Failure to clear intra-osteocytic *Staphylococcus aureus* is not the result of antibiotic-mediated autophagy dysregulation

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ABSTRACT:

Background: Staphylococcus aureus is a major causative pathogen of osteomyelitis. Intracellular infection of osteocytes and other bone cell types have been reported, with the bacteria able to survive and persist for an extended period, despite application of gold-standard clinical interventions. The mechanisms, by which intracellular *S. aureus* persists through antibiotic therapy are unknown. In this study we investigated if antibiotic-mediated dysregulation of autophagy is a contributing factor.

Methods: Human osteocyte-like cells were exposed to combinations of rifampicin, vancomycin and modulators of autophagy, in the presence or absence of *S. aureus*. Intracellular bacterial growth characteristics were assessed through CFU analysis, viable DNA abundance and the rate of escape into antibiotic-free medium, in parallel with measures of host cell autophagic flux.

Results: Rifampicin, alone or in combination with vancomycin, caused a rapid decrease in the culturability of the intracellular bacterial community concomitant with increased absolute bacterial DNA levels. Low dose vancomycin alone increased both measures but this was reversed at higher doses. Both antibiotics significantly inhibited autophagic flux. Whilst the modulation of autophagic flux affected bacterial culturability, this did not affect viable bacterial DNA levels.

Conclusions: Autophagy was shown to be a factor in the host-pathogen relationship in this model, as its modulation affected the growth phenotype of intracellular *S. aureus*. Whilst rifampicin and vancomycin treatments moderately suppressed autophagic flux acutely, this was not explanative of the paradoxical response of *S. aureus* to antibiotic treatment. As such, whilst rifampicin and vancomycin had off-target effects on autophagy, similar to those of *S. aureus*, these were not causally linked to the antibiotic-mediated pressure towards persistent infection.

Key Words

Autophagy; *Staphylococcus aureus*; osteocyte; antibiotics; host-pathogen

INTRODUCTION

Staphylococcus (S.) aureus exists as both a commensal and an opportunistic pathogen, capable of causing varied and severe diseases through the colonisation of different human cell types and anatomical niches (1). These infections are commonly resistant to antibiotic therapies and whilst this is often mediated through defined resistance mechanisms, *S. aureus* is also capable of displaying antibiotic tolerance in the absence of resistance, often through the adoption of alternative growth phenotypes (2, 3).

Whilst it is likely the case that *S. aureus* employs many of the same mechanisms of pathogenesis to persist within different anatomical niches, host cell-pathogen dynamics vary based on the tissue in question This raises the possibility that bacteria that reside within different host cell types may exhibit distinct phenotypic variations. These differences may span from the expression of different virulence factors to entirely different modalities of persistence, for example extracellular biofilm formation or intracellular residence (4, 5). Having a detailed, contextual understanding of the host-pathogen interaction in question could differentiate the therapeutic clearance of an infection from its suppression into a chronic state; different pharmacokinetic profiles and therapeutic strategies are required both to facilitate antibiotic treatment within specific tissues and to target different pathogen growth phenotypes. The requirement for this depth of understanding of host-pathogen interaction and bacterial persistence is laid bare when comparing the therapeutic strategies required to treat an established biofilm against those of an intracellularly resident bacterial population (6, 7). Additionally, the large variance in bone penetrance and bone:serum concentration ratios for different antibiotics (7) brings further therapeutic challenges. Currently, this level of knowledge is lacking, and as such, it is important that we investigate intracellular pathogen-antibiotic interactions in the context of specific host cell types, as this may give insight into the mechanisms of treatment failure. Periprosthetic joint infection (PJI) presents with a high rate of treatment failure and infection chronicity (8). Our group and others have previously highlighted the important role that the intracellular infection of osteocytes plays in persistent PJI with the most common causative organism, *S. aureus* (9, 10). As such, we

focused our analysis on the host-pathogen-antibiotic tripartite interaction of intra-osteocytic *S. aureus* with antibiotics that are commonly used to treat this type of infection, namely rifampicin and vancomycin. It is worth noting that all currently utilised interventions critically rely on antibiotics for the clearance of intracellular infection, while biofilms upon the abiotic implant surface are removed during staged revision surgery. Regardless of therapeutic strategy, infection relapse remains as high as 8.8-31% within two years after treatment (11-13). The more conservative surgical approach of debridement, antibiotics and implant retention (DAIR) with or without long-term (>1 year) suppressive antibiotic treatment, demonstrated similar to worse outcomes, with reported rates of failure to clear infection of 25% and 35.3% of patients (14, 15). As it could be the case that this underlying therapeutic strategy-independent failure rate is caused by the persistence of intracellular infection outside of the zone of debridement, we have sought to address this hypothesis by evaluating whether specific antimicrobial treatments are independently capable of clearing these infections *in vitro*.

The efficacy of antibiotics that target intracellular pathogens must be greater than the sum of the off-target effects that may compromise host cell innate immunity for the treatment to be beneficial in this context. Autophagy is the major intracellular catabolic pathway. As such, it is also the primary mechanism through which host cells clear intracellular infections, in a process termed xenophagy (16). Given that pathogenic bacteria have evolved means to subvert, disrupt or even make use of components of the xenophagic pathway to reside intracellularly, it is important that antibiotics do not contribute to this outcome (17). Indeed, several antibiotics used for the treatment of Staphylococcal PJI have been identified to elicit this off-target effect (9). Gentamicin, which is commonly used within antimicrobial-loaded polymethylmethacrylate spacers during staged revisions, has been demonstrated to inhibit autophagosome-lysosome fusion, an effect that is also mediated by intracellular *S. aureus* (18, 19). Further to this, aminoglycosides, including gentamicin, tobramycin and daptomycin, when fully protonated within the acidic environment of lysosomes cause aggregation of the aminoglycoside and inhibition of lysosomal phospholipase activity (20). In addition, vancomycin-induced kidney injury is in part mediated through autophagic dysregulation, potentially through the

accumulation within and subsequent rupture of autophagolysosomes (21). Indeed, vancomycin has also been demonstrated to block autophagic flux in macrophage cell lines (22). Rifampicin has been shown to inhibit rapamycin-induced autophagy through the suppression of the positive regulator of autophagy, PP2A (23).

These observations point to a scenario whereby the efficacy of antibiotics in clearing extracellular infection may mask undesirable off-target effects that promote intracellular residence of *S. aureus* that then relapse following cessation of antimicrobial treatment or lead to the development of resistance. In the current study, we sought to determine the involvement of autophagy in *S. aureus* infections in a human osteocyte model, and the impact of two commonly co-administered antibiotics on this interaction.

MATERIALS & METHODS

Cell Culture

Cell culture of SaOS2 cells and differentiation under osteogenic growth conditions to an osteocyte-like stage (SaOS2-OY) was performed as previously reported (24, 25). Briefly, SaOS2 cells were cultured for 28 days in differentiation medium consisting of αMEM (Gibco, NY, USA) with 10% v/v foetal calf serum, 10 mM HEPES, 2mM L-Glutamine (Thermo-Fisher, VIC, Australia) and 50 µg/ml ascorbate-2-phosphate and 1.8 mM potassium di-hydrogen phosphate (Sigma, MO, USA). Media were refreshed twice weekly during the 28 day differentiation period, and weekly thereafter. During differentiation the medium was supplemented with penicillin/streptomycin (Thermo-Fisher, VIC, Australia), both at 1 unit/ml. Following infection, where relevant, media were either antibiotic-free or supplemented with 10µg/ml lysostaphin (AMBI Products LLC, NY, USA). Routine light microscopy showed that the differentiated osteocyte-like cultures exhibited the characteristic mineralised extracellular matrix consistent with this model (24, 25).

Bacterial Culture and SaOS2-OY Infection

Growth and quantification of the wild-type, methicillin-resistant *S. aureus* (MRSA) strain WCH-SK2 was performed, as reported (24). Briefly, bacteria were streaked onto nutrient broth agar (NBA) from glycerol stocks to ensure the culture was a monoculture of the intended strain. Isolated colonies were sub-cloned into nutrient broth twice. Log phase broth was pelleted (2000 x g, 10 minutes, room temperature) and resuspended in phosphate buffered saline (PBS) to approximate the target CFU/ml using spectrometry (OD_{600nm}) with a standard curve. Differentiation medium was removed from SaOS2-OY cultures and the wells washed twice with PBS before the addition of the suspended bacteria. The accurate CFU/ml was determined by plating serial dilutions of the inoculate onto NBA. Following a 2-hour infection period, the wells were washed twice with PBS and exposed to medium containing 10 µg/ml lysostaphin for 2 hours to clear extracellular bacteria. Following this period, if an antibiotic above the concentration required to clear planktonic bacteria was to be used for a treatment, lysostaphin would be removed to reduce confounding variables.

Quantification of Bacterial Burden by qPCR

For infection studies, host cells were lysed in water for 20 minutes at 37°C. Following this, to extract DNA exclusively from viable bacteria, the lysate was treated with the photoreactive DNA-binding dye PMAxx (Biotium, CA, USA). Briefly, PMAxx was added to the lysates to a concentration of 20 µM and this was incubated for 10 minutes at 37°C under blue light. After this, or in the case of total DNA extraction wherein the PMAxx treatment was not used then following host cell lysis, the bacterial DNA from the lysates was extracted using the Gram-positive bacterial cell lysate protocol from the PureLink™ genomic DNA mini kit (Thermo-Fisher), except using 20 µg/ml lysostaphin instead of the suggested lysozyme. Real time qPCR was performed to quantify relative bacterial gene abundance

with RT2 SYBR Green Fluor qPCR Mastermix chemistry (Qiagen, Limburg, Netherlands) and the CFX Connect Real Time PCR platform (Biorad, CA, USA). Where appropriate, bacterial DNA quantity was normalised to the magnitude of human *ACTB* DNA using the $2^{-\Delta Ct}$ method. When PMAxx treatment was used, as it inhibits extraction of human DNA from the lysates, human DNA abundance was determined from replicate cultures extracted in the absence of PMAxx. The sequences of the oligonucleotide primer sets used are listed in **Supplementary Table 1.**

Quantification of bacterial gene expression

Bacterial transcripts were isolated using the Trizol reagent method (Life Technologies, NY, USA) and RNA reverse transcribed using the iScript gDNA clear cDNA synthesis system (Biorad, CA, USA). qPCR of complementary DNA was performed as previously described and normalised to human *ACTB* mRNA quantity using the $2^{-\Delta Ct}$ method (10).

Measurement of SaOS2-OY cell viability

Cell viability was assessed using the lactate dehydrogenase (LDH) enzyme activity method (Cytotoxicity Detection Kit (LDH) version 11; Sigma-Aldrich) to quantify compromised cells in response to exposure to antibiotics. SaOS2-OY cultures seeded into 48-well tissue culture plates were treated with a dose range of either rifampicin (0.75-75 µg/ml) or vancomycin (3-800 µg/ml) diluted in differentiation medium. LDH activity quantification was performed 24 hours after the initiation of treatments, following the manufacturer's protocol.

Measurement of Culturable Bacteria per Well

Bacterial outgrowth was routinely monitored by observation of media colour. For experiments where bacterial outgrowth was an outcome measure, this was further monitored by daily plating of 1.5 µl of media onto NBA.

For the purposes of measuring culturable intracellular bacteria per well, after thorough rinsing in sterile media to remove antimicrobial agents, SaOS2-OY cells were lysed by 20 minute exposure to pure water at 37°C. Following lysis, the cell layer was scraped into solution and agitated by repeated passage through a 200 µl pipette tip. Serial dilutions of the lysate free of clumped cellular debris were made with sterile PBS and plated onto NBA before being left to dry in a biosafety cabinet to prepare for incubation at 37°C. Plate counts were performed under sterile conditions after 48 hours of plate incubation. All plates were left for a minimum of 14 days in the incubator to ensure that all culturable bacteria have time to generate macroscopically visible colonies. These later plate images were cross referenced to the plate images used for counting to ensure that late emerging colonies were not omitted from the reported CFU/well.

Antibiotic treatments

When analysing and comparing the effects of antibiotics on bacterial outcomes we used minimum bactericidal concentrations (MBC) for planktonic growth to normalise the comparison. As the intracellular niche provides natural antibiotic resistance through varied mechanisms, we used both the MBC itself and a 100-fold higher concentration to probe the effects of both theoretically sub-lethal and potentially lethal stress. For vancomycin, the planktonic MBC for *S. aureus* WCH-SK2 was measured to be 8 µg/ml. The equivalent concentration for rifampicin was 0.75 µg/ml. When interrogating the effects of antibiotics on the host cells we used concentrations that included the 1x MBC to maintain intra-study comparisons as well as other doses that roughly fell into clinically relevant ranges. We also ensured that the ranges were relatively proportionate to each other to maintain similar comparative dynamics. For vancomycin, in this context, we used 3, 8 and 20 µg/ml whilst

osteocyte exposure ranges are predicted to be 1.1-6 µg/ml and serum trough concentrations for *S. aureus* infections are recommended to be 15-20 µg/ml (7, 26). The equivalent range used herein for rifampicin was 0.75, 2.5 and 6 µg/ml. The concentrations osteocytes are expected to be exposed to are 1.3-6.5 µg/ml (7).

Quantification of autophagic flux

Protein was isolated from differentiated cultures using M-PER™ mammalian cell protein lysis reagent and Halt® protease and phosphatase inhibitor cocktail (both from Thermo Scientific, Victoria, Australia). Protein samples were quantified using the BCA protein assay method (Bio-Rad, Victoria, Australia), and 10 µg (unless specified otherwise) electrophoresed using Novex® 4–12 % gradient Bis-Tris denaturing gels (Life Technologies, Victoria, Australia) and electroblotted to Trans-Blot® Turbo nitrocellulose membranes (Bio-Rad). Membranes were blocked in 5% diploma skim milk for 1 hour and then probed overnight at 4°C with primary antibodies directed to LC3A/B I-II (# 4108 in 5% BSA), Sequestosome (#5114 in 5% skim milk; both Cell Signaling Technology, Boston, MA), and β-actin (#A1978 in 5% skim milk; Sigma-Aldrich Co.) followed by matching horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature (R&D Systems, MN, USA). Chemiluminescent imaging was performed using the LAS-3000 platform and histogram densitometry was performed using Multi Gauge software (V3.1 Fujifilm, Tokyo, Japan). Density scores were normalised to the β-actin control and analysis performed consistent with relevant guidelines (27, 28). Briefly, bafilomycin is used to block degradation of autophagosomal membrane bound LC3A/B-II and SQSTM1. As such, the levels of these proteins measured in bafilomycin treated cells in reference to untreated cells indicate the amount of these proteins that would otherwise have been degraded by flux.

Intracellular infection escape assay

In order to assess resuscitation of intracellular *S. aureus* infections, a bacterial outgrowth or escape assay was generated. SaOS2-OY in 48-well plates were infected with a dilute suspension of WCH-SK2 (~0.05 MOI; in PBS). The infection was maintained for 2 weeks in a solely intracellular state under the pressure of lysostaphin supplemented differentiation medium, as reported (Gunn et al., 2021) to shift the bacterial community into a chronic-like, predominantly non-culturable state. At 15 days post-infection media were replaced with antimicrobial-free differentiation media containing the modulators of autophagy Ku-0063794 (autophagy promoter; Sigma-Aldrich), bafilomycin (autophagy blocker; Jomar, VIC, Australia) or untreated control media. Additionally at this time, host cell lysates were assessed for culturability and an average of 8 CFU/well was measured. Following the initiation of treatment, bacterial content was assessed daily (excluding 6 days post-treatment) by drying 2 µl/well of supernatant onto NBA plates to ascertain whether bacteria with a culturable phenotype had escaped into the media.

Transmission Electron Microscopy (TEM)

Uninfected SaOS2-OY cells were prepared for TEM, as previously described (10, 24). Briefly, they were cultured in 75 cm^2 cell culture flasks before 24 hour, 37°C exposure to fixative containing 1.25% v/v glutaraldehyde, 4% w/v sucrose and 4% w/v paraformaldehyde in PBS. After fixation the cells were demineralised to facilitate sectioning in Osteosoft™ solution (Sigma-Aldrich). The fixed and demineralised cells were processed for imaging in 2% w/v osmium tetroxide before being embedded in resin and cut into 5 µm sections.

RESULTS

A model of human osteocyte infection exposed to rifampicin and/or vancomycin paradoxically increases bacterial burden but not culturability

WCH-SK2 intracellular infections were established in SaOS2-OY cells and exposed to rifampicin and vancomycin at 1x and 100x MBC for the strain in planktonic growth conditions. CFU were measured from replicate cell lysates at 1, 3 and 7 days post-infection (DPI). Half of the lysate from each replicate within a group were pooled together to assess the average abundance of the bacterial gene *sigB* to model total bacterial population size within these samples. Outcomes from the control treatment (**Fig. 1A**) show that the SaOS2-OY model has the capacity to clear a significant proportion of invasive WCH-SK2 during the observation interval with a time-dependent decrease in intracellular CFU consistent with our previous report (24).

Rifampicin treatment was effective in clearing culturable intracellular bacteria (**Fig. 1B**), and this effect was heightened with co-exposure to vancomycin (**Fig. 1D**). Vancomycin treatment alone failed to cause a significant decrease in CFU/well (**Fig. 1C**). Interestingly, low dose vancomycin, rifampicin and the combination of the two increased detectable intracellular bacterial DNA levels compared to untreated controls. Across this experiment only the 100xMBC vancomycin treatment, which is a supra-clinical dose, appeared to cause a reduction in bacterial gene abundance, however rifampicin co-treatment countered this apparent beneficial effect of high dose vancomycin exposure.

The bacteria recovered from these lysates after treatment with rifampicin were almost entirely capable of growing in broth containing 1x MBC rifampicin (all 6 isolates tested were capable of growth). Whilst this alone may suggest that there was some acquired resistance, when an isolate that outgrew from the antibiotic-free group was tested, this was also capable of growth in 1x MBC rifampicin, unlike the parental strain under planktonic growth conditions. As such, it seems that the intracellular environment may promote a phenotypic change that facilitates greater rifampicin resistance or potentially it selects for a mutation that coincidentally increases rifampicin resistance,

although discriminating between these possibilities was outside of the scope of this work. In contrast to rifampicin treatment, no isolate tested, be it from the vancomycin-treated, antibiotic-free control or the parental strain, was capable of growth in broth with 1x MBC vancomycin.

To determine if the apparent paradoxical effect of antibiotics was also characteristic of chronic infections, infected SaOS2-OCy cells were cultured in the presence of lysostaphin until the host cell lysates were no longer culturable. These cells, deemed to be infected with a non-culturable community of persistent bacteria, were exposed to MBC doses of antibiotic treatments for 24 h. To ensure that the effect of antibiotics was measured on viable bacterial genome equivalents, the photoreactive viability PCR reagent PMAxx™ was used to rule out DNA signal from non-viable bacteria. This revealed a significant increase in the absolute abundance of *sigB* DNA signal for the cells exposed to either rifampicin or vancomycin, with no change recorded for the untreated cells during this treatment period (**Fig. 2**).

Effects of rifampicin and vancomycin on autophagic flux in SaOS2-OY cells

The SaOS2-OY model demonstrated significant autophagic potential, as indicated by the differential LC3A/B-II signal for the untreated cells (i.e. basal LC3A/B-II) vs its accumulation with the autophagy inhibitor, bafilomycin (+2.76-fold/flux of LC3A/B-II for bafilomycin vs no-exposure, β-actin normalised, P = 0.025, n = 3, **Fig. 3C/D**). Autophagic/xenophagic potential within SaOS2-OY cells is further supported by structures resembling lysosomes with membrane whorls under TEM (**Fig. 3A)** (29) and our previously published observations of *S. aureus* within this cell model in various stages of trafficking towards lysosomal degradation, including during the dissolution of the bacterial cell wall (24). Off-target effects imparted by the antibiotics may interfere with this bacterial clearance mechanism and promote intracellular residence of *S. aureus*. To examine this, SaOS2-OY cells were exposed to clinically relevant dose ranges of rifampicin or vancomycin and assessed for alterations of autophagic flux to assess whether xenophagy-related processes were modified by the treatments alone.

The abundance of LC3A/B-II positively correlated with increasing concentrations of either antibiotic (**Fig. 3C & D**, p = 0.044 & 0.047, respectively) and appeared to plateau at a similar proportion of total flux for rifampicin and vancomycin (34% and 37%, respectively, of the theoretical maximum increase determined by bafilomycin treatment). The range of antibiotic exposures used herein did not negatively impact cell viability (**Fig. 3G & H**, hollow data points), which is consistent with the retention of some level of autophagic potential (a pro-survival mechanism) after exposure to rifampicin or vancomycin and indicates that the effects on bacteria and autophagy observed were not the consequence of host cell death. The same association was not seen when analysing the relationship between Sequestosome levels and the concentration of antibiotic exposure (**Fig. 3E/F**; p = 0.392 & 0.643 for rifampicin and vancomycin respectively). This was likely influenced, at least in part, by the relatively high basal abundance of Sequestosome (vs LC3A/B-II), that when compared with observations for the bafilomycin exposure, were beyond the limit of assay saturation after a 6-hour exposure period. Hence, LC3A/B was used to examine autophagic dysregulation imparted by rifampicin and vancomycin.

Autophagic modulation impacts the culturability but not size of the intracellular *S .aureus* community

Given that we observed antibiotic modulation of autophagy, we next examined whether this is a primary mechanism responsible for the paradoxical effect observed. SaOS2-OY cultures infected with *S. aureus* WCH-SK2 were subjected to exogenous potentiation of autophagic flux with KU-0063794 (inhibitor of mTORC1 and mTORC2), which significantly reduced the number of culturable bacteria per well relative to the control exposure (**Fig. 4A**, 95% CI 14.8%-161% CFU/well relative to the control mean, p = 0.015). In line with this, treatment with bafilomycin resulted in a significant increase in the number of CFU within the host cell lysates (**Fig. 4A**, 95% CI 37-190% CFU/well increase relative to the control mean, p = 0.004). **Figures 4C-E** provide evidence that the outcomes related to bacterial

culturability correlate with exogenous manipulation of autophagy as a mechanism central to the modulation of intracellular bacteria in this system. For example, promotion of autophagic flux corresponds to the degradation of LC3A/B-II and Sequestosome, concomitant with the *S. aureus* WCH-SK2 cargo. However, examination of viable WCH-SK2 DNA levels indicated a persistent pool of bacteria that maintain residence despite effective autophagic activity in the host cells (**Fig. 4B**). Of note, while KU-0063794 dramatically reduced the frequency of culturable bacteria (**Fig. 4A**), the same intervention when analysing actual bacterial abundance produced outcomes that were statistically similar to the control conditions. Together these outcomes point to an adaptive response in at least a proportion of the WCH-SK2 community, which confers significant protection from autophagic degradation and that this resulting phenotype is non-culturable.

Modulation of autophagic flux reduces bacterial culturability and escape from the intracellular niche but not bacterial burden in a chronic infection model

Following characterisation of the interaction between intra-osteocytic *S. aureus* during the early stage of infection, we next investigated whether these dynamics were modified at a late stage, mimicking a chronic infection, where the bacteria were predominantly non-culturable. As with the acute infection scenario, bafilomycin treatment caused a significant increase in the culturability of the intracellular bacteria (**Fig. 5**A, p = 0.03). In contrast, potentiation of autophagic flux with KU-0063794 did not significantly affect culturability (p = 0.26) although as this was low at the start of treatment by design, this result was as expected. Neither treatment affected the relative quantity of viable bacterial DNA (**Fig. 5B**). Protein quantification of LC3A/B-II and SQSTM1 demonstrated similar outcomes of treatment with both autophagy modulators (**Fig. 5C-E**) to those observed for acute treatments (see **Fig. 4C-E**).

To further test the involvement of the autophagic pathway on bacterial persistence in osteocytes, we designed a bacterial outgrowth assay, where a non-culturable infection in a multi-well plate format

was treated with autophagy modulators in an antimicrobial-free medium and the percentage of wells containing bacterial outgrowth monitored over time. When testing the ability of long-term, intracellularly resident WCH-SK2 to spontaneously escape this niche in a culturable state – analogous to relapse of a chronic infection – we found that autophagic potentiation with KU-0063794, when compared to blockade with bafilomycin, increased the rate of bacterial escape and growth over time (**Fig. 6A**, $p = 0.03$). No difference in colony morphology was seen between the groups (**Fig. 6B**): all groups presented with unpigmented colonies after 24 hours of plate incubation, which developed pigmentation and haemolytic capability during further incubation.

DISCUSSION

As the infection of osteocytes is hypothesised to represent an important niche of persistence within PJI (10, 30), we sought to test if clinically utilised antibiotics could reduce the abundance of bacteria within a relevant cell model with the predominant causative organism of hip and knee osteomyelitis/PJI, *S. aureus*. The prevalence and severity of non-culturable infections in PJI and osteomyelitis informed the use of methods that would be sensitive to modification of non-culturable bacterial communities, and which would allow analysis of the effect of the treatment on the culturability of the population (31-33). We found that it is possible for the two antibiotics trialled to, in fact, increase the quantity of intracellular bacteria and that the measurement of bacterial abundance by culture alone would have masked this paradoxical effect. Ultimately, a bacterial population size is the consequence of a dynamic interaction between rates of death and replication. Consequently, our observations suggest either antibiotic-mediated bacterial adaptation that facilitates greater rates of replication or survival within the intracellular niche, or alternatively, an inhibition of the host cell capacity to clear infection or inhibit bacterial replication. It should be noted that whilst it is generally found to be the case, a bacterial strain being non-culturable doesn't necessitate that its baseline metabolism has fallen, just that its growth phenotype does not form

macroscopically visible colonies on the specific growth media used. As such, the lack of culturability does not necessarily exclude that the effect was due to an increased replication rate. Indeed, supporting that this outcome could be mechanistically caused by increased metabolic activity, this paradoxical response has a precedent for rifampicin treatment; previous results in mycobacterium found that it caused inhibition of one RNA polymerase promoter that resulted in greater total expression from an alternative promoter (34). Our observation that this response is at least consequentially consistent in *S. aureus* is novel.

When considering the similarity of observations in response to rifampicin and vancomycin, it may be surprising since the mechanisms of action of these are quite different. However, there is correlative evidence of vancomycin tolerance coinciding with *rpoB* mutations and rifampicin resistance, potentially indicating the involvement of *rpoB* mutations in vancomycin tolerance in *S. aureus* (35). Further, evidence exists linking *rpoB* mutations to increased capsule production that mediated greater resistance to antimicrobial peptides and whole blood bactericidal effects (36). This same mechanism could be mediating resistance to other arms of the innate immune system, such as lysosomal killing, although our investigation did not support that this pathway is capable, at least in this model and with this strain, of acting efficiently as modulation of autophagic flux did not cause modulation of the bacterial DNA quantity in the absence of any further resistance mechanism.

Complete clearance of culturable intracellular bacteria was not achieved in the absence of antibiotic treatment across the 7-day period tested herein. Notably, between 1 and 3 days post-infection (DPI) the relationship between the CFU/well and mean bacterial DNA quantity in the control was not proportional, representing an upward shift in the proportion of the population that was non-culturable. These measures in antibiotic-treated wells also did not commonly correlate over time, with the bacterial culturability dropping at times by many orders of magnitude, whilst this scale of change was not seen in the mean bacterial DNA quantity within the same cultures. Most notably, rifampicin treatment appeared to cause a dose-dependent increase in bacterial DNA abundance over time, coupled with a rapid and complete loss of culturability.

Our observations show that vancomycin and rifampicin were unable to eliminate viable intracellular *S. aureus* (see **Fig 1**), especially at the respective MBCs for the strain tested under planktonic conditions, which fall within the ranges we expect osteocytes to be exposed to *in vivo* during clinical use (7). The fact that the intracellular environment provided protection from the planktonic MBC of antibiotics used was anticipated, as the intracellular environment provides multiple known mechanisms of antibiotic resistance, most notably a reduction in concentration across the host cell membrane (37). However, concerningly, while a supra-clinical and supra-lethal dose (100x MBC) of rifampicin had greater effects on bacterial culturability, this was coupled with a marked increase in bacterial DNA levels. Curiously, a supra-clinical vancomycin concentration appeared to return the number of culturable bacteria and bacterial DNA quantity to similar or lower levels as those in the untreated controls.

The presence of a non-culturable but metabolically active phenotype of *S. aureus* was evidenced by the relative increase in bacterial DNA within intact bacterial membranes following the lysis of the host cells after rifampicin treatment of an established non-culturable infection, as well as the ability of a chronically non-culturable infection to spontaneously revert to a culturable state and outgrow into the medium when antimicrobial pressure was removed (see **Figs. 2 & 6**). Whilst the exact nature of these persister-like cells was not characterised herein, this reflects the viable but non-culturable state often described in the context of biofilm formation (38, 39).

To investigate whether the paradoxical effects of rifampicin and vancomycin treatment were mediated through disruption of innate immunity, we measured the ability of these antibiotics to modulate autophagic flux. We observed a significant dose-dependent inhibition of autophagic flux with clinically relevant doses of both antibiotics. Given that the antibiotic-mediated increase in LC3A/B-II was abrogated by bafilomycin co-treatment, the underlying mechanism is likely the inhibition or disruption of lysosomal maturation or autophagolysosomal degradation or fusion.

As autophagy responds to signals elicited by cellular stress, we sought to identify if the observed effects were modulated through the cytotoxicity of the respective treatments. Whilst rifampicin was shown have a dose-dependent cytotoxic effect on SaOS2-OY cells, this was observed at doses that exceed what was used within the experiments investigating the impact of the antibiotics on autophagic flux. Vancomycin showed no cytotoxic effect upon SaOS2-Ocy at any concentration tested. These results are consistent with reports for osteoblasts or osteoblast-like cells (40, 41).

The lack of modification of relative SQSTM1 quantity by bafilomycin treatment in uninfected SaOS2-OY cells implies that this adaptor protein is not involved in the basal flux being investigated in this cell type. Interestingly, SQSTM1 was also not significantly increased by infection itself. Potential explanations for this observation are negative regulation beyond basal levels, functional redundancy via other autophagy adaptors, a proportional increase in the rate of SQSTM1 degradation or via a yet to be identified effect exerted by the infecting bacteria. Nonetheless SQSTM1 appeared to retain its function, as the autophagy potentiators and blockers were capable of significantly altering relative SQSTM1 quantities when bacteria were present (see **Figs. 4 & 5)**.

Lysosomal maturation and autophagosome-lysosome fusion represent key steps in the xenophagic pathway, which facilitate the clearance of intracellular bacteria, and *S. aureus* has previously been shown to be capable of inducing the formation of a replicative niche through the dispruption of these processes in other cell contexts (42, 43). One possibility is that mechanisms that limit the cell's ability to traffic bacteria into the autophagolysosome may facilitate resident bacteria in generating a niche of persistence and/or replication within the host cell. As there was indeed some degree of autophagic flux inhibition, we sought to investigate whether the paradoxical increase in bacterial DNA quantity observed with antibiotic treatment could be explained by this effect.

As such, we tested the effects of an autophagic flux blocker or potentiator on microbiological outcomes in intracellularly infected cells. Neither treatment when applied either immediately following infection or after chronicity was established resulted in a significant change in the quantity

of viable bacterial DNA, whilst culturability was significantly modulated. This did not support our expectation that autophagic blockade would facilitate greater bacterial replication and persistence by reducing intracellular stress. The observation of the modulated culturability of the bacterial community suggests mechanisms beyond evading xenophagy, as if that was the case we would expect autophagic flux modification to have no effect on the intracellular bacterial population. As neither bacterial replication, nor death rate were directly measured, we cannot rule out whether the lack of change in population size was due to autophagy having no effect on either of these variables or if there was a proportional change in both to maintain total community size. Of note, xenophagy acting as a stress that induces a change in the growth phenotype but not the growth rate itself is conceptually similar to other reports of *S. aureus* being well adapted to overcome this stress in other contexts (19, 44). The most likely explanation for the interaction of autophagy with *S. aureus* in the context of flux blockers or potentiators is summarised in **Figure 7**.

In the period immediately following infection, bafilomycin treatment was expected to result in increased culturability by reducing the proportion of bacteria that were successfully trafficked into autophagolysosomes, and as such inhibiting the reduction in culturability triggered by this stress. Surprisingly, in the context of a chronic infection, bafilomycin treatment appeared to facilitate a bacterial sub-population returning to a culturable state, as opposed to simply inhibiting the induction of non-culturability. This has interesting implications for the mechanisms of recurrence in dormant infections, whereby the dysfunction in the host cell contributes to the regeneration of a culturable subpopulation. At the same time, our results when modelling recurrence of a chronic infection showed that the potentiation (but not blockage) of autophagic flux facilitated a greater rate of bacterial escape and growth into the culture media. This was unexpected, given that potentiation lowered the culturability of the bacteria when the host cell was lysed and the lysate spread on nutrient agar. However when the bacteria were escaping from the host cells into antibiotic-free media they retained the ability to grow in that context. Whilst only a single CFU would be required to escape into the media

in this experimental design, our findings do raise the possibility that the context of escape from the host cell could play a role in the reversion of the growth phenotype to a culturable state.

Limitations of this study include that xenophagic activity was measured by proxy via the flux of LC3A/B and SQSTM1; this analysis was performed in a targeted way and would miss potential changes in other pathways and proteins involved in xenophagy. In addition, the scope and clinical applicability of the observations is limited by the fact that a single bacterial strain was used within a cell line model. This was done for consistency between readouts and it is possible that other clinical *S. aureus* isolates might respond differently. The use of SaOS2-OY was also used for consistency, as donor variability is commonly an issue with primary cell models, however this is the only validated human osteocyte-like cell line model described to date.

CONCLUSIONS

Autophagic flux modulation in both the acute and chronic-like infection contexts tested herein were capable of significantly affecting the proportion of culturable intracellular *S. aureus* but not the total size of intracellular bacterial population. In contrast, rifampicin and vancomycin affected both the proportion of culturable bacteria and the total population size. Additionally, whilst these antibiotics caused some degree of autophagic flux inhibition, the effect on culturability could not be explained by this effect, as autophagic flux inhibition caused an increase, not the observed decrease, in culturability. As such, it appears that this paradoxical increase in intracellular bacterial population size in response to the antibiotics tested was not solely a result of autophagic modulation but was either mediated through some other host mechanism or, more likely, was caused by the bacteria in response to the antibiotic stress. It is interesting that, whilst there were differences in the efficacy of the two antibiotics at reducing culturability, both were capable of causing an increase in the total bacterial population size in a manner that would be overlooked by current routine clinical diagnostic procedures, even though they operate through entirely distinct mechanisms.

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FIGURE LEGENDS

Figure 1. Effects of rifampicin or vancomycin on intracellular CFU and bacterial DNA levels. SaOS2-OY cultures were infected with WCH-SK2 at an MOI of 1. Cultures were either untreated (A) or treated with 1x (0.75 and 8 µg/ml for rifampicin and vancomycin, respectively) or 100x the MBC of Rifampicin (B), Vancomycin (C) or Rifampicin + vancomycin (D). CFU counts from lysates of triplicate wells at either 1, 3 or 7 DPI in the presence or absence of antibiotic treatments were measured. Differences between treatments and antibiotic-free controls were determined at each time point using Student's t-tests. Half of the lysate from each well were physically pooled within groups and bacterial DNA abundance was measured from this by PCR for *sigB*, expressed as 2^{-CT}. The antibiotic-free group only was exposed to lysostaphin for 2 hours following infection to clear extracellular bacteria. DPI = days post-infection, CFU = Colony forming units, MBC = Minimum bactericidal concentration. Significant difference to controls is indicated by * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Figure 2. Effects of antibiotics on intracellular CFU and bacterial DNA levels in a chronic infection model. SaOS2-OY cultures were infected with WCH-SK2 at an MOI of 1. Cultures were maintained in lysostaphin supplemented media until bacterial culture was not detected in host cell lysate (17 days post-infection). Cultures were then exposed to antibiotic-supplemented (1x MBC of vancomycin or rifampicin) or control media for 24 hours. Host cell lysates were then prepared for total DNA isolation following treatment with the PMAxx™ reagent. The relative quantity of *sigB* DNA from bacteria with intact membranes following host cell lysis was then determined by qPCR. Significant difference to untreated controls is indicated by ** $p < 0.01$.

Figure 3. Rifampicin and vancomycin modulate autophagy in the SaOS-2 model. A) TEM images from within SaOS-2 cells showing a form of canonical autophagosome. Relative LC3A/B-II quantity per well was tracked across a dose range roughly representative of what osteocytes would be expected to be

exposed to clinically in vivo for either antibiotic. Measurements were taken at 6 hours following the start of treatment which were performed either with or without bafilomycin. (B) Representative example of a blot from which data was quantified. Quantification of the relative abundance of (C) LC3A/B-II and (D) *SQSTM1* with or without bafilomycin. Significance values associated with the linear regressions indicate whether the slope of the relationship was significantly non-zero. Potential flux remaining was determined by subtracting the treatment values from the treatment + bafilomycin values. (E) Percent cytotoxicity was determined using an LDH enzyme activity assay following 24 hours of exposure to a dose range of either antibiotic. Hollow data points represent doses within the range that osteocytes would be expected to be exposed to during clinical treatment in vivo and as such are also representative of the dose range used within this figure. The 95% confidence interval is presented along with the line of best fit which was either a linear regression for rifampicin or a one-phase association curve for vancomycin. Cont. = Control, Rif = Rifampicin, Vanc = Vancomycin, Baf = Bafilomycin, PFR= Potential Flux Remaining. Significant association between the variables are indicated by * $p < 0.05$.

Figure 4. Analysis of the impact of autophagic flux promoters or blockers on bacteriological and autophagic outcomes immediately following the initiation of infection. (A) Colony forming units per well from host cell lysates (B) relative *sigB* DNA quantity from within intact bacterial membranes extracted from host cell lysates. (C) Representative image of a western blot from which figures D & E were quantified. (D) Relative LC3A/B-II protein quantity per well from host cell lysates. (E) Relative SQSTM1 quantity per well from host cell lysates.

Cells were pre-treated for 4 hours before the infection begun. After the 2 hour infection period at an MOI of 1, treatments were re-added in media containing lysostaphin to clear any extracellular bacteria. All samples were collected the next day. Bacterial DNA abundance was measured by PCR for *sigB*, expressed as 2^{-CT}. Treatment doses were 4.3µM for KU-0063794 and 10nM for bafilomycin.

Protein levels were normalised to ACTB. Significant difference to infected controls is indicated by *$p < 0.05$ and **$p < 0.01$.

Figure 5. Analysis of the impact of autophagy modulators on bacteriological and autophagic outcomes in an established intracellular infection. Following 24-hour (A-E) or continuous(F) treatments of SaOS2-OY cells infected with WCH-SK2 for either 8(A-E) or 16(F) days. (A) Colony forming units per well from host cell lysate (B) relative *sigB* DNA quantity from within intact bacterial membranes extracted from host cell lysates. (C) Representative image of western blots from which figures D & E were quantified. (D) Relative LC3A/B-II quantity from host cell lysates. (E) Relative SQSTM1 quantity from host cell lysates. Bacterial DNA abundance was measured by PCR for *sigB*, expressed as 2^{-CT}. Protein quantification was normalized to ACTB. Significant difference to infected controls is indicated by *$p < 0.05$ and **$p < 0.01$.

Figure 6. Effect of autophagic modulation on bacterial escape from the intracellular niche. (A) Inverted Kaplan-Meier estimator tracking the percent of wells from which predominantly non-culturable intracellular WCH-SK2 escaped and grew from SaOS2-OY into antibiotic-free media following the cessation of antimicrobials and the initiation of autophagic flux modulation. (B) Gross colony morphological assessment with a comparable example from each experimental group after 1 day of plate incubation. Statistical differences between survival curves were analysed with a log-rank test and results are indicated by *$p < 0.05$.

Figure 7. Schematic of the proposed interaction of Ku-0063794 and bafilomycin-mediated modulation of autophagy on intracellular *S. aureus* within SaOS2-OY cells. *LC3* is given as an example of an autophagic gene whose transcription is modulated by mTOR blockade, although this also functions on a range of genes. After being trafficked into a phagolysosome, the phenotype switching that occurs

and results in a decline in culturability on nutrient agar appears to protect the cells adequately from the stress, such that there is no significant change in bacterial population size, likely through an unchanged death and growth rate in comparison to bacteria residing within unfused phagosomes in the context of bafilomycin treatment. In the absence of either treatment, we see an intermediate effect on culturability but the same population size as the bacteria are likely being trafficked in part into both contexts. Ub = Ubiquitin. *Image created using Biorender.*

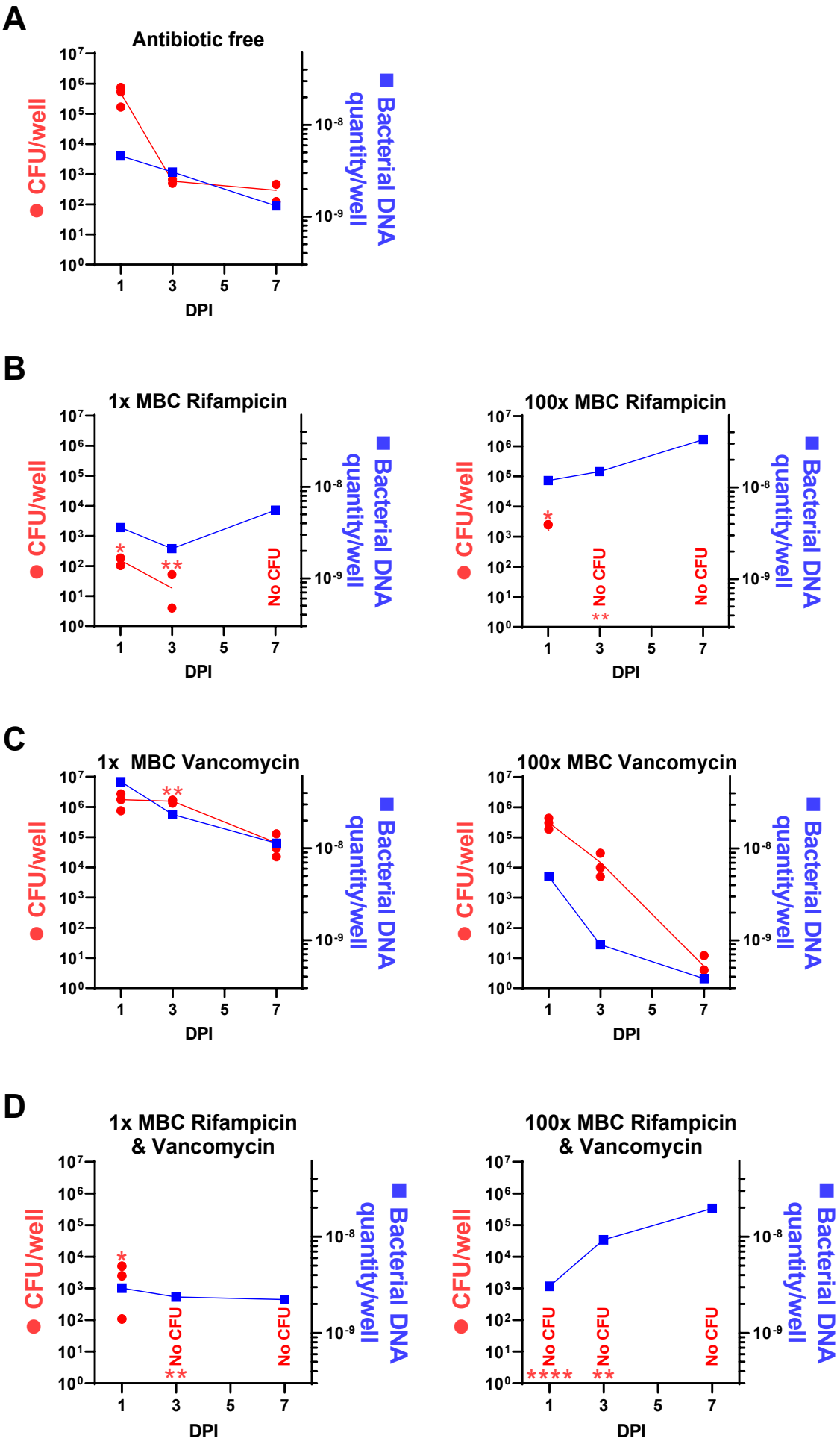
A
Antibiotic free
CFU/well
Bacterial DNA quantity/well
DPI
B
1x MBC Rifampicin
100x MBC Rifampicin
No CFU
C
1x MBC Vancomycin
100x MBC Vancomycin
D
1x MBC Rifampicin & Vancomycin
100x MBC Rifampicin & Vancomycin

Figure 1

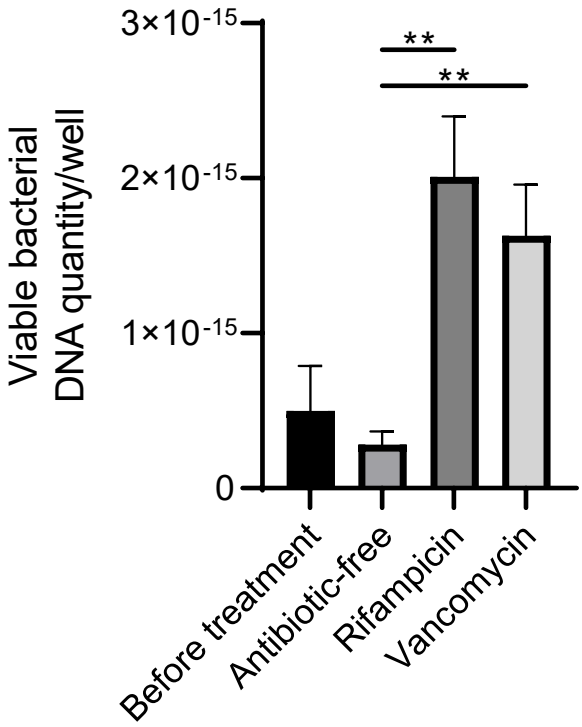
Viable bacterial
DNA quantity/well
3×10-15
2×10-15
1×10-15
0
**
**
Before treatment
Antibiotic-free
Rifampicin
Vancomycin

Figure 2

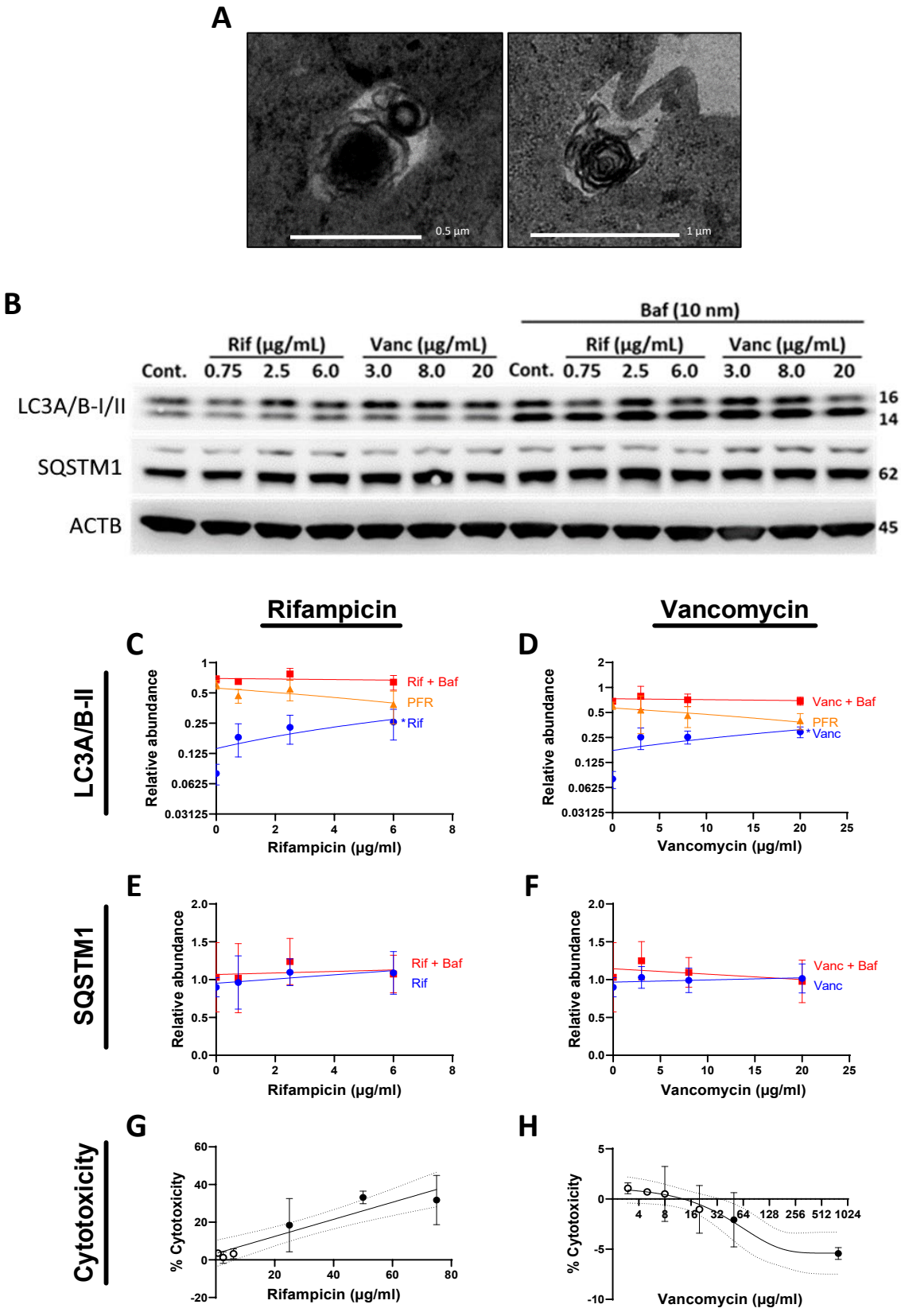
A
0.5 µm
1 µm
B
Baf (10 nm)
Rif (µg/mL)
Vanc (µg/mL)
Cont. 0.75 2.5 6.0 3.0 8.0 20 Cont. 0.75 2.5 6.0 3.0 8.0 20
LC3A/B-I/II
SQSTM1
ACTB
16
14
62
45
Rifampicin
Vancomycin
C
D
E
F
G
H
LC3A/B-II
SQSTM1
Cytotoxicity
Relative abundance
Rif + Baf
PFR
*Rif
Rif
Vanc + Baf
*Vanc
Vanc
Rifampicin (µg/ml)
Vancomycin (µg/ml)
% Cytotoxicity

Figure 3

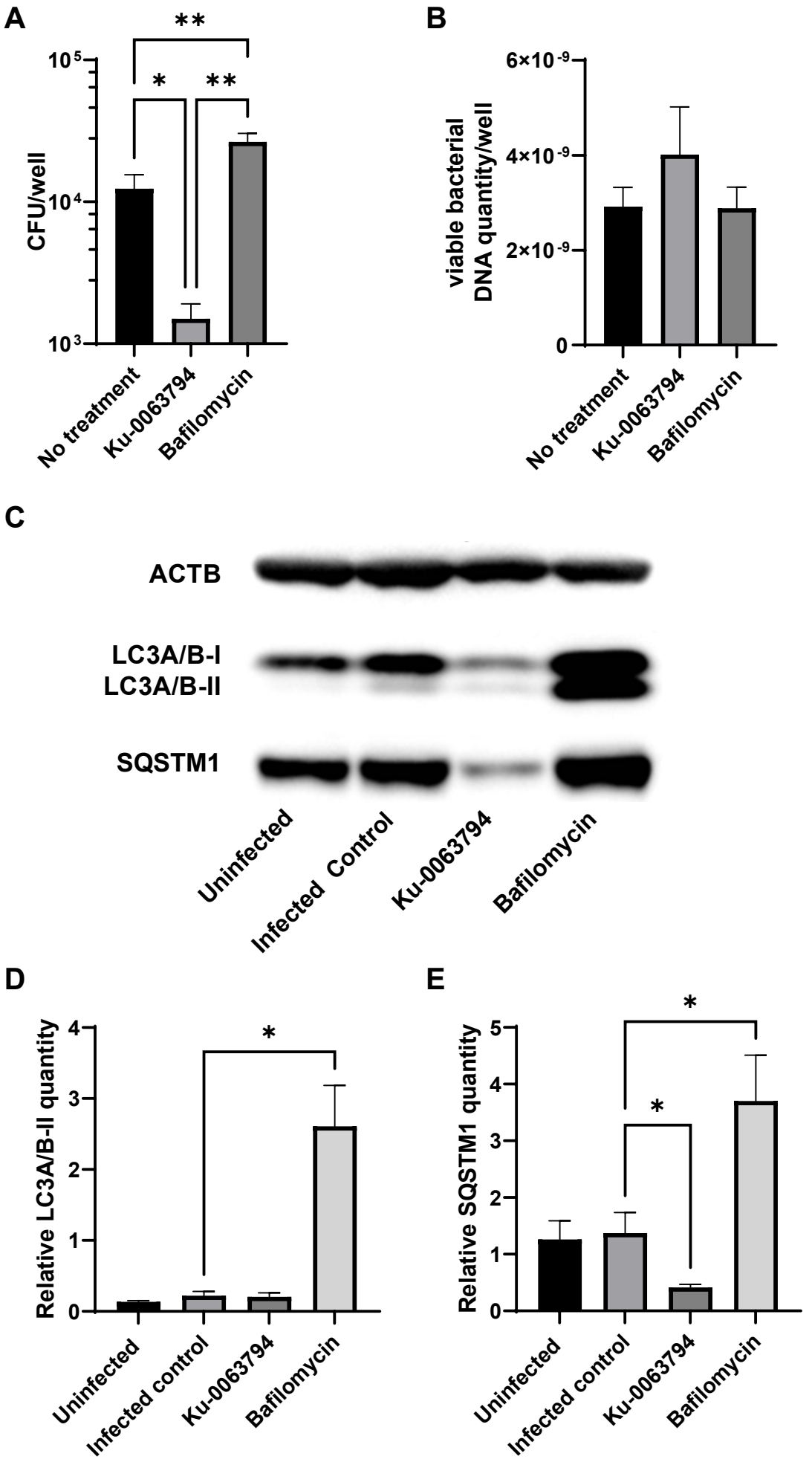
A
**
*
**
CFU/well
10^5
10^4
10^3
No treatment
Ku-0063794
Bafilomycin
B
viable bacterial DNA quantity/well
6×10^-9
4×10^-9
2×10^-9
0
No treatment
Ku-0063794
Bafilomycin
C
ACTB
LC3A/B-I
LC3A/B-II
SQSTM1
Uninfected
Infected Control
Ku-0063794
Bafilomycin
D
Relative LC3A/B-II quantity
*
Uninfected
Infected control
Ku-0063794
Bafilomycin
E
Relative SQSTM1 quantity
*
*
Uninfected
Infected control
Ku-0063794
Bafilomycin

Figure 4

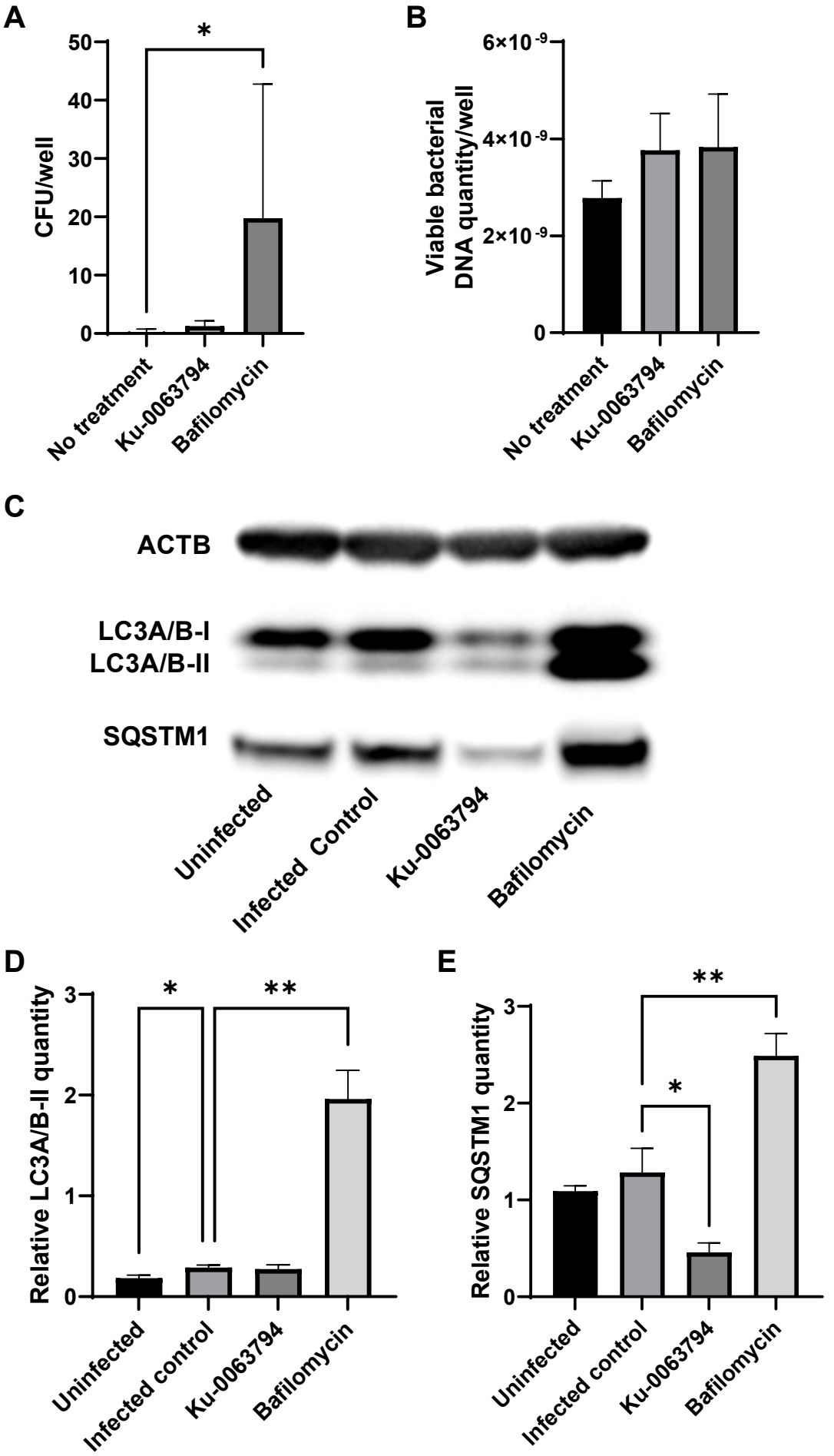
A
*
50
40
30
20
10
0
CFU/well
No treatment
Ku-0063794
Bafilomycin
B
6×10 -9
4×10 -9
2×10 -9
0
Viable bacterial DNA quantity/well
No treatment
Ku-0063794
Bafilomycin
C
ACTB
LC3A/B-I
LC3A/B-II
SQSTM1
Uninfected
Infected Control
Ku-0063794
Bafilomycin
D
*
**
3
2
1
0
Relative LC3A/B-II quantity
Uninfected
Infected control
Ku-0063794
Bafilomycin
E
**
*
3
2
1
0
Relative SQSTM1 quantity
Uninfected
Infected control
Ku-0063794
Bafilomycin

Figure 5

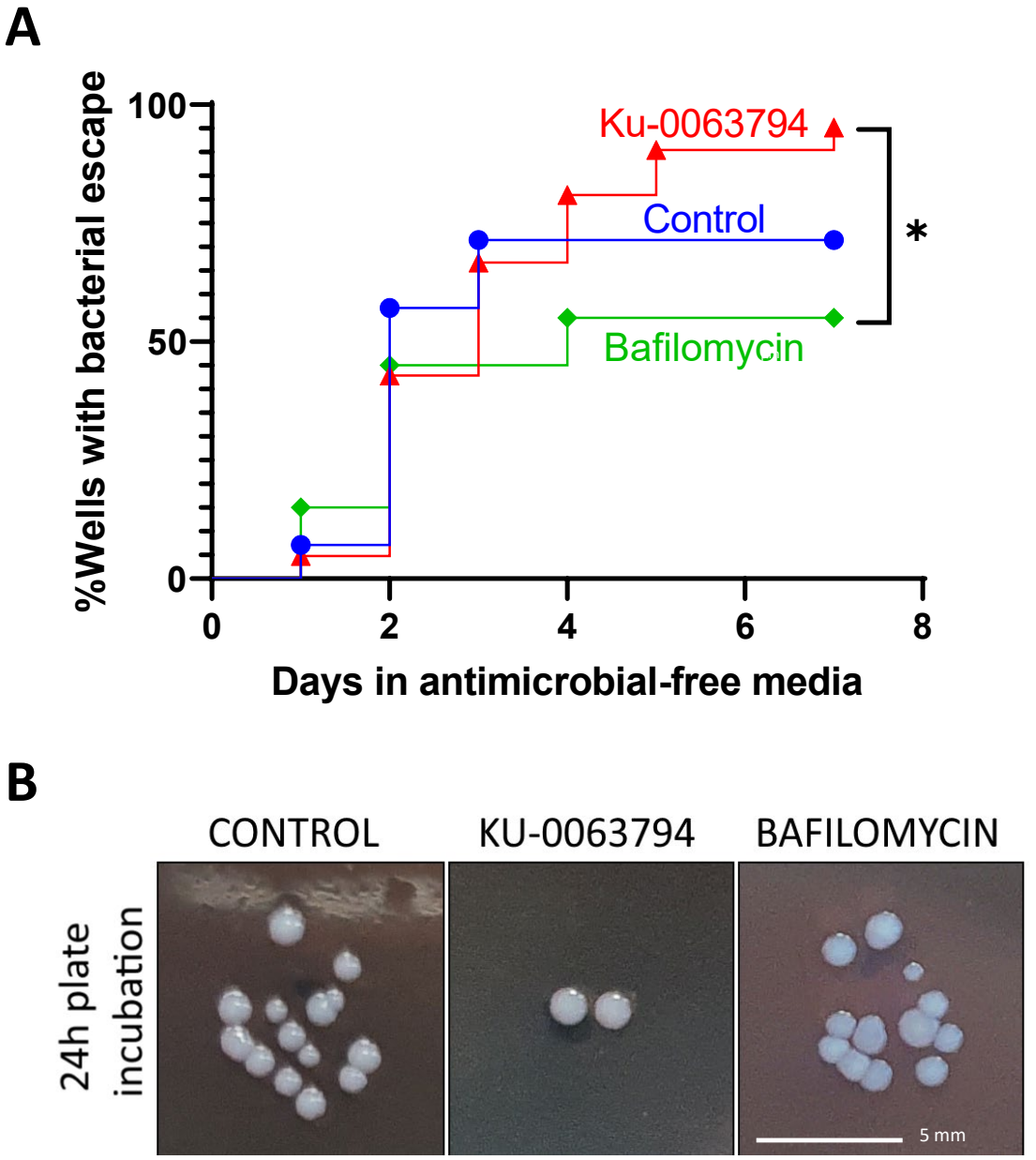
A
%Wells with bacterial escape
100
50
0
0
2
4
6
8
Days in antimicrobial-free media
Ku-0063794
Control
Bafilomycin
*
B
CONTROL
KU-0063794
BAFILOMYCIN
24h plate incubation
5 mm

Figure 6

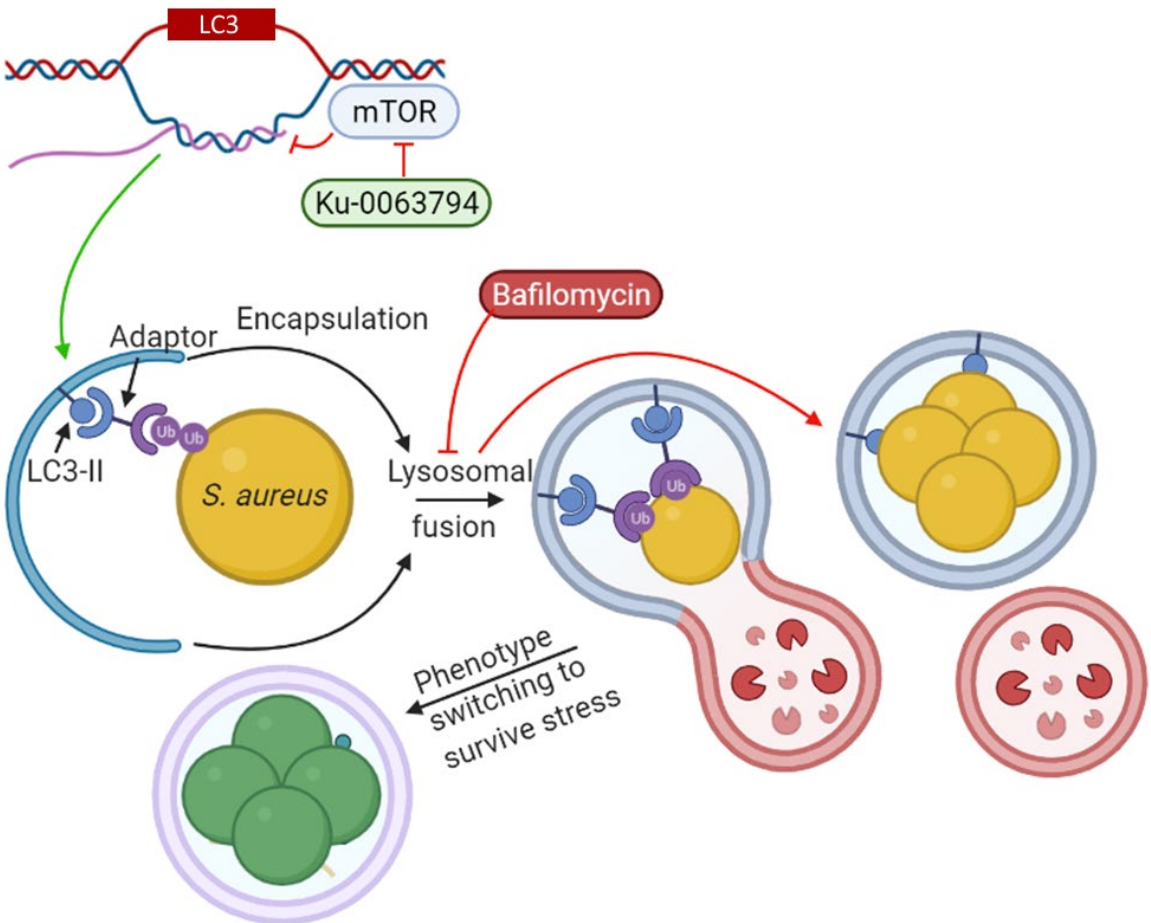
LC3
mTOR
Ku-0063794
Bafilomycin
Adaptor
Encapsulation
LC3-II
Ub
Ub
S. aureus
Lysosomal
fusion
Ub
Ub
Phenotype
switching to
survive stress

Figure 7

Supplemental Table 1.

Gene	Primer direction	Oligonucleotide Sequence (5'→ 3')
ACTB	forward	CGCGAGAAGATGACCCAGATC
	reverse	TCACCGGAGTCCATCACG
sigB	forward	GGGGCAACAAGATGACCATT
	reverse	TGCCGTTCTCTGAAGTCGTG