

Regulation and Impact of Secondary Metabolites produced by Staphylococci

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Leonie Reetz

aus Stuttgart

Tübingen

2024

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	16.10.2024
Dekan:	Prof. Dr. Thilo Stehle
1. Berichterstatter/-in:	Prof. Dr. Andreas Peschel
2. Berichterstatter/-in:	Prof. Dr. Christiane Wolz

Table of contents

TABLE OF CONTENTS	1
ABSTRACT	2
ZUSAMMENFASSUNG	4
CHAPTER 1	6
General Introduction	6
CHAPTER 2	20
The human microbiome-derived antibiotic lugdunin self-regulates its biosynthesis in a feed forward-like fashion.....	20
CHAPTER 3	54
Ulbaetin F represents a chalkophore facilitating copper homeostasis in <i>Staphylococcus lugdunensis</i>	54
CHAPTER 4	81
Horizontal transfer of bacteriocin biosynthesis genes requires metabolic adaptation to improve compound production and cellular fitness.....	81
CHAPTER 5	135
General Discussion.....	135
CONTRIBUTIONS TO PUBLICATIONS	145
CURRICULUM VITAE.....	148

Abstract

Every surface of the human body is colonised by bacteria, viruses, fungi, and other members of the microbiome. The diversity and fluctuation of the microbiome in distinct niches like the gut, or the nares plays a crucial role in health and disease of every individual. During colonisation, bacteria have to overcome eliminating factors like the human innate immune system as well as the pre-existing microbiome. Hence, many bacteria have evolved various strategies to overcome this limitation. For instance, the production and uptake of secondary metabolites, which are not necessary for normal growth and division of bacterial cells. Amongst them are for example molecules with a high-affinity for distinct transition metals, like siderophores for iron uptake, or the production of antimicrobial compounds to inhibit competing cells in close vicinity to the producer. Recent studies have shown that in various staphylococci, such secondary metabolites are not only used for competition between different species but are also shared, not only as molecules but also on a DNA level as biosynthetic gene clusters. However, the regulation of many biosynthetic gene clusters of such, often metabolically costly, secondary metabolites remain often unknown. Here, we describe the regulation of the lugdunin biosynthetic gene cluster of *Staphylococcus lugdunensis* as well as the expression of transporters for self-immunity. We furthermore elucidate the product of another undescribed biosynthetic gene cluster of *S. lugdunensis* and show that the previously described ulbactin F is synthesized. Thirdly, we report that micrococcin P1 can be shared in communities at a high metabolic cost impairing bacterial fitness and found, that the reduced fitness can be omitted by adaptive mutations of the receiving strain.

We demonstrate, that the production of lugdunin and the expression of the transporters LugEF and LugGH are dependent on a positive feedback regulation. Both regulators, LugR and LugJ, interact with distinct promoter sequences and hence repress transcription initiation. Bioinformatic analysis reveals that both regulators belong to different families, however, upon binding of lugdunin to either regulator, transcription is initiated.

Furthermore, we show that *S. lugdunensis* produces another previously unidentified secondary metabolite. We demonstrate that the produced compound is ulbactin F. Our study shows that in *S. lugdunensis* ulbactin F does not exhibit an iron-binding affinity even though its regulation is iron-dependent. Preliminary results strongly support a copper-binding affinity, identifying it as a chalkophore.

Additionally, we investigate the metabolic burden of micrococcin P1 production after horizontal transfer into the lab strain *Staphylococcus aureus* RN4220, leading to a growth deficiency and

disturbance in metabolic activity. Fitness disadvantages however could be identified to be reversible by adaptive mutation in the *citZ* gene encoding for the citrate synthase, leading to an evolutionary advantage.

Our studies further elucidate the impact of bacterial secondary metabolites produced by *Staphylococcus* spp. on various microbe-microbe interactions.

Zusammenfassung

Jede Oberfläche des menschlichen Körpers ist von Bakterien, Viren, Pilzen und anderen Mitgliedern des Mikrobioms besiedelt. Die Diversität und Fluktuation des Mikrobioms in bestimmten Nischen wie dem Darm oder den Nasenlöchern spielt eine fundamentale Rolle für die Gesundheit und Krankheit jedes Einzelnen. Während der Kolonisation müssen Bakterien eliminierende Faktoren wie das humane angeborene Immunsystem oder das bereits vorhandene Mikrobiom überwinden. Daher entwickelten viele Bakterien verschiedene Strategien um diese Limitierung zu überwinden, unter anderem die Produktion und Aufnahme sekundärer Metabolite, die für das normale Wachstum und die Zellteilung einer bakteriellen Zelle nicht essentiell sind. Hierzu gehören zum Beispiel Moleküle mit einer hohen Affinität für einzelne Übergangsmetalle, beispielsweise Siderophore für die Eisenaufnahme. Ebenso zugehörig ist die Produktion antimikrobieller Wirkstoffe um konkurrierende Bakterien in naher Umgebung zur produzierenden Zelle zu inhibieren. Jüngste Studien haben gezeigt, dass einige Staphylokokken solche sekundären Metabolite nicht nur im Wettbewerb gegen andere Spezies produzieren, sondern sie auch sowohl als Molekül als auch auf DNA-Ebene als biosynthetisches Gencluster teilt. Die Regulation solcher biosynthetischen Gencluster, die häufig einen metabolischen Aufwand für produzierende Bakterien bedeutet, ist jedoch oft weitestgehend unbekannt.

Hier beschreiben wir die Regulation des biosynthetischen Genclusters von Lugdunin, produziert von *Staphylococcus lugdunensis*, sowie der Expression der Transporter für dessen eigene Immunität. Beide Regulatoren interagieren mit distinktiven Promotorsequenzen und verhindern somit die Initiation der Transkription. Bioinformatische Analysen zeigen, dass beide Regulatoren zu unterschiedlichen Familien gehören, jedoch kann bei beiden eine Initiation der Transkription nach der Bindung von Lugdunin festgestellt werden.

Weiterhin zeigen wir, dass *S. lugdunensis* ein weiteres, bisher unidentifiziertes sekundäres Metabolit produziert. Wir legen dar, dass es sich hierbei um Ulbactin F handelt. Unsere Studie weist auf, dass in *S. lugdunensis* Ulbactin F keine Eisen-bindende Eigenschaft zeigt wenn auch die Produktion eisenabhängig reguliert ist. Erste Ergebnisse weisen auf eine Affinität für Kupfer hin und identifizieren es daher als Chalkophore.

Zusätzlich untersuchen wir den metabolischen Aufwand von Micrococcin P1 nach horizontalem Transfer des biosynthetischen Genclusters in den Laborstamm *Staphylococcus aureus* RN4220. Dies führt zu einem Wachstumsdefizit und einer Störung in der metabolischen Aktivität des Stammes. Jedoch konnte dies jedoch durch eine adaptive Mutation im *citZ* Gen,

kodierend für die Citratsynthase, als reversibel identifiziert werden und führt daher zu einem evolutionären Vorteil.

Durch unsere Studien klären wir weiterhin die Auswirkungen von bakteriellen sekundären Metaboliten, produziert von *Staphylococcus* spp., auf Interaktionen zwischen Mikroben auf.

Chapter 1

General Introduction

Human nasal microbiota in health and disease

The human body is colonized by trillions of microorganisms, of which bacteria represent the majority next to fungi, protozoans, and archaea. These bacteria living on and inside the human body form the human microbiome – also referred to as microbiota [1, 2]. For bacteria, the human body features a variety of specific ecological niches, such as the gastrointestinal tract, the skin, or nasal cavities. As a consequence of nutrient and oxygen availability, the microbial composition varies in different parts of the body [3-5]. In the human nasal cavity for instance, nutrient availability is scarce and therefore harbours a low species diversity compared to other, nutrient rich niches like the gut. In healthy human adults the microbiome of the nares is dominated by three phyla: Actinomycetota (previously Actinobacteria), Bacillota (previously Firmicutes), and Pseudomonadota (previously Proteobacteria) (Figure 1). Further, also members of the phyla Bacteroidota (previously Bacteroidetes) and Fusobacteriota (previously Fusobacteria) shape the human nasal microbiome [6-8]. Dominating genera within their respective phylum are *Bifidobacterium*, *Corynebacterium*, *Dolosigranulum*, *Moraxella*, *Staphylococcus* as well as *Streptococcus* [7, 9].

Besides the common microbiota, the nasal cavities are a major habitat for various infective agents, e.g., viruses like SARS-CoV-2 or pathogenic bacteria like *Haemophilus influenzae*, *Streptococcus pneumoniae* as well as the important opportunistic pathogen *Staphylococcus aureus* [6, 10, 11]. Microbiome imbalance is considered an important marker for disease onset, often associated with *S. aureus* colonisation [12, 13]. Approximately 20, 30 and 50% of the population have been identified as persistent, intermediate and non-carriers of *S. aureus* [13]. A high colonisation burden of *S. aureus* is associated with a shift from a highly diversified microbiome to a species-depleted one [11, 12, 14]. Colonisation with *S. aureus* increases the risk for infection of the respective individuals, ranging from small wound infections to life-threatening infections like bacteraemia or endocarditis [15]. The nasal microbiome participates in crucial host-associated functions like metabolism of nutrients or even immune system-like activity to help maintain a balance between the host and other microbes. Human health has been described to be dependent on the symbiosis between commensal members of the microbiome and their host [16, 17].

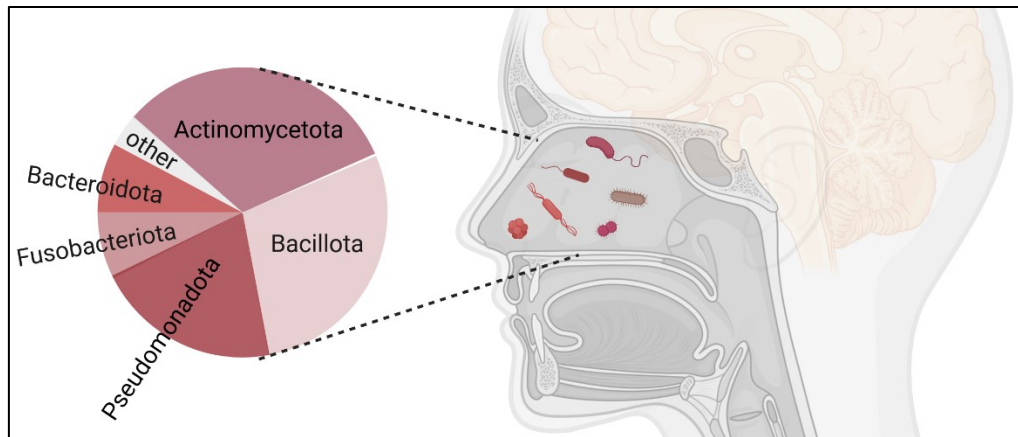


Figure 1 Nasal microbiome shaped by distinct phyla. Actinomycetota, Bacillota as well as Pseudomonadota represent the dominating phyla. Further members of the nasal microbiome appertain to the phyla of Bacteroidota as well as Fusobacteriota [7, 9, 18, 122].

Staphylococcal interactions with the host and the human microbiome

Host-microbe interactions at the human nasal surface layer

In order to colonise the nasal passage of the human host, bacteria need to enforce adhesion to the epithelial layer against the common microbiota, withstand attacks by the host immune system and overcome the limited amount of nutrients.

One of the most important factors in host infection is adherence of pathogenic bacteria to the epithelial cell barrier. The first line of defence in the human airway system is the mucus layer, which traps bacteria and other particles and, through ciliary motion, transports them towards the oral cavity [19, 20]. The mucus layer also contains immunoglobulin A (IgA), produced by activated B cells, preventing pathogens from interacting with epithelial surface receptors [21]. The epithelial cell layer also plays a vital role in pathogen defence. Epithelial cells as well as dendritic cells and macrophages express various receptors, so called pattern recognition receptors (PRRs) which enable rapid pathogen recognition and subsequent initiation of immune response (Figure 2A) [17]. After adherence to the host cells, pathogenic bacteria need to prevail against the microbiome which form a microbial barrier against 'foreign' bacteria. This mechanism has been shown for the human gut and skin, however, similar effects can be presumed for other niches in the human body [22, 23].

Bacterial interactions inside the host

Competition for nutrients and space is often associated with the production of secondary metabolites, which are not essential for the primary metabolism of bacterial cells, however, often allocate advantageous traits to bacteria [24, 25]. Three classes are defined for such secondary metabolites in staphylococci: polyketides (PKs), non-ribosomal peptides (NRPs), or

ribosomal synthesized and post-translationally modified peptides (RiPPs). Important types of secondary metabolites for microbe-microbe interactions are amongst other: signal molecules for cell-cell communication, metallophores for acquisition of essential trace metals and antimicrobial compounds for competition against other microbes.

Quorum sensing

The quorum sensing (QS) system is involved in bacterial cell-to-cell communication (Figure 2B). QS is responsible for regulation of various cellular functions like stress adaptation, virulence factor expression, biofilm formation or production of secondary metabolites [26, 27]. Quorum sensing bacteria express and secrete different autoinducers (AIs), hence the concentration is depending on bacterial density. When sufficient bacteria are present, the concentration of AIs increases and upon reaching a certain threshold, target genes are either activated or repressed. This has first been shown for the expression of *lux* genes responsible for luminescence in *Vibrio fischeri* and *Vibrio harveyi*, but has also been shown to have an important role in regulation of virulence factors of various human pathogens, for example *Pseudomonas aeruginosa* or *Burkholderia cepacia* [27-29]. In *S. aureus* virulence is regulated by QS where the AI, named autoinducer peptide (AIP) is encoded and sensed by the accessory gene regulator (*agr*) system [30]. In *S. aureus* four different AIPs have been described leading to the definition of four *agr*-types. Interestingly, AIs produced by one *agr*-type are able to cross-inhibit quorum sensing in *S. aureus* species with other *agr*-type, resulting in a specific intraspecies signalling and competition. This phenomenon is called 'agr interference' [31].

Metallophores

Another group of secondary metabolites which play a major role in microbe-microbe interactions are metallophores. Under transition metal-restricted conditions these low molecular weight compounds are produced, in order to scavenge transition metals from the environment and deliver them back to the cell. Various metallophores have been described with different metal binding affinities, such as siderophores with a high affinity for iron, zincophores, or copper-binding chalkophores [32-34]. Staphyloferrin A and staphyloferrin B (*S. aureus*) [35, 36], yersiniabactin (*Yersinia pestis*) [37], as well as methanobactin (*Methylosinus trichosporium*) [38] are well described examples metallophores. Siderophores, formerly known as siderochromes, have a major impact on inter- and intrabacterial interactions [39-41]. Three types of bacterial interactions have evolved for siderophore production and acquisition: cooperation, competition, and cheating (Figure 2C). As siderophores produced by one species can be shared in communities, they are sometimes considered as common goods for intra- or interspecies cooperation [42-44]. Interspecies cooperation has been extensively studied for *P. aeruginosa* and *S. aureus* [45, 46]. Co-colonization of the human airways by both *S. aureus* and *P. aeruginosa* is often associated with a detrimental disease outcome. A dual biofilm experiment, has shown that pyochelin, a siderophore produced by *P. aeruginosa*,

is converted to pyochelin methyl ester (PME) in *S. aureus* resulting in a lower iron affinity molecule [46, 47]. It is assumed, that this might have two causes: Firstly, iron availability for *P. aeruginosa* is reduced, leading to a higher survival rate of *S. aureus*. Secondly, *P. aeruginosa* can also sense PME, therefore PME is assumed to be a signalling molecule between *P. aeruginosa* and *S. aureus* [46].

Micronutrients however, are also a limiting factor. Therefore, some bacteria have developed specific receptors for the uptake of siderophores to compete for iron under limited availability [44]. This specific competition has been defined as exploitation, where nutrients are rapidly sequestered or consumed to outcompete competitors [48]. As the human nasal environment is low in nutrients, many interaction studies of the human nasal microbiota have been carried out [11, 49-51]. It has been shown that *Corynebacterium propinquum* inhibits growth of *S. aureus* by the production of a siderophore under iron-limiting conditions [49]. Further, also *Acinetobacter baumannii* was able to inhibit *S. aureus* as well as other Staphylococci by the production of the siderophore acinetobactin [52].

Cheating has emerged between siderophore non-producing strains which have evolved uptake mechanisms for siderophores of producing strains [44]. As the production is often costly, the cheaters' fitness expands at the expense of the producers' fitness, also described as 'the tragedy of the commons' [43, 53-55]. A well-studied example is pyoverdine production by *P. aeruginosa*. Pyoverdine is considered a 'public good' as it excreted and diffuses between cells, thus it can be taken up by producers as well as non-producers [43, 55, 56].

Antimicrobial peptides

An important role in microbe-microbe interactions play secreted antimicrobial peptides (AMPs) (Figure 2D). These are often toxic to a specific set of species, and therefore additionally determine niche colonization. A broad range of AMPs have been isolated from microbiome-derived bacteria and have been divided into various subclasses. AMPs belonging to the subclass of lantibiotics have been extensively described as RiPPs [57, 58]. Lantibiotics are short peptides with a length of <40 amino acids. Through post-translational modification, a cross-link between a cysteine and a serine or threonine is formed, resulting in the non-proteinogenic amino acid lanthionine [59]. Extensively studied examples are epidermin and its derivative gallidermin produced by individual strains of *S. epidermidis* and *S. gallinarum*, respectively [60-62]. Both are effective against *Micrococcus luteus* and *Lactococcus lactis*, and partially against *S. simulans* [63]. Both epidermin and gallidermin interact with lipid I and lipid II, peptidoglycan precursors, inhibiting the peptidoglycan crosslinking of growing cells. Further, interaction between epidermin/gallidermin and lipid II leads to membrane thickness-dependent pore formation in target cells. Interaction was also shown for cell wall precursors lipid III and lipid IV, resulting in inhibition of cell wall biosynthesis [63-66]. In contrast, NRPs are

synthesized by large multi modular enzyme complexes, so called non ribosomal peptide synthetases (NRPSs) [67]. NRPs can not only consist of the 20 proteinogenic L-amino acids, but also of other non-proteinogenic amino acids as well as fatty acids, sugars and heterocyclic domains, leading to an immense range of bioactive peptides [68-74]. A recently described NRP class are fibupeptides with lugdunin produced by *Staphylococcus lugdunensis*, being the first described AMP of this class [75, 76]. It was shown, that lugdunin-producing *S. lugdunensis* can outcompete *S. aureus* *in vitro* as well as *in vivo*, by a protonophore-like mode of action [75, 76].

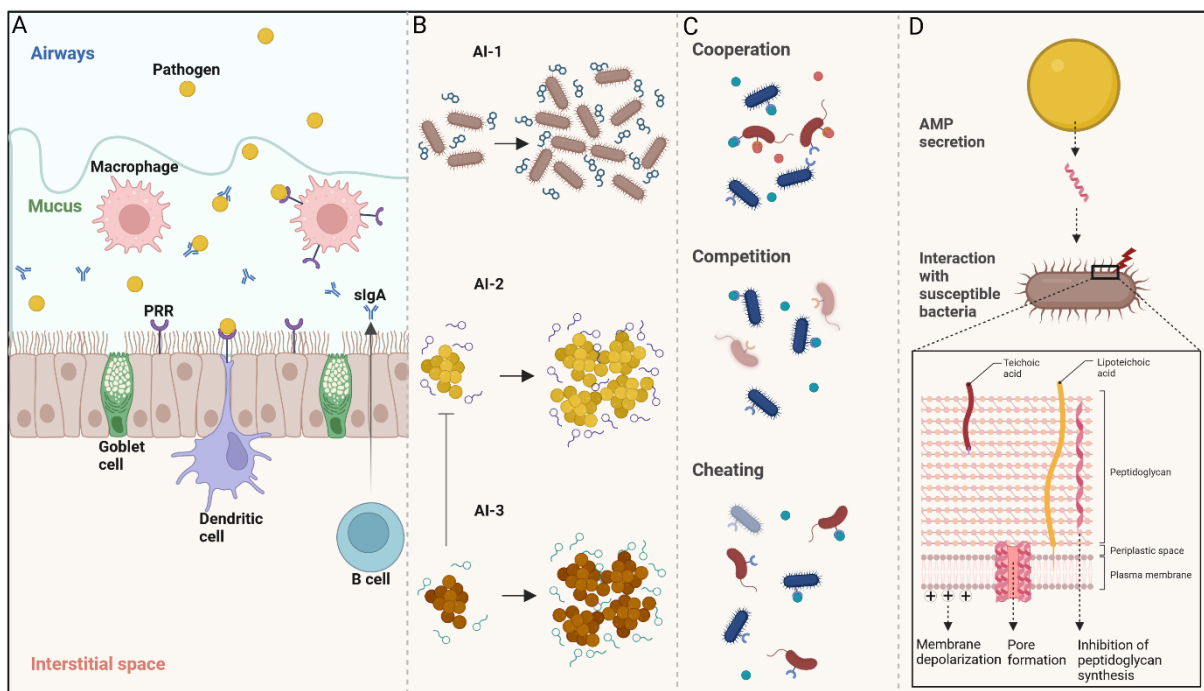


Figure 2 Microbe-host and microbe-microbe interactions. A) Host's innate immune system at the epithelial surface layer. PRR: Pattern recognition receptor, slgA: soluble immunoglobulin A. Microbe-microbe interactions are versatile. B) Quorum sensing: Communication within or between bacterial species by secreted autoinducers (AI). (C) Siderophores as shareable or non-shareable public goods. Interbacterial interactions can lead to cooperation, where siderophores are shared between different bacterial species. (D) Direct inhibition of susceptible bacterial species by the production of antimicrobial peptides (AMP) [122].

Regulation of biosynthetic gene clusters

Regulation of gene expression is crucial for every living organism. Every stage from DNA to a fully functional protein - the transcription of DNA to RNA, translation of RNA into a peptide chain and folding into a protein, as well post-translational modifications of proteins - can be regulated. The transcription initiation in prokaryotes is dependent on the formation of the

initiation complex, which comprises the DNA-dependent RNA polymerase (RNAP), a holoenzyme consisting of five subunits ($\alpha_2\beta\beta'\omega$), plus a sigma (σ) factor. The complete initiation complex is capable to interact with respective DNA regions – the promoters [77-80]. The core components of a promoter are the -10 region (also referred to as TATA-Box) and the -35 region, which are essential for initiation and transcription rate, respectively [81-83]. However, further elements like the upstream (UP) element, upstream of the -35 region with an AT-rich sequence, and the core recognition element (CRE), downstream of the -10 region, are necessary to fully establish the interaction between the initiation complex and the promoter (Figure 3) [83-85]. Upon successful binding, the σ factor is discharged from the RNAP, also referred to as core RNAP, and the DNA is transcribed to RNA [86-88]. Gene expression is mainly regulated by growth phase or environmental conditions of the respective bacteria through the binding of distinct σ factors to the RNAP [77, 80]. Exemplified for *S. aureus*, the primary sigma factor σ^{SA} is responsible for the transcription of housekeeping genes (necessary for normal growth of bacterial cells), whereas the alternative sigma factor σ^{SB} is responsible for the regulation of virulence genes by regulation of the global regulator SarA [89, 90].

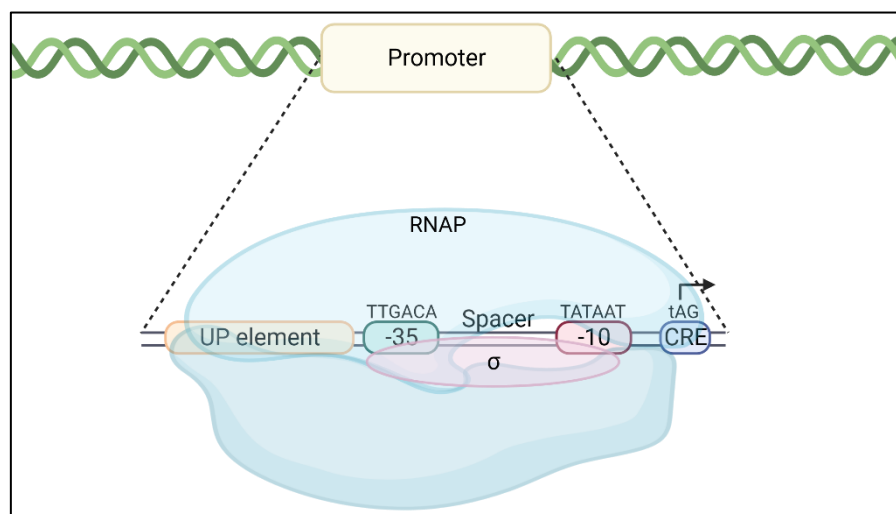


Figure 3 Interaction of the initiation complex with promoter sequences. The fully assembled initiation complex can successfully interact with promoter regions on the prokaryotic genome. The promoter region consists of an UP element, a -35 region, a -10 region and a core recognition element (CRE). Consensus sequences are shown above the respective regions of the promoter [122]. RNAP: DNA-dependent RNA Polymerase, σ : sigma factor.

Transcriptional regulators – regulatory proteins of gene expression

To ensure a fine-tuned, differential response to variations in environmental conditions, bacteria have developed further regulatory mechanisms on the DNA to RNA level, amongst them transcriptional regulators. They determine whether a gene is expressed or not and affect the transcription rate. These regulators comprise a wide family of proteins and consist of at least two domains: One for the interaction with a ligand, the regulatory-domain, and one for the interaction with the operator region on the DNA, the DNA-binding domain [91-94]. Binding of either a ligand or the operator sequence leads to conformational changes in the regulatory protein [94-96]. Transcriptional regulators can either act as repressors, blocking the binding site of RNAPs, or activators, enhancing the binding of RNAPs to the promoter [93, 94].

Feedback and feed forward regulation

Feedback regulation is a fundamental but complex mechanism, responsible for the reaction to changes in the environment of a bacterial cell by positively or negatively adjusting gene expression. When gene expression is regulated via a feedback loop, the regulated biosynthetic gene product acts as a signalling component on its own biosynthesis by binding to its own promoter region [97-99]. Feedback loops can be limited to the regulator and the gene product or include further proteins, for example transporters [98, 99]. Positive feedback loops lead to an amplification of gene expression and therefore to an enhanced intracellular response, by repressing the DNA-binding of transcriptional repressors or by activation of transcriptional activators [98]. Positive feedback loops for instance, play an important role in the context of response to an antimicrobial resistance, as positive feedback regulation can lead to an increase of expression of efflux pumps or antibiotic-degrading enzymes leading to antimicrobial resistance [100, 101]. Contrariwise, negative feedback loops lead to a reduction of biosynthetic pathway product and therefore down-regulate response pathways by the inhibition of gene expression by binding to the respective promoter regions [98]. This is well-described for metabolic pathways, where the gene product negatively regulates the activity of enzymes involved in its own synthesis. The biosynthesis of amino acid is one well-studied example [102].

Another complex regulation mechanism is the feed forward regulation. With this mechanism, bacteria can react to changes in the environment before an environmental change occurs. Feed forward regulation relies on an upstream metabolite or other signal which has a direct influence on the downstream gene expression and therefore metabolic processes [103-105]. With this, bacteria can also quickly adapt to changing environmental conditions, like nutrient supply. One well-studied example is the lactose metabolism by the *lac* operon [103, 106].

Metal responsive regulators

Transition metals like iron, zinc, manganese, cobalt or copper are vital for all forms of life, as they often play a pivotal role as catalytical or structural cofactors for enzymes, however, excess of these metals is often toxic to cells [107]. Therefore, the acquisition and homeostasis of transition metals is highly regulated in prokaryotic cells. Metal responsive regulators are key elements of metalloregulation in prokaryotes, they recognize and bind distinct operator DNA sequences in the promoter, so called 'boxes' [108-111]. One well-studied example is the Fur (ferric uptake regulator) box, with the following consensus sequence for *Staphylococcus* spp. 5'-GATAATgATTaTcAttat-3' [110, 112, 113]. Cytosolic metal-sensing regulators have been clustered into seven families so far, named after their first-described member [114]. These seven families can be further clustered into three distinct groups featuring different modes of action in response to binding of their cognate metal. The first group describes co-repressors, which bind and therefore inhibit their cognate promoter when the respective metal is bound. Regulators belonging to the group of co-repressors are for example Fur [115], nickel-responsive regulator (NikR) [116], and diphtheria toxin regulator (DtxR) [117]. The second group belongs to the activators, which also bind their cognate promoter when their respective metal is bound, but on the contrary the gene expression is then activated. The mercury-responsive regulator (MerR) belongs to the group of activators [118]. The third group comprises the de-repressors. Regulators belonging to this group bind to their respective promoter region, when the binding pocket for their cognate metal is free. Upon binding of the cognate metal to their respective regulators, the regulator is released from the promoter region, leading to gene expression. De-repressors are for example the copper-sensitive operon regulator (CsoR) [119], the cobalt and nickel repressor (RcnR) [120], as well as the arsenical regulator (ArsR) [121].

Aim of the study

As illustrated above, the regulation and therefore production of secondary metabolites plays a tremendous role for staphylococcal colonisation and its pathogenic potential. Still, the understanding of the potential of secondary metabolites produced by members of the human nasal microbiome is lacking behind. Through recent studies, staphylococcal competition by metallophores or antimicrobial compounds is highlighted. However, the regulation and metabolic costs of such often remains unknown. In this study, we aim to investigate the regulation of the lugdunin biosynthetic gene cluster as well as another, previously unknown compound produced by *S. lugdunensis*. Furthermore, the metabolic burden of horizontally transferred micrococcin P1 and its evolutionary adaptation in *S. aureus* is analysed. Thus, this work was supposed to gain more insight in the regulation of secondary metabolites of nasal microbiome shaping members of the genus *Staphylococcus*.

References

1. Hayes, W. and S. Sahu, *The Human Microbiome: History and Future*. J Pharm Pharm Sci, 2020. **23**: p. 404-411.
2. Davenport, E.R., et al., *The human microbiome in evolution*. BMC Biol, 2017. **15**(1): p. 127.
3. Lloyd-Price, J., G. Abu-Ali, and C. Huttenhower, *The healthy human microbiome*. Genome Med, 2016. **8**(1): p. 51.
4. Human Microbiome Project, C., *Structure, function and diversity of the healthy human microbiome*. Nature, 2012. **486**(7402): p. 207-14.
5. Costello, E.K., et al., *Bacterial community variation in human body habitats across space and time*. Science, 2009. **326**(5960): p. 1694-7.
6. Kumpitsch, C., et al., *The microbiome of the upper respiratory tract in health and disease*. BMC Biol, 2019. **17**(1): p. 87.
7. Bassis, C.M., et al., *The nasal cavity microbiota of healthy adults*. Microbiome, 2014. **2**: p. 27.
8. Lemon, K.P., et al., *Comparative analyses of the bacterial microbiota of the human nostril and oropharynx*. mBio, 2010. **1**(3).
9. Stearns, J.C., et al., *Culture and molecular-based profiles show shifts in bacterial communities of the upper respiratory tract that occur with age*. ISME J, 2015. **9**(5): p. 1246-59.
10. Bomar, L., S.D. Brugger, and K.P. Lemon, *Bacterial microbiota of the nasal passages across the span of human life*. Curr Opin Microbiol, 2018. **41**: p. 8-14.
11. Laux, C., A. Peschel, and B. Krismer, *Staphylococcus aureus Colonization of the Human Nose and Interaction with Other Microbiome Members*. Microbiol Spectr, 2019. **7**(2).
12. Frank, D.N., et al., *The human nasal microbiota and Staphylococcus aureus carriage*. PLoS One, 2010. **5**(5): p. e10598.
13. den Heijer, C.D., et al., *Prevalence and resistance of commensal Staphylococcus aureus, including meticillin-resistant S aureus, in nine European countries: a cross-sectional study*. Lancet Infect Dis, 2013. **13**(5): p. 409-15.
14. Yan, M., et al., *Nasal microenvironments and interspecific interactions influence nasal microbiota complexity and S. aureus carriage*. Cell Host Microbe, 2013. **14**(6): p. 631-40.
15. Cheung, G.Y.C., J.S. Bae, and M. Otto, *Pathogenicity and virulence of Staphylococcus aureus*. Virulence, 2021. **12**(1): p. 547-569.
16. Vayssier-Taussat, M., et al., *Shifting the paradigm from pathogens to pathobiome: new concepts in the light of meta-omics*. Front Cell Infect Microbiol, 2014. **4**: p. 29.
17. Man, W.H., W.A. de Steenhuijsen Piters, and D. Bogaert, *The microbiota of the respiratory tract: gatekeeper to respiratory health*. Nat Rev Microbiol, 2017. **15**(5): p. 259-270.
18. Krismer, B., et al., *The commensal lifestyle of Staphylococcus aureus and its interactions with the nasal microbiota*. Nat Rev Microbiol, 2017. **15**(11): p. 675-687.
19. Linden, S.K., et al., *Mucins in the mucosal barrier to infection*. Mucosal Immunol, 2008. **1**(3): p. 183-97.
20. Kuek, L.E. and R.J. Lee, *First contact: the role of respiratory cilia in host-pathogen interactions in the airways*. Am J Physiol Lung Cell Mol Physiol, 2020. **319**(4): p. L603-L619.

21. Hapfelmeier, S., et al., *Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses*. Science, 2010. **328**(5986): p. 1705-9.
22. Ducarmon, Q.R., et al., *Gut Microbiota and Colonization Resistance against Bacterial Enteric Infection*. Microbiol Mol Biol Rev, 2019. **83**(3).
23. Harris-Tryon, T.A. and E.A. Grice, *Microbiota and maintenance of skin barrier function*. Science, 2022. **376**(6596): p. 940-945.
24. Berdy, J., *Bioactive microbial metabolites*. J Antibiot (Tokyo), 2005. **58**(1): p. 1-26.
25. Jenke-Kodama, H., R. Muller, and E. Dittmann, *Evolutionary mechanisms underlying secondary metabolite diversity*. Prog Drug Res, 2008. **65**: p. 119, 121-40.
26. Mukherjee, S. and B.L. Bassler, *Bacterial quorum sensing in complex and dynamically changing environments*. Nat Rev Microbiol, 2019. **17**(6): p. 371-382.
27. Deep, A., U. Chaudhary, and V. Gupta, *Quorum sensing and Bacterial Pathogenicity: From Molecules to Disease*. J Lab Physicians, 2011. **3**(1): p. 4-11.
28. Fuqua, W.C., S.C. Winans, and E.P. Greenberg, *Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators*. J Bacteriol, 1994. **176**(2): p. 269-75.
29. Lewenza, S., et al., *Quorum sensing in Burkholderia cepacia: identification of the LuxRI homologs CepRI*. J Bacteriol, 1999. **181**(3): p. 748-56.
30. Recsei, P., et al., *Regulation of exoprotein gene expression in Staphylococcus aureus by agar*. Mol Gen Genet, 1986. **202**(1): p. 58-61.
31. Ji, G., R. Beavis, and R.P. Novick, *Bacterial interference caused by autoinducing peptide variants*. Science, 1997. **276**(5321): p. 2027-30.
32. Neumann, W., A. Gulati, and E.M. Nolan, *Metal homeostasis in infectious disease: recent advances in bacterial metallophores and the human metal-withholding response*. Curr Opin Chem Biol, 2017. **37**: p. 10-18.
33. Reitz, Z.L. and M.H. Medema, *Genome mining strategies for metallophore discovery*. Curr Opin Biotechnol, 2022. **77**: p. 102757.
34. Mikhaylina, A., et al., *Bacterial zinc uptake regulator proteins and their regulons*. Biochem Soc Trans, 2018. **46**(4): p. 983-1001.
35. Konetschny-Rapp, S., et al., *Staphyloferrin A: a structurally new siderophore from staphylococci*. Eur J Biochem, 1990. **191**(1): p. 65-74.
36. Haag, H., et al., *Isolation and biological characterization of staphyloferrin B, a compound with siderophore activity from staphylococci*. FEMS Microbiol Lett, 1994. **115**(2-3): p. 125-30.
37. Perry, R.D., et al., *Yersiniabactin from Yersinia pestis: biochemical characterization of the siderophore and its role in iron transport and regulation*. Microbiology (Reading), 1999. **145** (Pt 5): p. 1181-1190.
38. Kim, H.J., et al., *Methanobactin, a copper-acquisition compound from methane-oxidizing bacteria*. Science, 2004. **305**(5690): p. 1612-5.
39. Leong, J. and J.B. Neilands, *Mechanisms of siderophore iron transport in enteric bacteria*. J Bacteriol, 1976. **126**(2): p. 823-30.
40. Knusel, F., J. Nuesch, and H.J. Treichler, *[Siderochrome and iron metabolism in microorganisms]*. Naturwissenschaften, 1967. **54**(10): p. 242-7.
41. Frost, G.E. and H. Rosenberg, *Relationship between the tonB locus and iron transport in Escherichia coli*. J Bacteriol, 1975. **124**(2): p. 704-12.
42. Buckling, A., et al., *Siderophore-mediated cooperation and virulence in Pseudomonas aeruginosa*. FEMS Microbiol Ecol, 2007. **62**(2): p. 135-41.

43. Sexton, D.J. and M. Schuster, *Nutrient limitation determines the fitness of cheaters in bacterial siderophore cooperation*. Nat Commun, 2017. **8**(1): p. 230.
44. Kramer, J., O. Ozkaya, and R. Kummerli, *Bacterial siderophores in community and host interactions*. Nat Rev Microbiol, 2020. **18**(3): p. 152-163.
45. Biswas, L. and F. Gotz, *Molecular Mechanisms of Staphylococcus and Pseudomonas Interactions in Cystic Fibrosis*. Front Cell Infect Microbiol, 2021. **11**: p. 824042.
46. Uzi-Gavrilov, S., et al., *Chemical Modification of a Bacterial Siderophore by a Competitor in Dual-Species Biofilms*. Angew Chem Int Ed Engl, 2023. **62**(29): p. e202300585.
47. Jenul, C., et al., *Pyochelin biotransformation by Staphylococcus aureus shapes bacterial competition with Pseudomonas aeruginosa in polymicrobial infections*. Cell Rep, 2023. **42**(6): p. 112540.
48. Stubbendieck, R.M. and P.D. Straight, *Multifaceted Interfaces of Bacterial Competition*. J Bacteriol, 2016. **198**(16): p. 2145-55.
49. Stubbendieck, R.M., et al., *Competition among Nasal Bacteria Suggests a Role for Siderophore-Mediated Interactions in Shaping the Human Nasal Microbiota*. Appl Environ Microbiol, 2019. **85**(10).
50. Brugger, S.D., et al., *Dolosigranulum pigrum Cooperation and Competition in Human Nasal Microbiota*. mSphere, 2020. **5**(5).
51. Lemon, K.P., *Human nasal microbiota*. Curr Biol, 2020. **30**(19): p. R1118-R1119.
52. Knauf, G.A., et al., *Acinetobactin-Mediated Inhibition of Commensal Bacteria by Acinetobacter baumannii*. mSphere, 2022. **7**(1): p. e0001622.
53. Hardin, G., *The tragedy of the commons. The population problem has no technical solution; it requires a fundamental extension in morality*. Science, 1968. **162**(3859): p. 1243-8.
54. Garcia-Contreras, R. and D. Loarca, *The bright side of social cheaters: potential beneficial roles of "social cheaters" in microbial communities*. FEMS Microbiol Ecol, 2020. **97**(1).
55. Shao, J., et al., *Siderophore-mediated iron partition promotes dynamical coexistence between cooperators and cheaters*. iScience, 2023. **26**(9): p. 107396.
56. Figueiredo, A.R.T., A. Wagner, and R. Kummerli, *Ecology drives the evolution of diverse social strategies in Pseudomonas aeruginosa*. Mol Ecol, 2021. **30**(20): p. 5214-5228.
57. van der Donk, W.A. and S.K. Nair, *Structure and mechanism of lanthipeptide biosynthetic enzymes*. Curr Opin Struct Biol, 2014. **29**: p. 58-66.
58. Hegemann, J.D. and R.D. Sussmuth, *Matters of class: coming of age of class III and IV lanthipeptides*. RSC Chem Biol, 2020. **1**(3): p. 110-127.
59. Guder, A., I. Wiedemann, and H.G. Sahl, *Posttranslationally modified bacteriocins--the lantibiotics*. Biopolymers, 2000. **55**(1): p. 62-73.
60. Kellner, R., et al., *Gallidermin: a new lanthionine-containing polypeptide antibiotic*. Eur J Biochem, 1988. **177**(1): p. 53-9.
61. Schnell, N., et al., *Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings*. Nature, 1988. **333**(6170): p. 276-8.
62. Gotz, F., et al., *Epidermin and gallidermin: Staphylococcal lantibiotics*. Int J Med Microbiol, 2014. **304**(1): p. 63-71.
63. Bonelli, R.R., et al., *Insights into in vivo activities of lantibiotics from gallidermin and epidermin mode-of-action studies*. Antimicrob Agents Chemother, 2006. **50**(4): p. 1449-57.

64. Brotz, H., et al., *Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics*. Mol Microbiol, 1998. **30**(2): p. 317-27.
65. Christ, K., et al., *Membrane lipids determine the antibiotic activity of the lantibiotic gallidermin*. J Membr Biol, 2008. **226**(1-3): p. 9-16.
66. Muller, A., et al., *Interaction of type A lantibiotics with undecaprenol-bound cell envelope precursors*. Microb Drug Resist, 2012. **18**(3): p. 261-70.
67. Stein, T., et al., *The multiple carrier model of nonribosomal peptide biosynthesis at modular multienzymatic templates*. J Biol Chem, 1996. **271**(26): p. 15428-35.
68. Pfeffer, S., et al., *X-Ray structure of the antibiotic bacitracin A*. FEBS Lett, 1991. **285**(1): p. 115-9.
69. Vanittanakom, N., et al., *Fengycin--a novel antifungal lipopeptide antibiotic produced by Bacillus subtilis F-29-3*. J Antibiot (Tokyo), 1986. **39**(7): p. 888-901.
70. Kluge, B., et al., *Studies on the biosynthesis of surfactin, a lipopeptide antibiotic from Bacillus subtilis ATCC 21332*. FEBS Lett, 1988. **231**(1): p. 107-10.
71. Mootz, H.D. and M.A. Marahiel, *The tyrocidine biosynthesis operon of Bacillus brevis: complete nucleotide sequence and biochemical characterization of functional internal adenylation domains*. J Bacteriol, 1997. **179**(21): p. 6843-50.
72. Du, L., et al., *The biosynthetic gene cluster for the antitumor drug bleomycin from Streptomyces verticillus ATCC15003 supporting functional interactions between nonribosomal peptide synthetases and a polyketide synthase*. Chem Biol, 2000. **7**(8): p. 623-42.
73. Paulus, H. and E. Gray, *The Biosynthesis of Polymyxin B by Growing Cultures of Bacillus Polymyxa*. J Biol Chem, 1964. **239**: p. 865-71.
74. Shimura, K., T. Sasaki, and K. Sugawara, *Biosynthesis of Bacitracin. I. Formation of Bacitracin by a Subcellular Fraction of Bacillus Licheniformis*. Biochim Biophys Acta, 1964. **86**: p. 46-55.
75. Zipperer, A., et al., *Human commensals producing a novel antibiotic impair pathogen colonization*. Nature, 2016. **535**(7613): p. 511-6.
76. Schilling, N.A., et al., *Synthetic Lugdunin Analogues Reveal Essential Structural Motifs for Antimicrobial Action and Proton Translocation Capability*. Angew Chem Int Ed Engl, 2019. **58**(27): p. 9234-9238.
77. Burgess, R.R., et al., *Factor stimulating transcription by RNA polymerase*. Nature, 1969. **221**(5175): p. 43-6.
78. Darst, S.A., *Bacterial RNA polymerase*. Curr Opin Struct Biol, 2001. **11**(2): p. 155-62.
79. Borukhov, S. and E. Nudler, *RNA polymerase holoenzyme: structure, function and biological implications*. Curr Opin Microbiol, 2003. **6**(2): p. 93-100.
80. Feklistov, A., et al., *Bacterial sigma factors: a historical, structural, and genomic perspective*. Annu Rev Microbiol, 2014. **68**: p. 357-76.
81. Pribnow, D., *Bacteriophage T7 early promoters: nucleotide sequences of two RNA polymerase binding sites*. J Mol Biol, 1975. **99**(3): p. 419-43.
82. Reznikoff, W.S., et al., *The regulation of transcription initiation in bacteria*. Annu Rev Genet, 1985. **19**: p. 355-87.
83. Mejia-Almonte, C., et al., *Redefining fundamental concepts of transcription initiation in bacteria*. Nat Rev Genet, 2020. **21**(11): p. 699-714.
84. Benoff, B., et al., *Structural basis of transcription activation: the CAP-alpha CTD-DNA complex*. Science, 2002. **297**(5586): p. 1562-6.
85. Zhang, Y., et al., *Structural basis of transcription initiation*. Science, 2012. **338**(6110): p. 1076-80.

86. Stackhouse, T.M., A.P. Telesnitsky, and C.F. Meares, *Release of the sigma subunit from Escherichia coli RNA polymerase transcription complexes is dependent on the promoter sequence*. Biochemistry, 1989. **28**(19): p. 7781-8.
87. Hansen, U.M. and W.R. McClure, *Role of the sigma subunit of Escherichia coli RNA polymerase in initiation. II. Release of sigma from ternary complexes*. J Biol Chem, 1980. **255**(20): p. 9564-70.
88. Mekler, V., et al., *Structural organization of bacterial RNA polymerase holoenzyme and the RNA polymerase-promoter open complex*. Cell, 2002. **108**(5): p. 599-614.
89. Deora, R. and T.K. Misra, *Characterization of the primary sigma factor of Staphylococcus aureus*. J Biol Chem, 1996. **271**(36): p. 21828-34.
90. Deora, R., T. Tseng, and T.K. Misra, *Alternative transcription factor sigmaSB of Staphylococcus aureus: characterization and role in transcription of the global regulatory locus sar*. J Bacteriol, 1997. **179**(20): p. 6355-9.
91. Aravind, L., et al., *The many faces of the helix-turn-helix domain: transcription regulation and beyond*. FEMS Microbiol Rev, 2005. **29**(2): p. 231-62.
92. Maddocks, S.E. and P.C.F. Oyston, *Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins*. Microbiology (Reading), 2008. **154**(Pt 12): p. 3609-3623.
93. Fernandez-Lopez, R., et al., *Transcription factor-based biosensors enlightened by the analyte*. Front Microbiol, 2015. **6**: p. 648.
94. Chen, J., H. Boyaci, and E.A. Campbell, *Diverse and unified mechanisms of transcription initiation in bacteria*. Nat Rev Microbiol, 2021. **19**(2): p. 95-109.
95. Huffman, J.L. and R.G. Brennan, *Prokaryotic transcription regulators: more than just the helix-turn-helix motif*. Curr Opin Struct Biol, 2002. **12**(1): p. 98-106.
96. Reyes-Caballero, H., G.C. Campanello, and D.P. Giedroc, *Metalloregulatory proteins: metal selectivity and allosteric switching*. Biophys Chem, 2011. **156**(2-3): p. 103-14.
97. Cinquin, O. and J. Demongeot, *Positive and negative feedback: striking a balance between necessary antagonists*. J Theor Biol, 2002. **216**(2): p. 229-41.
98. Mitrophanov, A.Y. and E.A. Groisman, *Positive feedback in cellular control systems*. Bioessays, 2008. **30**(6): p. 542-55.
99. Femerling, G., et al., *Sensory Systems and Transcriptional Regulation in Escherichia coli*. Front Bioeng Biotechnol, 2022. **10**: p. 823240.
100. Foulston, L. and M. Bibb, *Feed-forward regulation of microbisporicin biosynthesis in Microbispora corallina*. J Bacteriol, 2011. **193**(12): p. 3064-71.
101. Vingadassalon, A., et al., *Transcriptional Regulation of Congocidine (Netropsin) Biosynthesis and Resistance*. Appl Environ Microbiol, 2021. **87**(24): p. e0138021.
102. Umbarger, H.E., *Evidence for a negative-feedback mechanism in the biosynthesis of isoleucine*. Science, 1956. **123**(3202): p. 848.
103. Geisel, N., *Constitutive versus responsive gene expression strategies for growth in changing environments*. PLoS One, 2011. **6**(11): p. e27033.
104. Mangan, S. and U. Alon, *Structure and function of the feed-forward loop network motif*. Proc Natl Acad Sci U S A, 2003. **100**(21): p. 11980-5.
105. Henrion, L., et al., *Exploiting Information and Control Theory for Directing Gene Expression in Cell Populations*. Front Microbiol, 2022. **13**: p. 869509.
106. Kalisky, T., E. Dekel, and U. Alon, *Cost-benefit theory and optimal design of gene regulation functions*. Phys Biol, 2007. **4**(4): p. 229-45.
107. Andreini, C., et al., *Metal ions in biological catalysis: from enzyme databases to general principles*. J Biol Inorg Chem, 2008. **13**(8): p. 1205-18.

108. Gabriel, S.E., et al., *Regulation of the Bacillus subtilis yciC gene and insights into the DNA-binding specificity of the zinc-sensing metalloregulator Zur*. J Bacteriol, 2008. **190**(10): p. 3482-8.
109. Deng, Z., et al., *Mechanistic insights into metal ion activation and operator recognition by the ferric uptake regulator*. Nat Commun, 2015. **6**: p. 7642.
110. Price, E.E. and J.M. Boyd, *Genetic Regulation of Metal Ion Homeostasis in Staphylococcus aureus*. Trends Microbiol, 2020. **28**(10): p. 821-831.
111. Yang, X., et al., *Structural basis of Streptomyces transcription activation by zinc uptake regulator*. Nucleic Acids Res, 2022. **50**(14): p. 8363-8376.
112. Horsburgh, M.J., E. Ingham, and S.J. Foster, *In Staphylococcus aureus, fur is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis*. J Bacteriol, 2001. **183**(2): p. 468-75.
113. Pich, O.Q., et al., *Detailed analysis of Helicobacter pylori Fur-regulated promoters reveals a Fur box core sequence and novel Fur-regulated genes*. Mol Microbiol, 2012. **84**(5): p. 921-41.
114. Waldron, K.J. and N.J. Robinson, *How do bacterial cells ensure that metalloproteins get the correct metal?* Nat Rev Microbiol, 2009. **7**(1): p. 25-35.
115. Steingard, C.H. and J.D. Helmann, *Meddling with Metal Sensors: Fur-Family Proteins as Signaling Hubs*. J Bacteriol, 2023. **205**(4): p. e0002223.
116. Chivers, P.T. and R.T. Sauer, *NikR repressor: high-affinity nickel binding to the C-terminal domain regulates binding to operator DNA*. Chem Biol, 2002. **9**(10): p. 1141-8.
117. Schmitt, M.P. and R.K. Holmes, *Iron-dependent regulation of diphtheria toxin and siderophore expression by the cloned Corynebacterium diphtheriae repressor gene dtxR in C. diphtheriae C7 strains*. Infect Immun, 1991. **59**(6): p. 1899-904.
118. O'Halloran, T.V., et al., *The MerR heavy metal receptor mediates positive activation in a topologically novel transcription complex*. Cell, 1989. **56**(1): p. 119-29.
119. Liu, T., et al., *CsoR is a novel Mycobacterium tuberculosis copper-sensing transcriptional regulator*. Nat Chem Biol, 2007. **3**(1): p. 60-8.
120. Iwig, J.S., J.L. Rowe, and P.T. Chivers, *Nickel homeostasis in Escherichia coli - the rcnR-rcnA efflux pathway and its linkage to NikR function*. Mol Microbiol, 2006. **62**(1): p. 252-62.
121. Osman, D. and J.S. Cavet, *Bacterial metal-sensing proteins exemplified by ArsR-SmtB family repressors*. Nat Prod Rep, 2010. **27**(5): p. 668-80.
122. Figures were created using BioRender.com

Chapter 2

The human microbiome-derived antibiotic lugdunin self-regulates its biosynthesis in a feed forward-like fashion

Leonie Reetz^{1,2,3}, Thales Kronenberger^{2,3,4,5}, Khaled A. Selim^{2,6}, Timm Schaeffle^{2,7}, Taulant Dema^{2,7}, Alexander Zipperer^{1,2,3,#}, Antti Poso^{2,3,4,5}, Stephanie Grond^{2,7}, Andreas Peschel^{1,2,3}, Bernhard Krismer^{1,2,3,*}

¹ Department of Infection Biology, Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, Tübingen, Germany; ² Cluster of Excellence EXC 2124 Controlling Microbes to Fight Infections; ³ German Center for Infection Research (DZIF), partner site Tübingen; ⁴ Department of Pharmaceutical and Medicinal Chemistry and Tuebingen Center for Academic Drug Discovery & Development (TüCAD2), University of Tübingen, Tübingen, Germany. ⁵ School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, Kuopio, Finland; ⁶ Organismic Interactions Department, University of Tübingen, Tübingen, Germany (current address: Molecular Physiology of Prokaryotes, Institute of Biology II, University of Freiburg, Freiburg, Germany), ⁷ Institute of Organic Chemistry, University of Tübingen, Tübingen, Germany

current address: F. Hoffmann-La Roche Ltd, 4070 Basel, Switzerland

*Correspondence: b.krismer@uni-tuebingen.de

Abstract

Many human microbiome members inhibit bacterial competitors by production of antimicrobial compounds whose expression needs to be tightly controlled to balance costs and benefits of compound biosynthesis. The nasal commensal *Staphylococcus lugdunensis* outcompetes *Staphylococcus aureus* using the antimicrobial lugdunin. The lugdunin biosynthetic gene cluster (BGC) encodes two potential regulators whose role has remained unknown. Deletion of the regulator genes *lugR* or *lugJ* led to increased lugdunin production and/or immunity. Whereas LugR was found to repress transcription of the biosynthetic *lugRABCTDZ* operon, LugJ repressed the *lugIEFGH* export and immunity genes. Both regulators bound to different inverted repeats in the controlled promoter regions. Notably, both repressors were released from cognate promoters to allow transcription upon addition of exogenous lugdunin. Even minor structural changes disabled lugdunin derivatives to induce expression of its BGC, which is consistent with inferior binding to the predicted LugR and LugJ binding pockets. Thus, lugdunin controls its own biosynthesis in a feed forward-like fashion.

Introduction

Fitness of bacteria in complex microbial communities is governed by different types of interactions, including mutualistic and antagonistic mechanisms. Many bacteria produce antimicrobial compounds, bacteriocins and related molecules, to inhibit microbial competitors. Bacteriocin production is a highly variable and dynamic trait, which is often used by bacteria in nutrient-poor environments (1). Since the production of antimicrobial molecules and establishment of resistance in the producer requires substantial energy, bacteria usually regulate expression of biosynthetic gene clusters (BGCs) for antimicrobials according to environmental cues (2, 3). The signals and signal transduction mechanisms governing the production of bacteriocin-related antimicrobials have remained unknown for most of the BGCs and are a neglected field of research.

In contrast to primary metabolites, bacteriocins and other secondary metabolites are not essential for the survival of bacteria but contribute to the fitness in specific ecological niches (4, 5). Bacterial secondary metabolites are, for example, important for bacterial intra- and inter-species competition or cooperation as well as for evasion of host defence mechanisms in host-associated habitats (6, 7). Bacteriocins and other bacterial antimicrobial molecules are also of potential importance as alternatives to antibiotics for the prevention or treatment of antibiotic-resistant infections, which are on the rise (5, 8, 9). In addition to the specific antimicrobial molecules, commensal bacteria producing such molecules are also considered for probiotic microbiome-targeted interventions (10). In nutrient-poor natural habitats, such as the human

nares, members of the microbiome need to compete effectively to prevail using a large variety of bacteriocin-like molecules (4, 11, 12). The most frequent members of the adult human nasal microbiome belong to the genera *Staphylococcus*, *Corynebacterium*, *Cutibacterium*, *Dolosigranulum* as well as *Moraxella* and *Finnegoldia* (12-14). Population-based studies have shown that about 20% of human individuals permanently carry the aggressive pathogen *Staphylococcus aureus* in their nose (15, 16). This carriage is a major risk factor for invasive *S. aureus* infections. *S. aureus*-non-carriers are probably protected from *S. aureus* because their nasal microbiomes include *S. aureus*-eradicating commensals (14). We are only beginning to understand, which commensals are beneficial against *S. aureus* colonization and which types of antimicrobials they use. One previously identified member of the nasal microbiome is *Staphylococcus lugdunensis* which outcompetes *S. aureus* by the production of the cyclic peptide antibiotic lugdunin (17-19).

Lugdunin is the founding member of a new class of cyclic thiazolidine peptides. It acts as a protonophore against Gram-positive bacteria and is especially effective against *S. aureus*, which occupies the same ecological niche as *S. lugdunensis* (20). In fact, the presence of lugdunin-producing *S. lugdunensis* in the human nose reduces the risk of *S. aureus* colonization about six-fold (19). Although the relative abundance of *S. lugdunensis* is usually low compared to *S. aureus*, the production of lugdunin and its synergistic effect with human-derived antimicrobial peptides are sufficient to eradicate *S. aureus* from the human nose (21). Which environmental conditions may lead to lugdunin production and how expression of the lugdunin BGC could be improved remains an important research topic.

We report here that the lugdunin BGC encodes the two repressors, LugJ and LugR, to control the major transcriptional units of the BGC. The product lugdunin itself serves as an inducer of the expression of the lugdunin biosynthesis and transport genes in a feed forward-like fashion. The two repressors are unrelated, but both are released from their operator motifs upon binding of lugdunin. Structural models identify potential distinct binding sites within LugJ and LugR dimers. These findings will help to engineer lugdunin-producing bacteria to ensure high-enough lugdunin production for long-term protection against *S. aureus* colonization.

Results

LugR and LugJ are regulators of the lugdunin BGC

The lugdunin gene cluster is composed of 13 genes with multiple functions in lugdunin synthesis, export, immunity, and, potentially, regulation. Those genes for lugdunin biosynthesis (*lugRABCTDZ*) as well as transport and immunity (*lugIEFGH*) have previously been characterized (19, 22). Additionally, two putative regulators, LugR and LugJ, whose roles have

remained unknown, are encoded in the BGC. LugR belongs to the TetR-like regulators (23). Its gene is located directly upstream of the biosynthetic genes, most likely together forming the *lugRABCTDZ* transcriptional unit. Upstream of the immunity and transport genes, transcribed in the opposite orientation, the second putative regulator, LugJ, is encoded, a winged helix-turn-helix (HTH)-type regulator (24). To analyse putative functions of LugR and LugJ as regulators and to identify their impact on lugdunin biosynthesis, secretion, and immunity, clean deletion mutants for both genes were constructed by allelic recombination. As shown in Figure 1A, both mutants, ΔlugR and ΔlugJ , showed stronger inhibition of the *S. aureus* indicator strain than the wild type, indicating a significant impact of the two genes on lugdunin biosynthesis or export. Whereas ΔlugR showed a 1.38-fold increased width of the inhibition zone, ΔlugJ exhibited a 2.08-fold larger inhibitory distance. Quantification of lugdunin production in liquid culture by wild-type and mutant strains revealed that ΔlugR and ΔlugJ secreted 1.67-fold and 1.82-fold more lugdunin than the wild type, respectively (Figure 1B). To elucidate if the regulators also impact the lugdunin immunity of the strains, susceptibilities of the strains to lugdunin were compared. Whereas ΔlugR showed no change in susceptibility, the ΔlugJ mutant exhibited a more than two-fold increase in the lugdunin MIC (Figure 1C) compared to the parental strain. These data suggested a critical function of LugR and LugJ in lugdunin biosynthesis or transport and of LugJ in lugdunin immunity.

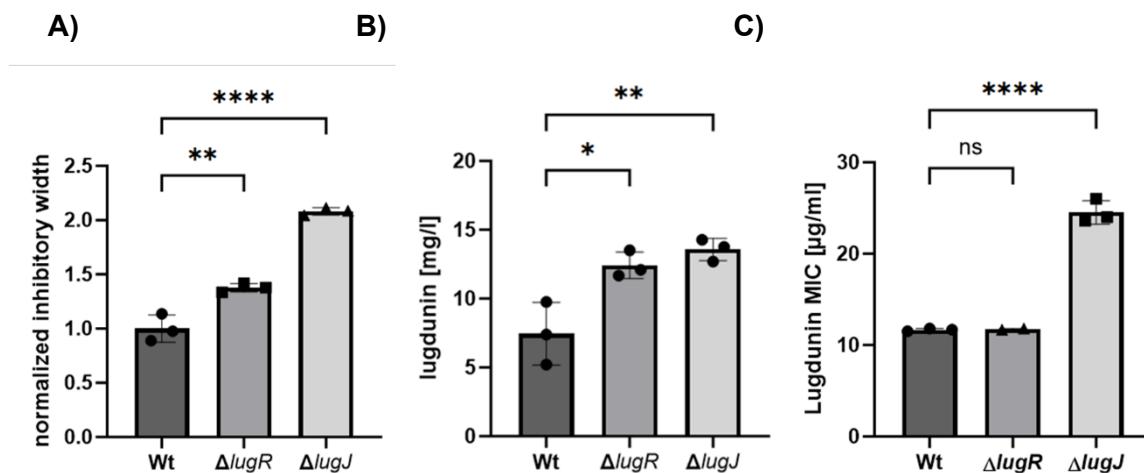


Figure 1. A) Both *S. lugdunensis* IVK28 ΔlugR and ΔlugJ elicit larger inhibition zones on *S. aureus* USA300 LAC lawns than the wild type. B) *S. lugdunensis* ΔlugR and ΔlugJ show 1.67-fold and 1.82-fold higher lugdunin production in liquid culture, respectively. C) Whereas *S. lugdunensis* IVK28 ΔlugR has the same lugdunin MIC as the wild type (wt), *S. lugdunensis* ΔlugJ tolerates ca. 2-fold higher lugdunin concentrations. Data points represent mean values $\pm$ SD of three independent experiments. All data were analysed using One-Way ANOVA, ns=not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

LugR and LugJ negatively regulate lugdunin biosynthesis and export

To elucidate the precise roles of the two regulators, the putative promoter regions of the transcription units *lugIEFGH* and *lugRABCTDZ* were examined. The very short 90-base pair (bp) intergenic region between *lugJ* and *lugI* (positions 823452-823541 of GenBank accession number CP063143) should contain two promoters in opposing orientations to drive expression of *lugJ* or *lugIEFGH*. However, they appear to share the -35 region 5'-TGTACA-3', which differs in two positions from the consensus sequence 5'-TTGACA-3' and represents a palindrome that could serve for both directions (Figure 2A). The putative -10 promoter regions occur in appropriate distance for both directions, 5'-TATTAT-3' as the -10 region of *lugJ* and 5'-AATAAT-3' as the -10 region of *lugIEFGH*, both differing by one nucleotide from the consensus sequence 5'-TATAAT-3'. Analysis of the sequence further revealed a large, nearly perfect 26-bp palindrome (5'-**CATTATCATTATCAATAATGATAATG**-3') between the -35 region and the *lugI* ribosomal binding site, which could serve as a repressor binding site (Figure 2A).

The 227-bp intergenic region between the 3'-end of *lugH* and the 5'-start of *lugR* was also analysed for putative promoter and operator sequences (positions 827680-827906 of GenBank accession number CP063143). Immediately upstream of *lugR*, putative -35 (5'-GTGACT-3') and -10 (5'-CATAAA-3') regions could be identified, interestingly forming part of a 24-bp palindromic sequence (5'-**gTGACTAGAAAAATTTATTTGGTCA**taaa-3'), which might also serve as a repressor binding motif (Figure 2B).

To confirm that the noncoding DNA regions between the *lug* operons contain promoters that are indeed potentially regulated by LugR or LugJ, the three putative promoter regions were cloned in front of a yellow-fluorescent protein (YFP) reporter gene and used for transformation of the restriction-modification deficient *S. aureus* strain PS187 Δ *hsdR* Δ *sauUSI* (25) (further referred as *S. aureus* PS187 $\Delta\Delta$). The putative promoter sequences were cloned either alone or together with one of the two regulator genes. A schematic overview of the constructs that were used for transformation of *S. aureus* PS187 $\Delta\Delta$ is given in Figure 2C. Indeed, the putative *lugI* promoter led to strong expression of YFP. Its activity was reduced to background level (2-3% of maximum expression) in the presence of the regulator gene *lugJ* but not *lugR* (Figure 3A). Likewise, the putative *lugR* promoter also led to strong YFP expression, which was downregulated by the presence of *lugR*, (3-4% of maximum expression) but not of *lugJ* (Figure 3B).

These results indicated that LugJ and LugR both act as repressors albeit for different promoters. LugJ regulates the transporter operon *lugIEFGH*, whereas LugR has an auto-regulatory function, controlling its own expression and that of the co-transcribed biosynthesis genes *lugABCTDZ*.

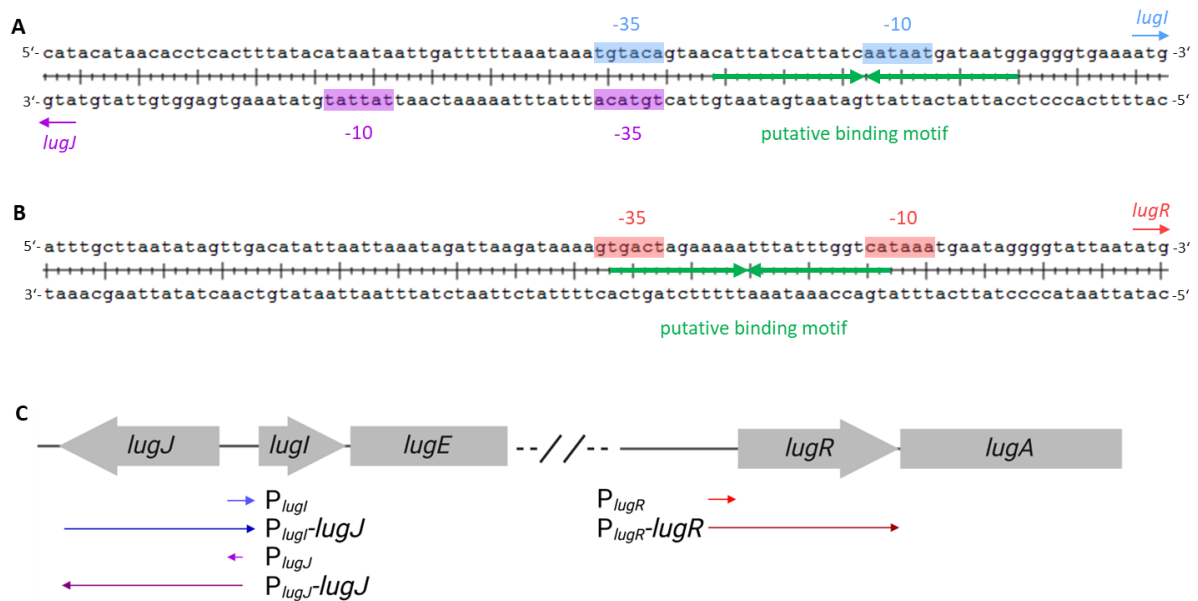


Figure 2. Genetic organization of the promoter regions of the *S. lugdunensis* IVK28 *lugdunin* BGC. A) Intergenic region between *lugJ* and *lugI*. Highlighted are the start codons of *lugJ* (purple) and *lugI* (blue) as well as the -10 and -35 promoter motifs. A palindrome that could be a putative regulator binding motif is shown in green (46). B) Promoter region of *lugR* with the predicted -10 and -35 motifs upstream of *lugR* (red). A palindromic putative regulator binding motif is shown in green. C) Schematic overview of the genomic operon of the *lugdunin* BGC. Shown below are the genetic constructs for the YFP reporter system. Arrows indicate the promoter orientation upstream of *yfp* on the pCG725 plasmid. P signifies the putative promoter sequence of the gene in subscript, which was cloned either alone (no regulator) or followed by the gene encoding the putative regulator *LugJ* or *LugR*, as indicated.

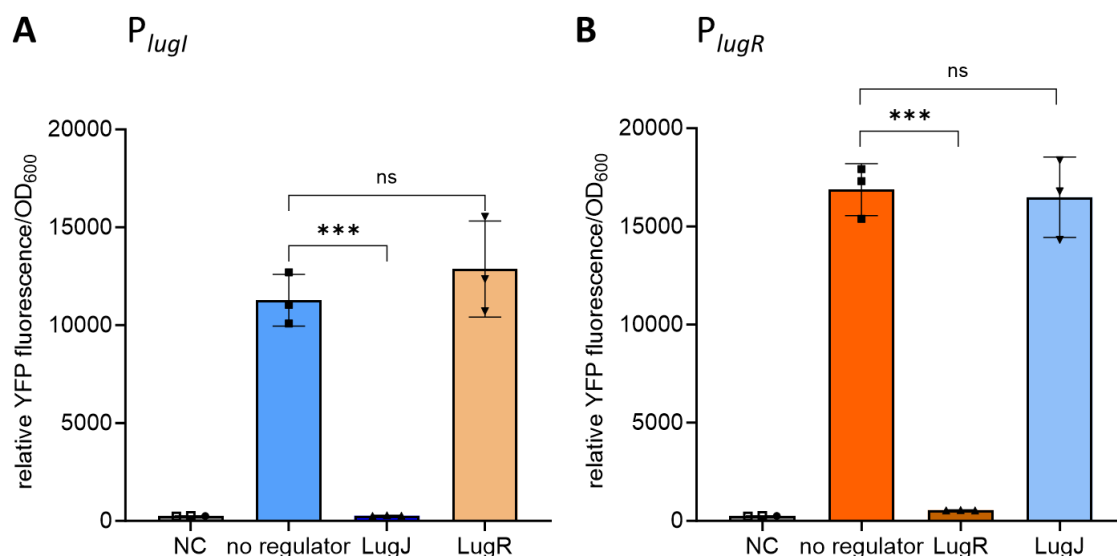


Figure 3. Effect of LugJ and LugR on the activity of the putative promoters as determined by YFP reporter assays. Promoter strength measured by endpoint YFP fluorescence activity of *S. aureus* PS187 $\Delta\Delta$ strains carrying the *yfp* reporter constructs indicated in Figure 2C. The fluorescence was normalized to OD₆₀₀ = 1 after 24 h of growth. A) Strength of P_{lugI} in absence (no regulator) or presence of either of the putative regulators LugJ or LugR. B) Promoter strength of P_{lugR} in the absence (no regulator) or presence of either of the putative regulators LugJ or LugR. NC: negative control. Experiments were performed in three independent biological replicates. All data were analysed using One-Way ANOVA, ns=not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Native lugdunin controls its own biosynthesis and transport

Since LugR and LugJ are repressors, we analyzed *S. lugdunensis* IVK28 culture for putative inducers, which would derepress the promoters in a *lugR* or *lugJ*-dependent fashion. To this end, whole *S. lugdunensis* IVK28 cultures were extracted with 1-butanol and subfractions, obtained by high-performance liquid chromatography (HPLC), were analysed for influences on the *lugI* or *lugR* promoter activities in the presence of LugJ or LugR, respectively. Strong derepression of both promoters by distinct HPLC fractions obtained from the *S. lugdunensis* IVK28 WT strain was observed (Figure S1A). Analysis of these fractions by HPLC-coupled mass spectrometry (HPLC-MS) revealed the presence of lugdunin as a common constituent of the inducing fractions, suggesting that lugdunin itself might be an inducer of *lugI* and *lugR* promoter activities. As a control, a culture of the lugdunin non-producing mutant *S. lugdunensis* IVK28 $\Delta lugD$ (19) was extracted and microfractionated as described above, but none of its fractions showed derepression capabilities (Figure S1B). This finding supports the hypothesis that lugdunin induces its own biosynthesis and transport.

Assuming that lugdunin itself might activate the genes of the lugdunin BGC by interaction with the regulatory proteins, the specificity of such interactions was analysed in the heterologous reporter gene expression system using native lugdunin (**1**) or synthetic lugdunin variants. To this end, three previously published derivatives (structures in Figure S2), enantio-lugdunin (**2**), which has opposing conformation but the same antimicrobial activity as native lugdunin, fourfold less antimicrobially active 2-Ala-lugdunin (**3**), and antimicrobially inactive N-methylthiazolidine-lugdunin (**4**) (20, 26) were selected.

To minimize antimicrobial effects on the growth of the heterologous reporter strain *S. aureus*, inhibitory concentrations of lugdunin of 5 µg/ml (22) and its derivatives were avoided and limited to sub-inhibitory 2 µg/ml. This concentration of native lugdunin was sufficient to de-repress the *lugI* promoter in the presence of LugJ to ca. 75% of the unregulated promoter activity in the absence of *lugJ* (Figure 4A), thereby confirming that it is indeed lugdunin, which induces *lugI* transcription. The same lugdunin concentration also increased the LugR-repressed *lugR* promoter activity albeit only to 10% of the promoter activity measured in the absence of the regulator gene (Figure 4B). De-repression was not observed by enantio-lugdunin (**2**). 2-Ala-lugdunin (**3**) increased YFP expression slightly from 3 to 8% for the LugJ-repressed P_{lugI} promoter, but no increase was detected for the $P_{lugR-lugR}$ construct. Only the addition of N-methylthiazolidine-lugdunin (**4**) resulted in significant induction of $P_{lugI-lugJ}$ leading to 32% of the fluorescence of the unrepressed promoter. In contrast, no significant increase could be detected for the $P_{lugR-lugR}$ construct with compound (**4**) (Figure 4A,B). This pattern indicates a high specificity of the regulators for the natural inducer lugdunin, which is obviously unrelated to the antimicrobial activity.

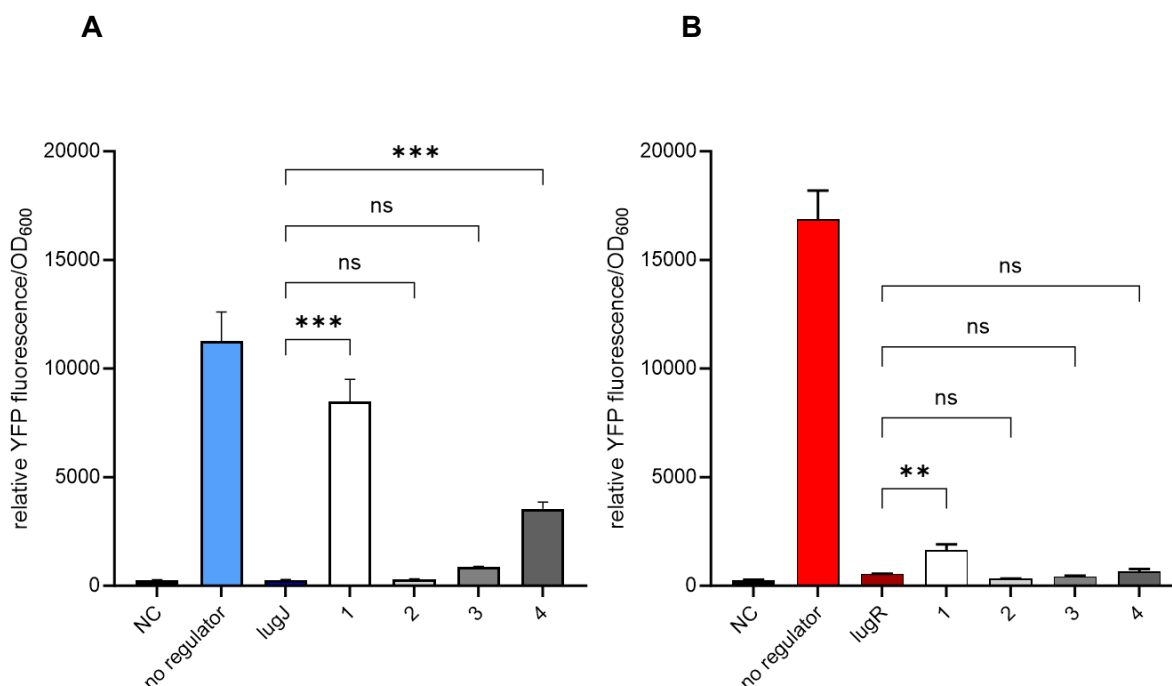


Figure 4. Specificity of inducers for promoter activity of P_{lugI} and P_{lugR} in the YFP reporter assay. Endpoint measurements of YFP fluorescence in strains carrying the reporter construct P_{lugI} - $lugJ$ (A) and P_{lugR} - $lugR$ (B) after induction with 2 μ g/ml lugdunin (1) or the derivatives enantio-lugdunin (2), 2-Ala-lugdunin (3), N-methylthiazolidine-lugdunin (4). The measured YFP intensities were correlated to the respective OD₆₀₀ after 24 h of growth at 37°C. NC; negative control. Experiments were performed in three independent biological replicates. All data were analysed using One-Way ANOVA, ns=not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

LugJ and LugR act as homodimers on the respective promoters

To further examine the structure and function of LugR and LugJ, both proteins were expressed with an N-terminal 8xHis-tag in *E. coli* Rosetta-gami™. After purification, proteins were used for size-exclusion chromatography coupled to multiangle light scatter (SEC-MALS) analysis to assess the oligomerization state of proteins (27). Proteins in the eluted peaks were collected and precipitated by addition of trichloroacetic acid (TCA) and analysed by SDS-PAGE followed by Western Blot analysis. Retention volumes of 14.2 and 14.5 ml were determined for 8xHis-LugJ and 8xHis-LugR, respectively (Figure 5) corresponding to masses of 47.3 kDa (8xHis-LugJ) and 46.8 kDa (8xHis-LugR) for the native proteins as indicated by MALS analysis. However, monomeric masses of 24 kDa and 26.3 kDa were detected by Western Blot of denaturing SDS-PAGE gels using an anti-His antibody, indicating that both proteins in their native state associate as dimers.

To confirm that LugR and LugJ impact the expression of lugdunin biosynthesis and transport genes by directly binding to the respective promoters, the purified proteins were combined with

promoter DNA fragments encompassing the *lugI* or *lugR* promoters, which were then analysed by electrophoretic mobility shift assays (EMSA). Both, LugJ and LugR, bound only the cognate promoter sequences they had been found to regulate in the reporter gene assay, in a concentration-dependent manner (Figure 6A, D), whereas none of the proteins bound to the other promoter (Figure 6B, E). To analyse if the palindromes in the promoter sequences represent indeed the regulator binding motifs, promoter DNA fragments were modified to lack the palindromes (P_{lugI}^c and P_{lugR}^c) without affecting the -10 and -35 motifs. No shift of these promoter fragments was visible in the EMSA (Figure 6B, E), which confirms that the palindromes are indeed operator sequences for LugJ or LugR. Addition of increasing concentrations of lugdunin to unmodified, regulator-bound promoter fragments led to a dose-dependent release of free DNA. Equimolar ratios of lugdunin and regulators resulted in an almost complete detachment of DNA from the proteins (Figure 6C, F), confirming that lugdunin has a direct impact on the capacity of the regulators to bind to their specific recognition sequences.

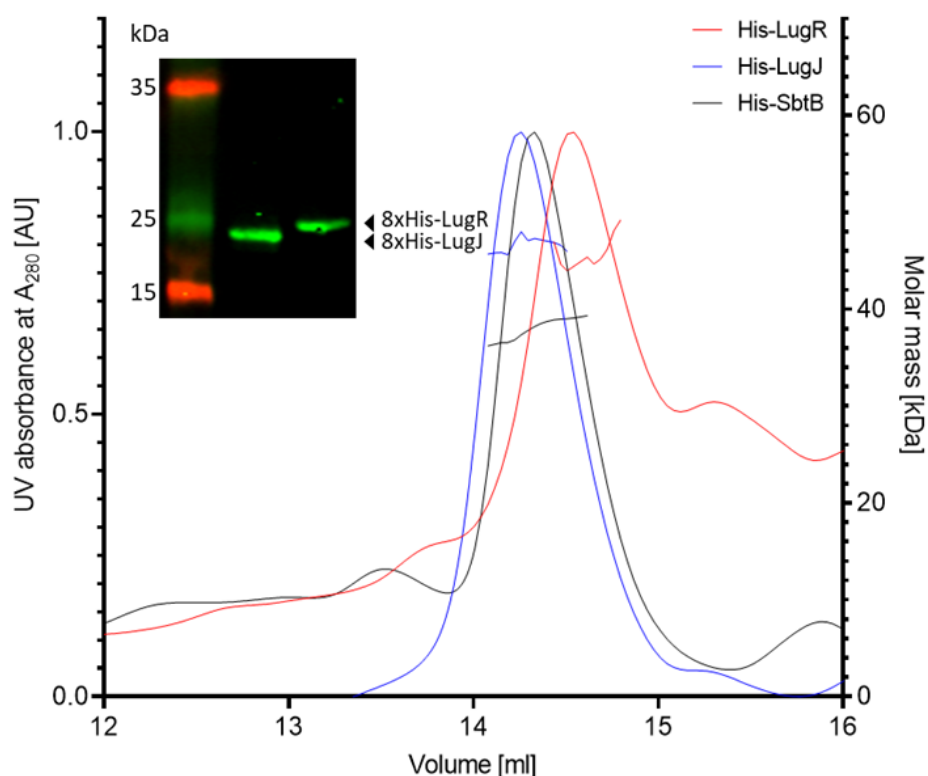


Figure 5. Characterization of native LugR and LugJ oligomerisation states. SEC-MALS analysis of the purified proteins revealed that both exist in a dimeric state with a calculated mass of approximately 44.9 kDa (His-LugR) and 46.7 kDa (His-LugJ). The theoretical size of 26.3 kDa (His-LugR) and 24 kDa (His-LugJ) could be detected in Western blot analysis (Insert). Trimeric His-SbtB (P(II)-like signaling protein), eluted around the same retention time and with molar mass of approximately 40 kDa, was used as a positive control.

These results indicate that both regulators, LugR and LugJ, probably act as homodimers on distinct lugdunin BGC promoters, where specific palindromic sequences are recognized and bound by the regulators. Addition of lugdunin, the biosynthetic product of the BGC, abrogates the binding and results in increased expression of the biosynthetic genes *lugABCTDZ* as well as the transporter genes *lugIEFGH*.

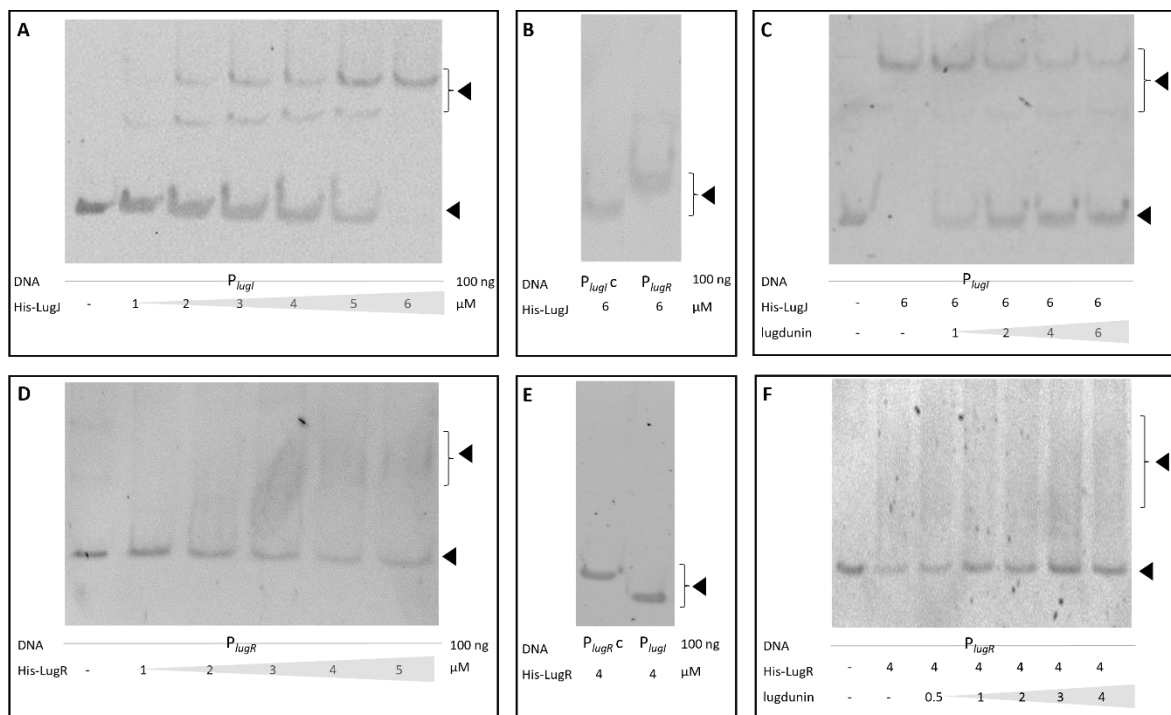


Figure 6. Interaction of promotor sequences and regulators verified by EMSA analysis. DNA fragments, proteins, and lugdunin were incubated in EMSA buffer, subjected to native PAGE and stained using SYBR Safe. A-F) 100 ng of the P_{lugJ} or P_{lugR} PCR fragment was used for interaction studies with LugJ or LugR, respectively. A) Increasing amounts of LugJ (0 to 6 μ M) were added to P_{lugJ} , leading to a shift. B) 100 ng of P_{lugJ} without the palindromic sequence ($P_{lugJ} c$) as well as of the promoter sequence of *lugR* (P_{lugR}) were used as controls showing no shift upon incubation with the regulator LugJ. C) Addition of lugdunin (0 to 6 μ M) led to a concentration-dependent release of the P_{lugJ} fragment. D) Increasing amounts of LugR (0 to 5 μ M) were added to P_{lugR} leading to a shift. E) 100 ng of P_{lugR} without the palindromic sequence ($P_{lugR} c$) as well as of the promoter sequence of *lugJ* (P_{lugJ}) were used as controls showing no shift upon incubation with the regulator LugR. F) Addition of lugdunin (0 to 4 μ M) led to a concentration-dependent release of the P_{lugR} fragment.

Lugdunin can stably bind to predicted binding sites in LugR and LugJ structure models

We generated LugJ and LugR monomeric structure models using AlphaFold and inferred homodimeric states of the two proteins based on selected homologues. The LugJ dimeric structure was modelled based on a putative transcription factor from *Kribbella flavida* (PDB ID: 4G6Q), which was the highest-ranked structure in terms of resolution and secondary structure

similarity (Figure 7A). LugR was modelled after the homodimeric apostructure of FadR (PDB ID: 5GPC), an acyl-CoA or fatty acid-binding member of the TetR regulator family, bound to dsDNA from *Bacillus halodurans* (Figure 7B) (28). The inferred dimeric structures of LugJ and LugR were congruent with those of other dimeric DNA-binding proteins.

We identified potentially drug-binding (druggable) binding pockets (DScores>0.9, anything above 0.8 is considered druggable) in each monomer of LugJ (Figure 7C), within the alpha-helix bundle (DScore 1.12). In contrast, the druggable pocket of LugR, whose homodimeric fold is Ω -shaped, resides in the middle of the dimerization interface (Figure 7D). The predicted pocket of LugR superimposes with the tetracycline binding site of TetR orthologues (29). Interestingly, comparison of different structures of the LugR-related FadR indicated that ligand binding prevents the interaction with DNA.

Since lugdunin exists in a racemic mixture of the thiazolidine's chiral centre (20), docking and further calculations were performed for both, (*r*)-lugdunin and (*s*)-lugdunin. Selected docking poses were submitted to short molecular dynamics simulations (see Supporting information section on the extra methods), in order to evaluate the stability of the proposed interactions and calculate lugdunin's binding energy. Interestingly, (*s*)-lugdunin displays a more stable predicted binding mode on LugJ (Figure 7E,G), with lower predicted binding energy and more abundant stable polar interactions than (*r*)-lugdunin. In contrast, (*r*)-lugdunin seems to be the preferred ligand of LugR (Figure 7F,G). Our models propose potential binding modes for all combinations of lugdunin configurations and protein binding (Supporting information, Figure S3A). However, the key interactions between lugdunin and LugJ or LugR are rather transient, which is reflected in the overall binding energy along the simulations.

Single-point energy calculations generated with the inactive or less active lugdunin variants **2**, **3**, and **4** (Supporting information, Figure S2) suggests a much worse binding energy for all (*r*)-configuration of the (*r*)-lugdunin analogues *r*-2, -3 and -4, for LugR. LugJ binding of the various *s*-isomers also worsened, compared to its affinity for unaltered lugdunin (Figure S3B). This increase in the binding energies is likely due to distinct reasons, such as the lost hydrophobic contacts between LugR's Met127 and the methyl group from **3** (an isopropyl moiety in the original lugdunin) or loss of hydrogen bond interactions with Ser164 (LugR) and Asn143 (LugJ) due to the amino-methylation present in **4**.

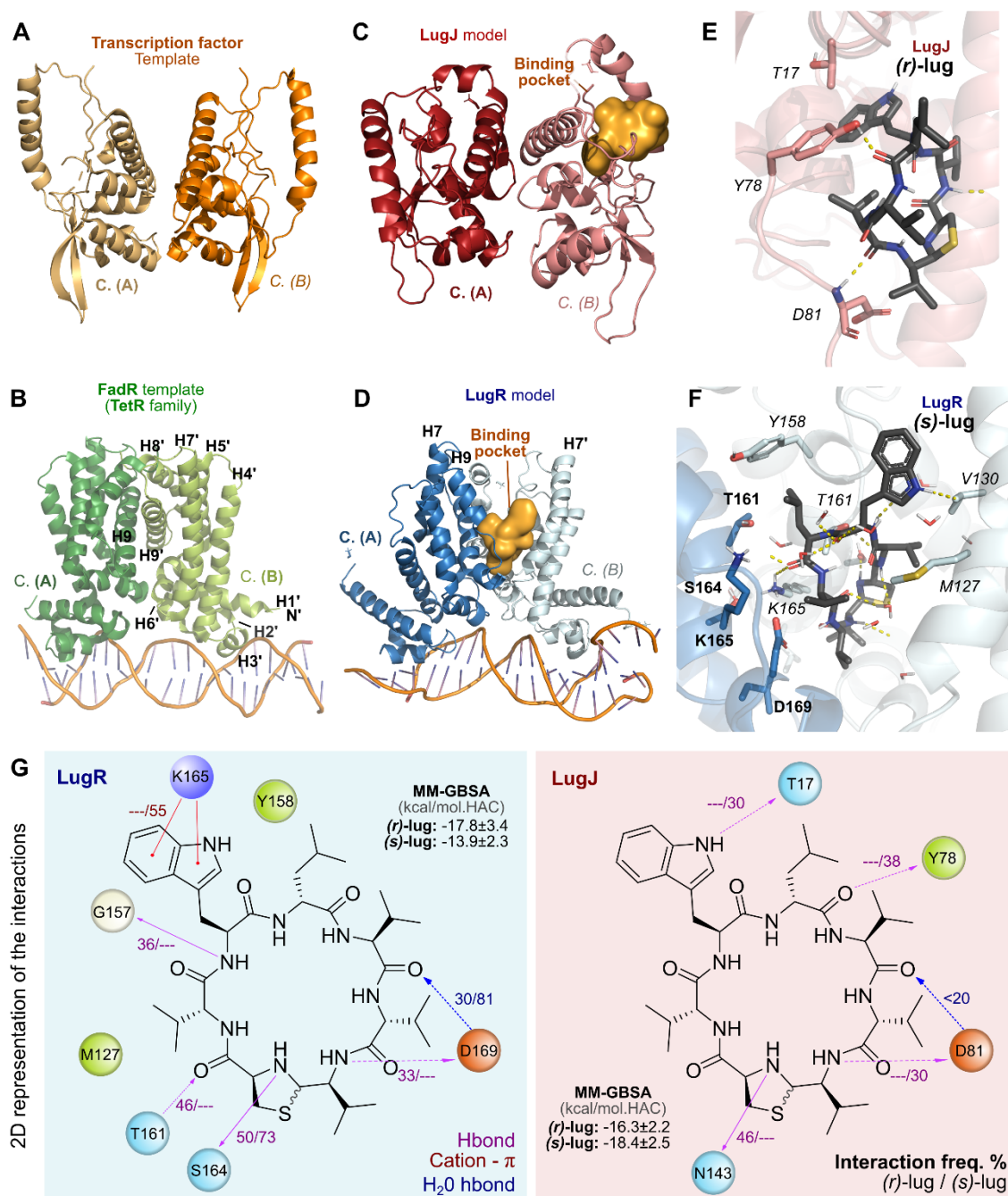


Figure 7. (r,s)-lugdunin can stably bind to the predicted binding sites in LugR and LugJ. Templates utilized in model generation for LugJ (A) and FadR (B). Final model and highlighted druggable binding pockets (orange) for LugJ (C) and LugR (D). 3D representation of lugdunin binding mode for LugJ (E) and LugR (F), polar contacts are highlighted as yellow dashed lines, bold and italic labels represent amino acids from different chains performing interactions. These structures represent the most populated cluster in the molecular dynamics (MD) simulations. G) 2D schematic representation of lugdunin interactions along the simulations (at least 1 μ s, i.e. 5 x 200 ns) in both configurations (r) and (s). Binding energy, estimated using MM-GBSA, is displayed as the mean of the simulation and standard deviation, both normalized by the number of heavy atoms in the ligand.

Discussion

We demonstrate that the two major transcription units of the lugdunin BGC are regulated by repressors that bind to the BGC promoter regions. The operons for lugdunin biosynthesis and for lugdunin export and immunity are controlled by different repressors, LugR and LugJ, of which LugR also controls its own expression. LugJ did not affect transcription of the *lugR* promoter. Likewise, LugR did not repress the *lugI* promoter, and it is unlikely to affect *lugJ* transcription since the promoter sequence does not contain the LugR operator motif. The two repressors belong to different families of DNA-binding proteins and do not share any similarity, but they respond to the same inducer, the end product lugdunin. It seems odd that the BGC uses two different regulators for the same purpose. Nevertheless, based on reporter expression data and modelled binding pockets, LugR and LugJ appear to have different binding modes for lugdunin. Derepression of the *lugIEFGH* promoter by particularly low lugdunin amounts may be of importance since immunity gene expression should be secured even at moderate lugdunin concentrations, while maximum expression of biosynthesis genes may be required at later stages, when full-level immunity has been established.

It was surprising to find that the end product lugdunin does not inhibit its biosynthesis genes, as often found in metabolic regulatory negative feedback loops, but that lugdunin activates its own expression. Such a concept of feed forward regulation or autoinduction (30) seems to make sense for *S. lugdunensis* considering the varying habitats of the bacteria and the fact that lugdunin production is only reasonable for the producer in environments where lugdunin can accumulate, to reach concentrations that allow inhibition of bacterial competitors. In habitats with continuous liquid secretion, lugdunin will be strongly diluted and its costly biosynthesis will be useless for *S. lugdunensis*. In habitats with limited diffusion such as epithelial micro-niches and hair follicles, however, lugdunin may easily reach sufficient concentrations to inhibit competitors such as *S. aureus*. Instead of harnessing a typical *Staphylococcus* house-keeping quorum sensing system, such as *agr* (31), the lugdunin BGC employs its product lugdunin both, as an autoinducer and as an antimicrobial effector (Figure 8). Although lugdunin has a high tendency to integrate into membranes because it acts as a protonophore (20), the lugdunin BGC does not encode a trans-membrane sensory protein, which could report lugdunin amounts in the cytoplasmic membrane of the producer. We demonstrate that LugJ and LugR both bind lugdunin directly, which suggests that lugdunin that accumulates extracellularly, finds its way back into the cytoplasmic membrane of the producer cells, and may enter the cytoplasm to derepress the *lugIEFGH* and *lugRABCTDZ* operons. This regulation principle can also limit excessive lugdunin production because lugdunin increases expression of the exporter proteins, which will reduce intracellular lugdunin

concentrations, eventually restoring the repressor function of LugR and LugJ to silence the *lug* promoters (Figure 8).

Recently, we have shown that the transporters *LugIEFGH* confer resistance to native lugdunin in *S. aureus* but not or only to a lesser extent to lugdunin derivatives such as enantio-lugdunin (**2**) or 6-Trp-lugdunin, even though these congeners have antimicrobial activity (22). Here we show that antibacterially active enantio-lugdunin and 2-Ala-lugdunin (**3**) are also no ligands for LugJ and LugR and are unable to derepress the *lug* promoters. Only the antibacterially inactive N-methylthiazolidine-lugdunin (**4**) exhibited some inducing capacity on LugJ, indicating that not only transport and immunity but also induction of the BGC-encoded genes is highly specific for natural lugdunin.

Whereas addition of lugdunin led to strong de-repression of the *lugIEFGH* promoter (ca. 75% of the unregulated promoter), the same lugdunin concentration led to only 10% activity of the unregulated *lugRABCTDZ* operon in our heterologous reporter gene system. One explanation for the weak de-repression of the *lugR* promoter could be that LugR is less sensitive to lugdunin derepression than LugJ. The calculations suggest the highest affinity for (*r*)-lugdunin in the binding pocket of the LugR dimer contact zone when the repressor is DNA-bound. As soon as the (*r*)-lugdunin-LugR complex dissociates from the DNA, the calculated free-binding energy for (*r*)-lugdunin changes from -17.8 to -9.3 kcal/mol, which would result in dissociation of (*r*)-lugdunin and the repressor would bind back to the DNA. In contrast, LugJ has a high affinity for (*s*)-lugdunin of -18.4 kcal/mol even when it is not bound to DNA. The structure of the LugR orthologue FadR has previously been determined both in its active dimer apo form, and in its inactive monomeric state, co-crystallized with palmitic acid. Structural comparison between these different forms confirms that ligand binding prevents DNA interaction (28), suggesting that LugR might also lose its DNA binding capacity upon lugdunin binding. The used lugdunin concentration was therefore probably not sufficient to achieve stronger de-repression of the *lugRABCTDZ* promoter, but we had to keep it at subinhibitory amounts. An alternative explanation lies in the genetic organization, since LugR represses its own transcription. Induction of transcription necessarily increases the concentration of LugR, which will again repress transcription in the absence of sufficient amounts of the inducer. These possible explanations might reflect very efficient mechanism to ensure that the lugdunin concentration never exceeds a specific critical concentration since also the producer, *S. lugdunensis*, is not fully resistant against its own product (22).

Regulatory proteins are found in most of the BGCs for antimicrobial compounds, but in most of the cases the regulatory signals have remained unknown. Actinobacteria, the typical and best-studied antibiotic producers, appear to link antibiotic gene expression to global cellular circuits in incompletely understood ways. Quorum-sensing linked LuxR-type regulators appear

to play a major role in actinobacterial antibiotic producers (2, 32). In some cases, intracellular second messengers such as ppGpp are involved (33). It remains to be investigated how lugdunin gene expression may be linked to global regulatory mechanisms of *S. lugdunensis*. Feed forward regulation has also been reported for the actinobacterial lanthipeptides microbisporicin (34) and planosporicin (35), the angucycline jadomycin B (36), the anthracycline daunorubicine (37), and the pyrrolamide congocidine (38). Moreover, characterization of antibacterial BGCs in Bacillota has led to additional examples of auto-inducing antimicrobial compounds. The lanthipeptides nisin and subtilin, for instance, induce their own expression via the NisRK and SpaRK two-component regulator system, respectively (39, 40). Thus, autoinduction of antibiotic production may be more common than currently acknowledged.

Our study is one of the first to confirm direct binding of an antimicrobial product to a regulatory protein governing expression of an antibiotic BGC and it will help to unravel general mechanisms of regulated antibacterial production. Our finding will also help to assess under which environmental conditions lugdunin may be effectively produced to support the fitness of *S. lugdunensis* in competition with other bacteria.

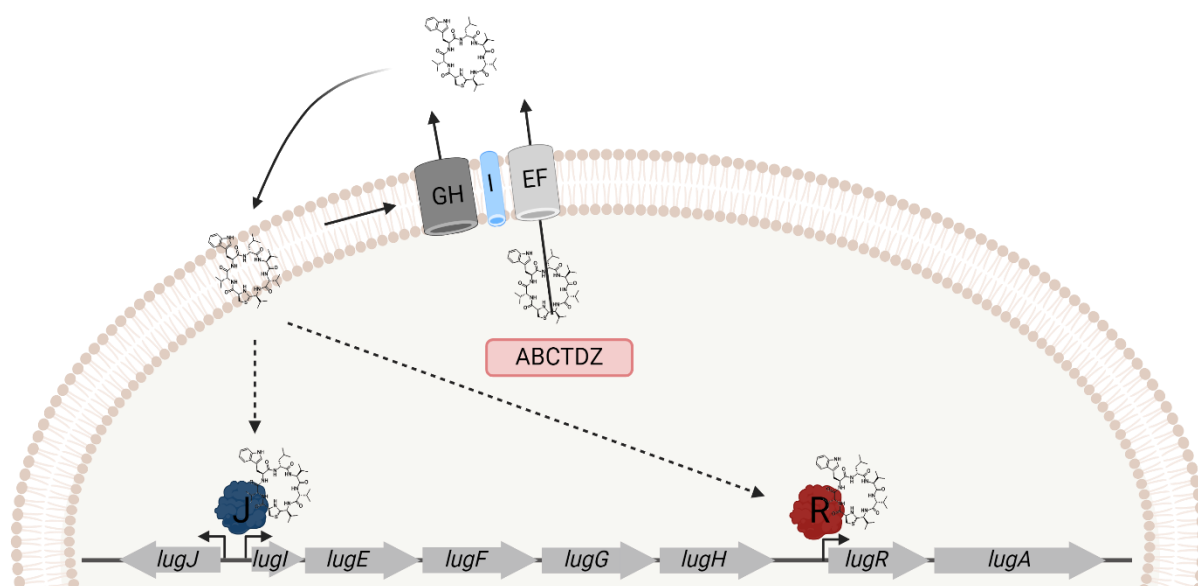


Figure 8 (and Graphical summary). LugJ and LugR act as homodimeric repressors of the *lugIEFGH* and *lugRABCTDZ* operons, respectively. Lugdunin acts as an inducer to release the repressors from the promoters leading to the expression of the lugdunin biosynthetic and transport genes. This effect seems to be highly specific as the lugdunin enantiomer and other derivatives cannot or can only slightly induce the system. For simplicity only part of the lugdunin BGC is shown [46].

Material and Methods

Bacterial strains and culture conditions. *Escherichia coli* DC10B and *E. coli* Rosetta-gami 2(DE3) were used for plasmid construction and protein expression, respectively. *S. aureus* PS187 Δ hsdR Δ sauUSI (25) was used as a heterologous host for reporter gene expression, *S. lugdunensis* IVK28 wild type and Δ lugD mutant were used for extraction of putative activators of lugdunin BGC transcription. All strains were cultivated at 37°C under continuous shaking. *E. coli* strains were incubated in lysogeny broth (Lennox, LB (Carl Roth GmbH, Germany)), *S. lugdunensis* and *S. aureus* in basic medium (BM: 1% soy peptone (Organotechnie SAS, France), 0.5% yeast extract (Deutsche Hefewerke GmbH, Germany), 0.5% NaCl, 0.1% glucose, and 0.1% K₂HPO₄). Appropriate selection was achieved using antibiotics ampicillin at 100 µg ml⁻¹ or kanamycin at 25 µg ml⁻¹ for *E. coli* and chloramphenicol 10 µg ml⁻¹, for *S. aureus*. MIC assays were performed as described before (22).

Construction of the YFP reporter system and His-tagged regulators. For the construction of the plasmid based YFP reporter system, genomic DNA of *S. lugdunensis* IVK28 was isolated using the NucleoSpin Microbial DNA Kit® (Macherey & Nagel, Germany). To obtain the different DNA fragments of promotor regions and putative regulators of *lugI*, *lugJ*, and *lugR*, genomic DNA was amplified using the primers listed in Table 1. For exchange of the native P_{cap} promoter located upstream of *yfp* in the reporter plasmid pCG725 (derivative of pCG717 (41) with altered ribosomal binding site) the indicated restriction enzymes were used. For the construction of the promoterless Δ P_{cap} plasmid, restriction enzymes SphI and Sall were used to delete the P_{cap} promoter. Subsequently, the digest was treated with Klenow fragment (ThermoFisher Scientific) to generate blunt ends and religated. All cloning was performed in *E. coli* DC10B and sequence-verified plasmids were transferred to *S. aureus* PS187 Δ hsdR Δ sauUSI by electroporation. To generate 6xHis-tagged proteins, *lugR* and *lugJ* were amplified using His-lugR for and rev or His-lugJ for and rev primers, respectively. PCR products and the plasmid pET-28a were digested with BamHI and XhoI and subsequently ligated. To extend the plasmid-encoded 6xHis-tag to an 8xHis-Tag, whole plasmids containing the regulator genes were amplified using 8xHis for and 8xHis rev primers. Plasmids were assembled using Gibson Assembly Master Mix (New England Biolabs GmbH, Germany) and further used for transformation of *E. coli* DC10B and *E. coli* Rosetta-gami 2(DE3). Plasmids used are listed in Table 1.

Construction of *lugR* and *lugJ* deletion mutants. Deletion of *lugR* and *lugJ* was achieved via allelic replacement. To this end, flanking regions of *lugR* were amplified by PCR with primer pairs lugH SacI-lugH Acc65I u and lugA Acc65I d-lugA BglII, digested with the indicated restriction enzymes, ligated into the SacI and BglII-digested shuttle-vector pBASE6 (42), and transferred into *E. coli* DC10B. Flanking regions of *lugJ* were PCR-amplified with primer pairs

lugJ K.O. upstream SacI- lugJ K.O. upstream Acc65I and lugJ K.O. downstream Acc65I- lugJ K.O. downstream BglII, digested with the indicated restriction enzymes and ligated into pBASE6 as described before. The resulting plasmids were confirmed by sequencing, transferred into *S. aureus* PS187 $\Delta hsdR \Delta sauUSI$ by electroporation and subsequently transduced into *S. lugdunensis* IVK28 as previously described (43). Homologous recombination was achieved as published earlier (42).

Table 1: Primers and plasmids used in this study with restriction sites underlined.

Primer	Sequence	Assignment
lugH SacI	TTGGAGCTCCCTTTATTACAACGTTTCATAG	lugR knock-out
lugH Acc65I u	TTCGGTACCTATTCATTTATGACCAAATAA	lugR knock-out
lugA Acc65I d	GACGGTACCGTTGATAGGATTTTTGAATAAG	lugR knock-out
lugA BglII	TTTAGATCTGCCAGTCAATTCAAACCTTTGC	lugR knock-out
lugJ K.O. upstream SacI	ATCGAGCTCACTGGTAACAAAGAACAAG	lugJ knock-out
lugJ K.O. upstream Acc65I	GATGGTACCTACTAGCAAAAGTTAAAAC	lugJ knock-out
lugJ K.O. downstream Acc65I	TTAAATGGTACCTCAATAATGATAATGGAG	lugJ knock-out
lugJ K.O. downstream BglII	ATTAGATCTGGCTTTTCGACTGGTTACA	lugJ knock-out
lugI Prom Sph	ATTGGCATGCATAATAGAAAGTCCTTCTGG	P_{lugI}
lugI Prom Sal	TTCATTTTCGTCGACCATTATCATTATTG	P_{lugI} and $P_{lugI-lugJ}$
lugJ Prom Sph	TTCATTTTCGCATGCCATTATCATTATTG	P_{lugJ}
lugJ Prom Sal	AACAGTCGACTTTATACATAATAATTGATTT	P_{lugJ}
lugJ Sph	GCTAGTAGCATGCATCAAAATTGGTTATGG	$P_{lugI-lugJ}$
lugR Prom Sph	TAAAGCATGCAATAAGAAATACAATGTAAATG	P_{lugR} and $P_{lugR-lugR}$
lugR Prom Sal	AATACCCCTAGTGCAGCTATGACCAAATAAAT	P_{lugR}
lugA Prom Sal	ACCTCCGTCGACTAAAACTTATTCAAAAATCC	$P_{lugR-lugR}$
PlugJ-lugJ Sal	CTAAGTGCAGCTTATGGAATAATAACAAG	$P_{lugJ-lugJ}$
lugR_prom_EcoRV	TAAAGATATCAATAAGAAATACAATGTAAATG	$P_{lugI-lugR}$
lugR_prom_PstI	ACCTCCCTGCAGTAAAACTTATTCAAAAATCC	$P_{lugI-lugR}$

lugJ_prom_EcoRV	TTTTGATATCTCATTATTGATAATGAT	<i>P_{lugR-lugJ}</i>
lugJ_prom_PstI	TAGCAGGATCCCCTAGGATTAACCTT	<i>P_{lugR-lugJ}</i>
His-lugJ for	GTCGCGGATCCATGGAATTCACTT	<i>His-lugJ</i>
His-lugJ rev	GGTGCTCGAGACATTATGGAATAAT	<i>His-lugJ</i>
His-lugR for	GTCGCGGATCCATGGCATTCTCTA	<i>His-lugR</i>
His-lugR rev	GGTGCTCGAGACTTATTCAAAAATCCT	<i>His-lugR</i>
8xHis for	GGCAGCAGCCATCATCATCATCATCATCAT AGCAGCGGCCTGGTGCCGC	8xHis-Tag
8xHis rev	GCGGCACCAGGCCGCTGCTATGATGATGATGA TGATGATGATGGCTGCTGCC	8xHis-Tag
YFP_up	GGTAAGTTTTCCGTATGTTGCATC	Sequencing upstream YFP
pCG725_down	CCTTTTGCTCACATGTTCTTTCC	Sequencing upstream YFP
pCG725_Seq_ insert_for	GTATTATATTTTGTATTATCGTTGA	Sequencing downstream YFP
pCG725_Seq_ insert_rev	TTCTGTAACTTACTAACTC	Sequencing downstream YFP
IR800 lugI_Prom_for	ACATAACACCTCACTTTATACATAA	<i>lugR</i> promotor sequence for EMSA
IR800 lugI_Prom_rev	CATTTTCACCCTCCATTATCATTATTG	<i>lugR</i> promotor sequence for EMSA
IR800_ lugR_Prom_for	CAATGTAAATGTAGTATAAAATCCAC	<i>lugI</i> promotor sequence for EMSA
IR800_ lugR_Prom_rev	ATTAATACCCCTATTCAATTTATGACC	<i>lugI</i> promotor sequence for EMSA
Plasmid	Description	
pCG725	YFP reporter assay	
pET-28a	Protein expression	

YFP reporter assay. To determine promotor activities of the lugdunin biosynthesis and transporter operons, YFP expression was measured. Strains were grown overnight in Mueller-Hinton Broth (Carl Roth GmbH, Germany) with chloramphenicol. Each strain was adjusted to

an optical density at 600 nm (OD_{600}) of 1 in BM. 500 μ l BM with chloramphenicol, and 2 μ g/ml lugdunin, if required, were added to a 48-well microtiter plate. Each well was inoculated with 2.5 μ l of the OD_{600} of 1 bacterial stock solution. The plates were incubated at 37°C with orbital shaking in a Spark Multimode Plate Reader (Tecan Trading AG). Optical density at 600 nm (OD_{600}) and YFP fluorescence (λ_{ex} 485 nm, λ_{em} 535 nm, gain 60) were measured after 24 h. Relative fluorescence units (rel. fl.) were determined after 24 h and normalized to the respective OD_{600} .

Expression and purification of 8xHis-LugR and 8xHis-LugJ. For the expression and purification of 8xHis-tagged regulators LugR and LugJ *E. coli* Rosetta-gami 2(DE3) pET-28a-8xhis-lugR and pET-28a-8xhis-lugJ were grown overnight in LB medium with kanamycin. Main cultures (1 L medium in 5 L baffled Erlenmeyer flasks) were inoculated to an OD_{600} of 0.1 and incubated at 37°C with shaking at 130 rpm until an OD_{600} of 0.7 was reached. Protein expression was induced by the addition of 1 mM isopropyl-D-1-thiogalactopyranoside (IPTG) at subsequent culture at 20°C for 16 h. Cells were harvested by centrifugation at 5,000xg for 20 min at 4°C and washed once with phosphate-buffered saline (pH 7.4). Pellets were resuspended in lysis buffer (20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 40 mM imidazole, 100 μ g/ml lysozyme, 1 cOmplete™ EDTA-free proteinase inhibition cocktail tablet) to a final OD_{600} of 10. After incubation on ice for 30 min, cells were disrupted by sonication. Cell lysate was centrifuged at 9,000xg for 30 min at 4°C, and the supernatant was filter sterilized (0.22 μ m pore size). Clear supernatant was loaded onto HisTrap HP 5 ml columns (GE Healthcare) with a flow of 2.5 ml/min. Columns were washed with buffer (20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl; 6 column volumes (CVs)) without imidazole at a flow rate of 10 ml/min and with a buffer containing 200 mM imidazole (4 CVs) at a flow rate of 5 ml/min. Finally, proteins were eluted with 4 CVs buffer containing 500 mM imidazole at a flow rate of 5 ml/min.

SDS-PAGE and Western Blot analysis. For denaturing protein analysis 20 μ l samples were mixed with 4 μ l 6x Laemmli Sample Buffer (Bio-Rad Laboratories), incubated at 95°C for 5 min, loaded onto 12% Mini-Protean TGX Gel (Bio-Rad Laboratories), and subjected to electrophoresis for 1 h at 120 V. After transfer of the proteins onto a nitrocellulose membrane, proteins were first probed with Penta-His Antibody (QIAGEN, Germany). As the secondary antibody, IRDye® 680RD Goat anti-Rabbit IgG (LI-COR Biosciences) was used. Proteins were visualized using the Odyssey CLx (LI-COR Biosciences, USA).

Butanol extraction. Overnight cultures of the *S. lugdunensis* IVK28 wild type or Δ lugD mutant were used to inoculate BM 1:1 000. The cultures were incubated at 37°C for 24 h. After incubation, the whole cultures were extracted with 1-butanol at a ratio of 3:1 for 1 h. Subsequently, the culture-butanol mixture was centrifuged at 5,000xg for 30 min, and the upper butanol phase was evaporated at 42°C under reduced pressure.

Microfractionation of *S. lugdunensis* IVK28 wild type and Δ *lugD* mutant. Butanol extracts with a set concentration of 10 mg/ml were microfractionated by HPLC (Agilent 1260 Infinity II). HPLC-grade water and HPLC-grade acetonitrile were used, HPLC was performed with a gradient from 10% to 100% acetonitrile over 20 min and the flow rate was set to 1 ml/min. A Kromasil 100 C18 column with a length of 250 mm, an inner diameter of 4 mm, pore size of 100 Å, and a particle size of 5 µm was used.

Quantification of lugdunin in samples using HPLC-MS. A lugdunin calibration curve was created by solving purified synthetic lugdunin in MeOH at a concentration of 1 mg/ml. The sample was serially diluted at 10-fold steps and HPLC-MS data were acquired on a Bruker MaXis 4G ESI-QTOF instrument coupled to a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific). Each analysis was calibrated using sodium formate as internal calibrant. The nebulizer pressure of the ESI source was set to 2.0 bar and dry gas was set to 8.0 L/min at an operating temperature of 200°C. For MS-MS spectra acquisition, the auto MS/MS mode with collision energy stepping was used. The mobile phase consisted of solvent A (H₂O + 0.1% formic acid (FA)) and solvent B (methanol (MeOH) + 0.06% FA). The routine gradient was 10% methanol (0.06 % FA) to 100% methanol in 20 min with a flow rate of 0.3 mL/min on a Nucleoshell® EC RP-C18 (150 x 2 mm, 2.7 µm) column from Macherey-Nagel.

Electrophoretic mobility shift assay (EMSA). Promotor DNA fragments of *lugI* and *lugR* were amplified with primers listed in Table 1. 100 ng of purified DNA was incubated with appropriate amounts of protein and lugdunin if required in EMSA buffer (10 mM Tris/HCl pH 7.4, 10 mM NaCl for LugJ or 100 mM NaCl for LugR, 0.1 mg/ml BSA, 1 mM DTT, 1 mM EDTA, 10% glycerol) and incubated for 30 min at 25°C. 5% native gels (1 ml 40% acrylamide (29:1), 400 µl 1 M Tris/HCl pH 7.5, 1.5 ml 1 M glycine, 32 µl 0.5 M EDTA, 5.2 ml ddH₂O, 40 µl 10% ammonium persulfate, 6 µl N,N,N',N'-trimethylethylenediamine) were pre-run in Tris-glycine buffer (25 mM Tris/HCl pH 8.3, 193 mM glycine) for 30 min at 100 V. 20-µl samples were loaded per lane and subjected to electrophoresis at 100 V for 45 min. Afterwards, the gel was stained using SYBR Safe (Thermo Fisher Scientific) for 30 min.

Size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS). SEC-MALS was carried out using a Micro-Äkta chromatography system (GE Healthcare) with a 24-ml Superose 6 Increase 10/300 GL column (GE Healthcare). For MALS analysis, an optilab T-rEX refractometer and a miniDawn treos detector (Wyatt technology) were attached to the Micro-Äkta system for accurate molar mass determination. The runs were performed as described before (44). The flow rate was set to 0.5 ml/min, and after equilibration of the column with running buffer (20 mM sodium phosphate pH 7.4, 0.5 M NaCl), 100 µl of purified 8xHis-LugR or 8xHis-LugJ, each concentrated to 0.5 mg/ml, were applied. Peak fractions were collected for precipitation with trichloroacetic acid (TCA) and used later for Western blotting analysis. The elution volume was plotted against the UV signal at 280 nm and against the

molecular masses derived from the MALS data. Data analysis and molecular weight calculations were done using ASTRA software (Wyatt). Trimeric His-SbtB, with a molar mass of approximately 40 kDa, was used as a positive control (45).

TCA precipitation. Samples from SEC-MALS were mixed with 30% TCA in a 3:1 ratio (sample:TCA) and incubated on ice for 30 min. Afterwards, samples were centrifuged at 20,000xg for 15 min at 4°C. After removal of the supernatant the pellet was washed three times with 80% acetone, dried for 10 min at 95°C, and finally resuspended in 20 µl water.

Molecular modelling. LugR and LugJ dimeric models were generated using AlphaFold and all lugdunin isomers were docked into predicted binding sites. The proposed binding mode systems were then simulated, in an all-atom and explicit solvent system for several microseconds and had their protein-ligand interaction frequencies and binding energy variation monitored along the simulated trajectory. (See Supporting information methods for the entire pipeline description).

Associated content

Supporting Information. All molecular dynamics trajectories and raw data related to the protein-ligand interactions within the simulations are available in the repository: [10.5281/zenodo.7648534](https://doi.org/10.5281/zenodo.7648534)

Acknowledgments

The authors thank Vera Augsburger and Manuel Beltran for excellent technical support and Evi Stegmann, Wolfgang Wohlleben, Karl Forchhammer and Christiane Wolz for helpful discussions.

Author contributions

Conceptualization, L.R., A.Pe., and B.K.; Methodology, L.R., T.K., A.Po., and K.A.S.; Investigation, L.R., K.A.S., T.S., T.D., and A.Z.; Writing – Original Draft, L.R., A.Pe., and B.K.; Writing - Review & Editing, L.R., T.K., T.S., A.Pe., and B.K.; Visualisation, L.R., T.K., and A.Pe.; Funding Acquisition, S.G. and A.Pe.; Resources, T.K., T.S., T.D.; Supervision, S.G., A.Pe., and B.K.

Declaration of interests

Eberhard Karls University Tübingen holds a patent (EP3072899B1) covering the compound lugdunin, derivatives thereof and the bacterial infection prevention by lugdunin-producing bacteria. The patent has also been filed in the USA (US2018/0155397A1). The authors declare no other competing interests.

Funding

The authors' work was financed by grants from Deutsche Forschungsgemeinschaft (DFG) TRR261 (A.P. and S.G; project ID 398967434), GRK1708 (S.G., A.P.) and Cluster of Excellence EXC2124 Controlling Microbes to Fight Infection (CMFI, S.G., B.K., T.K., and A.P; project ID 390838134), TRR156 (A.P.; project ID 246807620), and ZUK 63 (N.A.S.); from the German Center of Infection Research (DZIF) to B.K. and A.P.; from the German Ministry of Research and Education (BMBF) Culture Challenge to A.P.; and from the European Innovative Medicines Initiative IMI (COMBACTE) to A.P.. T.K. is funded by the fortune initiative and from TüCAD₂ and CMFI. TüCAD₂ is funded by the Federal Ministry of Education and Research (BMBF) and the Baden-Württemberg Ministry of Science as part of the Excellence Strategy of the German Federal and State Governments. The authors would like to thank CSC-Finland for the generous computational resources.

References

1. Janek D, Zipperer A, Kulik A, Krismer B, Peschel A. 2016. *High Frequency and Diversity of Antimicrobial Activities Produced by Nasal Staphylococcus Strains against Bacterial Competitors*. PLoS Pathog 12:e1005812.
2. Liu G, Chater KF, Chandra G, Niu G, Tan H. 2013. *Molecular regulation of antibiotic biosynthesis in streptomyces*. Microbiol Mol Biol Rev 77:112-43.
3. Rebuffat S. 2022. *Ribosomally synthesized peptides, foreground players in microbial interactions: recent developments and unanswered questions*. Nat Prod Rep 39:273-310.
4. Hardy BL, Merrell DS. 2021. *Friend or Foe: Interbacterial Competition in the Nasal Cavity*. J Bacteriol 203.
5. Torres Salazar BO, Heilbronner S, Peschel A, Krismer B. 2021. *Secondary Metabolites Governing Microbiome Interaction of Staphylococcal Pathogens and Commensals*. Microb Physiol 31:198-216.
6. Heilbronner S, Krismer B, Brotz-Oesterhelt H, Peschel A. 2021. *The microbiome-shaping roles of bacteriocins*. Nat Rev Microbiol doi:10.1038/s41579-021-00569-w.
7. O'Brien J, Wright GD. 2011. *An ecological perspective of microbial secondary metabolism*. Curr Opin Biotechnol 22:552-8.
8. Gould IM. 2009. *Antibiotic resistance: the perfect storm*. Int J Antimicrob Agents 34 Suppl 3:S2-5.
9. Mbaveng AT, Sandjo LP, Tankeo SB, Ndifor AR, Pantaleon A, Nagdju BT, Kuete V. 2015. *Antibacterial activity of nineteen selected natural products against multi-drug resistant Gram-negative phenotypes*. Springerplus 4:823.
10. Nakatsuji T, Hata TR, Tong Y, Cheng JY, Shafiq F, Butcher AM, Salem SS, Brinton SL, Rudman Spergel AK, Johnson K, Jepson B, Calatroni A, David G, Ramirez-Gama M, Taylor P, Leung DYM, Gallo RL. 2021. *Development of a human skin commensal microbe for bacteriotherapy of atopic dermatitis and use in a phase 1 randomized clinical trial*. Nat Med 27:700-709.
11. Granato ET, Meiller-Legrand TA, Foster KR. 2019. *The Evolution and Ecology of Bacterial Warfare*. Curr Biol 29:R521-R537.
12. Stubbendieck RM, May DS, Chevrette MG, Temkin MI, Wendt-Pienkowski E, Cagnazzo J, Carlson CM, Gern JE, Currie CR. 2019. *Competition among Nasal Bacteria Suggests a Role for Siderophore-Mediated Interactions in Shaping the Human Nasal Microbiota*. Applied and Environmental Microbiology 85.
13. Frank DN, Feazel LM, Bessesen MT, Price CS, Janoff EN, Pace NR. 2010. *The human nasal microbiota and Staphylococcus aureus carriage*. PLoS One 5:e10598.
14. Liu CM, Price LB, Hungate BA, Abraham AG, Larsen LA, Christensen K, Stegger M, Skov R, Andersen PS. 2015. *Staphylococcus aureus and the ecology of the nasal microbiome*. Sci Adv 1:e1400216.
15. Kluytmans J, van Belkum A, Verbrugh H. 1997. *Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks*. Clin Microbiol Rev 10:505-20.
16. Nouwen JL, Ott A, Kluytmans-Vandenbergh MFQ, Boelens HAM, Hofman A, van Belkum A, Verbrugh HA. 2004. *Predicting the Staphylococcus aureus nasal carrier state: Derivation and validation of a "culture rule"*. Clinical Infectious Diseases 39:806-811.
17. Bieber L, Kahlmeter G. 2010. *Staphylococcus lugdunensis in several niches of the normal skin flora*. Clinical Microbiology and Infection 16:385-388.

18. Rasmussen TT, Kirkeby LP, Poulsen K, Reinholdt J, Kilian M. 2000. *Resident aerobic microbiota of the adult human nasal cavity*. *Apmis* 108:663-675.
19. Zipperer A, Konnerth MC, Laux C, Berscheid A, Janek D, Weidenmaier C, Burian M, Schilling NA, Slavetinsky C, Marschal M, Willmann M, Kalbacher H, Schitteck B, Brötz-Oesterhelt H, Grond S, Peschel A, Krismer B. 2016. *Human commensals producing a novel antibiotic impair pathogen colonization*. *Nature* 535:511-516.
20. Schilling NA, Berscheid A, Schumacher J, Saur JS, Konnerth MC, Wirtz SN, Beltrán-Beleña JM, Zipperer A, Krismer B, Peschel A, Kalbacher H, Brötz-Oesterhelt H, Steinem C, Grond S. 2019. *Synthetic Lugdunin Analogues Reveal Essential Structural Motifs for Antimicrobial Action and Proton Translocation Capability*. *Angewandte Chemie International Edition* 58:9234-9238.
21. Bitschar K, Sauer B, Focken J, Dehmer H, Moos S, Konnerth M, Schilling NA, Grond S, Kalbacher H, Kurschus FC, Gotz F, Krismer B, Peschel A, Schitteck B. 2019. *Lugdunin amplifies innate immune responses in the skin in synergy with host- and microbiota-derived factors*. *Nat Commun* 10:2730.
22. Krauss S, Zipperer A, Wirtz S, Saur J, Konnerth MC, Heilbronner S, Salazar BOT, Grond S, Krismer B, Peschel A. 2021. *Secretion of and Self-Resistance to the Novel Fibupeptide Antimicrobial Lugdunin by Distinct ABC Transporters in Staphylococcus lugdunensis*. *Antimicrobial Agents and Chemotherapy* 65.
23. Cuthbertson L, Nodwell JR. 2013. *The TetR family of regulators*. *Microbiol Mol Biol Rev* 77:440-75.
24. Aravind L, Anantharaman V, Balaji S, Babu MM, Iyer LM. 2005. *The many faces of the helix-turn-helix domain: transcription regulation and beyond*. *FEMS Microbiol Rev* 29:231-62.
25. Winstel V, Liang C, Sanchez-Carballo P, Steglich M, Munar M, Broker BM, Penades JR, Nubel U, Holst O, Dandekar T, Peschel A, Xia G. 2013. *Wall teichoic acid structure governs horizontal gene transfer between major bacterial pathogens*. *Nat Commun* 4:2345.
26. Saur JS, Wirtz SN, Schilling NA, Krismer B, Peschel A, Grond S. 2021. *Distinct Lugdunins from a New Efficient Synthesis and Broad Exploitation of Its MRSA-Antimicrobial Structure*. *J Med Chem* 64:4034-4058.
27. Selim KA, Lapina T, Forchhammer K, Ermilova E. 2020. *Interaction of N-acetyl-l-glutamate kinase with the PII signal transducer in the non-photosynthetic alga Polytomella parva: Co-evolution towards a hetero-oligomeric enzyme*. *FEBS J* 287:465-482.
28. Yeo HK, Park YW, Lee JY. 2017. *Structural basis of operator sites recognition and effector binding in the TetR family transcription regulator FadR*. *Nucleic Acids Res* 45:4244-4254.
29. Kisker C, Hinrichs W, Tovar K, Hillen W, Saenger W. 1995. *The Complex Formed between Tet Repressor and Tetracycline-Mg²⁺ Reveals Mechanism of Antibiotic-Resistance*. *Journal of Molecular Biology* 247:260-280.
30. Wall ME. 2011. *Structure-function relations are subtle in genetic regulatory networks*. *Math Biosci* 231:61-8.
31. Le KY, Otto M. 2015. *Quorum-sensing regulation in staphylococci-an overview*. *Front Microbiol* 6:1174.
32. Zhang N, Dong Y, Zhou H, Cui H. 2022. *Effect of PAS-LuxR Family Regulators on the Secondary Metabolism of Streptomyces*. *Antibiotics (Basel)* 11.

33. Fernandez-Martinez LT, Gomez-Escribano JP, Bibb MJ. 2015. *A relA-dependent regulatory cascade for auto-induction of microbisporicin production in Microbispora corallina*. Mol Microbiol 97:502-14.
34. Foulston L, Bibb M. 2011. *Feed-forward regulation of microbisporicin biosynthesis in Microbispora corallina*. J Bacteriol 193:3064-71.
35. Sherwood EJ, Bibb MJ. 2013. *The antibiotic planosporicin coordinates its own production in the actinomycete Planomonospora alba*. Proc Natl Acad Sci U S A 110:E2500-9.
36. Wang W, Ji J, Li X, Wang J, Li S, Pan G, Fan K, Yang K. 2014. *Angucyclines as signals modulate the behaviors of Streptomyces coelicolor*. Proc Natl Acad Sci U S A 111:5688-93.
37. Vasanthakumar A, Kattusamy K, Prasad R. 2013. *Regulation of daunorubicin biosynthesis in Streptomyces peucetius - feed forward and feedback transcriptional control*. J Basic Microbiol 53:636-44.
38. Vingadassalon A, Lorieux F, Juguet M, Noel A, Santos LDF, Marin Fernandez L, Pernodet JL, Bury-Mone S, Lautru S. 2021. *Transcriptional Regulation of Congocidine (Netropsin) Biosynthesis and Resistance*. Appl Environ Microbiol 87:e0138021.
39. de Ruyter PG, Kuipers OP, de Vos WM. 1996. *Controlled gene expression systems for Lactococcus lactis with the food-grade inducer nisin*. Appl Environ Microbiol 62:3662-7.
40. Spiess T, Korn SM, Kotter P, Entian KD. 2015. *Autoinduction Specificities of the Lantibiotics Subtilin and Nisin*. Appl Environ Microbiol 81:7914-23.
41. Keinhorster D, Salzer A, Duque-Jaramillo A, George SE, Marincola G, Lee JC, Weidenmaier C, Wolz C. 2019. *Revisiting the regulation of the capsular polysaccharide biosynthesis gene cluster in Staphylococcus aureus*. Molecular Microbiology 112:1083-1099.
42. Geiger T, Francois P, Liebeke M, Fraunholz M, Goerke C, Krismer B, Schrenzel J, Lalk M, Wolz C. 2012. *The stringent response of Staphylococcus aureus and its impact on survival after phagocytosis through the induction of intracellular PSMs expression*. PLoS Pathog 8:e1003016.
43. Winstel V, Kuhner P, Rohde H, Peschel A. 2016. *Genetic engineering of untransformable coagulase-negative staphylococcal pathogens*. Nature Protocols 11:949-959.
44. Walter J, Selim KA, Leganes F, Fernandez-Pinas F, Vothknecht UC, Forchhammer K, Aro EM, Gollan PJ. 2019. *A novel Ca(2+)-binding protein influences photosynthetic electron transport in Anabaena sp. PCC 7120*. Biochim Biophys Acta Bioenerg 1860:519-532.
45. Selim KA, Haase F, Hartmann MD, Hagemann M, Forchhammer K. 2018. *P(II)-like signaling protein SbtB links cAMP sensing with cyanobacterial inorganic carbon response*. Proc Natl Acad Sci U S A 115:E4861-E4869.
46. Figures were created using BioRender.com

Supplementary Figures

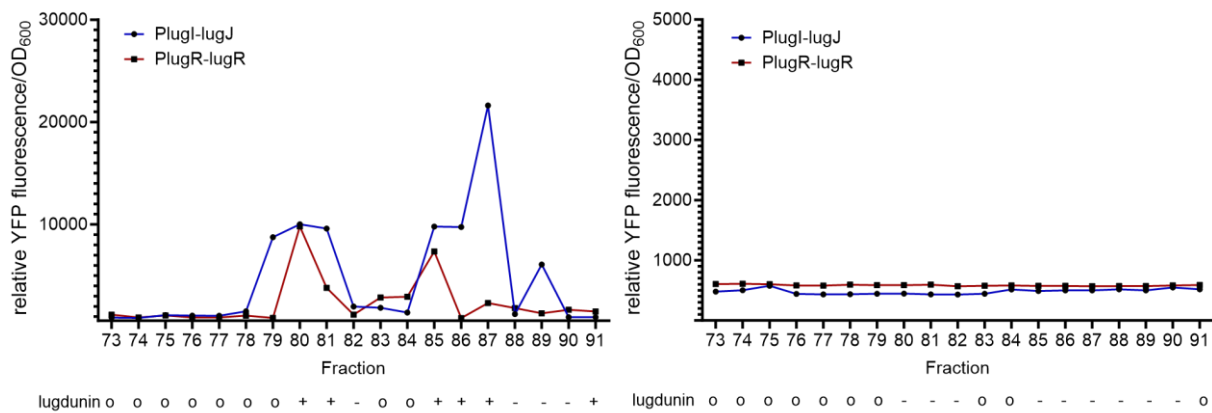


Figure S1. YFP signal intensity of *S. aureus* PS187 pCG725-P_{lugI}-*lugJ* or pCG725-P_{lugR}-*lugR* in response to components from *S. lugdunensis* IVK28 superatant. An overnight culture of (A) *S. lugdunensis* IVK28 or (B) *S. lugdunensis* IVK28 Δ *lugD* was 1-butanol extracted and microfractionated in a 96-well plate. The samples were dried and reconstituted with BM media containing (red) *S. aureus* PS187 $\Delta\Delta$ pCG725-P_{lugI}-*lugJ* or (blue) *S. aureus* PS187 $\Delta\Delta$ pCG725-P_{lugR}-*lugR*. Relative fluorescence intensities and optical densities were determined after 24 h. Fractions were then analysed for the presence of lugdunin with HR-MS, o = not analysed, - = no lugdunin identified, + = lugdunin identified.

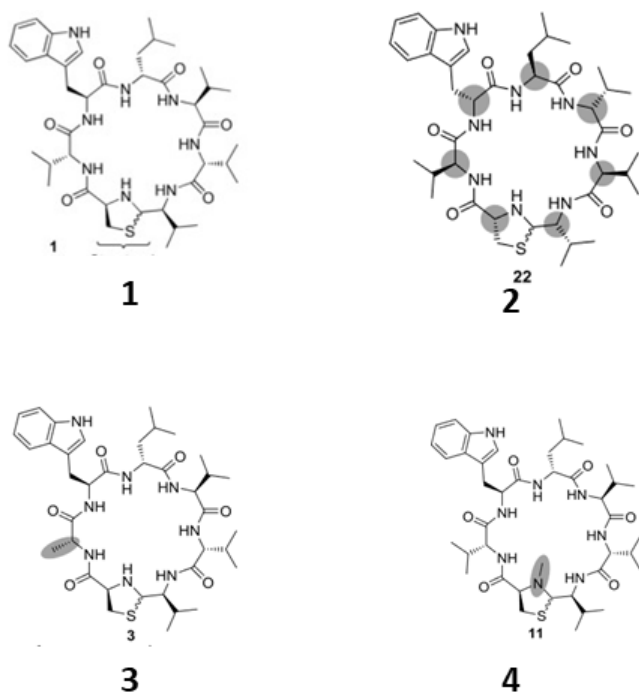


Figure S2. Chemical structures of lugdunin and its derivatives. Lugdunin (1), enantio-lugdunin (2), 2-Ala-lugdunin (3) and N-methylthiazolidine-lugdunin (4).

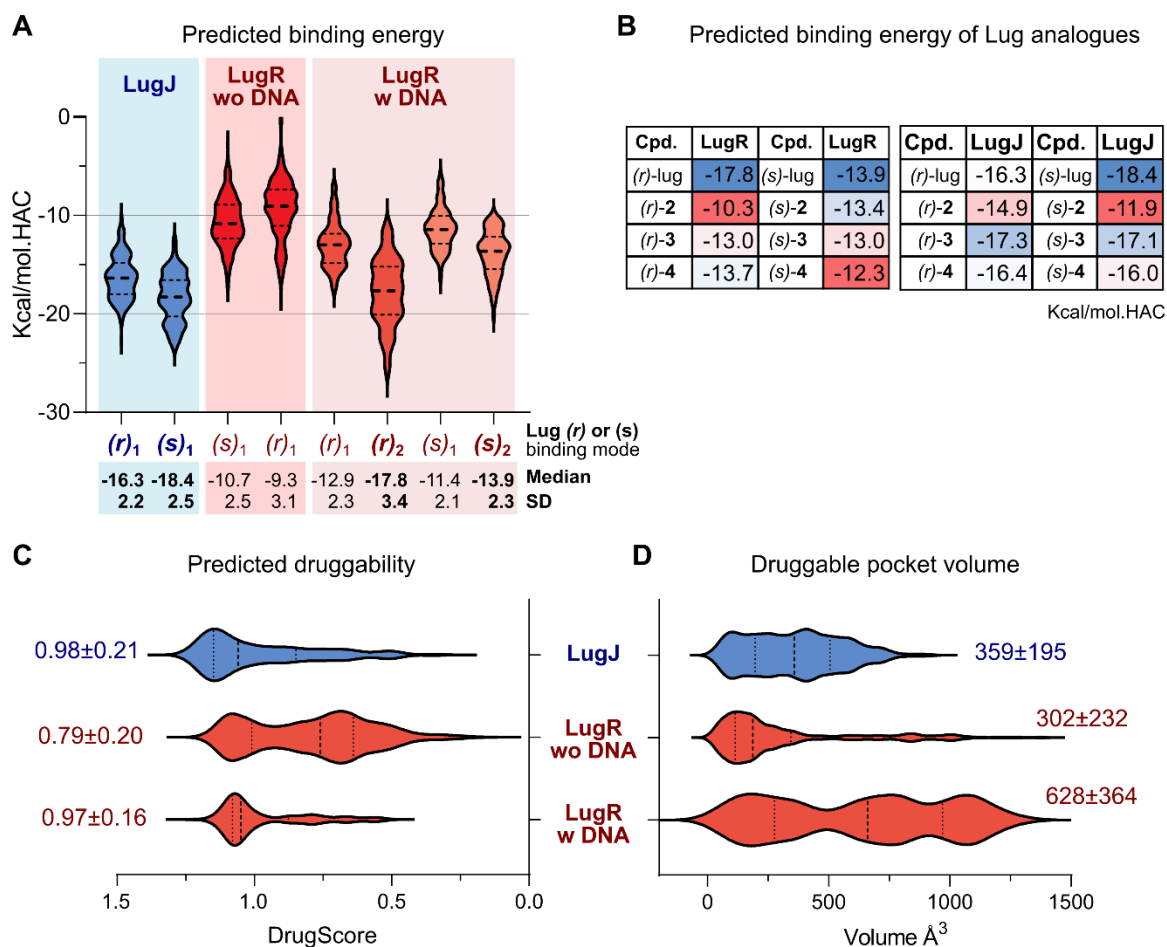


Figure S3. Characterization of the predicted binding pockets of LugJ and LugR. A) ligand efficiency prediction distribution for the simulations displayed as violin plots. Ligand efficiency was calculated using MM-GBSA's predicted binding energy (see methods) and is represented by its mean + standard deviation. HAC: heavy atom count; r: (r)-lugdunin (R-lug; also represented by dashed boxes) and s: (s)-lugdunin (S-lug; plain boxes), numbers represent potential binding modes generated as alternative solutions results of docking. B) single point binding energy calculations for distinct lugdunin analogues within the cluster-representative binding pocket for each transcription factor. Predicted druggable binding pocket monitored along the simulation time for LugJ and LugR models (with and without DNA), in terms of predicted druggability (DrugScores, C) and volume (D, volume is expressed in cubic Angstroms), with their respective median and standard deviation values. Calculated free-binding energies are represented by the MM/GBSA and normalized by the number of heavy atoms (HAC), according to the following formula: ligand efficiency = (energy) / (1 + ln(HAC)).

Supplementary information

Molecular modelling.

Homology model and protein preparation. The *S. lugdunensis* IVK28's LugJ (accession number: WP_002460039) and LugR (WP_002460032) monomeric homology models were generated using the ColabFold5 implementation of the AlphaFold6 with standard options.

LugJ showed an HTH-containing regulator folding and its multimeric structure was modelled after the (PDB ID: 4G6Q, resolution: 2.1 Å, a transcription factor from *K. flavida*, unpublished but deposited). The DNA would bind in the lower helix-wing portion of the template's N-termini, similar to the MarR family⁷. However, the absence of the typical arginine patch in the potential DNA surface (blocked by the dimerization interface) did not allow us to place a nucleic acid chain by superimposition. Therefore, we proceed with a final model without dsDNA.

On the other hand, given the high similarity in terms of folding between LudR and the TetR/FadR transcription factor family, as well as the availability of high-quality structural data, we decided to include dsDNA coordinates from FadR (PDB ID: 5GPC1, resolution: 2.8 Å), in our final models by superimposing the structures and retrieving their structure. Control LugR models without dsDNA were generated based on the ketosteroid transcriptional regulator of *Mycobacterium tuberculosis* (PDB ID: 4W97, Resolution: 1.60 Å⁸), which was selected due to its high resolution and completeness. Though it would sound logical to generate our LugR working model after the inhibited state, its binding pocket was too constricted to generate relevant binding mode poses, despite the similar drugability potential (Supporting information, Figure S3C,D).

Final models were validated by checking its Ramachandran plot and overall energy levels, low confidence level regions in the N- and C-termini (as already suggested by the ColabFold scores) indeed displayed higher energy and underwent energy minimization as a last step of preparation. All protein structures were prepared using the Protein Wizard Preparation tool (Schrödinger LCC), with standard options and the homology model was further refined to remove sterical clashes. For all structures protonation states of amino acids were optimized with PROPKA (Schrödinger, LLC, New York, NY, 2022.4), where we selected the most likely ionization state as proposed by the software, and the structures were minimized using steep descent (cut-off for heavy atoms 1 Å).

Ligand preparation and molecular docking. Three-dimensional ligand structures were generated with LigPrep, using Epik9 to predict their protonation in pH 7.0 ± 1.0. The diastereoisomer configuration was derived from the synthesis (10), except for the thiazolidine

ring, which was simulated for both, (*r*)-lugdunin (herein R-lug) and (*s*)-lugdunin (S-lug) configurations. The OPLS4 force field (11) was employed for structure generation. Each prepared ligand underwent extensive conformer generation (using MacroModel with implicit solvent and standard options), and all conformers were submitted to docking.

Ligands were docked within a grid around 13 Å from the centroid of the potential binding pocket, identified using SiteMap2 (Schrödinger LCC). Docking was performed using Glide (12,13) using the SiteMap-identified pocket and employing XP scoring function. All docking poses were visually inspected, independently from the docking score, and those with the highest number of consistent interactions were selected for simulation.

Molecular dynamics (MD) simulations. MD simulations were carried out by using the Desmond engine (14) with the OPLS4 force-field (11). The system encompassed the protein-ligand/cofactor complex, a predefined water model (TIP3P) (15) as a solvent and counterions (Na^+ or Cl^- adjusted to neutralize the overall system charge). The system was treated in a cubic box with a periodic boundary condition (PBC) specifying the shape and the size of the box as 13 Å distance from the box edges to any atom of the protein. Short-range coulombic interactions were calculated using 1-fs time steps and 9.0-Å cut-off value, whereas long-range coulombic interactions were estimated using the Smooth Particle Mesh Ewald (PME) method (16,17).

Initially, the relaxation of the system was performed using Steepest Descent and the limited-memory Broyden-Fletcher-Goldfarb-Shanno algorithms in a hybrid manner, according to the established protocol available in the Desmond standard settings. During the equilibration step, the simulation was performed under the NPT ensemble for 5 ns implementing the Berendsen thermostat and barostat methods (18). A constant temperature of 310 K was kept throughout the simulation using the Nose-Hoover thermostat algorithm (19) and Martyna-Tobias-Klein Barostat algorithm (20,21) to maintain 1 atm of pressure, respectively.

After minimization and relaxation of the system, each protein+lugdunin system was subjected to at least 1-μs simulations (five replicas of 200 ns each) with random seeds, unless stated otherwise. All the trajectories, interaction data, and RMSD/RMSF reports are available on the Zenodo repository (code: 10.5281/zenodo.7648534). MD trajectories were visualized, and figures were generated using PyMOL v.2.5.2 (Schrödinger LCC, New York, NY, USA).

MD simulation trajectory analyses. Protein-ligand interactions and atomic distances were calculated using the Simulation Event Analysis pipeline as implemented in Maestro (v2022.4,

Schrödinger LCC) using standard options. Representative frames of the simulations were retrieved using hierarchical clustering analyses. Trajectories were clustered using the script `trj_cluster.py` (implemented in Maestro 2022.4, Schrödinger LCC) according to the RMSD of ligand's heavy atoms, using 1 Å as the cut-off. RMSD values of the protein backbone were used to monitor simulation equilibration and protein folding changes (all raw data is available in the repository link).

MM-GBSA binding energy calculations. Molecular mechanics with generalized Born and surface area (MM-GBSA) predicts the binding free energy of protein-ligand complexes and the ranking of ligands based on the free energy could be correlated to the experimental binding affinities, especially in a congeneric series. Every 10th frame from the simulations was considered for the calculations. These were used as input files for the MM-GBSA calculations with `thermal_mmgbsa.py` script from the Schrödinger package. Calculated free-binding energies are represented by the MM/GBSA and normalized by the number of heavy atoms (HAC), according to the following formula: $\text{ligand efficiency} = \ln(\text{docking score}) / (1 + \ln(\text{HAC}))$.

Pocket volume and druggability scores were calculated using SiteMap along the trajectory with the script `trajectory_binding_site_volumes.py`, calculating every 10th frame from the simulations.

Supplementary References

1. Yeo HK, Park YW, Lee JY. 2017. *Structural basis of operator sites recognition and effector binding in the TetR family transcription regulator FadR*. Nucleic Acids Research 45:4244-4254.
2. Halgren TA. 2009. *Identifying and Characterizing Binding Sites and Assessing Druggability*. Journal of Chemical Information and Modeling 49:377-389.
3. Hinrichs W, Kisker C, Duvel M, Muller A, Tovar K, Hillen W, Saenger W. 1994. *Structure of the Tet Repressor Tetracycline Complex and Regulation of Antibiotic-Resistance*. Science 264:418-420.
4. Kisker C, Hinrichs W, Tovar K, Hillen W, Saenger W. 1995. *The Complex Formed between Tet Repressor and Tetracycline-Mg²⁺ Reveals Mechanism of Antibiotic-Resistance*. Journal of Molecular Biology 247:260-280.
5. Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M. 2022. *ColabFold: making protein folding accessible to all*. Nature Methods 19:679-+.
6. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Zídek A, Potapenko A, Bridgland A, Meyer C, Kohl SAA, Ballard AJ, Cowie A, Romera-Paredes B, Nikolov S, Jain R, Adler J, Back T, Petersen S, Reiman D, Clancy E, Zielinski M, Steinegger M, Pacholska M, Berghammer T, Bodenstein S, Silver D, Vinyals O, Senior AW, Kavukcuoglu K, Kohli P, Hassabis D. 2021. *Highly accurate protein structure prediction with AlphaFold*. Nature 596:583-+.
7. Corbella M, Liao QH, Moreira C, Parracino A, Kasson PM, Kamerlin SCL. 2021. *The N-terminal Helix-Turn-Helix Motif of Transcription Factors MarA and Rob Drives DNA Recognition*. Journal of Physical Chemistry B 125:6791-6806.
8. Crowe AM, Stogios PJ, Casabon I, Evdokimova E, Savchenko A, Eltis LD. 2015. *Structural and Functional Characterization of a Ketosteroid Transcriptional Regulator of*. Journal of Biological Chemistry 290:872-882.
9. Shelley JC, Cholleti A, Frye LL, Greenwood JR, Timlin MR, Uchimaya M. 2007. *Epik:: a software program for pK prediction and protonation state generation for drug-like molecules*. Journal of Computer-Aided Molecular Design 21:681-691.
10. Schilling NA, Berscheid A, Schumacher J, Saur JS, Konnerth MC, Wirtz SN, Beltrán-Beleña JM, Zipperer A, Krismer B, Peschel A, Kalbacher H, Brötz-Oesterhelt H, Steinem C, Grond S. 2019. *Synthetic Lugdunin Analogues Reveal Essential Structural Motifs for Antimicrobial Action and Proton Translocation Capability*. Angewandte Chemie-International Edition 58:9234-9238.
11. Lu C, Wu CJ, Ghoreishi D, Chen W, Wang LL, Damm W, Ross GA, Dahlgren MK, Russell E, Von Bargen CD, Abel R, Friesner RA, Harder ED. 2021. *OPLS4: Improving Force Field Accuracy on Challenging Regimes of Chemical Space*. Journal of Chemical Theory and Computation 17:4291-4300.
12. Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT, Repasky MP, Knoll EH, Shelley M, Perry JK, Shaw DE, Francis P, Shenkin PS. 2004. *Glide: A new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy*. Journal of Medicinal Chemistry 47:1739-1749.
13. Friesner RA, Murphy RB, Repasky MP, Frye LL, Greenwood JR, Halgren TA, Sanschagrin PC, Mainz DT. 2006. *Extra precision glide: Docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes*. Journal of Medicinal Chemistry 49:6177-6196.

14. Bowers KJ, Chow E, Xu H, Dror RO, Eastwood MP, Gregersen BA, Klepeis JL, Kolossvary I, Moraes MA, Sacerdoti FD, Salmon JK, Shan Y, Shaw DE. 2006. *Scalable algorithms for molecular dynamics simulations on commodity clusters*, *abstr Proceedings of the 2006 ACM/IEEE conference on Supercomputing, Tampa, Florida*, Association for Computing Machinery,
15. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. 1983. *Comparison of Simple Potential Functions for Simulating Liquid Water*. *Journal of Chemical Physics* 79:926-935.
16. Darden T, York D, Pedersen L. 1993. *Particle Mesh Ewald - an $N \cdot \log(N)$ Method for Ewald Sums in Large Systems*. *Journal of Chemical Physics* 98:10089-10092.
17. Cheatham TE, Miller JL, Fox T, Darden TA, Kollman PA. 1995. *Molecular-Dynamics Simulations on Solvated Biomolecular Systems - the Particle Mesh Ewald Method Leads to Stable Trajectories of DNA, Rna, and Proteins*. *Journal of the American Chemical Society* 117:4193-4194.
18. Berendsen HJC, Postma JPM, Vangunsteren WF, Dinola A, Haak JR. 1984. *Molecular-Dynamics with Coupling to an External Bath*. *Journal of Chemical Physics* 81:3684-3690.
19. Martyna GJ, Klein ML, Tuckerman M. 1992. *Nose-Hoover Chains - the Canonical Ensemble Via Continuous Dynamics*. *Journal of Chemical Physics* 97:2635-2643.
20. Martyna GJ, Tuckerman ME, Tobias DJ, Klein ML. 1996. *Explicit reversible integrators for extended systems dynamics*. *Molecular Physics* 87:1117-1157.

Chapter 3

Ulbactin F represents a chalkophore facilitating copper homeostasis in *Staphylococcus lugdunensis*

Leonie Reetz^{a,b,c}, Heiko Pfeffer^{b,d}, Lukas Schulze^{a,b,c}, Hannah Simonis^a, Angelika Müller-Jochim^a, Stephanie Grond^{b,d}, Simon Heilbronner^{a,b,c}, Andreas Peschel^{a,b,c}, Bernhard Krismer^{a,b,c*},

^a Department of Infection Biology, Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, Tübingen, Germany; ^b Cluster of Excellence EXC 2124 Controlling Microbes to Fight Infections; ^c German Center for Infection Research (DZIF), partner site Tübingen; ^d Institute of Organic Chemistry, University of Tübingen, Tübingen, Germany

*Corresponding author: b.krismer@uni-tuebingen.de

Abstract

Metal acquisition in metal-restricted environments is crucial for bacterial survival and multiplication. Secretion and uptake of metallophores play important roles in metal homeostasis for many bacteria. *Staphylococcus lugdunensis* is known for the production of the non-ribosomal peptide (NRP) lugdunin, founding-member of cyclic thiazolidine peptides, with antimicrobial activity against the major pathogen *Staphylococcus aureus*. Here, we analysed the second biosynthetic gene cluster (BCG) encoded by *S. lugdunensis*. It turned out to be responsible for the production of the previously described ulbactin F, produced by the marine sponge-associated *Brevibacillus brevis*. Ulbactin F has a broad metal binding spectrum with high affinity to iron and has therefore been considered as a siderophore. However, we could not observe any siderophore activity of ulbactin F in *S. lugdunensis*. In contrast, we observed the highest binding affinity of ulbactin F for copper ions. Deletion of biosynthesis and transport genes led to increased tolerance to inhibitory copper concentrations, supporting the notion that ulbactin F is a chalkophore responsible for copper import into *S. lugdunensis* with an important role in copper homeostasis.

Introduction

Metals are vital for all forms of life, as about 30% of proteins are metal-dependent [1, 2]. Manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu) and zinc (Zn) ions are metal cofactors for multiple metalloproteins [1]. These transition metals have unfilled *d*-orbitals, except for Zn, allowing them to change their oxidation state. Therefore, these transition metals often have catalytic roles in biological processes such as DNA replication and transcription, enzymatic reactions, respiration, or response to oxidative stress [3]. Bacterial intracellular homeostasis of transition metals is crucial, as an excess can lead to the generation of damaging reactive oxygen species through Fenton chemistry or mis-metalation of metalloproteins, thereby abrogating their enzymatic function [4]. To ensure metal homeostasis, metal-dependent regulatory proteins, such as the ferric uptake regulator (Fur) [5], sense the bioavailability and regulate genes involved in metal import or export as well as storage and distribution transcriptionally [6]. For instance, in many bacteria Cu homeostasis is regulated by the *cop* operon. This operon consists of four genes encoding CopY, a repressor, CopZ, an intracellular chaperone for Cu, as well as CopA and CopB, two CPx-type Cu-transporting membrane-bound ATPases, responsible for the export of toxic metals from cells [7-12]. Intracellular Cu is sensed in bacteria by *cop* operons under the regulation of the copper-sensitive operon repressor (CsoR). Excess of Cu leads to de-repression of the *cop* operon and hence to translation of the Cop proteins and Cu export [13-15]. Cu resistance is an important trait for overcoming the host immune system during phagocytosis by macrophages, in which

Cu is transported into the phagolysosome by the Cu transporter CTR1 and the Cu ATPase ATP7A. This process is a host defence mechanism against pathogens in addition to pH stress and the generation of reactive oxygen species [3, 16, 17].

During infection, pathogenic bacteria must not only deal with metal intoxication but also with low metal availability as the host innate immune system sequesters metals from invading pathogens, a phenomenon called 'nutritional immunity' [18-20]. This mechanism is achieved by expression of various plasma proteins. For Fe, the most abundant transition metal in the human body, a variety of different proteins is used to sequester free Fe. While the majority of extracellular Fe is bound to haem and hemoglobin, further free ferric Fe circulating in the human plasma is bound to transferrin [21]. Intracellular Fe is bound to metalloproteins or ferritin to prevent concentration-dependent Fe toxicity and Fe uptake by intracellular pathogens [22, 23]. The human organism also secretes calprotectin to bind the transition metals Fe, Mn, Zn or Ni [24-27]. Extracellular binding and transport of Cu is accomplished mostly by the protein ceruloplasmin. However, albumin which is the most abundant plasma protein, also has a high Cu affinity [28, 29].

To bypass the host's nutritional immunity, staphylococci and other bacteria have developed several mechanisms for metal uptake. Siderophores for iron uptake represent the archetype for metal acquisition. Siderophores are small molecules with a low molecular weight and a high affinity for Fe(III). The pathogen *Staphylococcus aureus* synthesises the two siderophores staphyloferrin A and staphyloferrin B [30-32], while enterobactin (also named enterochelin) can be found, for example, in *Escherichia coli* or *Salmonella enterica* [33]. Besides siderophores, metallophores that facilitate the uptake of other essential metal ions, such as pseudopaline from *Pseudomonas aeruginosa* or staphylopin from *S. aureus*, both facilitating zinc uptake, have evolved [34, 35]. *Staphylococcus* spp. are known to produce a variety of secondary metabolites, and staphylopin-like biosynthetic gene clusters (BGCs) have been detected in genomes of the majority of *Staphylococcus* species [36]. For *S. lugdunensis*, a commensal that colonizes the human nose and is known to produce the antimicrobial lugdunin - a fibupeptide with antimicrobial activity against *S. aureus* [37, 38]. Neither siderophores nor other metallophores have been described in *S. lugdunensis* yet. Nevertheless, the iron-regulated surface determinant (Isd) system as well as an energy-coupling factor (ECF) type transporter for the uptake of iron via heme named Lha have been identified [39, 40].

The genomes of most *S. lugdunensis* isolates encode three non-ribosomal peptide synthetase (NRPS) BGCs, of which the first encodes the aureusimines, cyclic dipeptides which do not have antibiotic activity, instead serve as regulators of virulence expression [41]. The second is responsible for lugdunin production [42]. In this study we aimed to investigate the function of

the third, yet unknown, non-ribosomal peptide (NRP) cluster. We show that this BGC exhibits significant homology to the siderophore BGCs for equibactin. Unexpectedly, *S. lugdunensis* was not able to grow under iron restricted conditions, indicating that the unknown NRP might fulfil an alternative function. Purification and structure elucidation of the molecule identified it as ulbactin F, previously identified in *Brevibacillus brevis*. In contrast to *B. brevis*, for which ulbactin F has been proposed to serve as a siderophore, we show that ulbactin F functions as a copper-binding molecule, so called “chalkophore” in *S. lugdunensis*, with lower affinities to other metal ligands. Moreover, we elucidate the interplay between ulbactin F synthesis and copper tolerance.

Results and Discussion

S. lugdunensis N920143 produces an unknown secondary metabolite via a non-ribosomal peptide cluster

Non-ribosomally synthesized peptide products are very rare in the genus *Staphylococcus* except for the aureusimines from *S. aureus* [41], lugdunin from *S. lugdunensis* [37], and the recently published epifadin from *Staphylococcus epidermidis* [43]. In this context it is exceptional that *S. lugdunensis* alone harbours three NRPS systems. The NRPIII BGC of *S. lugdunensis* N920143 (accession number: NC_017353.1, nt 2,042,913 - 2,022,714) was analysed using antiSMASH (bacterial version 7.0) and yielded 28% similarity of the entire BGC with that of the siderophore NRP equibactin from *Streptococcus equi subsp. equi* (Figure 4) [44, 45].

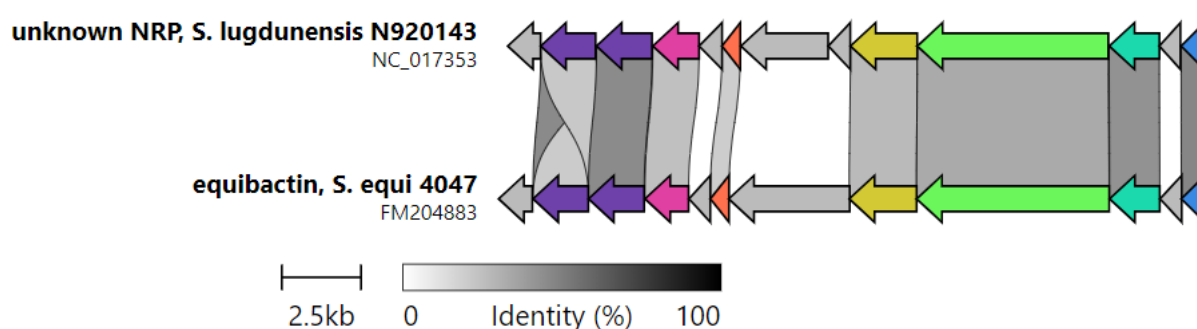


Figure 4 Gene cluster comparison of the unknown NRP from *S. lugdunensis* N920143 and the equibactin BGC from *S. equi* 4047 using clinker from the comparative gene cluster analysis toolbox (CAGECAT) [46].

The BGC in *S. lugdunensis* comprised 13 open reading frames (ORFs), comprising the biosynthetic genes *lgbA* – *lgbM* (Figure 5A). The BGC encoded a putative thioesterase (LgbA) and 4'-phosphopantheyl transferase (LgbB) required for the NRP synthases (LgbD), an AMP-binding protein (LgbC), a oxidoreductase/methyltransferase (LgbE), an unknown gene (LgbF), a polyketide synthase (LgbG), a putative ECF transporter with LgbH as substrate binding domain, LgbI as transmembrane domain, and LgbJ as ATP-binding domain, two additional transporters (LgbK, LgbL) as well as an unusual saccharopine dehydrogenase homologue (LgbM). In contrast, the equibactin BGC consists of 14 ORFs, with *egbA* as a predicted iron-dependent regulator, whereas in the *lgb* BGC no regulator could be identified. Interestingly, the *S. lugdunensis* *lgbG* encodes a polyketide synthase whereas the homologous protein in *S. equi*, *EqbG*, lacks the N-terminal domain involved in the biosynthesis of the polyketide [47]. This indicates, that the synthesized molecule differs structurally from equibactin, even though the final structure for equibactin has remained unsolved.

To facilitate identification and analysis of the product of the *lgb* BGC in *S. lugdunensis*, the genes *lgbCD* were deleted (Δ *lgbCD*). As equibactin is proposed to be a siderophore that is only expressed under iron-limiting conditions, the iron chelator EDDHA was added to liquid culture of the *S. lugdunensis* N920143 wildtype (WT) and the *S. lugdunensis* N920143 Δ *lgbCD* mutant [47]. The liquid cultures were extracted using 1-butanol and further analysed via reversed-phase high-pressure liquid chromatography (HPLC). Comparison of both extracts revealed one distinct signal missing peak in the Δ *lgbCD* mutant compared to the WT, in the base peak chromatogram (BPC), as well as in the corresponding UV chromatogram at 254 nm with a retention time of approximately 16.5 min (Figure 5B).

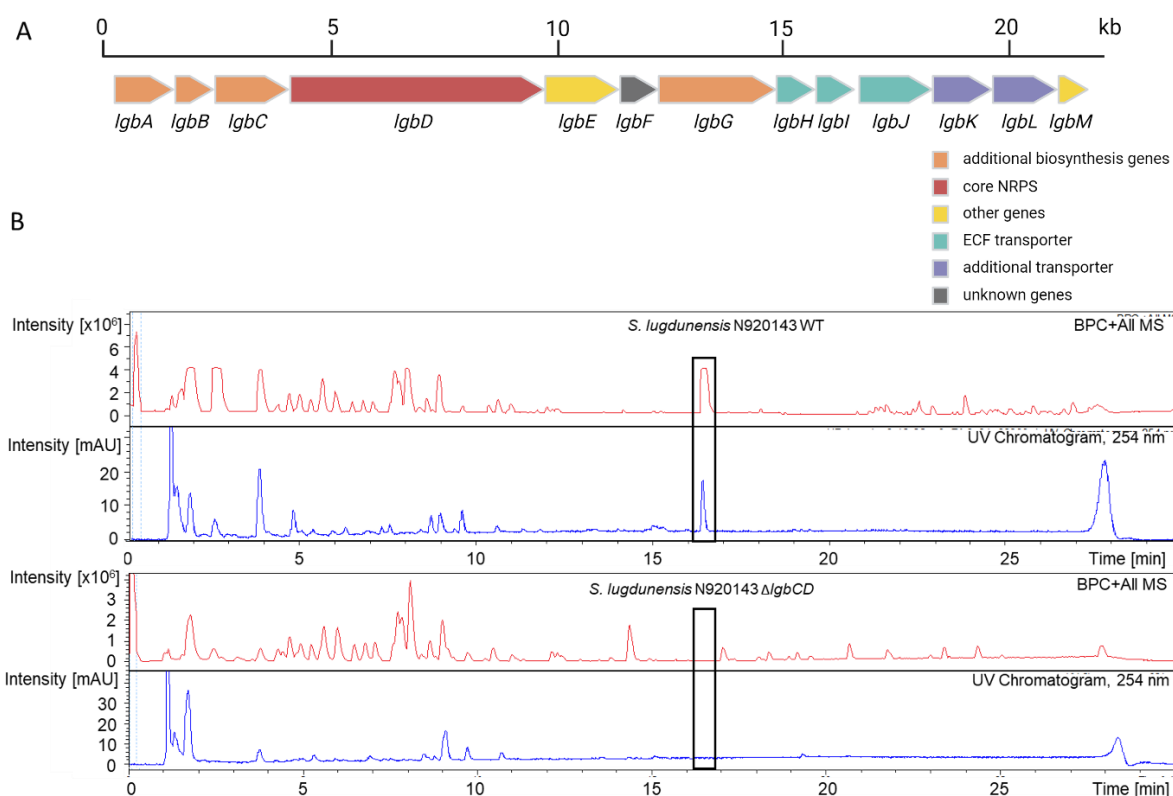


Figure 5 Characterisation of the *S. lugdunensis* N920143 NRPIII. A) Schematic illustration of the genomic organisation of the NRPIII BGC. Annotated protein functions are shown in distinct colours. B) HPLC chromatogram of butanol extracted cultures of *S. lugdunensis* N920143 WT (upper chromatogram) and Δ *lgbCD* mutant (lower chromatogram). Shown are the base peak chromatogram (BPC) in red and the UV chromatogram at 254 nm in blue. Retention time in minutes [min] is shown on the x-axis, intensity in milli arbitrary units [mAU] of the peaks is shown on the y-axis.

S. lugdunensis produces ulbactin F

In a next step, we aimed to identify the molecule from the distinct peak at 16.5 min retention time. To this end high-resolution mass spectrometry (HR-MS) was performed, which yielded a mass of $m/z = 394.0711$ suggesting the molecular formula $C_{17}H_{19}N_3O_2S_3$ calculated $m/z = 394.0712$ $[M+H]^+$, $\Delta 0.2$ parts per million (ppm) and a relative molecular mass of 393.5380 g/mol. Surprisingly, the calculated m/z of 394.0712 has already been described for the metallophore ulbactin F, found in *Brevibacillus brevis* [48].

The purified compound was further analysed via nuclear-magnetic resonance (NMR). This analysis showed that it represents a NRP resembling ulbactin F, consisting of one hydroxyphenylalanine and three cysteines, which are cyclized to form one thiazoline and two thiazolidine rings. It has a 2-(2'-hydroxyphenyl)-2-thiazolidine building block with an N-bridged 1,4-thiazepan-5-on ring including a N-terminal methylation at position 10". The suggested structure is shown in Figure 6A. To examine the similarity between the unknown compound

and ulbactin F, ulbactin F was chemically synthesised according to the previously published protocol of Shapiro et al., 2018 [49]. For both, the synthetic ulbactin F and the similar *S. lugdunensis* compound, the MS/MS fragmentation patterns were analysed (Figure S1), which turned out to be identical, indicating that the unknown compound produced by *S. lugdunensis* represents ulbactin F. Next, the chemical shifts obtained by NMR analysis from both molecules were analysed and found to be the same, showing that *S. lugdunensis* produces the metallophore ulbactin F (Figure 6B). Both ulbactin F BGCs from *S. lugdunensis* and *B. brevis* were compared, revealing only minor sequence homologies (Figure S2).

The 2-(2'-hydroxyphenyl)-2-thiazolidine moiety of ulbactin F is known from other metallophores such as pyochelin or yersiniabactin, synthesized by *Pseudomonas* spp. or *Yersinia* spp., respectively [50, 51]. Both molecules are proven siderophores. However, for yersiniabactin a non-canonical role in the uptake of other metals, including Zn or Cu, has been described [50, 52-54].

The BGCs for ulbactin F and yersiniabactin require salicylic acid as starter building block for biosynthesis, encode a salicylate synthase [55, 56]. However, a homologue for this protein could not be identified in the genome of *S. lugdunensis*, suggesting that *S. lugdunensis* requires external salicylic acid for ulbactin F synthesis. To assess this hypothesis, an extract from the liquid culture of *S. lugdunensis* N920143 WT grown in chemically defined medium (CDM) with or without salicylic acid was analysed (Figure S3). In the absence of external salicylic acid, no ulbactin F was found, indicating that salicylate is a building block for ulbactin F biosynthesis in *S. lugdunensis*, which needs to be acquired from external sources.

Interestingly, this dependency has also been observed for biosynthesis of the siderophore equibactin produced by *S. equi* [47]. Equibactin has been proposed to increase survival of *S. equi* during host infection, as it increases iron acquisition [57].

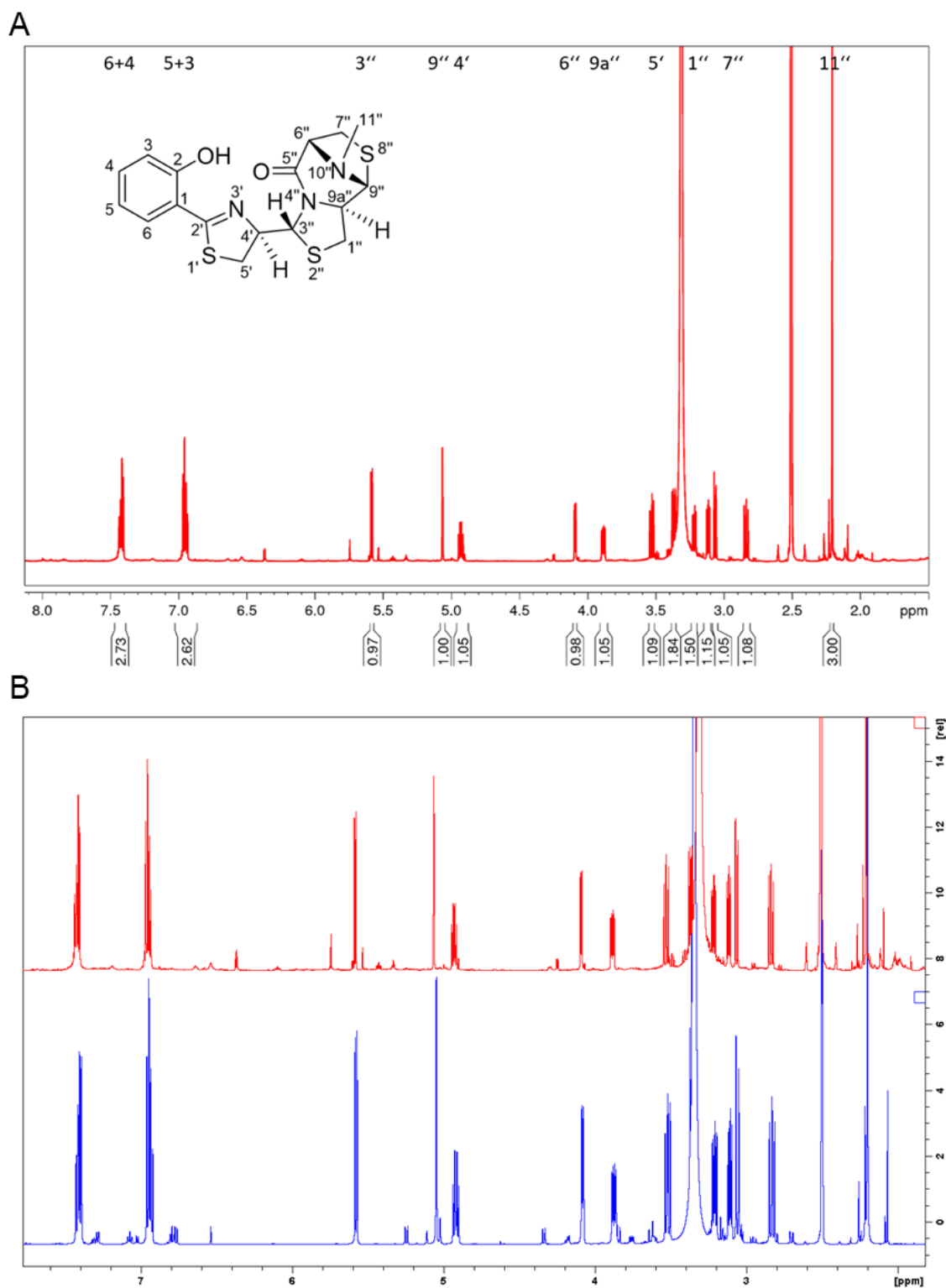


Figure 6 Nuclear-magnetic resonance (NMR) analysis of ulbactam F. A) NMR of purified ulbactam F, DMSO- d_6 303K 700 MHz. The chemical structure is depicted with marked chemical shifts. B) Comparative NMR of ulbactam F purified from *S. lugdunensis* (red), DMSO- d_6 303K 700 MHz and chemically synthesized ulbactam F (blue), DMSO- d_6 303K 600 MHz.

Ulbactin F biosynthesis is controlled by transcriptional uptake regulators for iron and zinc

To determine the effect of metal-sensing regulators on the expression of ulbactin F, three mutants were generated in *S. lugdunensis* N920143 lacking transcriptional regulators of metal ion uptake including Δzur (zinc uptake regulator), Δfur (ferric uptake regulator), and $\Delta csoR$ (copper-sensing operon repressor). The production of ulbactin F of these *S. lugdunensis* mutants after growth in either BHI broth or iron-depleted BHI+ broth (containing 100 μ M EDDHA), both supplemented with the ulbactin F building blocks cysteine and salicylic acid, was analysed using HPLC-MS.

In the *S. lugdunensis* N920143 WT the production of ulbactin F was observed to be approximately 100x increased under iron-limited conditions compared to the BHI broth control. This indicates, that iron inhibits the production of ulbactin F in *S. lugdunensis*. In the *S. lugdunensis* mutants Δzur and Δfur the presence of iron no longer diminished ulbactin F production, reaching 10- and 100-fold expression compared to the *S. lugdunensis* N920143 WT grown in BHI broth, respectively. The $\Delta csoR$ mutant, however, exhibited the same baseline expression as the WT. In iron-depleted BHI+ broth, ulbactin F production by the WT and the $\Delta csoR$ mutant was identical, whereas the Δzur and Δfur mutants exhibited a 10-fold lower production compared to the WT (Figure 7A). Growth of the Δfur mutant in BHI broth was significantly lower after 24 h compared to the WT. In contrast, Δzur and $\Delta csoR$ mutants showed comparable growth to the WT in BHI broth. However, growth of the WT, and the $\Delta csoR$ and Δfur mutants was significantly reduced in BHI+. Only the Δzur mutant showed no growth defect in iron-depleted BHI+ medium compared to BHI after 24 h (Figure 7B).

Iron is known to be essential for many bacteria to colonize or infect their hosts. Iron acquisition and homeostasis are controlled in many bacterial species mainly by Fur, a global regulator of hundreds of genes, several of which are not only involved in iron acquisition but also in respiration or other metabolic processes [58, 59]. Interestingly, the nickel uptake regulator (Nur), manganese uptake regulator (Mur), the peroxide response regulator (PerR), as well as Zur all belong to the transcriptional regulators of the Fur family [6, 60]. Siderophore production regulated by Fur has also been shown for many different bacteria including *Chromobacterium violaceum* [61], *E. coli* [62] or *P. aeruginosa* [63], and *S. aureus* [64]. The zincophore pseudopaline produced by *P. aeruginosa* has been reported to be regulated by the global regulator Zur [65]. Ulbactin F production in *S. lugdunensis* seems to be regulated by both global regulators, Fur and Zur. Cross-talking between Fur and Zur has been described for the cyanobacterium *Anabena sp.* [66]. However, it is generally believed that DNA binding of the global regulator proteins to their respective sequence motifs is highly specific, even when there are only a few nucleotides different in their respective operator sequences [60]. Growth defects

of the *S. lugdunensis* Δfur mutant compared to the WT were observed (Figure 7B). This phenotype has been described before for mutants of yersiniabactin producing uropathogenic *E. coli* [62], or *Bordetella pertussis*, and *Bordetella bronchiseptica* [67]. The growth defect occurred specifically in iron-replete media, presumably as a result of excessive iron uptake leading to iron intoxication.

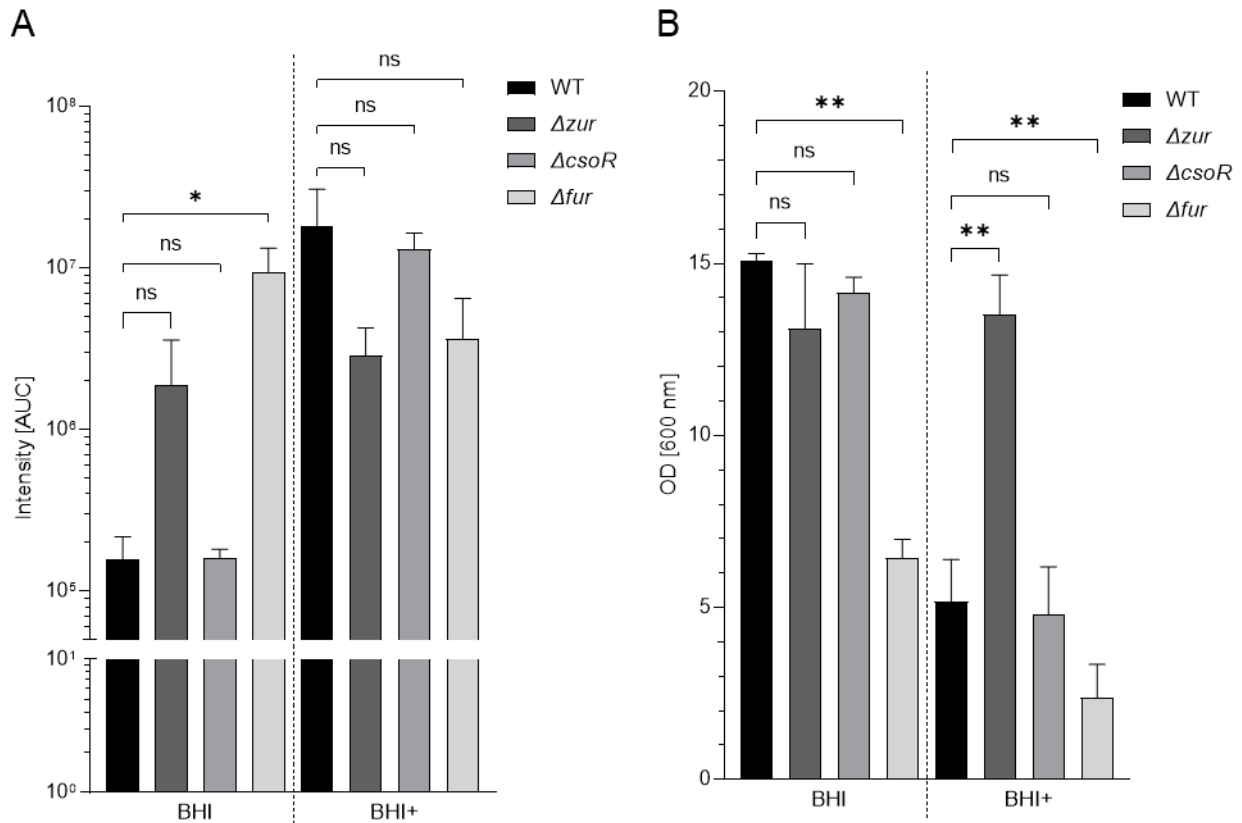


Figure 7 Impact of global regulator mutants on the production of ulbactin F. A) Ulbactin F production of *S. lugdunensis* N920143 WT, Δfur , Δzur and $\Delta csoR$ was measured using butanol extracts of whole liquid cultures. Shown are the area under the curve (AUC) values from HPLC chromatograms of the distinct ulbactin F peaks. B) Growth of *S. lugdunensis* N920143 WT and the mutants was determined by measuring the optical density (OD) at 600 nm after 24 h of growth in the respective culture broth. Statistical significance was determined for each condition, ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$.

S. lugdunensis uses ulbactin F as a chalkophore

S. lugdunensis does not produce siderophores for iron uptake such as staphyloferrin A or staphyloferrin B, which can be found, for instance, in *S. aureus*. However, transporters for uptake of these two siderophores are expressed by *S. lugdunensis*, thereby debunking *S. lugdunensis* as a potential “social cheater” [68]. As equibactin and ulbactin F have both been assumed to be iron-binding molecules, siderophore activity was analysed using chrome-azurol S (CAS). This assay is based on a colorimetric change, where a siderophore

scavenges the iron from an Fe-CAS-hexadecyltrimethylammonium bromide complex. Upon release of the CAS dye a colour change from blue to orange is detectable [69]. In this experiment, no colour change after the addition of culture supernatant of the *S. lugdunensis* N920143 WT grown in iron-limited medium could not be observed (Figure S4), confirming the current knowledge, that *S. lugdunensis* does not produce siderophores. This finding indicates however, that ulbactin F produced by *S. lugdunensis* may have other physiological functions than iron ion binding.

Fluorescence titration experiments using chemically synthesized ulbactin F were performed. This experiment is based on the autofluorescence of the compound of interest, in our case excitation at 316 nm and emission at 462 nm was used. Metal ion binding to ulbactin F can either leads to enhancement or quenching of the fluorescent signal, indicating complex formation [70]. The highest affinity of ulbactin F was observed for Cu(II) (CuCl_2). Here the titration curve showed an 1:2 (ulbactin F: CuCl_2) stoichiometry. Also, Fe(III) (FeCl_3) and cobalt Co(II) (CoCl_2) led to a plateau of fluorescence intensity after 9 or 12 equivalents, respectively. This experiment indicates that, under the chosen conditions, ulbactin F has only minor affinity for iron and cobalt compared to copper. For Zn(II) (ZnCl_2), however, no plateau in the fluorescence intensity was observed, even after addition of 20 equivalents of zinc. No binding was detected for manganese Mn(II) (MnCl_2) or calcium Ca(II) (CaCl_2) (Figure 8).

Ulbactin F production has been first described for *B. brevis* by Shapiro et al. [49], who observed the highest affinity of ulbactin F for Fe, and a lower affinity for Cu, whereas no binding could be observed for Ni or Zn [49]. Therefore, they regarded ulbactin F as a siderophore. In contrast, our data identify ulbactin F as a high-affinity chalkophore and a low-affinity broad-spectrum metallophore. Such unspecific, non-canonical binding capacities of metallophores have been described before. The siderophore yersiniabactin is produced by *Yersinia pestis* as well as uropathogenic *E. coli* to overcome Fe limitations during infection in the human host. However, yersiniabactin also binds Zn to overcome Zn limitations [71]. Further, Cu bound to yersiniabactin led to a stimulation of yersiniabactin biosynthesis [62]. *S. aureus*, however, produces the zincophore staphylopin. This metallophore is utilized mainly for Zn acquisition, however the opine structure enables the molecule to additionally bind several other metals like Ni, Co, Fe or Cu. Therefore, it is considered as a broad-spectrum metallophore [72, 73]. Other metallophores bind predominantly Cu ions – so-called chalkophores. Examples can be found in methanotrophs such as *Methylosinus trichosporium* or *Methylocystis* sp., both producing methanobactin [74]. However, methanobactins can bind other metals than Cu, including Fe or Mn as well as gold or mercury with different binding affinities [74]. Moreover, also pyoverdine and pyochelin from *P. aeruginosa* are known to bind

Cu but only the Fe-bound compounds are efficiently taken up by *P. aeruginosa*. The *in vivo* relevance of metal binding by ulbactin F needs to be evaluated for *S. lugdunensis* to assess its actual fitness function [75, 76].

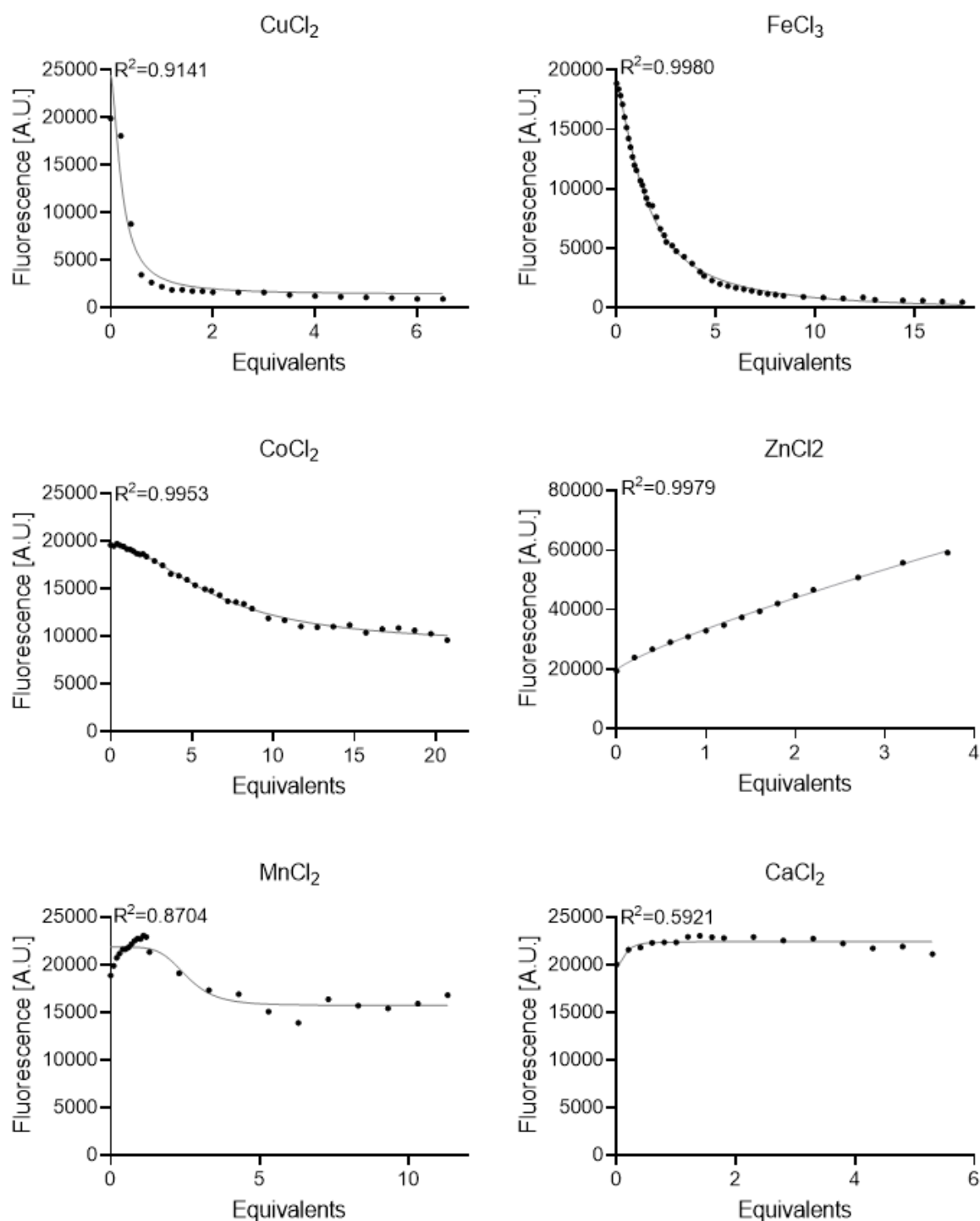


Figure 8 Fluorescence titration for binding of ulbactin F to specific transition metals. Copper (Cu), iron (Fe), cobalt (Co), zinc (Zn), manganese (Mn) or calcium (Ca) were used as chloride ions for fluorescence titration with synthetic ulbactin F. Change in fluorescence is depicted in arbitrary units (A.U.). Representative measurement is shown.

To assess a potential chalkophore activity of ulbactin F, growth of *S. lugdunensis* under copper-limiting conditions was tested, using the copper(I)-specific chelator bathocuproine

disulfonate (BCS). Addition of BCS led to reduced growth of both, the *S. lugdunensis* N920143 WT and the $\Delta lgbCD$ mutant, and no significant growth differences between both strains were detected (Figure S5). Nevertheless, potential roles of ulbactin F in copper homeostasis or copper resistance were further investigated.

Copper homeostasis in *S. lugdunensis* is achieved by ulbactin F and the *copAZ* operon

Like most bacteria, also *S. lugdunensis* encodes the copper resistance operon *copAZ* in which *copA* encodes a copper exporter and *copZ* encodes a copper-binding chaperone, together accomplishing the excretion of excess copper amounts [77]. To elucidate the interplay of ulbactin F with CopA and CopZ in copper homeostasis, mutants deficient in the *copAZ* operon (herein called $\Delta copAZ$) were generated in the *S. lugdunensis* wild type and $\Delta lgbCD$ mutant. Further, liquid cultures of *S. lugdunensis* N920143 WT, $\Delta lgbCD$, $\Delta copAZ$, or $\Delta lgbCD \Delta copAZ$ mutants were treated with increasing concentrations of Cu. Treatment of *S. lugdunensis* N920143 WT and $\Delta lgbCD$ mutant with up to 160 μM $CuCl_2$ in liquid culture showed similar growth effects, no differences between the WT and the mutant could be observed (Figure 9A). However, Cu addition to liquid cultures of *S. lugdunensis* N920143 $\Delta copAZ$ and $\Delta lgbCD \Delta copA$ mutants led to a 10-fold reduction in optical density of the $\Delta copAZ$ mutant at 160 μM $CuCl_2$ compared to the untreated culture, whereas the $\Delta lgbCD \Delta copA$ mutant showed approximately a 6-fold reduction (Figure 9B). Plating experiments, to evaluate the colony forming units, of the *S. lugdunensis* N920143 WT or the $\Delta lgbCD$ mutant on $CuCl_2$ -containing RPMI-1640 plates also revealed no differences in colony forming units (CFU). However, a toxic effect for both strains could be detected at lower Cu concentrations compared to liquid culture. At 60 μM $CuCl_2$, CFUs were reduced by 50 % in both strains, compared to 0 μM $CuCl_2$ (Figure 9C). This indicated that both the *S. lugdunensis* N920143 WT as well as the $\Delta lgbCD$ mutant have the same susceptibility to Cu. On solid medium, no growth could be observed at concentrations of 60 μM $CuCl_2$ indicating an increased toxicity of Cu to the cells. At 20 μM $CuCl_2$ the *S. lugdunensis* N920143 $\Delta lgbCD \Delta copAZ$ mutant showed one log more growth than the $\Delta copAZ$ mutant (Figure 9D). This difference demonstrated that the *copAZ* operon plays a crucial role in copper homeostasis of *S. lugdunensis* and supports the hypothesis that ulbactin F is used for copper uptake. In the $\Delta lgbCD \Delta copAZ$ mutant, where no ulbactin F could mediate Cu import into the cells, copper-intoxication is significantly lower than in the $\Delta copAZ$ mutant.

Intracellular copper concentrations have been shown to be strictly regulated in *E. coli* with approximately 10^4 Cu atoms per cell (approximately 10 μM Cu) [78, 79]. In *E. coli* Cu resistance is achieved by the efflux pump CopA as well as the multicopper oxidase CueO, responsible for the oxidation of Cu^+ to the less toxic Cu^{2+} in the periplasm [79]. In Gram-positive

bacteria such as *Listeria monocytogenes* Cu resistance is mainly regulated by the repressor CsoR, which changes its conformation upon binding to Cu, thus allowing transcription of the *copAZ* operon. However, also plasmids encoding a multicopper oxidase as well as a Cu-translocating P-type ATPase have been identified, further contributing to Cu resistance in *L. monocytogenes* [80]. In *S. aureus*, Cu resistance is maintained by the efficient export of Cu through the CopAZ system as well as the CopBL system, consisting of CopB, the P-type ATPase necessary for Cu export and CopL, an extracellular Cu-binding lipoprotein [80, 81]. CsoR is also responsible for regulation of the *copAZ* as well as the *copBL* operons in *S. aureus*. Upon Cu binding, CsoR completely dissociates from the promotor regions thus allowing transcription [80]. As no other Cu uptake mechanisms have been described in *S. lugdunensis* so far, we assume that the production of ulbactin F is sufficient for the acquisition of Cu and saturation of Cu-dependent proteins.

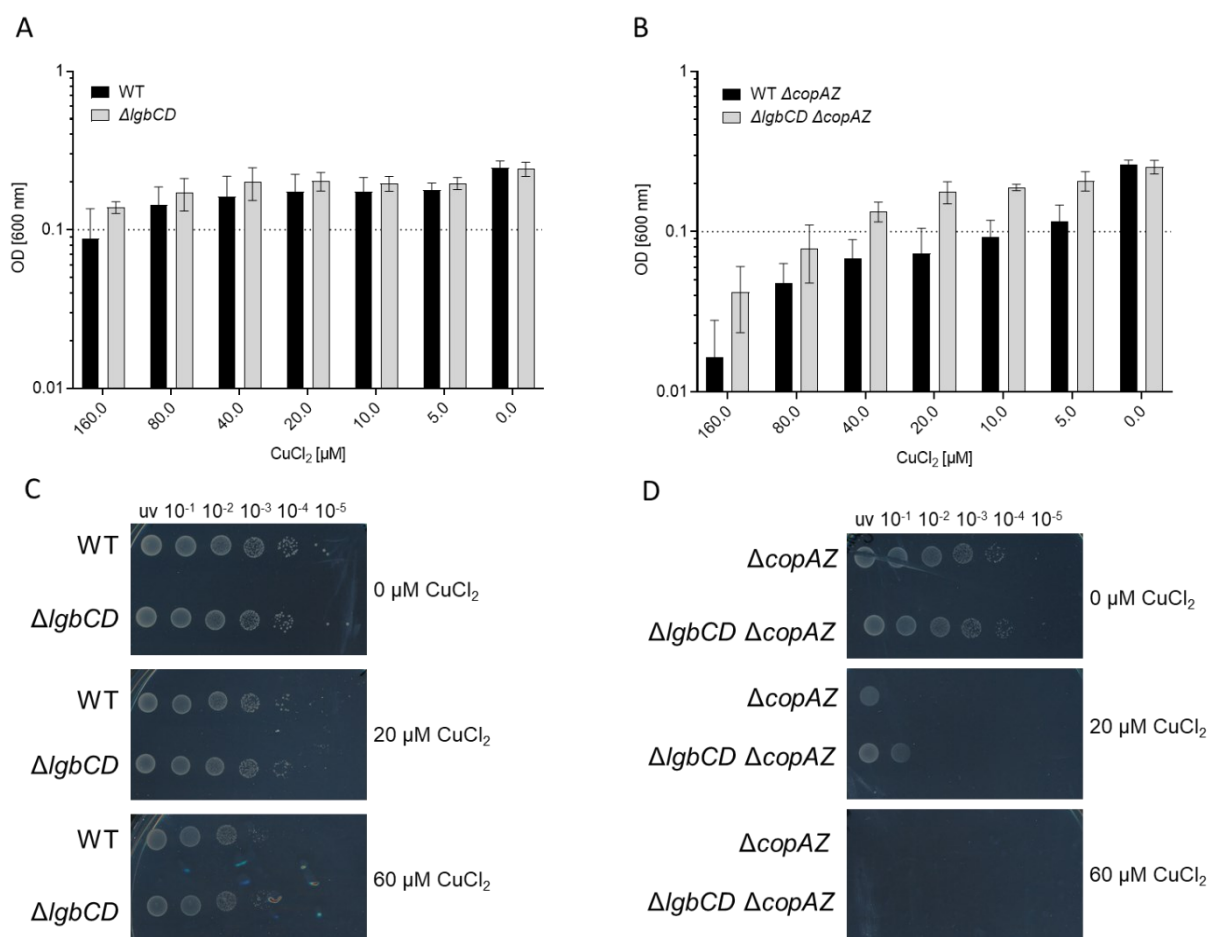


Figure 9 Copper resistance of *S. lugdunensis* N920143 WT, $\Delta lgbCD$, $\Delta copAZ$, or $\Delta lgbCD \Delta copAZ$ mutants. A,B) Minimal inhibitory concentration of copper chloride ($CuCl_2$) in liquid culture, growth was measured as optical density (OD) at 600 nm after 21 h at 37°C, n=3. C,D) Minimal inhibitory concentration of $CuCl_2$ using spot assays on $CuCl_2$ -containing RPMI-1640 agar plates. Overnight culture of the WT or the respective mutants were adjusted to an OD₆₀₀ of 1 and serially diluted to 10⁻⁵. 10 μl each were spotted, plates were incubated at 37°C for 24 h, n=3, representative images are shown.

Conclusion

In this study we could demonstrate that *S. lugdunensis* produces the copper-binding metallophore ulbactin F using a previously uncharacterised NRPS that differs substantially from the ulbactin BGC of *B. brevis*. Purification followed by HPLC-MS/MS and NMR analysis revealed the production of ulbactin F in *S. lugdunensis*. This compound was only known from the marine sponge-derived bacterium *B. brevis*. We demonstrate that ulbactin F has the highest *in vitro* binding affinity to copper, indicating that it may act as a chalkophore. Nevertheless, deletion mutants of global metal responsive regulators indicated that the transcription is iron-dependent.

An impact on copper uptake by ulbactin F could be shown only indirectly by analysing how it influences *S. lugdunensis* susceptibility to inhibitory copper concentrations. We could also demonstrate that in *S. lugdunensis* copper resistance is achieved by the CopAZ system, known from many other bacteria. Production of ulbactin F leads to a higher copper sensitivity in a *copAZ* deletion mutant, suggesting higher ulbactin F-dependent copper uptake in *S. lugdunensis*.

Material and Methods

Bacterial strains and culture conditions. *S. lugdunensis* N920143 was used in this study for all experiments. For the generation of knockout mutants, *E. coli* DC10B was used as a cloning intermediate (Table 2). For growth of *E. coli* tryptic soy broth (TSB, Carl Roth GmbH) was used, supplemented with 10 µg ml⁻¹ chloramphenicol if necessary. All experiments with *S. lugdunensis* were performed in either brain heart infusion broth (BHI, Carl Roth GmbH) supplemented with 10 µg ml⁻¹ chloramphenicol if necessary, Roswell Park Memorial Institute (RPMI-1640) medium without phenol red (R8755, Sigma-Aldrich) supplemented with 0.1 % (w/v) casamino acids (Cas, BD Diagnostics) or chemically defined medium (CDM: 1x M9 minimal salts [Sigma-Aldrich], 1x phosphate-buffered saline [PBS, ThermoFisher Scientific], each 1 mM L-alanine, L-arginine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophane, L-tyrosine, L-valine, 5 mM MgSO₄, 200 µg/l biotin, 2 mg/l nicotinic acid, 1 mg/l pantothenic acid, 3 mg/l pyridoxamine, 2 mg/ml thiamine, 2 mg/l riboflavin, 700 µM glucose) prepared in HPLC grade water (ThermoFisher Scientific). Bacteria were cultured at 37 °C and shaking at 160 rpm if not stated otherwise.

Table 2: Bacterial strains used in this study.

Strain	Genotype
<i>E. coli</i> DC10B	<i>E. coli</i> K12 DH10B Δdcm
<i>S. lugdunensis</i> N920143	WT
<i>S. lugdunensis</i> N920143 $\Delta lgbCD$	$\Delta SLUG_RS09650 \Delta SLUG_RS09645$
<i>S. lugdunensis</i> N920143 $\Delta copAZ$	$\Delta SLUG_RS02070 \Delta copZ$
<i>S. lugdunensis</i> N920143 $\Delta lgbCD \Delta copAZ$	$\Delta SLUG_RS09650 \Delta SLUG_RS09645$ $\Delta SLUG_RS02070 \Delta copZ$
<i>S. lugdunensis</i> N920143 Δfur	$\Delta SLUG_RS07120$
<i>S. lugdunensis</i> N920143 Δzur	$\Delta SLUG_RS06850$
<i>S. lugdunensis</i> N920143 $\Delta csoR$	$\Delta csoR$
<i>S. lugdunensis</i> N920143 $\Delta perR$	$\Delta perR$

Generation of knockout mutants. For the deletion of genes of interest, flanking regions with 500 bp in size were amplified using the primers listed in Table 3. Gene fragments were digested using suitable restriction enzymes (ThermoFisher Scientific; Table 3) and further cloned into the plasmid pIMAY. *E. coli* DC10B was transformed as an intermediate host, further plasmids were verified by sequencing. Validated plasmids were then used to transform *S. lugdunensis* N920143, plates were incubated at 30 °C. Integration into the genome was

achieved after incubation at 37 °C for 24 h with chloramphenicol. Single colonies were passaged 3x in BM media at 30 °C, afterwards plated on BM plates containing 1 µg/ml anhydrotetracycline and incubated for 2 days at 30 °C. Single colonies were checked for loss of plasmids by streaking on BM plates with and without chloramphenicol. Chloramphenicol-sensitive strains were confirmed by sequencing for the loss of gene of interest [82].

Table 3: Primers used in this study. Restriction sites are underlined.

Primer	Sequence
KO-lgbCD_kpnI	GGACATATGTTTATTAAC <u>GGTACCT</u> CCGAAGAAG
KO-lgbCD_ATG	CATTTTGGAACCTCCATCATTGAAATACC
KO-lgbCD_TAA	GGTTCCAAAATGTAAATATGAAAAATTGTATTATATGTGG
KO-lgbCD_SacI	GGTCAATATCATCAC <u>GAGCTC</u> CATTGTACGC
KO_csoR_up_for	GCTG <u>GGTACC</u> ACCATATCTTTATATTTAAC
KO_csoR_up_rev	CCTAGTCTTTTAGCATTTACTCAATTACTTACT
KO_csoR_dw_for	TAAGTAATTGAGTAAATGCTAAAAGACTAGG
KO_csoR_dw_rev	ATTG <u>GAGCTC</u> GACGCACTCGAAC
KO_fur_up_for	CTCGAGG <u>TCGAC</u> CCCCTGAGAATGAAGT
KO_fur_up_rev	CGGGATGTAACAAAGTGTCTGGAAGCTTCACCCC
KO_fur_dw_for	GGGGTGAAGCTTCCAGACACTTTGTTACATCCCG
KO_fur_dw_rev	AATTG <u>GAGCTC</u> TTCAATATGCACGAGTT
KO_zur_up_for	CCCTCGAGG <u>TCGAC</u> ATATCTTTGGCAGATG
KO_zur_up_rev	ATAAATATGATTGATGAAAAGAATTAAACACCCTTTTT
KO_zur_dw_for	AAAAAGGGTGTTTAATTCTTTTCATCAATCATATTTAT
KO_zur_dw_rev	GAATTG <u>GAGCTC</u> GAATTAGGAGTTAAAAATTC
KO_copAZ_up_for	CTCGAGG <u>TCGAC</u> TTAGTTTCATTTTAATGA
KO_copAZ_up_rev	TCTTAACACAACGATTAGAAATTTCAATCCTT
KO_copAZ_dw_for	AAGGATTGAAATTTCTAATCGTTGTGTTAAGA
KO_copAZ_dw_rev	CGAATTG <u>GAGCTC</u> GAAGATATTATTTTAACGC

Growth curves. For every experiment a fresh overnight culture was prepared in RPMI-Cas media. *S. lugdunensis* N920143 strains were adjusted to an OD₆₀₀ of 1 in PBS. A 48-well plate was filled with 500 µl of RPMI-Cas supplemented with 50 µM cysteine and 72 µM salicylic acid (RPMI+) and inoculated with 2.5 µl of OD₆₀₀-adjusted strains. Further either bathocuproinedisulfonic acid (BCS) or metals were added as indicated. Plates were sealed with a SealPlate (Excel Scientific) and incubated in a plate reader at 37°C with shaking for 24 h, the OD₆₀₀ was measured every 15 min.

Production and purification of ulbactin F. From an overnight culture of *S. lugdunensis* the main culture was inoculated 1:100. As main cultures either brain heart infusion (BHI, Carl Roth) broth supplemented with 100 µM ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid) (EDDHA), 50 µM cysteine and 72 µM salicylic acid (BHI+) or RPMI+ or CDM were used. The main cultures were incubated at 37°C for 24 h prior to butanol extraction. Extracts were performed using 1-butanol at a ratio of 3:1, with shaking at 37°C for 1 h. Afterwards, the culture-butanol:mixture was centrifuged at 5,000xg for 30 min. The upper butanol phase was evaporated at 42°C under reduced pressure. For purification of ulbactin F the butanol extracts were solved in methanol and further subjected to gel filtration using Sephadex® LH20 (Sigma-Aldrich) on an Äkta system. Methanol was used as running buffer, flow rate was set to 0.5 ml/min, fractions of 10 ml were collected. For further purification via HPLC a Thermo Scientific dionex Ultimate 3000 (column: Dr. Maisch GMBH, Kromasil-100 C18, 7 µ, 250x20 mm) was used. Gradient was set to 50-100 % in 30 min. As running buffer methanol with 0.1 % formic acid was used. Samples were manually injected and collected.

Mass spectrometry analysis. Dried ulbactin F samples from butanol extracts were resolved with LC-MS methanol to a concentration of 3 mg/ml. 3 µl of the sample were injected to a Thermo Fisher scientific UltiMate 3000 HPLC (column: Nucleoshell® EC RP- C18, 150 x 2 mm, 2,7 µm, Macherey-Nagel) coupled with a Bruker daltonics maXis 4G ESI-QTOF mass spectrometer. Water (0.1 % formic acid) and methanol (0.06 % formic acid) with a flow rate of 3 ml/min and gradient 10 to 100% methanol in 20 minutes followed by 5 minutes 100 % and 5 min 10 % methanol was used as solvent.

Synthesis of ulbactin F. Chemical synthesis of ulbactin F was performed as described elsewhere [49].

Nuclear magnetic resonance spectroscopy analysis. For NMR analysis, dried ulbactin F samples were dissolved in deuterated chloroform or dimethylsulfoxid. 600 MHz NMR spectrum was analysed using the Bruker Avance III HDX 600. 700 MHz NMR spectrum was analysed using the Bruker Avance III HDX 700. Signals were expressed as chemical shifts (δ) in parts

per million (ppm) relative to the residual proton signal of the solvent as an internal standard. Reference signals CDCl_3 : 7.26 ppm (^1H) and 77.26 ppm (^{13}C), $(\text{CD}_3)_2\text{SO}$: 2.50 ppm (^1H) and 39.52 ppm (^{13}C).

Fluorescence titration. Synthetic ulbactin F was used for fluorescence titration experiments. 150 μl of a 400 μM ulbactin F solution in MeOH was added to 20 μl of 0.1 M MOPS buffer, pH 7.0. Metals were serially added from a 6 mM stock solution. Fluorescence was measured after every addition (ex. 316 nm / em. 462 nm) using a Tecan Infinite 200 Pro.

Siderophore detection. *S. lugdunensis* strains were grown over night in BHI + 100 μM EDDHA, 50 μM cysteine and 72 μM salicylic acid. On the next day 1 ml culture was centrifuged at 15 000 $\times g$ for 5 min, the supernatant was used for siderophore detection using the SideroTec AssayTM (Emergen Bio) after manufacturer's protocol.

Copper resistance using Cu(II)-enriched plates. The respective cultures were grown over night for 21 h in RPMI, RPMI+ plates containing specific CuCl_2 concentrations were prepared freshly. 1 ml of the overnight cultures were washed 2x with PBS and then adjusted to an OD_{600} of 1 and further serially diluted. 10 μl of each dilution was spotted on Cu-containing RPMI+ plates and incubated at 37°C over night for 24 h. After incubation growth was examined.

Minimal inhibitory concentration of Cu(II). The respective cultures were grown over night in RPMI. 1 ml of the overnight cultures were washed 2x with PBS and then adjusted to an OD_{600} of 0.0625. 100 μl of the OD_{600} -adjusted cultures were added in triplicates to a 96-well plate with defined CuCl_2 concentrations as indicated. The culture was incubated at 37 °C over night for 21 h prior to measuring the final OD_{600} .

References

1. Andreini, C., et al., *Metal ions in biological catalysis: from enzyme databases to general principles*. J Biol Inorg Chem, 2008. **13**(8): p. 1205-18.
2. Waldron, K.J., et al., *Metalloproteins and metal sensing*. Nature, 2009. **460**(7257): p. 823-30.
3. Besold, A.N., E.M. Culbertson, and V.C. Culotta, *The Yin and Yang of copper during infection*. J Biol Inorg Chem, 2016. **21**(2): p. 137-44.
4. Foster, A.W., D. Osman, and N.J. Robinson, *Metal preferences and metallation*. J Biol Chem, 2014. **289**(41): p. 28095-103.
5. Steingard, C.H. and J.D. Helmann, *Meddling with Metal Sensors: Fur-Family Proteins as Signaling Hubs*. J Bacteriol, 2023. **205**(4): p. e0002223.
6. Lee, J.W. and J.D. Helmann, *Functional specialization within the Fur family of metalloregulators*. Biometals, 2007. **20**(3-4): p. 485-99.
7. Solioz, M. and J.V. Stoyanov, *Copper homeostasis in Enterococcus hirae*. FEMS Microbiol Rev, 2003. **27**(2-3): p. 183-95.
8. Magnani, D. and M. Solioz, *Copper chaperone cycling and degradation in the regulation of the cop operon of Enterococcus hirae*. Biometals, 2005. **18**(4): p. 407-12.
9. Penaud, S., et al., *Induction of heavy-metal-transporting CPX-type ATPases during acid adaptation in Lactobacillus bulgaricus*. Appl Environ Microbiol, 2006. **72**(12): p. 7445-54.
10. Toes, A.M., et al., *Expression of copA and cusA in Shewanella during copper stress*. Microbiology (Reading), 2008. **154**(Pt 9): p. 2709-2718.
11. Al-Tameemi, H., et al., *Staphylococcus aureus lacking a functional MntABC manganese import system has increased resistance to copper*. Mol Microbiol, 2021. **115**(4): p. 554-573.
12. Shafeeq, S., et al., *The cop operon is required for copper homeostasis and contributes to virulence in Streptococcus pneumoniae*. Mol Microbiol, 2011. **81**(5): p. 1255-70.
13. Smaldone, G.T. and J.D. Helmann, *CsoR regulates the copper efflux operon copZA in Bacillus subtilis*. Microbiology (Reading), 2007. **153**(Pt 12): p. 4123-4128.
14. Chaplin, A.K., et al., *Copper trafficking in the CsoR regulon of Streptomyces lividans*. Metallomics, 2015. **7**(1): p. 145-55.
15. Marcus, S.A., et al., *CsoR Is Essential for Maintaining Copper Homeostasis in Mycobacterium tuberculosis*. PLoS One, 2016. **11**(3): p. e0151816.
16. Stafford, S.L., et al., *Metal ions in macrophage antimicrobial pathways: emerging roles for zinc and copper*. Biosci Rep, 2013. **33**(4).
17. Ladomersky, E., et al., *Host and Pathogen Copper-Transporting P-Type ATPases Function Antagonistically during Salmonella Infection*. Infect Immun, 2017. **85**(9).
18. Weinberg, E.D., *Nutritional immunity. Host's attempt to withhold iron from microbial invaders*. JAMA, 1975. **231**(1): p. 39-41.
19. Hood, M.I. and E.P. Skaar, *Nutritional immunity: transition metals at the pathogen-host interface*. Nat Rev Microbiol, 2012. **10**(8): p. 525-37.
20. Murdoch, C.C. and E.P. Skaar, *Nutritional immunity: the battle for nutrient metals at the host-pathogen interface*. Nat Rev Microbiol, 2022. **20**(11): p. 657-670.
21. Barber, M.F. and N.C. Elde, *Escape from bacterial iron piracy through rapid evolution of transferrin*. Science, 2014. **346**(6215): p. 1362-6.
22. Haschka, D., A. Hoffmann, and G. Weiss, *Iron in immune cell function and host defense*. Semin Cell Dev Biol, 2021. **115**: p. 27-36.

23. Shi, H., et al., *A cytosolic iron chaperone that delivers iron to ferritin*. Science, 2008. **320**(5880): p. 1207-10.
24. Nakashige, T.G., et al., *Human calprotectin is an iron-sequestering host-defense protein*. Nat Chem Biol, 2015. **11**(10): p. 765-71.
25. Damo, S.M., et al., *Molecular basis for manganese sequestration by calprotectin and roles in the innate immune response to invading bacterial pathogens*. Proc Natl Acad Sci U S A, 2013. **110**(10): p. 3841-6.
26. Nakashige, T.G., et al., *The Hexahistidine Motif of Host-Defense Protein Human Calprotectin Contributes to Zinc Withholding and Its Functional Versatility*. J Am Chem Soc, 2016. **138**(37): p. 12243-51.
27. Nakashige, T.G., et al., *Nickel Sequestration by the Host-Defense Protein Human Calprotectin*. J Am Chem Soc, 2017. **139**(26): p. 8828-8836.
28. Hellman, N.E. and J.D. Gitlin, *Ceruloplasmin metabolism and function*. Annu Rev Nutr, 2002. **22**: p. 439-58.
29. Linder, M.C., *Ceruloplasmin and other copper binding components of blood plasma and their functions: an update*. Metallomics, 2016. **8**(9): p. 887-905.
30. Beasley, F.C., et al., *Characterization of staphyloferrin A biosynthetic and transport mutants in Staphylococcus aureus*. Mol Microbiol, 2009. **72**(4): p. 947-63.
31. Cheung, J., et al., *Molecular characterization of staphyloferrin B biosynthesis in Staphylococcus aureus*. Mol Microbiol, 2009. **74**(3): p. 594-608.
32. Beasley, F.C. and D.E. Heinrichs, *Siderophore-mediated iron acquisition in the staphylococci*. J Inorg Biochem, 2010. **104**(3): p. 282-8.
33. Raymond, K.N., E.A. Dertz, and S.S. Kim, *Enterobactin: an archetype for microbial iron transport*. Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3584-8.
34. Ghssein, G. and Z. Ezzeddine, *A Review of Pseudomonas aeruginosa Metallophores: Pyoverdine, Pyochelin and Pseudopaline*. Biology (Basel), 2022. **11**(12).
35. Ghssein, G. and Z. Ezzeddine, *The Key Element Role of Metallophores in the Pathogenicity and Virulence of Staphylococcus aureus: A Review*. Biology (Basel), 2022. **11**(10).
36. Morey, J.R. and T.E. Kehl-Fie, *Bioinformatic Mapping of Opine-Like Zincophore Biosynthesis in Bacteria*. mSystems, 2020. **5**(4).
37. Zipperer, A., et al., *Human commensals producing a novel antibiotic impair pathogen colonization*. Nature, 2016. **535**(7613): p. 511-6.
38. Schilling, N.A., et al., *Synthetic Lugdunin Analogues Reveal Essential Structural Motifs for Antimicrobial Action and Proton Translocation Capability*. Angew Chem Int Ed Engl, 2019. **58**(27): p. 9234-9238.
39. Jochim, A., et al., *An ECF-type transporter scavenges heme to overcome iron-limitation in Staphylococcus lugdunensis*. Elife, 2020. **9**.
40. Flannagan, R.S., et al., *In vivo growth of Staphylococcus lugdunensis is facilitated by the concerted function of heme and non-heme iron acquisition mechanisms*. J Biol Chem, 2022. **298**(5): p. 101823.
41. Wyatt, M.A., et al., *Staphylococcus aureus nonribosomal peptide secondary metabolites regulate virulence*. Science, 2010. **329**(5989): p. 294-6.
42. Heilbronner, S., et al., *Genome sequence of Staphylococcus lugdunensis N920143 allows identification of putative colonization and virulence factors*. FEMS Microbiol Lett, 2011. **322**(1): p. 60-7.

43. Torres Salazar, B.O., et al., *Commensal production of a broad-spectrum and short-lived antimicrobial peptide polyene eliminates nasal Staphylococcus aureus*. Nat Microbiol, 2024. **9**(1): p. 200-213.
44. Blin, K., et al., *antiSMASH 6.0: improving cluster detection and comparison capabilities*. Nucleic Acids Res, 2021. **49**(W1): p. W29-W35.
45. Blin, K., et al., *antiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation*. Nucleic Acids Res, 2023. **51**(W1): p. W46-W50.
46. Gilchrist, C.L.M. and Y.H. Chooi, *clinker & clustermap.js: automatic generation of gene cluster comparison figures*. Bioinformatics, 2021. **37**(16): p. 2473-2475.
47. Heather, Z., et al., *A novel streptococcal integrative conjugative element involved in iron acquisition*. Mol Microbiol, 2008. **70**(5): p. 1274-92.
48. Igarashi, Y., et al., *Ulbactins F and G, Polycyclic Thiazoline Derivatives with Tumor Cell Migration Inhibitory Activity from Brevibacillus sp.* Org Lett, 2016. **18**(7): p. 1658-61.
49. Shapiro, J.A., et al., *Biologically Inspired Total Synthesis of Ulbactin F, an Iron-Binding Natural Product*. Org Lett, 2018. **20**(18): p. 5922-5926.
50. Youard, Z.A., N. Wenner, and C. Reimann, *Iron acquisition with the natural siderophore enantiomers pyochelin and enantio-pyochelin in Pseudomonas species*. Biometals, 2011. **24**(3): p. 513-22.
51. Perry, R.D., et al., *Yersiniabactin from Yersinia pestis: biochemical characterization of the siderophore and its role in iron transport and regulation*. Microbiology (Reading), 1999. **145** (Pt 5): p. 1181-1190.
52. Kaplan, A.R., D.G. Musaev, and W.M. Wuest, *Pyochelin Biosynthetic Metabolites Bind Iron and Promote Growth in Pseudomonads Demonstrating Siderophore-like Activity*. ACS Infect Dis, 2021. **7**(3): p. 544-551.
53. Perry, R.D. and J.D. Fetherston, *Yersiniabactin iron uptake: mechanisms and role in Yersinia pestis pathogenesis*. Microbes Infect, 2011. **13**(10): p. 808-17.
54. Koh, E.I., et al., *Metal selectivity by the virulence-associated yersiniabactin metallophore system*. Metallomics, 2015. **7**(6): p. 1011-22.
55. Gehring, A.M., et al., *Iron acquisition in plague: modular logic in enzymatic biogenesis of yersiniabactin by Yersinia pestis*. Chem Biol, 1998. **5**(10): p. 573-86.
56. Komaki, H., et al., *Draft genome sequence of a sponge-derived Brevibacillus sp. TP-B0800, a producer of ulbactins with tumor cell migration inhibitory activity*. Gene Reports, 2016. **5**: p. 140-143.
57. Harris, S.R., et al., *Genome specialization and decay of the strangles pathogen, Streptococcus equi, is driven by persistent infection*. Genome Res, 2015. **25**(9): p. 1360-71.
58. Ochsner, U.A., et al., *GeneChip expression analysis of the iron starvation response in Pseudomonas aeruginosa: identification of novel pyoverdine biosynthesis genes*. Mol Microbiol, 2002. **45**(5): p. 1277-87.
59. Jackson, L.A., et al., *Iron-regulated small RNA expression as Neisseria gonorrhoeae FA 1090 transitions into stationary phase growth*. BMC Genomics, 2017. **18**(1): p. 317.
60. Sevilla, E., et al., *Fur-like proteins: Beyond the ferric uptake regulator (Fur) paralog*. Arch Biochem Biophys, 2021. **701**: p. 108770.
61. Santos, R., B.B. Batista, and J.F. da Silva Neto, *Ferric Uptake Regulator Fur Coordinates Siderophore Production and Defense against Iron Toxicity and Oxidative Stress and Contributes to Virulence in Chromobacterium violaceum*. Appl Environ Microbiol, 2020. **86**(21).

62. Katumba, G.L., H. Tran, and J.P. Henderson, *The Yersinia High-Pathogenicity Island Encodes a Siderophore-Dependent Copper Response System in Uropathogenic Escherichia coli*. mBio, 2022. **13**(1): p. e0239121.
63. Pasqua, M., et al., *Ferric Uptake Regulator Fur Is Conditionally Essential in Pseudomonas aeruginosa*. J Bacteriol, 2017. **199**(22).
64. Dale, S.E., et al., *Role of siderophore biosynthesis in virulence of Staphylococcus aureus: identification and characterization of genes involved in production of a siderophore*. Infect Immun, 2004. **72**(1): p. 29-37.
65. Wang, S., et al., *Strategies for Zinc Uptake in Pseudomonas aeruginosa at the Host-Pathogen Interface*. Front Microbiol, 2021. **12**: p. 741873.
66. Napolitano, M., et al., *Characterization of the response to zinc deficiency in the cyanobacterium Anabaena sp. strain PCC 7120*. J Bacteriol, 2012. **194**(10): p. 2426-36.
67. de Jonge, E.F. and J. Tommassen, *Conditional growth defect of Bordetella pertussis and Bordetella bronchiseptica ferric uptake regulator (fur) mutants*. FEMS Microbiol Lett, 2022. **369**(1).
68. Brozyna, J.R., J.R. Sheldon, and D.E. Heinrichs, *Growth promotion of the opportunistic human pathogen, Staphylococcus lugdunensis, by heme, hemoglobin, and coculture with Staphylococcus aureus*. Microbiologyopen, 2014. **3**(2): p. 182-95.
69. Himpsl, S.D. and H.L.T. Mobley, *Siderophore Detection Using Chrome Azurol S and Cross-Feeding Assays*. Methods Mol Biol, 2019. **2021**: p. 97-108.
70. Ryan, D.K. and J.H. Weber, *Fluorescence quenching titration for determination of complexing capacities and stability constants of fulvic acid*. Analytical Chemistry, 1982. **54**(6): p. 986-990.
71. Price, S.L., et al., *Yersiniabactin contributes to overcoming zinc restriction during Yersinia pestis infection of mammalian and insect hosts*. Proc Natl Acad Sci U S A, 2021. **118**(44).
72. Grim, K.P., et al., *The Metallophore Staphylopin Enables Staphylococcus aureus To Compete with the Host for Zinc and Overcome Nutritional Immunity*. mBio, 2017. **8**(5).
73. Ghsssein, G., et al., *Biosynthesis of a broad-spectrum nicotianamine-like metallophore in Staphylococcus aureus*. Science, 2016. **352**(6289): p. 1105-9.
74. Kenney, G.E. and A.C. Rosenzweig, *Methanobactins: Maintaining copper homeostasis in methanotrophs and beyond*. J Biol Chem, 2018. **293**(13): p. 4606-4615.
75. Brandel, J., et al., *Pyochelin, a siderophore of Pseudomonas aeruginosa: physicochemical characterization of the iron(III), copper(II) and zinc(II) complexes*. Dalton Trans, 2012. **41**(9): p. 2820-34.
76. Braud, A., et al., *Presence of the siderophores pyoverdine and pyochelin in the extracellular medium reduces toxic metal accumulation in Pseudomonas aeruginosa and increases bacterial metal tolerance*. Environ Microbiol Rep, 2010. **2**(3): p. 419-25.
77. Radford, D.S., et al., *CopZ from Bacillus subtilis interacts in vivo with a copper exporting CPx-type ATPase CopA*. FEMS Microbiol Lett, 2003. **220**(1): p. 105-12.
78. Finney, L.A. and T.V. O'Halloran, *Transition metal speciation in the cell: insights from the chemistry of metal ion receptors*. Science, 2003. **300**(5621): p. 931-6.
79. Changela, A., et al., *Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR*. Science, 2003. **301**(5638): p. 1383-7.
80. Kaur, I., et al., *Role of horizontally transferred copper resistance genes in Staphylococcus aureus and Listeria monocytogenes*. Microbiology (Reading), 2022. **168**(4).

81. Rosario-Cruz, Z., et al., *The copBL operon protects Staphylococcus aureus from copper toxicity: CopL is an extracellular membrane-associated copper-binding protein.* J Biol Chem, 2019. **294**(11): p. 4027-4044.
82. Heilbronner, S., et al., *Sortase A promotes virulence in experimental Staphylococcus lugdunensis endocarditis.* Microbiology (Reading), 2013. **159**(Pt 10): p. 2141-2152.

Supplementary Data

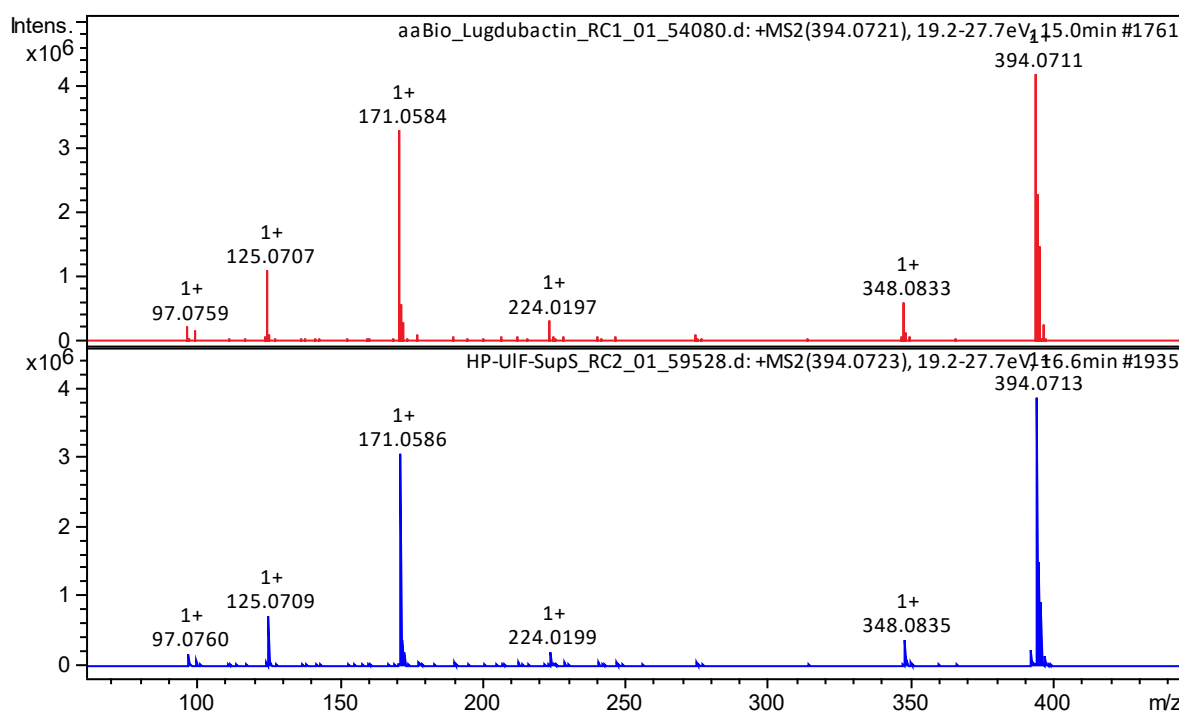


Figure S1 MS/MS fragmentation of purified ulbactin F from *S. lugdunensis* N920143 (red) and chemically synthesized ulbactin F (blue). Masses and charges are depicted.

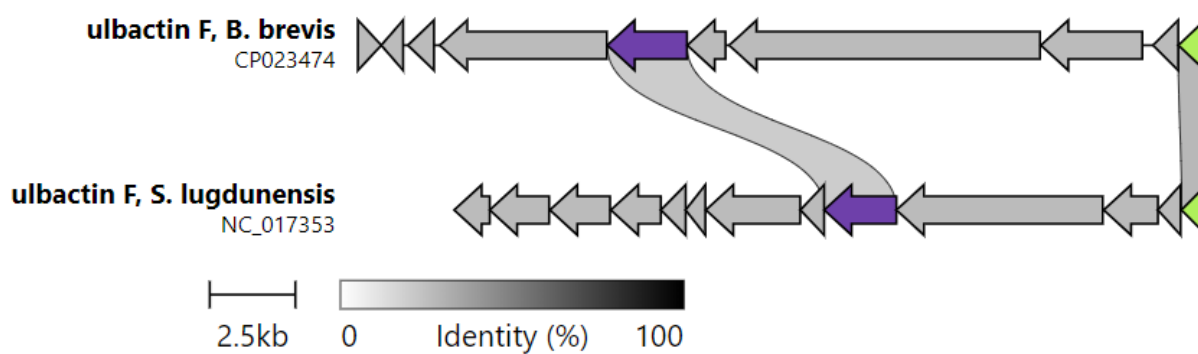


Figure S2 Ulbactin F gene cluster comparison of *B. brevis* X23 and *S. lugdunensis* N920143 using clinker from the comparative gene cluster analysis toolbox (CAGECAT) [46].

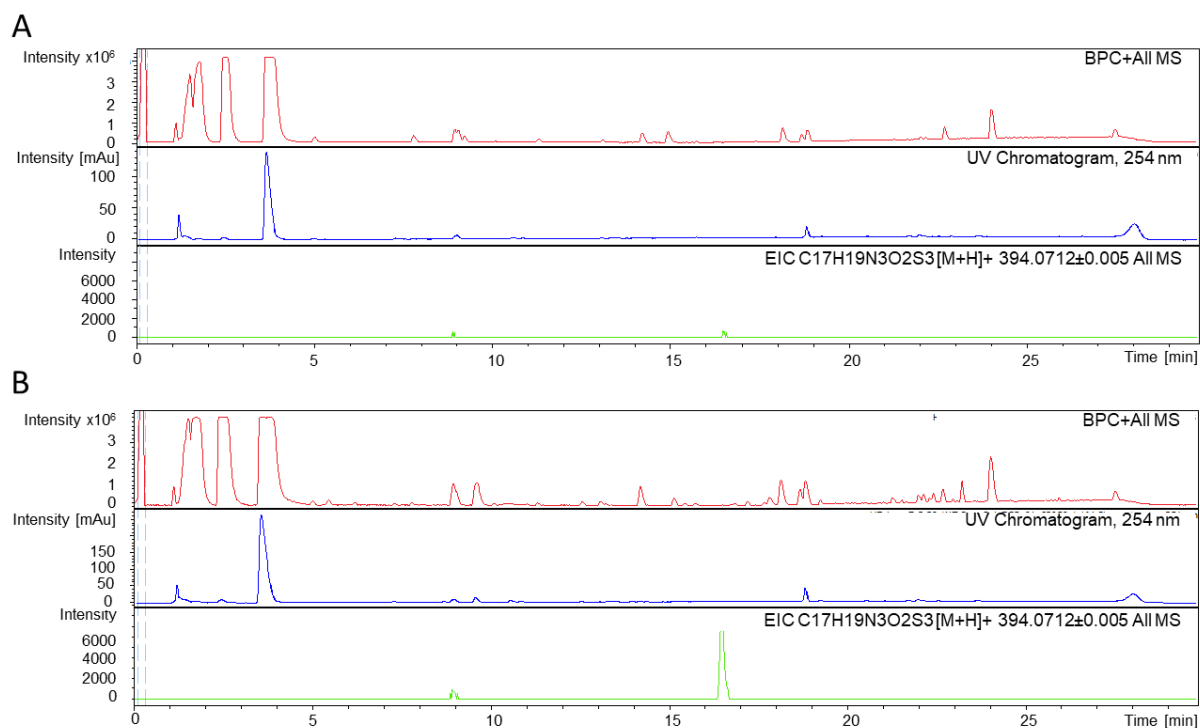


Figure S3 HPLC chromatogram of *S. lugdunensis* strains grown in CDM. (A) *S. lugdunensis* N920143 $\Delta lgbCD$ mutant and (B) *S. lugdunensis* N920143 WT. Base peak chromatogram (BPC, red), UV Chromatogram at 254 nm (blue) and extracted ion chromatogram (EIC, green). Representative chromatograms are shown.

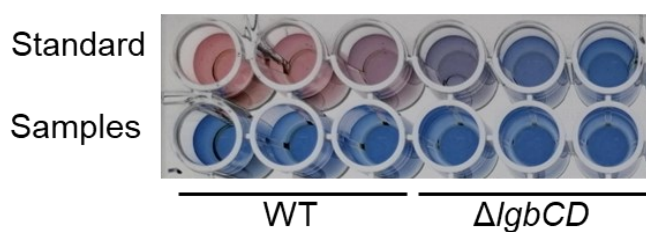


Figure S4 Siderophore detection using chrome azurol S (CAS) detection. Standard for calorimetric change is shown in the upper lane, triplicates of samples from the *S. lugdunensis* N920143 WT and *S. lugdunensis* N920143 $\Delta lgbCD$ mutants are shown in the lower lane.

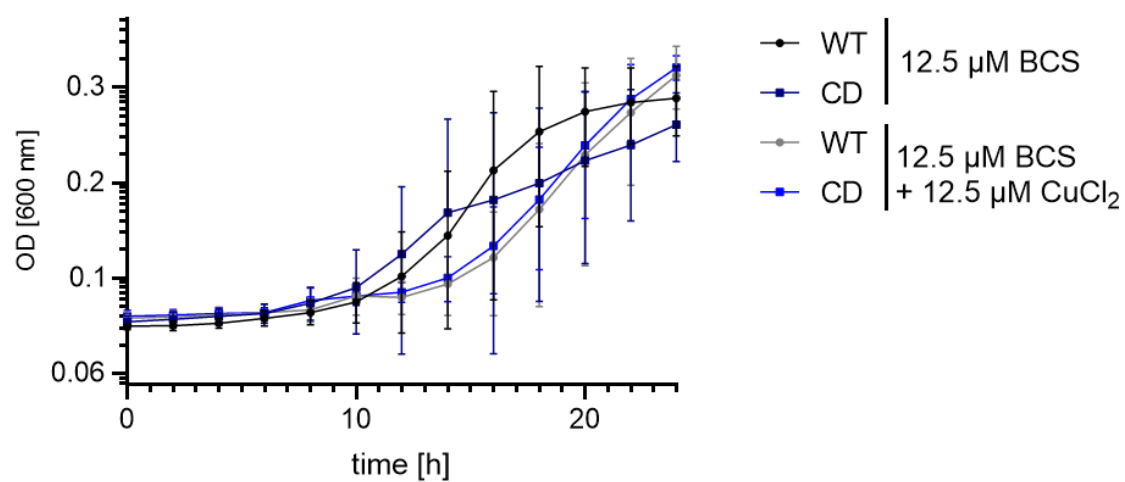


Figure S5 Growth in copper-limited media. *S. lugdunensis* N920143 WT and the *S. lugdunensis* N920143 Δ *lgbCD* mutant were grown in RPMI minimal media supplemented with 50 μ M cysteine and 72 μ M salicylic acid. The RPMI media was either treated with bathocuproinedisulfonic acid (BCS) or BCS and CuCl_2 as indicated. The experiment was performed in triplicates.

Chapter 4

Horizontal transfer of bacteriocin biosynthesis genes requires metabolic adaptation to improve compound production and cellular fitness

Sophia Krauss^{a,b,c}, Theresa A. Harbig^d, Johanna Rapp^{b,e}, Timm Schaeffle^{b,f}, Mirita Franz-Wachtel^g, Leonie Reetz^{a,b,c}, Ahmed M. A. Elsherbini^{a,b,c}, Boris Macek^g, Stephanie Grond^{b,f}, Hannes Link^{b,e}, Kay Nieselt^d, Bernhard Krismer^{a,b,c}, Andreas Peschel^{a,b,c}, Simon Heilbronner^{b,c,h} *

^a Department of Infection Biology, Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, 72076 Tübingen, Germany.

^b Cluster of Excellence EXC 2124 Controlling Microbes to Fight Infections, 72076 Tübingen, Germany.

^c German Center for Infection Research (DZIF), partner site Tübingen.

^d Interfaculty Institute for Bioinformatics and Medical Informatics (IBMI), University of Tübingen, 72076 Tübingen, Germany.

^e Department of Bacterial Metabolomics, Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, 72076 Tübingen, Germany.

^f Institute of Organic Chemistry, University of Tübingen, 72076 Tübingen, Germany.

^g Proteome Center Tübingen, University of Tübingen, 72076 Tübingen, Germany.

^h Interfaculty Institute of Microbiology and Infection Medicine, Institute for Medical Microbiology and Hygiene, UKT Tübingen, 72076 Tübingen, Germany.

* Corresponding author: simon.heilbronner@uni-tuebingen.de

Running Title: Metabolic adaptation to micrococcin P1 production

Microbiol Spectr. 2023 Jan-Feb; 11(1): e03176-22

Abstract

Biosynthetic gene clusters (BGCs) encoding the production of bacteriocins are widespread amongst bacterial isolates and are important genetic determinants of competitive fitness within a given habitat. Staphylococci produce a tremendous diversity of compounds and the corresponding BGCs are frequently associated with mobile genetic elements, suggesting gain and loss of biosynthetic capacity. Pharmaceutical biology has shown that compound production in heterologous hosts is often challenging and many BGC recipients produce initially low compound amounts or show reduced growth rates. To assess whether transfer of BGCs between closely related *S. aureus* strains can be instantly effective or requires elaborate metabolic adaptation, we investigated the intra species transfer of a BGC encoding the ribosomally synthesized and post-translationally modified peptide (RiPP) micrococcin P1 (MP1). We found that acquisition of the BGC by *S. aureus* RN4220 enabled immediate MP1 production but also imposed a metabolic burden, which was relieved after prolonged cultivation by adaptive mutation. We used a multiomics approach to study this phenomenon and found adaptive evolution to select for strains with increased activity of the tricarboxylic acid cycle (TCA), which enhanced metabolic fitness and levels of compound production. Metabolome analysis revealed increases of central metabolites including citrate and α -ketoglutarate in the adapted strain, suggesting metabolic adaptation to overcome the BGC-associated growth defects. Our results indicate that BGC acquisition requires genetic and metabolic predispositions allowing the integration of bacteriocin production into the cellular metabolism. Inappropriate metabolic characteristics of recipients can entail physiological burdens, negatively impacting the competitive fitness of recipients within natural bacterial communities.

Importance

Human microbiomes are critically associated with human health and disease. Importantly, pathogenic bacteria can hide in human associated communities and can cause disease when the composition of the community becomes unbalanced. Bacteriocin producing commensals are able to displace pathogens from microbial communities, suggesting that their targeted introduction in human microbiomes might prevent pathogen colonisation and infection. However, in view of future probiotic approaches, strains are needed that produce high levels of bioactive compounds and retain cellular fitness within mixed bacterial communities. Our work offers insights into the metabolic burdens associated with the production of the bacteriocin micrococcin P1 and highlights evolutionary strategies that increase cellular fitness in the context of production. Most likely metabolic adaptations are

broadly relevant for bacteriocin producers and need to be considered for the future development of effective microbiome editing strategies.

Introduction

It is increasingly recognized that biosynthetic gene clusters (BGCs) allowing the production of antibacterial compounds are omnipresent in bacterial communities (1). These antibacterial compounds are frequently referred to as bacteriocins. They can either be produced ribosomally or by non-ribosomal enzymatic systems and are hugely diverse in terms of molecular size and structure (2). In line with their structural diversity bacteriocins have diverse molecular targets and killing mechanisms and therefore exhibit toxicity towards diverse spectra of bacterial species (2, 3). Bacteriocin-producing bacterial lineages have recently gained increasing attention for their potential to displace pathogens from various human body sites thereby preventing infection (4-6).

Gram-positive staphylococci are one example in this regard. The genus comprises human commensals that are opportunistic pathogens, such as *Staphylococcus epidermidis*, *Staphylococcus capitis*, *Staphylococcus lugdunensis* and *Staphylococcus haemolyticus*, but also the frequently invasive pathogen *Staphylococcus aureus*. Many staphylococcal isolates show inhibitory activity against a diverse range of human nasal commensals and pathogens (7-9). Interestingly, most staphylococcal BGCs appear to be associated with mobile genetic elements such as plasmids, transposons, IS-elements, lysogenic phages or chromosomal islands with G+C contents diverging from the genome average (1, 4, 7, 10). This suggests that the BGCs are transferred between strains and lineages and create strain rather than species specific antimicrobial properties (1, 11, 12).

Transfer of antibiotic BGC between strains or species represents a natural system for heterologous expression of antibacterial compounds. Acquisition of a BGC provides a direct benefit to the recipient as it allows inhibition of competitors. However, acquisition might also be metabolically challenging for the novel host. Indeed, transfer of BGCs between classical antibiotic-producing bacterial species (e.g., streptomycetes) often results in the production of limited amounts of compound in heterologous hosts, and adaptive mutations or changes in nutritional supplies are needed to optimise compound production (13-16). The same might be true in the context of naturally occurring transfer of BGCs between staphylococcal strains and species. Acquisition of bacteriocin BGCs places a burden on the recipient cell. The novel genetic material needs to be propagated and, if functionally expressed, it entails production and secretion of high amounts of toxic secondary metabolites. Precursor molecules need to be channelled from primary metabolism and cellular energy levels might consequently be reduced, entailing metabolic costs for the producer (17). Finally, suboptimal producer-immunity against the compound can further entail physiological burdens (18). Accordingly, it seems

plausible that BGC acquisition might represent a mixed blessing for bacterial cells. On the one hand, compound production will provide a competitive advantage when susceptible competitors are present. On the other hand, BGC-associated burdens might reduce fitness of the producer and might require adaptive evolution to optimize compound production and fitness. The physiological costs and the mechanisms of integration of bacteriocin synthesis into primary metabolism in staphylococci remain unclear. However, knowledge about this phenomenon is key to understand why BGCs are largely strain specific and not species-wide conserved traits. Future approaches for the use of bacteriocin-producing bacterial strains to displace pathogens from human microbiomes will crucially depend on the availability of “healthy” strains stably producing high levels of antibiotic molecules.

In this work we used multiomic approaches to study the transfer of the naturally occurring plasmid pD4-19 between closely related *S. aureus* strains. This plasmid encodes the BGC for the biosynthesis of the thiopeptide bacteriocin micrococcin P1 (MP1). Transfer of pD4-19 to *S. aureus* RN4220 allowed immediate production of MP1 but caused growth defects. Genome analysis showed that long-term *in vitro* evolution experiments altered the sequence of the citrate synthase-encoding gene to increase translation of this core metabolic enzyme. Metabolome analysis revealed significant changes in the levels of central metabolic molecules including citrate, α -ketoglutarate and several amino acids. Transcriptome analysis showed that adaptive evolution also increases the expression of ribosomal proteins as well as of enzymes involved in cofactor biosynthesis and protein turnover, all suggesting increased cellular fitness in the context of augmented MP1 production. Phenotypically, the adaptation enhanced compound production and allowed overcoming BGC-associated growth defects. Our data indicate that strain-specific genetic and metabolic predispositions will determine the levels of bacteriocin production and fitness of BGC-recipients. Hence, this will most likely determine the success of a BGC recipient in the context of competitive environments.

Results

MP1 BGC as a model system

We sought to investigate the effects of BGC-acquisition on the cellular fitness of the recipient cell. Screening of our extensive collection of nearly 1500 nasal isolates of diverse bacterial species resulted in the identification of two *S. aureus* strains showing intra-species inhibition against the test strain *S. aureus* USA300 LAC. Whole genome sequencing (WGS) and antiSMASH (bacterial version 5.0) analysis revealed the presence of plasmids encoding BGCs for aureocin A70 (19) in strain *S. aureus* P1-22 and for the thiopeptide MP1 (20) in strain *S. aureus* D4-19. The plasmid pD4-19 has a size of 28 391 bp (Fig. 1A) and, besides the 11 kb MP1-encoding BGC (Fig. 1B), encodes a β -lactamase, which we considered useful for *in vitro* plasmid transfer experiments. Therefore, we focused on pD4-19 as a model BGC.

The thiopeptide MP1 is a RiPP-type (ribosomally synthesized and post-translationally modified peptide) bacteriocin targeting the bacterial ribosome (21), and an alternative ribosomal subunit gene (*tc/Q*) provides resistance to the producer (21-23). The MP1 BGC on pD4-19 showed an overall similarity of 99% to the gene cluster identified by Liu et al. in an *S. hominis* isolate (20), suggesting the production of the same compound. To validate this, we performed whole-cell methanol extraction and analysed the extract by high performance liquid chromatography high resolution electrospray (+)-ionisation mass spectrometry (HPLC HR ESI(+)-MS). This analysis revealed the presence of the mass of MP1 (1144.4 Da with the assigned sum formula of MP1: C₄₈H₄₉N₁₃O₉S₆) (Fig. 1C), confirming production of MP1 by *S. aureus* D4-19. To prove that the identified BGC is responsible for the observed antimicrobial activity, we performed random transposon mutagenesis. Strains without antibiotic activity were found to carry the transposon within the MP1 BGC (Fig. S1) confirming that MP1 alone was responsible for the antibiotic activity of *S. aureus* D4-19.

Acquisition of the MP1 BGC enables MP1 production but imposes a metabolic burden.

We sought to investigate whether pD4-19 can be transferred to a related *S. aureus* strain and whether this entails significant physiological changes. The plasmid isolated from *S. aureus* D4-19 was used to transform *S. aureus* RN4220 by electroporation. A recovered transformant (RN-T) showed antimicrobial activity against *S. aureus* USA300 LAC suggesting the production of MP1 (Fig. 2A). However, the zone of inhibition was smaller than that of the original producer *S. aureus* D4-19. HPLC-MS and NMR-analysis of cell extracts showed that RN-T produced MP1 as well as a putative derivative with a mass of 1163.2116 Da (Fig. 2B, S2, S3, S4). However, the combined amount of MP1 and the putative derivative was threefold lower than the amount of MP1 produced by *S. aureus* D4-19 (Fig. 2C).

Growth curve analysis showed that *S. aureus* RN-T reached lower optical densities than the parental strain *S. aureus* RN4220 (RN) (Fig. 2D) suggesting that MP1 production may limit the proliferation of the strain. To verify that the reduced growth was caused by MP1 production and not by other plasmid-associated effects, a mutant lacking the entire MP1 operon (*tc/ESQIJKLMNPU* + *orf18*) was constructed by allelic replacement. The resulting strain *S. aureus* pD4-19 ΔMP1 lacked antimicrobial activity against *S. aureus* USA300 LAC (Fig. S5B) and reached a similar OD₆₀₀ as *S. aureus* RN4220 (Fig. S5A). This supports the idea that acquisition of the MP1 BGC and the associated MP1 production impose a physiological burden on *S. aureus* RN4220. It has been reported that insufficient immunity to an antimicrobial product can limit production of the compound and growth of the producer (18). However, *S. aureus* D4-19 and RN-T mutant strains lacking the MP1 structural gene *tcIE* and are therefore deficient in MP1 production were highly resistant to purified MP1 (MIC > 100 µg/ml). This finding indicates that the immunity to MP1 conferred by the alternative ribosomal

subunit TcdQ is strong enough in both strain backgrounds to allow effective growth even during high-level MP1 production (Fig. S5C).

Adaptive evolution increases MP1 production and relieves the metabolic burden

We speculated that MP1 biosynthesis might perturb the primary metabolism of *S. aureus* RN4220, thereby limiting bacterial growth and propagation. To analyse whether RN4220 can adapt its metabolism to MP1 production, RN-T was passaged daily in BM medium over 28 consecutive cultures. Colonies arising on solid medium at the end of the experiment were found to be larger than those formed by the original transformant suggesting improved growth. This adapted strain was named RN-A (*S. aureus* RN4220 pD4-19 adapted). Growth curve analysis confirmed that the new strain reached OD₆₀₀ values similar to those of the *S. aureus* RN (Fig. 2D). Additionally, *S. aureus* RN-A produced comparable levels of MP1 as the native MP1 producer *S. aureus* D4-19 and 2.86-fold more than the non-adapted, parental strain RN-T (Fig. 2C).

To identify mutations explaining the phenotypic differences between RN-T and RN-A, the strains were subject to whole genome sequencing and single nucleotide polymorphisms (SNPs) were extracted. We identified a SNP in RN-A, which was located upstream of the gene *citZ* (RN4220 ACCFDFCE_01589) encoding citrate synthase. The mutation created a functional in-frame start codon (ATG instead of ATA) extending the annotated open reading frame of *citZ* by 75 nucleotides (Fig. 3A). An “AG”-rich motif resembling a Shine-Dalgarno sequence is present six base pairs upstream of the new start codon creating a canonical translational start site. In contrast, the annotated shorter allele of *S. aureus* RN4220 lacks an obvious Shine-Dalgarno sequence and relies on a non-canonical “TTG” start codon, which is rarely used in *S. aureus* (24). Accordingly, we speculated that the *citZ* gene of *S. aureus* RN4220 is truncated and potentially non-functional. When the *citZ* allele of *S. aureus* RN4220 was compared with those of other *S. aureus* strains, the shorter *citZ* allele was found in the entire clonal lineage of *S. aureus* RN4220 including the ancestral strains NRS146, NRS133 and VC40 derived from *S. aureus* NCTC8325, which was originally described in 1965 (25). All other 3 834 *S. aureus* genome sequences of the NCBI database, including the original host of the plasmid *S. aureus* D4-19 carry the same full-length *citZ* allele as found in the adapted strain RN-A.

Accordingly, we hypothesised that RN4220 possesses a malfunctioning *citZ* allele whose functionality is restored by the adaptive mutation. To test this, the full-length *citZ* gene of the adapted strain plus its Shine-Dalgarno sequence as well as the corresponding sequence of RN4220 were cloned with a C-terminal His-tag in the *S. aureus* vector pRB473-XylIR enabling xylose-inducible expression in RN4220. Upon induction, protein levels were assessed by infra-red Western Blotting. Interestingly, we found that expression of both alleles resulted in the

production of apparently full length CitZ proteins (43 kDa) (Fig. 3B). This suggested that the malfunctioning allele is translated using the ATA codon as a non-canonical start codon to produce full length CitZ, a phenomenon that has been described in *E. coli* (26). However, the ATG mutation of the same codon in the adapted allele increased CitZ levels dramatically (Fig. 3B), supporting the idea that the adaptive mutation increases the cellular levels of CitZ by creating an appropriate translational start. This hypothesis was further confirmed by subsequent proteome analysis. An eleven amino acid motif (GLEGVIAAETK) that can only result from the fragmentation of full length CitZ protein was found in both strains. However, this fragment was 20-fold more abundant in RN-A than in RN-T (Fig. 3C). This confirmed that both strains translate the CitZ mRNA using the ATA/ATG codon affected by the point mutation. However, the efficiency of translation is strongly increased by the adaptive mutation entailing increased levels of CitZ.

Increased citrate levels allow overcoming the MP1-associated burden

To investigate the effects of the mutation on the cellular metabolism, we measured the intracellular concentration of citrate using a colorimetric assay. We found increased levels in RN-A compared to RN-T (Fig. 4A), reflecting the increased amounts of CitZ upon adaptation. Notably, addition of 5 mM sodium citrate to the culture medium allowed the original transformant RN-T to overcome the bacteriocin-associated growth defects (Fig. 4B), indicating that intracellular citrate levels represent the limiting factor for growth of MP1-producing *S. aureus* RN4220.

The adaptive mutation increases the metabolic activity of the MP1 producer

To gain insights into the overall levels of primary metabolites, we performed Flow Injection Mass Spectrum (FI-MS)-based untargeted metabolomics of cell lysates of *S. aureus* RN, the initial transformant RN-T and the adapted strain RN-A. Hierarchical clustering showed that the metabolic profiles of RN and of RN-A were more similar to each other than to those of RN-T (Fig. 5). This finding indicated that the acquisition of the BGC-encoding plasmid impacts the general metabolism of the recipient, and these effects are largely corrected by the adaptive mutation of *citZ*.

RiPP-type bacteriocins rely on the availability of appropriate amino acids as well on availability of ATP. Precursors for the biosynthesis of amino acids are derived from intermediates of glycolysis, pentose phosphate pathway and tricarboxylic acid (TCA) cycle. Therefore, we used a combination of LC-MS/MS and FI-MS analysis to investigate the levels of the central metabolites with the highest possible accuracy (Fig. 6, S6, S7, S8, S9). Relative to the WT, the levels of glycolysis intermediates (3-phosphoglycerate, phosphoenolpyruvate, pyruvate, acetyl-CoA) increased upon plasmid acquisition and decreased to or even below RN

levels upon adaptive mutation (Fig. 6, S7B). This suggests that the TCA cycle is insufficiently fed upon plasmid acquisition and that the adaptive mutation restores efficient feeding. In line with this, we found increased levels of citrate (citrate and its two isobars isocitrate, 5-dehydro-4-deoxy-D-glucarate cannot be discriminated in this analysis) as well as of α -ketoglutarate upon adaptive mutation (Fig. 6, S7A), supporting the stimulative effect.

Interestingly, we found that pD4-19 acquisition led to the accumulation of various amino acids (aspartate and asparagine, alanine, tyrosine and tryptophan, threonine, glycine, and histidine). Amino acids did not accumulate in the CitZ adapted strain. In contrast, levels of most amino acids were reduced compared to RN, pointing to a reduced rate of protein biosynthesis (27). Amino acids did not accumulate in the CitZ adapted strain. In contrast, levels of most amino acids were reduced compared to RN, suggesting their efficient usage in protein biosynthesis (Fig. 6, S6). An exception were glutamate, glutamine and proline, which increased in both RN-T and RN-A relative to RN. Glutamate and glutamine serve as precursors for several amino acids (including proline) and bacteria form glutamate or glutamine by condensation of α -ketoglutaric acid or glutamate with ammonium as a means to acquire environmental nitrogen for anabolic processes. Accordingly, their levels are regarded as an indicator of nitrogen availability (28) and they connect the urea cycle to amino acid biosynthesis. Accumulation of urea cycle intermediates (ornithine, citrulline, argininosuccinate) was only observed in RN-T but not upon adaptive mutation, suggesting that the intermediates are efficiently used to feed the TCA cycle and with it, amino acid biosynthesis in the adapted strain (Fig. 6, S8).

Besides the provision of precursors for metabolic processes, the activity of the TCA cycle is crucial for the aerobic generation of ATP. In the presence of glucose, *S. aureus* produces ATP by substrate-level phosphorylation even in the presence of oxygen and produces predominantly acetate, which is secreted. Only after glucose is depleted, acetate is consumed and decarboxylated using the TCA cycle and the respiratory chain (29, 30). This metabolic switch has been reported to occur after approximately five hours of growth in glucose-containing complex media (30), which matches the observed starting point of growth deficiency in our experiments. We noted that the intracellular levels of acetate were increased in RN-T compared to RN (Fig. 6, S9). We therefore speculated that CitZ and TCA cycle activity might not allow sufficient ATP generation when acetate needs to be catabolized. Interestingly, we detected accumulation of acetate in the culture supernatants of *S. aureus* RN4220 and of RN-T, while accumulation was strongly reduced in RN-A and dropped quickly after six hours of growth (Fig. 7A). This suggests an increased rate of acetate consumption and oxidative decarboxylation upon restoration of the *citZ* allele. However, ATP levels measured by metabolome analysis did not support energy depletion as the reason of the growth defect of RN-T (Fig. 7B). Compared to the WT strain, ATP-levels were only moderately decreased in

RN-T and the decrease intensified upon adaptive mutation. As RN-A does not show abnormal growth, these data suggest that even the lowest ATP levels observed in RN-A are sufficient to support maximal growth.

Transcriptomic signatures support increased overall metabolic activity upon adaptive mutation.

We performed RNA-seq analysis of RN-T and RN-A to investigate the effects of the adaptive mutation on the transcriptome. We extracted differentially regulated genes and assigned them to Gene Ontology (GO) groups to identify the general cellular functions altered in response to the adaptive mutation in *citZ*. The adaptive mutation entailed increased transcription of the translational machinery (30S and 50S ribosomal subunits), pointing to generally enhanced protein biosynthesis as a consequence of overall metabolic alterations (Fig. 8). This change was accompanied by upregulation of pathways for the biosynthesis of the cofactors folate (*folPBK*), thiamine (*thiEM*), and riboflavin (*ribAB*). Increased amino acid turnover was reflected by upregulation of the L-tryptophan biosynthesis pathway (*trpCDEFG*) as well as of catabolic pathways for threonine (*ilvA*) and alanine (*ald1*). Additionally, upregulation of the genes encoding the urea transporter Utp and of each gene of the urease operon (*ureABCEFGD*), catalysing the hydrolysis of urea into carbon dioxide and ammonia, was observed (Fig. 8, S10A). Furthermore, upon plasmid acquisition, several genes associated with iron homeostasis as well as the genes encoding the twin-arginine translocation (Tat) pathway were differently expressed (Fig. 8). The Tat system is responsible for the translocation of the iron-dependent peroxidase (FepB), which is also involved in iron uptake (31). Accordingly, one can speculate that the increased metabolism upon adaptive mutation does also influence iron homeostasis. Reasons for this are unclear, but TCA-derived citrate is the central molecule needed for production of the *S. aureus* siderophore *staphyloferrin A* (32). It seems likely that a secondary effect of the adaptive mutation is optimization of siderophore production resulting in increased cellular iron levels.

Of note, expression of plasmid associated genes did not differ significantly between RN-T and RN-A (Fig. S10B).

Inclusion of the transcriptomic profile of *S. aureus* RN into this analysis allowed us to extract several additional effects of plasmid acquisition on the transcriptome. We found transcription of the carnitine transporter *opuC* (*opuCA/CB/CC/CD*) to be downregulated in *S. aureus* RN-T compared to RN. This is in line with reduced carnitine levels detected via metabolome analysis (Fig. S9) (33). Our transcriptome analysis identified several genes that were differentially expressed upon pD4-19 acquisition and whose expression remained altered upon adaptive evolution, arguing for intrinsic effects of the plasmid. Among those genes several were associated with virulence and immune interference including hemolysins (*hlgB/C*, *hly*), the capsule biosynthesis operon *cap*, the serine protease locus *spl*, and the type seven

secretion system *ess* (Fig. S10A). Also, transcription of the *icaADBC* operon was increased upon plasmid acquisition and all genes except for *icaD* remained highly expressed upon adaptive mutation. The *ica* operon allows the synthesis of the polysaccharide intercellular adhesin (PIA) and is thereby responsible for biofilm formation (Fig. S10A). In line with this, RN-T showed a significant increase in biofilm formation (Fig. 9). Interestingly, this phenotype was reverted upon adaptive evolution.

Discussion

Using our collection of nasal staphylococcal isolates, we identified the *S. aureus* strain D4-19 carrying a plasmid encoding the MP1 BGC. A number of studies have identified MP1-BGCs in a variety of staphylococcal species, including *S. aureus* (34), *S. epidermidis* (35), *Staphylococcus equorum* (36), *Staphylococcus pseudintermedius* (37), *Staphylococcus agnetis*, and *S. hominis* (20). Also, an isolate from *Mammaliicoccus sciuri* (former *Staphylococcus sciuri*) (38) has been shown to produce MP1. Moreover, the NCBI database includes various homologous sequences in *S. aureus* genomes as well as many coagulase-negative staphylococci. The abundance of this BGC in different strain backgrounds suggests intra- and inter-species horizontal transfer, which is supported by the fact that the so far identified MP1 gene clusters in staphylococci are all located on plasmids. The cellular consequences of BGC acquisition in staphylococci are profound and we studied the reasons for metabolic constraints along with mechanisms overcoming them using evolutionary adaptation and multiomics approaches.

It is generally recognised that bacteriocin production is associated with metabolic costs for the producing cell and that expression is therefore frequently regulated, often in complex ways for instance depending on producer cell densities or on the detection of bacterial competitors (39, 40). Compound production generates metabolic burdens as the responsible gene cluster needs to be propagated, precursors need to be channelled from the primary metabolism and sufficient self-immunity needs to be established. This concept might be of special relevance for RiPPs such as MP1 as their production relies on cellular tRNA pools and sudden expression upon acquisition of the BGC might therefore disturb central metabolic fluxes. It seems therefore likely that cellular fitness in the context of bacteriocin production requires adjustments of metabolic pathways. Our experiments showed that acquisition of the plasmid pD4-19 enabled production of bioactive MP1. However, the BGC-recipient produced significantly less compound than the original plasmid host (*S. aureus* D4-19) while simultaneously displaying a growth defect compared to the plasmid free parental strain RN4220. Increased availability of citrate, of exogenous or endogenous origin, improved compound production and abrogated any growth defects. Interestingly, we found the recipient

S. aureus RN4220 to carry a malfunctional *citZ* allele, limiting the levels of citrate synthase activity, of intracellular citrate and most likely, of the entire TCA cycle. Importantly, the entire lineage of *S. aureus* NCTC8325 (parental lineage of *S. aureus* RN4220) developed and maintained the malfunctioning *citZ* allele and it has been observed previously that NCTC8325 enters stationary phase earlier than other *S. aureus* strains (41). Accordingly it would be interesting to investigate if also NCTC8325 (or RN4220 WT) would benefit from an increased translation of CitZ. However, the acquisition of pD4-19 made metabolic shortcomings of RN4220 apparent. The most prominent phenotype of TCA cycle-deficient mutants is a growth defect that manifests after approximately five hours of growth in glucose-containing medium (30). This is due to the fact that *S. aureus* preferentially degrades glucose via glycolysis or the pentose phosphate pathway and fermentation to produce acetate (29, 30). Concurrently, catabolite repression of glucose inhibits the TCA cycle (42). Only after glucose is consumed, acetate catabolism demands TCA cycle activity to create ATP and to sustain growth (30). *S. aureus* RN4220 does not show signs of TCA cycle deficiency. However, we observed that acquisition of pD4-19 and the associated MP1 production entailed premature growth arrest as well as increased levels of intracellular pyruvate and acetyl-CoA as well as extracellular accumulation of acetate, all of which are hallmarks of TCA cycle-deficient strains (30). Adaptive mutation reverted these signs of TCA deficiency. Acetyl-CoA levels dropped, and the levels of citrate and α -ketoglutarate increased suggesting efficient feeding of the TCA cycle. Simultaneously, accumulation of extracellular acetate was limited. Interestingly, we did not find that improvement of TCA cycle activity upon adaptive mutation increases the cellular ATP pools. In contrast, we found ATP levels in the adapted strain to be lower than in any other strain, despite the fact that the adapted strain showed a prolonged growth phase and produced more MP1 than the other strains. Accordingly, it is unlikely that increased needs for ATP upon plasmid acquisition were causative for the observed growth deficiency of the original recipient strain RN-T.

It is tempting to speculate that MP1 production entailed excessive channelling of TCA cycle intermediates into the biosynthesis of amino acids, ultimately leading to premature growth arrest of the strain. Along this line, BGC-acquisition had a wide impact on the metabolome of *S. aureus* and adaptive mutation shifted the general pattern a second time to reflect largely that of the plasmid free parental strain. This realization suggested that metabolic fluxes needed to be normalized by improvement of *citZ* expression and the associated increased availability of citrate. Interestingly, deletion of bacteriocin biosynthesis genes in enterococci has been associated with improved growth characteristics and altered expression of ribosomal proteins, vitamins, and glycolysis enzymes (43). Similarly, we found that restoration of the *citZ* allele also entailed a strong increase in expression of the translational machinery, cofactor biosynthesis and of the urea cycle, all suggesting an increase in protein

biosynthesis and turnover, supporting the positive effect of the mutation on the cellular metabolism.

The impact of BGC acquisition on TCA cycle activity in *S. aureus* RN4220 is further underlined by the fact that the initial transformant produced high levels of biofilm, which was abrogated upon adaptive mutation in *citZ*. Several studies have associated TCA cycle activity with the staphylococcal capacity to form biofilms. A major factor determining staphylococcal biofilm formation is the production of the PIA (44, 45). We found the responsible *ica* genes to be strongly upregulated upon pD4-19 acquisition. Vuong et al. described that reduced TCA cycle activity increased PIA production in *S. epidermidis* (46), and TCA-deficient mutants were shown to derepress the *ica* genes and to channel carbohydrates into PIA-synthesis (47). We found strongly increased expression of *ica* genes upon pD14-19 acquisition, supporting the general finding that MP1 production induces a TCA deficient phenotype in RN4220. Additionally, it was described that citrate as well as TCA cycle intermediates can stimulate expression of the fibronectin-binding proteins FnbA and FnbB entailing *ica*-independent biofilm formation (48). However, we did not observe differences in *fnbA/fnbB* expression levels in our experiments, suggesting that this mechanism is not relevant for biofilm formation in *S. aureus* RN-T.

Restoration of the *citZ* allele also enhanced MP1 production by the RN4220 lineage. It is well accepted, that expression of antibiotic BGCs in heterologous hosts can be limited due to inappropriate supply of cellular precursors. This is of special relevance for compounds produced by non-ribosomal peptide synthetases or by polyketide synthases which frequently rely on special precursors such as non-proteinogenic amino acids or unusual carbohydrates. For example, overexpression of rhamnose and forosamine biosynthetic pathways improved the biosynthesis of the polyketide antibiotic spinosad 1000-fold (49), and medium optimisation to provide appropriate precursors has proven to be an efficient strategy to enhance compound production (13). Similarly, optimisation of the microbial central metabolic processes including glucose, amino acid, or fatty acid metabolism can boost compound production in heterologous hosts (50). For RiPPs like MP1, the relevance of this concept is less clear, as the compounds are produced using the ribosomal machinery, which relies on the cellular pool of canonical aminoacyl-tRNAs. This might ensure that precursor molecules are generally available, and compound production is possible. However, it seems plausible that RiPP production can drain the pool of aminoacyl-tRNAs. This hypothesis is supported by the finding that addition of the amino acids glutamate, glycine, serine, and threonine as well as the addition of maltose enhanced the production of the RiPP gallidermin (51). However, our metabolomic analysis showed that, in the context of MP1 production, amino acid levels were not depleted in the BGC recipient at the timepoint of growth arrest arguing against amino acid limitation as a sole underlying reason for the decreased compound production.

Finally, it has to be considered that effects that are independent of cellular metabolites might impact the fitness of antibiotic producers. For instance, production of the lantibiotics epidermin and gallidermin imposes a physiological burden on the producing staphylococcal strains because of insufficient immunity of the producer strains leading to increased cell lysis (18). In contrast, we did not observe imperfect resistance of MP1 producing strains, suggesting that insufficient immunity does not substantially contribute to the observed growth defect of RN-T. Most likely the different findings for MP1 and epidermin are due to the different modes of action and the associated resistance mechanisms. Lantibiotics target lipid I, II, III in the bacterial membrane, ultimately damaging the integrity of the cell envelope and inducing lysis (18, 52). Resistance is imperfect and relies on the active expulsion of lantibiotics (53, 54). In contrast, MP1 targets the bacterial ribosome (21) and full resistance is provided by the expression of an alternative L11 ribosomal subunit (55). Interestingly, accumulation of amino acids similar to that observed in the pD4-19 recipient is known to be induced by antibiotic compounds targeting the bacterial ribosome (27). However, expression of the plasmid-associated resistance determinant did not change upon adaptive mutation in *citZ* suggesting that altered levels of autoimmunity are not causative for the observed increase of cellular fitness. However, it seems possible that the malfunctioning *citZ* allele might not be translated in the context of the alternative ribosomal subunit which might explain the TCA-deficient phenotype of the transformant. In general, our observations support a model in which pD4-19 acquisition and associated compound production drain the levels of TCA-cycle intermediates and cause pleiotropic effects including growth deficiency, reduced compound production and increased biofilm formation. These findings enhance our understanding of how antibiotic production is integrated into and optimized by the cellular central metabolism. Interestingly variation of TCA cycle activity is known to cause heterogeneity in staphylococcal populations (56) and TCA cycle deficient mutants are reported to arise during invasive infection (57). These phenomena are known to cause formation of antibiotic tolerant cells, and it also suggests that our findings might be relevant in staphylococcal strains in natural communities.

Even in environments such as the nasal microbiome bacteriocin producing strains remain rare, although the horizontal transfer of the responsible BGCs is possible and competitive benefits of inhibiting competitors should be immense (1, 10). Our study can explain why most BGC remain rare among isolates of a given bacterial species. As the consequence of a substantial metabolic burden imposed by bacteriocin production, a BGC-carrying mobile genetic element will most likely be counter selected. Accordingly, The BGCs will only be maintained if adaptive evolution events such as that reported in our study are possible and quickly occurring while simultaneously bacteriocin sensitive competitors are efficiently cleared from the ecological niche. However, further experimental evidence is needed to support this idea.

Material and Methods

Strains and growth conditions

The *Staphylococcus* strains used in this study were *S. aureus* D4-19, *S. aureus* RN4220 (RN), and *S. aureus* USA300 LAC. Further strains generated during this study were *S. aureus* RN4220 pD4-19 (RN-T), *S. aureus* RN4220 pD4-19 adapted (RN-A), *S. aureus* D4-19 TN1/2/3, *S. aureus* RN4220 pD4-19 Δ MP1, *S. aureus* D4-19 Δ PP, and *S. aureus* RN4220 pD4-19 Δ PP. Overexpression strains were constructed in *S. aureus* RN4220 background carrying the plasmid pRB473-*xyIR*-6xHis-*citZ*, with *citZ* deriving from either *S. aureus* RN4220 pD4-19 or *S. aureus* RN4220 pD4-19 adapted. The construction of the plasmids and knockouts is described below. *Escherichia coli* DC10B or *E. coli* Sa08B were used as the cloning host for further transformation in *S. aureus* D4-19, *S. aureus* RN4220 or *S. aureus* RN4220 pD4-19.

Basic medium (BM; 1% soy peptone A3 [Organotechnie SAS, France], 0.5% Ohly Kat yeast extract [Deutsche Hefewerke GmbH, Germany], 0.5% NaCl, 0.1% glucose, and 0.1% K₂HPO₄, pH 7.2) was used as the standard growth medium. If necessary, antibiotics were supplemented at a concentration of 10 μ g ml⁻¹ for chloramphenicol, 2.5 μ g ml⁻¹ for erythromycin or 0.5 μ g ml⁻¹ for penicillin G. *E. coli* transformants were grown in lysogeny broth (LB; Lennox) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl; Carl Roth GmbH, Germany) supplemented with 10 μ g ml⁻¹ chloramphenicol or on BM agar with 10 μ g ml⁻¹ chloramphenicol. BM without glucose (B₀) was used for expression of *citZ*.

To monitor growth over time, strains were grown overnight in BM under continuous shaking at 37°C. Each strain was adjusted to an optical density at 600 nm (OD₆₀₀) of 1 in BM, and 5 μ l of the bacterial stock solutions were pipetted to 1 ml BM into a 24-well microtiter plate. If necessary, 5 mM sodium citrate was added to each well. The plates were incubated for 24 h under continuous shaking in a microplate reader, and OD₆₀₀ was measured every 15 minutes. For all experiments assessing growth, transcription or metabolic profiles, bacterial strains were grown in 10 ml volumes of medium in 50 ml Erlenmeyer flasks containing a single baffle. Cultures were incubated on 37 °C with 160 rpm agitation. CO₂-levels were not controlled.

Cloning

DNA manipulation, isolation of plasmid DNA, and transformation of *E. coli* and *S. aureus* were performed by using standard procedures. Enzymes for molecular cloning were obtained from Thermo Fisher Scientific.

Transposon mutagenesis

To identify the biosynthetic gene cluster responsible for antimicrobial activity of *S. aureus* D4-19, the strain was transformed with the plasmid pBTn and mutants were

generated by transposon insertion as described previously (58). To identify the insertion site of the transposon in clones, that had lost antimicrobial activity, genomic DNA was isolated, and an inverse PCR was performed. Therefore, 5 µg gDNA were digested with the restriction enzyme BspHI for 3h at 37°C. After purification of the digest, 2 µg of DNA were religated in 100 µl total volume for 2-3 hours and 2 µl of the ligation mixture were used for standard PCR (in 25 µl volume) with pBTn up and down primers (Table 1). Analyse the PCR products on an analytical gel, isolate strong bands, and sequence with pBTn up/down primers.

Generation of *S. aureus* RN4220 pD4-19 and adapted mutant

S. aureus D4-19 belongs to clonal complex 8, and, to circumvent restriction modification (RM) barriers, we used the laboratory strain *S. aureus* RN4220 as recipient strain. This RM-deficient strain belongs to the same clonal complex, exhibits high transformation rates, and does not show inhibitory effects on other staphylococci. Finally, *S. aureus* RN4220 is highly sensitive to penicillin G (MIC = 0.015 µg/ml) allowing for the selection of pD4-19 which encodes a β-lactamase. Plasmid DNA was isolated from *S. aureus* D4-19 and was used to transform *S. aureus* RN4220 by electroporation. Positive transformants were selected on BM plates containing 0.5 µg ml⁻¹ Penicillin G and screened by PCR with primers binding in the BGC and the β-lactamase gene cluster (Table 1). Plasmid isolation and restriction digestion confirmed that the strain *S. aureus* RN4220 had acquired the plasmid pD4-19 and propagated it as an extrachromosomal element.

To generate a mutant that had adapted to MP1 production, *S. aureus* RN4220 pD4-19 was passaged for 28 days by inoculating fresh BM medium every day with 10 µl of overnight culture.

Generation of knockout mutants

For the generation of knockout mutants, the temperature-sensitive shuttle vector pIMAY was used, and mutants were generated by allelic replacement as described previously (59). Flanking regions of the genes to be deleted were amplified by PCR (Table 1) and ligated into pIMAY after digestion with suitable restriction enzymes. Cloning was performed in *E. coli* DC10B or *E. coli* Sa08B. Sequence-verified plasmids were transferred in the target strains, *S. aureus* D4-19 and *S. aureus* RN4220 pD4-19. Successful knockouts were confirmed by PCR with respective primers and sequencing of the PCR product covering the area of the knocked-out gene.

Overexpression of *citZ*

To overexpress *citZ*, a novel xylose-inducible plasmid was constructed on the basis of the shuttle vector pRB473. The regulatory unit containing the *xyIR* repressor gene and about 200 nucleotides at its 3'-end encompassing the regulated promoter were amplified with primers

pTX15 Hind and pTX15 Sma and plasmid pTX15 as template (Table 1). From the resulting PCR product, the required fragment was generated via HindIII and BamHI digestion, which was subsequently ligated to the equally digested pRB473 resulting in pRB473-xyIR. Furthermore, a 6xHis tag was inserted into the plasmid to get a C-terminally tagged protein. Therefore, plasmid pPSHG3 was used as template to amplify the 6xHis tag with the primers His-term down and His-term up (Table 1). The insert and pRB473-xyIR were digested with EcoRI and BamHI and after ligation, *E. coli* DC10B was transformed. *citZ* was amplified from *S. aureus* RN4220 pD4-19 and *S. aureus* RN4220 pD4-19 adapted using the primers *citZ*_overex_fwd and *citZ*_overex_rev_new (Table 1). After restriction digestion with BamHI and SacI, the inserts were ligated into pRB473-xyIR-6xHis. Sequence verified plasmids were transferred into *S. aureus* RN4220. To check the expressed protein levels, strains were grown overnight in BM, inoculated to OD₆₀₀=0.1 in B₀ and grown to OD₆₀₀=0.5. After induction with 0.5% xylose, samples were taken at time points 0 h, 2 h and 4 h. Samples were loaded on an SDS-Page (12% pre-cast gel, BioRad) after incubation with 4x Laemmli Sample Buffer (BioRad, add. 355 mM 2-mercaptoethanol) at 95°C for 5 min and centrifugation for 10 min at 16 000 xg. PageRuler Prestained (ThermoScientific) was used, and the gel was run at 120 V for 1 h. For Western Blotting, a nitrocellulose membrane was cut to the size of the gel and the blotting paper, and the membrane were equilibrated in transfer buffer. Blotting paper, gel, membrane, and blotting paper were stacked in a blotting chamber of the BioRad Transblot Turbo. To detect the His-Tag, the membrane was incubated in blocking buffer and then washed with PBS-T (PBS-Tween) and PBS. After incubation with the primary antibody from QIAGEN (Penta-His Antibody, 1:1000 dilution) at RT for 1 h under shaking, the membrane was washed again, 3x with PBS-T and 1 with PBS. The secondary antibody from LICOR (IRDye® 680RD Goat anti-Rabbit IgG, 1:10000 dilution) was added and after incubation for 1h at RT with shaking, washed again as mentioned before. The membrane was analysed using LI-COR Odyssey. Protein amounts were calculated using the LI-COR Odyssey imaging tools.

MIC assay

Strains used for MIC determinations were grown overnight in BM under continuous shaking at 37°C. Each strain was adjusted to OD₆₀₀=0.00125 in BM and 200 µl were pipetted in the first well of each row of a 96-well microtiter plate. 100 µl were pipetted in the remaining wells, one well per row was used as blank control containing 100 µl BM. The MP1 stock solution was serially diluted in this 96-well microtiter plate and the plates were incubated at 37°C for 21 h under continuous shaking (160 rpm). OD₆₀₀ of cultures in each well was measured with a microplate reader, and the lowest concentration of MP1 leading to no bacterial growth was defined as the MIC value.

Citrate assay

Citrate levels were measured using the Citrate Assay Kit (MAK057) from Sigma-Aldrich. Strains were grown over night in BM, diluted into fresh BM to OD=0.1 and grown for 5 h at 37°C under continuous shaking. For each strain, 1×10^8 cells in 100 μ l citrate assay buffer were homogenised in a 1.5 ml microcentrifuge tube containing 100 μ l glass beads with a Fast prep at 6.5 m/s for 60 s. After centrifugation for 10 min at maximum speed, 30 μ l of supernatant were pipetted in a 96 well plate. 20 μ l of citrate assay buffer was added to reach the final volume of 50 μ l described in the kit manual. Reaction mixes were prepared, and the analysis was carried out as described in the manufacturer's manual.

Acetate assay

Acetate levels were measured using the Acetate Colorimetric Assay Kit (MAK086) from Sigma-Aldrich. Strains were grown over night in BM, fresh BM was inoculated to OD=0.1 and grown for 8 h at 37°C under continuous shaking. At time points 0, 2, 4, 6 and 8 hours, 1 ml of culture was centrifuged at 11 000 $\times g$ for 5 min and the supernatant was transferred into a fresh tube. Samples were 1000-fold diluted, reaction mixes were prepared, and the analysis was carried out as described in the manufacturer's manual.

DNA isolation and sequencing

DNA isolation, library preparation and sequencing were performed by the Institute for Medical Microbiology (part of the NGS Competence Center NCCT, Tübingen, Germany). DNA was extracted using the Qiagen Genomic Tip 20/G Kit, following the manufacturer's instructions. The genomic DNA was quantified with a Qubit dsDNA BR Assay Kit (Thermo Fisher).

ONT library preparation was performed following the instructions manual Native barcoding genomic DNA (with EXP-NBD196 and SQK-LSK109, Oxford Nanopore) with an input of 250 ng DNA. 12 μ l template DNA was supplemented with the required reagents from the NEBNext Ultra II End Repair/dA Tailing kit (E7546S, NEB) and was first incubated at 20°C for 5 minutes and then at 65°C for 5 minutes. For the barcode ligation 3 μ l of nuclease-free water, 0.75 μ l End-prepped DNA, 1 μ l Native Barcode (Native Barcoding Expansion 96, EXP-NBD196) and 5 μ l Blunt/TA Ligase Master Mix (NEB Blunt/TA Ligase Master Mix, M0367) were combined in a new reaction vessel and incubated for 20 minutes at room temperature. 1 μ l of 0.5 M EDTA was added and samples were pooled in a new reaction tube. The pool was cleaned-up by using AMPure XP Beads (Agencourt), washed twice with 70% ethanol and resuspended in nuclease-free water. For barcode ligation 5 μ l Adapter Mix II, 10 μ l NEBNext Quick Ligation Reaction Buffer (5X) and 5 μ l Quick T4 DNA Ligase were added to the pool and incubated for 10 minutes at room temperature. The pool was cleaned up using AMPure XP Beads, washed

twice with Long Fragment Buffer and eluted in Elution Buffer. The library pool was loaded on a MinION device (Oxford Nanopore Technology, ONT) and stopped at 39 Gb output. Base calling was performed using the ONT's Guppy basecaller version 4.1.1.

Libraries for Illumina short-read sequencing were prepared using the Illumina Nextera™ DNA Flex Library Preparation Kit with IDT for Illumina DNA/RNA UD indexes, tagmentation according to the manufacturer's protocol with 500 ng DNA input and 5 cycles indexing PCR. Libraries were checked for correct fragment length on an Agilent 2100 Bioanalyzer and pooled equimolarly and quantified with Qubit DNA HS Assay Kit (ThermoFisher). Equimolarly pooled libraries were sequenced on a MiSeq Reagent Kit v2 (300 cycles) flow cell (Illumina) with 2 x 150 bp read length. For demultiplexing bcl2fastq v2.19.0.316 was used (<https://emea.support.illumina.com/downloads/bcl2fastq-conversion-software-v2-20.html>).

DNA data assessment and analysis

Sequencing statistics including the quality per base and adapter content assessment of Illumina reads were conducted with FastQC v0.11.8 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>, accessed June 2022). Unicycler v0.5.0 (60) with default parameters was used for a hybrid assembly of the Oxford Nanopore and Illumina reads of the *S. aureus* RN4220 pD4-19 genome. The resulting genome was annotated using prokka v1.14.6 (61) with the additional parameters to add gene features in the annotation and searching for non-coding RNAs (parameters --addgenes and --rfam). The quality of the assembly was assessed using quast v5.1.0 (62). The genome of the Illumina reads of *S. aureus* RN4220 pD4-19 adapted was assembled using EAGER v1.92.56 (63) and MUSIAL v1.0 (<https://github.com/Integrative-Transcriptomics/MUSIAL/tree/v1.0>). As a reference the assembly of *S. aureus* RN4220 pD4-19 was used. In EAGER parameters were set to not merge the paired-end reads and to use bwa-mem (64) for the mapping. For SNP calling GATK HaplotypeCaller was chosen (65).

Whole-genome sequence of *S. aureus* D4-19 was determined by Illumina short-read sequencing as described above. Illumina reads were *de-novo* assembled with SPAdes (version: 3.9.0) (66) and the plasmid contig was identified with MAUVE (67). The MP1 BGC was analyzed with antiSMASH 5.0 (bacterial settings) (68).

Spot assay

Antimicrobial activity was assessed, by resuspending the sensitive *S. aureus* USA300 LAC in 200 µl BM and spreading it with a cotton swab on a BM plate. Producer strains were also resuspended in BM and 10 µl of the suspension were spotted on the prepared indicator plate. Once the spots were dry, the plates were incubated at 37°C overnight.

Purification of MP1

Cell bound MP1 can be isolated as follows. 50 ml overnight culture were centrifuged at 6 000 xg for 10 min, the pellet was washed two times with 15 ml PBS and then resuspended in 3 ml methanol. After incubation on a spinning wheel for 1 h and a centrifugation step at 6 000 xg for 10 min, the supernatant was transferred in a fresh falcon and used for HPLC or MS/MS analysis.

LC-MS analysis of MP1

LC-MS analyses were performed with a HPLC (Ultimate 3000, Thermo Fischer) and subsequent HR-ESI(+)-TOF-MS (Maxis 4G, Bruker). For HPLC analysis, LC-MS-grade water (with 0.01 % formic acid) and LC-MS-grade methanol (with 0.06 % formic acid) were used and fractionation was performed with a gradient from 10% to 100% over 20 min and a flow rate of 0.3 ml/min. A nucleoshell RP18 column with a column length of 150 mm, an inner diameter of 2 mm and a particle size of 2.7 μm , prewarmed to 40°C was used. For HR-ESI(+)-TOF-MS sodium formiate was used as calibrant.

NMR analysis of MP1

NMR analyses were performed on a Bruker AvanceIII-700 instrument. ^1H NMR spectra were recorded with a frequency of 700 MHz and ^{13}C NMR spectra were recorded with a frequency of 176 MHz, both at a temperature of 303 K.

Sample preparation for metabolome analysis

For the preparation of samples for metabolome analysis, strains were grown over night, inoculated in fresh medium (20 ml) to $\text{OD}_{600}=0.1$ and grown for 5 h at 37°C under continuous shaking. OD_{600} was measured and all strains were set to the lowest OD measured. The culture was filtered through a 0.22 μm bottle-top sterile filter (250 ml, Nalgene) via vacuum. The filter was washed with 0.6 % ice-cold NaCl, cut in 4 pieces and incubated at -20°C for 20 min with 5 ml ice-cold 40:40:20 (v/v/v) methanol:acetonitrile:water in a glass bottle. 1 ml of filtrate was transferred into a microcentrifuge tube and bead beaten 2x 30 s at 6.5 m/s with 0.5 ml glass beads. Samples were kept on ice in between. After centrifugation for 5 min at 4°C at maximum speed, 600 μl of supernatant were stored at -80°C.

Metabolite analysis by flow injection MS (FI-MS)

Metabolites were analyzed by flow injection into a high-resolution quadrupole time-of-flight (QTOF) mass spectrometer (Agilent QTOF 6546) as described previously (69). 3 μL of the sample was injected with an Agilent 1290 Bio Multisampler (G7137A) into the mobile phase

that was a 60:40 (v/v-%) mixture of isopropanol (LiChrosolv Supelco hypergrade for LC-MS, 1.02781.2500) and ultrapure water (Omnia Pure, stakpure), buffered with 10 mM ammonium carbonate ((NH₄)₂CO₃, Sigma-Aldrich, 3799-10 G) and 0.04% ammonium hydroxide (NH₄OH, Honeywell/Fluka TraceSELECT Ultra, 16748-250 ML). The flow rate of the mobile phase was 0.15 ml*min⁻¹. Mass spectra were separately recorded in positive- and negative ionization profile mode from *m/z* 50 to *m/z* 1700 with an acquisition rate of 1.4 ms/spectrum using the highest resolving power (10 GHz-High Sensitivity). Online mass axis correction was performed with purine and hexakis(1H,1H,3H- tetrafluoropropoxy)phosphazine (HP-0921, Agilent Technologies). The source gas temperature of the ESI ion source was 225 °C, with 11 l*min⁻¹ drying gas and a nebulizer pressure of 20 psi. The sheath gas temperature was 350 °C, and the flow rate was 10 l*min⁻¹. Electrospray nozzle and capillary voltages were 2,000 and 3,500 V, respectively. Fragmenter and skimmer voltages were 120 and 65 V, respectively. Ion peaks were annotated by matching the mass-to-charge ratios to calculated, single (de-) protonated masses of metabolites listed in a genome scale model of *Escherichia coli* K12 (iML1515) (70). Hierarchical cluster plot was generated using MATLAB. Metabolites obtained via FI-MS, which were used for further analysis, are listed in table 2.

Metabolite analysis by LC-MS/MS

LC-MS/MS was performed with an Agilent 6495 triple quadrupole mass spectrometer (Agilent Technologies) as described previously (71). An Agilent 1290 Infinity II UHPLC system (Agilent Technologies) was used for liquid chromatography using two columns: i) an Acquity UPLC BEH Amide (Waters) for acidic conditions and ii) an iHILIC-Fusion(P) (HILICON AB) for basic conditions. The column oven was at 30°C. LC solvents were: solvent A: water with ammonium formate (10 mM) and formic acid (0.1 % v/v) for acidic conditions, and water with ammonium carbonate (10 mM) and ammonium hydroxide (0.2 %) for basic conditions; solvents B: acetonitrile with formic acid (0.1% v/v) for acidic, and acetonitrile for basic conditions. The LC gradient was: 0 min 90% B, 1.3 min 40% B, 1.5 min 40% B, 1.7 min 90% B, 2 min 90 % B. The flow rate was 0.4 ml/min. The injection volume was 3 µL. Settings of the ESI source were: 200 °C source gas, 14 L/min drying gas and 24 psi nebulizer pressure. The sheath gas temperature was at 300 °C and flow at 11 L/min. The electrospray nozzle was set to 500 V and capillary voltage to 2500 V. All samples were mixed with a 13-C labelled internal standard and the ratio of 12-C and 13-C peak heights was used to quantify metabolites. 12C/13C ratios were normalized to the OD-specific cell volume at the time point of sampling. Alanine, arginine, asparagine, aspartate, glutamate, glutamine, glycine, histidine, leucine/isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan, valine, acetyl-CoA, succinate, 3-phosphoglycerate, phosphoenolpyruvate, urea, ornithine, argininosuccinate and ATP were measured via LC-MS/MS.

Sample preparation for proteome analysis

Strains were grown over night, inoculated in fresh medium to $OD_{600}=0.1$ and grown for 5 h at 37°C under continuous shaking. 1.5 ml were centrifuged, the pellet was resuspended in 1 ml SDS buffer (4% w/v sodium dodecyl sulfate (SDS), in 100 mM tris(hydroxymethyl)aminomethane (Tris)/HCl; pH 8). Cells were homogenised at 6.5 m/s for 2x40s by using a Fast Prep, and incubated on ice for 2 min in between. Samples were centrifuged at maximum speed for 1 min and the supernatant was transferred into a fresh Eppendorf tube. To reduce cysteine disulfide bonds, 10 mM Dithiothreitol (DTT) was added to the samples and incubated for 45 min shaking at 650 rpm at room temperature (RT). 5.5 mM Iodoacetamide (IAA) were added to alkylate reduced cysteine thiol groups. Incubation for 45 min at RT with shaking at 650 rpm in the dark were followed by centrifugation of the samples at 12 000 xg for 15 min. The supernatant was transferred in a new tube and one volume of supernatant was mixed with 7 volumes of ice cold 8:1 acetone:methanol, vortexed and incubated over night at -20°C. Centrifugation of the precipitated proteins for 5 min at 1 000 xg was followed by two washing steps with 80% acetone at RT. The protein pellet was air dried for 10-15 min and rehydrated in denaturation buffer (6 M urea, 2 M thiourea in 10 mM Tris/HCl; pH 7.5).

LC-MS/MS analysis of proteome samples

Ten microgram of proteins per sample were digested in solution with trypsin as described previously (72). Desalted peptides (73) were separated on an Easy-nLC 1200 system coupled to a quadrupole Orbitrap Exploris 480 mass spectrometer (all Thermo Fisher Scientific) as described previously (74) with slight modifications: peptides were separated using an 87-minute segmented gradient from 10-33-50-90% of HPLC solvent B (80% acetonitrile in 0.1% formic acid) in HPLC solvent A (0.1% formic acid) at a flow rate of 200 nl/min. The mass spectrometer was operated in data-dependent mode, collecting MS spectra in the Orbitrap mass analyser (60,000 resolution, 300-1750 m/z range) with an automatic gain control (AGC) set to standard and a maximum ion injection time set to automatic. The 20 most intense precursor ions were sequentially fragmented with a normalized collision energy of 28 in each scan cycle using higher energy collisional dissociation (HCD) fragmentation. In all measurements, sequenced precursor masses were excluded from further selection for 30 s. MS/MS spectra were recorded with a resolution of 15,000, whereby fill time was set to automatic. Acquired MS spectra were processed with MaxQuant software package version 1.6.14.0 (75) with integrated Andromeda search engine (76). Database search was performed against a *Staphylococcus aureus* (allStrains) protein database (downloaded on 7th of October 2020, 216,059 entries), and 286 commonly observed contaminants. Endoprotease trypsin was

defined as protease with a maximum of two missed cleavages. Oxidation of methionine, and protein N-terminal acetylation were specified as variable modifications. Carbamidomethylation on cysteine was set as fixed modification. Initial maximum allowed mass tolerance was set to 4.5 parts per million (ppm) for precursor ions and 20 ppm for fragment ions. Peptide, protein and modification site identifications were reported at a false discovery rate (FDR) of 0.01, estimated by the target-decoy approach (77). The iBAQ (Intensity Based Absolute Quantification) and LFQ (Label-Free Quantification) algorithms were enabled, as was the “match between runs” option (78, 79).

RNA isolation for transcriptome analysis

Strains were grown over night, inoculated in fresh medium to $OD_{600}=0.1$ and grown for 5 h at 37°C under continuous shaking. 1/10 volume EtOH/Phenol was added to 500 µl of sample and mixed for 1 min. After incubation on ice for 5 min, the samples were centrifuged for 1 min at 20 000 xg at 4°C. The supernatant was discarded, and the pellet was resuspended in 1 ml TRIzol. Each sample was transferred to one screw-cap tube with glass beads and cells were lysed via bead-beating for 2x 6.5 ms/s for 30 s. In between the two runs the cells were kept on ice for 2 min. 200 µl chloroform were added to the samples, mixed and incubated 2-3 min before centrifugation of the samples for 15 min, 12 000 xg , 4°C. The aqueous supernatant was taken, mixed with 500 µl isopropanol and samples were centrifuged for 10 min, 21 000 xg , 4°C. The supernatant was discarded, and the pellet was resuspended in 500 µl 75% EtOH. After centrifugation for 5 min, 20 000 xg , 4°C, the supernatant was discarded, and the pellet was dried at RT. The pellet was resuspended in 100 µl RNA-grade water and RNA was concentrated via the MN RNA clean up kit (Machery-Nagel) and RNA was eluted in 60 µl RNA-grade water. After quantification with a Nanodrop, RNA was stored at -80°C. Library Prep and Sequencing was performed by the Institute for Medical Microbiology (part of the NGS Competence Center NCCT (Tübingen, Germany)). RNA samples were DNase I digested (DNase I recombinant, Rnase-free, Millipore Sigma), cleaned up (RNA Clean & Concentrator-5, Zymo Research), quantified (Qubit RNA BR Assay Kit, ThermoFisher) and normalized to 100 ng in 11 µl nuclease-free water. Library preparation was performed according to the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus Reference Guide. Library concentration was measured with Qubit DNA HS Assay Kit, (ThermoFisher) on a Qubit Fluorometer (Invitrogen) and fragment length was assessed with an Agilent 2100 Bioanalyzer (Agilent High sensitivity DNA Kit, Agilent). Samples were equimolarly pooled and sequenced on NextSeq™ 500 High Output Kit v2.5 (75 cycles) flow cell (Illumina) with 1 x 75 bp read length.

RNA-Seq data assessment and analysis

Sequencing statistics including the quality per base and adapter content assessment of resulting transcriptome sequencing data were conducted with FastQC v0.11.8 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>, accessed June 2022). All reads mappings were performed against the previously assembled reference strain *S. aureus* RN4220 pD4-19 (SRA Bioproject ID PRJNA855446). The mappings of all samples were conducted with HISAT2 v2.1.0 (80). As parameters spliced alignment of reads was disabled and strand-specific information was set to reverse complemented (HISAT2 parameter `--no-spliced-alignment` and `--rna-strandness "R"`). The resulting mapping files in SAM format were converted to BAM format using SAMtools v1.9 (81). Mapping statistics, including strand specificity estimation and percentage of mapped reads, were conducted with the RNA-Seq module of QualiMap2 v2.2.2-a (82). Gene counts for all samples were computed with featureCounts v1.6.4 (83) based on the prokka annotation of the assembled *S. aureus* RN4220 pD4-19 genome, where the selected feature type was set to transcript records (featureCounts parameter `-t transcript`). A quality check for ribosomal rRNA was performed with a self-written script based on the absolute counts of annotated rRNAs. To assess variability of the replicates of each time series, a principal component analysis (PCA) was conducted with the DESeq2 package v1.28.1 (84).

Normalization and differential gene expression

For the computation of genes differentially expressed between the two different strains (*S. aureus* RN4220 pD4-19 and pD4-19 adapted) and the wild type strain *S. aureus* RN4220, DESeq2 v1.20.0 (84) was applied to the absolute gene counts as computed with featureCounts. For differences between the two strains and the wildtype strain, genes with an adjusted p-value (FDR) < 0.05 and absolute log2 fold change (FC) > 1 were reported as differentially expressed.

Gene set enrichment analysis

The assembled *S. aureus* RN4220 pD4-19 genome was functionally annotated using FAcOP (<http://facop.molgenrug.nl/>). Gene set enrichment analysis was performed on differentially expressed genes using FUNAGE-Pro (<http://gseapro.molgenrug.nl/>).

Biofilm assay

Strains were grown over night in tryptic soy broth and adjusted to the OD₆₀₀ of the strain with the lowest OD₆₀₀. 5 µl were added to 995 µl of BM containing 1% (v/v) glucose. 200 µl were transferred into wells of a fibrinogen coated Nunclon Delta surface microtiter plate. The

plate was incubated at 37°C for 24 h, in non-shaking conditions. Control wells with broth and no bacteria were included. The supernatant was discarded, and wells were washed three times with 200 µl PBS. The plate was inverted for 30 min to dry. 100 µl crystal violet was added to each well and washed off after 1 min (3-5 times with PBS). 100 µl of 5% acetic acid was added to the wells and the plate was placed on a shaker for 5 min to dissolve the cells. Subsequently the absorbance at 570 nm was measured using the FLUOstar Optima.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.02.

Data availability

ONT and Illumina reads for *S. aureus* RN4220 pD4-19 and *S. aureus* RN4220 pD4-19 adapted can be found at SRA Bioproject ID PRJNA855446 and the plasmid sequence of pD4-19 (from *S. aureus* D4-19) has the GenBank accession number ON936820. All RNA-Seq Illumina read files as well as the raw counts have been deposited in NCBI's Gene Expression Omnibus and are accessible under accession number GSE208001. Metabolome data can be accessed at Metabolights via the identifier MTBLS5196. Proteome data can be accessed at PRIDE via the accession number PXD035193.

Acknowledgements

The authors thank Libera Lo Presti for critically reading and editing this manuscript and the NCCT (NGS Competence Center Tübingen, Germany) for whole genome and transcriptome sequencing of our strains. Furthermore, we authors thank the Quantitative Biology Center of the University of Tübingen for project and data management.

Our work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) in the frame of the TRR261 project ID 398967434 (S.H., A.P., S.G., K.N., H.L., B.M.), Germany's Excellence Strategy – EXC 2124 – 390838134 (S.H., A.P., B.K., H.L.), and the TRR156 (A.P.). Further we acknowledge support by the High Performance and Cloud Computing Group at the Zentrum für Datenverarbeitung of the University of Tübingen, the state of Baden-Württemberg through bwHPC and DFG grant no INST 37/935-1 FUGG. Finally, we acknowledge funding by the German Center of Infection Research (DZIF) to S.H., A.P.

References

1. Heilbronner S, Krismer B, Brotz-Oesterhelt H, Peschel A. 2021. The microbiome-shaping roles of bacteriocins. *Nat Rev Microbiol* doi:10.1038/s41579-021-00569-w.
2. Cotter PD, Ross RP, Hill C. 2013. Bacteriocins - a viable alternative to antibiotics? *Nat Rev Microbiol* 11:95-105.
3. Simons A, Alhanout K, Duval RE. 2020. Bacteriocins, Antimicrobial Peptides from Bacterial Origin: Overview of Their Biology and Their Impact against Multidrug-Resistant Bacteria. *Microorganisms* 8.
4. Zipperer A, Konnerth MC, Laux C, Berscheid A, Janek D, Weidenmaier C, Burian M, Schilling NA, Slavetinsky C, Marschal M, Willmann M, Kalbacher H, Schitteck B, Brotz-Oesterhelt H, Grond S, Peschel A, Krismer B. 2016. Human commensals producing a novel antibiotic impair pathogen colonization. *Nature* 535:511-6.
5. Claesen J, Spagnolo JB, Ramos SF, Kurita KL, Byrd AL, Aksenov AA, Melnik AV, Wong WR, Wang S, Hernandez RD, Donia MS, Dorrestein PC, Kong HH, Segre JA, Linington RG, Fischbach MA, Lemon KP. 2020. A *Cutibacterium acnes* antibiotic modulates human skin microbiota composition in hair follicles. *Sci Transl Med* 12.
6. Kim SG, Becattini S, Moody TU, Shliha PV, Littmann ER, Seok R, Gjonbalaj M, Eaton V, Fontana E, Amoretti L, Wright R, Caballero S, Wang ZX, Jung HJ, Morjaria SM, Leiner IM, Qin W, Ramos R, Cross JR, Narushima S, Honda K, Peled JU, Hendrickson RC, Taur Y, van den Brink MRM, Pamer EG. 2019. Microbiota-derived lantibiotic restores resistance against vancomycin-resistant *Enterococcus*. *Nature* 572:665-669.
7. Janek D, Zipperer A, Kulik A, Krismer B, Peschel A. 2016. High Frequency and Diversity of Antimicrobial Activities Produced by Nasal *Staphylococcus* Strains against Bacterial Competitors. *PLoS Pathog* 12:e1005812.
8. Newstead LL, Varjonen K, Nuttall T, Paterson GK. 2020. Staphylococcal-Produced Bacteriocins and Antimicrobial Peptides: Their Potential as Alternative Treatments for *Staphylococcus aureus* Infections. *Antibiotics (Basel)* 9.
9. Carson DA, Barkema HW, Naushad S, De Buck J. 2017. Bacteriocins of Non-aureus *Staphylococci* Isolated from Bovine Milk. *Appl Environ Microbiol* 83.
10. Dragos A, Andersen AJC, Lozano-Andrade CN, Kempen PJ, Kovacs AT, Strube ML. 2021. Phages carry interbacterial weapons encoded by biosynthetic gene clusters. *Curr Biol* 31:3479-3489 e5.
11. Tang X, Kudo Y, Baker JL, LaBonte S, Jordan PA, McKinnie SMK, Guo J, Huan T, Moore BS, Edlund A. 2020. Cariogenic *Streptococcus mutans* Produces Tetramic Acid Strain-Specific Antibiotics That Impair Commensal Colonization. *ACS Infect Dis* 6:563-571.
12. Ceotto H, Dias RC, Nascimento Jdos S, Brito MA, Giambiagi-Demarval M, Bastos Mdo C. 2012. Aureocin A70 production is disseminated amongst genetically unrelated *Staphylococcus aureus* involved in bovine mastitis. *Lett Appl Microbiol* 54:455-61.
13. Huo L, Hug JJ, Fu C, Bian X, Zhang Y, Muller R. 2019. Heterologous expression of bacterial natural product biosynthetic pathways. *Nat Prod Rep* 36:1412-1436.
14. Barbuto Ferraiuolo S, Cammarota M, Schiraldi C, Restaino OF. 2021. Streptomyces as platform for biotechnological production processes of drugs. *Appl Microbiol Biotechnol* 105:551-568.
15. Bekiesch P, Basitta P, Apel AK. 2016. Challenges in the Heterologous Production of Antibiotics in *Streptomyces*. *Arch Pharm (Weinheim)* 349:594-601.
16. McCloskey D, Xu S, Sandberg TE, Brunk E, Hefner Y, Szubin R, Feist AM, Palsson BO. 2018. Adaptive laboratory evolution resolves energy depletion to maintain high

- aromatic metabolite phenotypes in *Escherichia coli* strains lacking the Phosphotransferase System. *Metab Eng* 48:233-242.
17. Blanchard AE, Liao C, Lu T. 2016. An Ecological Understanding of Quorum Sensing-Controlled Bacteriocin Synthesis. *Cellular and Molecular Bioengineering* 9:443-454.
 18. Ebner P, Reichert S, Luqman A, Krismer B, Popella P, Gotz F. 2018. Lantibiotic production is a burden for the producing staphylococci. *Sci Rep* 8:7471.
 19. Netz DJ, Sahl HG, Marcelino R, dos Santos Nascimento J, de Oliveira SS, Soares MB, do Carmo de Freire Bastos M. 2001. Molecular characterisation of aureocin A70, a multi-peptide bacteriocin isolated from *Staphylococcus aureus*. *J Mol Biol* 311:939-49.
 20. Liu Y, Liu Y, Du Z, Zhang L, Chen J, Shen Z, Liu Q, Qin J, Lv H, Wang H, He L, Liu J, Huang Q, Sun Y, Otto M, Li M. 2020. Skin microbiota analysis-inspired development of novel anti-infectives. *Microbiome* 8:85.
 21. Rosendahl G, Douthwaite S. 1994. The antibiotics micrococcin and thiostrepton interact directly with 23S rRNA nucleotides 1067A and 1095A. *Nucleic Acids Res* 22:357-63.
 22. Porse BT, Cundliffe E, Garrett RA. 1999. The antibiotic micrococcin acts on protein L11 at the ribosomal GTPase centre. *J Mol Biol* 287:33-45.
 23. Ciufolini MA, Lefranc D. 2010. Micrococcin P1: structure, biology and synthesis. *Nat Prod Rep* 27:330-42.
 24. Starmer J, Stomp A, Vouk M, Bitzer D. 2006. Predicting Shine-Dalgarno sequence locations exposes genome annotation errors. *PLoS Comput Biol* 2:e57.
 25. Novick RP, Richmond MH. 1965. Nature and Interactions of the Genetic Elements Governing Penicillinase Synthesis in *Staphylococcus Aureus*. *J Bacteriol* 90:467-80.
 26. Hecht A, Glasgow J, Jaschke PR, Bawazer LA, Munson MS, Cochran JR, Endy D, Salit M. 2017. Measurements of translation initiation from all 64 codons in *E. coli*. *Nucleic Acids Res* 45:3615-3626.
 27. Hancock R. 1960. Accumulation of pool amino acids in *Staphylococcus aureus* following inhibition of protein synthesis. *Biochim Biophys Acta* 37:47-55.
 28. Kloosterman TG, Hendriksen WT, Bijlsma JJE, Bootsma HJ, van Hijum SAFT, Kok J, Hermans PWM, Kuipers OP. 2006. Regulation of Glutamine and Glutamate Metabolism by GlnR and GlnA in *Streptococcus pneumoniae**. *Journal of Biological Chemistry* 281:25097-25109.
 29. Blumenthal HJ, Huettner CF, Montiel FA. 1974. Comparative aspects of glucose catabolism in *Staphylococcus aureus* and *S. epidermidis*. *Ann N Y Acad Sci* 236:105-14.
 30. Somerville GA, Chaussee MS, Morgan CI, Fitzgerald JR, Dorward DW, Reitzer LJ, Musser JM. 2002. *Staphylococcus aureus* aconitase inactivation unexpectedly inhibits post-exponential-phase growth and enhances stationary-phase survival. *Infect Immun* 70:6373-82.
 31. Biswas L, Biswas R, Nerz C, Ohlsen K, Schlag M, Schafer T, Lamkemeyer T, Ziebandt AK, Hantke K, Rosenstein R, Gotz F. 2009. Role of the twin-arginine translocation pathway in *Staphylococcus*. *J Bacteriol* 191:5921-9.
 32. Sheldon JR, Marolda CL, Heinrichs DE. 2014. TCA cycle activity in *Staphylococcus aureus* is essential for iron-regulated synthesis of staphyloferrin A, but not staphyloferrin B: the benefit of a second citrate synthase. *Mol Microbiol* 92:824-39.
 33. Schuster CF, Bellows LE, Tosi T, Campeotto I, Corrigan RM, Freemont P, Grundling A. 2016. The second messenger c-di-AMP inhibits the osmolyte uptake system OpuC in *Staphylococcus aureus*. *Sci Signal* 9:ra81.

34. Kassem MA, Saafan AE, Bayomy F, El-Gendy AO. 2021. Exploring clinically isolated *Staphylococcus* sp. bacteriocins revealed the production of amonabactin, micrococcin, and alpha-circulocin. *Iran J Microbiol* 13:212-224.
35. Bennallack PR, Burt SR, Heder MJ, Robison RA, Griffiths JS. 2014. Characterization of a novel plasmid-borne thiopeptide gene cluster in *Staphylococcus epidermidis* strain 115. *J Bacteriol* 196:4344-50.
36. Ovchinnikov KV, Kranjec C, Telke A, Kjos M, Thorstensen T, Scherer S, Carlsen H, Diep DB. 2021. A Strong Synergy Between the Thiopeptide Bacteriocin Micrococcin P1 and Rifampicin Against MRSA in a Murine Skin Infection Model. *Front Immunol* 12:676534.
37. O'Neill AM, Worthing KA, Kulkarni N, Li F, Nakatsuji T, McGrosso D, Mills RH, Kalla G, Cheng JY, Norris JM, Pogliano K, Pogliano J, Gonzalez DJ, Gallo RL. 2021. Antimicrobials from a feline commensal bacterium inhibit skin infection by drug-resistant *S. pseudintermedius*. *Elife* 10.
38. Van der Veken D, Hollanders C, Verce M, Michiels C, Ballet S, Weckx S, Leroy F. 2022. Genome-Based Characterization of a Plasmid-Associated Micrococcin P1 Biosynthetic Gene Cluster and Virulence Factors in *Mammaliococcus sciuri* IMDO-S72. *Appl Environ Microbiol* 88:e0208821.
39. Maldonado-Barragan A, West SA. 2020. The cost and benefit of quorum sensing-controlled bacteriocin production in *Lactobacillus plantarum*. *J Evol Biol* 33:101-111.
40. Gonzalez D, Mavridou DA. 2019. Making the Best of Aggression: The Many Dimensions of Bacterial Toxin Regulation. *Trends Microbiol* 27:897-905.
41. Somerville GA, Said-Salim B, Wickman JM, Raffel SJ, Kreiswirth BN, Musser JM. 2003. Correlation of acetate catabolism and growth yield in *Staphylococcus aureus*: implications for host-pathogen interactions. *Infect Immun* 71:4724-32.
42. Strasters KC, Winkler KC. 1963. Carbohydrate Metabolism of *Staphylococcus Aureus*. *J Gen Microbiol* 33:213-29.
43. Ladjouzi R, Lucau-Danila A, Drider D. 2020. Metabolic Shift of an Isogenic Strain of *Enterococcus faecalis* 14, Deficient in Its Own Bacteriocin Synthesis, as Revealed by a Transcriptomic Analysis. *Int J Mol Sci* 21.
44. Mack D, Fischer W, Krokotsch A, Leopold K, Hartmann R, Egge H, Laufs R. 1996. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J Bacteriol* 178:175-83.
45. Cramton SE, Gerke C, Schnell NF, Nichols WW, Gotz F. 1999. The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect Immun* 67:5427-33.
46. Vuong C, Kidder JB, Jacobson ER, Otto M, Proctor RA, Somerville GA. 2005. *Staphylococcus epidermidis* polysaccharide intercellular adhesin production significantly increases during tricarboxylic acid cycle stress. *J Bacteriol* 187:2967-73.
47. Sadykov MR, Olson ME, Halouska S, Zhu Y, Fey PD, Powers R, Somerville GA. 2008. Tricarboxylic acid cycle-dependent regulation of *Staphylococcus epidermidis* polysaccharide intercellular adhesin synthesis. *J Bacteriol* 190:7621-32.
48. Shanks RM, Meehl MA, Brothers KM, Martinez RM, Donegan NP, Graber ML, Cheung AL, O'Toole GA. 2008. Genetic evidence for an alternative citrate-dependent biofilm formation pathway in *Staphylococcus aureus* that is dependent on fibronectin binding proteins and the GraRS two-component regulatory system. *Infect Immun* 76:2469-77.

49. Tan GY, Deng K, Liu X, Tao H, Chang Y, Chen J, Chen K, Sheng Z, Deng Z, Liu T. 2017. Heterologous Biosynthesis of Spinosad: An Omics-Guided Large Polyketide Synthase Gene Cluster Reconstitution in *Streptomyces*. *ACS Synth Biol* 6:995-1005.
50. Olano C, Lombo F, Mendez C, Salas JA. 2008. Improving production of bioactive secondary metabolites in actinomycetes by metabolic engineering. *Metab Eng* 10:281-92.
51. Kempf M, Theobald U, Fiedler HP. 1999. Correlation between the consumption of amino acids and the production of the antibiotic gallidermin by *Staphylococcus gallinarum*. *Biotechnology Letters* 21:959-963.
52. Brotz H, Josten M, Wiedemann I, Schneider U, Gotz F, Bierbaum G, Sahl HG. 1998. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. *Mol Microbiol* 30:317-27.
53. Peschel A, Gotz F. 1996. Analysis of the *Staphylococcus epidermidis* genes *epiF*, *-E*, and *-G* involved in epidermin immunity. *J Bacteriol* 178:531-6.
54. Otto M, Peschel A, Gotz F. 1998. Producer self-protection against the lantibiotic epidermin by the ABC transporter EpiFEG of *Staphylococcus epidermidis* Tu3298. *FEMS Microbiol Lett* 166:203-11.
55. Baumann S, Schoof S, Bolten M, Haering C, Takagi M, Shin-ya K, Arndt HD. 2010. Molecular determinants of microbial resistance to thiopeptide antibiotics. *J Am Chem Soc* 132:6973-81.
56. Zalis EA, Nuxoll AS, Manuse S, Clair G, Radlinski LC, Conlon BP, Adkins J, Lewis K. 2019. Stochastic Variation in Expression of the Tricarboxylic Acid Cycle Produces Persister Cells. *mBio* 10.
57. Chen E, Eghbal M, Shaffer M, Culyba MJ. 2021. 128. Mutations that Inactivate the Tricarboxylic Acid Cycle in *Staphylococcus aureus* Arise During Persistent MRSA Bacteremia. *Open Forum Infectious Diseases* 8:S78-S79.
58. Li M, Rigby K, Lai Y, Nair V, Peschel A, Schitteck B, Otto M. 2009. *Staphylococcus aureus* mutant screen reveals interaction of the human antimicrobial peptide dermcidin with membrane phospholipids. *Antimicrob Agents Chemother* 53:4200-10.
59. Monk IR, Shah IM, Xu M, Tan MW, Foster TJ. 2012. Transforming the Untransformable: Application of Direct Transformation To Manipulate Genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Mbio* 3.
60. Wick RR, Judd LM, Gorrie CL, Holt KE. 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol* 13:e1005595.
61. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30:2068-9.
62. Gurevich A, Saveliev V, Vyahhi N, Tesler G. 2013. QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 29:1072-5.
63. Peltzer A, Jager G, Herbig A, Seitz A, Kniep C, Krause J, Nieselt K. 2016. EAGER: efficient ancient genome reconstruction. *Genome Biol* 17:60.
64. Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv: Genomics*.
65. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res* 20:1297-303.
66. Nurk S, Bankevich A, Antipov D, Gurevich AA, Korobeynikov A, Lapidus A, Prjibelski AD, Pyshkin A, Sirotkin A, Sirotkin Y, Stepanauskas R, Clingenpeel SR, Woyke T,

- McLean JS, Lasken R, Tesler G, Alekseyev MA, Pevzner PA. 2013. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. *J Comput Biol* 20:714-37.
67. Darling AC, Mau B, Blattner FR, Perna NT. 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. *Genome Res* 14:1394-403.
 68. Blin K, Shaw S, Steinke K, Villebro R, Ziemert N, Lee SY, Medema MH, Weber T. 2019. antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Res* 47:W81-W87.
 69. Fuhrer T, Heer D, Begemann B, Zamboni N. 2011. High-throughput, accurate mass metabolome profiling of cellular extracts by flow injection-time-of-flight mass spectrometry. *Anal Chem* 83:7074-80.
 70. Monk JM, Lloyd CJ, Brunk E, Mih N, Sastry A, King Z, Takeuchi R, Nomura W, Zhang Z, Mori H, Feist AM, Palsson BO. 2017. iML1515, a knowledgebase that computes *Escherichia coli* traits. *Nat Biotechnol* 35:904-908.
 71. Guder JC, Schramm T, Sander T, Link H. 2017. Time-Optimized Isotope Ratio LC-MS/MS for High-Throughput Quantification of Primary Metabolites. *Anal Chem* 89:1624-1631.
 72. Zittlau KI, Lechado-Terradas A, Nalpas N, Geisler S, Kahle PJ, Macek B. 2022. Temporal Analysis of Protein Ubiquitylation and Phosphorylation During Parkin-Dependent Mitophagy. *Mol Cell Proteomics* 21:100191.
 73. Rappsilber J, Mann M, Ishihama Y. 2007. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* 2:1896-906.
 74. Bekker-Jensen DB, Martinez-Val A, Steigerwald S, Ruther P, Fort KL, Arrey TN, Harder A, Makarov A, Olsen JV. 2020. A Compact Quadrupole-Orbitrap Mass Spectrometer with FAIMS Interface Improves Proteome Coverage in Short LC Gradients. *Mol Cell Proteomics* 19:716-729.
 75. Cox J, Mann M. 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 26:1367-72.
 76. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. 2011. Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res* 10:1794-805.
 77. Elias JE, Gygi SP. 2007. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat Methods* 4:207-14.
 78. Schwanhauser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M. 2011. Global quantification of mammalian gene expression control. *Nature* 473:337-42.
 79. Lubner CA, Cox J, Lauterbach H, Fancke B, Selbach M, Tschopp J, Akira S, Wiegand M, Hochrein H, O'Keefe M, Mann M. 2010. Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. *Immunity* 32:279-89.
 80. Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 12:357-60.
 81. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing S. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25:2078-9.
 82. Okonechnikov K, Conesa A, Garcia-Alcalde F. 2016. Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics* 32:292-4.

83. Liao Y, Smyth GK, Shi W. 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30:923-30.
84. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15:550.

Tables and Figures

Table 1: Primers used for transposon mutagenesis and generation of knockout mutants.

Primer	Sequence (5'-3')	assignment
pBTn seq up	ACCAACATGACGAATCCCTCC	inverse PCR and sequencing
pBTn seq down	CCCGCCATACCACAGATGTT	inverse PCR and sequencing
TclS	GCATCAGACACCTGTTCACTTA TT	pD4-19 sequencing
BlaR1-fwd	CTATGGCTGAATGGGATGTTAT	pD4-19 sequencing
WT_KO_PP_up_fwd	CGAGGGTCGACTAAACTTAAA CCTGACTGTCATTGT	Deletion of MP1 precursor peptide (<i>tclE</i>) and MP1 BGC
WT_KO_PP_up_rev	CCTAACTAAATTATTATTACGA GCACCACCTTTACTTAGAT	Deletion of MP1 precursor peptide (<i>tclE</i>)
WT_KO_PP_dw_fw d	GGTGGTGCTCGTAATAATAATT TAGTTAGGTATAAATTA	Deletion of MP1 precursor peptide (<i>tclE</i>)
WT_KO_PP_dw_rev	CTGCAGGAATTCAGAATATCTA GTATCGAAGATTT	Deletion of MP1 precursor peptide (<i>tclE</i>)
KO_MP1_up_rev	TTTAATATAATTGTATGGAGGT TAGTTACGAGCACCACTTTAC TTAGAT	Deletion of MP1 BGC
KO_MP1_down_fwd	ATCTAAGTAAAGGTGGTGCTC GTAACCTAACCTCCATACAATTA TATTAAA	Deletion of MP1 BGC
KO_MP1_down_rev	ATATGAATTCATCGAGTTGTCTG AAATGTTAGAA	Deletion of MP1 BGC
pTX15 Hind	CGTTATCACAAGTGGTCACCA CTCT	Construction of pRB473-xylR
pTX15 Sma	GCTTCCGGCTCGTATGTTGTG TGG	Construction of pRB473-xylR
His-term down	GGCTAGCGGATCCTCGAGTCG ACTACCGAGCTCAGATCTCAT C	Insertion of 6xHis in pRB473- xylR
His-term up	AGTTAGAATTCTGCAGTTTCAT GAATATTACAAACAAAAAGC	Insertion of 6xHis in pRB473- xylR
citZ_overex_fwd	AATGTAGGATCCAATTGTTGCA CATATAATGCCAGTT	Amplification of <i>citZ</i> with promotor region

citZ_overex_rev_ne w	AGATCTGAGCTCTTTTCTTTCT TCAAGCGGGATATAC	Amplification of <i>citZ</i> with promotor region
-------------------------	---	--

Table 2: Metabolites and their isobars measured via FI-MS.

metabolite(s)	m/z
citrate/isocitrate/5-dehydro-4-deoxy-D-glucarate [M-H] ⁻	191.020
α-ketoglutarate [M-H] ⁻	145.014
malate [M-H] ⁻	133.014
fumarate [M-H] ⁻	115.004
pyruvate [M-H] ⁻	87.009
lactate/glyceraldehyde/dihydroxyacetone [M-H] ⁻	89.024
acetate [M-H] ⁻	59.014
glucose, galactose, allose, fructose, mannose and myo-inositol [M-H] ⁻	179.056
citrulline [M+H] ⁺	176.103
butyrate [M+H] ⁺	89.060
carnitine [M+H] ⁺	162.112
choline [M+H] ⁺	104.107

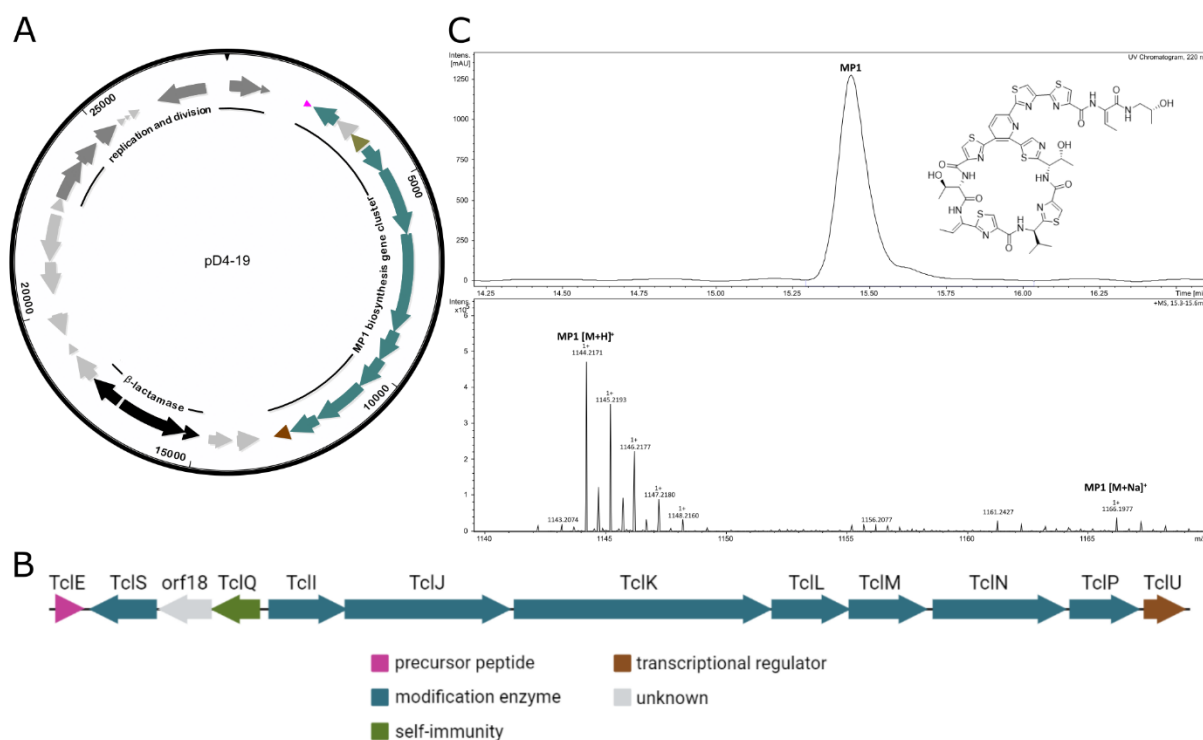


Figure 1. Plasmid pD4-19 encodes the BGC for MP1 production. **A)** Map of plasmid pD4-19 (28 391 bp) that was identified in *S. aureus* D4-19 and that encoded the MP1 BGC (colour), a β -lactamase operon (black), several genes encoding for replication and division factors (dark grey) and hypothetical genes (light grey). **B)** Detailed view of the MP1 BGC. The annotated protein functions are indicated by different colours. Created with BioRender.com. **C)** HPLC-UV-chromatogram (220 nm) of methanol extract from *S. aureus* D4-19 culture pellet. Absorbance was measured in milli absorption units (mAU). The chemical structure of MP1 is depicted. MS spectrum confirming the sum formula of MP1 ($[M+H]^+$, $m/z = 1144.2171$ (found), $m/z = 1144.2173$ (calculated for $C_{48}H_{50}N_{13}O_9S_6$)).

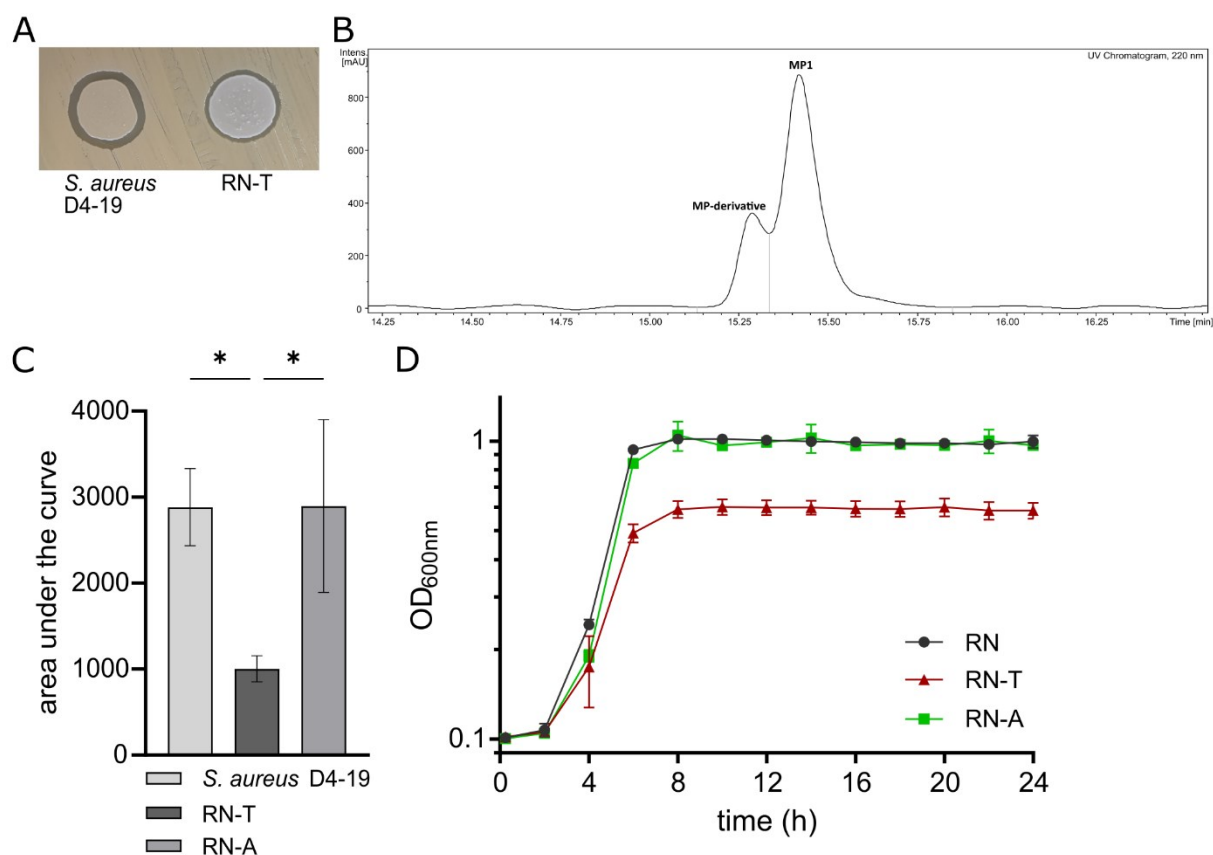


Figure 2. Acquisition of BGC allows production of MP1 but imposes a fitness cost which can be overcome through adaptive evolution. A) Spot assay of the nasal isolate *S. aureus* D4-19 and the *S. aureus* recipient strain RN-T on *S. aureus* USA300 LAC, demonstrating antimicrobial activity of both strains against the indicator strain. **B)** HPLC-UV-chromatogram (220 nm) of cell pellet extracts of RN-T, confirming the production of MP1. An MP-derivative (exact mass $m/z = 1163.2116$), with retention time 15.3 min, is also produced by this strain. **C)** Estimation of the relative quantity of MP1 produced by *S. aureus* strains D4-19, RN-T and RN-A. MP1 amount is estimated as curve integral ($n=3$). The adapted strain RN-A produces similar amounts as the nasal isolate. MP1 production is significantly reduced in the newly transformed strain RN-T. **D)** Growth curves of *S. aureus* RN, RN-T and RN-A grown in BM over 24 hours in a 24 well plate ($n=4$). Statistical significance was determined using an ordinary One-way ANOVA (Tukey's multiple comparisons test) (* $p < 0.05$).

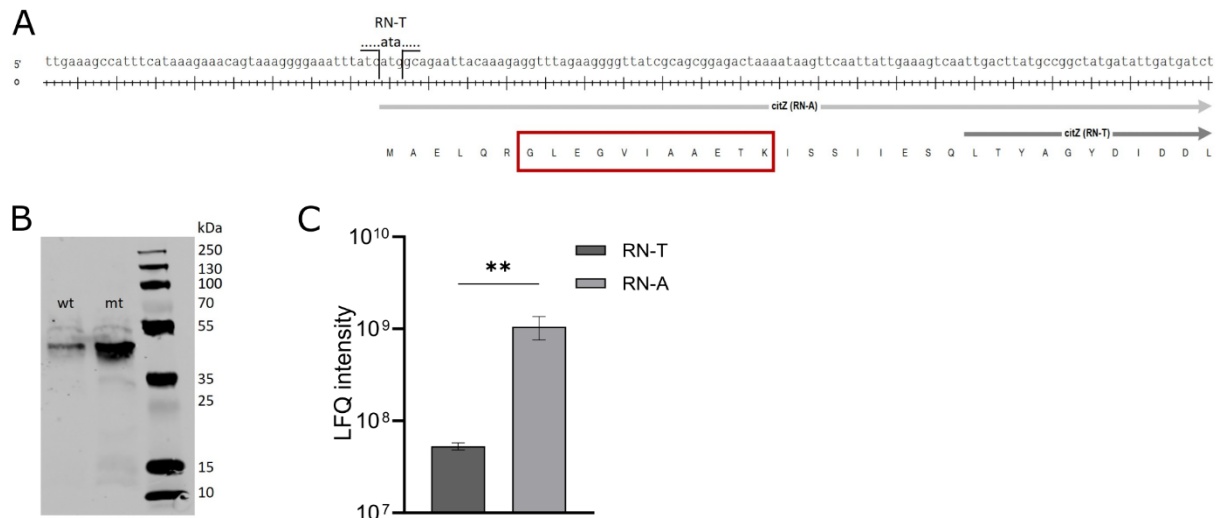


Figure 3. An adaptive mutation in the *citZ* gene increases the levels of the citrate synthase CitZ.

A) Promoter region and start site of *citZ* (ACCFDFCE_01589) gene from RN-T (wt) (dark grey) and RN-A (mt) (light grey). The wt *citZ* has a delayed start codon compared to the mt allele, where ATA is exchanged to ATG, leading to an earlier transcription start of *citZ* in RN-A. This mutation reverts CitZ to a full-size protein, similar to that of other staphylococci. The red box highlights the part of the amino acid sequence of CitZ that was used for the analysis shown in C). **B)** Western blot of cell extracts from *S. aureus* RN4220 pRB473-XylR-6xHis-*citZ* (wt) and *S. aureus* RN4220 pRB473-XylR-6xHis-*citZ* (mt) expressing the wt CitZ protein and the mt CitZ protein, respectively. CitZ has a size of 42.6 kDa and can be detected in both extracts, but in higher amounts in the mt CitZ expressing strain, indicating leaky expression of wt CitZ. **C)** Proteome data were analysed for the presence of the protein fragment GLEGVIAAETK (depicted in A) (n=3) to confirm leaky expression of wt CitZ with ATA as startcodon. The indicated fragment can be found in both proteomes, but with a 20-fold increase in RN-A. Statistical significance was determined using an unpaired t-test (**p < 0.01).

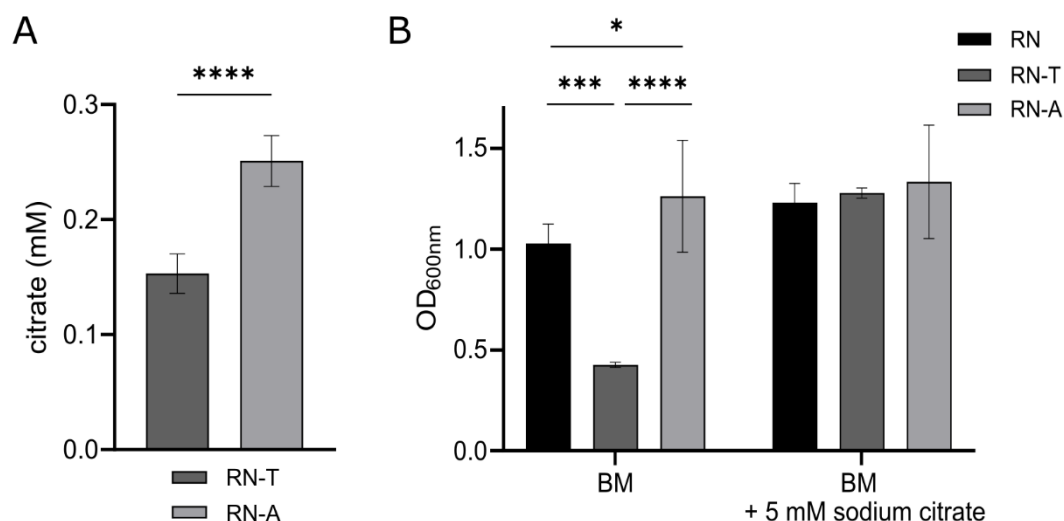


Figure 4. Increased levels of citrate can restore wild type levels of growth in the MP1 BGC recipient strain RN-T. A) Significantly reduced citrate levels in RN-T compared to RN-A were determined using the citrate assay kit from Sigma-Aldrich (n=3). Statistical significance was calculated using an unpaired t-test (****p < 0.0001). **B)** Endpoint OD_{600nm} of *S. aureus* RN, RN-T and RN-A in standard growth medium or medium supplemented with 5 mM sodium citrate. Strains were grown in a 24 well plate for 24 hours (n=3). Statistical significance was calculated using an ordinary One-way ANOVA (Tukey's multiple comparisons test) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

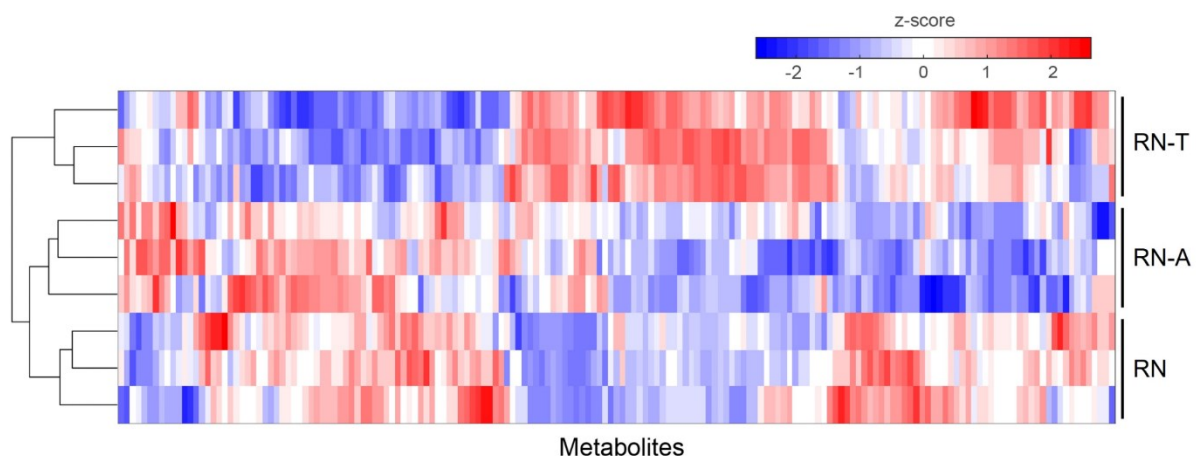


Figure 5. Acquisition of the MP1 BGC alters the metabolite profile of the recipient strain RN-T, an effect that can be reverted by the adaptive mutation. The heatmap shows data from FI-MS analysis for the indicated strains. Depicted are z-score normalized intensities of m/z features in positive and negative ionization mode that were annotated to metabolites (mean of $n = 3$ replicates). Samples were grouped by hierarchical clustering as indicated by dendrograms.

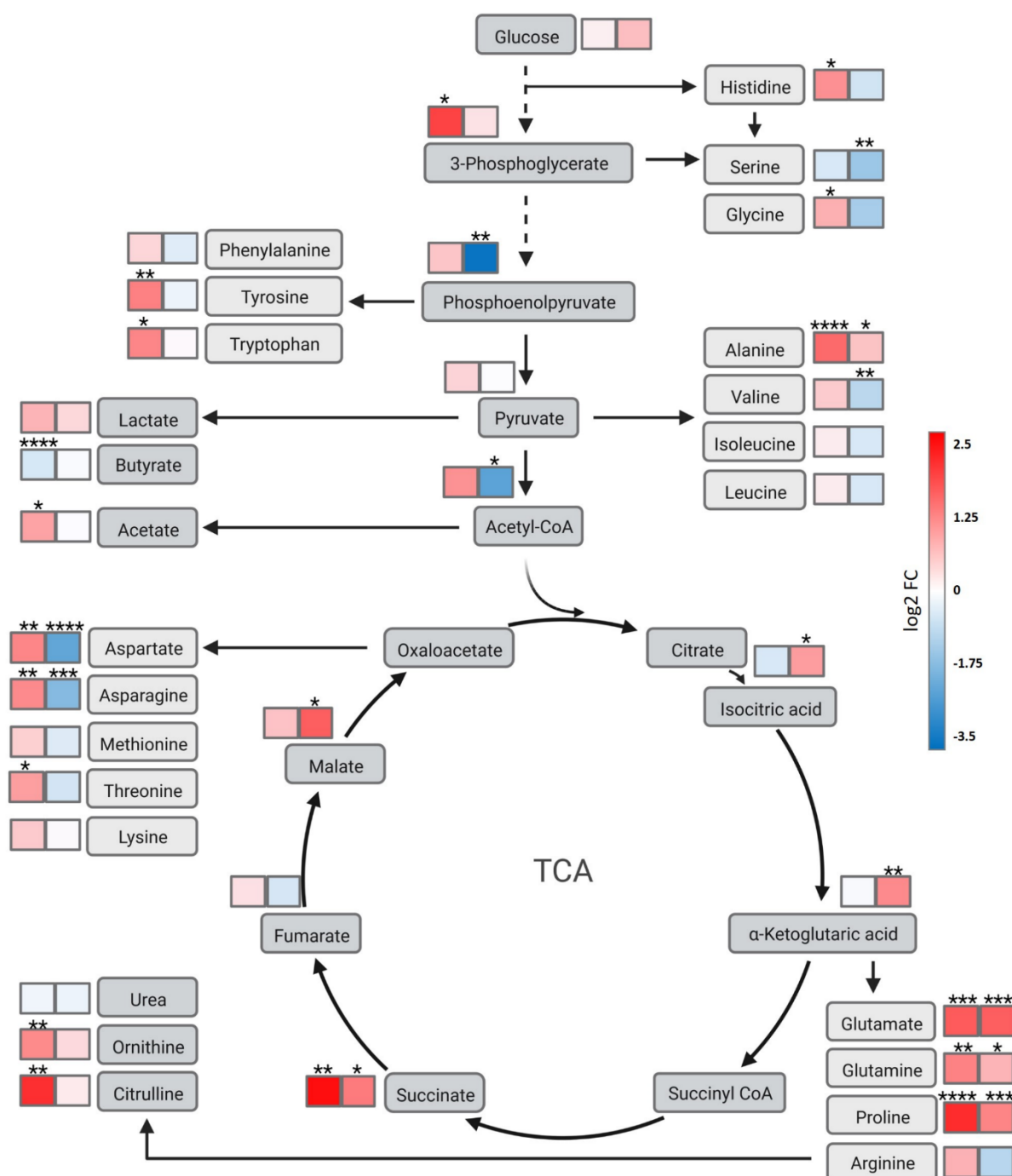


Figure 6. Overview of the metabolic differences between the recipient strain RN-T and the adapted strain. Metabolic differences of RN-T (left box) and RN-A (right box) are shown as log2 fold changes relative to *S. aureus* RN4220. Red: Increase in metabolite levels compared to *S. aureus* RN4220. Blue: Decrease of metabolite levels compared to *S. aureus* RN4220. Metabolic pathways are not depicted in detail. Metabolites were measured via FI-MS and LC-MS/MS. The p-values were calculated using an ordinary One-way ANOVA (Tukey's multiple comparisons test). Statistical significance is indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Created with BioRender.com.

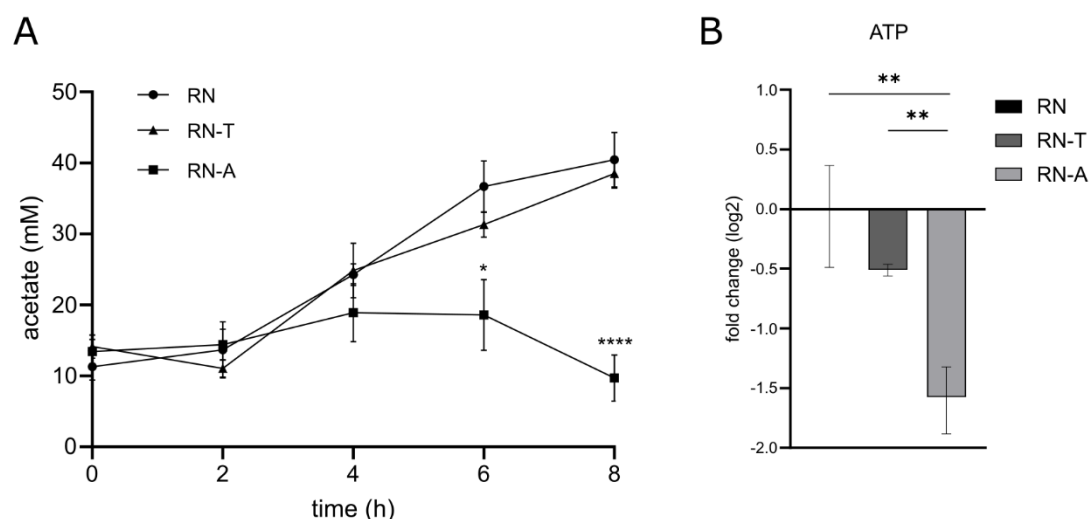


Figure 7. The increased intracellular levels of acetate in RN-T cannot be ascribed to reduced levels of ATP. A) Extracellular acetate levels were measured in *S. aureus* RN, RN-T and RN-A after 0, 2, 4, 6 and 6 hours of growth in BM with the acetate assay kit from Sigma-Aldrich (n=3). **B)** Changes (log₂-fold) of cytoplasmic ATP levels in *S. aureus* RN-T (dark grey) and RN-A (light grey) compared to RN (black) (n=3). ATP was measured via LC-MS/MS. The p-values were calculated using an ordinary One-way ANOVA (Tukey's multiple comparisons test). Statistical significance is indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

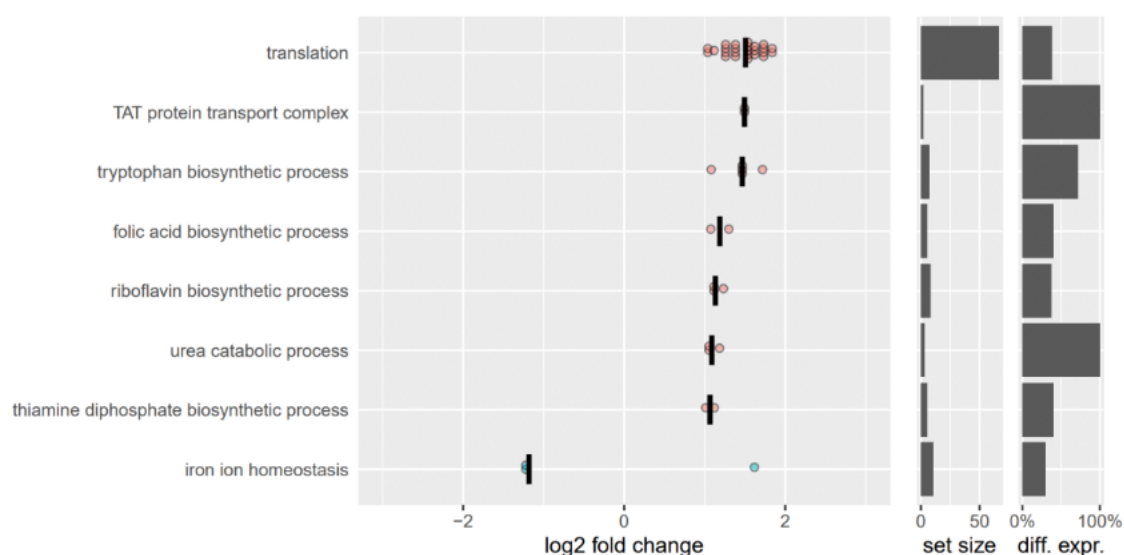


Figure 8. The adaptive mutation increases the overall metabolic activity of strain RN-A as confirmed by transcriptomic analysis. Selection of significantly enriched GO terms and the corresponding differentially expressed genes obtained via RNAseq. Depicted is the log2-fold change in expression levels of *S. aureus* RN-A compared to RN-T. Increased median expression is indicated in red, decreased median expression in blue. The set size indicates the number of genes covered by the GO term. The last column indicates how many genes of the set are differentially expressed (in %).

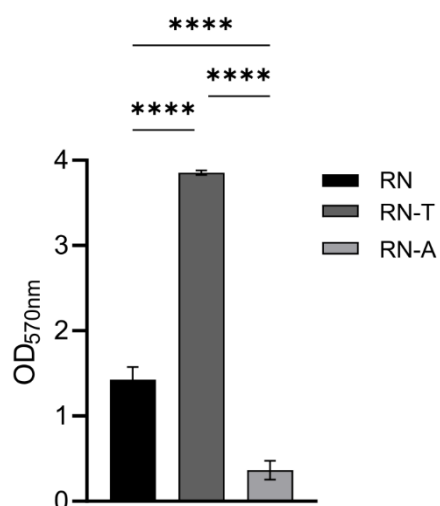


Figure 9. Acquisition of the MP1 BGC is associated with increased levels of biofilm formation.

Biofilm formation of *S. aureus* RN, RN-T and RN-A grown in BM (n=3). Statistical significance was calculated using an ordinary One-way ANOVA (Tukey's multiple comparisons test) (****p < 0.0001).

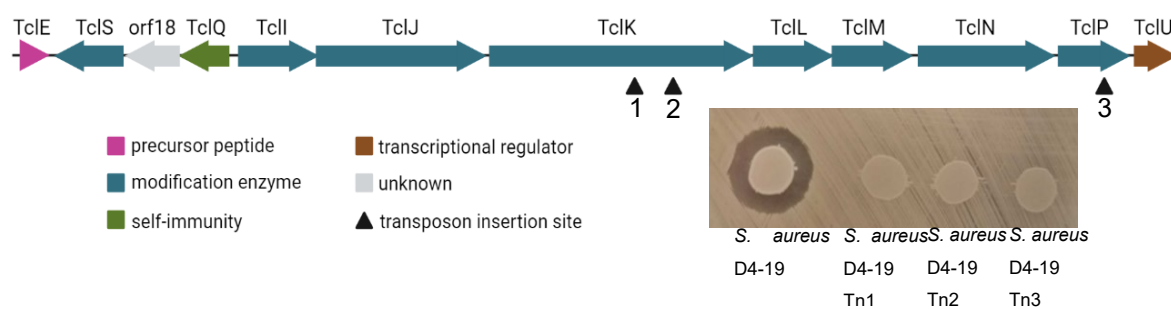


Figure S1: Transposon insertion sites in the MP1 gene cluster of *S. aureus* D4-19 as indicated by arrowheads and spot assay of these mutants on *S. aureus* USA300 LAC to confirm loss of antimicrobial activity.

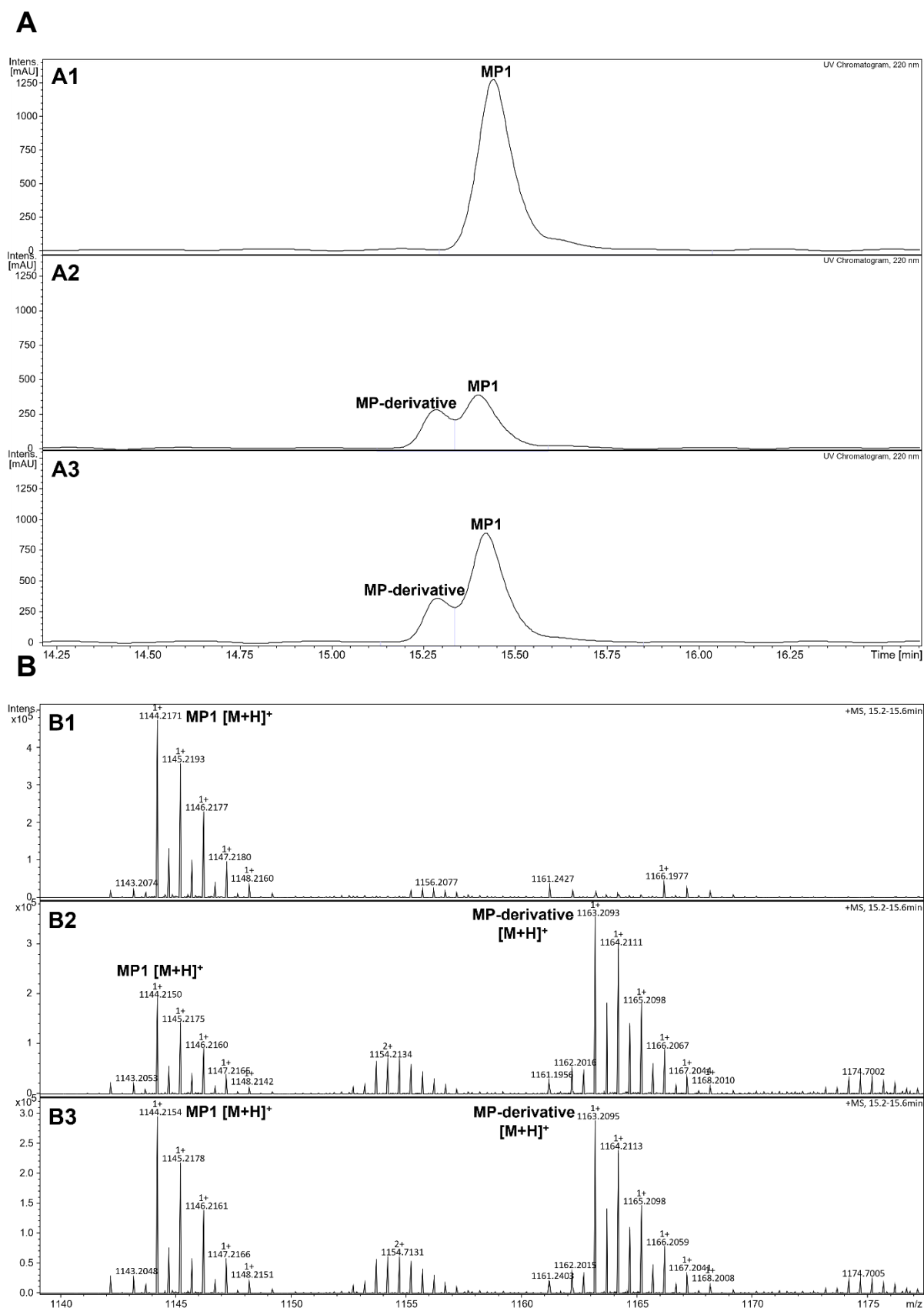


Figure S2: A) HPLC-UV-chromatograms of extracts of *S. aureus* D4-19 (A1), *S. aureus* RN-T (A2) and RN-A (A3). **B)** Mass spectra of micrococci (MP1 ($[M+H]^+$, calculated $m/z = 1144.2173$) and MP-derivative ($[M+H]^+$, calculated $m/z = 1163.2116$)) detected in *S. aureus* D4-19 (B1), *S. aureus* RN-T (B2) and RN-A (B3).

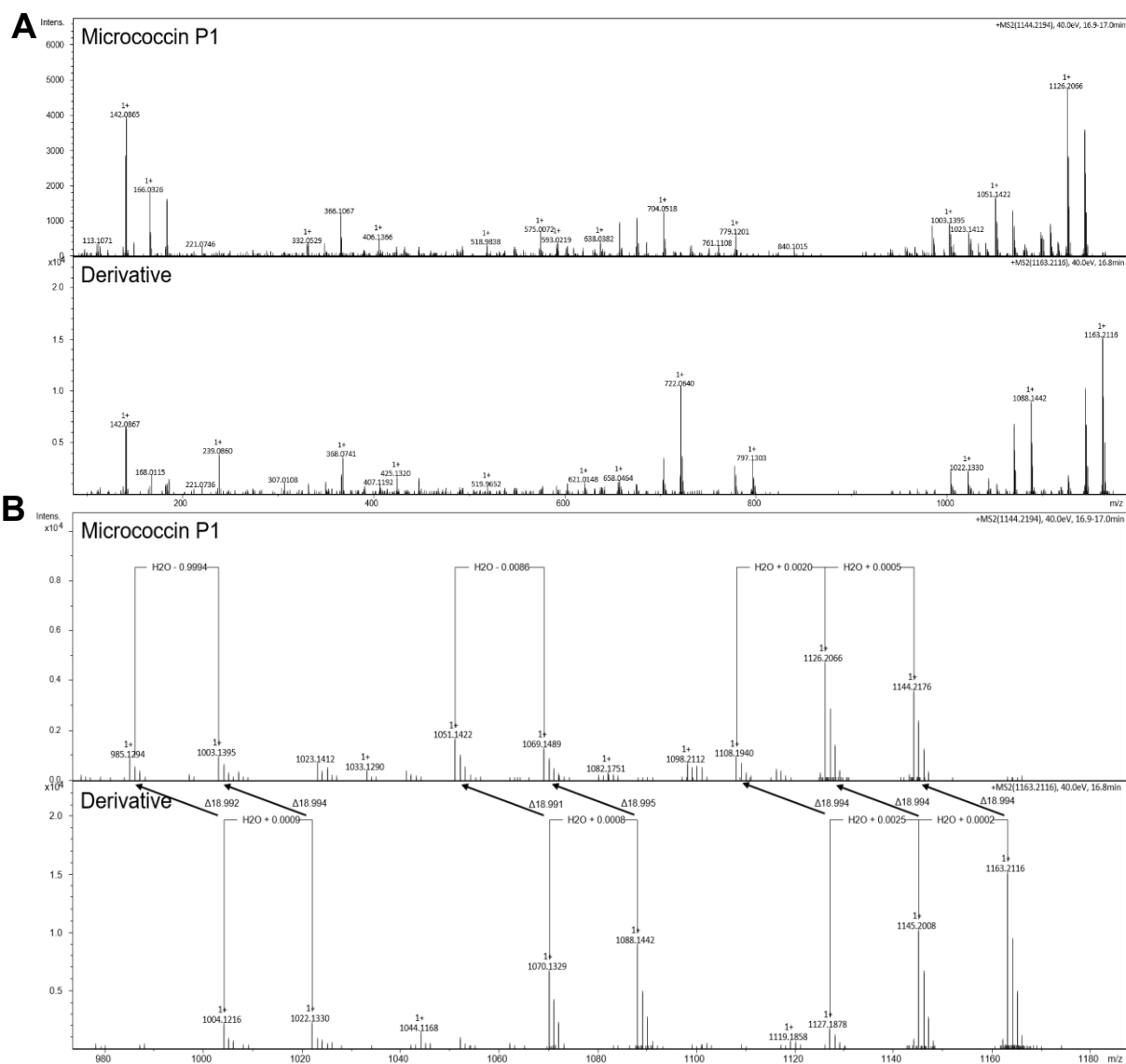


Figure S3: High performance liquid chromatography coupled tandem mass spectra (HPLC-ESI(+)-MS/MS) of MP1 and MP-derivative. **A)** Full tandem mass spectra (MS/MS) of MP1 and the derivative. **B)** Tandem mass spectra (MS/MS) of MP1 ($[M+H]^+$, $m/z = 1144.2171$ (found), 1144.2173 (calculated for $C_{48}H_{50}N_{13}O_9S_6$) and MP-derivative ($m/z = 1163.2116$) with focus on the first fragmentations showing losses of water and mass differences between both compounds. Tandem mass spectra were recorded with high performance liquid chromatography coupled high resolution electrospray mass spectrometer with time-of-flight detection (HPLC-HR-ESI(+)-TOF-MS).

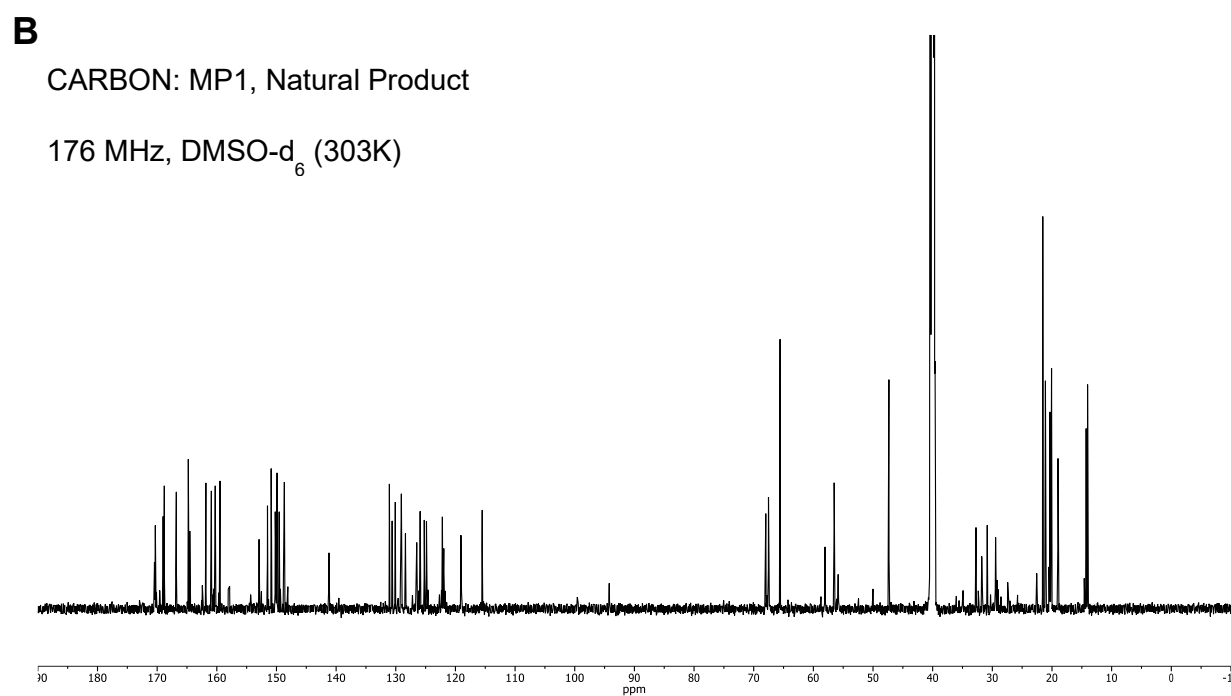
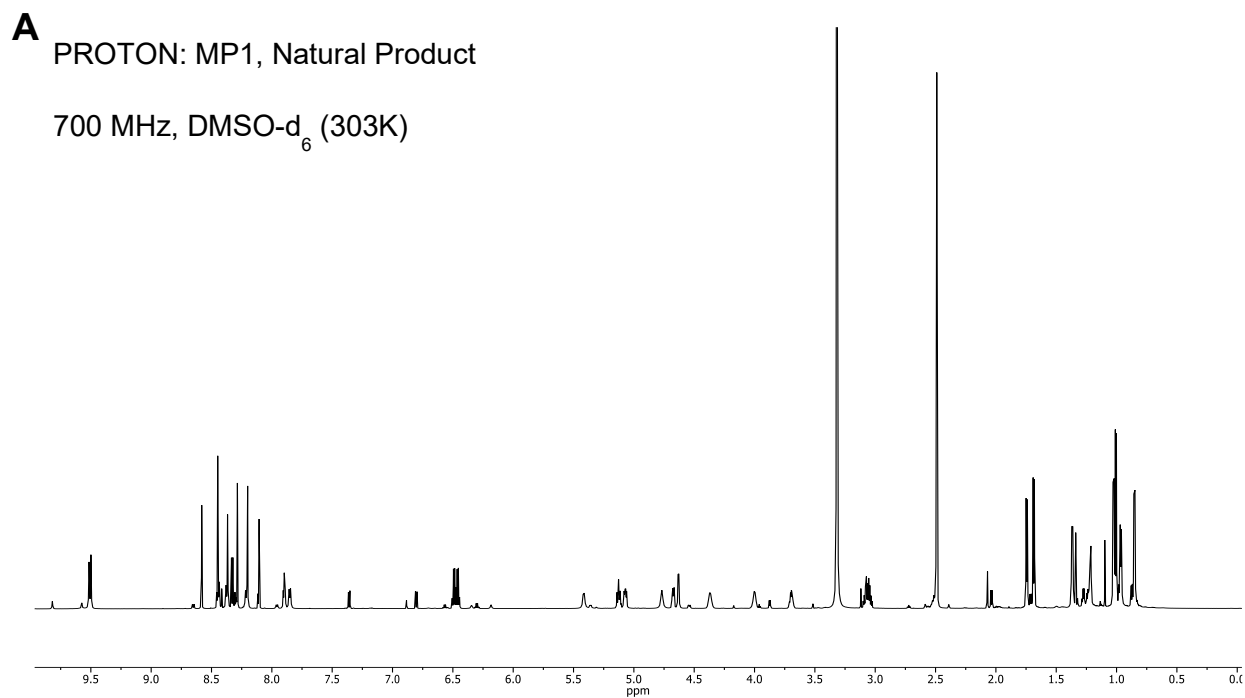


Figure S4: **A)** ^1H NMR spectrum of isolated natural MP1 (10 mg) in DMSO-d₆ (303K). **B)** ^{13}C NMR spectrum of isolated natural M1 (10 mg) in DMSO-d₆ (303K). NMR spectra were recorded on a Bruker AvanceIII-700 (^1H : 700 MHz, ^{13}C : 176 MHz).

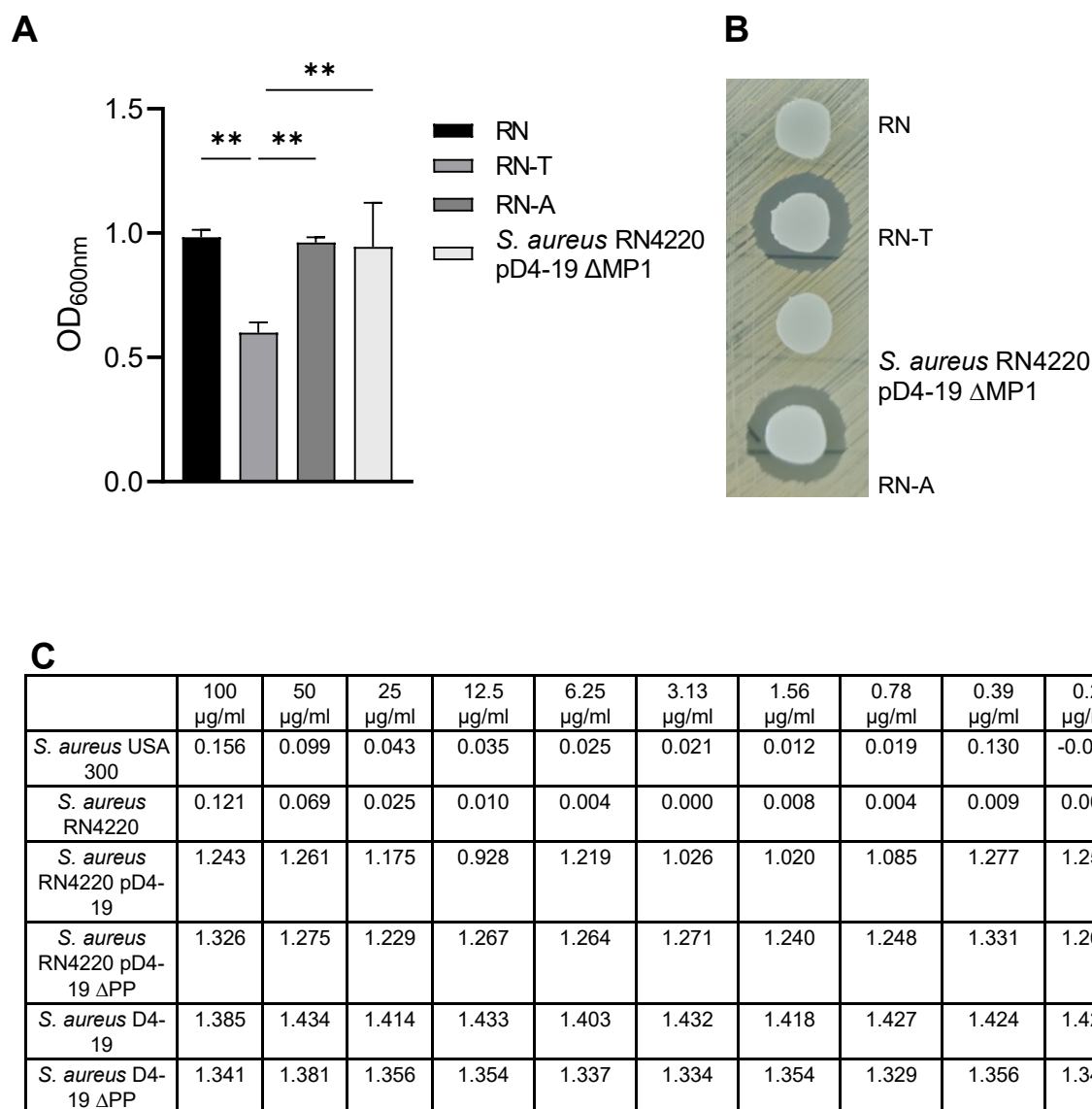


Figure S5: A) Endpoint OD_{600nm} of *S. aureus* RN, RN-T, RN-A and *S. aureus* RN4220 pD4-19 ΔMP1 after 20 h in a 24-well microtiter plate (n=3). **B)** Spot assay of *S. aureus* RN, RN-T, RN-A and *S. aureus* RN4220 pD4-19 ΔMP1 on BM plate with *S. aureus* USA300 LAC as indicator strain. Statistical significance was calculated using an ordinary One-way ANOVA (Tukey's multiple comparisons test) (****p < 0.0001). **C)** MIC values of purified MP1 were assessed against *S. aureus* USA300, *S. aureus* RN4220, *S. aureus* RN4220 pD4-19, *S. aureus* RN4220 pD4-19 ΔPP, *S. aureus* D4-19, *S. aureus* D4-19 ΔPP. Mean of three replicates is shown. The highest concentration of MP1 (100 μg/ml) leads to the formation of a precipitate which explains the higher OD values for the two wild type strains at this MP1 concentration. A growth control (GC) is included where no MP1 was added to the well.

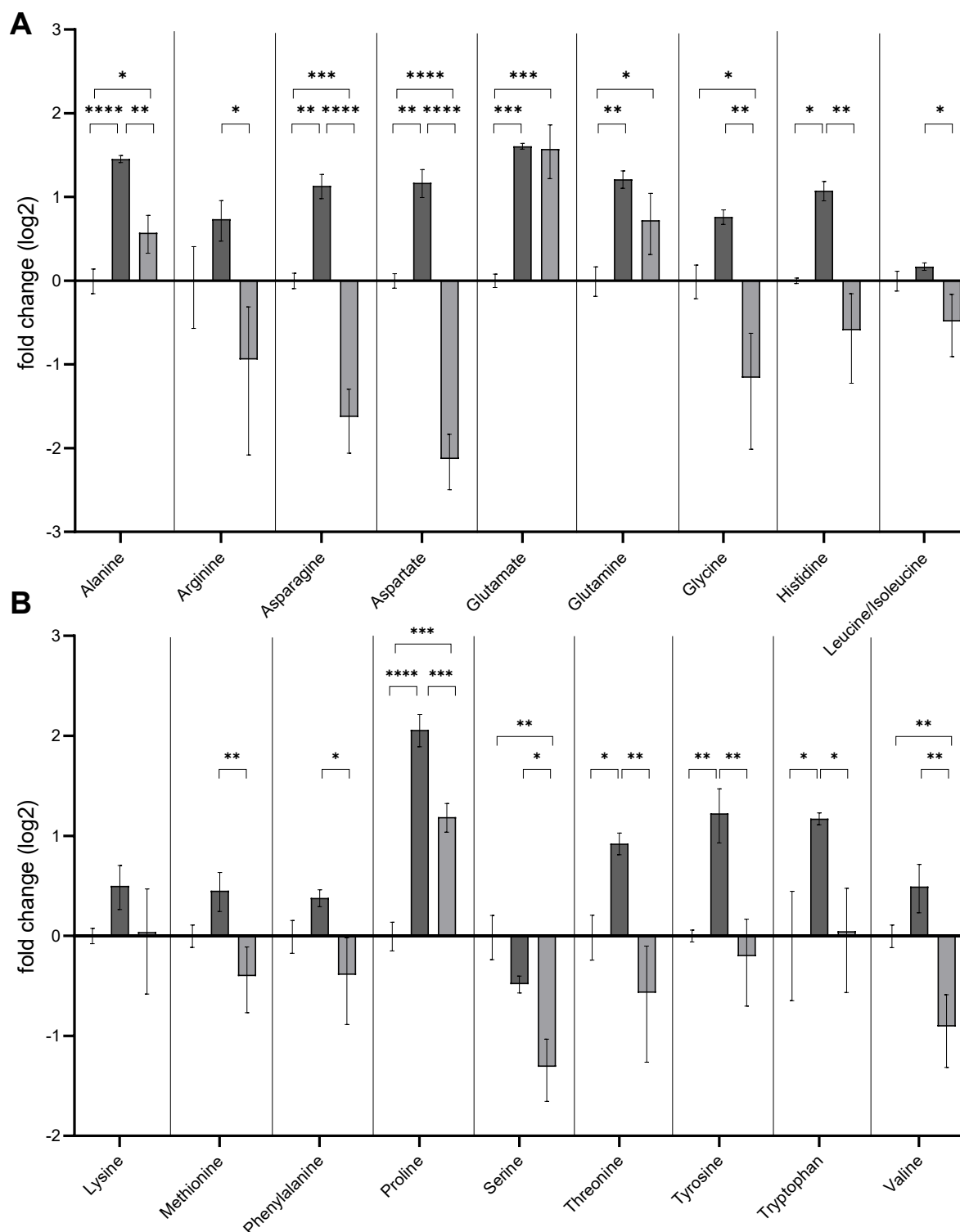


Figure S6: A) Amino acid levels as measured via metabolome analysis in *S. aureus* RN (black), RN-T (dark grey) and RN-A (light grey). Metabolite concentrations are shown as log₂-fold changes that are normalized to *S. aureus* RN4220. All amino acids were measured using LC-MS/MS. **B)** Amino acid levels as detected via metabolome analysis in *S. aureus* RN (black), RN-T (dark grey) and RN-A (light grey). Metabolite concentrations are shown as log₂-fold changes that are normalized to *S. aureus* RN4220. All amino acids were measured using LC-MS/MS. The p-values were calculated using an ordinary One-way ANOVA (Tukey's multiple comparisons test). Statistical significance is indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

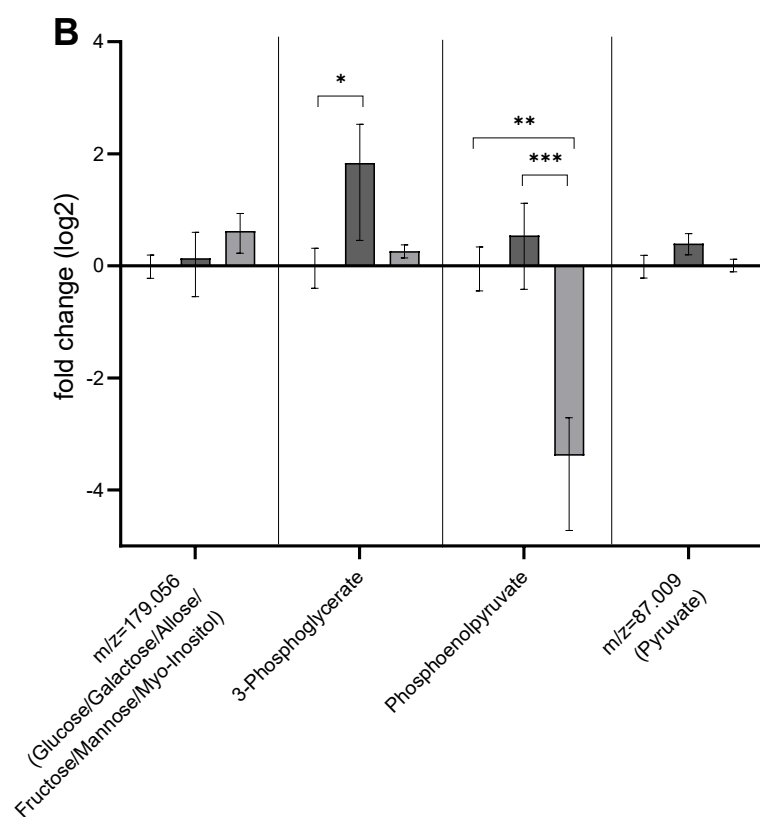
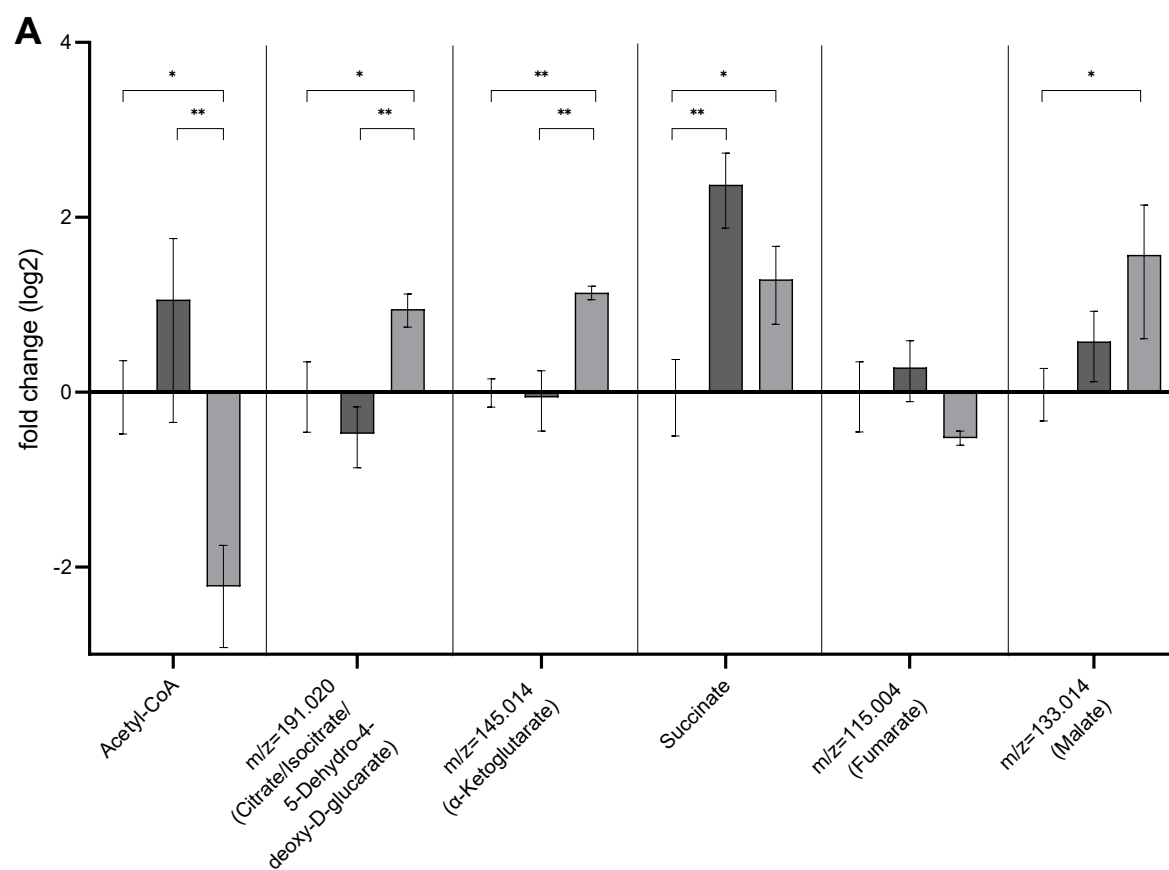


Figure S7: A) TCA intermediates as measured via metabolome analysis in *S. aureus* RN (black), RN-T (dark grey) and RN-A (light grey). Metabolite concentrations (acetyl-CoA and succinate) or ion intensities of *m/z* features (*m/z*=191.020 matches deprotonated citrate and its isobars isocitrate and 5-dehydro-4-deoxy-D-glucarate, *m/z*=145.014 matches deprotonated α -ketoglutarate, *m/z*=115.004 matches deprotonated fumarate and *m/z*=133.014 matches deprotonated malate) are shown as log₂-fold changes that are normalized to *S. aureus* RN4220. **B)** Glycolysis intermediates detected via metabolome analysis in *S. aureus* RN (black), RN-T (dark grey) and RN-A (light grey). Metabolite concentrations (3-phosphoglycerate and phosphoenolpyruvate) or ion intensities of *m/z* features (*m/z*=179.056 matches deprotonated glucose and its isobars galactose, allose, fructose, mannose and myo-inositol and *m/z*=87.009 matches deprotonated pyruvate) are shown as log₂-fold changes that are normalized to *S. aureus* RN4220. The p-values were calculated using an ordinary One-way ANOVA (Tukey's multiple comparisons test). Statistical significance is indicated by **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

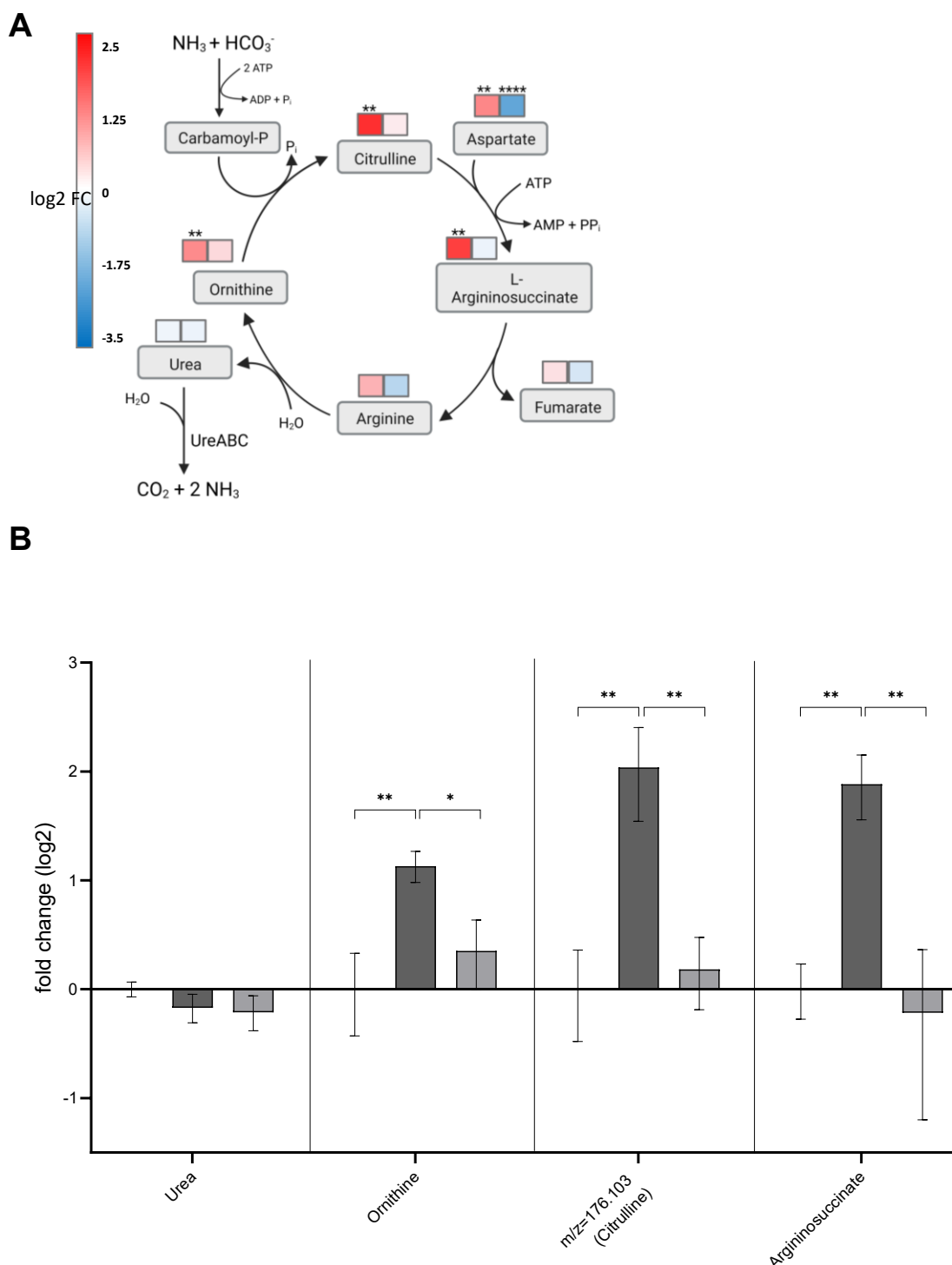


Figure S8: A) Summary of the metabolic differences in urea cycle intermediates between *S. aureus* RN-T and RN-A. The indicated log2 fold changes were calculated for RN-T (left box) and RN-A (right box) vs. *S. aureus* RN4220, respectively. The fold change is indicated by different colours: a decrease in metabolite levels compared to *S. aureus* RN4220 is depicted in blue, an increase in metabolite levels compared to *S. aureus* RN4220 is depicted in red. Created with BioRender.com.

B) Urea cycle intermediates as measured via metabolome analysis in *S. aureus* RN (black), RN-T (dark grey) and RN-A (light grey). Metabolite concentrations (urea, ornithine, argininosuccinate) or ion intensities of m/z features (m/z=176.103 matches protonated citrulline) are shown as log2-fold changes that are normalized to *S. aureus* RN4220. The p-values were calculated using an ordinary

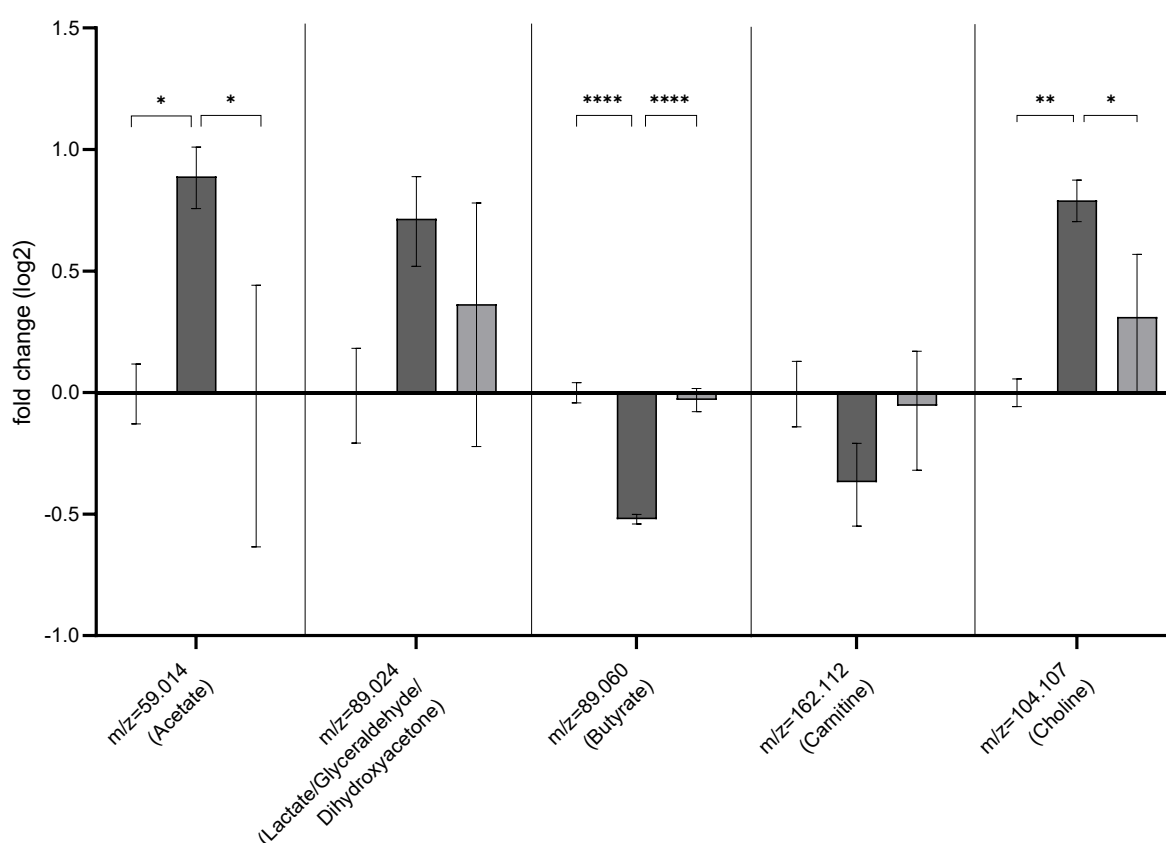
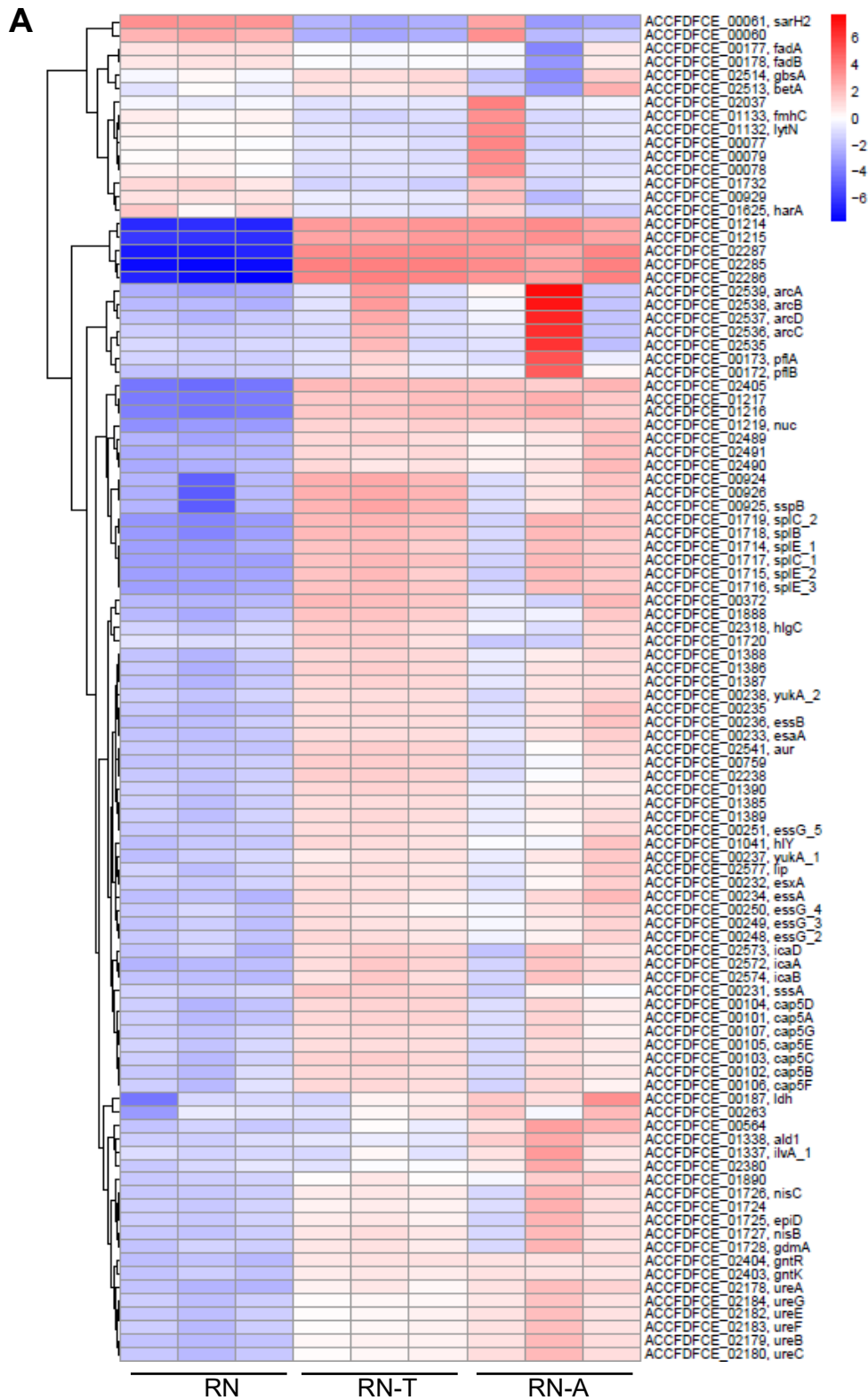


Figure S9: Fermentation products and osmoprotectants as measured via metabolome analysis in *S. aureus* RN (black), RN-T (dark grey) and RN-A (light grey). Ion intensities of m/z features (m/z=59.014 matches deprotonated acetate, m/z=89.024 matches deprotonated lactate and its isobars glyceraldehyde and dihydroxyacetone, m/z=89.060 matches protonated butyrate, m/z=162.112 matches protonated carnitine and m/z=104.107 matches protonated choline) are shown as log₂-fold changes that are normalized to *S. aureus* RN4220. The p-values were calculated using an ordinary One-way ANOVA (Tukey's multiple comparisons test). Statistical significance is indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.



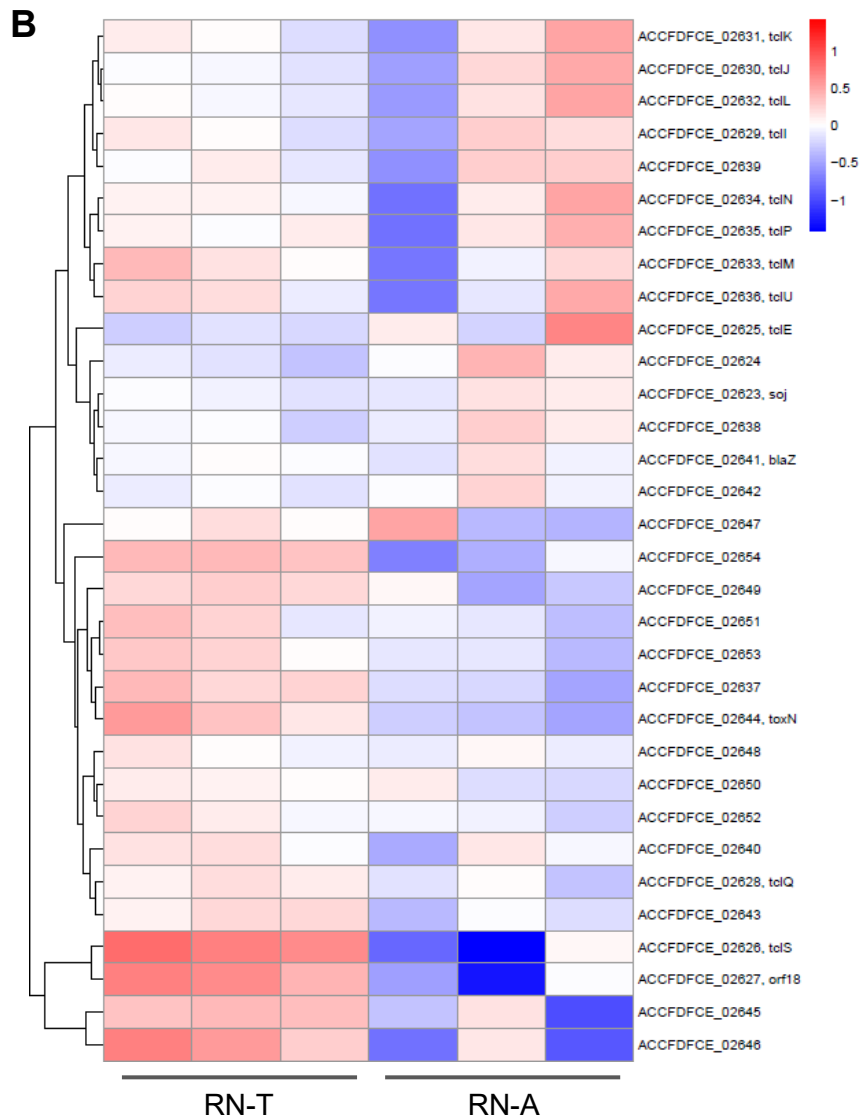


Figure S10: Heat maps showing normalized expression (variance stabilizing transformation) centred on the mean of each row. **A)** Heat map of the 100 most variant genes in *S. aureus* RN, RN-T and RN-A. **B)** Heat map of all genes expressed on pD4-19 in *S. aureus* RN-T and RN-A.

Chapter 5

General Discussion

Feed forward regulation of the microbiome-associated antibiotic lugdunin

The search for new antimicrobial compounds is inevitable, as antimicrobial resistances are on the rise. Therefore, Janek et al. investigated the antimicrobial potential of human nasal *Staphylococcus* spp. strains against other members of the human nasal microbiome and found that 84% of those nasal staphylococcal isolates produce antimicrobial compounds [1]. Further investigation of the antimicrobial potential of *S. lugdunensis* led to the identification of the first microbiome-associated antimicrobial fibupeptide lugdunin [2]. Studies on secretion and self-resistance of *S. lugdunensis* showed, that the transporter LugEF is mainly responsible for secretion of lugdunin outside of the cytoplasm, whereas the transporter LugGH had a pivotal role in self-immunity [3].

In bacteria, functionally related genes often show a specific gene arrangement and are co-transcribed, so-called operons. Since a few decades different studies have been performed on the organization and evolution of such operons [4-7]. It has been shown that transcription of genes in close vicinity to each other are assumptively showing less noise in gene expression and also show a more accurate stoichiometry [5, 7], indicating a putative explanation why many BGCs encode their own regulators. In our study, we aimed to investigate the role of two putative regulators, LugJ and LugR, on the expression of the transporter operon *lugIEFGH* as well as on the biosynthesis operon *lugRABCTDZ*, respectively. Analysis of single-deletion mutants of either *lugJ* or *lugR* led to an increase of lugdunin production and an elevated inhibition of the *S. aureus* test strain, identifying both putative regulators as repressors of their respective operons. This was further confirmed by heterologous expression of *yfp* under the control of either the promoter of *lugI* or *lugR* respectively. This intriguing first result shows an unconventional regulation mechanism, as for other antimicrobial peptides like nisin Z produced by *Lactococcus lactis* [8], congocidine produced by *Streptomyces ambofaciens* ATCC23877 [9] as well as epidermin/gallidermin produced by *Staphylococcus epidermidis* / *Staphylococcus gallinarum* [10, 11] the encoded regulator(s) act as activators on their respective BGCs.

In search for putative inducers for LugR and LugJ, a culture of *S. lugdunensis* IVK28 was extracted and analysed, revealing the end product lugdunin as inducer for both regulators.

Interestingly, the inducing potential on the promoter of *lugI* is comparably higher than the inducing potential on the promoter of *lugR*. Here, the genomic structure of both operons might be pivotal. As for the *lugIEFGH* region, *lugJ* is oppositely orientated to the *lugIEFGH* operon, leading to an increase in transporter expression upon induction but not of the regulator LugJ. Whereas in the *lugRABCTDZ* operon, the first transcribed gene is *lugR* leading to a direct increase of regulator upon induction of the lugdunin biosynthesis operon. Two possible explanations can be found for this phenomenon. Firstly, Krauss, et al. have shown that *S. lugdunensis* is not fully resistant against its own antimicrobial peptide lugdunin [3]. Therefore, having a strict regulation of lugdunin biosynthesis might be a self-protecting trait. Secondly, lugdunin belongs to the leaderless peptides, meaning that during biosynthesis no N-terminal leader peptide is attached to an inactive pre-peptide, which is only cleaved off directly before export out of the producing cell [2]. Hence, the producing cell is not protected from its own antimicrobial peptide [12]. It can be argued, that it might be another self-protecting trait of *S. lugdunensis* to have a fast and reliable inducible transport system for the export of lugdunin.

Interestingly, lugdunin itself serves as the inducer for both regulators, this means that the transcriptional activation of lugdunin biosynthesis and transport seems to be more dependent on population density of *S. lugdunensis*, than on the presence of other competing members of the nasal microbiome. Self-regulation has been described before, e.g., for the biosynthesis of nisin. However, as a two-component system depending on signal transduction instead of direct interaction as for lugdunin. Encoded in the nisin BGC are a histidine sensor kinase (NisK) and a transcriptional regulator (NisR). NisK is localized in the cytoplasmic membrane of *L. lactis* and is the receptor for nisin [8, 13]. Upon extracellular binding of nisin to NisK, a signal transduction cascade is initiated leading to autophosphorylation of NisK [13, 14]. The phosphoryl-group is further transferred to NisR, which is thereby activated and able to bind to the respective promoter region. This leads to further transcriptional activation of nisin biosynthesis and immunity genes [8, 13].

The three derivatives enantio-lugdunin, 2-Ala-lugdunin as well as N-methylthiazolidine-lugdunin were tested for inducing potential and showed no or comparably lesser induction of both regulators. The extremely high specificity of both regulators LugR and LugJ to lugdunin is surprising, as there are some well-known examples for exchangeable inducers. For instance, the regulation of lactose metabolism within the *lac* operon, which consists of *lacZ*, *lacY*, and *lacA* encoding for a β -galactosidase, a permease, and a transferase. Further, *lacI* encoding for an unregulated repressor. When no inducer is present, LacI binds to the promoter of the *lac* operon. Upon binding of the inducer allolactose to LacI, the promoter is released [15, 16]. However, not only allolactose serves as an inducer but also other molecules

like isopropyl- β -D-1-thiogalactoside (IPTG) as well as methyl-1-thio- β -D-galactopyranoside (TMG), both showing a comparable affinity to LacI as allolactose [17-19].

In this study we showed, that still not every lesson is learned about lugdunin production by *S. lugdunensis*. Interestingly, the regulation is based on a feed forward-like mechanism by the end product lugdunin. However, these studies were initially performed in a heterologous *S. aureus* strain. To further elucidate the regulation of lugdunin biosynthesis and transport, further experiments in *S. lugdunensis* are needed.

Unidentified metallophore in *S. lugdunensis* with copper-binding properties

Staphylococci are known producers of various secondary metabolites like antimicrobial peptides or metallophores [20]. Examples are capidermin produced by *S. capitis* CIT060 [21], romsacin produced by *S. haemolyticus* [22], staphylopine produced by *S. aureus* [23], as well as the recently identified epifadin produced by *S. epidermidis* [24].

Whereas the lugdunin BGC of *S. lugdunensis* was extensively characterized as novel NRPS system, Heilbronner et al. showed by sequencing of two *S. lugdunensis* strains and other staphylococci, that both *S. lugdunensis* strains harbour three NRPS cluster of which only one is comparable to clusters found in other staphylococci [25]. In our study we aimed to identify the product of the NRPSIII (named *lgb* BGC) produced by *S. lugdunensis* N920143. We were able to identify ulbactin F as the product of this NRPS BGC. Bioinformatic studies have shown, that the *lgb* BGC comprises 13 genes encoding proteins necessary for biosynthesis and transport, however no regulator was found. Komaki et al. analysed the draft genome sequence of ulbactin F-producing *Brevibacillus brevis* TP-B0800. The study only revealed the presence of a biosynthesis apparatus consisting of seven genes, with no hints on possible transporters [26]. We found, that the ulbactin F BGC from *S. lugdunensis* shows partial homology to the equibactin BGC (*eqb*) produced by *Streptococcus equi*, which indicated a putative role for iron acquisition in *S. lugdunensis* by the unknown metabolite. Yersiniabactin is structurally comparable metallophore to equibactin, produced by *Y. pestis*. The yersiniabactin BGC comprises 11 genes including a salicylate synthase and a regulator, both missing in the ulbactin F gene clusters [27]. A recently identified salicylate-containing metallophore is anthrochelin (*ant*) produced by *Luteibacter anthropi* [28]. The *ant* BGC comprises 15 genes and it was compared to various other BGCs, among them ulbactin F BGC from *B. brevis*, where only little homology was found. Furthermore, encoded in the *ant* BGC are an isochorismate synthase and an isochorismate lyase, providing *L. anthropi* with the needed salicylate for anthrochelin in comparison to the *lgb* and *eqb* BGC where genes encoding for salicylate biosynthesis are missing [28]. Therefore, *S. lugdunensis* is dependent on the salicylate uptake for ulbactin F production.

As previously mentioned, the *lgb* BGC does not encode for a regulator. Therefore, metal-dependent master regulators were tested for a regulatory effect on the *lgb* BGC. We found, that both FUR and ZUR have a regulatory influence on the expression of ulbactin F in *S. lugdunensis*, indicating that ulbactin F is only expressed under iron- and/or zinc-limiting conditions. Fluorescence titration of metals to analyse the metal affinity of ulbactin F however has shown, that ulbactin F does only fairly bind iron and no binding of zinc was observed, in contrast to the previously described ulbactin F from *B. brevis*. On the other hand, we observed the highest affinity for copper, identifying ulbactin F as a putative chalkophore. One of the best studied examples of chalkophores are methanobactins produced by methanotrophic bacteria [29-31]. Chalkophores have also been identified in other bacteria, for example chalkophomycin from *Streptomyces* sp. CB00271 [32]. Methanobactins have been shown to feature a high affinity to both oxidative states of copper (Cu^+ and Cu^{2+}) [33]. A study of Choi et al. revealed, that methanobactins not only bind copper but are also able to bind silver, aluminium, or lead, whereas only poor binding was observed for cadmium, iron, manganese, or zinc [34]. Indicating, that ulbactin F might have chalkophore activity in *S. lugdunensis*. For other metallophores binding of other metals next to the one with the highest affinity has been observed. The siderophore yersiniabactin was found to play a role in acquisition of nickel [35], copper [36] as well as gallium or cobalt [37]. Also, binding affinities for various divalent metals have been described for pseudopaline produced by *P. aeruginosa* [38]. Ghsssein et al., have shown that staphylopine from *S. aureus* is a broad-spectrum metallophore able to bind iron, zinc or copper [23]. Also, the recently discovered anthrochelin has been identified as a broad-spectrum metallophore for *L. anthropic*, including binding of iron, copper, nickel or gallium [28].

Our studies were mainly performed using synthetically-derived ulbactin F. Therefore, these results still need to be confirmed using natural ulbactin F produced by *S. lugdunensis*. Further *in vivo* studies are needed to comprehensively understand the role of ulbactin F-production and its *in vivo* relevance.

Metabolic burden after horizontal gene transfer of micrococcin P1 in staphylococci

Bacteria are not only able to vertically transmit their DNA from an ancestor to a descendant cell during cell division, but also to transmit their DNA horizontally to more or less closely related organisms [39, 40]. The three main forms of horizontal gene transfer (HGT) are transformation (uptake of free DNA), transduction (uptake of DNA provided by bacteriophages) as well as conjugation (transfer of single stranded DNA from a donor to a recipient by cell-cell-contact). Plasmids as well as gene clusters from the bacterial chromosome can be shared via HGT [41]. Plasmids often play an evolutionary role, as they can encode BGCs which positively contribute to their hosts fitness [41-43].

Production of micrococcin P1 (MP1) has been described for many staphylococcal species, amongst them *S. equorum* [44], *S. felis* [45], *S. hominis* [46, 47], *S. aureus* [47], or *S. epidermidis* [48]. The MP1 BGC is known to mainly reside on a plasmid in association with mobilization genes, therefore displaying distinct features for transmission.

The burden of acquiring such huge plasmids encoding antimicrobial compounds or other secondary metabolites on prokaryotes has not yet been extensively studied. Plasmid acquisition and carriage is often associated with a loss of fitness for the host, meaning that in theory plasmids should be eliminated from of a bacterial population. However, plasmids remain a common genetic element in prokaryotes, a phenomenon called ‘the plasmid paradox’ [49-52]. In our study, acquisition of the MP1 plasmid pD4-19 by *S. aureus* RN4220 led to a significant decrease in bacterial fitness, which was reverted by a mutation in the citrate synthase gene *citZ*. Dorado-Morales et al. have shown that the uptake of the plasmid pUR2940, encoding various multi-resistance genes but no antimicrobial compound, by *S. aureus* was associated with high fitness costs for the host [50]. Passaging experiments led to an evolution in the plasmid leading to a loss of antibiotic resistance genes [50]. Arguably, the pD4-19 plasmid encodes a toxin-antitoxin system enabling *S. aureus* RN4220 to possibly withstand microbiome-mediated pressure whilst protecting the producers, indicating a possible explanation for higher plasmid stability than pUR2940.

Adaptive evolution between acquired plasmids and their hosts has been described as ‘compensatory evolution’ leading to a stable interaction between bacteria and plasmids [52-54]. Biosynthetic burdens of plasmids are highly associated with elevated expression levels of plasmid-encoded genes as they tend to have a higher dosage effect than genes encoded on the chromosome [55, 56]. This leads to higher energy costs due to the increase of tRNA and amino acid consumption and therefore ultimately to a reduced fitness of the host [56-58]. In order to keep such a costly plasmid, bacteria adopt to successfully establish the plasmid within their genome [52-54]. Deletion or rearrangement of genetic regions have been mainly described for metabolic adaptation of a receiving strain [59, 60] further also compensatory mutations either within the plasmid or the chromosome [61-63].

Final conclusion

In this study we showed, that *S. lugdunensis* IVK28 regulates lugdunin biosynthesis and transport to outcompete *S. aureus* in a feed-forward mechanism. We found that lugdunin itself acts as a ligand on both regulators. In addition to the lugdunin NRPS, a further BGC in *S. lugdunensis* N920143 was analysed and found to encode ulbactin F, a previously unidentified metallophore in *S. lugdunensis*. First experiments revealed that ulbactin F features a copper-binding affinity. Further, we could show that *S. aureus* is able to integrate further

BGCs in its genome via horizontal gene transfer and adaptive evolution to overcome high metabolic costs of the newly acquired BGC. We can conclude, that the biosynthetic potential of *S. lugdunensis* and *S. aureus* is complex and still not fully understood. We can expect comprehensive and interesting studies about nasal microbiome defining interactions between staphylococci and other nasal microbiome-members.

References

1. Janek, D., et al., *High Frequency and Diversity of Antimicrobial Activities Produced by Nasal Staphylococcus Strains against Bacterial Competitors*. PLoS Pathog, 2016. **12**(8): p. e1005812.
2. Zipperer, A., et al., *Human commensals producing a novel antibiotic impair pathogen colonization*. Nature, 2016. **535**(7613): p. 511-6.
3. Krauss, S., et al., *Secretion of and Self-Resistance to the Novel Fibupeptide Antimicrobial Lugdunin by Distinct ABC Transporters in Staphylococcus lugdunensis*. Antimicrob Agents Chemother, 2020. **65**(1).
4. Dandekar, T., et al., *Conservation of gene order: a fingerprint of proteins that physically interact*. Trends Biochem Sci, 1998. **23**(9): p. 324-8.
5. Swain, P.S., *Efficient attenuation of stochasticity in gene expression through post-transcriptional control*. J Mol Biol, 2004. **344**(4): p. 965-76.
6. Price, M.N., A.P. Arkin, and E.J. Alm, *The life-cycle of operons*. PLoS Genet, 2006. **2**(6): p. e96.
7. Rocha, E.P., *The organization of the bacterial genome*. Annu Rev Genet, 2008. **42**: p. 211-33.
8. Kuipers, O.P., et al., *Autoregulation of nisin biosynthesis in Lactococcus lactis by signal transduction*. J Biol Chem, 1995. **270**(45): p. 27299-304.
9. Vingadassalon, A., et al., *Transcriptional Regulation of Congocidine (Netropsin) Biosynthesis and Resistance*. Appl Environ Microbiol, 2021. **87**(24): p. e0138021.
10. Peschel, A., et al., *Secretion of the lantibiotics epidermin and gallidermin: sequence analysis of the genes gdmT and gdmH, their influence on epidermin production and their regulation by EpiQ*. Mol Gen Genet, 1997. **254**(3): p. 312-8.
11. Gotz, F., et al., *Epidermin and gallidermin: Staphylococcal lantibiotics*. Int J Med Microbiol, 2014. **304**(1): p. 63-71.
12. Oman, T.J. and W.A. van der Donk, *Follow the leader: the use of leader peptides to guide natural product biosynthesis*. Nat Chem Biol, 2010. **6**(1): p. 9-18.
13. Engelke, G., et al., *Regulation of nisin biosynthesis and immunity in Lactococcus lactis 6F3*. Appl Environ Microbiol, 1994. **60**(3): p. 814-25.
14. Kleerebezem, M., *Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis*. Peptides, 2004. **25**(9): p. 1405-14.
15. Beckwith, J.R., *Regulation of the lac operon. Recent studies on the regulation of lactose metabolism in Escherichia coli support the operon model*. Science, 1967. **156**(3775): p. 597-604.
16. Jacob, F. and J. Monod, *Genetic regulatory mechanisms in the synthesis of proteins*. J Mol Biol, 1961. **3**: p. 318-56.
17. Barkley, M.D., et al., *Interaction of effecting ligands with lac repressor and repressor-operator complex*. Biochemistry, 1975. **14**(8): p. 1700-12.
18. Kercher, M.A., P. Lu, and M. Lewis, *Lac repressor-operator complex*. Curr Opin Struct Biol, 1997. **7**(1): p. 76-85.
19. Marbach, A. and K. Bettenbrock, *lac operon induction in Escherichia coli: Systematic comparison of IPTG and TMG induction and influence of the transacetylase LacA*. J Biotechnol, 2012. **157**(1): p. 82-8.
20. Torres Salazar, B.O., et al., *Secondary Metabolites Governing Microbiome Interaction of Staphylococcal Pathogens and Commensals*. Microb Physiol, 2021. **31**(3): p. 198-216.

21. Lynch, D., et al., *Identification and characterisation of capidermicin, a novel bacteriocin produced by Staphylococcus capitis*. PLoS One, 2019. **14**(10): p. e0223541.
22. Wolden, R., et al., *The novel bacteriocin romsacin from Staphylococcus haemolyticus inhibits Gram-positive WHO priority pathogens*. Microbiol Spectr, 2023. **11**(6): p. e0086923.
23. Ghssein, G., et al., *Biosynthesis of a broad-spectrum nicotianamine-like metallophore in Staphylococcus aureus*. Science, 2016. **352**(6289): p. 1105-9.
24. Torres Salazar, B.O., et al., *Commensal production of a broad-spectrum and short-lived antimicrobial peptide polyene eliminates nasal Staphylococcus aureus*. Nat Microbiol, 2024. **9**(1): p. 200-213.
25. Heilbronner, S., et al., *Genome sequence of Staphylococcus lugdunensis N920143 allows identification of putative colonization and virulence factors*. FEMS Microbiol Lett, 2011. **322**(1): p. 60-7.
26. Komaki, H., et al., *Draft genome sequence of a sponge-derived Brevibacillus sp. TP-B0800, a producer of ulbactins with tumor cell migration inhibitory activity*. Gene Reports, 2016. **5**: p. 140-143.
27. Perry, R.D. and J.D. Fetherston, *Yersiniabactin iron uptake: mechanisms and role in Yersinia pestis pathogenesis*. Microbes Infect, 2011. **13**(10): p. 808-17.
28. Buttner, H., et al., *Discovery and Biosynthesis of Anthrochelin, a Growth-Promoting Metallophore of the Human Pathogen Luteibacter anthropi*. Chembiochem, 2023. **24**(17): p. e202300322.
29. Kenney, G.E. and A.C. Rosenzweig, *Chemistry and biology of the copper chelator methanobactin*. ACS Chem Biol, 2012. **7**(2): p. 260-8.
30. Farhan Ul-Haque, M., et al., *Methanobactin from Methylocystis sp. strain SB2 affects gene expression and methane monooxygenase activity in Methylosinus trichosporium OB3b*. Appl Environ Microbiol, 2015. **81**(7): p. 2466-73.
31. Kenney, G.E., et al., *The biosynthesis of methanobactin*. Science, 2018. **359**(6382): p. 1411-1416.
32. Gong, B., et al., *Characterization of Chalkophomycin, a Copper(II) Metallophore with an Unprecedented Molecular Architecture*. J Am Chem Soc, 2021. **143**(49): p. 20579-20584.
33. El Ghazouani, A., et al., *Variations in methanobactin structure influences copper utilization by methane-oxidizing bacteria*. Proc Natl Acad Sci U S A, 2012. **109**(22): p. 8400-4.
34. Choi, D.W., et al., *Spectral and thermodynamic properties of Ag(I), Au(III), Cd(II), Co(II), Fe(III), Hg(II), Mn(II), Ni(II), Pb(II), U(IV), and Zn(II) binding by methanobactin from Methylosinus trichosporium OB3b*. J Inorg Biochem, 2006. **100**(12): p. 2150-61.
35. Robinson, A.E., et al., *Uropathogenic enterobacteria use the yersiniabactin metallophore system to acquire nickel*. J Biol Chem, 2018. **293**(39): p. 14953-14961.
36. Koh, E.I., et al., *Copper import in Escherichia coli by the yersiniabactin metallophore system*. Nat Chem Biol, 2017. **13**(9): p. 1016-1021.
37. Koh, E.I., et al., *Metal selectivity by the virulence-associated yersiniabactin metallophore system*. Metallomics, 2015. **7**(6): p. 1011-22.
38. Zhang, J., et al., *De novo synthesis, structural assignment and biological evaluation of pseudopaline, a metallophore produced by Pseudomonas aeruginosa*. Chem Sci, 2019. **10**(27): p. 6635-6641.
39. Tatum, E.L. and J. Lederberg, *Gene Recombination in the Bacterium Escherichia coli*. J Bacteriol, 1947. **53**(6): p. 673-84.

40. Griffith, F., *The Significance of Pneumococcal Types*. J Hyg (Lond), 1928. **27**(2): p. 113-59.
41. Rankin, D.J., E.P. Rocha, and S.P. Brown, *What traits are carried on mobile genetic elements, and why?* Heredity (Edinb), 2011. **106**(1): p. 1-10.
42. San Millan, A., et al., *Multicopy plasmids potentiate the evolution of antibiotic resistance in bacteria*. Nat Ecol Evol, 2016. **1**(1): p. 10.
43. Saati-Santamaria, Z., *Global Map of Specialized Metabolites Encoded in Prokaryotic Plasmids*. Microbiol Spectr, 2023. **11**(4): p. e0152323.
44. Carnio, M.C., et al., *Pyridinyl polythiazole class peptide antibiotic micrococcin P1, secreted by foodborne Staphylococcus equorum WS2733, is biosynthesized nonribosomally*. Eur J Biochem, 2001. **268**(24): p. 6390-401.
45. O'Neill, A.M., et al., *Antimicrobials from a feline commensal bacterium inhibit skin infection by drug-resistant S. pseudintermedius*. Elife, 2021. **10**.
46. Liu, Y., et al., *Skin microbiota analysis-inspired development of novel anti-infectives*. Microbiome, 2020. **8**(1): p. 85.
47. Fernandez-Fernandez, R., et al., *Detection and evaluation of the antimicrobial activity of Micrococcin P1 isolated from commensal and environmental staphylococcal isolates against MRSA*. Int J Antimicrob Agents, 2023. **62**(5): p. 106965.
48. Bennallack, P.R., et al., *Characterization of a novel plasmid-borne thiopeptide gene cluster in Staphylococcus epidermidis strain 115*. J Bacteriol, 2014. **196**(24): p. 4344-50.
49. San Millan, A. and R.C. MacLean, *Fitness Costs of Plasmids: a Limit to Plasmid Transmission*. Microbiol Spectr, 2017. **5**(5).
50. Dorado-Morales, P., et al., *Fitness Cost Evolution of Natural Plasmids of Staphylococcus aureus*. mBio, 2021. **12**(1).
51. Prenskey, H., et al., *Conjugation dynamics depend on both the plasmid acquisition cost and the fitness cost*. Mol Syst Biol, 2021. **17**(3): p. e9913.
52. Harrison, E. and M.A. Brockhurst, *Plasmid-mediated horizontal gene transfer is a coevolutionary process*. Trends Microbiol, 2012. **20**(6): p. 262-7.
53. Bouma, J.E. and R.E. Lenski, *Evolution of a bacteria/plasmid association*. Nature, 1988. **335**(6188): p. 351-2.
54. San Millan, A., et al., *Positive selection and compensatory adaptation interact to stabilize non-transmissible plasmids*. Nat Commun, 2014. **5**: p. 5208.
55. Hall, J.P.J., et al., *Plasmid fitness costs are caused by specific genetic conflicts enabling resolution by compensatory mutation*. PLoS Biol, 2021. **19**(10): p. e3001225.
56. San Millan, A., et al., *Integrative analysis of fitness and metabolic effects of plasmids in Pseudomonas aeruginosa PAO1*. ISME J, 2018. **12**(12): p. 3014-3024.
57. Sorek, R., et al., *Genome-wide experimental determination of barriers to horizontal gene transfer*. Science, 2007. **318**(5855): p. 1449-52.
58. Tuller, T., et al., *Association between translation efficiency and horizontal gene transfer within microbial communities*. Nucleic Acids Res, 2011. **39**(11): p. 4743-55.
59. Zhang, J.F., et al., *A Trade-Off for Maintenance of Multidrug-Resistant IncHI2 Plasmids in Salmonella enterica Serovar Typhimurium through Adaptive Evolution*. mSystems, 2022. **7**(5): p. e0024822.
60. Takahashi, Y., et al., *Carbazole-degradative IncP-7 plasmid pCAR1.2 is structurally unstable in Pseudomonas fluorescens Pf0-1, which accumulates catechol, the intermediate of the carbazole degradation pathway*. Appl Environ Microbiol, 2009. **75**(12): p. 3920-9.

61. Pansegrau, W., et al., *Complete nucleotide sequence of Birmingham IncP alpha plasmids. Compilation and comparative analysis.* J Mol Biol, 1994. **239**(5): p. 623-63.
62. Harrison, E., et al., *Parallel compensatory evolution stabilizes plasmids across the parasitism-mutualism continuum.* Curr Biol, 2015. **25**(15): p. 2034-9.
63. San Millan, A., et al., *Interactions between horizontally acquired genes create a fitness cost in Pseudomonas aeruginosa.* Nat Commun, 2015. **6**: p. 6845.

Contributions to publications

Chapter 2 - The human microbiome-derived antibiotic lugdunin self-regulates its biosynthesis in a positive feedback-like fashion

For this manuscript I established the method to perform reporter experiments for the evaluation of promoter activities and the impact of putative regulators on the lugdunin BGC. With the support of Khaled A. Selim I performed the SEC-MALS experiment and further analysis of the proteins. I purified the proteins LugR and LugJ, established the EMSA protocol and performed all biological assays. I performed the corresponding biological assays (Figure 3, Figure 4, Figure 5, Figure 6, and Figure S1) Further, I wrote the manuscript under guidance of Andreas Peschel and Bernhard Krismer and prepared the according figures (Figure 2, Figure 8, and Figure S2).

Thales Kronenberger and Antti Poso established the mathematical model for the binding of the promoters to the lugdunin BGC (Figure 7, and Figure S3) and strongly supported the writing of this part of the manuscript (Supplementary information, Molecular modelling). Thales Kronenberger revised the manuscript.

Khaled A Selim gave strong support for the SEC-MALS experiment (Figure 5) as well as for further experiment designs and for purification of the regulators. Further he revised the manuscript.

Timm Schaeffle performed the microfractionation of *S. lugdunensis* IVK28 (Figure S1).

Taulant Dema performed the analysis of lugdunin production via HPLC-MS (Figure 1B).

Alexander Zipperer constructed the plasmids and strains of the regulator-deficient mutants for evaluation of lugdunin production and self-resistance (Figure 1).

Stephanie Grond contributed to the design of the chemical experiments in this study and supported with fruitful discussion of the data and the manuscript.

Andreas Peschel fundamentally supervised my work. He gave pivotal contributions to the concept of the experiments and the manuscript. Further, he gave intellectual through critical revision of the manuscript.

Bernhard Krismer performed the biological assays on lugdunin production and MIC, further he prepared the corresponding figure (Figure 1). He supported this work by permanent

contributions to the design of the experiments and the manuscript. He gave intellectual support to the manuscript by critical revision.

Chaper 3 - Ulbactin F represents a chalkophore facilitating copper homeostasis in *Staphylococcus lugdunensis*

I performed *in silico* analysis of the ulbactin F gene cluster to the other gene clusters (Figure 1, Figure 2a, and Figure S2) and performed all biological experiments (Figure 4, Figure 6, Figure S4, and Figure S5) with technical support from Lukas Schulze and Hannah Simonis. I constructed all plasmids and strains except for one provided by Angelika Müller-Jochim. Further I wrote the manuscript under guidance of Andreas Peschel and Bernhard Krismer and prepared all figures in the manuscript.

Heiko Pfeffer performed all chemical experiments in this manuscript (Figure 2B, Figure 3, and Figure 5). He gave intellectual support to the chemical results and discussion in the manuscript.

Angelika Müller-Jochim constructed the production deficient mutant *S. lugdunensis* N920143 $\Delta lgbCD$.

Stephanie Grond gave intellectual input to the biological experiments and the manuscript by fruitful discussions. Furthermore, she contributed to the design and evaluation of the chemical experiments.

Simon Heilbronner gave intellectual support to the design and evaluation of the biological experiments.

Andreas Peschel fundamentally supervised my work. He gave pivotal contributions to the concept of the experiments and the manuscript. Further, he gave intellectual support through critical revision of the manuscript.

Bernhard Krismer supported this work by permanent contributions to the design of the experiments and the manuscript. He gave intellectual support to the manuscript by critical revision.

Chapter 4 - Horizontal transfer of bacteriocin biosynthesis genes requires metabolic adaptation to improve compound production and cellular fitness

I supported the biological experiments for evaluation of the adaptive mutation of the *cizZ* gene (Figure 3), metabolite level (Figure 4 and Figure 7A). Furthermore, I supported Sophia Kraus with the experiments for proteomics, metabolomics and transcriptomics. I gave intellectual support to the manuscript and helped with the design of the figures.

Sophia Krauss designed and performed the biological experiments in this manuscript, prepared the figures and wrote the manuscript.

Theresa A. Harbig performed the transcriptomic analysis (Figure 8, and Figure S10).

Johanna Rapp performed the metabolomic analysis (Figure 5, Figure 6, Figure 7B, Figure S6, Figure S7, Figure S8 and Figure S9)

Timm Schaeffle performed all chemical analysis in this manuscript (Figure 1C, Figure 2B and 2C, Figure S2, Figure S3, and Figure S4).

Mirita Franz-Wachtel performed the proteomic analysis (Figure 3C).

Ahmed M. A. Elsherbini performed phylogenetic analysis.

Boris Macek supervised Mirita Franz-Wachtel and gave intellectual support to the evaluation of the data and the manuscript.

Stephanie Grond gave intellectual input to the biological experiments and the manuscript by fruitful discussions. Furthermore, she contributed to the design and evaluation of the chemical experiments.

Hannes Link supervised Johanna Rapp and gave intellectual support to the evaluation of the data and the manuscript.

Kay Nieselt supervised Theresa A. Harbig and gave intellectual support to the evaluation of the data and the manuscript.

Bernhard Krismer supported the work by permanent contributions to the design of the experiments and the manuscript. He gave intellectual support to the manuscript by critical revision.

Andreas Peschel supervised the work. He gave pivotal contributions to the concept of the experiments and the manuscript. Further, he gave intellectual support through critical revision of the manuscript.

Simon Heilbronner gave intellectual support to the design and evaluation of the biological experiments. He critically revised the manuscript.

Curriculum vitae

removed