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Discovery of MRSA active antibiotics using primary sequence from the human microbiome

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Here we present a natural product discovery approach, whereby structures are bioinformatically predicted from primary sequence and produced by chemical synthesis (synthetic-bioinformatic natural products, syn-BNPs), circumventing the need for bacterial culture and gene expression. When we applied the approach to nonribosomal peptide synthetase gene clusters from human-associated bacteria, we identified the humimycins. These antibiotics inhibit lipid II flippase and potentiate β-lactam activity against methicillin-resistant *Staphylococcus aureus* in mice, potentially providing a new treatment regimen.

The characterization of small molecules produced by bacteria in laboratory culture has been a key step to understanding bacterial physiology and developing small-molecule therapeutics[1]. As successful as this approach has been for identifying novel bioactive small molecules, extensive sequencing of bacterial genomes and metagenomes has revealed that the bacterial biosynthetic diversity traditionally accessed in the laboratory represents only a small fraction of what is predicted to exist in nature[2,3]. This shortcoming arises from our inability to culture most bacteria in the laboratory and from the fact that most biosynthetic gene clusters remain silent under laboratory fermentation conditions[4]. Here we present pipeline for the discovery of bioactive small molecules that circumvents the requirement for either bacterial culture or gene cluster expression. In our approach, natural product structures are bioinformatically predicted from primary sequence data and produced by chemical synthesis. As natural products often appear in nature as families of related structures with the same biological activity, we reasoned that even if our structural predictions were not perfect, many syn-BNPs would be sufficiently accurate representations of nature to elicit the intended bioactivities. We named these bioinformatically inspired compounds syn-BNPs (**Fig. 1a**).

The human microbiome is an exemplary test case for a syn-BNP discovery approach. Tremendous resources have been allocated to the sequencing and bioinformatic analysis of the human microbiome[5,6]. Nevertheless, functional characterization of these data, including commensal-bacteria-encoded natural product biosynthetic gene clusters, remains rare. Our interest in exploring syn-BNPs encoded by the human microbiota stems from the potential use of these metabolites as therapeutics and as tools for improving our understanding of human microbiome functions. Because antibiotics can serve as medicines and as modulators of the composition of the human microbiome, we chose to screen syn-BNPs predicted from the human microbiome for antibacterial activity against human-associated commensal and pathogenic bacteria.

Systematic bioinformatic analysis of sequenced bacterial genomes indicate that nonribosomal peptides (NRPs) are one of the most common and diverse families of complex secondary metabolites produced by bacteria[7,8]. Over the past two decades, a number of models have been developed for predicting the identity, order and modification of the amino acids comprising an NRP, based solely on the primary sequence of NRP megasynthetases[9–12]. Concurrently, solid phase peptide synthesis (SPPS) of structurally diverse peptides has become rapid and economical, making NRP gene clusters an ideal test case for a syn-BNP approach (**Fig. 1b**).

We bioinformatically queried genomic sequence data from human (commensal and pathogenic)-associated bacteria for gene clusters predicted to encode large NRPs (≥5 residues), as short NRPs are often highly modified and are therefore not easily accessible using SPPS alone. This analysis led to the identification of 57 unique NRP synthetase (NRPS) gene clusters, from which we removed those that appeared to be incomplete in the existing sequence data and those containing more than one PKS module, a thioreductase domain or any heterocyclization domains. We predicted the chemical outputs of the remaining 25 gene clusters, which we believed to be amenable to SPPS, using three published NRPS prediction algorithms (Stachelhaus, Minowa and NRPSPredictor2) to produce syn-BNP targets[9–12]. When bioinformatic predictions for human microbiome-associated gene clusters diverged strongly between algorithms (e.g., side chains were predicted to carry opposite charges), we designed and synthesized multiple syn-BNP peptides. In all cases where NRPS gene clusters were bioinformatically predicted to encode an N-terminally acylated peptide, we elected to design the syn-BNP to be N-acylated with β-hydroxymyristic acid (HMA), a fatty acid commonly observed in NRPs[13]. We designed 30 syn-BNPs targets on the basis of the gene clusters found in human commensal bacterial sequence data. After two rounds of SPPS using standard Fmoc chemistry, we obtained pure samples for 25 of the 30 targeted syn-BNPs (**Supplementary Results**, **Supplementary Table 1**).

To identify new antibiotic scaffolds with potential *in vivo* roles in shaping the ecology of the human microbiome, we assayed this collection of syn-BNPs for antibacterial activity against a panel of common human commensal and pathogenic bacteria. This led to the identification of two antibiotics we named humimycin A (**1**) and humimycin B (**2**) (for 'human microbiome mycin'; **Fig. 2a**). The humimycins were predicted from closely related NRPS gene clusters found in the genomes of *Rhodococcus equi* and *R. erythropolis*, respectively. Bioinformatic analyses of these two NRPS gene clusters indicated that they encoded hepta-peptides that differed at only the fourth and the sixth residues (F versus Y and V versus I, respectively; **Fig. 2b**). We synthesized both syn-BNPs with N-terminal HMA modifications, owing to the presence of

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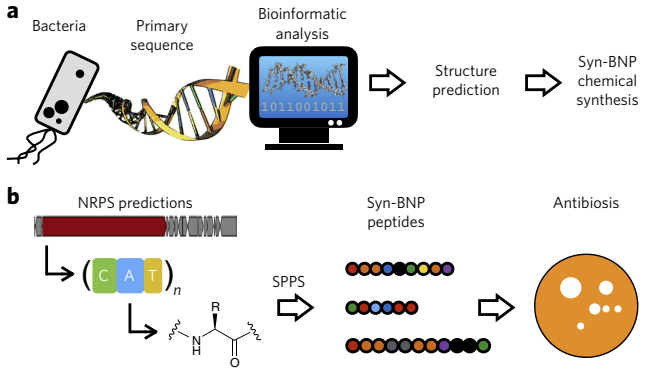

Figure 1 | Overview of the syn-BNP approach. (**a**) Advances in our understanding of natural product biosynthesis have enabled the prediction of natural product structures from primary sequence data alone. In a syn-BNP approach, these structures are accessed through chemical synthesis instead of biosynthesis. (**b**) Here we applied a syn-BNP approach to NRPs predicted from human microbiome sequence data and assayed these new molecules for antibiosis activities.

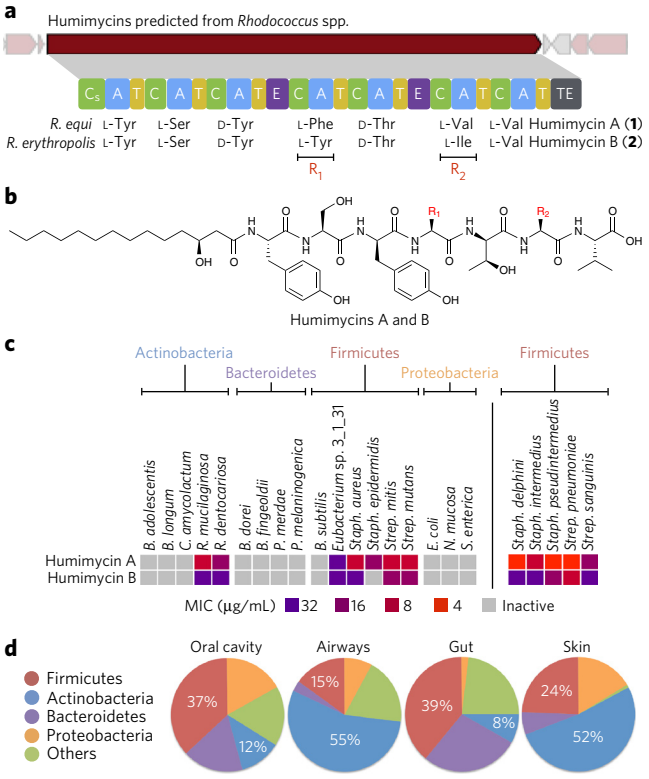

Figure 2 | Discovery and screening of the humimycins. (**a**) The humimycins were predicted from closely related gene clusters found in two *Rhodococcus* spp. cultured from human subjects. (**b**) Chemical structures of humimycin A (**1**) and B (**2**). The two antibiotics differ only at the fourth (F or Y) and sixth (V or I) residues. (**c**) MIC values for the humimycins against a panel of human commensal and pathogenic bacteria (left; $n = 2$). The humimycins were particularly active against bacteria in the *Staphylococcus* and *Streptococcus* genus (right; $n = 3$). (**d**) Human microbiota composition at various body sites.

starter condensation domains (Cs) in the gene clusters, which are associated with acylation of the first amino acid of an NRP[13].

Rhodococcus species have been extensively studied for natural product production using traditional fermentation-based discovery methods. None of those studies report the identification of a metabolite resembling the humimycins[14]. Likewise, our extensive analysis of *Rhodococcus* species culture broth extracts by both liquid chromatography (LC)-UV and LC-mass spectrometry (MS) analyses did not reveal any metabolites related to the humimycins, suggesting that the humimycin gene cluster is silent under laboratory fermentation conditions.

The humimycins were broadly active against Firmicutes and showed some activity against Actinobacteria, when we screened them for antibiosis against commensal and pathogenic bacteria (**Fig. 2c**). The humimycins were particularly active against *Staphylococcus* and *Streptococcus* species, including common members of the normal human flora such as *S. aureus* (minimum inhibitory concentration (MIC) of 8 μg/mL) and *S. pneumoniae* (MIC of 4 μg/mL). This spectrum of activity is interesting in light of the fact that Firmicutes and Actinobacteria dominate the human microbiota of the gut[15] (**Fig. 2d**). In a structure-activity relationship study, we found that no residue in **1** could be replaced with alanine without dramatically impacting the potency of the antibiotic (**Supplementary Table 3**).

Humimycin A MIC values were 8–128 μg/mL against methicillin-resistant *S. aureus* (MRSA) clinical isolates (**Supplementary Table 4**). To study the antibacterial mode of action of the humimycins, we selected *S. aureus* USA300 mutants that could survive on 2.5 times the MIC (20 μg/mL) and sequenced the genomes of 23 resistant mutants. Upon comparison to the parent strain, we found that all 23 mutants contained one nonsynonymous mutation in *SAV1754*, an essential gene in *S. aureus* (**Supplementary Fig. 3** and **Supplementary Table 5**). Fifteen of these strains contained no other detectable mutations. Overexpression of *SAV1754* in *S. aureus* conferred resistance to humimycin A (MIC >128 μg/mL), further supporting inhibition of *SAV1754* as a likely mode of action of the humimycins (**Supplementary Table 6**). The gene product of *SAV1754* is believed to be a homolog of MurJ, a flippase responsible for the translocation of peptidoglycan precursors from the inside to the outside of the cell[16].

Whereas MurJ is essential in many bacteria, including many important pathogens, it remains an underexplored antibacterial target[17]. In a high-throughput screen for molecules that could potentiate β-lactam antibiosis against otherwise resistant strains, Merck & Co. identified synthetic small-molecule inhibitors of SAV1754 (refs. 18,19). The ability of SAV1754 inhibitors to potentiate β-lactam antibiosis is thought to arise from the fact that both antibiotics target the same essential pathway, peptidoglycan biosynthesis (**Fig. 3a**). Humimycin A exhibits a similar ability to restore β-lactam sensitivity to β-lactam-resistant bacteria. For example, the MIC of carbenicillin (carboxypenicillin) decreased from 32 μg/mL to 1 μg/mL in the presence of 2 μg/mL humimycin A (0.25× MIC) against MRSA USA300 (**Fig. 3b**), one of the most predominant community-associated MRSA strains in the United States.

We also observed the ability of humimycin A to potentiate β-lactam activity in strains where it alone showed no detectable antibacterial activity. For example, although the MRSA COL strain was not susceptible to humimycin A (MIC > 512 μg/ml) and exhibited a very high MIC for the β-lactam dicloxacillin (MIC of 256 μg/mL), it was sensitive to dicloxacillin at 8 μg/mL in the presence of as little as 4 μg/mL humimycin A (**Fig. 3c**). The ability of humimycin A to potentiate β-lactam activity *in vitro* led us to explore the possibility that it might do the same *in vivo*. In mouse tolerability studies, humimycin A was tolerated at concentrations (>50 mg/kg), far exceeding those expected to be necessary for β-lactam potentiation. In a mouse peritonitis-sepsis model, treatment of a MRSA COL infection with dicloxacillin and humimycin together dramatically increased survival compared to treatment with either humimycin or dicloxacillin alone (**Fig. 3d**), potentially providing a new MRSA treatment regimen.

Although *R. equi* has historically been regarded as an opportunistic pathogen, seen in animals and immunocompromised patients[20],

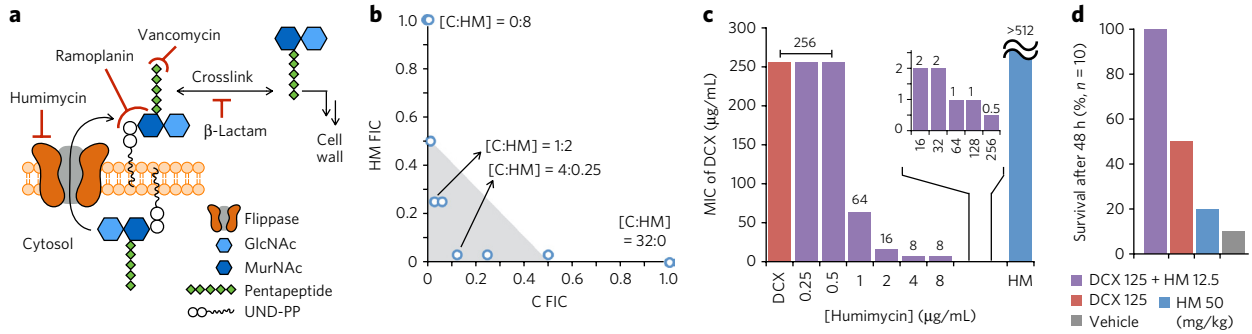

Figure 3 | Humimycin A and β-lactam act in synergy. (**a**) SAV1754 is the *S. aureus* homolog of MurJ, which is a flippase responsible for the transportation of peptidoglycan precursors across the cytoplasmic membrane. (**b**) Carbenicillin (C) and humimycin A (HM) acted synergistically to inhibit the growth of MRSA USA300 ($n = 2$). Fraction inhibitory concentration (FIC) values ≤ 0.5 defines synergy between two agents (shaded area); [C:HM] denotes the respective inhibitory concentrations at each data point (μg/mL). (**c**) MIC of humimycin A with dicloxacillin (DCX) alone and at various humimycin A concentrations against MRSA COL ($n = 2$) are shown in red and purple, respectively. Humimycin A alone did not inhibit MRSA COL growth (MIC > 512 μg/mL; blue). (**d**) Survival data for mice treated with humimycin A or dicloxacillin either alone or together using a MRSA COL peritonitis model ($n = 10$ mice per cohort). In this model humimycin potentiated β-lactam activity *in vivo*.

R. erythropolis is found as a part of the normal human nasal, mouth and eye microbiota[21,22]. The occurrence of *Rhodococcus* species in the gut increases dramatically to a median of 30% in some patients diagnosed with ulcerative colitis (UC)[23]. The production of an antibiotic with activity against Firmicutes and Actinobacteria could have a role in establishing the overpopulation of *R. erythropolis* in the UC gut, as Firmicutes and Actinobacteria normally represent nearly half of the gut microbiota[15]. In addition to the potential to provide new small molecule therapeutics, characterization of molecules inspired by commensal bacteria biosynthetic gene clusters can provide a means for developing hypotheses about how commensal bacteria affect human physiology. For example, the discovery of the humimycins provides a testable mechanistic hypothesis for how dysbiosis of gut microbiota might evolve in UC[24].

Our identification of the humimycins using a syn-BNP approach validates this as a strategy for identifying bioactive metabolites and highlights the unique state of the field of natural product chemistry today. Extensive biosynthetic studies have culminated in our emerging ability to predict the structures of many natural products from primary sequence alone. Although in this study we focused on linear peptides because of the ease with which they can be generated by SPPS, there are many ways to expand this approach to more topologically and functionally complex NRPs. For example, the construction of cyclic peptides using purified thioesterase domains is compatible with SPPS[25]. Based on our analysis of high-quality sequenced bacterial genomes in GenBank not associated with the human microbiome, there are currently more than 1,500 unique large NRPS gene clusters (encoding ≥5 amino acids) amenable to a SPPS-based syn-BNP approach. As the sequencing of microbial genomes is still in an early exponential growth phase, this number should only continue to grow for the foreseeable future (**Supplementary Fig. 2**). With the development of improved bioinformatic prediction algorithms for biosynthetic gene cluster families beyond NRPSs and the incorporation of more sophisticated chemical and chemo-enzymatic synthesis steps, we believe this approach will enable broad and rapid access to diverse bioactive compounds inspired by gene clusters found in the ever-growing assemblage of microbial sequence data.

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Methods

Methods and any associated references are available in the online version of the paper.

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Author contributions

S.F.B. conceived of the project. J.C. and X.V.-F. carried out antibiosis assays, spectrum of activity screening and resistant mutant selection. D.I., H.A.Z., R.G.-M., M.J., S.S. and J.S.F. carried out peptide synthesis on large scale. M.T. carried out genome sequencing. L.J.C. and E.A.G. screened anaerobic bacteria. B.V.B.R. and Z.C.-P. carried out bioinformatic analysis. S.P. and D.S.P. carried out mouse studies.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Any supplementary information, chemical compound information and source data are available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to S.F.B.

ONLINE METHODS

Bioinformatic prediction of NRPs. Genome sequences of the human microbiota were downloaded from the NIH Human Microbiome Project (HMP; ftp://ftp.ncbi.nlm.nih.gov/genomes/HUMAN_MICROBIOM/Bacteria)[26] and the Human Oral Microbiome Database (HOMD; ftp://ftp.homd.org/HOMD_annotated_genomes)[27]. The software package Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) v2.0 was used for the identification and prediction of NRP biosynthetic gene clusters encoded by these genomes[28]. Syn-NRPs originating from the HMP and HOMD databases were named serially as [Human.N] and [Oral.N]. All syn-NRPs discussed in this manuscript are listed in **Supplementary Table 1**. AntiSMASH consults three prediction algorithms to call the amino acid substrate specificity of an adenylation domain (NRPSPredictor2, Stachelhaus code, and Minowa). A consensus prediction refers to the situation wherein two (or all three) algorithms make consistent substrate predictions for a given adenylation domain. In this case the predicted amino acid was used in the synthesis of the syn-BNP. In case of a minor conflict between prediction algorithms we opted for the amino acid with the smaller side chain (underlined), e.g., Val/Leu/Ile and Ser/Thr. In case of major conflicts (for example, where side chains were predicted to carry opposite charges), both peptides were synthesized. Tyrosine and phenylalanine prediction made by NRPSPredictor2 or Stachelhaus code were chosen over Trp predictions made Minowa, as we noticed that Trp is overrepresented in Minowa predictions. Lastly, Tyr was used at the first residue in place of *p*-hydroxyphenylglycine (Hpg) in Human.8v1 and v2. To check the robustness of these NRPS prediction algorithms, we carried out a similar analysis of NRPS gene clusters deposited in the MiBIG database and found that the core peptide encoded by the vast majority NRPS gene clusters was predicted correctly (**Supplementary Fig. 2**).

Peptide synthesis. Resins for peptide synthesis were purchased from AnaSpec. Coupling reagents (PyBOP) and *N*α-Fmoc/side-chain-protected amino acids were purchased from P3BioSystems. 3-Hydroxymyristic acids were purchased from TCI America (racemic mixture) and Santa Cruz Biotechnology (pure enantiomers). All other chemical reagents and solvents were purchased from Sigma-Aldrich. Reaction vessels were custom-made in the Scientific Glassblowing Laboratory at the Department of Chemistry of Yale University.

Pure samples were obtained for 25 of the 30 syn-BNP peptides targeted for chemical synthesis (**Supplementary Table 1**). 20 of these peptides were purchased through the custom peptide synthesis service of GenScript Biotech Corporation and five were synthesized in-house. Peptides from GenScript were delivered as lyophilized materials that had been HPLC-purified and MS-verified (MALDI). All pure peptides were dissolved in DMSO at 12.8 mg/mL as stock solutions and stored at −20 °C. In-house peptide syntheses, including humimycin A and B, were built on Wang resin[29] following standard Fmoc/tBu SPPS methods. The first amino acid (6 equiv.) was activated using DIC (3 equiv.) in 10% DMF/DCM (0 °C), added to the resins in the presence of DMAP as a catalyst (0.1 equiv.) and shaken under nitrogen (4 h at 0 °C). Unreacted resins were capped using acetic anhydride in pyridine (1 h). Fmoc removal was accomplished using three rounds of treatment with 20% piperidine in DMF (15 min, 10 min and 5 min. each). All ensuing amino acids were coupled twice. In each coupling an *N*α-Fmoc and side-chain-protected amino acid was activated using a mixture of PyBOP (4 equiv.) and DIEA (8 equiv.), followed by reaction with the peptide on-resin (1 h). Peptides were cleaved by 95% TFA supplemented with TIS and H_2O (2.5% of each, v/v) for 2 h, concentrated to ~10% of the original volume, diluted with aqueous MeCN (75%, v/v), passed through a 0.45 μm filter and HPLC-purified. All purified peptides were examined by LC-MS (electrospray ionization; ESI).

Characterization of the humimycins. A racemic mixture of 3-hydroxymyristic acid was used for N-terminal modification in our initial syntheses of all syn-BNPs. Humimycin A diastereomers showed different MIC values when tested against MRSA USA300 (**Supplementary Table 3**). The absolute stereochemistry of the most active diastereomer was determined by comparing HPLC-purified peptides from the bulk synthesis to independent batches of small-scale syntheses using enantiopure (R)- and (S)-3-hydroxymyristic acid (**Supplementary Fig. 1**). The more potent (S)-isomer is referred to as humimycin A (**1**). In the case of humimycin B, the analogous (S)-isomer was purified and is referred to as compound **2**. Humimycin A (**1**) high-resolution mass spectrometry (HRMS): mass to charge ratio (*m/z*) calculated for $[M - H]^-$ ($C_{58}H_{84}N_7O_{14}$) was 1,102.6076; found was 1,102.6075. Humimycin B (**2**) HRMS: *m/z* calculated for $[M - H]^-$ ($C_{59}H_{86}N_7O_{15}$) was 1,132.6182; found was 1132.6194.

Syn-BNP screening. Syn-BNPs were screened against a panel of commensal and pathogenic bacteria covering the four major phyla associated with the human microbiome. This included five Actinobacteria, four Bacteroidetes, six Firmicutes and three Proteobacteria species. All peptides were tested in duplicate for antibiosis activity. Assays were performed in microtiter plates, wherein each well contained 100 μL growth medium (see **Supplementary Table 2** for a list of growth media), 32 μg/mL syn-BNP and bacteria diluted 1,000-fold from a stationary phase culture. Binary antibiosis results for most bacteria were determined by visual inspection after static incubation at 37 °C for 18 h. *P. melaninogenica* and *Eubacterium* sp. 3_1_31 were grown for 36 h, and *C. amycolactum* was grown for 60 h. Specific MICs were determined for syn-BNPs that inhibited bacterial growth in this initial screen (see below). Bacteria species associated with the human flora were obtained from BEI Resources.

Susceptibility assays. *Standard assays.* MIC assays were performed in duplicate in 96-well microtiter plates based on the protocol recommended by Clinical and Laboratory Standards Institute[30]. All presented data are the average of at least two independent assays, where *n* denotes the number of replicate. DMSO stock solutions of syn-BNPs (12.8 mg/mL) were added to the first well in a row and serially diluted (twofold per transfer) across the microtiter plate. The last well was reserved for a peptide-free control. Overnight cultures of bacteria were diluted 5,000-fold, and 50 μL was used as an inoculum in each well. MIC values were determined by visual inspection after 18 h incubation (37 °C, static growth).

Synergy assays. Synergistic β-lactam-humimycin activities were assessed through a two-dimensional (2D) susceptibility assay. Twofold serial dilutions were carried out as described above. Carbenicillin (a β-lactam antibiotic) was diluted serially from left to right, and humimycin was diluted serially from top to bottom. The highest concentration tested for both antibiotics was 32 μg/mL. Fractional inhibitory concentration (FIC) is defined as the ratio of the apparent synergistic MIC divided by the MIC of the antibiotic measured alone[31].

Selection of humimycin-A-resistant mutants. A single *S. aureus* USA300 colony (the parent) from a freshly struck plate was inoculated into LB medium and grown overnight at 37 °C. Part of the overnight culture (4 mL) was spun down and kept frozen at −20 °C. The rest of the overnight culture was diluted 100-fold, supplemented with humimycin A at 20 μg/mL (2.5× MIC) and 100 μL aliquots was distributed into 200 unique microtiter plate wells. Growth was observed in 50 wells after overnight incubation, indicating the presence of bacteria with mutation(s) conferring humimycin A resistance. Approximately 2 μL of culture from each of these wells was used to inoculate freshly prepared 100 μL aliquots of LB media supplemented with humimycin A (20 μg/mL). The resulting cultures after overnight incubation were struck out for single colonies on LB agar plates supplemented with humimycin A (20 μg/mL) for single colonies.

Genome sequencing. Single colonies of 23 humimycin-A-resistant mutants as well as the USA300 parent were individually inoculated into 4 mL of LB media free of any antibiotics. After overnight incubation cells were collected by centrifugation. DNA extractions were performed using a MasterPure Purification Kit (EpiCentre Biotechnologies). Multiplex sequencing libraries were prepared from the resulting genomic DNA using a Nextera XT DNA Sample Preparation Kit (FC-131-1024) with Nextera XT Index kit (FC-131-1001) based on protocols provided by the manufacturer (Illumina). Briefly, the genomic DNA was treated with RNase and quantified using the Qubit dsDNA HS Assay System (Q32854, ThermoFisher Scientific). Tagmentation and PCR amplification proceeded according to the manufacturer's protocol, after which the quality and size of the libraries were verified using HS D1000 ScreenTape

(TapeStation 2200, Agilent Technologies). Libraries were pooled at equimolar concentrations and column purified by NucleoSpin Gel and PCR Clean-up (MN-750609-250, Macherey-Nagel). The resulting tagged DNA library was size-selected by E-Gel (Life Technologies), and the 450-base-pair band was excised. The final library pool was checked for molarity on TapeStation and sequenced using MiSeq Reagent Kit v3 (MS-102-3003, Illumina).

Mutation (single-nucleotide polymorphism) identification. De-barcoded MiSeq reads were assessed for mutations by comparing each read against the reference genome of *S. aureus* USA300_FPR3757 (RefSeq assembly accession: GCF_000013465.1). All reads were mapped to the reference genome using SNIPPY (https://github.com/tseemann/snippy) for the identification of variants. SNIPPY is a wrapper of several programs including freebayes (https://github.com/ekg/freebayes)[32]. Single-nucleotide polymorphisms observed in the parent strain were then subtracted from those observed in the humimycin A resistant strains, resulting in a final list of single-nucleotide polymorphisms (**Supplementary Table 5**).

Cloning and overexpression of SAV1754. The *SAV1754* gene was PCR-amplified from wild type *S. aureus* USA300 and a mutant resistant to humimycin A (mutant no. 8, **Supplementary Table 5**). PCR products and the pRMC2 (ref. 33) vector were digested (SacI and KpnI) and ligated, followed by transformation into *S. aureus* RN4220 and selection on BHI agar plates containing chloramphenicol (10 μg/mL). The recombinant plasmids were verified by DNA sequencing. Overnight cultures of the resulting *S. aureus* strains were used to inoculate LB containing chloramphenicol (10 μg/mL). Late log-phase cultures (OD_{600} of ~0.8) were induced by anhydrotetracycline (50 ng/mL) for 5 h and then tested in the presence of anhydrotetracycline (5 ng/mL) for susceptibilities against humimycin A. Primer sequences, PCR conditions and susceptibility data are listed in **Supplementary Table 6**.

Mouse peritonitis-sepsis model. Female outbred Swiss Webster mice were used for this study. MRSA COL was grown in Mueller-Hinton broth at 37 °C overnight and diluted with 5% hog mucin and 0.9% NaCl to provide challenge inoculum of ~5×10^8 CFU per mouse in a volume of 0.5 mL via intraperitoneal injection. Forty mice were randomly grouped into ten per cohort, and each group was given single doses of vehicle (20% DMA, 40% PEG, 40% D5W), humimycin (HM) at 50 mg/kg, dicloxacillin (DCX) at 125 mg/kg, and HM:DCX combination at 12.5 mg/kg HM:125 mg/kg DCX 1 h after infection via intravenous injection. Mice were maintained in accordance with American Association for Accreditation of Laboratory Care criteria. The Rutgers University Institutional Animal Care and Use Committee approved all animal procedures.

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