Human antimicrobial peptide, LL-37, induces non-inheritable reduced susceptibility to vancomycin in *Staphylococcus aureus*

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ABSTRACT

Antimicrobial peptides (AMPs) are central components of the innate immune system providing protection against pathogens. Yet, serum and tissue concentrations vary between individuals and with disease conditions. We demonstrate that the human AMP LL-37 lowers the susceptibility to vancomycin in the community-associated methicillin-resistant *S. aureus* (CA-MRSA) strain FPR3757 (USA300). Vancomycin is used to treat serious MRSA infections, but treatment failures occur despite MRSA strains being tested susceptible according to standard susceptibility methods. Exposure to physiologically relevant concentrations of LL-37 increased the minimum inhibitory concentration (MIC) of *S. aureus* towards vancomycin by 75%, and resulted in shortened lag-phase and increased colony formation at sub-inhibitory concentrations of vancomycin. Computer simulations using a mathematical antibiotic treatment model indicated that a small increase in MIC might decrease the efficacy of vancomycin in clearing a *S. aureus* infection. This prediction was supported in a *Galleria mellonella* infection model, where exposure of *S. aureus* to LL-37 abolished the antimicrobial effect of vancomycin. Thus, physiological relevant concentrations of LL-37 reduce susceptibility to vancomycin, indicating that tissue and host specific variations in LL-37 concentrations may influence vancomycin susceptibility *in vivo*.

33 ## Introduction

34 Bacterial resistance to antibiotics is generally associated with genetic changes, such as point mutations in the
35 core genome or acquisition of resistance genes present on mobile genetic elements[1] or intrinsic resistance to
36 antimicrobials, such as in-permeability of the outer membrane of Gram-negative bacteria or the active efflux of
37 antimicrobials provided by chromosomally encoded efflux pumps[2]. Previously, we showed that exposure to the
38 synthetic antimicrobial peptide (AMP) colistin reduces susceptibility to vancomycin in the clinically important,
39 methicillin resistant *Staphylococcus aureus* (MRSA) strain USA300 via induction of the GraRS cell wall regulon[3].
40 *S. aureus* is a human pathogen that causes a variety of diseases and particularly the MRSA clones are becoming
41 widely distributed both in the hospital environment and in the community[4]. MRSA infections are often treated
42 with the glycopeptide antibiotic vancomycin. The CLSI susceptibility breakpoint for vancomycin is 2 µg/ml[5].
43 However, in the last decade vancomycin-intermediate *S. aureus* strains (VISA) have emerged with reduced
44 vancomycin susceptibility (MIC = 4-8 µg/ml)[6]. These strains are characterized by chromosomal mutations that
45 reduce negative cell wall charge[7], increase cell wall thickness[8] or reduce Triton-X mediated autolysis[9]. Even for
46 non-VISA strains vancomycin treatment failures have been linked to strains displaying MIC in the higher end of
47 the susceptibility range (≥1.5 µg/ml)[5,10-13]. Our previous work revealed that upon colistin exposure, *S. aureus*
48 cells transiently and reversibly induce cellular changes that result in a phenotypic VISA-like appearance[3]. This
49 reduced vancomycin susceptibility state will remain undetectable by routine clinical testing and thus the MIC of
50 vancomycin upon colistin exposure *in vivo* may be higher than expected from such testing.

51 Colistin is a cationic AMP used in treatment of Gram-negative infections, however AMPs are also major
52 components of the innate immune system and a critical defence against a large number of pathogens[14]. Human
53 AMPs are small, often cationic peptides with broad-spectrum antimicrobial activity caused by membrane-
54 damaging pore-formation[14]. An example is the cathelicidin, LL-37[15], which is expressed in a variety of immune
55 cells, including polymorphonuclear leukocytes (PMN), the first line of defence against invading bacterial

56 pathogens. In the present study, we demonstrate that the human AMP LL-37 transiently reduces susceptibility

57 to vancomycin.

58 ## Materials and methods

59 *Bacterial strains and growth conditions*

60 *S. aureus* USA300 FPR3757[16] was obtained from the American Type Culture Collection (ATCC). *S. aureus* strains

61 were routinely grown in Mueller-Hinton (MH) medium (Sigma) at 37°C in Erlenmeyer flasks with aeration.

62

63 *Minimum inhibitory concentration*

64 To assess vancomycin susceptibility of *S. aureus* exposed to LL-37 (Sigma), *S. aureus* FPR3757 was diluted to 10^5

65 CFU/ml in Mueller-Hinton (MH) media and incubated for 1 h (200 rpm, 37 °C). Pre-treatment with either 10 or

66 20 µg/ml LL-37 for 1 h was followed by supplementation of vancomycin (Sigma) and the minimum inhibitory

67 concentration (MIC) was determined in a 96-well microtiter plates (200 rpm, 37 °C, 24 h using technical

68 duplicates and biological triplicates).

69

70 *Vancomycin susceptibility testing by plating*

71 Exponential phase *S. aureus* FPR3757 cultures were adjusted to OD_{600} = 0.25 in pre-heated MH broth and was

72 then treated with sub-inhibitory concentrations of LL-37 (0, 5, 10, 20 and 40 µg/ml) for 1 h. The cultures were

73 adjusted to OD_{600} = 0.1 before spotting 10 µl aliquots of serial four-fold dilutions on freshly prepared MH agar

74 plates containing increasing concentrations of vancomycin (0, 1, 1.25 and 1.5 µg/ml).

75 In one experiment, cultures of *S. aureus* FPR3757 were grown with 10 µg/ml LL-37 for 60 min and LL-37 was

76 removed from the medium by spinning (5,000g, 5 min, room temperature) and re-suspended in pre-heated MH

77 broth in a subset of the culture, while exposure to LL-37 was maintained in the other subset. After 0, 60 and 90

78 min, the cultures where plated on agar plates containing vancomycin (1.5 µg/ml).

79 The agar plates were inspected for growth following 24 h incubation.

80 *Growth experiments*

81 Overnight cultures were diluted to approx. 10^5 CFU/ml in fresh MH broth, and incubated for 1 h at 37°C and
82 200 rpm. The culture was then split in appropriate aliquots and treated with LL-37 or left untreated for 1 h at
83 37°C and 200 rpm. The untreated and pre-treated cultures were then split in appropriate aliquots and treated
84 with vancomycin (Sigma) or left untreated. These were then further aliquoted into the wells of a 100-well
85 honeycomb plate and incubated in a Bioscreen C Analyzer (Oy Growth Curves Ab Ltd, Helsinki, Finland) at 37°C
86 and medium continuous shaking.

87

88 *Determination of Triton X-100 induced autolysis*

89 Strains were grown in MH broth at 37°C, 180 rpm, to mid-exponential phase. The cultures were diluted to an
90 OD_{600} of 0.15 in warm MH, grown to an OD_{600} of 0.25, and 10 µg/ml LL-37 was added to one subset of cultures
91 while another subset of cultures served as the unexposed control. After 1 h, the cultured cells were washed
92 twice in ice-cold sterile distilled water and resuspended in the same volume of 0.05 M Tris-HCl (pH 7.2)
93 containing 0.05% Triton X-100. Cells were incubated at 30°C, and the OD_{600} was measured every 30 min. Data
94 are expressed as the percent loss of OD_{600} at the indicated times compared to findings at time zero. Each data
95 point represents the mean and standard deviation from three independent experiments.

96

97 *A model of antibiotic treatment*

98 Using previous models[17], the following model was derived to test the effect of a higher MIC on clearance when
99 treated with the same antibiotic concentration. In a continuous flow at rate *w* a bacterial population (P) is
100 limited by the concentration of a resource (R; µg/ml). An antibiotic of concentration (A; µg/ml) is dosed every 8
101 hours and the concentration declines exponentially at rate *d* per day, due to flow from the site of infection. The
102 population replicates at maximum rates V_{MAX} and the rates of growth are proportional to the concentration of

103 the resource and antibiotic. For the antibiotic-mediated killing, we assume hill functions so that at an antibiotic

104 concentration of A and resource concentration of R, the net growth rate of the P population is,

$$\psi_P(A,R) = \left(V_{MAX} - \left(\frac{(V_{MAX} - V_{MIN})\left(\frac{A}{MIC}\right)^k}{\left(\frac{A}{MIC}\right)^k - \left(\frac{V_{MIN}}{V_{MAX}}\right)} \right) \right) \frac{R}{(R+k)}$$

105 where V_{MIN} is the minimum growth (maximum kill) rates and MIC the corresponding minimum inhibitory

106 concentrations of the antibiotic. The parameter κ controls the shape of this function[17]. To account for the

107 resource limitation, we use the function R/(R+k) where k, the Monod constant, is the concentration of the

108 limiting resource where in the absence of the antibiotic the rate of is half its maximum value. The bacteria take

109 up resource at rates proportional to their maximum growth rates and a conversion efficiency parameter, e

110 (μg/cell). The population is maintained in continuous culture (chemostat-like) habitat into which resources

111 from a reservoir at a concentration of C μg/ml enter at a constant rate, w per hour, which is the same rate at

112 which excess resources, bacteria and antibiotics are removed. With these definitions and assumption, the rate

113 of change in the densities of the different populations of bacteria and concentration of antibiotics

$$\frac{dR}{dt} = w(C - R) - e\frac{R}{R+k}V_{MAX}P$$

$$\frac{dP}{dt} = \psi_P(A,R)P - wP$$

$$\frac{dA}{dt} = -A(w + d)$$

114 where d is the rate of decay in the concentration of the antibiotic.

115 The simulation was programmed in Berkeley Madonna™ using Euler.

116

117 *Galleria mellonella infection model*

118 Twenty healthy 5th instar larvae each weighing 250 mg were injected with 2.5 x 10^6 CFU of *S. aureus* FPR3757
119 followed by 10 µg/ml LL-37 or 0.9 % NaCl for controls. One hour post infection, the larvae were treated with 15
120 mg/kg bodyweight of vancomycin, corresponding to a relevant clinical dose. The LL-37 pre-treatment and
121 antimicrobial therapy was repeated every 24 h for a total of 72 h and survival was monitored. Controls for
122 toxicity of LL-37 combined with the antimicrobials as well as for non-treated *S. aureus* infection were included
123 with all larvae receiving an equal number of injections of either active compound or 0.9 % NaCl.

124

125 *Statistics*

126 The data were analyzed in GraphPad Prism 7 (GraphPad Software Inc.) using one-way analysis of variance
127 (ANOVA) with a *post hoc* analysis of Dunnett's multiple comparison tests, where $P < 0.05$ (*), $P < 0.01$ (**) and
128 $P < 0.001$ (***). The survival data was analyzed by using the Kaplan-Meier method and statistical difference
129 determined, using log-rank test.

130 ## Results

131 *Pre-exposure to LL-37 reduces S. aureus susceptibility to vancomycin*

132 We have previously shown that pre-exposure of *S. aureus* to the antimicrobial peptide, colistin, reduces
133 vancomycin susceptibility[3]. To address if exposure to the human antimicrobial peptide LL-37 also affects
134 vancomycin susceptibility we initially measured the minimum inhibitory concentration (MIC) towards
135 vancomycin in the presence or absence of LL-37. While untreated *S. aureus* FPR3757 displayed vancomycin MIC
136 of 0.8 µg/ml, pre-treatment with LL-37 (MIC_{LL-37} = >128 µg/ml) for one hour at sub-inhibitory concentrations, 10
137 or 20 µg/ml, displayed vancomycin MIC of 1.2 and 1.4 µg/ml, respectively. To further examine the effect of LL-
138 37 on vancomycin susceptibility, we spotted serial dilutions of cultures pre-treated with various sub-inhibitory
139 concentrations of LL-37 on agar plates containing increasing concentrations of vancomycin. Cultures exposed
140 to 40 µg/ml LL-37 were able to form colonies in the presence of 1.5 µg/ml vancomycin in contrast to untreated
141 cells, where no growth was observed (Figure 1).

142 When observing the growth dynamics of treated and untreated cells in liquid cultures, pre-treatment with LL-
143 37 notably reduced the vancomycin induced lag-phase measured as the time from inoculation until density
144 reached OD_{600} = 0.1 (Figure 2a and b). In the presence of vancomycin (at 0.75 µg/ml) the lag-phase was 24.5
145 (±1.9) h for untreated cells, whereas the lag-phase was 8.1 (±2.5) h following pre-exposure to 20 µg/ml LL-37
146 ($p<0.001$). Shortening of the lag phase was proportional to the concentration of LL-37 used for pre-treatment
147 (Figure 2a and b). In the absence of vancomycin, LL-37 treatment did not affect growth kinetics (Figure 2a).
148 When determining maximum growth rate, no effect of LL-37 was observed in the vancomycin-treated or
149 control cultures (Figure S1), indicating that shortening of lag-phase rather than growth rate is important for the
150 LL-37 induced reduction in vancomycin susceptibility.

151 *LL-37 induces reversible vancomycin tolerance*

152 To examine if continuous LL-37 exposure is needed for the reduced vancomycin susceptibility we monitored
153 the LL-37 exposure time required before being able to observe reduced susceptibility to vancomycin and how
154 long the cells retained the reduced susceptibility after removal of LL-37. We found that the phenotype was
155 observed already after 10 minutes of exposure to LL-37 and that it lasted approximately 60 minutes after LL-37
156 was removed from the growth medium (Figure 3). These results demonstrate that the reduced vancomycin
157 susceptibility induced by LL-37 exposure is transient and non-inherited.

158

159 *LL-37 reduces autolysis*

160 Reduced susceptibility to vancomycin in genetically stable VISA strains are characterized phenotypically by a
161 decrease in Triton X-100 induced autolysis[9]. We examined the effect of LL-37 exposure (10 µg/ml) on autolysis
162 as described previously[3,7]. Compared to an un-treated control, the pre-treated culture showed less Triton X-100
163 induced autolysis (Figure 4).

164

165 *In silico model*

166 Clinical isolates of vancomycin susceptible MRSA strains with MIC in the higher end of the susceptibility range
167 (≥1.5 µg/ml) are linked to vancomycin treatment failure[5]. Such treatment failures may be related to
168 unidentified host factors, which are causing an increase in *S. aureus* vancomycin MIC[18]. Since we observed an
169 increase in vancomycin MIC after exposure to relevant concentrations of human LL-37 in the laboratory, we
170 explored the potential clinical implications *in silico* using a mathematical antibiotic treatment model to
171 simulate situations of constant concentrations of antibiotic and variable MIC. As illustrated by this model, if the
172 concentration of antibiotic is significantly higher (≈5x) than the MIC, small increments in MIC do not markedly
173 affect the rate of clearance (Figure 5). However, the lower the antibiotic concentration is at the site of

174 infection, the more impact the increased MIC will have on the rate of clearance. Maximum allowable
175 vancomycin concentration during treatment is limited by the nephrotoxic properties of the drug[19] and thus
176 clinical data[5] and our computer simulations indicate that the relatively small increase in MIC observed when *S.*
177 *aureus* is exposed to clinically relevant LL-37 concentrations may have a negative impact on the efficacy of
178 vancomycin in clearing an infection.

179

180 *LL-37 abolishes antimicrobial effect of vancomycin in Galleria mellonella*

181 The observed *in vitro* reduction in vancomycin susceptibility after LL-37 exposure combined with the
182 predictions of treatment failure associated with modest increments in MIC from our computer model
183 prompted us to test the efficacy of vancomycin in treating *Galleria mellonella* wax moth larvae[20] infected with
184 *S. aureus* FPR3757 in the presence or absence of LL-37. In the absence of LL-37, vancomycin reduced the
185 mortality inflicted by the MRSA strain. However, in the presence of LL-37, the antimicrobial potential of
186 vancomycin was completely abolished ($P<0.001$), rendering the larvae as susceptible to *S. aureus* killing as the
187 non-treated *S. aureus* infection (Figure 6).

188 ## Discussion

189 Antimicrobial chemotherapy is currently based on standardized *in vitro* antimicrobial susceptibility testing,
190 however the assay can face limitations in predicting *in vivo* treatment efficacy[21]. The lack of predictive value is
191 partly attributed to the fact that the growth media in which the susceptibility tests are conducted, do not
192 sufficiently represent the *in vivo* environment. Several factors, such as nutrient availability, oxygen level, pH
193 and presence of innate immune components, have been identified to affect antibiotic susceptibility in both
194 Gram-positive and –negative species[22-25]. Here we show that the presence of the antimicrobial peptide LL-37
195 of the innate immune response antagonizes the activity of vancomycin against *S. aureus in vitro*. This effect is
196 transient and is also evident in the *G. mellonella* infection model, where the presence of LL-37 with vancomycin
197 significantly reduces survival of *G. mellonella* larvae compared with vancomycin treatment alone. Our data
198 suggest that the MIC values reported from *in vitro* assays may underestimate the vancomycin MIC *in vivo* in the
199 presence of LL-37.

200 Vancomycin is one of the most important antibiotics for treatment of MRSA infections[26]. The CLSI susceptibility
201 breakpoint for vancomycin is 2 µg/ml[5]. Treatment failures reported for infections with MRSA strains being
202 susceptible to vancomycin has led to discussions whether the MIC breakpoint at 2 µg/ml should be further
203 decreased[5]. A meta-analysis associated high-MIC (≥ 1.5 µg/ml) MRSA isolates with increased risk of mortality
204 compared with low-MIC (< 1.5 µg/ml) MRSA isolates[5], thus indicating that even minor increases in vancomycin
205 MIC can negatively affect treatment outcome and survival of patients with MRSA infections.

206 The impact of LL-37 on vancomycin susceptibility under *in vivo* conditions may vary from person to person.
207 Plasma levels of LL-37 in healthy individuals have been measured to be approximately 1 µg/ml, but with
208 variations between individuals from 0.5 to 3 µg/ml[27]. In areas of bacterial infection the local concentration will
209 reach even higher levels due to de-granulation of PMNs, e.g. in bronchoalveolar lavage fluid, where LL-37
210 concentrations from 5 to 25 µg/ml have been measured in infected individuals[28,29]. Furthermore, LL-37 levels

211 are also influenced by disease conditions, where for example psoriasis patients have increased LL-37 levels[30],

212 while atopic dermatitis patients have reduced levels[31]. Thus, it is conceivable that person-to-person variation in

213 LL-37 levels could influence susceptibility to vancomycin pointing to the importance of host factors in

214 antimicrobial chemotherapy.

215 Collectively, our results indicate that a better understanding of the host's role in the development of reduced

216 vancomycin susceptibility could be important to design treatment regimens and improve clinical outcome.

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C.F., J.H., B.L. and H.I. conceived and designed the study. Experiments were performed by C.F., A.F. and J.H. Analysis of data and drafting of the manuscript was performed by C.F., J.H., M.V., V.P., B.L. and H.I. All authors read and approved the final manuscript.

Competing interests

None to declare.

Ethical approval

Not required.

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312

313 ## Figure legends

314 **Figure 1. LL-37 influences vancomycin susceptibility of *S. aureus*.**

315 Exponential phase *S. aureus* FPR3757 cultures were pretreated with 0, 5, 10, 20 and 40 µg/ml LL-37 for 1 h
316 before spotting serial four-fold dilutions on MH agar containing increasing concentrations of vancomycin.

317

318 **Figure 2. LL-37 influences vancomycin susceptibility of *S. aureus* in liquid culture.**

319 (a) The growth of *S. aureus* FPR3757 with (black lines) or without (grey lines) exposure to 0.75 µg/ml
320 vancomycin after treatment for 1 h with 0, 5, 10 or 20 µg/ml LL-37. The curves are an average of five replicates.
321 (b) The lag-phase of the same cultures depicted in (a) (n=5). Error bars display 95% confidence intervals.
322 **P<0.01; *P<0.05.

323

324 **Figure 3. Transient induction of reduced vancomycin susceptibility by LL-37.**

325 (a) Cultures of *S. aureus* FPR3757 were grown without (left) or with (right) 10 µg/ml LL-37 for 10 minutes and
326 spotted on vancomycin plates (1.5 µg/ml). (b) Cultures of *S. aureus* FPR3757 were grown with 10 µg/ml LL-37
327 for 60 min and LL-37 was removed from the medium in a subset of the culture (left), while exposure to LL-37
328 was maintained in the other subset (right). After 0, 60 and 90 min, the cultures where plated on agar plates
329 containing vancomycin (1.5 µg/ml).

330

331 **Figure 4. LL-37 exposure reduces Triton X-100 mediated autolysis.**

332 LL-37 induced autolysis measured as reduction of bacterial density over time in a 0.05% Triton X-100 solution
333 containing treated (10 µg/ml LL-37) or untreated cultures. Error bars display 95% confidence intervals, (n=3).

334

335 **Figure 5. Computer simulation of the effects of increased vancomycin MIC on the clearance of an infection**

336 **treated with the same antibiotic concentration.**

337 The full, dashed or double lines represent populations with MICs of 0.8, 1.2 or 1.4 µg/ml, respectively. The

338 black, green and orange lines represent exposure to 10, 5 or 2.5 µg/ml antibiotic, dosed every 8 hours.

339 Parameter values: V_{MAX}=1, V_{MIN}=-3, k=1, e=5x10^{-7}, w=0.5 and C=1000.

340

341 **Figure 6. Influence of LL-37 on vancomycin treatment of *S. aureus* infecting *G. mellonella.***

342 Survival of *G. mellonella* wax moth larvae infected with *S. aureus* FPR3757 and with or without LL-37 (10 µg/ml)

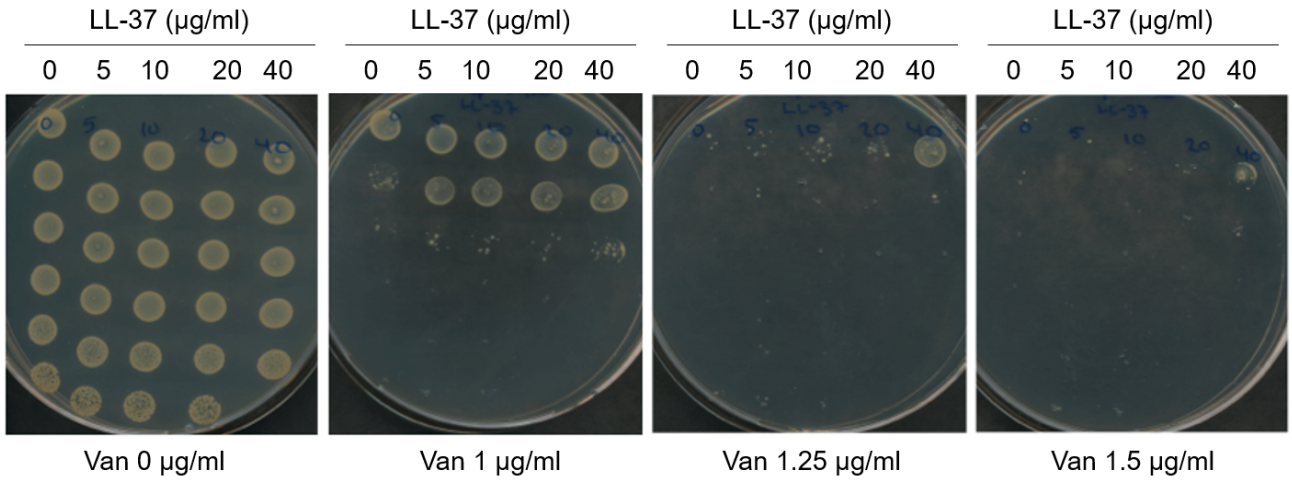
343 exposure followed by treatment with vancomycin (15 mg/kg bodyweight). Controls for non-treated *S. aureus*

344 infection and non-infected larvae exposed to combinations of the drugs were included. All larvae received

345 same number of injections. n=20 larvae for each condition.

346 Figures

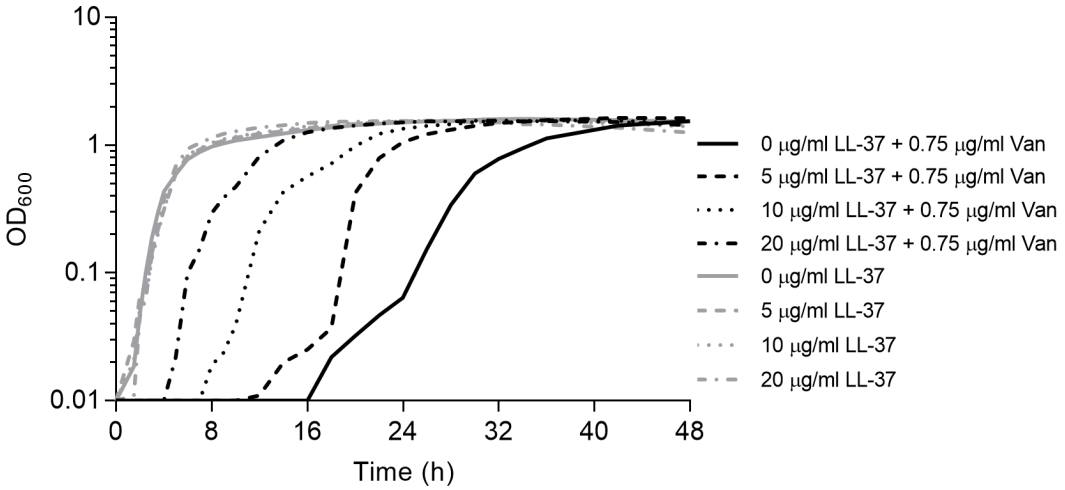
347 *Figure 1*



348

349 *Figure 2*

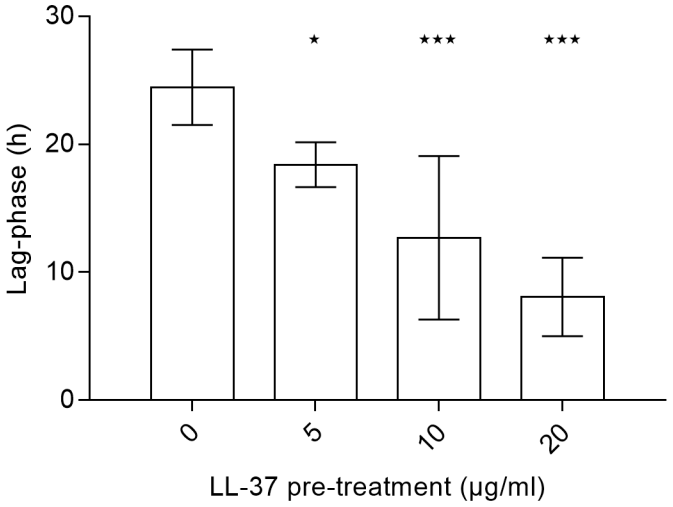
350 (a)



351

352

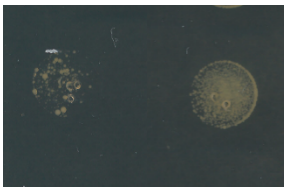
353 (b)



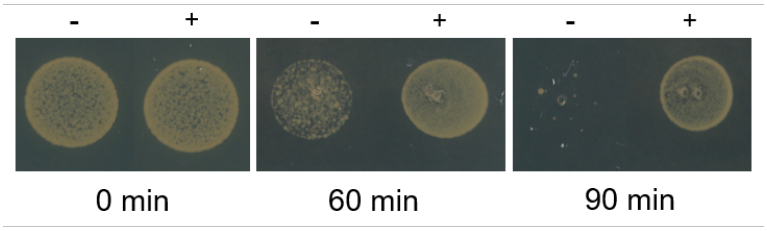
354

Figure 3

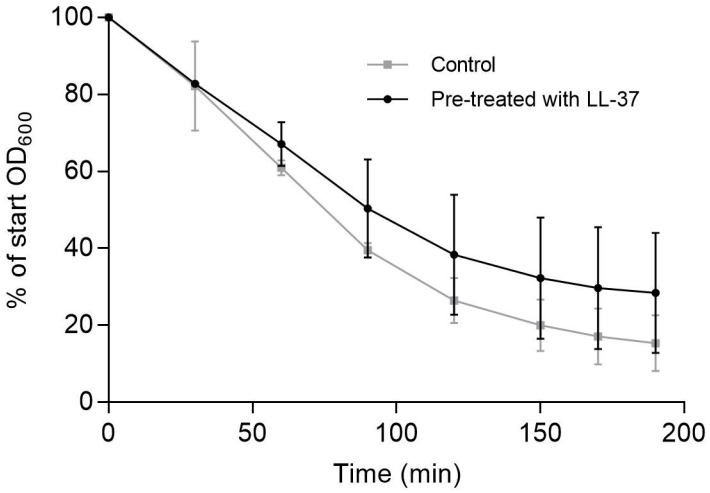
(a)



(b)

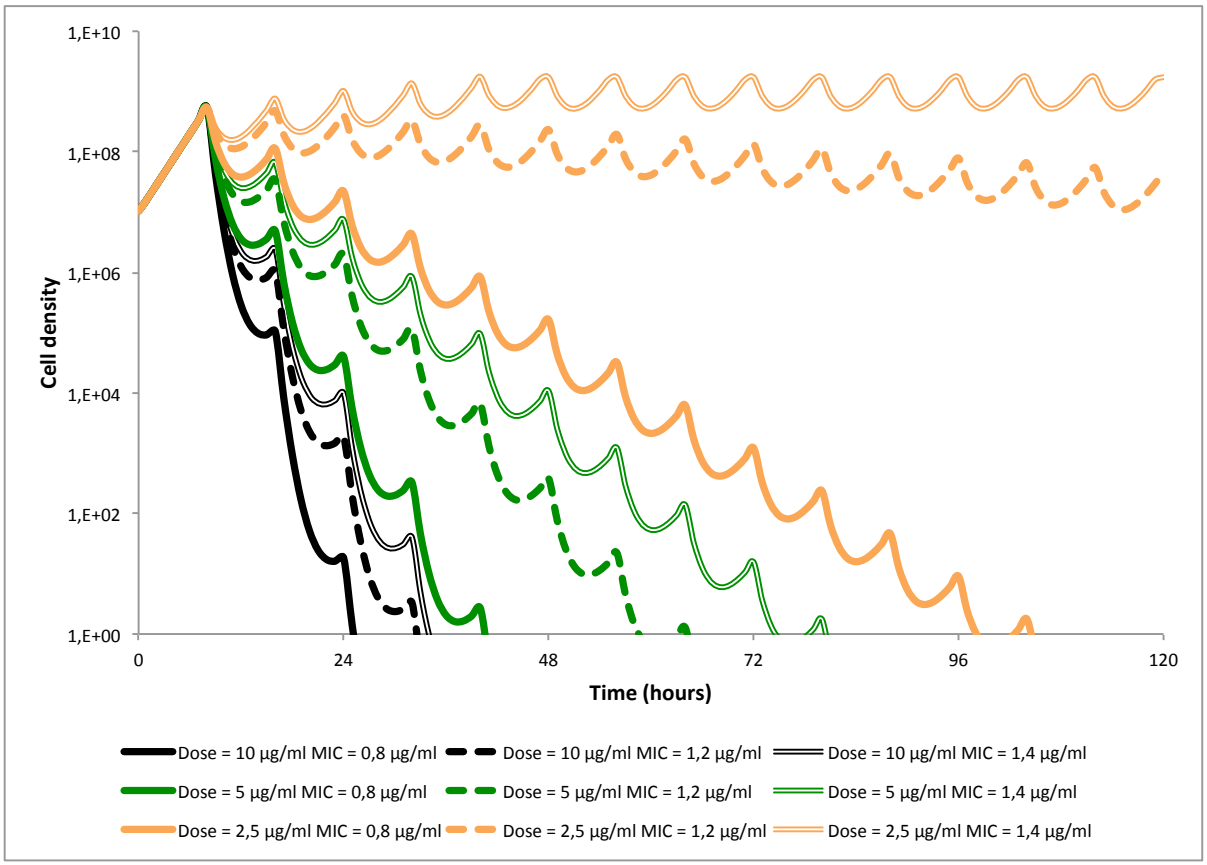


360 *Figure 4*



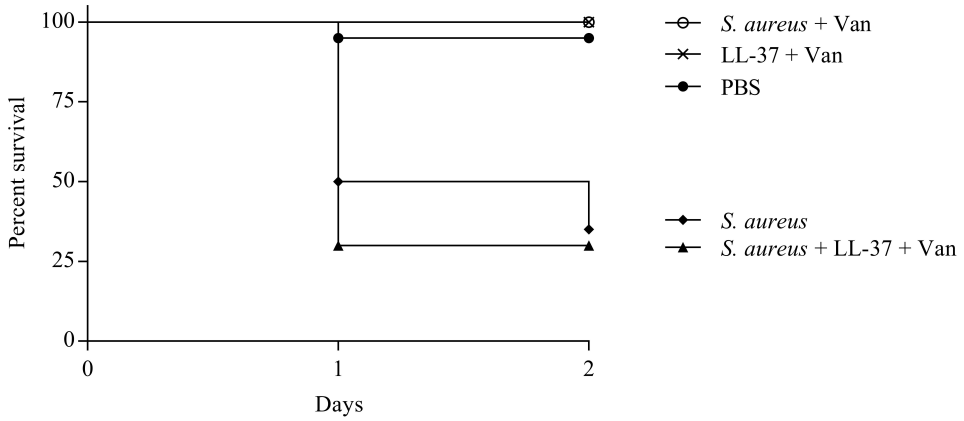
361

362 *Figure 5*



363

364 *Figure 6*

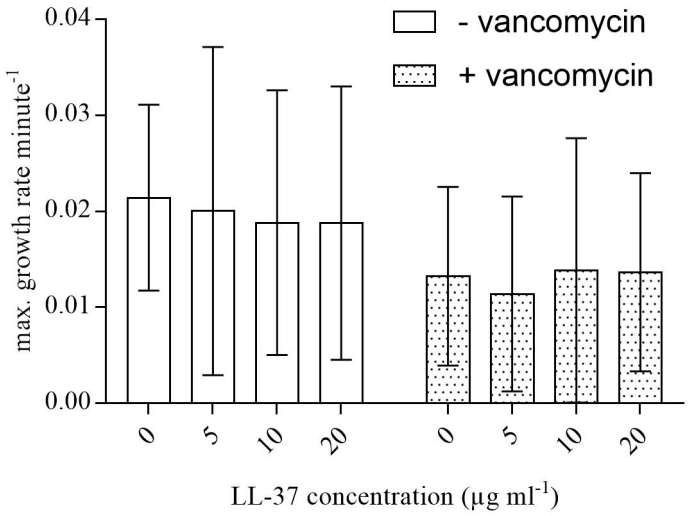


365

366

367 ## Supplementary figure

368 **Figure S1. LL-37 effect on maximum growth rate.**
369 From the growth experiments as described in Materials and Methods, the maximum growth rates were
370 determined for *S. aureus* cultures pre-exposed to LL-37 (0 to 20 µg/ml) for 1 h before being exposed to
371 0 or 0.75 µg/ml vancomycin. Error bars represent s.d. of 3 biological replicates each containing 3
372 technical replicates.
373



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