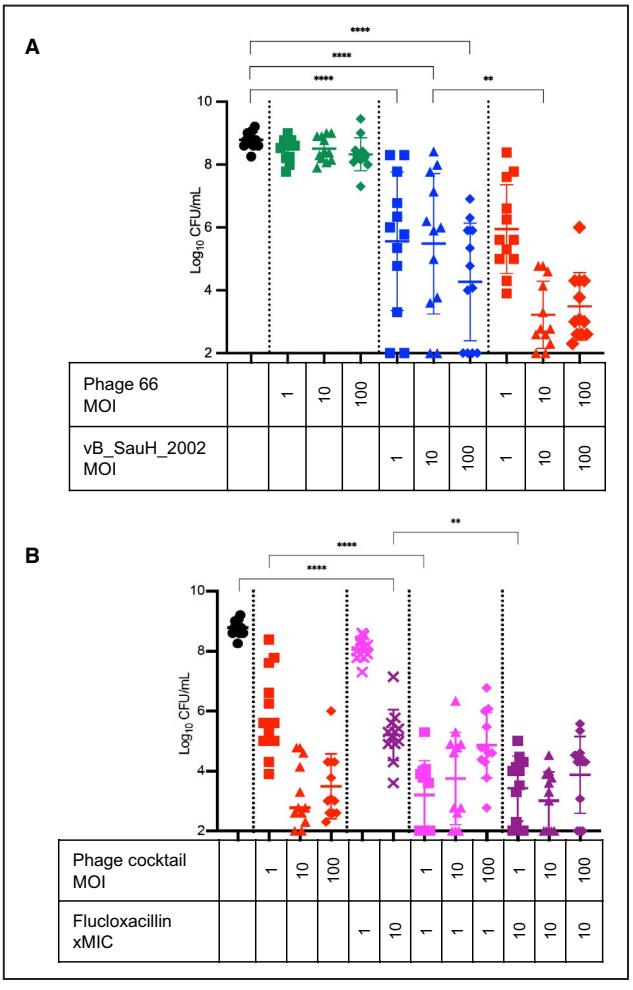


Figure 2. Activity of bacteriophages against *S. aureus* biofilms with and without flucloxacillin.
A, *S. aureus* Laus102 biofilms challenged for 24 hours with single phages or the phage cocktail. **B**, *S. aureus* Laus102 biofilms challenged for 24 hours with the phage cocktail (alone), flucloxacillin (alone), or the phage cocktail in combination with flucloxacillin. Means (±SDs) of 3 independent experiments performed in triplicate are depicted; **$P<0.01$; ****$P<0.0001$; 1-way ANOVA with Tukey's multiple comparisons test. CFU indicates colony forming unit; MIC, minimum inhibitory concentration; and MOI, multiplicity of infection.

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±0.92 $\log_{10}$ CFU/g), and mock therapy (saline) allowed the bacterial load to increase to 9.20±1.05 $\log_{10}$ CFU/g 24 hours later (Figure 3B). Bacteriostasis (bacterial load similar to before phage application, ie, 6.57±1.03 $\log_{10}$ CFU/g versus 7.22±0.92 $\log_{10}$ CFU/g at the onset of treatment) was observed 24 hours after administration of the phage cocktail (10^{10} PFU in 1 mL saline followed by 8×10^{10} PFU over 24 hours via continuous intravenous infusion at 0.3 mL/h). Similarly, bacteriostasis was observed 24 hours after administration of a low dose of flucloxacillin every 12 hours (simulating antibacterial treatment in human patients) with 5.35±3.16 $\log_{10}$ CFU/g versus 7.22±0.92 $\log_{10}$ CFU/g at the onset of treatment. In sharp contrast, the combination of both treatments had a highly bactericidal effect (2.62±1.01 $\log_{10}$ CFU/g, ie, Δ of −5.25 $\log_{10}$ CFU/g versus treatment onset, $P<0.0001$) owing to synergistic activity with the phage cocktail (Δ of −2.15 $\log_{10}$ CFU/g versus flucloxacillin alone, $P<0.01$).

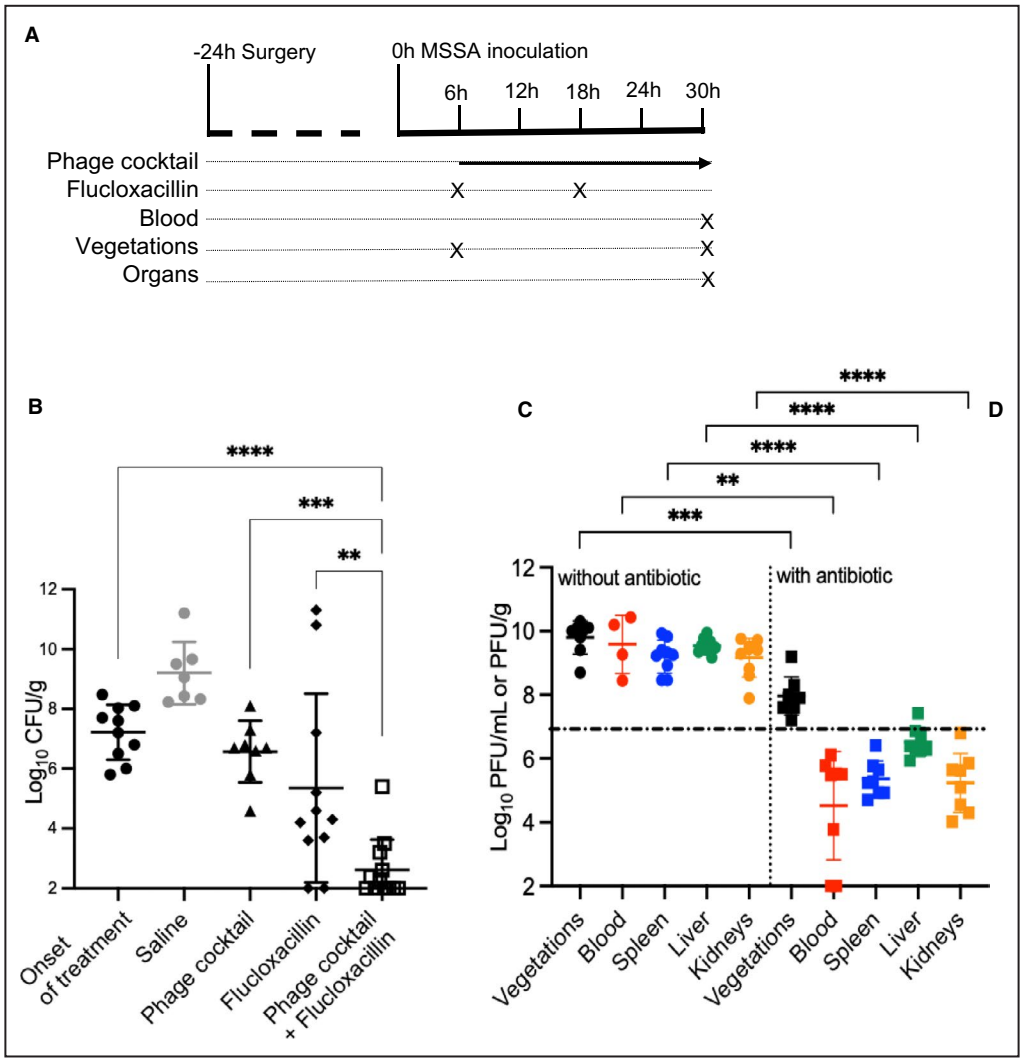


Figure 3. Treatment of EE with a two-phage cocktail in the presence or absence of low-dose of flucloxacillin.

A, Study design with sampling time points (in hours). **B**, Bacterial loads in cardiac vegetations measured at 6 hours post infection (onset of treatment) in the control rats and 24 hours after the onset of treatment in rats given a mock therapy (saline), the phage cocktail (alone), a low dose of flucloxacillin (alone), or the phage cocktail in combination with flucloxacillin. Each symbol represents an animal (N=10, 7, 8, 11, and 12, respectively). **C** and **D**, In vivo phage pharmacokinetics. The phage concentrations observed in cardiac vegetations (black), blood (red), spleen (blue), liver (green), and kidneys (orange) from rats 24 hours after initiation of treatment with either (**C**) the phage cocktail (alone) or (**D**) the phage cocktail and flucloxacillin combination. Each dot represents an animal ([**C**] N=9, 4, 9, 9, and 8, respectively; and [**D**] n=12, 11, 11, 12, and 12, respectively). The black dotted-dashed line represents the mean concentration of phages at 24 hours in the blood of healthy rats treated with the phage cocktail (alone) (N=3). Means (±SDs) are reported. ***P*<0.01; ****P*<0.001, *****P*<0.0001; 1-way ANOVA with Tukey's multiple comparisons test. CFU indicates colony forming unit; EE, experimental infective endocarditis; MSSA, methicillin-susceptible *Staphylococcus aureus*; and PFU, plaque forming unit.

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 Δ1.84±0.28 $\log_{10}$ PFU/mL, P<0.001; blood Δ5.06±0.75 $\log_{10}$ PFU/g, P<0.01; spleen Δ3.84±0.27 $\log_{10}$ PFU/g, P<0.0001; liver Δ3.02±0.18 $\log_{10}$ PFU/g, P<0.0001; and kidneys Δ3.92±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 (Δ2.35±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 *tar*S 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

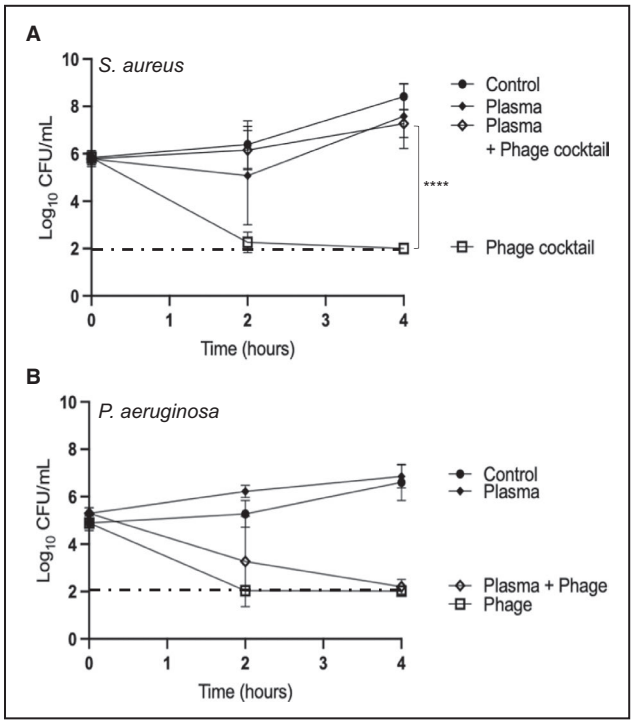


Figure 4. In vitro time-kill assays after 30-minute preincubation of the bacterial cells in 10% rat plasma.
A, Phage cocktail at MOI=100 on *S. aureus* Laus102. **B**, Phage vB_PaeM_4002 at MOI=100 on *P. aeruginosa* strain ATCC® 15442™. The mean±SD of 3 independent experiments performed in triplicates are shown. The black dotted-dashed line represents the limit of detection (100 CFU/mL). ****P<0.0001; 1-way ANOVA with Tukey's multiple comparisons test. CFU indicates colony forming unit; EE, experimental infective endocarditis; IE, infective endocarditis; MOI, multiplicity of infection; and PFU, plaque forming unit.

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.[24] 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.[25] 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),[26] 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.[17,27,28] 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.[29,30] 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.[31] Hence, inhibition of *S. aureus* phage activity by blood proteins[32] 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.[33–35] 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[36] 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.[37] 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.[26] When a subtherapeutic dosage of flucloxacillin 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, synergism 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 flucloxacillin (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*.[38] Whereas point mutations in *tarM* have been shown to alter susceptibility to *Podoviridae*,[38] 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.[39] 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.[40]

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.[41,42] 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,[43] 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,[44] based on the availability of the PhagoBurn phage cocktail.[45] The present model has far greater clinical relevance because *S. aureus* is the

predominant IE pathogen.[46] Combination therapy outperformed either phage or antibiotic treatment alone, consistent with recent publications on this topic.[15,47,48] Deciphering the mechanisms behind the observed synergy however would require a thorough investigation (eg, transcriptomic analysis[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. Synergism cannot be assumed and there are potential risks of adverse effects of combining phages with antibiotics.[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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Disclosures

GR worked as a consultant for Phagomed GmbH and is a former employee of the University of Lausanne, which licensed phage vB_SauH_2002 to PhagoMed GmBH.

Supplemental Material

Data S1
Tables S1–S3
Figure S1

REFERENCES

1. Fowler VG, Miro JM, Hoen B, Cabell CH, Abrutyn E, Rubinstein E, Corey GR, Spelman D, Bradley SF, Barsic B, et al. *Staphylococcus aureus* endocarditis: a consequence of medical progress. *JAMA*. 2005;293:3012–3021. doi: 10.1001/jama.293.24.3012
2. Wang A, Athan E, Pappas PA, Fowler VG Jr, Olaison L, Pare C, Almirante B, Munoz P, Rizzi M, Naber C, et al. Contemporary clinical profile and outcome of prosthetic valve endocarditis. *JAMA*. 2007;297:1354–1361. doi: 10.1001/jama.297.12.1354
3. Baddour LM, Wilson WR, Bayer AS, Fowler VG, Tleyjeh IM, Rybak MJ, Barsic B, Lockhart PB, Gewitz MH, Levison ME, et al. Infective endocarditis in adults: diagnosis, antimicrobial therapy, and management of complications: a scientific statement for healthcare professionals from the American Heart Association. *Circulation*. 2015;132:1435–1486. doi: 10.1161/CIR.0000000000000296
4. Chirouze C, Alla F, Fowler VG, Sexton DJ, Corey GR, Chu VH, Wang A, Erpelding M-L, Durante-Mangoni E, Fernández-Hidalgo N, et al. Impact of early valve surgery on outcome of *Staphylococcus aureus* prosthetic valve infective endocarditis: analysis in the International Collaboration of Endocarditis-Prospective Cohort Study. *Clin Infect Dis*. 2015;60:741–749. doi: 10.1093/cid/ciu871
5. Pirnay JP, Ferry T, Resch G. Recent progress towards the implementation of phage therapy in Western medicine. *FEMS Microbiol Rev*. 2021;fuab040:1–17. doi: 10.1093/femsre/fuab040
6. Rose T, Verbeken G, Vos DD, Merabishvili M, Vaneechoutte M, Lavigne R, Jennes S, Zizi M, Pirnay JP. Experimental phage therapy of burn wound infection: difficult first steps. *Int J Burns Trauma*. 2014;4:66–73.
7. Gupta P, Singh HS, Shukla VK, Nath G, Bhartiya SK. Bacteriophage therapy of chronic nonhealing wound: clinical study. *Int J Low Extrem Wounds*. 2019;18:171–175. doi: 10.1177/1534734619835115
8. Fadlallah A, Chelala E, Legeais JM. Corneal infection therapy with topical bacteriophage administration. *Open Ophthalmol J*. 2015;9:167–168. doi: 10.2174/1874364101509010167
9. Rubalskii E, Ruemke S, Salmoukas C, Boyle EC, Warnecke G, Tudorache I, Shrestha M, Schmitto JD, Martens A, Rojas SV, et al. Bacteriophage therapy for critical infections related to cardiothoracic surgery. *Antibiotics (Basel)*. 2020;9:232. doi: 10.3390/antibiotics9050232
10. Doub JB, Ng VY, Johnson AJ, Slomka M, Fackler J, Horne B, Brownstein MJ, Henry M, Malagon F, Salvage BB. Bacteriophage therapy for a chronic MRSA prosthetic joint infection. *Antibiotics*. 2020;9:241. doi: 10.3390/antibiotics9050241
11. Ferry T, Kolenda C, Batailler C, Gustave C-A, Lustig S, Malatray M, Fevre C, Josse J, Petitjean C, Chidiac C, et al. Phage therapy as adjuvant to conservative surgery and antibiotics to salvage patients with relapsing *S. aureus* prosthetic knee infection. *Front Med (Lausanne)*. 2020;7:570572. doi: 10.3389/fmed.2020.570572
12. Aslam S, Lampley E, Wooten D, Karris M, Benson C, Strathdee S, Schooley RT. Lessons learned from the first 10 consecutive cases of intravenous bacteriophage therapy to treat multidrug-resistant bacterial infections at a single center in the United States. *Open Forum Infect Dis*. 2020;7:ofaa389. doi: 10.1093/ofid/ofaa389
13. Petrovic Fabijan A, Lin RCY, Ho J, Maddocks S, Ben Zakour NL, Iredell JR, Khalid A, Venturini C, Chard R, Morales S, et al. Safety of bacteriophage therapy in severe *Staphylococcus aureus* infection. *Nat Microbiol*. 2020;5:465–472. doi: 10.1038/s41564-019-0634-z
14. Gilbey T, Ho J, Cooley LA, Petrovic Fabijan A, Iredell JR. Adjunctive bacteriophage therapy for prosthetic valve endocarditis due to *Staphylococcus aureus*. *Med J Aust*. 2019;211:142–143 e1. doi: 10.5694/mja2.50274
15. Oechslin F, Piccardi P, Mancini S, Gabard J, Moreillon P, Entenza JM, Resch G, Que YA. Synergistic interaction between phage therapy and antibiotics clears *Pseudomonas Aeruginosa* infection in endocarditis and reduces virulence. *J Infect Dis*. 2017;215:703–712. doi: 10.1093/infdis/jiw632
16. Resch G, Francois P, Morisset D, Stojanov M, Bonetti EJ, Schrenzel J, Sakwinska O, Moreillon P. Human-to-bovine jump of *Staphylococcus aureus* CC8 is associated with the loss of a beta-hemolysin converting prophage and the acquisition of a new staphylococcal cassette chromosome. *PLoS One*. 2013;8:e58187. doi: 10.1371/journal.pone.0058187
17. Prazak J, Iten M, Cameron DR, Save J, Grandgirard D, Resch G, Goepfert C, Leib SL, Takala J, Jakob SM, et al. Bacteriophages improve outcomes in experimental *Staphylococcus aureus* ventilator-associated pneumonia. *Am J Respir Crit Care Med*. 2019;200:1126–1133. doi: 10.1164/rccm.201812-2372OC
18. Tasse J, Cara A, Saglio M, Villet R, Laurent F. A steam-based method to investigate biofilm. *Sci Rep*. 2018;8:13040. doi: 10.1038/s41598-018-31437-y
19. Chin JN, Jones RN, Sader HS, Savage PB, Rybak MJ. Potential synergy activity of the novel ceragenin, CSA-13, against clinical isolates of

Pseudomonas aeruginosa, including multidrug-resistant *P. aeruginosa*. *J Antimicrob Chemother*. 2008;61:365–370. doi: 10.1093/jac/dkm457
20. Heraief E, Glauser MP, Freedman LR. Natural history of aortic valve endocarditis in rats. *Infect Immun*. 1982;37:127–131. doi: 10.1128/iai.37.1.127-131.1982
21. Que YA, Entenza JM, Francioli P, Moreillon P. The impact of penicillinase on cefamandole treatment and prophylaxis of experimental endocarditis due to methicillin-resistant *Staphylococcus aureus*. *J Infect Dis*. 1998;177:146–154.
22. Veloso TR, Chaouch A, Roger T, Giddey M, Vouillamoz J, Majcherczyk P, Que YA, Rousson V, Moreillon P, Entenza JM. Use of a human-like low-grade bacteremia model of experimental endocarditis to study the role of *Staphylococcus aureus* adhesins and platelet aggregation in early endocarditis. *Infect Immun*. 2013;81:697–703. doi: 10.1128/IAI.01030-12
23. Hernandez-Morales AC, Lessor LL, Wood TL, Migl D, Mijalis EM, Cahill J, Russell WK, Young RF, Gill JJ. Genomic and biochemical characterization of acinetobacter podophage petty reveals a novel lysis mechanism and tail-associated depolymerase activity. *J Virol*. 2018;92:1–18. doi: 10.1128/JVI.01064-17
24. Segall AM, Roach DR, Strathdee SA. Stronger together? Perspectives on phage-antibiotic synergy in clinical applications of phage therapy. *Curr Opin Microbiol*. 2019;51:46–50. doi: 10.1016/j.mib.2019.03.005
25. Hughes KA, Sutherland IW, Jones MV. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology*. 1998;144(Pt 11):3039–3047. doi: 10.1099/00221287-144-11-3039
26. Danis-Wlodarczyk K, Dabrowska K, Abedon ST. Phage therapy: the pharmacology of antibacterial viruses. *Curr Issues Mol Biol*. 2020;40:81–164. doi: 10.21775/cimb.040.081
27. Prazak J, Valente L, Iten M, Grandgirard D, Leib SL, Jakob SM, Haenggi M, Que YA, Cameron DR. Nebulized bacteriophages for prophylaxis of experimental ventilator-associated pneumonia due to methicillin-resistant *Staphylococcus aureus*. *Crit Care Med*. 2020;48:1042–1046. doi: 10.1097/CCM.0000000000004352
28. Prazak J, Valente L, Iten M, Federer L, Grandgirard D, Soto S, Resch G, Leib SL, Jakob SM, Haenggi M, et al. Benefits of aerosolized phages for the treatment of pneumonia due to methicillin-resistant *Staphylococcus aureus* (MRSA): an experimental study in rats. *J Infect Dis*. 2021;jiab112:1–8. doi: 10.1093/infdis/jiab112
29. Stulik L, Malafa S, Hudcova J, Rouha H, Henics BZ, Craven DE, Sonnevend AM, Nagy E. α-Hemolysin activity of methicillin-susceptible Staphylococcus aureus predicts ventilator-associated pneumonia. *Am J Respir Crit Care Med*. 2014;190:1139–1148. doi: 10.1164/rccm.201406-1012OC
30. Bubeck Wardenburg J, Bae T, Otto M, Deleo FR, Schneewind O. Poring over pores: α-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nat Med*. 2007;13:1405–1406. doi: 10.1038/nm1207-1405
31. Que YA, Moreillon P. Infective endocarditis. *Nat Rev Cardiol*. 2011;8:322–336. doi: 10.1038/nrcardio.2011.43
32. Rakieten ML, Zalkan G, Rakieten TL. Bacteriophage inhibition by serum. *Yale J Biol Med*. 1935;7:541–554.
33. Gratia A, Jaumain D. Dualité du principe lytique du colibacille et du staphylocoque. *C R Des Séances Soc Biol Fil*. 1921;85:882–884.
34. Gratia A, Mutsaars W. L'action inhibitrice du sérum normal sur la lyse du staphylocoque doré par les bactériophages staphylococciques polyvalents. *C R Des Séances Soc Biol Fil*. 1931;106:943–945.
35. Mutsaars W. De l'action protectrice exercée par le sérum normal sur les staphylocoques dorés, contre la fixation du bactériophage. *C R Des Séances Soc Biol Fil*. 1931;108:235–237.
36. Frati K, Malagon F, Henry M, Velazquez Delgado E, Hamilton T, Stockelman MG, Duplessis C, Biswas B. Propagation of *S. aureus* Phage K in presence of human blood. *Biomed J Sci Tech Res*. 2019;18:13815–13819.
37. Tagliaferri TL, Jansen M, Horz HP. Fighting pathogenic bacteria on two fronts: phages and antibiotics as combined strategy. *Front Cell Infect Microbiol*. 2019;9:22. doi: 10.3389/fcimb.2019.00022
38. Li X, Gerlach D, Du X, Larsen J, Stegger M, Kuhner P, Peschel A, Xia G, Winstel V. An accessory wall teichoic acid glycosyltransferase protects *Staphylococcus aureus* from the lytic activity of Podoviridae. *Sci Rep*. 2015;5:17219. doi: 10.1038/srep17219
39. Mistretta N, Brossaud M, Telles F, Sanchez V, Talaga P, Rokbi B. Glycosylation of *Staphylococcus aureus* cell wall teichoic acid is influenced by environmental conditions. *Sci Rep*. 2019;9:3212. doi: 10.1038/s41598-019-39929-1
40. Mangalea MR, Duerkop BA. Fitness trade-offs resulting from bacteriophage resistance potentiate synergistic antibacterial strategies. *Infect Immun*. 2020;88:1–15. doi: 10.1128/IAI.00926-19
41. Kebriaei R, Lev KL, Stamper KC, Lehman SM, Morales S, Rybak MJ. Bacteriophage AB-SA01 cocktail in combination with antibiotics against MRSA-VISA strain in an in vitro pharmacokinetic/pharmacodynamic model. *Antimicrob Agents Chemother*. 2020;65:1–5. doi: 10.1128/AAC.01863-20
42. Lerche CJ, Schwartz F, Theut M, Fosbol EL, Iversen K, Bundgaard H, Hoiby N, Moser C. Anti-biofilm approach in infective endocarditis exposes new treatment strategies for improved outcome. *Front Cell Dev Biol*. 2021;9:643335. doi: 10.3389/fcell.2021.643335
43. Xiong YQ, Kupferwasser LI, Zack PM, Bayer AS. Comparative efficacies of liposomal amikacin (MiKasome) plus oxacillin versus conventional amikacin plus oxacillin in experimental endocarditis induced by *Staphylococcus aureus*: microbiological and echocardiographic analyses. *Antimicrob Agents Chemother*. 1999;43:1737–1742.
44. Ramireddy S, Gudipati S, Zervos M. Expect the unexpected: a rare case of *Pseudomonas aeruginosa* endocarditis. *Idcases*. 2020;21:e00787. doi: 10.1016/j.idcr.2020.e00787
45. Jault P, Leclerc T, Jennes S, Pirnay JP, Que Y-A, Resch G, Rousseau AF, Ravat F, Carsin H, Le Floch R, et al. Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *Lancet Infect Dis*. 2019;19:35–45. doi: 10.1016/S1473-3099(18)30482-1
46. Vogkou CT, Vlachogiannis NI, Palaiodimos L, Kousoulis AA. The causative agents in infective endocarditis: a systematic review comprising 33,214 cases. *Eur J Clin Microbiol Infect Dis*. 2016;35:1227–1245. doi: 10.1007/s10096-016-2660-6
47. Morrisette T, Kebriaei R, Lev KL, Morales S, Rybak MJ. Bacteriophage therapeutics: a primer for clinicians on phage-antibiotic combinations. *Pharmacotherapy*. 2020;40:153–168. doi: 10.1002/phar.2358
48. Rodriguez-Gonzalez RA, Leung CY, Chan BK, Turner PE, Weitz JS. Quantitative models of phage-antibiotic combination therapy. *mSystems*. 2020;5:1–13. doi: 10.1128/mSystems.00756-19
49. Simon K, Pier W, Kruttgen A, Horz HP. Synergy between phage Sb-1 and oxacillin against methicillin-resistant *Staphylococcus aureus*. *Antibiotics*. 2021;10:849. doi: 10.3390/antibiotics10070849
50. Burmeister AR, Fortier A, Roush C, Lessing AJ, Bender RG, Barahman R, Grant R, Chan BK, Turner PE. Pleiotropy complicates a trade-off between phage resistance and antibiotic resistance. *Proc Natl Acad Sci USA*. 2020;117:11207–11216. doi: 10.1073/pnas.1919888117
51. CLSI: methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. In: *CLSI standards M07*. 11th, Wayne, PA: Clinical and Laboratory Institute; 2018.
52. Acs N, Gambino M, Brondsted L. Bacteriophage enumeration and detection methods. *Front Microbiol*. 2020;11:594868. doi: 10.3389/fmicb.2020.594868
53. Mastronarde DN. Automated electron microscope tomography using robust prediction of specimen movements. *J Struct Biol*. 2005;152:36–51. doi: 10.1016/j.jsb.2005.07.007
54. Kutter E. Phage host range and efficiency of plating. *Methods Mol Biol*. 2009;501:141–149.
55. Dell RB, Holleran S, Ramakrishnan R. Sample size determination. *ILAR J*. 2002;43:207–213. doi: 10.1093/ilar.43.4.207
56. Sakwinska O, Giddey M, Moreillon M, Morisset D, Waldvogel A, Moreillon P. *Staphylococcus aureus* host range and human-bovine host shift. *Appl Environ Microbiol*. 2011;77:5908–5915. doi: 10.1128/AEM.00238-11
57. Kos VN, Desjardins CA, Griggs A, Cerqueira G, Van Tonder A, Holden MT, Godfrey P, Palmer KL, Bodi K, Mongodin EF, et al. Comparative genomics of vancomycin-resistant *Staphylococcus aureus* strains and their positions within the clade most commonly associated with Methicillin-resistant *S. aureus* hospital-acquired infection in the United States. *MBio*. 2012;3:1–9. doi: 10.1128/mBio.00112-12
58. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. *J Clin Microbiol*. 2003;41:5113–5120. doi: 10.1128/JCM.41.11.5113-5120.2003
59. Cameron DR, Ramette A, Prazak J, Entenza J, Haenggi M, Que YA, Resch G. Draft genome sequence of methicillin-resistant

Staphylococcus aureus strain AW7, isolated from a patient with bacteremia. *Microbiol Resour Announc*. 2019;8:1–2. doi: 10.1128/MRA.00806-19

60. Gill SR, Fouts DE, Archer GL, Mongodin EF, Deboy RT, Ravel J, Paulsen IT, Kolonay JF, Brinkac L, Beanan M, et al. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol*. 2005;187:2426–2438. doi: 10.1128/JB.187.7.2426-2438.2005
61. Leuenberger A, Sartori C, Boss R, Resch G, Oechslin F, Steiner A, Moreillon P, Graber HU. Genotypes of *Staphylococcus aureus*: on-farm epidemiology and the consequences for prevention of intramammary infections. *J Dairy Sci*. 2019;102:3295–3309. doi: 10.3168/jds.2018-15181

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 [16], 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 [51].

Bacteriophages.

The *Podoviridae* phage 66 and *Herelleviridae* phage vB_SauH_2002 genomes are publicly available (Genbank accession no. NC_007046 and MW528836, respectively) [17]. 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 OD_{595nm} 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 ×*g* 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 $25e^-/A^2/s$) [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 200rpm. 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 [18]. Briefly, overnight cultures were diluted 1:100 in TSB, and 100-µL samples of the subsequent solution containing ca. 10^7 CFU/mL were used to inoculate 96-well polystyrene plates (Greiner Bio-One, Kremsmünster, Austria) (final concentration of bacteria ~10^6 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 [18].

Treatment of biofilm. Mature biofilms were treated for 24 h at 37 °C with 10^8 PFU/mL, 10^9 PFU/mL, or 10^{10} 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

Termination criteria:
Score of 0: no action.
Score of 1: animal is observed twice daily. If animal does not return to normal within 48 hours it will be euthanized.
Score of 2: animal is observed three times daily. If animal does not return to normal within 12 hours it will be euthanized.
When an animal reaches a score of 3, either cumulative or in one observable criteria, it will be immediately euthanized.
*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 vegetations, 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-β) = 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.

			EOP score			
***S. aureus* strain**	**Genbank access N°**	**ST**	**vB_SauH_2002**	**Phage 66**	**Covered by the phage cocktail**	**Reference**
Human carriage strains from healthy volunteers						
Laus102	JAETXI000000000.1	8	1	1	yes	[16]
Laus385	CP071350.1	8	1.5	1.25	yes	[16]
F60	NA	15	0.025	5.10^{-4}	yes	[56]
Human clinical strains						
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 (AIS1000505)*†	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)*†	AHBN00000000.1	5	0.025	0.075	yes	[57]
VRS3b (HIP13419)*†	AHBM00000000.1	5	0	0.9	yes	[57]
VRS2 (HIP11983)*†	AHBL00000000.1	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)*†	AHBO00000000.1	5	0.7	0.25	yes	[57]
ATCC 29213	LHUS00000000.2	5	0	0	no	Vicosa et al. unpublished
I37	CP071352.1	8	0.85	0.25	yes	[16]
USA300 FPR3747‡	JAFFHX000000000.1	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						
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]
M192	CP071339.1	8	0.025	0	yes	[16]
M385	CP071333.1	8	0.45	0	yes	[56]
M308	CP071336.1	8	0.65	0	yes	[16]
G03	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	[16]
M37	CP071347.1	8	0.45	0.25	yes	[16]
M5	CP071349.1	8	0.025	0	yes	[16]
M20	CP071348.1	8	0.45	1	yes	[16]
M319	CP071334.1	8	0.6	2	yes	[16]
M313	CP071335.1	8	0.6	0	yes	[16]
M124	CP071343.1	8	0.025	0	yes	[16]
M117	CP071344.1	8	0.35	0	yes	[16]
M184	NA	15	1	0	yes	[56]
M356	NA	71	0.025	0.25	yes	[16]
M159	NA	389	0.025	4.10^{-4}	yes	[16]
M323	NA	389	0.025	0	yes	[16]
M3	NA	395	0.025	0.025	yes	[16]
M75	NA	504	0.1	0.175	yes	[56]
M52	NA	504	0.025	0.9	yes	[16]
M86	CP071346.1	1650	0.6	0	yes	[16]
M126	NA	1651	0.025	3.5	yes	[16]
% 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

Non-synonymous mutations							
Contig	**Pos**	**Score**	**Ref_nt**	**Var_nt**	**Frameshift**	**Gene N°**	**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	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	aagaaagta	AAGAAAAGta	yes	2211	Transposase, IS4 family
0009	87789	17524.8	ttggtgcgg	ttTGTGTgg		2211	Transposase, IS4 family
0009	87812	17154.9	agt	aAt		2211	Transposase, IS4 family
0009	87830	10619.7	gataattcaatttttattgatggt	AATAATTCAATTTTTATTGATGGt		2211	Transposase, IS4 family
0009	87885	8389.01	ttctat	ttCCat		2211	Transposase, IS4 family
0009	88220	2276.45	atgacccaa	TTGATCCAa		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							
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	acctctgtt	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	aagaaagta	AAGAAAAGta	yes	2211	Transposase, IS4 family
0009	87789	20770.6	ttggtgcgg	ttTGTGTgg		2211	Transposase, IS4 family
0009	87812	21642.7	agt	aAt		2211	Transposase, IS4 family
0009	87830	11586.8	gataattcaatttttattgatggt	AATAATTCAATTTTTATTGATGGt		2211	Transposase, IS4 family
0009	87885	9779.11	ttctat	ttCCat		2211	Transposase, IS4 family
0009	88220	1090.55	atgacccaa	TTGATCCAa		2211	Transposase, IS4 family
0009	88235	17043.0	att	aAt		2211	Transposase, IS4 family
Synonymous mutations							
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**	**gatttttataga**	**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	aagaaagta	AAGAAAAGta	yes	2211	Transposase, IS4 family
0009	87789	19560.9	ttggtgcgg	ttTGTGTgg		2211	Transposase, IS4 family
0009	87812	20662.2	agt	aAt		2211	Transposase, IS4 family
0009	87830	11884.0	gataattcaatttttattgatggt	AATAATTCAATTTTTATTGATGGt		2211	Transposase, IS4 family
0009	87885	9920.24	ttctat	ttCCat		2211	Transposase, IS4 family
0009	88220	2783.96	atgacccaa	TTGATCCAa		2211	Transposase, IS4 family
0009	88235	17981.6	att	aAt		2211	Transposase, IS4 family
Synonymous mutations							
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	acctctgtt	acTTCTGtt		2211	Transposase, IS4 family
0010	42286	3500.84	act	acG		2285	Hypothetical protein, Lmo2313 homolog [phage A118]

Clone 18C1

Non-synonymous mutations							
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	aagaaagta	AAGAAAAGta	yes	2211	Transposase, IS4 family
0009	87789	19603.4	ttggtgcgg	ttTGTGTgg		2211	Transposase, IS4 family
0009	87812	20831.3	agt	aAt		2211	Transposase, IS4 family
0009	87830	12640.3	gataattcaatttttattgatggt	AATAATTCAATTTTTATTGATGGt		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							
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	acctctgtt	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	Gat		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	agt	aAt		2211	Transposase, IS4 family
0009	87830	7869.22	gataattcaatttttattgatggt	AATAATTCAATTTTTATTGATGGt		2211	Transposase, IS4 family
0009	87885	7835.95	ttctat	ttCCat		2211	Transposase, IS4 family
0009	88220	2190.76	atgacccaa	TTGATCCAa		2211	Transposase, IS4 family
0009	88235	15953.5	att	aAt		2211	Transposase, IS4 family
Synonymous mutations							
0001	525712	10074.7	att	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	acctctgtt	acTTCTGtt		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	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	aagaaagta	AAGAAAAGta	yes	2211	Transposase, IS4 family
0009	87789	17524.8	ttggtgcgg	ttTGTGTgg		2211	Transposase, IS4 family
0009	87812	17154.9	agt	aAt		2211	Transposase, IS4 family
0009	87830	10619.7	gataattcaatttttattgatggt	AATAATTCAATTTTTATTGATGGt		2211	Transposase, IS4 family
0009	87885	8389.01	ttctat	ttCCat		2211	Transposase, IS4 family
0009	88220	2276.45	atgacccaa	TTGATCCAa		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							
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	acctctgtt	acTTCTGtt		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.