Chemical Science

EDGE ARTICLE

View Article Online
View Journal | View Issue



Cite this: *Chem. Sci.*, 2014, **5**, 1158

# α-Methylene-γ-butyrolactones attenuate *Staphylococcus aureus* virulence by inhibition of transcriptional regulation†

Martin H. Kunzmann, Nina C. Bach, Bianca Bauer and Stephan A. Sieber*

Received 8th August 2013
Accepted 20th November 2013

DOI: 10.1039/c3sc52228h

www.rsc.org/chemicalscience

Bacterial pathogenesis is triggered by complex molecular mechanisms that sense bacterial density within an infected host and induce the expression of toxins for overriding the immune response. Virulence is controlled by a set of transcriptional regulators that directly bind to DNA promoter regions of toxin-encoding genes. Here, we identified an α-methylene-γ-butyrolactone as a potent inhibitor of *Staphylococcus aureus* virulence. Treatment of bacteria not only resulted in a markedly decreased expression of one of the most prominent virulence factors α-hemolysin (Hla) but also caused attenuated invasion efficiency. Mass spectrometry (MS) based target identification revealed this biological effect originating from the consolidated binding to three important transcriptional regulators SarA, SarR and MgrA. MS investigation of the binding site uncovered a conserved cysteine in all three proteins which gets covalently modified. Intriguingly, investigation of DNA binding demonstrated an impaired DNA-protein interaction upon compound treatment. The functional correlation between target binding, inhibition and the observed biological effect was proven by gene knockouts and confirmed the expected mode of action.

## Introduction

*Staphylococcus aureus* represents an opportunistic pathogen that infects a variety of human organs and causes severe diseases such as endocarditis, pneumonia and toxic shock syndrome.[1] Tissue infection is promoted by a variety of bacterial virulence factors including hemolysins, leukocidins and immune modulators that support bacterial propagation within the host and attenuation of the immune response.[2]

Due to the lack of novel and effective antibiotic drugs and the rising concern about multidrug-resistant bacteria, the targeting of virulence received increasing interest in the past years as an alternative treatment strategy.[3–6] Virulence factors are not essential for bacterial viability and thus inhibition of virulence reduces selective pressure, which is a major cause of resistance development. Recent genomic and proteomic studies have revealed insights into the complex pathways by which bacteria control the expression of several virulence factors.[7] Multiple two component systems sense environmental conditions and transduce extracellular signals inside the cell, thereby initiating selective molecular responses including virulence gene expression *via* transcriptional regulators.[8,9] In *S. aureus* virulence expression is controlled by global regulatory elements such as staphylococcal accessory regulators (Sar).[7,10,11] The SarA protein binds to several target gene promoters including α-hemolysin (Hla), protein A and fibronectin binding protein (FnBP).[12–14] Analysis of multiple *S. aureus* genomes revealed the existence of additional members of this regulatory family including SarR and MgrA which share a high structural similarity to SarA.[7,15–17] Recent studies emphasized the existence of a central Cys based redox sensor which is crucial for DNA binding and thus regulatory activity.[18] In addition, SarA activity is controlled by phosphorylation of the same Cys residue.[19] Genetic deletion of *sarA*, *sarR* and *mgrA* regulators revealed dramatic changes in the production of several toxins, including Hla, that result in strains that cannot cause infection within murine abscess models.[20,21] The regulation of toxin expression by transcriptional regulators, however, is highly complex and inactivation of individual proteins such as SarA can result in the up- or downregulation of Hla toxin expression depending on the corresponding *S. aureus* strain.[22–24]

In the search for novel therapies against multidrug-resistant *S. aureus* (MRSA) strains, anti-virulence concepts become more and more important. We previously described the inhibition of ClpP, a central bacterial protease, which in

*Center for Integrated Protein Science CIPSM, Institute of Advanced Studies IAS, Department Chemie, Lehrstuhl für Organische Chemie II, Technische Universität München, Lichtenbergstrasse 4, 85747 Garching, Germany. E-mail: stephan.sieber@tum.de; Fax: +49 8928913210; Tel: +49 8928913302*

† Electronic supplementary information (ESI) available: Synthesis, cloning, mutagenesis, protein expression, purification and biochemical assays. See DOI: 10.1039/c3sc52228h

addition to the transcriptional regulators, controls the expression of crucial virulence factors.[25,26] β-lactones were found to be specific inhibitors which downregulate the expression of several toxins including toxic shock syndrome toxin, Hla and enterotoxin B.[3] More recently, He *et al.* screened a library of compounds for dedicated MgrA inhibitors and identified 5,5-methylenedisalicylic acid (MDSA) that efficiently blocked DNA binding.[27]

In our search for novel antibacterial compounds we screened a distinct collection of α-methylene-γ-butyrolactones to reduce the expression of Hla (Fig. 1). α-Methylene-γ-butyrolactones represent a potent and privileged structural motif that exhibits a huge diversity of bioactivities and is present in about 3% of all known natural products.[28,29]

We identified two compounds that revealed a significant reduction in toxin expression and subsequently started target deconvolution by activity based protein profiling (ABPP) (Fig. 1).[30–32] Proteomic profiling and mass spectrometric analysis showed that all active compounds exhibited binding and reactivity preferences for the three transcriptional virulence regulators SarA, SarR and MgrA *via* the conserved Cys redox sensing residue. Moreover, subsequent EMSA assays demonstrated that covalent binding to these particular regulatory proteins induced protein-DNA dissociation. Concordantly with previous reports,[13,33–35] the relevance of these targets for virulence in *S. aureus* was verified by genetic knockouts. Thus, these results not only expand the scope of virulence inhibition but also emphasize the druggability of multiple response regulators for medical applications.

# Results and discussion

### Synthesis of α-methylene-γ-butyrolactones

Inspired by the diversity of α-methylene-γ-butyrolactones in nature we extended our existing collection of xanthatine inspired molecules (ESI, Scheme 1†) by four additional molecules that bear the core structure but are equipped with different side chains.[28] All molecular structures exhibit a terminal alkyne handle which serves as a tag for downstream target identification by activity based protein profiling (ABPP).[23,30,31,36,37] In this strategy the small probe molecules penetrate cells and bind to the dedicated target(s) within a native environment (Fig. 1). Subsequent cell lysis, Huisgen–Sharpless–Meldal cycloaddition (click chemistry) with functionalized azides allow the binding, enrichment and visualization of proteins of interest for subsequent identification by SDS-gel electrophoresis and mass spectrometry, respectively (Fig. 1).[38–40]

The synthesis followed two different procedures in order to account for aliphatic as well as aromatic substituted α-methylene-γ-butyrolactones (Scheme 1).

For aliphatic substituted lactones, compound **11** was synthesized as described by Kamijo *et al.* and a subsequent reaction with the indicated Grignard reagent lead to the precursors **12** and **13**.[41] A Reformatsky reaction with ethyl 2-(bromo-methyl)acrylate yielded the final aliphatic substituted lactone probes **1** and **2** as racemic mixtures.[42] In the case of phenyl substituted lactones, a Wittig reaction of benzaldehyde with (carbethoxyethylidene) triphenylphosphorane led to the formation of compound **14** which was brominated at its allylic position by using NBS and AIBN to yield lactone precursor **15**.[43] The final phenyl substituted lactones

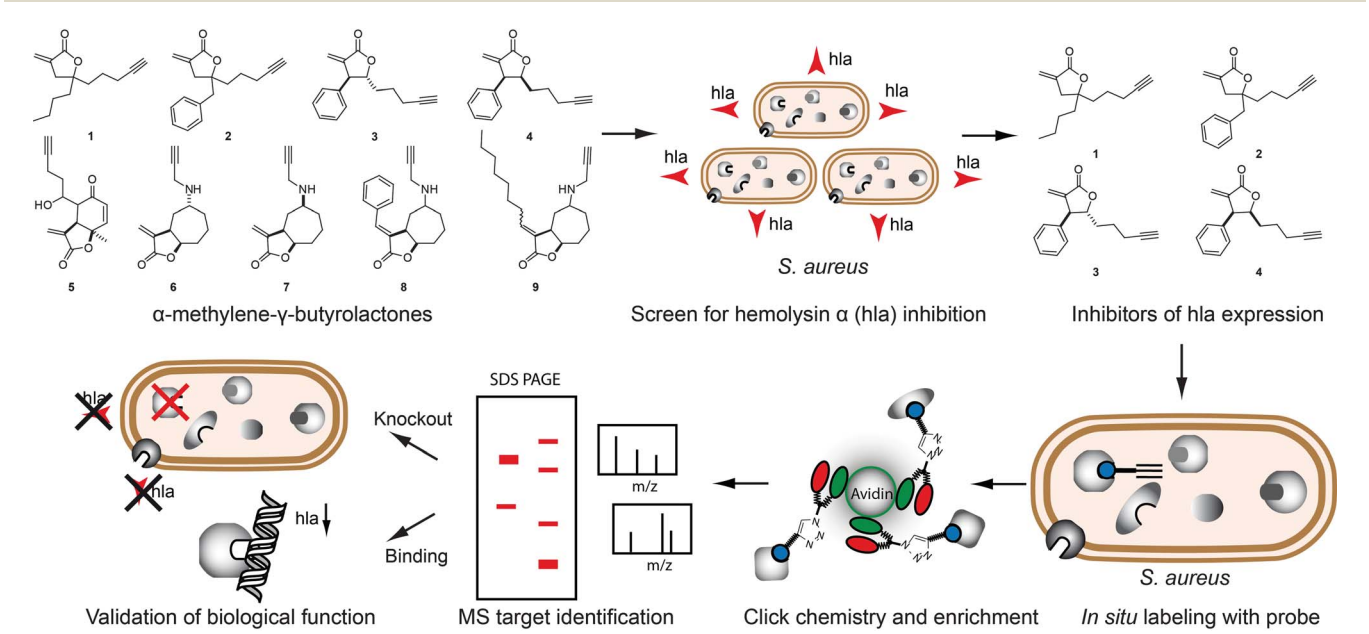
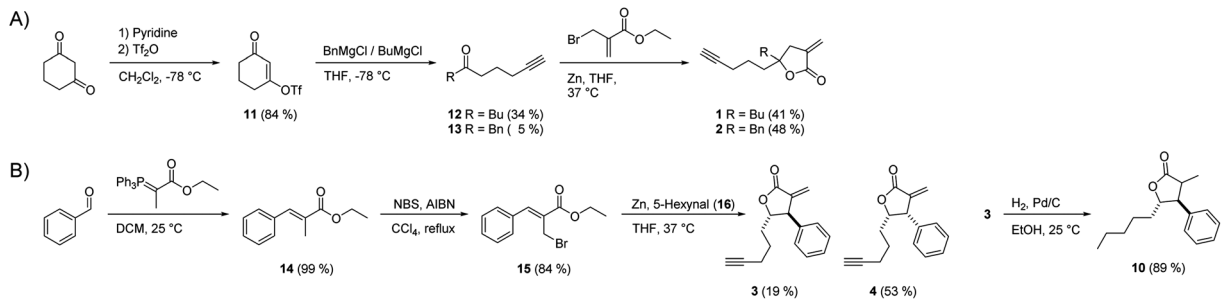



Fig. 1 An α-methylene-γ-butyrolactone library was screened for hemolysis inhibition of *S. aureus*. The cellular targets of potent compounds were identified by incubation with living *S. aureus* cells, subsequent lysis, click chemistry with rhodamine biotin azide and enrichment with biotin-avidin beads followed by SDS page and mass spectrometric analysis (MS). *In vitro* assays and knockout studies were performed to verify the identified proteins and the biological effect.




Scheme 1 Synthesis of (A) aliphatic substituted and (B) phenyl substituted α-methylene-γ-butyrolactone probes and the hydrated compound **10**. For synthetic details please refer to the ESI† section.



**3** and **4** (each racemic) were obtained by a Reformatsky reaction of **15** with 5-hexynal.[42] Compound **3** was reduced by hydration with Pd/C to yield probe **10**.

### Test for anti-hemolytic and antibiotic activity

All compounds including the previously established α-methylene-γ-butyrolactones were tested for bioactivities against several *S. aureus* strains including antibiotic sensitive (NCTC8325) as well as MRSA (Mu50 and USA300) (Table S1†). First, all compounds were investigated for their ability to inhibit *S. aureus* hemolysis of sheep derived erythrocytes. Compound **3** exhibited the most potent $IC_{50}$ value of 4 μM in NCTC8325 and 2 μM in USA300 followed by compound **4** that was slightly less active (Fig. 2A and ESI, Fig. 1†). In order to verify if the anti-hemolytic effect is based on the direct inhibition of virulence rather than an indirect consequence of antibiotic activity, we additionally determined the minimal inhibitory (MIC) concentration of all molecules (Fig. 2B). Interestingly, only weak antibiotic activity could be obtained for compound **3** (100 μM) which is 25–50 fold higher than the $IC_{50}$ for hemolytic activity. All other compounds did not show any antibiotic effects below 100–500 μM. In addition, compounds **3** and **4** were added to NCTC8325 at various concentrations close to their hemolysis $IC_{50}$. No adverse effect on the bacterial growth was observed that could explain their anti-hemolytic effects (Fig. 2C) emphasizing that the molecules directly target virulence gene expression.

Although all structures share the same α-methylene-γ-butyrolactone scaffold, the side chain decoration seems to significantly alter and influence the corresponding bioactivity. The anti-hemolytic compounds **3** and **4** for instance represent the only scaffolds with a substituent in 4-position. Interestingly, the conversion of **3** from *trans* to *cis* **4** reduces the anti-hemolytic effect emphasizing a precise structure activity relationship (SAR) for target interactions.

All molecules were further tested for cytotoxicity in human HeLa cells *via* the MTT assay (ESI, Fig. 2†). Compound **3** showed a toxic effect with an $EC_{50}$ of 12 μM, which is 3–6-fold above its anti-hemolytic effect. Thus further optimization of the compounds is desired in order to improve their toxicity profile for pharmacological application. This could be achieved by a fine tuning of the electrophilic exocyclic double bond as a fully reduced α-methyl group in compound **10** revealed a toxicity $EC_{50}$ of > 100 μM. However, the presence of an electrophile seems mandatory since no reduction of Hla expression with compound **10** was observed (ESI, Fig. 1†). A better understanding of this SAR data would be achieved by the knowledge of the corresponding cellular targets.

### Target deconvolution by ABPP

To unravel the molecular basis for the anti-hemolytic activity of **3** we utilized a proteomic approach by which the compounds were incubated with living bacteria of three different strains (antibiotic sensitive NCTC8325, and two MRSA strains: Mu50 and USA300) at various concentrations. The cells were lyzed and the alkyne was subsequently modified with a rhodamine azide tag by click chemistry. The labeled cytosolic and cell envelope proteomes were individually separated by SDS-gel electrophoresis and analyzed by fluorescent scanning. Due to the reactive Michael acceptor system, several fluorescently labeled proteins could be detected and a concentration of 5–10 μM seemed optimal for saturated labeling (Fig. 3A, ESI, Fig. 3A and C†). The target pattern can roughly be divided in to high molecular weight (hmw) proteins and low molecular weight proteins (lmw). While some lmw protein bands were characteristic for the majority of compounds others were specific to **3** and **4**, the compounds that exhibited the best anti-hemolytic activity (ESI, Fig. 3B†). This emphasizes that the corresponding proteins could be involved in virulence. Another interesting observation derived from the comparison of all three strains in the hmw segment of the gel. One additional protein band at a molecular weight of 60 kDa appears only in Mu50 and thus might be resistance associated (ESI, Fig. 4†).

In order to identify these particular cytosolic protein bands we next applied gel-based and gel-free MS identification methods. Labeled proteins were attached to a trifunctional tag consisting of azide, rhodamine and biotin *via* click chemistry.[44] Biotinylated proteins were enriched on avidin beads, washed and released by heat denaturation. The proteins were then either separated *via* SDS-PAGE, isolated, tryptically digested and analyzed by LC-MS/MS or directly digested and analyzed by LC-MS/MS. MS/MS fragmentation revealed peptide sequences that were investigated by the SEQUEST search algorithm in order to obtain protein identities. Analysis of the lmw protein IDs suggested thioredoxin A (TrxA), a staphylococcal disulfide

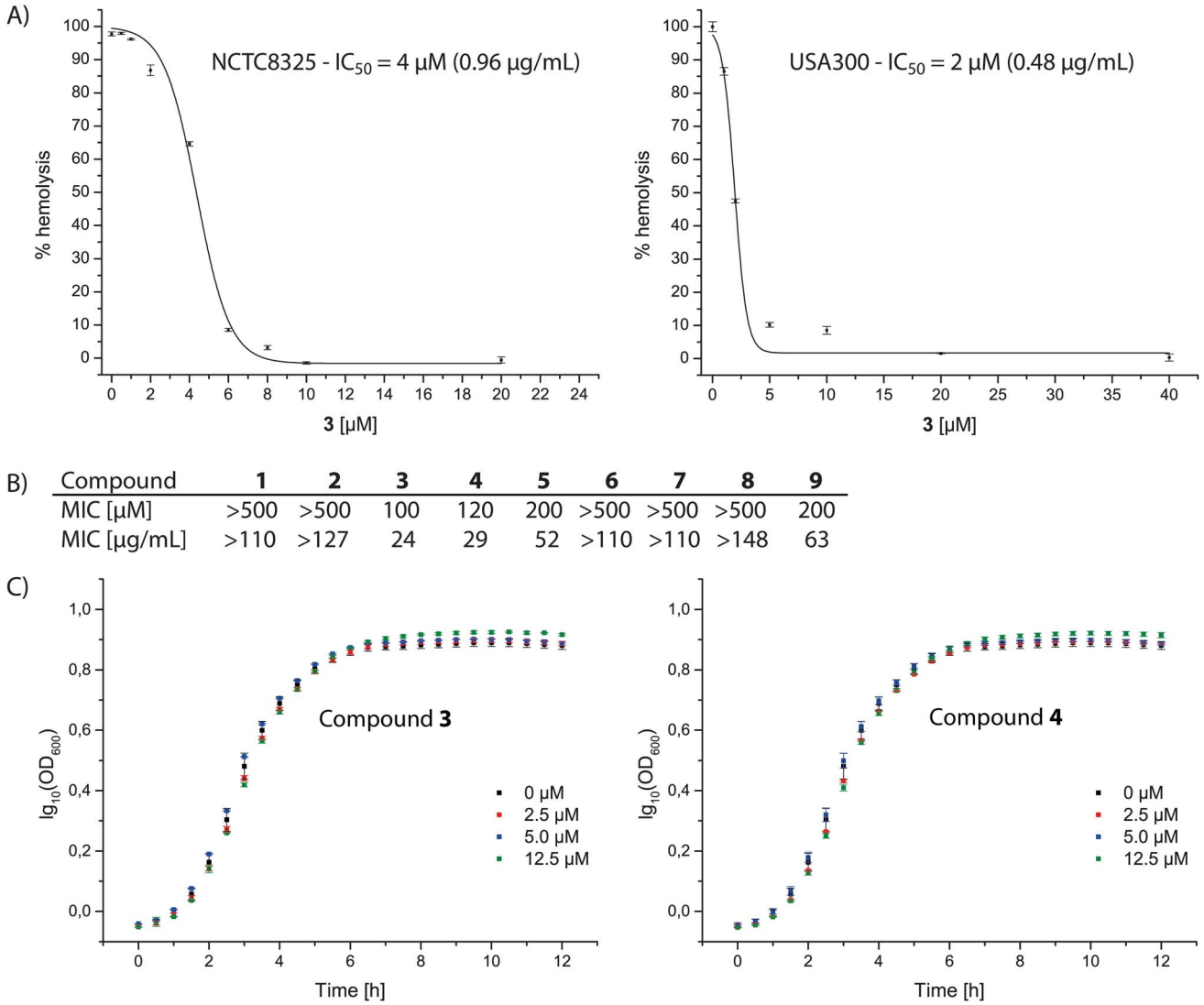



Fig. 2 (A) Inhibition of sheep blood hemolysis caused by *S. aureus* NCTC8325 and USA300 by probe **3**. Data were fitted to the dose-response function $f(x) = A_1 + (A_2 - A_1)/(1 + 10\text{^}(\log(x_0 - x))p)$ with a variable Hill-slope given by parameter $p$. (B) MIC values for NCTC8325 strain of the corresponding lactone probes in µM and µg mL$^{-1}$. (C) NCTC8325 growth curves based on $OD_{600}$ with compounds **3** or **4**. Each compound was tested in at least three independent trials in triplicates; average values are shown and error bars display standard deviations from the mean.

reductase, as well as three transcriptional regulators, SarA, SarR and MgrA as the most likely hits over several runs (ESI, Tables 2 and 3†).

This result is in line with the observed phenotype of compounds **3** and **4** as SarA, SarR and MgrA are involved in virulence regulation and *hla* expression.[21–24,45,46] Interestingly, all other compounds did not or only weakly label SarA, SarR and/or MgrA in the proteome which provides a link to the observed phenotype (ESI, Fig. 3 and 4†). In addition, the distinct 60 kDa protein specific for Mu50 was identified as bifunctional AAC/APH, an enzyme that is involved in propagating resistance to several antibiotics including gentamycin and kanamycin (ESI, Fig. 4†).[47] The protein band below at about 55 kDa was identified as MurA1 and MurA2 – two enzymes that are involved in cell wall biosynthesis and are crucial for bacterial viability.[48,49] Their putative inhibition by α-methylene-γ-butyrolactones could explain the observed antibiotic activity at higher concentrations. All other bands in the 90 kDa region correspond to characteristic reactive proteins in *S. aureus* that are unspecifically labeled at concentrations above 20 µM.[50] One predominant member has been assigned as formate acetyltransferase before by several MS experiments.[49,50] Since this study focuses on the anti-hemolytic activity of α-methylene-γ-butyrolactones we commenced with target validation for proteins that are characteristic for the observed biological effects.

## Target validation

Before we started with the functional studies of the transcriptional regulators we first analyzed MurA1 and MurA2 inhibition by **3** in order to evaluate their potential contribution to the

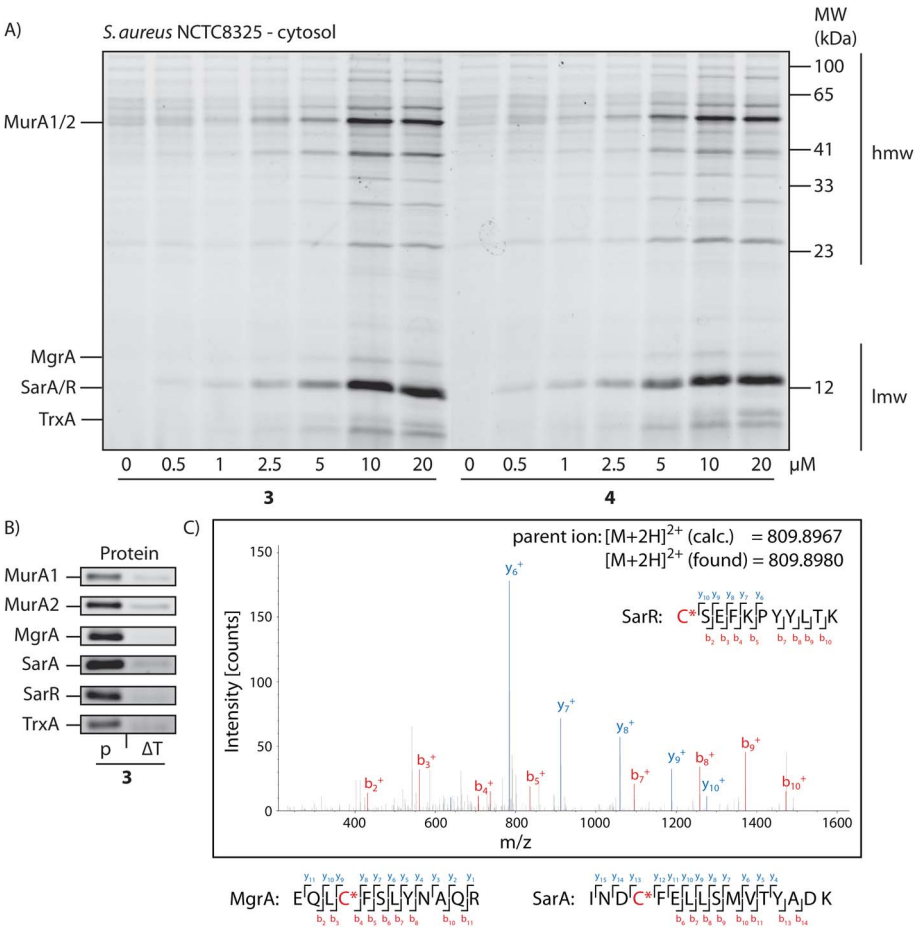



Fig. 3 Target identification of α-methylene-γ-butyrolactones. (A) *In situ* labeling of *S. aureus* NCTC8325 with probes **3** and **4** at indicated concentrations. Identified proteins of the corresponding gel bands are shown on the left. For details about the protein abbreviations please refer to Table S2†. (B) Target validation by labeling of recombinant proteins with the probes (p = recombinant protein, $\Delta T$ = heat denatured protein). (C) MS/MS spectrum for the identification of the modified binding site (C* = compound **3** modified cysteine residue).

observed anti-hemolytic effects at sub-MIC concentrations. Incubation of recombinant MurA proteins[49] with **3** and subsequent modification with the fluorescent dye *via* click chemistry revealed a strong fluorescent band on SDS-PAGE (Fig. 3B). No labeling was observed if the proteins were heat denatured prior to compound addition emphasizing a specific interaction with the folded and active enzyme. Enzyme activity of the cell wall biogenesis regulating enzymes MurA1 and MurA2 was measured by monitoring the enolpyruvyl transfer reaction with phosphoenolpyruvate (PEP) and UDP-*N*-acetylglucosamine (UDPAG).[49] Here, 100 μM compound **3** was required to significantly impair enzyme activity (ESI, Fig. 5†). This is comparable to the corresponding MIC value (100 μM) suggesting that inhibition of these essential enzymes is related to the observed antibiotic activity. To evaluate the contribution of MurA1/2 inhibition to the reduction of hemolysis we utilized the well characterized MurA1/2 inhibitor fosfomycin and applied it at sub-MIC concentrations to *S. aureus* NCTC8325.[48] No effect on the hemolysis production was observed at a concentration of 0.55 μM (MIC < 2.75 μM) emphasizing that these targets are not responsible for the observed anti-hemolytic effects (ESI, Fig. 6†). Therefore, we focused our attention to the functional characterization of the lmw proteins with special emphasis on the transcriptional regulators.

*TrxA*, *sarA*, *sarR* and *mgrA* were cloned into strep-tag expression vector systems and expressed as recombinant proteins in *E. coli* BL21 DE3. Specific interactions of the probe molecules with the corresponding proteins were confirmed *via* *in situ* labeling with living cells before and after induction, *in vitro* labeling with heat denatured cell lysates containing the proteins as well as heat denaturation of the purified proteins prior to labeling (Fig. 3B, ESI, Fig. 7†). The denatured proteins did not bind the compound emphasizing the importance of a properly folded binding pocket for recognition.

In order to identify the site of binding we incubated the purified proteins with 4-fold excess of **3** for 30 min, digested with trypsin and analyzed the resulting peptides *via* HPLC-MS/MS. In case of SarA, SarR and MgrA a single cysteine residue (Cys9, Cys57 and Cys12) was identified as site of covalent attachment (Fig. 3C and ESI, Fig. 8†). The identified cysteines are highly conserved in all three structurally related proteins and located at the dimerization interface.[17,18,51] They form

H-bonds with residues from the adjacent monomer thus likely explaining their elevated nucleophilicity and reactivity towards the electrophilic α-methylene-γ-butyrolactones. These cysteine residues are important regulatory switches. For instance, Cys12 in MgrA is an important sensor for oxidative stress.[18] Peroxides cause oxidation of the thiol to sulfenic acid and subsequent dissociation from the DNA.[18,52] In case of SarA, recent studies show phosphorylation of Cys9 as an additional regulation principle reducing DNA binding affinity.[19,53]

It is known that *S. aureus* adapts to changes of external oxygen concentrations by redox dependent processes regulating the expression of genes. Small thiol specific proteins such as thioredoxin (TrxA) maintain the intracellular thiol-disulfide balance.[54,55] TrxA is an oxidoreductase enzyme featuring a conserved active site Trp-Cys-Gly-Pro-Cys sequence that forms or breaks disulfide bonds by inter-protein exchange reactions.[56] As this regulation also indirectly triggers the redox state of transcriptional regulators we were interested if **3** is able to inhibit the reductase activity of TrxA.[54] The inhibition of TrxA by **3** was evaluated by an assay system which measures the reduction of disulfide bonds within insulin.[57] Reduced insulin becomes insoluble and enzyme turnover is followed by increasing turbidity. Concentration-dependent inhibition of TrxA activity was observed and an $IC_{50}$ value of 17 μM obtained (ESI, Fig. 9†). Apart from its function in the cellular redox pathway not much is known about the biological impact of TrxA inhibition. A *trxA* deletion in *Salmonella enterica* exhibited a reduction in intracellular replication and mouse virulence emphasizing a possible role in virulence regulation that would correlate with the above-mentioned function.[58]

These examples highlight the importance of a fine tuned cellular redox mechanism that among other duties, maintains cysteine oxidation states in transcriptional regulators for DNA binding. In order to evaluate if covalent modification with a small molecule induces DNA displacement effects we utilized electrophoretic mobility shift assays (EMSA) and obtained corresponding $IC_{50}$ values (Fig. 4 and ESI, Fig. 10†).

Based on previous studies we selected the *agr* promoter DNA region as a confirmed binding site for SarA, SarR and MgrA.[45,59] A fluorescent AlexaFluor488 dye at the 5′site allowed us to monitor protein-DNA interaction *via* fluorescence scanning of the corresponding native polyacrylamide gels. All three proteins were incubated in the presence of the fluorescent DNA and various concentrations of compounds. Unspecific binding was reduced by the addition of an excess of salmon sperm DNA.

The corresponding mobility shift gels are shown in Fig. 4 and ESI, Fig. 10†. Interestingly, SarA, SarR and MgrA showed mobility shifts at low molar excess of **3** (0.5-fold for SarR and 4-fold for SarA and MgrA). This emphasizes that the binding to all three transcriptional regulators could be responsible for the phenotypic effect. Importantly, compounds not exhibiting any anti-virulence activity, except compound **5**, did not show mobility shifts with SarA, SarR and MgrA, thereby demonstrating a lack of interaction (Fig. 4 and ESI, Fig. 10,† right panel). This is also in line with labeling studies of the three recombinant proteins (ESI, Fig. 11†).

Several previous studies show that SarA, SarR and MgrA regulate *hla* expression.[7,10] This regulation principle, however, is highly complex and strain specific. For instance, a *sarA* knockout in *S. aureus* strain 8325-4 downregulates *hla* expression[13,60,61] while the same knockout in strains V8 or SH1000 upregulates Hla levels.[23] Several reasons for this observation have been discussed and a strong link to the Sar family protein amount (including SarS) has been suggested.[23,25] In order to further elucidate if the observed anti-hemolytic effect correlates indeed with reduced expression levels of Hla we utilized western blot analysis with anti-Hla antibodies. In agreement with the results of the blood hemolysis assay as well as the EMSA assay the expression of Hla was significantly diminished at a concentration of 2 μM **3** and abolished at concentrations above 4 μM (Fig. 4C). This strongly suggests that at least the cumulative inhibition of all three transcriptional regulators reduces *hla* expression in the *S. aureus* strains investigated here (NCTC8325 and USA3000). However, the individual role of each regulator has to be investigated with the corresponding *mgrA*, *sarA* and *sarR* knockout strains.

### Functional analysis by gene knockouts

In order to validate the relevance of transcriptional regulator inhibition by α-methylene-γ-butyrolactone **3** we obtained previously established knockout strains of *sarA*, *sarR*, *mgrA* as well as a *sarA*/*R* double knockout. Phage mediated transduction of the selective resistance cassettes into the corresponding genes of the *S. aureus* NCTC8325 genome revealed corresponding knockout strains for further functional investigations.[13,33–35]

Subsequent hemolysis assays with the parental strain and the knockouts revealed a significant reduction in Hla production in the case of Δ*sarR* and Δ*mgrA* (Fig. 5). Contrary, the Δ*sarA* strain showed an increase in hemolysis as described for several strains previously,[23] thereby indicating SarA as being not a responsible target for the anti-hemolytic effect of compound **3**. Interestingly, addition of compound **3** decreased hemolysis production of Δ*sarA* bacteria, thus emphasizing SarR and MgrA as the responsible anti-virulence-determining targets. In order to investigate which of the two effects–activation of hemolysis by Δ*sarA* or reduction of hemolysis by Δ*sarR* is dominating we investigated a Δ*sarA*Δ*sarR* double mutant. Interestingly, this double mutation resulted in no detectable hemolysis demonstrating that inactivation of at least these two transcriptional regulators is sufficient to match the chemical phenotype of α-methylene-γ-butyrolactone **3**. Thus, the results of ABPP, EMSA studies as well as functional knockouts clearly support a mechanism of action by which compound **3** covalently attaches to a functional relevant cysteine in all three transcriptional regulators, preventing DNA binding and downstream hemolysis production.

### Reduction of *S. aureus* invasion into human THP-1 cells

Previous studies with *sarA* deletion strains in *S. aureus* indicated a reduced binding to fibronectin, an important membrane

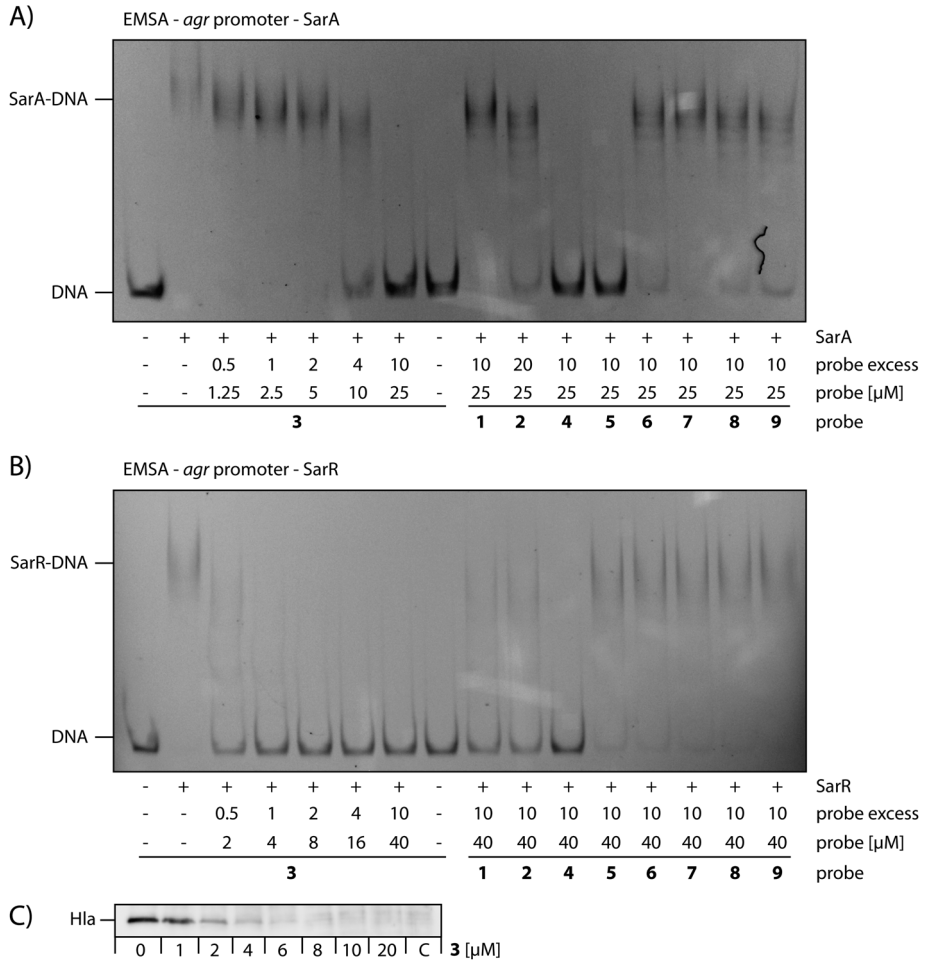



Fig. 4 Electrophoretic mobility shift assay (EMSA) with (A) SarA and (B) SarR (for MgrA please refer to ESI, Fig. 10†). The left part of the gel shows a concentration dependent analysis with anti-virulence compound **3**, the right part displays a single concentration analysis with all other compounds. DNA = unbound fluorescent promoter DNA; SarA/SarR-DNA = protein bound DNA. (C) Luminescent western blot of *S. aureus* NCTC8325 supernatant (grown with increasing concentrations of probe **3**) incubated with a primary anti-Hla (α-hemolysin) and a secondary (HRP) antibody.

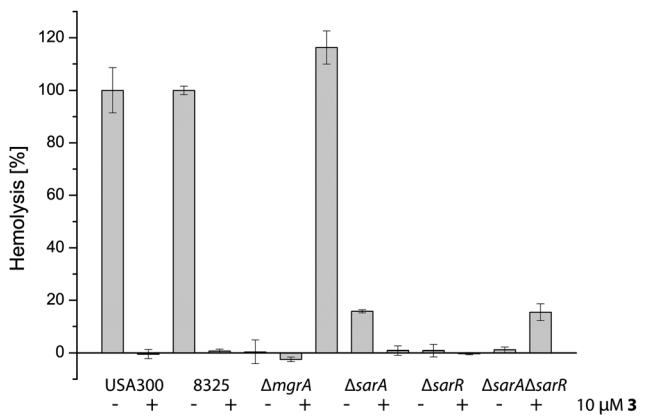



Fig. 5 Hemolysis of sheep red blood cells upon incubation with supernatants of *S. aureus* USA300, NCTC8325 and the corresponding NCTC8325 knockout strains Δ*mgrA*, Δ*sarA*, Δ*sarR* and Δ*sarA*Δ*sarR* with and without 10 μM probe **3**.

associated protein in eukaryotic cells and a trigger for bacterial invasion.[62,63] Therefore, we finally investigated if binding of **3** to the transcriptional regulators would reduce the phagocytosis mediated invasion of *S. aureus* into human macrophages for which we performed a compound-based assay.[64] Bacterial cells were grown in the presence of 20 μM **3** for 16 h, and incubated with differentiated THP-1 cells in the presence of 2 μM **3** for 2 h. In order to interrupt cellular uptake mechanisms cells were washed with cold PBS and subsequently incubated in medium supplemented with 50 μg $mL^{-1}$ gentamycin to kill all remaining extracellular, non-internalized bacteria. The cells were lyzed, lysates were plated and intracellular bacteria were counted as colony forming units (CFU) after one day of incubation at 37 °C. Interestingly, compound **3** significantly reduced the invasiveness of *S. aureus* by 40% emphasizing that the pathogen is not only disarmed in its Hla mediated virulence but also weakened in its general pathogenicity (ESI, Fig. 12†). This further highlights the relevance of these proteins as putative targets against bacterial infections.

# Conclusion

Inhibition of virulence is a powerful approach to reduce the pathogenicity of bacteria. The expression of toxins that contribute to the devastating effects of bacterial infections is regulated by a complex network of proteins that mediate a precise timing in the initiation of virulence. This timing is important, as bacteria need a certain density in order to overrun the host. Several transcriptional regulators are activated by quorum sensing and initiate toxin production. SarA, SarR and MgrA are DNA binding proteins that attach to a promoter region important for *hla* transcription. Once their central cysteine is modulated by *e.g.* phosphorylation or oxidation these proteins show reduced DNA affinity and therefore alter transcription of the target genes. We were able to show that the covalent modification of these particular central cysteine residues by suitably designed α-methylene-γ-butyrolactones not only reduced DNA affinity but induced DNA dissociation that resulted in a strong decrease of Hla expression. In addition, the invasion of *S. aureus* into human THP-1 cells was significantly reduced demonstrating that bacteria are weakened in multiple aspects of pathogenesis. This approach is novel and exhibits several advantages compared to a previous MgrA specific inhibitor (MDSA).[27] α-Methylene-γ-butyrolactone **3** abolishes Hla production at 2–4 μM concentration and is thus more potent compared to MSDA, which exhibits a hemolysis $IC_{50}$ of 0.2 mM. Combined inhibition of several targets might be an advantageous strategy as the effects are likely additive leading to a more efficient depletion of Hla mediated virulence. Due to the structural similarity of all three transcriptional regulators it is not surprising that one compound with a suitable decoration binds all proteins *via* the sensing cysteine. This irreversible binding also exhibits the advantage of a stable link that is long lasting and only reversed by protein turnover.

# Methods

### Determination of MIC-values

*S. aureus* was grown in B broth shaking at 37 °C until cell density reached $OD_{600}$ = 0.6–0.8. Fresh B broth (Yeast extract (5.0 g), tryptic peptone (10.0 g), NaCl (5.0 g), $K_2HPO_4$ (1.0 g), $H_2O$ (1.0 L)) was inoculated with $2 \times 10^6$ bacteria per mL and 99 μL of this culture were added to each well of a 96 well plate. Probe in DMSO (1 μL, varying concentrations) was added and control wells containing no probe (1 μL DMSO) or no bacteria (99 μL B broth) were included. The plate was incubated overnight (16 h) shaking at 37 °C and the resulting optical density was measured by a Tecan Infinite® M200 PRO plate reader.

### Hemolysis-assay

*S. aureus* was grown in B broth shaking at 37 °C until cell density reached $OD_{600}$ = 0.6–0.8. Fresh B broth was inoculated with $2 \times 10^6$ bacteria per mL and to 1 mL aliquots was added probe in DMSO (1 μL). The cultures were incubated overnight (16 h) shaking at 37 °C. The $OD_{600}$ was measured to check the effect of substances on bacterial growth. Cultures were centrifuged (10 min, 13 000 *g*) and 100 μL of each supernatant (triplicates for each culture) were pipetted into 96 well plates. Positive controls (1 μL DMSO) and negative controls (1 mL sterile broth) were included. Erythrocyte suspension (50 μL) was added to each well and the plate was incubated for 10 min shaking at 37 °C. The $OD_{600}$, which correlates with the amount of intact erythrocytes, was measured by a Tecan Infinite® M200 PRO plate reader.

### Electrophoretic Mobility Shift Assay (EMSA)

The Alexa Fluor 488 (AF488) labeled *agr* promoter fragment of NCTC8325 (ref. 24) was generated by PCR and then purified by an Omega Bio-Tek MicroElute Cycle-Pure Kit. MgrA, SarA and SarR were purified as described in the ESI† and aliquots were stored in 100 mM Tris–HCl pH 8.0, 150 mM NaCl and 1 mM ETDA at −80 °C. To 10.8 μL EMSA-Binding-Buffer (100 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 5% glycerol) containing MgrA (1.75 μM), SarA (2.50 μM) or SarR (4.00 μM) were added 0.2 μL probe of the desired concentration in DMSO and the mixture was incubated 1.5 h at room temperature. Then 1 μL of *agr* promoter fragment mixed with salmon sperm DNA (Carl Roth, Karlsruhe) was added leading to final concentrations of 5 nM (7.5 ng) *agr* promoter fragment and 2 μg $\mu L^{-1}$ salmon sperm DNA (Carl Roth, Karlsruhe). After incubation for 15 min at room temperature, 2 μL EMSA-Loading-Buffer (100 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 5% Glycerol, 0.1% bromphenol blue) were added and the samples were applied on 5% TBE polyacrylamide gels (150 V, 45 min). Fluorescence was recorded in a Fujifilm Las-4000 luminescent image analyzer with a Fujinon VRF43LMD3 lens and an Y515-Di filter. Forward primer: 5′-CAATTTTACACCACTCTCCTC (5′-AF488), Reverse primer: 5′-CATCAACTATTTTCCATCACATCT (5′-AF488).

### Invasion assay for *S. aureus* infected human macrophages

Human THP-1 monocytes were seeded in a 6-well plate ($1 \times 10^6$ cells per well in 2 mL RPMI1640 medium containing 10% fetal calf serum and 5 mM L-glutamine) and PMA (phorbol 12-myristate 13-acetate, 40 ng per well) was added. Cells were incubated for 2 days (37 °C; 5% $CO_2$), medium was removed, cells were washed three times with PBS (RT) and fresh medium was added. Bacteria (*S. aureus* NCTC8325) were inoculated in fresh B-medium (1 : 100), incubated to an $OD_{600}$ of 0.4–0.5 and diluted to $3.4 \times 10^7$ bacteria per mL (formula $y = 4E + 07e^{1.0958x}$). Bacteria were supplemented with respective inhibitory compounds (1000 × DMSO stocks) and incubated for 16 h (37 °C, 200 rpm). Bacterial culture was diluted in B-medium (1 : 10) and $OD_{600}$ was measured. CFU was calculated (see formula) and $5 \times 10^7$ bacteria (MOI 50, confluent monolayer in 6-well plate $1 \times 10^6$ cells) were diluted in 2 mL RPMI1640/10% FCS/5 mM L-glutamine. The respective compound (non-lethal concentration, 2 μM, 1000 × DMSO stocks) was added to bacterial suspensions and 2 mL of the bacterial suspension was added to the cells (2 mL without bacteria as control). Infected cells were incubated for 2 h (37 °C, 5% $CO_2$), supernatant was removed and collected for CFU-plating assay of the inoculum. Cells were washed four times

with ice cold PBS and 2 mL medium containing gentamycin (50 μg $mL^{-1}$) was added. Cells were incubated for 24 h with gentamycin to kill all extracellular bacteria and proceed intracellular replication of invaded bacteria. Afterwards cells were washed once with PBS (1 mL) and 0.5% saponin (1 mL)/B-medium was added. Incubation of cells proceeded for 10 min at 37 °C (5% $CO_2$) and then the lysate was plated out by using different dilutions. Quantitative analysis of bacterial concentrations was performed by counting CFUs one day after plating. Dilutions for CFU-plating assay: inoculum: $10^{-2}$, $10^{-3}$, $10^{-4}$; supernatants: $10^{0}$; lysed cells: $10^{-1}$, $10^{-2}$ in B-medium. Plates were incubated overnight (37 °C) and colonies counted subsequently.[64]

## Acknowledgements

We thank Mona Wolff for excellent technical support, David C. Hooper, Simon J. Foster and Erik Gustafsson for providing *S. aureus* knockout strains and Matt Nodwell and Katrin Lorenz-Baath for scientific discussions. S. A. Sieber was supported by the Deutsche Forschungsgemeinschaft SFB749, SFB1035, FOR1406, an ERC starting grant (259024) and the Center for Integrated Protein Science Munich CIPSM. M. H. Kunzmann thanks the TUM Graduate School for project funding and the ERC for financial support.

## References

1 F. D. Lowy, *N. Engl. J. Med.*, 1998, **339**, 520–532.
2 W. W. Navarre and O. Schneewind, *Microbiol. Mol. Biol. Rev.*, 1999, **63**, 174–229.
3 T. Böttcher and S. A. Sieber, *J. Am. Chem. Soc.*, 2008, **130**, 14400–14401.
4 L. Cegelski, G. R. Marshall, G. R. Eldridge and S. J. Hultgren, *Nat. Rev. Microbiol.*, 2008, **6**, 17–27.
5 A. E. Clatworthy, E. Pierson and D. T. Hung, *Nat. Chem. Biol.*, 2007, **3**, 541–548.
6 D. T. Hung, E. A. Shakhnovich, E. Pierson and J. J. Mekalanos, *Science*, 2005, **310**, 670–674.
7 A. L. Cheung, A. S. Bayer, G. Zhang, H. Gresham and Y. Q. Xiong, *FEMS Immunol. Med. Microbiol.*, 2004, **40**, 1–9.
8 R. P. Novick, *Mol. Microbiol.*, 2003, **48**, 1429–1449.
9 E. A. George and T. W. Muir, *ChemBioChem*, 2007, **8**, 847–855.
10 A. L. Cheung, K. A. Nishina, M. P. Trotonda and S. Tamber, *Int. J. Biochem. Cell Biol.*, 2008, **40**, 355–361.
11 P. M. Dunman, E. Murphy, S. Haney, D. Palacios, G. Tucker-Kellogg, S. Wu, E. L. Brown, R. J. Zagursky, D. Shlaes and S. J. Projan, *J. Bacteriol.*, 2001, **183**, 7341–7353.
12 Y. Chien, A. C. Manna, S. J. Projan and A. L. Cheung, *J. Biol. Chem.*, 1999, **274**, 37169–37176.
13 P. F. Chan and S. J. Foster, *J. Bacteriol.*, 1998, **180**, 6232–6241.
14 A. C. Manna, M. G. Bayer and A. L. Cheung, *J. Bacteriol.*, 1998, **180**, 3828–3836.
15 T. T. Luong, P. M. Dunman, E. Murphy, S. J. Projan and C. Y. Lee, *J. Bacteriol.*, 2006, **188**, 1899–1910.
16 T. T. Luong, S. W. Newell and C. Y. Lee, *J. Bacteriol.*, 2003, **185**, 3703–3710.
17 Y. Liu, A. C. Manna, C. H. Pan, I. A. Kriksunov, D. J. Thiel, A. L. Cheung and G. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 2392–2397.
18 P. R. Chen, T. Bae, W. A. Williams, E. M. Duguid, P. A. Rice, O. Schneewind and C. He, *Nat. Chem. Biol.*, 2006, **2**, 591–595.
19 F. Sun, Y. Ding, Q. Ji, Z. Liang, X. Deng, C. C. Wong, C. Yi, L. Zhang, S. Xie, S. Alvarez, L. M. Hicks, C. Luo, H. Jiang, L. Lan and C. He, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 15461–15466.
20 I.-M. Jonsson, C. Lindholm, T. T. Luong, C. Y. Lee and A. Tarkowski, *Microbes Infect.*, 2008, **10**, 1229–1235.
21 A. K. Zielinska, K. E. Beenken, L. N. Mrak, H. J. Spencer, G. R. Post, R. A. Skinner, A. J. Tackett, A. R. Horswill and M. S. Smeltzer, *Mol. Microbiol.*, 2012, **86**, 1183–1196.
22 R. Babai, B. E. Stern, J. Hacker and E. Z. Ron, *Infect. Immun.*, 2000, **68**, 5901–5907.
23 J. Oscarsson, A. Kanth, K. Tegmark-Wisell and S. Arvidson, *J. Bacteriol.*, 2006, **188**, 8526–8533.
24 J. Morschhauser, G. Kohler, W. Ziebuhr, G. Blum-Oehler, U. Dobrindt and J. Hacker, *Philos. Trans. R. Soc. London, Ser. B*, 2000, **355**, 695–704.
25 D. Frees, J. H. Andersen, L. Hemmingsen, K. Koskenniemi, K. T. Baek, M. K. Muhammed, D. D. Gudeta, T. A. Nyman, A. Sukura, P. Varmanen and K. Savijoki, *J. Proteome Res.*, 2012, **11**, 95–108.
26 D. Frees, K. Sorensen and H. Ingmer, *Infect. Immun.*, 2005, **73**, 8100–8108.
27 F. Sun, L. Zhou, B. C. Zhao, X. Deng, H. Cho, C. Yi, X. Jian, C. X. Song, C. H. Luan, T. Bae, Z. Li and C. He, *Chem. Biol.*, 2011, **18**, 1032–1041.
28 M. H. Kunzmann and S. A. Sieber, *Mol. BioSyst.*, 2012, **8**, 3061–3067.
29 R. R. Kitson, A. Millemaggi and R. J. Taylor, *Angew. Chem., Int. Ed.*, 2009, **48**, 9426–9451.
30 M. J. Evans and B. F. Cravatt, *Chem. Rev.*, 2006, **106**, 3279–3301.
31 M. Fonovic and M. Bogyo, *Expert Rev. Proteomics*, 2008, **5**, 721–730.
32 T. Böttcher, M. Pitscheider and S. A. Sieber, *Angew. Chem., Int. Ed.*, 2010, **49**, 2680–2698.
33 Q. C. Truong-Bolduc, Y. Ding and D. C. Hooper, *J. Bacteriol.*, 2008, **190**, 7375–7381.
34 J. Oscarsson, K. Tegmark-Wisell and S. Arvidson, *Int. J. Med. Microbiol.*, 2006, **296**, 365–380.
35 E. Gustafsson and J. Oscarsson, *FEMS Microbiol. Lett.*, 2008, **284**, 158–164.
36 J. Krysiak and R. Breinbauer, *Top. Curr. Chem.*, 2012, **324**, 43–84.
37 W. P. Heal and E. W. Tate, *Top. Curr. Chem.*, 2012, **324**, 115–135.
38 V. V. Rostovtsev, J. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599.
39 C. W. Tornøe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057–3064.
40 R. Huisgen, *Proc. Chem. Soc.*, 1961, 357–396.

41 S. Kamijo and G. B. Dudley, *J. Am. Chem. Soc.*, 2006, **128**, 6499–6507.
42 M. S. Sawant, R. Katoch, G. K. Trivedi and U. R. Desai, *J. Chem. Soc., Perkin Trans. 1*, 1998, **0**, 843–846.
43 L.-W. Ye, X. Han, X.-L. Sun and Y. Tang, *Tetrahedron*, 2008, **64**, 1487–1493.
44 J. Eirich, J. L. Burkhart, A. Ullrich, G. C. Rudolf, A. Vollmar, S. Zahler, U. Kazmaier and S. A. Sieber, *Mol. BioSyst.*, 2012, **8**, 2067–2075.
45 S. Ingavale, W. van Wamel, T. T. Luong, C. Y. Lee and A. L. Cheung, *Infect. Immun.*, 2005, **73**, 1423–1431.
46 A. K. Zielinska, K. E. Beenken, H. S. Joo, L. N. Mrak, L. M. Griffin, T. T. Luong, C. Y. Lee, M. Otto, L. N. Shaw and M. S. Smeltzer, *J. Bacteriol.*, 2011, **193**, 2948–2958.
47 D. D. Boehr, W. S. Lane and G. D. Wright, *Chem. Biol.*, 2001, **8**, 791–800.
48 W. Du, J. R. Brown, D. R. Sylvester, J. Huang, A. F. Chalker, C. Y. So, D. J. Holmes, D. J. Payne and N. G. Wallis, *J. Bacteriol.*, 2000, **182**, 4146–4152.
49 T. Böttcher and S. A. Sieber, *J. Am. Chem. Soc.*, 2010, **132**, 6964–6972.
50 M. B. Nodwell, H. Menz, S. F. Kirsch and S. A. Sieber, *ChemBioChem*, 2012, **13**, 1439–1446.
51 Y. Liu, A. Manna, R. Li, W. E. Martin, R. C. Murphy, A. L. Cheung and G. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 6877–6882.
52 G. A. Somerville and R. A. Proctor, *Microbiol. Mol. Biol. Rev.*, 2009, **73**, 233–248.
53 D. F. Fujimoto, R. H. Higginbotham, K. M. Sterba, S. J. Maleki, A. M. Segall, M. S. Smeltzer and B. K. Hurlburt, *Mol. Microbiol.*, 2009, **74**, 1445–1458.
54 A. Ballal and A. C. Manna, *J. Bacteriol.*, 2010, **192**, 336–345.
55 O. Uziel, I. Borovok, R. Schreiber, G. Cohen and Y. Aharonowitz, *J. Bacteriol.*, 2004, **186**, 326–334.
56 J. Messens, I. Van Molle, P. Vanhaesebrouck, M. Limbourg, K. Van Belle, K. Wahni, J. C. Martins, R. Loris and L. Wyns, *J. Mol. Biol.*, 2004, **339**, 527–537.
57 A. Holmgren, *J. Biol. Chem.*, 1979, **254**, 9627–9632.
58 E. Bjur, S. Eriksson-Ygberg, F. Aslund and M. Rhen, *Infect. Immun.*, 2006, **74**, 5140–5151.
59 A. C. Manna and A. L. Cheung, *Mol. Microbiol.*, 2006, **60**, 1289–1301.
60 A. S. Khan, I. Muhldorfer, V. Demuth, U. Wallner, T. K. Korhonen and J. Hacker, *Mol. Gen. Genet.*, 2000, **263**, 96–105.
61 M. Steinert, U. Hentschel and J. Hacker, *Naturwissenschaften*, 2000, **87**, 1–11.
62 Y. Q. Xiong, A. S. Bayer, M. R. Yeaman, W. Van Wamel, A. C. Manna and A. L. Cheung, *Infect. Immun.*, 2004, **72**, 1832–1836.
63 A. Karlsson, P. Saravia-Otten, K. Tegmark, E. Morfeldt and S. Arvidson, *Infect. Immun.*, 2001, **69**, 4742–4748.
64 M. P. Horn, S. M. Knecht, F. L. Rushing, J. Birdsong, C. P. Siddall, C. M. Johnson, T. N. Abraham, A. Brown, C. B. Volk, K. Gammon, D. L. Bishop, J. L. McKillip and S. A. McDowell, *J. Pharmacol. Exp. Ther.*, 2008, **326**, 135–143.