

Citation for published version:

Alves, D, Gaudion, A, Bean, J, Pérez Esteban, P, Arnot, TC, Harper, DR, Kot, W, Hansen, LH, Enright, MC & Jenkins, ATA 2014, 'Combined use of bacteriophage K and a novel bacteriophage to reduce *Staphylococcus aureus* biofilm', *Applied and Environmental Microbiology*, vol. 80, no. 21, pp. 6694-6703.
<https://doi.org/10.1128/AEM.01789-14>

DOI:

[10.1128/AEM.01789-14](https://doi.org/10.1128/AEM.01789-14)

Publication date:

2014

Document Version

Peer reviewed version

[Link to publication](#)

This is the author's accepted version of an article published by the American Society for Microbiology in Alves, D, Gaudion, A, Bean, J, Pérez Esteban, P, Arnot, TC, Harper, DR, Kot, W, Hansen, LH, Enright, MC & Jenkins, ATA 2014, 'Combined use of bacteriophage K and a novel bacteriophage to reduce *Staphylococcus aureus* biofilm' *Applied and Environmental Microbiology*, vol 80, no. 21, pp. 6694-6703., and available online via: <http://dx.doi.org/10.1128/AEM.01789-14>

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1 **Combined use of bacteriophage K and a novel bacteriophage to reduce *Staphylococcus***
2 ***aureus* biofilm**

3

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21 **Conflict of interest statement:**

22 The authors declare that they have no conflicts of interest.

23 **ABSTRACT**

24 Biofilms are major causes of impairment of wound healing and patient morbidity. One of
25 the most common and aggressive wound pathogens is *Staphylococcus aureus*, displaying a
26 a large repertoire of virulence factors and commonly reduced susceptibility to antibiotics,
27 such as the spread of methicillin-resistant *S. aureus* (MRSA). Bacteriophages are obligate
28 parasites of bacteria. They multiply intracellularly and lyse their bacterial host releasing
29 their progeny. We isolated a novel phage, DRA88 that has a broad host range amongst *S.*
30 *aureus*. Morphologically the phage belongs to the *Myoviridae* family and comprises a large
31 dsDNA genome of 141,907 bp. DRA88 was mixed with phage K to produce a high titre
32 mixture that showed strong lytic activity against a wide range of *S. aureus* isolates
33 including representatives of the major international MRSA clones and coagulase-negative
34 *Staphylococcus*. Its efficacy was assessed both in planktonic cultures and when
35 treating established biofilms produced by three different biofilm producing *S. aureus*
36 isolates. A significant reduction of biofilm biomass over 48 hours of treatment was
37 recorded in all cases. The phage mixture may form the basis of an effective treatment
38 for infectious caused by *S. aureus* biofilms.

39

40

41 **Key words:** *Staphylococcus aureus*, bacteriophage therapy, biofilm, antibiotic resistant
42 bacteria, MRSA, sequence analysis.

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47 **INTRODUCTION**

48 *Staphylococcus aureus* is an opportunistic human bacterial pathogen that primarily
49 colonizes the anterior nares (1) but is frequently shed onto skin surfaces. When an
50 opportunity occurs that facilitates its penetration of the skin surface, it is able to cause a
51 broad spectrum of human diseases, from skin and soft-tissue infections to systemic
52 infections such as pneumonia, meningitis and osteomyelitis (1, 2). Host invasion and immune
53 evasion is possible due to a myriad of virulence factors, including toxins, adhesins and
54 evasins (3). In addition, *S. aureus* infections often comprise strains with antibiotic resistance.
55 Examples of these are penicillin and methicillin resistant *S. aureus* (MRSA) and isolates with
56 decreased susceptibility to vancomycin (4).

57

58 *S. aureus* is one of the most common gram-positive causes of wound infectious (5, 6). The
59 wound environment is an ideal one for establishment of a bacterial infection as it contains
60 large aggregations of necrotic tissue and accumulations of protein exudate (7). It is also
61 observed that wound infections are strongly associated with the formation of biofilm
62 communities (8). Once in a biofilm, bacterial cells experience greater protection against
63 antibiotics and against elements of the host immune system, when compared to cells
64 growing in a planktonic state (9). For example, the exopolysaccharide matrix blocks
65 antibody penetration into biofilm (10) and phagocytes are unable to interact with bacterial
66 cells (11). Biofilm structures are believed to be present in many acute wounds, but are very
67 common in chronic wounds (12), and are a major factor delaying wound healing (12, 13).
68 Moreover, many biofilms colonize implanted medical devices (14) greatly increasing
69 patient morbidity and mortality and they are associated with larger health care costs (15).
70 Chronic wounds affect 6.5 million patients in the United States and more than US\$25
71 billion are spent every year on treatment (16).

72 Current antibiotic options to successfully treat *S. aureus* are becoming scarce despite the
73 development of some novel drugs (17), and there is a growing need for effective agents to
74 combat infections (18). Bacteriophage therapy is a viable alternative / adjunct to antibiotics in
75 treating bacterial infection (for review see (19)). Bacteriophages (phages) are viruses able to
76 infect highly specifically and kill the bacterial species targeted, but not eukaryotic cells. The
77 phage-encoded lysis proteins – endolysin and holin - cause the breakdown of the bacterial
78 membrane (20) resulting in cell death and release of phage particles.

79

80 Several studies have shown the potential of using phages to treat *S. aureus* infections (21–23)
81 and it has been demonstrated that phages also have the ability to disrupt bacterial biofilms
82 (24). Phages are increasingly recognized as serious candidates in the fight against antibiotic
83 resistant bacteria in human therapeutics and as prophylaxis (25). Phages with strictly lytic
84 life cycles, which result in a rapid killing of the target host and diminish the chances for
85 bacteria to evolve towards phage resistance, are preferred for bacteriophage therapy use
86 (26). It is also of value for the phage (or phage mixture) used to have a polyvalent nature,
87 i.e. is able to infect a large set of strains within a species and may show improved
88 applicability in situations where the etiological agent of an infectious disease has not been
89 identified.

90

91 Here we investigate the potential of using two lytic and polyvalent *S. aureus* phages: K, a
92 well-documented staphylococcal phage (27–29) that attaches specifically to the cell wall
93 teichoic acid (30) providing a wide host range; and a newly isolated phage, DRA88. Phage K
94 was, previously, shown to be able to disrupt biofilms produced by *S. aureus* strains (28).
95 Here, we characterized the antimicrobial activity of the phages alone and in combination in
96 planktonic and biofilm systems.

97 **MATERIALS AND METHODS**

98 **Bacteria and Growth Conditions**

99 *S. aureus* strains (listed in Table 1) used in this study were from our collection of >5000
100 clinical isolates and they were selected to be genetically diverse (by multilocus sequence
101 typing (31)) and also to contain members of the major MRSA and MSSA clones present
102 worldwide. Examples of eight coagulase-negative staphylococci: *S. xylosus*, *S. sciuri* subsp.
103 *sciuri*, *S. chromogenes*, *S. hyicus*, *S. arlettae*, *S. vitulinus*, *S. simulans* and *S. epidermidis*
104 were also included in this study. Bacteria from 5% (v/v) blood agar plates were grown at 37
105 °C with constant shaking (170 rpm) in Tryptic Soy Broth (TSB). Tryptic Soy Agar (TSA)
106 and TSB-soft-agar containing 0.65% of bacteriological agar were used for bacteriophage
107 propagation and plaque count assays. Note that media was supplemented with 1mM CaCl₂
108 and MgSO₄ to improve phage adsorption (32). Bacterial aliquots were stocked at -80°C in
109 broth containing 15% glycerol (v/v).

110

111 **Bacteriophage Isolation**

112 Bacteriophages were isolated from crude sewage (Thames Water PLC, Luton, UK).
113 Bacterial enrichments with *S. aureus* isolates were performed to increase phage numbers as
114 follows: 5 ml of actively growing *S. aureus* cells (from overnight liquid culture in TSB)
115 and TSB supplemented with 1 mM MgSO₄ and 1 mM CaCl₂. The enrichment was
116 incubated overnight at 37 °C. A 10 ml aliquot was taken from the overnight culture and 1 M
117 NaCl and 0.2% chloroform were added. The culture was then centrifuged (30 min, 3000 x
118 g) to remove bacteria and the supernatant was filtered sterilized (0.22 µm pore size,
119 Millipore filter). This lysate (supernatant) was used to check the presence of lytic phages
120 using the double layer method as described previously (33). Isolated single plaques were
121 picked into SM buffer (5 M NaCl, 1M MgSO₄, 1 M Tris-HCl [pH 7.5], gelatine solution

122 distilled water) and successive rounds of single plaque purification were carried out until
123 purified plaques were obtained, reflected by single plaque morphology. Purified phages
124 were stored in 50% glycerol (v/v) at -80 °C for long term use. Short term stock
125 preparations were maintained at 4 °C.

126

127 **Bacteriophage Propagation**

128 Phage lysates were propagated on their respective bacterial hosts. Briefly, 100 µl of phage
129 lysate and 100 µl of host growing culture were mixed and left for 5 min at room temperature.
130 Afterwards 3 ml of soft-agar was added and poured onto TSA plates. The following day,
131 after an overnight incubation at 37 °C, plates displaying confluent lysis were selected and 3
132 ml of SM buffer and 2% chloroform were added before incubating at 37 °C for 4 h. High
133 titre phage solution was removed from the plates, and centrifuged (8,000 x g, 10 min) to
134 remove cell debris, then filter sterilized (pore size, 0.22 µm) and stored at 4 °C. Both phages
135 were propagated in the prophage-free isolate *S. aureus* RN4220 (40) to avoid potential
136 contamination with mobilized phages (34).

137

138 **Sensitivity Assay**

139 To determine phage sensitivity of bacterial isolates spot tests were performed. Briefly, 3 ml
140 of TSB-soft-agar was added to 100 µl of host growing culture and poured onto TSA. Plates
141 were left to dry for 20 min at 37 °C. The different phage lysates were standardized to a
142 titre of 10⁹ PFU/ml and 10 µl of each lysate were spotted onto the bacterial lawns. This
143 assay was performed in triplicate. The plates were allowed to dry before incubating
144 overnight at 37 °C. The following day the sensitivity profiles of each of the bacterial strains
145 were determined: if the bacterial lawn was lysed; slightly disrupted; or not disrupted, the
146 bacterial isolate was labelled: sensitive; intermediate; or resistant to the phage infection.

147 **Bacteriophage adsorption**

148 The experiment was carried out at 37 °C under constant shaking (60 rpm) and a phage
149 inoculum of MOI (Multiplicity Of Infection) = 0.01. The number of free phages was
150 calculated from the PFU of chloroform-treated samples within 5 min after inoculation. The
151 adsorption rate, assuming a first order kinetics was calculated in terms of the percentage of
152 free phage loss by fitting phage decay curve (normalized as a percentage) to the rate
153 equation:

154

$$155 \ln (\% \text{ phage})_t = \ln (\% \text{ phage})_o - k't \quad (1)$$

156

157 Where k' is the pseudo 1st rate constant for free phage loss:

158

$$159 k' = k[\text{bacteria}] \quad (2)$$

160

161 From this, the percentage phage remaining at any time t can be easily calculated.

162

163 **One-step growth curve**

164 Phage growth cycle parameters - the latent period (L), eclipse period (E) and burst size (B),
165 were determined from the dynamical change of the number of free and total phages.
166 Hence, one-step growth curves were measured as described by Pajunen *et al.* (35) with
167 some modifications: 10 ml of a mid-exponential-phase culture were harvested by
168 centrifugation (7,000x g, 10 min, 4°C) and resuspended in 5 ml TSB to obtain OD₆₀₀ of 1.0.
169 To this suspension, 5 ml of phage solution were added to obtain a MOI (multiplicity of
170 infection) of 0.001. Phages were allowed to adsorb for 5 min at room temperature. The
171 mixture was then centrifuged as described above and the pellet was resuspended in 10 ml of

172 fresh TSB medium. Two samples were taken every 5 min over a period of 1 h at 37 °C
173 under constant shaking. The first samples were plated immediately without any treatment
174 and the second set of samples was plated after treatment with 1 % (v/v) chloroform to release
175 intracellular phages.

176

177 **Measurement of phage zeta potential and size**

178 The particle size and electrophoretic mobility of the phages was measured by Dynamic
179 Light Scattering (DLS) using a Zetasizer Z/S (Malvern, UK) at 37 °C. Cuvettes filled with
180 the sample were carefully inspected to avoid air bubbles. Phages were diluted in dH₂O to a
181 final concentration of 10⁸ PFU ml⁻¹. Measurements were repeated at least three times.

182

183 **Electron Microscopy**

184 Phage particles in water were deposited on carbon coated copper grids and negatively stained
185 with 1% uranyl acetate (pH 4). Visualization was performed using a transmission electron
186 microscope (TEM) (JEOL JEM1200EXII, Bath, UK) operated at 120 kV.

187

188 **Phage DNA Extraction**

189 Concentrated phage preparations were obtained by a caesium chloride (CsCl) (Sigma,
190 UK) gradient composed of three different solutions with densities of: 1.35, 1.50 and 1.7 g
191 ml⁻¹. They were prepared in a 36.5 ml ultracentrifuge tube (Beckman Coulter, Seton
192 Scientific, UK). For the preparation of CsCl solution at a given density, ρ (g ml⁻¹), the
193 following equation was used to calculate the final CsCl concentration, c (g ml⁻¹): $c =$
194 $0.0478\rho^2 + 1.23\rho - 1.27$ as well as the protocol described previously by Boulanger (36).
195 After ultracentrifugation for 3 hours (75,600 g at 4 °C) the phage band was collected and

196 dialysis was performed to remove CsCl residuals. Briefly, phage suspension was washed
197 into dialysis cassettes (Slide-A-Lyser, Fisher, UK), which in turn were introduced in 500
198 volumes of dialysis buffer (10 mM sodium phosphate, pH 7) for 30 min. After performing
199 the process three times the concentrated and purified phage suspension was collected.
200 Phenol/chloroform extraction was performed according to (37). 1.8 ml of phage lysates were
201 treated with 18µl DNase I (1 mg ml⁻¹) (Sigma Aldrich) and 8 µl RNase A (10 mg ml⁻¹)
202 (Sigma Aldrich) and incubated at 37 °C for 60 min. Subsequently, 18 µl proteinase K (10
203 mg ml⁻¹) (Sigma Aldrich), 1% of sodium dodecyl sulphate (SDS) and 1 mM EDTA.Na₂
204 were added to the samples and these were incubated at 65 °C for further 60 min. All
205 protein material was eliminated by using phenol:chloroform:isoamyl alcohol (25:24:1)
206 (Sigma Aldrich) and DNA extraction and precipitation was performed as described
207 previously elsewhere (38). Nucleic acid concentration and quality was assessed with a
208 Nanodrop spectrophotometer (Thermo Scientific, UK).

209

210 **DNA sequencing, analysis and assembly**

211 DNA sequencing libraries were prepared using the Nextera® XT DNA kit (Illumina, San
212 Diego, USA) according to the manufacturer's protocol. Individually tagged libraries were
213 sequenced as a part of a flowcell as 2x250 base paired-end reads using the Illumina MiSeq
214 platform (Illumina, San Diego, USA) at The Danish National High-Throughput DNA-
215 Sequencing Centre. Reads were analysed, trimmed and assembled using 6.5.1 CLC
216 Genomic Workbench as described before by Kot *et al.* (39). Genes were predicted and
217 annotated using RAST server (40).

218 **Planktonic cultures treated with phage mixture**

219 Planktonic cultures were performed in 96-well microtitre plates and their optical density
220 was measured. In summary, a 1:100 dilution was prepared in the wells of the microplate by

221 adding 2 μ l of an overnight culture to 198 μ l of TSB. After 2 hours of incubation at 37
222 $^{\circ}$ C, the single or mixed phage at a MOI of 0.1 was added and the microplates were
223 incubated for a further 24 hours. The incubation was followed on a plate reader (FLUOstar
224 Omega, BMG LabTech, UK) where the growth curves were established at an optical
225 density at 590 nm (OD_{590}). This approach allows observation of the phage-bacteria
226 interaction over time and also allows for monitoring of the appearance of resistant mutants
227 for each phage lysate.

228

229 **Biofilm formation**

230 Biofilm assay was performed similarly to previously described methods (41), but with
231 some modifications in order to optimize the system. Biofilm formation was performed
232 in 96-well polystyrene tissue culture microplates (NunclonTM Delta Surface, Nunc, UK) to
233 achieve an improved cell attachment. TSB supplemented with 1% D-(+)-glucose (TSBg)
234 and 1% NaCl (TSBg+NaCl) was used to perform this assay as this helps to improve
235 biofilm formation (37, 38). An overnight culture was diluted to a titre of 10^8 cfu ml⁻¹.
236 Briefly, in the microplate wells a 1:100 dilution was performed by adding 2 μ l of the
237 bacterial suspension to 198 μ l of TSBg+NaCl, making a starting inoculum of 10^6 cfu ml⁻¹.
238 200 μ l of broth were added to a set of wells as a negative control. All wells were replicated
239 three times. Afterwards, microplates were incubated at 37 $^{\circ}$ C for 48 hours with no shaking
240 for biofilm formation. During the incubation time (~24 hours after incubation), 50 μ l of
241 fresh TSBg + NaCl was added to all control and test wells. Following incubation, medium
242 was poured off and wells were carefully washed twice with sterile phosphate buffered
243 saline (PBS) solution (Sigma Aldrich, UK) to remove any planktonic cells. Microplates were
244 allowed to dry for 1 hour at 50 $^{\circ}$ C. To determine total biofilm biomass microplate wells
245 were stained with 0.1% Crystal Violet (CV). After staining the wells were washed twice

246 with PBS solution and dried. Biofilm formation was determined by visual comparison of the
247 stained wells and photographed.

248

249 **Biofilm treatment with phage mixture**

250 Biofilm formation was carried out as described above. Once biofilms were established and
251 washed once with PBS, 100 µl of phage mixture in PBS was added to a set of wells. Two
252 different MOIs were set up for the single or mixed phage: 1 and 10. 100 µl of PBS were
253 added both to the isolate positive and negative controls. All the experiments were performed
254 three times. After static incubation at 37 °C microplates were washed and stained, as
255 described before, at pre-determined time-points. To perform optical density readings of the
256 staining intensity, 100 µl of 95% ethanol (v/v) was added to each well and optical density at
257 590 nm (OD590) was taken using a plate reader.

258

259 **Data analysis**

260 Comparisons between the different time points and the positive controls were made by
261 performing a *Student's t-test* and a *p-value* < 0.05 was considered statistically significant for
262 all cases. All tests were performed with a confidence level of 95%. Spread of data at the 95%
263 confidence interval (CI) was estimated using the Winpepi freeware statistical analysis
264 program (42).

265

266 **Nucleotide sequence accession number**

267 The genome sequence of phage DRA88 has been deposited in the GenBank database
268 under accession number KJ888149.

269

270

271 RESULTS

272 Isolation and host range determination

273 Several lytic phages were isolated in crude sewage samples from a water treatment facility
274 and tested against the *S. aureus* bacterial collection listed in Table 1 in order to isolate phages
275 with broad host ranges. One phage, DRA88, presented a host infectivity coverage of 60.0%
276 (95% CI: 50% - 69%; 57 isolates out of 95 were susceptible) and so was selected for further
277 studies. Phage K was also tested and showed host coverage of 64.2% (95% CI: 54% - 73%;
278 61 isolates out of 95 were susceptible). These two phages were mixed in a phage mixture
279 giving a total coverage of 73.7% (95% CI: 64% - 82%) of the *S. aureus* isolates assigned
280 to 14 different MLST (Multilocus Sequence Typing) types. Infectivity of both phages was
281 also tested on a group of coagulase-negative isolates, where DRA88 did not kill efficiently
282 any of the isolates. Phage K showed also a weakly infectivity, however two of the isolates (*S.*
283 *simulans* and *S. hyicus*) were sensitive (Table. 2).

284

285 Phage Growth Characteristics

286 The life cycle and adsorption affinity of phage DRA88 and phage K were assessed when
287 growing in RN4220 *S. aureus* host at 37 °C. Firstly, one-step growth studies were
288 performed to identify the different phases of a phage infection process. After infection,
289 phage growth cycle parameters (L - latent, E - eclipse, and B – burst size) were determined
290 (Fig. 1). In the system established, the eclipse and latent periods of DRA88 were, 15
291 min and 25 min, respectively. DRA88 yielded a burst size of 76PFU and phage K of 125
292 PFU per infected cell after 60 min. These phage life cycle values are in conformity with the
293 values normally observed for T7 group phages (43). The adsorption efficiency of phages to
294 the host was estimated with cells in the early logarithmic growth phase (Fig. 2). The
295 adsorption rate (adsorption affinity) of the phages, when infecting actively growing *S.*

296 *aureus* RN4220, was measured. From equation (1), the rate constant for the adsorption (loss
297 of free phage) for phage K and DRA88 were calculated (Fig. 2), $k' = 0.352 \text{ min}^{-1}$ and $k' =$
298 0.252 min^{-1} , respectively. Hence, although similar, after 5 minutes, 80.3% of phage K was
299 adsorbed to the bacteria and 71.6% of DRA88. After 10 min, values for free viral particles
300 were below 5% for both phages.

301

302 **Morphology of phage particles**

303 The isolated phage DRA88 was further characterized with regard to its morphology.
304 Images of the phage DRA88 were developed using Transmission Electron Microscopy
305 (TEM). The results revealed that phage DRA88 has, along with an icosahedral head of ~ 78
306 nm in diameter, a long contractile tail of ~ 179 nm with tail fibers (Fig. 3A). Therefore it
307 can be classified as belonging to the *Myoviridae* family (order *Caudoviridales*),
308 according to the classification system of Ackermann (44). It was observed for phage
309 DRA88 that besides single phage particles there were also various aggregates made
310 through contact of their tail fibers (Fig. 3B). Phage K was also observed under TEM (Fig.
311 S1), revealing a viral particle also belonging to the *Myoviridae* family (27). The zeta
312 potential of phage DRA88 and phage K calculated from the electrophoretic mobility was
313 -17 mV and -26.3 mV , respectively. The size measured was 122 nm for phage K, but
314 according to the TEM micrographs and the literature this phage has an average size of 280
315 nm total (45). This discrepancy could be due to contraction of tails, which was observed
316 frequently, and can interfere with the measurement. Note that Dynamic Light Scattering
317 (DLS) may show lower accuracy when measuring irregular shaped particles, such as tailed
318 phages, where the size is not uniform in all dimensions. Regarding phage DRA88 we were
319 not able to obtain an accurate size measurement and this can be related to the phenomena of
320 aggregation observed under TEM.

321 **Genomic characterization of DRA88 and comparison with phage K**

322 To gain a more ample understanding of the phage DRA88, its DNA was extracted and
323 genome sequencing performed. Upon assembly and annotation it was found that phage
324 DRA88 has a large double stranded DNA genome with terminal redundancy, which
325 suggests that phage DRA88 has a headful packaging system (46). The genome comprises
326 141,907 bp and can be grouped into the class III of Staphylococcal phages (>125kbp) (47);
327 204 putative coding regions and four tRNA genes were identified (Fig. 5 and Table 1 in
328 Supplementary Data). The gene coding potential with 1.44 genes per kb, exhibits a high
329 gene density. The majority of genes, 145 (71%), are found in the forward strand and 59
330 (29%) in the opposite strand. tRNA genes are all located in the reverse strand of the
331 genome. Regarding the GC% content it shows 30.4%, a lower percentage than the one
332 found in the *S. aureus* host – 32.9%. The amino acid sequence was found to share strong
333 similarities (>95%) with several other phages, such as JD007 and GH15 previously
334 sequenced (48, 49). A comparison between phage DRA88 and phage K was performed
335 using the BLAST algorithm (48). DRA88 genome seems to be organized into functional
336 modules – cell lysis, DNA replication and structural elements – highly similar to the
337 organization of phage K and other staphylococcal *Myoviridae* phages belonging to the
338 Twort-like viruses (47, 50, 51). Between these modules we can find several putative coding
339 regions that are not yet found in the NCBI database or have no attributed function
340 (phage and hypothetical proteins). These unknown functions represent 84.81% of the
341 coding capacity. Three potential coding regions (orf178, orf192 and orf195) did not have
342 any identical match with phage genes in the NCBI database. DRA88 lysin and DNA
343 polymerase are not interrupted by introns (49), contrary to phage K, but similarly to phage
344 GH15. At the end of the genome (between orf164 and orf182) there is a large coding
345 region with unidentified functions inserted into the DRA88 genome that is not

346 observed in phage K. Also, DRA88 genome analysis did not reveal similarity to known
347 virulent associated or toxin proteins.

348

349 **Bacteriophage mixture inhibits planktonic bacterial growth**

350 The efficacy of single DRA88 and phage K and their combination was assessed when treating
351 bacterial cultures. Single phages and the phage mixture (MOI = 0.1) were introduced in a
352 bacterial culture already growing for two hours under planktonic conditions and let to
353 incubate for further 22 hours (Fig. 5). Single phage treatment showed to be less
354 successful in general than the phage mixture and this was clearly observed for *S. aureus*
355 15981 isolate (Fig 5A). For all three *S. aureus* bacterial cultures – 15981, MRSA252 and
356 H325 – no cell growth was observed when the phage mixture was present compared to
357 bacterial growth only. In fact, we observed efficient inhibition of the bacterial growth for
358 15981 and H325. The same was not observed for MRSA 252, where we observed
359 bacterial growth after 18 hours of treatment (time = 20 hours).

360

361 **Biofilm eradication**

362 Biofilms produced by the three *S. aureus* isolates were established in microtitre plates.
363 All isolates were strong biofilm producers, however MRSA 252 followed by H325 isolates
364 produced lower biofilm biomass and more fragile structure (when performing the
365 mechanical washing steps the biofilm was more susceptible to disruption). The established
366 biofilms were treated with single and mixed phage at MOI 10 and the biofilm was assessed
367 (Figs 6 and 7). Measurement of biofilm density made using crystal violet / OD590 nm
368 over 48 hours showed a clear reduction following phage inoculation compared with non-
369 treated controls ($p = < 0.05$). A decrease in biofilm biomass from the mixture compared
370 with the single phages after 48 hours of treatment was seen in all cases, although not

371 significant it can be observed by eye (along with CV stained wells). Established biofilms
372 were also treated with the phage mix at two different MOIs: 1 and 10, and the biofilm was
373 assessed at the time points: 0h, 2h, 4h, 24h and 48h (Fig 8). Unsurprisingly, phage mixtures
374 with higher MOIs (10 compared with 1) gave a more rapid reduction in biofilm density,
375 although both MOIs of 1 and 10 resulted in the same endpoint after 48 hours. For *S.*
376 *aureus* 15981 biofilms treated with MOI 10, 4 hours after treatment, there was already
377 more than 50% biofilm reduction ($p\text{-value} < 0.05$) and after 48 hours of treatment the
378 biofilm biomass was almost completely disrupted (MOI 10, $p\text{-value} = 4.82 \times 10^{-3}$; MOI 1,
379 $p\text{-value} = 1.47 \times 10^{-5}$) (Fig. 8A). Figure 8B shows that biofilms produced by MRSA 252
380 were eliminated by more than 50% (MOI 10, $p\text{-value} = 0.003$; MOI 1, $p\text{-value} = 0.012$) after
381 48 hours of phage treatment. At last, biofilms produced by H325 were not initially as strong
382 as the other isolates. However we were able to observe a reduction of the biofilms as well
383 over 48 hours for MOI 10 ($p\text{-value} = 0.049$). For MOI 1 a reduction of the biofilm
384 structure was observed after 24 hours ($p\text{-value} = 0.034$). However such reduction was not
385 observed after 48 hours.

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395 **DISCUSSION**

396 *S. aureus* biofilms in wounds and catheter sites present particular problems to patients ,
397 increasing morbidity, mortality and difficulty in delivering effective chemotherapy. For
398 wound healing to occur, treatment of the biofilm infection is essential and often
399 requires selection of the correct antibiotic. The choice of appropriate chemotherapy is
400 however made more difficult due to the increasing prevalence of antibiotic resistance.
401 Hence, solutions are required to avoid treatment delay or ineffectiveness.

402

403 The host range infectivity of DRA88 against a genetically diverse collection of *S. aureus*
404 isolates was broad, showing that this is a polyvalent phage. Based on TEM analysis
405 DRA88 belongs to the *Myoviridae* tailed-phage family. DRA88 adsorption occurred
406 rapidly and at a similar rate to that observed for phage K, which is in accordance to several
407 *S. aureus Myoviridae* infecting phages (52, 53). An interesting observation for phage
408 DRA88 was the possible formation of phage aggregates. Phage aggregation is observed
409 occasionally in nature (54, 55) and is dependent on pH, ionic strength and the composition
410 of ions. Such phenomena could have been influenced by uranyl acetate (pH 4) used to
411 stain the phages for TEM observation. However, DLS measurements (in d.H₂O: ~pH 7)
412 were unsuccessful and possibly due to several sizes found in the sample (singles and
413 several aggregates), hence, we suspect that phage DRA88 is prone to form aggregates. When
414 interacting with bacterial cells, aggregation could impede phage access to the cells and
415 hence slow the rate of adsorption. Phage aggregation can be inhibited by optimization of
416 growth media composition or stabilised in nano-emulsions resulting in phages that more
417 efficiently attach to bacterial cells (56–58), consequently this could affect estimation of the
418 PFU which then may not directly correspond with the number of infective particles (MOI).

419

420 At the genome level, DRA88 was revealed to be a large dsDNA phage, usually a
421 common characteristic of *Myoviridae* virus (50), carrying a high gene density and low
422 GC%. It exhibits a high degree of relatedness to several other phages of the Twort-like
423 group, including phage K. No virulence factors in the genome were identified, according to
424 data available, suggesting that this phage could be safely used to treat *S. aureus* infections.
425 The majority of putative coding regions of DRA88 genome do not have yet any function
426 attributed yet, which is generally observed for the numerous phages being sequenced at the
427 current time (59). Consequently, there is a crucial need for a more comprehensive
428 investigation of phage genomes. Considering phage therapy as a therapeutic approach
429 option, it is extremely important that we expand our knowledge regarding phage genes and
430 proteins, and their respective functions and potentialities, as they can be involved in phage-
431 host interaction and even code for novel virulence determinants (60).

432

433 The novel isolated phage was mixed into a phage mixture with phage K and as a result their
434 lytic potential was increased (74 % of host coverage). The use of a phage mixture is
435 largely preferred over use of single phage as it results in a decreased rate of bacteria exhibiting
436 resistance (61, 62). Bacterial broth cultures growing with phage mixtures showed an
437 elimination of bacterial cells and suppression of resistant mutants. However, this was only
438 observed for two strains; after 18 hours of growth, MRSA 252 was able to counteract the
439 infectivity of the phage particles present in the culture, possibly due to the presence of phage
440 receptor mutated clones present in the culture. This situation could be overcome or delayed by
441 including more phage types to the therapeutic mixture, a task far easier (more rapid and likely
442 to succeed) to undertake compared to the discovery of a new antibiotic. It has been
443 demonstrated that phage therapy could be delivered in synchronization with another
444 antibacterial therapy, such as antibiotics (63). Reducing the initial bacterial load could be

445 sufficient to bring the bacterial numbers under control so that the drug and also the action of
446 the immune system can clear the infection (63–65) in an effective way.

447

448 More than 60% of all infections are related to the formation of biofilms (12). Hence, it is
449 important that biofilm model studies are investigated when testing a new antibacterial phage
450 mixture. Some studies already suggest the significant potential of phages to reduce and/or
451 eliminate biofilms. This is the case of phage K, where biofilms produced by *S. aureus* isolates
452 showed a remarkable decrease in their biofilm biomass after addition of the phage (28, 41).
453 Here, we showed that the phage mixture was able to reduce significantly the biofilm load on
454 the polystyrene surface a microtitre plate. For isolate 15981 the eradication effect started
455 readily after addition of the killing mixture and after 48 hours of treatment the biofilm was
456 almost completely disrupted. Higher MOI showed a more rapid effect of biofilm reduction and
457 a better prevention of biofilm regrowth was also observed for isolate H325. Comparing the
458 effect of phage mixture on broth cultures to biofilm we observed a more positive effect on
459 eliminating the bacterial load of broth cultures than that on biofilm, which is disrupted more
460 slowly. This scenario was hypothesized to be due the metabolic state of the cells. In biofilms,
461 cells can be in a low metabolic activity stage and phages cannot proliferate as efficiently as in
462 active growing cells (41). To date, very few studies have been performed regarding phage
463 mixture treatments, especially to treat *S. aureus* biofilms and in particular MRSA-caused ones.
464 Kelly *et al* (28) have already shown the efficacy of a phage K and derivatives mixture
465 on the eradication of *S. aureus* biofilms produced by non-human clinical isolates. Here we go
466 further by treating biofilms produced by prevalent human clinical isolates that also include
467 MRSA types. This study suggests that the utilisation of a mixture of bacteriophage i.e. phage
468 K and DRA88 in this case, could provide a practical alternative to antibiotic / antimicrobial
469 treatments for combating some *S. aureus* infections and in particular the devastating effects of

470 MRSA infections and biofilms related, such as burn-wound or catheter infection. Even with
471 the contribution of this study on the effectiveness of bacteriophage therapy to fight established
472 bacterial infections, there is still a long way to go and several barriers to address. The safety
473 of phages and ethical and regulatory issues, for example, must be overcome in order to phages
474 become an available alternative therapeutic (see review (66)).

475

476 In summary, here we describe the isolation and characterization of a novel bacteriophage
477 against pathogenic *S. aureus* bacteria. The phage, in combination with phage K,
478 showed an improved range of infectivity of *S. aureus* isolates and a potent effect in
479 biofilm dispersion making it a good candidate for further therapeutic development.

480

481 **ACKNOWLEDGEMENTS**

482 The authors thank Thames Water for providing the water samples and Ursula Potter for her
483 help with TEM analysis and sample preparation. The authors also thank AmpliPhi
484 Biosciences Corp for their important collaboration and technical assistance and the
485 Engineering and Physical Sciences Research Council (EPSRC) Healthcare Partnership
486 fund for their funding and support. This work was partly supported by the MetaPhageLAB
487 project financed by The Danish Research Council for Technology and Production
488 (Project: 11-106991, Doc#: 2105568).

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672

673 **TABLE LEGENDS**

674 **Table 1:** Sensitivity screening[†] of phage mixture against 95 *S. aureus* isolates.

675 Further information regarding the isolate sequence type (ST) and origin are supplied.

676 [†] Bacterial isolates were Susceptible – clear spot (S, light grey), Intermediate – turbid

677 spot (I, medium grey) or Resistant – no disturbance of bacterial lawn (R, dark grey) to

678 phage infection.

679 **Table 2:** Sensitivity screening of phage mixture against coagulase-negative staphylococci

680 isolates. [†] Bacterial isolates were Susceptible – clear spot (S, light grey), Intermediate –

681 turbid spot (I, medium grey) or Resistant – no disturbance of bacterial lawn (R, dark grey) to

682 phage infection.

683

684 **FIGURE LEGENDS**

685 **Figure 1:** Curve One-step growth of phage DRA88 (left) and phage K (right) in

686 RN4220 *S. aureus* at 37 °C. Shown are the PFU per infected cell in untreated

687 cultures (●) and in chloroform-treated cultures (○) at several time points over 60

688 minutes. The phage growth parameters are indicated in the figure and correspond to:

689 E - eclipse period; L - latent period and B - burst size. Each data point is the mean

690 of three independent experiments and error bars indicate the mean ± standard

691 deviation.

692 **Figure 2:** Percentage of free (A) DRA88 and (B) Phage K phages after infection

693 of actively growing RN4220 *S. aureus* at a MOI of 0.001 at several time points over

694 10 minutes. Rate constants for loss of phage are 0.352 min⁻¹ for Phage K and 0.252

695 min⁻¹ for DRA88. Each data point is the mean of three independent experiments and

696 error bars indicate the mean ± standard deviation.

697 **Figure 3:** Electron micrograph images of phage DRA88 negatively stained with

698 1% uranyl acetate, (A) showing the tail in a contracted position and (B)
699 formation on phage particles aggregates. Scale bar is indicated.

700 **Figure 4:** Comparative genomic analysis of phage DRA88 and phage K. Nucleotide
701 sequences were compared using the Artemis Comparison Tool (ACT). Predicted ORFs
702 are denoted by arrows, tRNAs are indicated (vertical blue dashed line) and genes encoding
703 proteins with at least 69% amino acid identity between the two genomes are indicated by
704 shaded regions.

705 **Figure 5:** Dynamic of bacteria with single and phage mixture in liquid cultures over 24
706 hours incubation at 37 °C. Absorbance readings at 590nm were taken in a microtitre plate
707 reader. *S. aureus* isolates: A – 15981; B – MRSA 252; C – H325 growing with only SM
708 buffer (○), with single DRA88 (▼), with single phage K (△) and with the phage mixture in
709 SM buffer (■) and also a negative control only SM buffer (●) are shown in the figure. Assays
710 were performed four times and OD590 was expressed as the mean ± standard deviation.

711 **Figure 6:** Normalized biofilm biomass treated with single phage K, DRA88 and the phage
712 mixture after 48 hours at MOI 10 (OD590 reading after CV staining). *S. aureus* isolates: 1 –
713 15981; 2 – MRSA 252; 3 – H325. Mean values for the three strains treated with: phage K =
714 0.63 (SD±0.10), 0.30 (SD±0.16), 0.27 (SD±0.06); DRA88 = 0.11 (SD±0.04), 0.18
715 (SD±0.08), 0.29 (SD±0.03) phage mixture = 0.06 (SD±0.02), 0.15 (SD±0.02), 0.23
716 (SD±0.02). Assays were performed three times and the mean ± standard deviation is
717 indicated. Statistically significance of biofilm reduction was assessed by performing a
718 *Student's t-test*. *p-values* are indicated (* : <0.05).

719 **Figure 7:** Visualization of wells stained with 0.1% of crystal violet after 48 hours of phage
720 treatment at MOI 10. Shown are the biofilm wells treated with PBS, phage K, DRA88 and
721 phage mixture at 0h (A) and (B) 48 hours after. Experiments were performed in triplicate.

722 **Figure 8:** Normalized biofilm biomass treated with the phage mixture over 48 hours at two

723 different MOIs (OD590 reading after CV staining). *S. aureus* isolates: A – 15981; B –
724 MRSA 252; C – H325. Assays were performed three times and the mean ± standard
725 deviation is indicated. Statistical significance of biofilm reduction was assessed by
726 performing a *Student's t-test*. *p-values* are indicated (* : <0.05).

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748 **Table 1:** Sensitivity screening[†] of phage mixture against 95 *S. aureus* isolates. Further
 749 information regarding the isolate sequence type (ST) and origin are supplied.

Isolates	ST	Country	Phage K	DRA88	Phage mixture
WBG8343	1	Australia	S	I	S
MSSA H476	1	England	S	S	S
HT2001749	1	USA	S	I	S
H148	3	UK	S	S	S
CDC980193-USA800	5	USA	S	S	S
Mu3	5	Japan	S	R	S
963Small	5	USA	S	S	S
97.1948.S.	5	Scotland	R	R	R
C56	6	UK	S	S	S
C2	7	UK	I	I	I
CDC201114-USA300	8	USA	S	S	S
15981	8	Spain	I	S	S
HT20030203	8	USA	I	S	S
HT20030206	8	USA	S	I	S
C125	8	UK	I	I	I
Fra97392	8	France	I	R	I
EMRSA6	8	UK	I	I	I
99st22111	8	-	S	S	S
H169	9	UK	S	S	S
D316	11	UK	I	I	I
D329	12	England	S	S	S
H117	12	UK	S	S	S
H402	13	UK	S	S	S
C154	14	UK	S	S	S
C357	15	UK	S	S	S
H291	18	UK	S	I	S
H42	20	UK	S	S	S
HO50960412	22	UK	I	S	S
H182	22	England	S	S	S
C720	22	England	R	S	S
C13	22	Eire	S	S	S
C49	23	UK	S	S	S
D279	25	UK	I	I	I
Not116	27	UK	S	S	S
H118	28	UK	S	I	S
SwedenAO17934/97	30	Sweden	S	I	S
Cuba4030	30	Cuba	I	I	I

C390	31	UK	S	S	S
H399	33	UK	S	S	S
C160	34	UK	I	I	I
Btn766	36	UK	S	S	S
MRSA 252	36	England	S	S	S
H325	36	UK	S	S	S
EMRSA16	36	UK	S	S	S
H137	38	UK	I	I	I
H137MRSA	38	UK	S	S	S
C253	40	UK	I	I	I
C427	42	UK	S	S	S
Fin76167	45	Finland	S	S	S
C316	49	UK	S	S	S
H417	50	UK	S	S	S
C3	51	UK	S	S	S
D49	53	UK	S	S	S
D98	54	UK	I	I	I
D318	57	UK	S	S	S
D535	59	UK	I	I	I
HT20050306	59	Australia	I	I	I
H40	60	UK	S	S	S
D473	69	UK	I	I	I
CDC201078-USA700	72	USA	I	I	I
HT20040991	80	Algeria	S	R	S
SwedN8890/99	80	Sweden	I	I	I
BK1563	88	USA	I	S	S
HT20020635	93	Australia	S	I	S
HT2001634	93	Australia	I	S	S
HT20020635	93	USA	I	S	S
Cuba4005	94	Cuba	S	S	S
D302	97	UK	S	S	S
Not38	101	UK	S	S	S
D472	109	UK	S	S	S
H560	121	UK	S	S	S
D139	145	UK	I	I	I
D22	182	UK	I	I	I
Can6428-011	188	Canada	S	S	S
D470	207	UK	I	I	I
98/10618	217	UK	S	S	S
CDC12	225	USA	S	S	S
Germany131/98	228	Germany	S	S	S
CDC16	231	USA	S	S	S

99.3759V	235	Scotland	S	I	S
SwedenON408/99	246	Sweden	I	I	I
KD121618	250	Switzerland	S	I	S
KD12943	257	UK	I	I	I
Not271	264	UK	I	I	I
Not380	266	UK	I	S	S
Not98-53	280	UK	I	S	I
CAN6820-0616	289	Canada	I	I	I
Fin62305	296	Finland	S	I	S
Not266	301	UK	S	S	S
Btn2164	312	UK	S	S	S
Btn2299	322	UK	I	I	S
Btn2289	322	UK	S	S	S
515/09	398		S	S	S
Not161	517	UK	S	I	S
Not290	529	UK	S	S	S

750 † Bacterial isolates were Susceptible – clear spot (S, light grey), Intermediate – turbid spot (I,
751 medium grey) or Resistant – no disturbance of bacterial lawn (R, dark grey) to phage
752 infection.

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764 **Table 2:** Sensitivity screening of phage mixture against coagulase-negative staphylococci

765 isolates.

Species	Isolates	k	DRA88	Phage Cocktail
<i>S. xylosum</i>	ATCC 29971	I	I	I
<i>S. sciuri</i> subsp. <i>sciuri</i>	ATCC 29062	I	I	I
<i>S. chromogenes</i>	CCM 3387	I	I	I
<i>S. hyicus</i>	CCM 29368	S	I	S
<i>S. arlettae</i>	N910 254	I	I	I
<i>S. vitulinus</i>	ATCC 51145	I	I	I
<i>S. simulans</i>	N920 197	S	I	S
<i>S. epidermidis</i>	ATCC 14990	I	R	I

766 † Bacterial isolates were Susceptible – clear spot (S, light grey), Intermediate – turbid spot (I,
767 medium grey) or Resistant – no disturbance of bacterial lawn (R, dark grey) to phage
768 infection.

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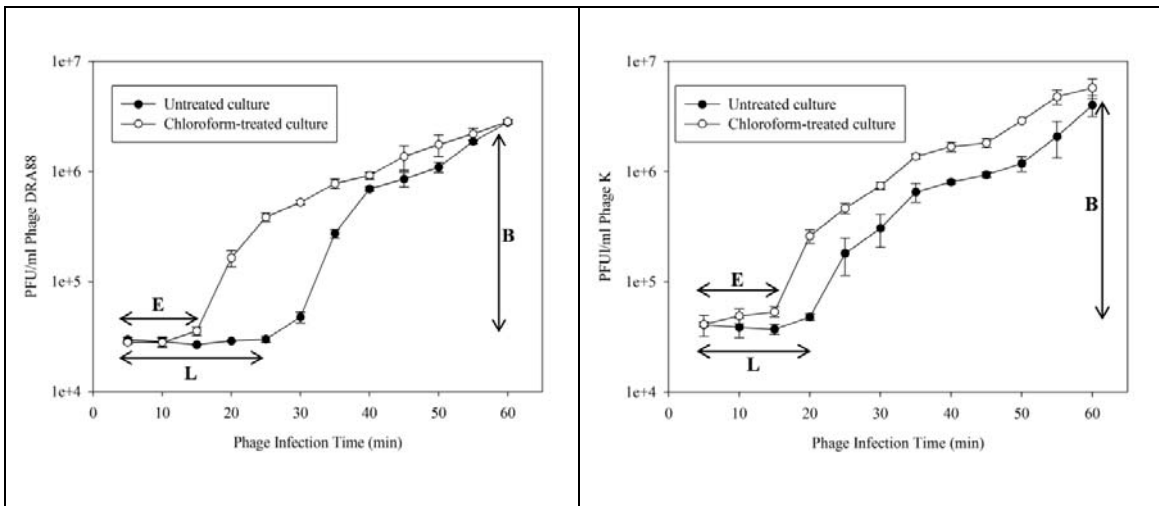


Figure 1: Curve One-step growth of phage DRA88 (left) and phage K (right) in RN4220 *S. aureus* at 37 °C. Shown are the PFU per infected cell in untreated cultures (●) and in chloroform-treated cultures (○) at several time points over 60 minutes. The phage growth parameters are indicated in the figure and correspond to: E - eclipse period; L - latent period and B - burst size. Each data point is the mean of three independent experiments and error bars indicate the mean \pm standard deviation.

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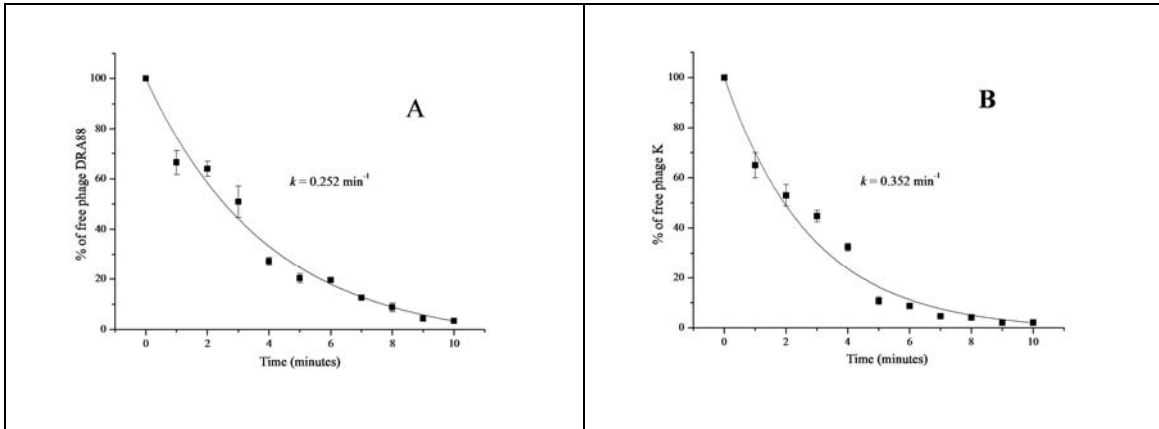


Figure 2: Percentage of free (A) DRA88 and (B) Phage K phages after infection of actively growing RN4220 *S. aureus* at a MOI of 0.001 at several time points over 10 minutes. Rate constants for loss of phage are 0.352 min^{-1} for Phage K and 0.252 min^{-1} for DRA88. Each data point is the mean of three independent experiments and error bars indicate the mean \pm standard deviation.

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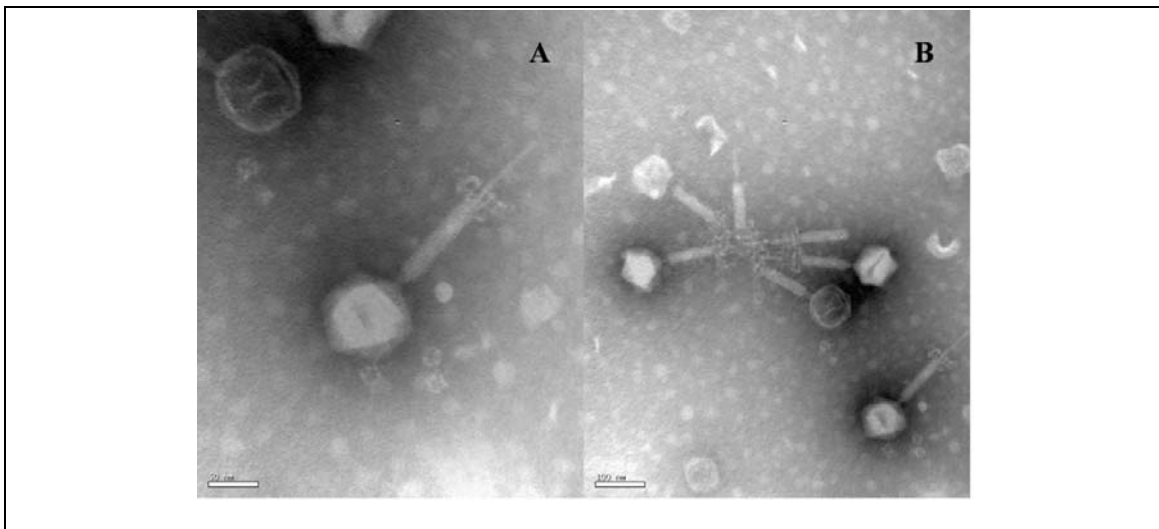
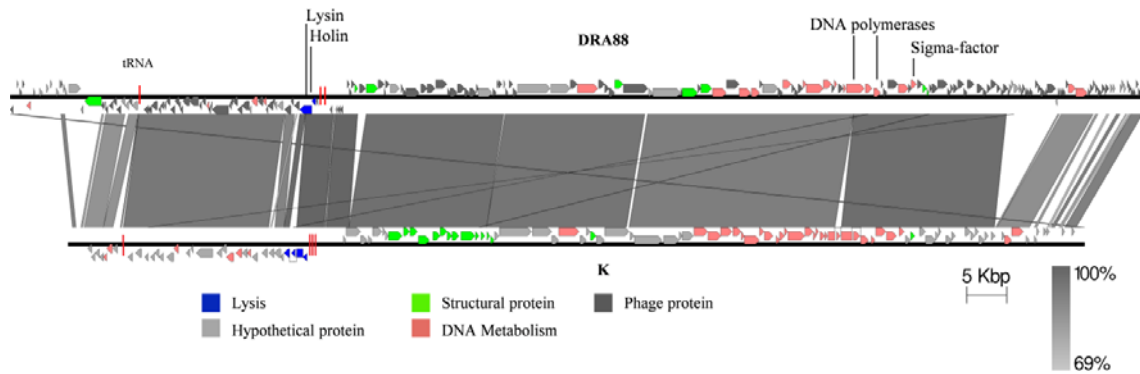


Figure 3: Electron micrograph images of phage DRA88 negatively stained with 1% uranyl acetate, (A) showing the tail in a contracted position and (B) formation on phage particles aggregates. Scale bar (50 nm (A) and 100 nm (B) is indicated.

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794 **Figure 4:** Comparative genomic analysis of phage DRA88 and phage K. Nucleotide
795 sequences were compared using the Artemis Comparison Tool (ACT). Predicted ORFs
796 are denoted by arrows, tRNAs are indicated (vertical blue dashed line) and genes encoding
797 proteins with at least 69% amino acid identity between the two genomes are indicated by
798 shaded regions.

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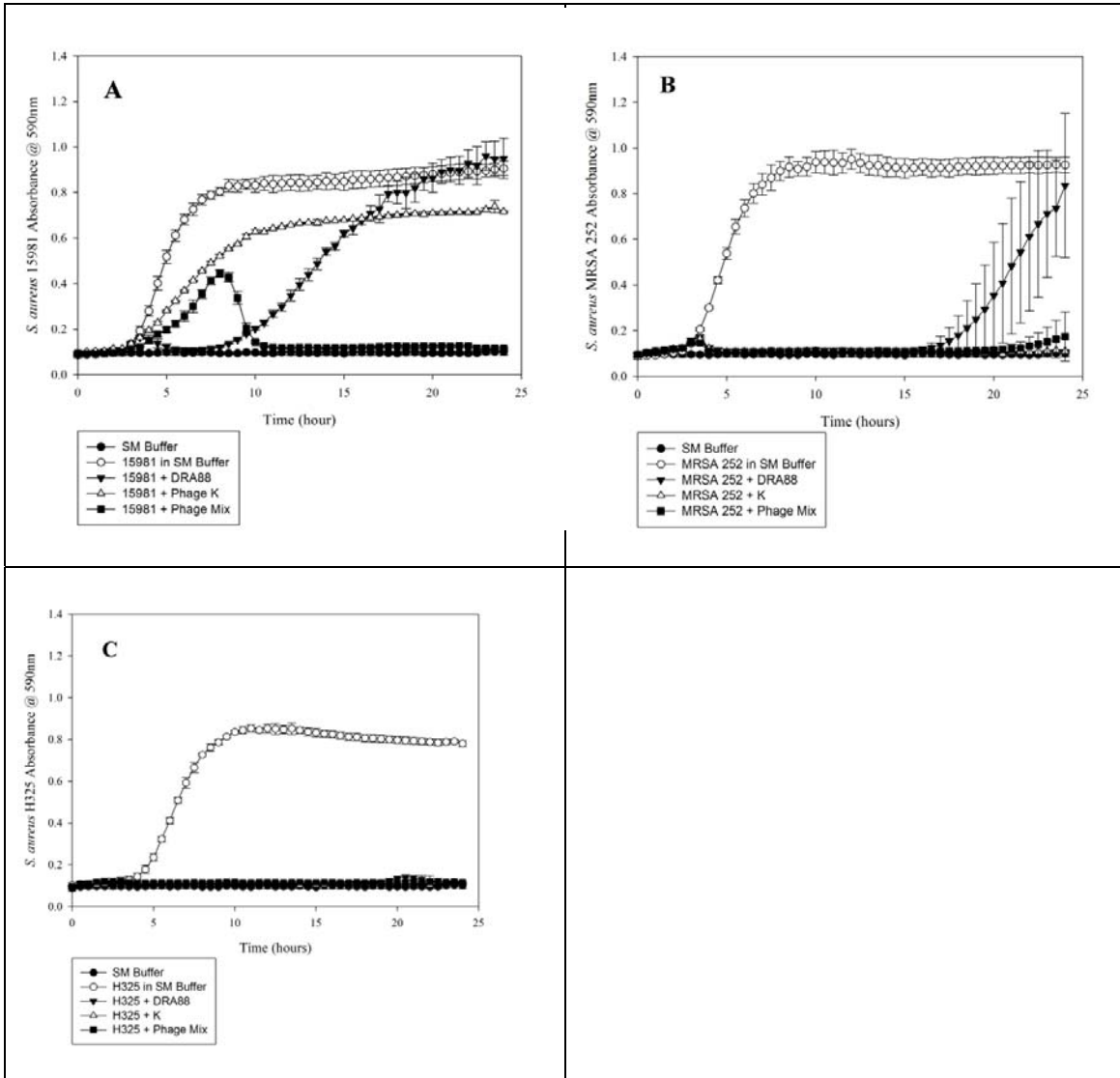


Figure 5: Dynamic of bacteria with single and phage mixture in liquid cultures over 24 hours incubation at 37 °C. Absorbance readings at 590nm were taken in a microtitre plate reader. *S. aureus* isolates: A – 15981; B – MRSA 252; C – H325 growing with only SM buffer (○), with single DRA88 (▼), with single phage K (△) and with the phage mixture in SM buffer (■) and also a negative control only SM buffer (●) are shown in the figure. Assays were performed four times and OD590 was expressed as the mean ± standard deviation.

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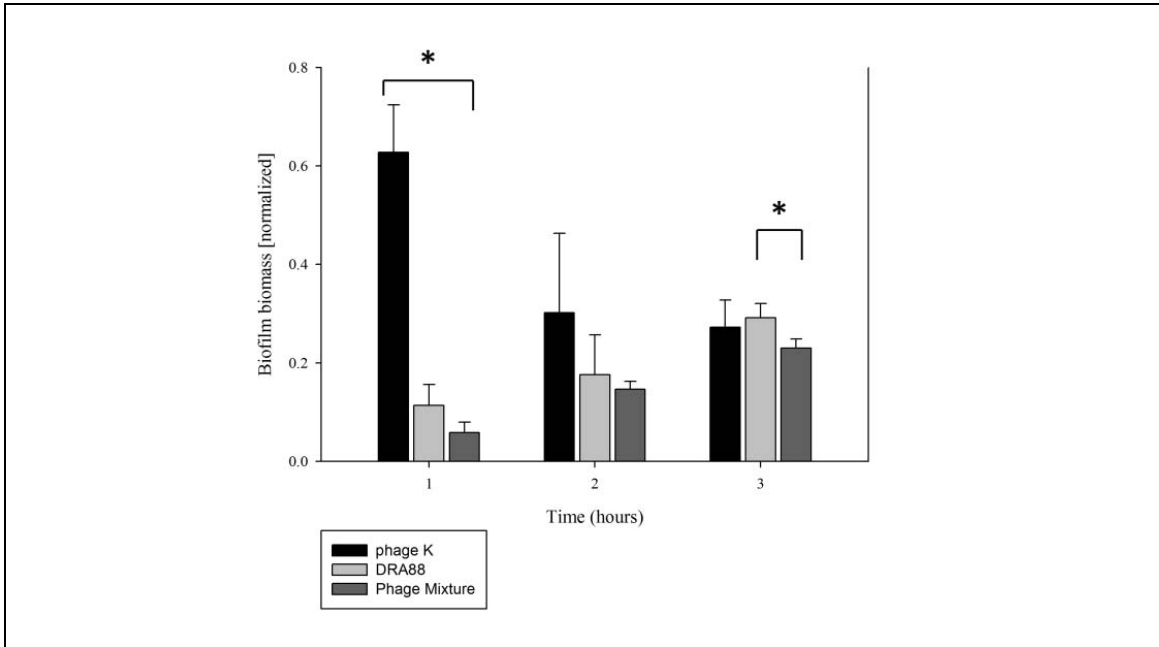


Figure 6: Normalized biofilm biomass treated with single phage K, DRA88 and the phage mixture after 48 hours at MOI 10 (OD590 reading after CV staining). *S. aureus* isolates: 1 – 15981; 2 – MRSA 252; 3 – H325. Mean values for the three strains treated with: phage K = 0.63 (SD±0.10), 0.30 (SD±0.16), 0.27 (SD±0.06); DRA88 = 0.11 (SD±0.04), 0.18 (SD±0.08), 0.29 (SD±0.03) phage mixture = 0.06 (SD±0.02), 0.15 (SD±0.02), 0.23 (SD±0.02). Assays were performed three times and the mean ± standard deviation is indicated. Statistical significance of biofilm reduction was assessed by performing a *Student's t-test*. *p-values* are indicated (* : <0.05).

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	15981		MRSA 252		H325	
Biofilm Treatment	A	B	A	B	A	B
PBS						
Phage K						
DRA88						
Phage Combination						

Figure 7: Visualization of wells stained with 0.1% of crystal violet after 48 hours of phage treatment at MOI 10. Shown are the biofilm wells treated with PBS, phage K, DRA88 and phage mixture at 0h (A) and (B) 48 hours after. Experiments were performed in triplicate.

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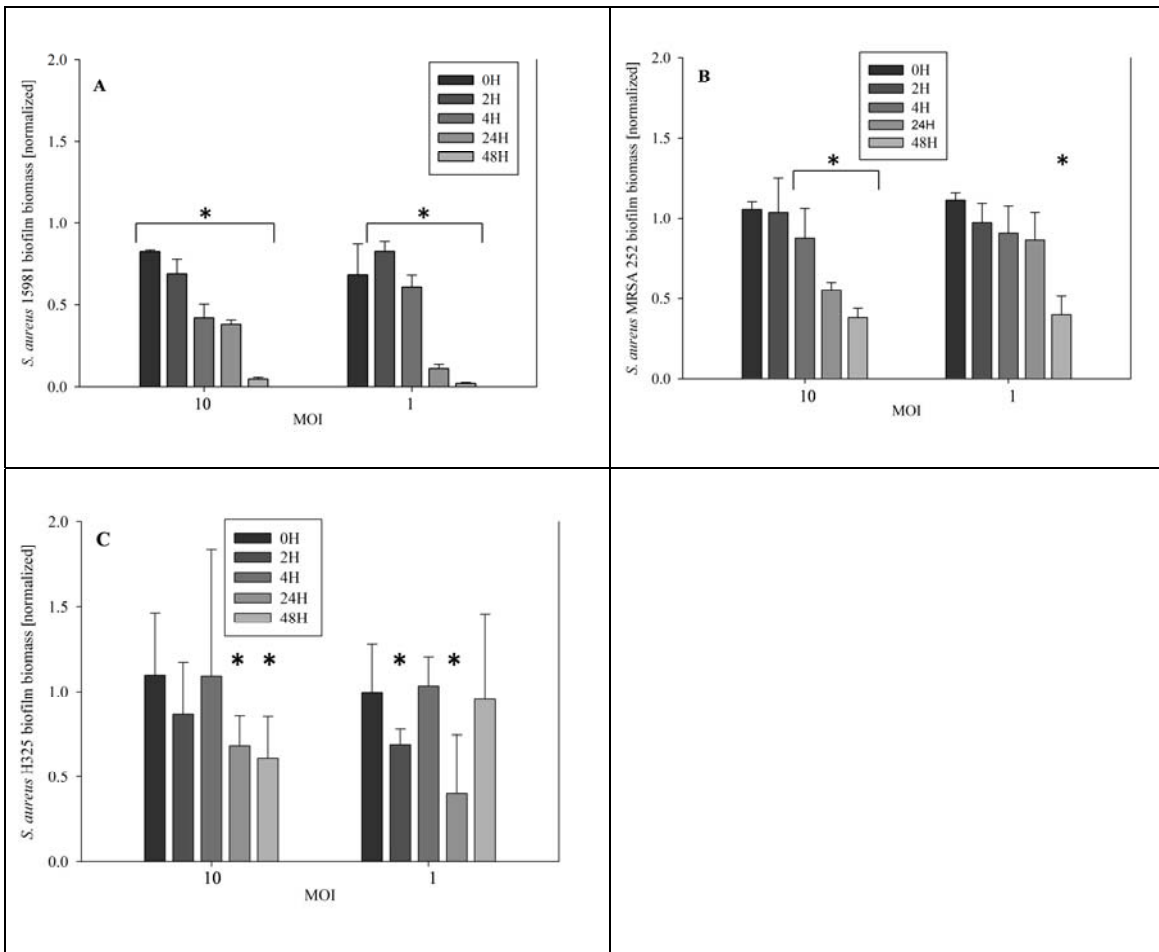


Figure 8: Normalized biofilm biomass treated with the phage mixture over 48 hours at two different MOIs (OD590 reading after CV staining). *S. aureus* isolates: A – 15981; B – MRSA 252; C – H325. Assays were performed three times and the mean \pm standard deviation is indicated. Statistical significance of biofilm reduction was assessed by performing a *Student's t-test*. *p-values* are indicated (* : <0.05).

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