nature chemistry

ARTICLES

https://doi.org/10.1038/s41557-019-0256-3

Identification of autoinducing thiodepsipeptides from staphylococci enabled by native chemical ligation

Bengt H. Gless[1], Martin S. Bojer[2], Pai Peng[2], Mara Baldry[2], Hanne Ingmer[2] and Christian A. Olsen[1*]

Staphylococci secrete autoinducing peptides (AIPs) as signalling molecules to regulate population-wide behaviour. AIPs from non-*Staphylococcus aureus* staphylococci have received attention as potential antivirulence agents to inhibit quorum sensing and virulence gene expression in the human pathogen *Staphylococcus aureus*. However, only a limited number of AIP structures from non-*S. aureus* staphylococci have been identified to date, as the minute amounts secreted in complex media render it difficult. Here, we report a method for the identification of AIPs by exploiting their thiolactone functionality for chemoselective trapping and enrichment of the compounds from the bacterial supernatant. Standard liquid chromatography mass spectrometry analysis, guided by genome sequencing data, then readily provides the AIP identities. Using this approach, we confirm the identity of five known AIPs and identify the AIPs of eleven non-*S. aureus* species, and we expect that the method should be extendable to AIP-expressing Gram-positive bacteria beyond the *Staphylococcus* genus.

Cell-to-cell communication in staphylococci is mediated by secreted peptide signalling molecules—the so-called autoinducing peptides (AIPs)[1]. These peptides are 7–12 amino acids long, contain a carboxy (C)-terminal cyclic thiolactone (or lactone) and are part of the quorum-sensing machinery, which is expressed by the chromosomal locus known as the accessory gene regulator (*agr*)[2–4]. The *agr* system regulates group behaviour, such as virulence gene expression and biofilm formation, in response to cell density via a regulatory RNAIII. Once the AIP concentration reaches a certain threshold, the cognate AIP activates its transmembrane receptor AgrC, resulting in upregulated expression of all quorum-sensing-controlled virulence factors[2–4]. The *agr* has mostly been characterized in *Staphylococcus aureus* but is also present in the coagulase-negative staphylococci, and more than one specificity group may be present in a given species, forming unique AIP–AgrC pairs, such as for *S. aureus* where four *agr* groups exist (*agr*-I–IV)[2].

AIPs potently activate their cognate AgrC receptor and often inhibit AgrC receptors of other staphylococcal species or subgroups within their own species[5,6]. This bacterial cross-talk is not fully understood from a biological standpoint but offers a platform for the development of quorum-sensing inhibitors. The AIPs of *S. aureus agr*-I–IV (**1**–**4**) have been investigated extensively for this purpose[7–16], and more recently, AIPs of non-*S. aureus* staphylococci have received considerable attention[17–21]. A limiting factor for these studies has been the challenging identification of new AIP molecules.

The low concentrations of secreted AIPs and the complex nature of the growth medium represent a significant obstacle. The AIPs known to date have been identified through activity-guided high-performance liquid chromatography (HPLC) purifications[1,5,22,23] or enabled by advanced mass spectrometry[20,24–26]. Even though the first AIPs were discovered more than 20 years ago, only 11 AIPs from 6 species have been characterized thus far. Herein, we present an alternative approach that allows rapid identification of AIPs using standard laboratory equipment, enabled by trapping the peptides through their thiolactone functionality and performing subsequent genome sequence-guided liquid chromatography mass spectrometry (LC-MS) analysis (Fig. 1).

Results and discussion

Development of native chemical ligation (NCL) trapping. C-terminal thiolactones are reactive intermediates in NCL reactions[27], and AIPs readily react with thiols to form linear C-terminal thioesters[28]. We therefore envisioned that an acid-labile Rink-amide resin[29] loaded with unprotected cysteine would covalently trap AIPs from the bacterial supernatant through NCL reaction. This allows enrichment through extensive washing, cleavage from the solid support, and reconstitution of the resulting cysteine-modified AIP for LC-MS analysis. AIP identification is then guided by the genomic sequence of the AIP precursor peptide AgrD, which has a conserved overall structure in all staphylococci[3]. The AIP-containing AgrD sequence is flanked by a C-terminal recognition sequence and an amino (N)-terminal leader peptide, which can be identified by highly conserved residues (shown in blue boxes in Fig. 2b). However, the cleavage site between the leader peptide and the thiolactone-forming cysteine cannot be predicted based on the genome. Therefore, examination of extracted ion chromatograms (EICs) for all seven possible linear AIP-Cys analogues is necessary (Fig. 2c).

First, we successfully tested our hypothesis with synthetic AIPs and high-swelling polyethylene glycol polyacrylamide (PEGA) resin loaded with Rink-amide linker and cysteine (Cys-Rink-PEGA resin; **5**) (see Supplementary Fig. 1). Next, we performed NCL trapping with lyophilized AIP-II (**2**)-containing bacterial supernatant that was reconstituted in phosphate buffer (0.1 M, pH = 7.4)

[1]Center for Biopharmaceuticals, Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark. [2]Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark. *e-mail: cao@sund.ku.dk

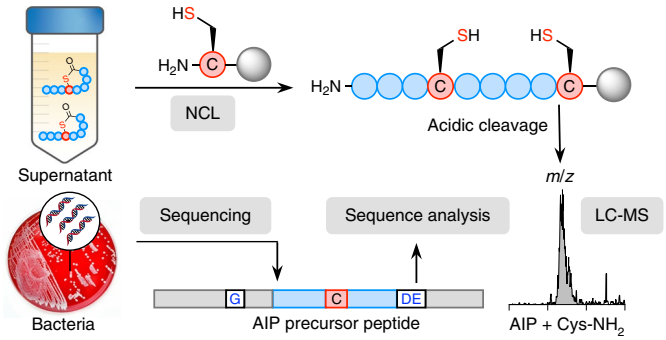


Fig. 1 | Overview of the reported workflow. Supernatants of bacterial overnight cultures contain minute amounts of secreted AIPs. The AIPs contain a thiolactone moiety, which enables chemoselective reaction with resin-bound cysteine through NCL, and thereby covalent trapping of the AIPs as linear peptide derivatives. The trapped AIPs may be released from the solid support using TFA and subsequently analysed by LC-MS. The LC-MS analysis is guided by the amino acid sequence of the AIP precursor peptide, which is obtained by genomic sequencing. The identity of the excreted AIP is thus confirmed by an *m/z* signal corresponding to the mass of the AIP + cysteine amide.

containing 20% *N*,*N*-dimethylformamide (DMF) and tris(2-carboxyethyl)phosphine (TCEP; 30 mM) (Fig. 2a). The resin was then washed extensively, and resin-bound AIP-II (**6**) was released using trifluoroacetic acid (TFA) to give linear AIP-II with a C-terminal cysteine amide residue (**7**). Examination of the sequence of AgrD-II provided 7 possible AIPs ranging from 6–12 amino acids (Fig. 2b). We displayed the *m/z* $[M+H]^+$ values corresponding to the 7 possible linear peptides containing a C-terminal cysteine amide as individual EICs and found a strong signal for the expected *m/z* $[M+H]^+ = 999.4$ (Fig. 2c). The applicability of our NCL trapping strategy was confirmed in a control experiment, where synthetic AIP-II (**2**) was added to fresh tryptic soy broth (TSB) medium—the medium used to grow *S. aureus*—and trapped using the same method, giving identical results (Fig. 2c). The protocol was further validated by confirming the identity of the remaining AIPs from *S. aureus* (**1**, **3** and **4**) (Table 1 and Supplementary Figs. 2–5).

Identified AIPs. Next, we focused on the identification of new non-*S. aureus* staphylococcal AIPs from a collection of diverse *Staphylococcus* species (Table 1, Supplementary Tables 1–3 and Supplementary Figs. 6–17). First, the structure of the recently identified AIP of the coagulase-negative strain *S. saprophyticus*

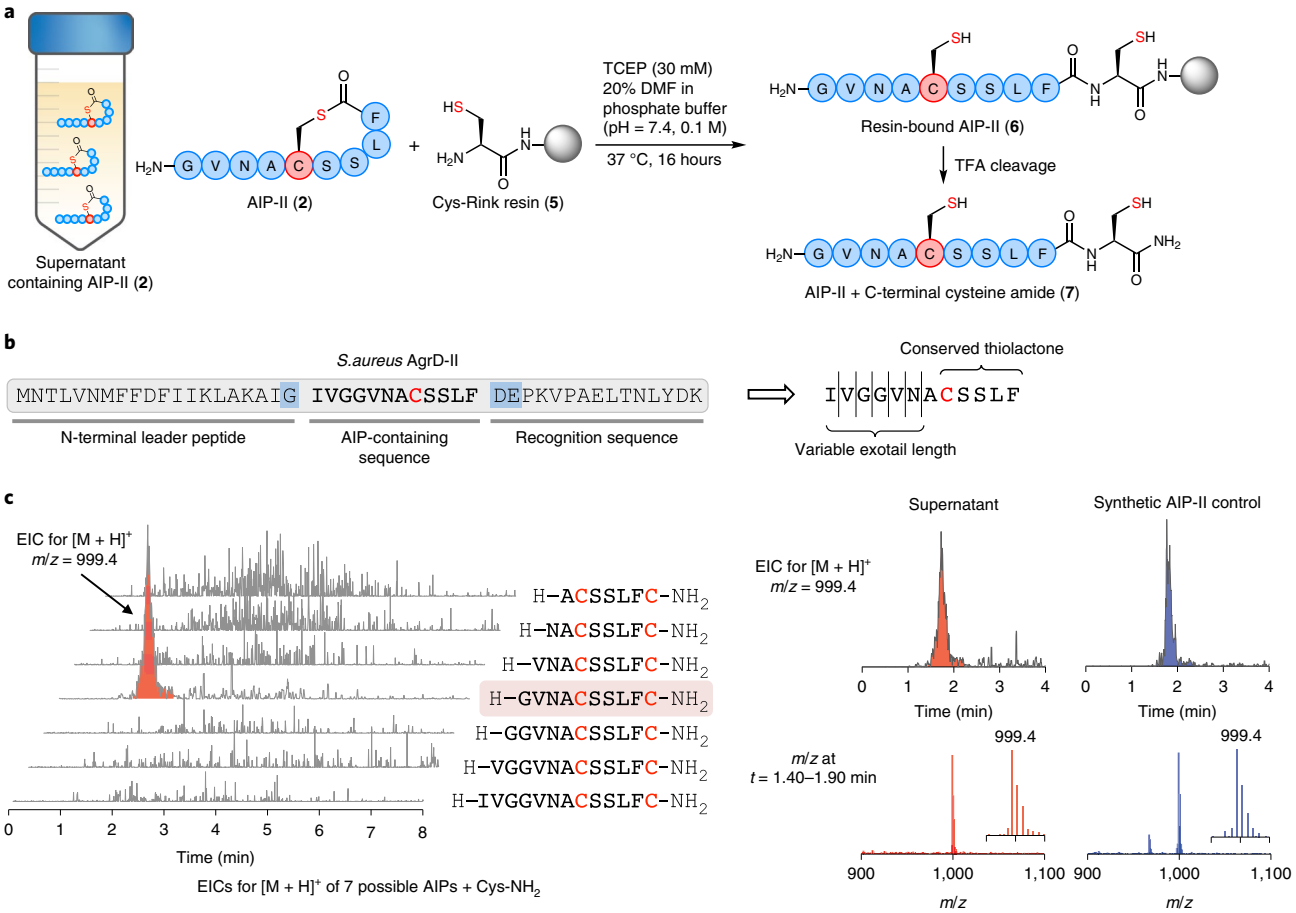
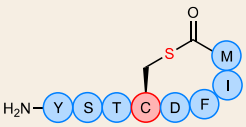
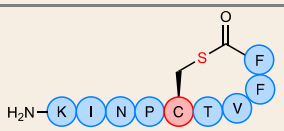
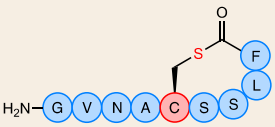
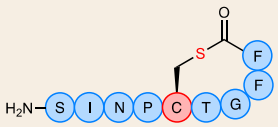
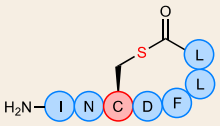
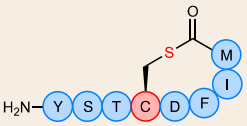
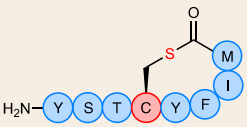
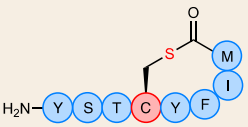
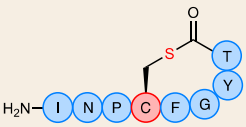
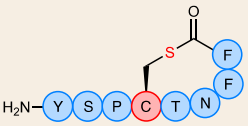
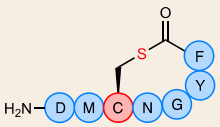
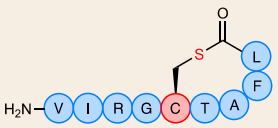
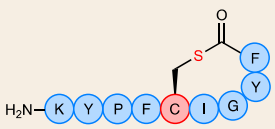
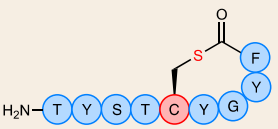
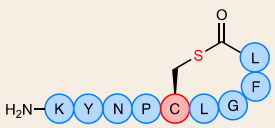
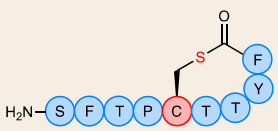


Fig. 2 | NCL trapping and sequence-guided identification of AIP-II (2). a, Spent medium (supernatant) containing AIP-II (**2**) was lyophilized and reconstituted in trapping buffer, to which Cys-Rink-PEGA resin (**5**) was also added. After incubation at 37 °C overnight, the resin was washed, and trapped molecules were released by treatment with TFA and collected for LC-MS analysis. **b**, The AIP-II (2) precursor AgrD-II consists of three segments: an N-terminal leader peptide; an AIP-containing sequence; and a C-terminal recognition sequence. The segments can be identified by the highly conserved residues highlighted in blue boxes. The unpredictable length of the exotail of the AIP is defined by the seven-amino-acid sequence before the conserved thiolactone moiety, giving rise to seven possible AIP structures. **c**, LC-MS analysis of the cleaved AIP-II-Cys-NH_2 conjugate (**7**) through examination of the EICs for *m/z* = $[M+H]^+$ of the seven possible linear AIP-II derivatives containing the C-terminal cysteine amide confirmed the identity of AIP-II (**2**). This was further validated by performing a control experiment with synthetic AIP-II (**2**).

Table 1 | Identified AIPs

Staphylococcus species Sequence	AIP Structure	Staphylococcus species Sequence	AIP Structure
S. aureus agr- I[a] YST-[CDFIM] AIP-I (**1**)		*S. hyicus*[b] KINP-[CTVFF] S_{hy}-AIP (**12**)	
S. aureus agr -II[a] GVNA-[CSSLF] AIP-II (**2**)		*S. chromogenes*[b] SINP-[CTGFF] S_{ch}-AIP (**13**)	
S. aureus agr -III[a] IN-[CDFLL] AIP-III (**3**)		*S. argenteus*[b] YST-[CDFIM] AIP-I (**1**)	
S. aureus agr -IV[a] YST-[CYFIM] AIP-IV (**4**)		*S. schweitzeri*[b] YST-[CYFIM] AIP-IV (**4**)	
S. saprophyticus[a] INP-[CFGYT] S_{sa}-AIP (**8**)		*S. warneri*[b] YSP-[CTNFF] S_{wa}-AIP (**14**)	
S. lugdunensis agr -II[b] DM-[CNGYF] S_{lu}-AIP-II (**9**)		*S. vitulinus*[b] VIRG-[CTAFL] S_{vi}-AIP (**15**)	
S. schleiferi[b] KYPF-[CIGYF] S_{sc}-AIP (**10**)		*S. hominis*[b] TYST-[CYGYF] S_{ho}-AIP (**16**)	
S. simulans[b] KYNP-[CLGFL] S_{si}-AIP (**11**)		*S. haemolyticus*[b] SFTP-[CTTYF] S_{ha}-AIP (**17**)	

[a]Known AIP identity confirmed in this study. [b]AIP characterized for the first time from the mentioned species.

(S_{sa}-AIP; **8**)[26] was confirmed, and the predicted AIP of the second specificity group of the human pathogen *S. lugdunensis agr*-II (S_{lu}-AIP-II; **9**)[30] was identified to be seven amino acids in length. The AIP of the *agr*-I-inhibiting *S. schleiferi* strain (S_{sc}-AIP; **10**) was then identified as a nonapeptide, in contrast with the previously predicted octapeptide (S_{sc}-AIP$_8$, YPF-[CIGYF]; **10a**)[18,19], thus correcting its structure and highlighting the importance of direct AIP identification from the biological source.

We further identified the AIPs of three animal pathogens—namely *S. simulans*[31] (S_{si}-AIP; **11**), *S. hyicus*[32] (S_{hy}-AIP; **12**) and *S. chromogenes*[31] (S_{ch}-AIP; **13**)—as being nonapeptides, and classified the recently described[33] *S. schweitzeri* and *S. argenteus* as strains that express AIP-IV (**4**) and AIP-I (**1**), respectively. Applying the protocol further confirmed the predicted AIP[34] of the human pathogen *S. warneri*[35] (S_{wa}-AIP; **14**) to be the octapeptide structure. Finally, the structures of the AIPs of three coagulase-negative strains (that is, *S. vitulinus*[36] (S_{vi}-AIP; **15**), the antimicrobial peptide-producing strain *S. hominis*[37] (S_{ho}-AIP; **16**) and the clinically relevant strain *S. haemolyticus*[38] (S_{ha}-AIP; **17**)) were found to be nine amino acids in length, revealing a total of seven new AIPs that were different from the often previously predicted octapeptide structures[3].

Quorum-sensing modulation. All newly identified AIPs were synthesized using a previously reported on-resin cyclization–cleavage protocol[19] in acceptable-to-good yields (Fig. 3).

The new AIPs were then tested for their potential as quorum-sensing modulators of all four *S. aureus agr* groups (Table 2). Activation assays were performed in case there was no detectable inhibition at a peptide concentration of 10 μM. The trends of previously reported half-maximal inhibitory concentration (IC_{50}) and half-maximal effective concentration (EC_{50}) values, employing

Table 2 | IC_{50} and EC_{50} values (nM) for quorum-sensing modulation measured in a β-lactamase assay using *S. aureus* AgrC-I–IV reporter strains

AIP	AgrC-I	AgrC-II[a]	AgrC-III[a]	AgrC-IV[a]
AIP-I (**1**)	**5.4 ± 0.5**[b]	700 ± 126	80 ± 11	**790 ± 66**[b]
AIP-II (**2**)	110 ± 12	**3.7 ± 0.5**[b]	40 ± 15	230 ± 19
AIP-III (**3**)	120 ± 15	150 ± 63	**11 ± 1**[b]	>1,000
AIP-IV (**4**)	**59 ± 2**[b]	47 ± 9	9.8 ± 0.2[c]	**2.6 ± 0.2**[b]
S_{sa}-AIP (**8**)	360 ± 30	–[d]	–[d]	–[d]
S_{lu}-AIP-II (**9**)	>1,000	–[d]	–[d]	–[d]
S_{sc}-AIP (**10**)	2.8 ± 0.8	86 ± 6	80 ± 16[c]	**31 ± 6**[b]
S_{si}-AIP (**11**)	8.6 ± 0.5	23 ± 2	50 ± 11	280 ± 66
S_{hy}-AIP (**12**)	3.3 ± 0.5	350 ± 90	4.0 ± 0.8	180 ± 33
S_{ch}-AIP (**13**)	15 ± 1	200 ± 13	60 ± 13	350 ± 85
S_{wa}-AIP (**14**)	100 ± 10	1,000 ± 105	460 ± 59	>1,000
S_{vi}-AIP (**15**)	190 ± 15	800 ± 116	690 ± 46	–[d]
S_{ho}-AIP (**16**)	134 ± 8	580 ± 61	220 ± 46[c]	**54 ± 5**[b]
S_{ha}-AIP (**17**)	340 ± 25	–[d]	340 ± 91	–[d]
AIP-III D4A (**3a**); IN-[CAFLL]	3.5 ± 0.8	34 ± 1	1.6 ± 0.2[c]	10 ± 0.2
S_{sc}-AIP$_8$ (**10a**); YPF-[CIGYF]	3.3 ± 0.7	62 ± 4	22 ± 4	**29 ± 5**[b]

[a]P3-fused β-lactamase reporter strains expressing AgrC-II–IV were constructed (see Supplementary Information). [b]EC_{50} values. [c]Only partial inhibition observed. All inhibition assays were performed in the presence of 100 nM cognate AIP and the results represent means ± s.e.m. of at least duplicate determinations performed in biological triplicate. [d]No inhibition recorded at the highest AIP concentration tested.

similar assays, are in agreement with our results[7,10]. Several newly identified AIPs (**8**, **9** and **14**–**17**) exhibited weak-to-undetectable inhibition of the AgrC-I–IV activation in *S. aureus*. Medium-to-high potencies against AgrC-I–IV activation were exhibited by S_{si}-AIP (**11**), S_{hy}-AIP (**12**) and S_{ch}-AIP (**13**). Compounds **10**–**13** were more potent than AIP-I–IV (**1**–**4**) against several subgroups, and in some cases equipotent to the optimized, global, pan-inhibitor AIP-III D4A (**3a**)[12], rendering them promising candidates for further quorum-sensing inhibitor development.

Interestingly, three tested AIPs (**10**, **10a** and **16**) were potent activators of AgrC-IV, which is an intriguing discovery, keeping in mind that the sequence similarity of AgrC-I and AgrC-IV is 87% and that their native AIPs are cross-activators[2]. To the best of our knowledge, S_{sc}-AIP (**10**) and S_{ho}-AIP (**16**) constitute the first staphylococcal interspecies agonists discovered. Furthermore, no compounds except AIP-III D4A (**3a**) exhibit potent inhibition against AgrC-IV. The *S. aureus agr* group IV is considered an evolutionary divergence from group I[39] and is known to have early or hyperactive activation kinetics[23]. While a previous testing of compound **10a** was in agreement with the current results[19], we measured it to be an inhibitor of AgrC-IV in the original account[18]. We suspect that this is due to the mentioned activation kinetics, which makes the potency measurements highly sensitive to assay conditions. Based on the collective evidence, we are confident that both **10** and **10a** should be considered activators of the *S. aureus agr* group IV.

Investigation of *Listeria monocytogenes*. Having identified and characterized 16 staphylococcal AIPs, we were interested in applying the protocol to other Gram-positive bacteria known to have *agr*-like systems[40]. In *L. monocytogenes*, the *agr* system has been shown to control biofilm formation, virulence and carbohydrate metabolism[41–44], and an AIP of *L. monocytogenes* was recently identified directly in spent medium by advanced LC-MS/mass spectrometry to be the thiolactone-containing hexapeptide L_{mo}-AIP (**20**; Fig. 4a)[26]. To further test our protocol, three different isolates of *L. monocytogenes* were therefore grown in either TSB or brain heart infusion media (a commonly used medium for the growth of *Listeria* strains). NCL trapping experiments were then performed on the lyophilized and reconstituted supernatants, as outlined above. Surprisingly, no signal for the linear heptapeptide **21** (m/z $[M+H]^+ = 819.3$) or other masses corresponding to potential AIP sequences, could be detected in any of these experiments, including the mass resulting from trapping the pentapeptide thiolactone previously described[44] (see Supplementary Fig. 18). However, this thiolactone would be expected to undergo spontaneous S–N shift under our trapping conditions to furnish the corresponding cysteine-containing homodetic pentapeptide, which would not be prone to undergoing NCL with resin (**5**).

To examine whether the hexapeptide L_{mo}-AIP (**20**) would be trapped using our protocol, and to investigate the detection limit in the two different media, we synthesized L_{mo}-AIP (**20**) and performed NCL trapping experiments using the synthetic AIP in growth media at varying concentrations (Fig. 4 and Supplementary Fig. 18). We were pleased to find that, regardless of the medium, concentrations as low as 0.063 μM still allowed the detection of the linear heptapeptide **21** or its disulfide **22**, corresponding to a concentration of L_{mo}-AIP (**20**) of 12.5 nM in the supernatant before lyophilization and reconstitution in a trapping experiment (Fig. 4a,b and Supplementary Fig. 18). This should be seen in the light that *S. aureus* AIPs are estimated to reach a concentration of around 1 μM in the supernatant[45]. We further tested synthetic L_{mo}-AIP (**20**), as well as the supernatants of our three *L. monocytogenes* isolates, for their ability to inhibit or activate *S. aureus* AgrC-I–IV. None of the supernatants showed measurable inhibition, and L_{mo}-AIP (**20**) only partially inhibited AgrC-III at the highest tested concentration (Fig. 4c and Supplementary Fig. 18). We conclude that L_{mo}-AIP (**20**) can be trapped and identified at concentrations corresponding to just 12.5 nM in bacterial supernatants, and that if this AIP were expressed under our growth conditions, it must have been secreted in a concentration below this very low threshold.

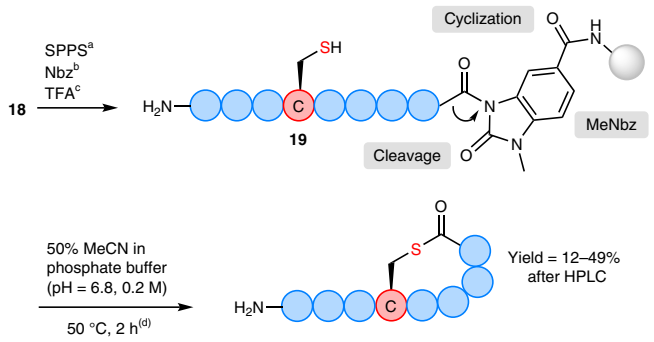


Fig. 3 | Synthesis of AIPs. Reagents and conditions were as follows. [a]Automated fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) on MeDbz-Gly resin (**18**) using Fmoc-Xaa-OH (5 equivalents), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (5 equivalents), *i*-Pr_2EtN (10 equivalents) and DMF for 40 min. [b]4-Nitrophenolchloroformate (5 equivalents) in CH_2Cl_2, then *i*-Pr_2EtN (25 equivalents) in DMF. [c]TFA–*i*-Pr_3SiH–H_2O for 2 hours. [d]Deprotected peptidyl-resin **19** was swollen in cyclization buffer for 2 hours at 50 °C, furnishing the desired AIPs in 12–49% yield after HPLC purification (length varying from hepta- to nona-petide).

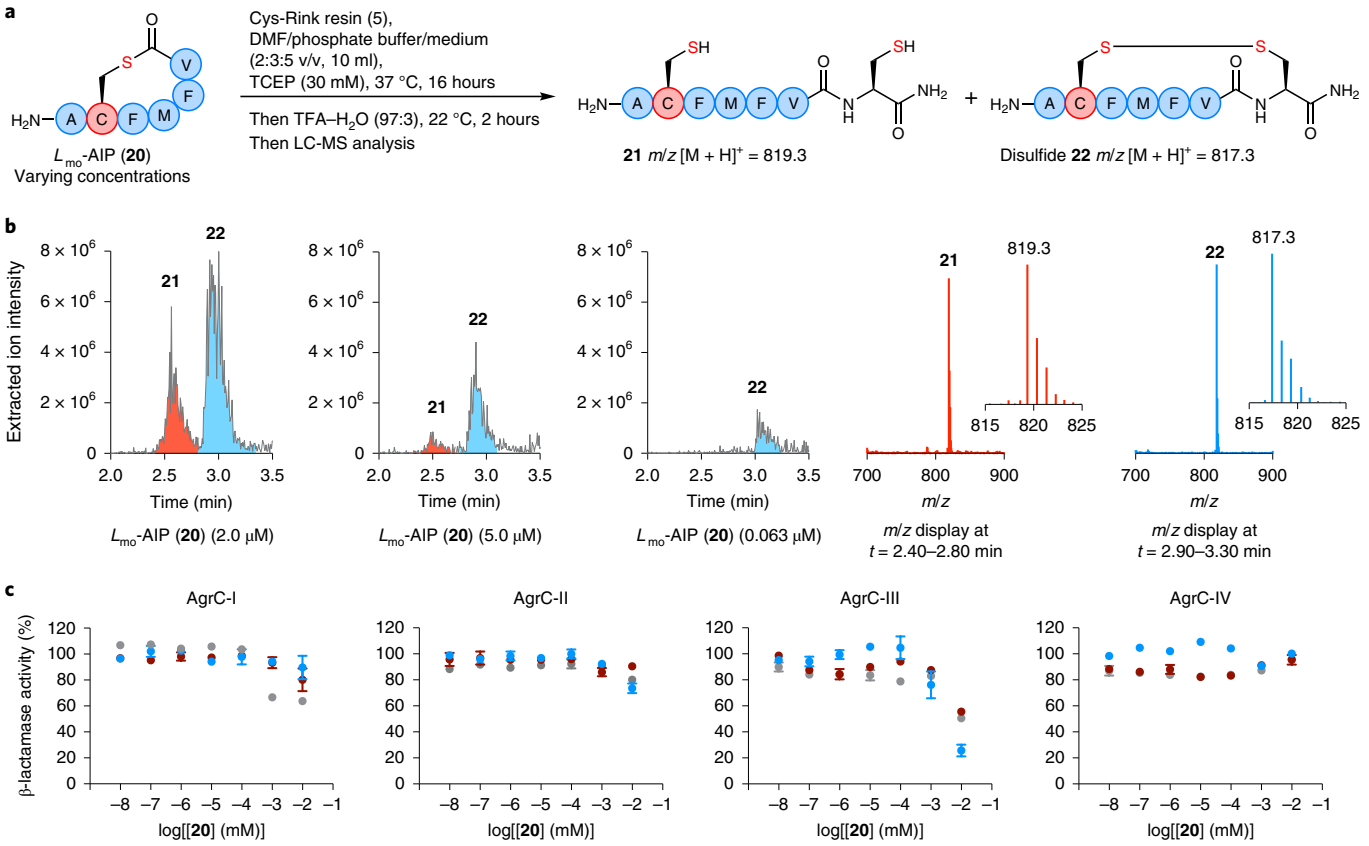


Fig. 4 | Detection limit of NCL trapping for synthetic *L. monocytogenes* AIP (20). a, NCL trapping of synthetic L_{mo}-AIP (**20**) dissolved at varying concentrations in 50% TSB medium (total volume, 10 ml). Although TCEP is present during trapping, some oxidation to form the cyclic disulfide occurs, presumably during TFA cleavage. **b**, Display of overlaid EICs for the linear peptide **21** ($[M+H]^+$ $m/z = 819.3$) and its corresponding disulfide **22** ($[M+H]^+$ $m/z = 817.3$) after TFA cleavage and LC-MS analysis for three concentrations (2.0 μM, 0.5 μM and 0.063 μM). This clearly shows a signal for **22** at the lowest concentration (0.063 μM). **c**, *β*-Lactamase assay inhibition curves of L_{mo}-AIP (**20**) against AgrC-I–IV show no or very weak inhibition. The error bars represent the s.e.m. of two individual assays performed in biological triplicate.

Considering these results, we are confident that our protocol will be useful for the investigation of quorum-sensing systems of Gram-positive bacteria that rely on thiolactone-containing signalling molecules beyond the staphylococci.

Conclusion

The data presented here provide a readily applicable protocol that exploits the chemoselective reaction between thiolactones and a resin-bound cysteine, which allows trapping of AIPs from bacterial supernatants via NCL. The developed method facilitated rapid doubling of the number of known staphylococcal AIPs, and thus provides the means to start mapping of the staphylococcal AIP signalling molecule landscape. The *Staphylococcus* genus currently includes 52 species and 28 subspecies[46], where several species have more than one specificity group, such as *S. aureus agr*-I–IV. Thus, a conservative estimate of the potential number of staphylococcal AIPs is likely to comprise more than 100 peptides still to be discovered. We furthermore expect the methodology to be broadly applicable to other Gram-positive bacteria that use thiolactone-containing auto inducers and therefore to enable the achievement of a better understanding of quorum sensing in general. Finally, access to new AIPs paves the way to detailed studies of quorum sensing and its impact on important pathogen properties such as biofilm formation, colonization, virulence and interactions with commensal staphylococci.

Methods

NCL trapping of AIPs from bacterial supernatants. Fmoc-Cys(StBu)-Rink-PEGA resin (**5**) (50 mg) was placed in a 10 ml fritted polypropylene syringe, swelled in DMF for 30 min and washed with DMF (5 × 1 min). The resin was treated with piperidine in DMF (1:4 *v/v*, 2.0 ml) (1 × 2 min and 1 × 20 min) and washed with DMF (3 × 1 min), MeOH (3 × 1 min) and H_2O (3 × 1 min). The resin was treated with a TCEP solution (0.5 M, pH = 7.00, 0.5 ml) for 15 min and subsequently washed with H_2O (3 × 1 min), MeOH (3 × 1 min) and DMF (3 × 1 min). Lyophilized supernatant (original volume 50 ml) was dissolved in 10 ml trapping buffer (phosphate buffer (0.1 M, pH = 7.4)–DMF–TCEP solution (0.5 M, pH = 7.0) = 7.4:2:0.6 *v/v/v*) and the solution was added to the resin. The syringe containing the trapping mixture was agitated at 37 °C overnight. The trapping solution was removed from the resin and the resin was subsequently washed with DMF (3 × 1 min), MeOH (3 × 1 min) and H_2O (3 × 1 min). The resin was then treated with a TCEP solution (0.5 M, pH = 7.00, 0.5 ml) for 10 min and subsequently washed with H_2O (3 × 2 ml). A solution of DMF in H_2O (10 ml, 1:1 *v/v*) was added to the resin and the resin was agitated at 37 °C. After 30 min, the resin was washed with DMF (3 × 1 min), MeOH (3 × 1 min) and DCM (3 × 1 min) and dried under suction for 15 min. The dried resin was treated with a cleavage cocktail (1.5 ml TFA–Milli-Q water, 97:3 *v/v*) for 2 h at room temperature. The peptide containing cleavage solution was removed from the resin and collected, and the resin was rinsed with neat TFA (1.0 ml). The combined TFA fractions were evaporated under an N_2 stream to near-dryness, redissolved in a solution of MeCN in H_2O (100 μl, 1:1 *v/v*) and filtered (0.22 μm). The solution was analysed by LC-MS as described.

Sequence-guided LC-MS analysis. The filtered TFA cleavage solution was analysed using a Waters Acquity ultra-HPLC system equipped with a Phenomenex Kinetex column (1.7 μm, 100 Å, 50 × 2.10 mm) applying a gradient with eluent C (0.1% HCOOH in water) and eluent D (0.1% HCOOH in MeCN) rising linearly from 0–95% of D over 10.0 min at a flow rate of 0.6 ml min^{-1} and an injection

volume of 40 µl. The total ion chromatograms were analysed by displaying EICs of m/z $[M+H]^+$ values of the possible linear peptides with an additional C-terminal cysteine amide residue compared to the AgrD sequence.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Primary sequencing data are deposited at the National Centre for Biotechnology Information (NCBI GenBank). All other data generated and analysed during this study are available in the article and its Supplementary Information. Further details are available from the corresponding author on request.

Received: 23 September 2018; Accepted: 14 March 2019;
Published online: 22 April 2019

References

1. Ji, G., Beavis, R. C. & Novick, R. P. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proc. Natl Acad. Sci. USA* **92**, 12055–12059 (1995).
2. Novick, R. P. & Geisinger, E. Quorum sensing in staphylococci. *Annu. Rev. Genet.* **42**, 541–564 (2008).
3. Thoendel, M., Kavanaugh, J. S., Flack, C. E. & Horswill, A. R. Peptide signaling in the staphylococci. *Chem. Rev.* **111**, 117–151 (2011).
4. Wang, B. & Muir, T. W. Regulation of virulence in *Staphylococcus aureus*: molecular mechanisms and remaining puzzles. *Cell Chem. Biol.* **23**, 214–224 (2016).
5. Ji, G., Beavis, R. & Novick, R. P. Bacterial interference caused by autoinducing peptide variants. *Science* **276**, 2027–2030 (1997).
6. Otto, M., Süßmuth, R., Vuong, C., Jung, G. & Götz, F. Inhibition of virulence factor expression in *Staphylococcus aureus* by the *Staphylococcus epidermidis* agr pheromone and derivatives. *FEBS Lett.* **450**, 257–262 (1999).
7. Mayville, P. et al. Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. *Proc. Natl Acad. Sci. USA* **96**, 1218–1223 (1999).
8. McDowell, P. et al. Structure, activity and evolution of the group I thiolactone peptide quorum-sensing system of *Staphylococcus aureus*. *Mol. Microbiol.* **41**, 503–512 (2001).
9. Lyon, G. J., Mayville, P., Muir, T. W. & Novick, R. P. Rational design of a global inhibitor of the virulence response in Staphylococcus aureus, based in part on localization of the site of inhibition to the receptor-histidine kinase AgrC. *Proc. Natl Acad. Sci. USA* **97**, 13330–13335 (2000).
10. Lyon, G. J., Wright, J. S., Muir, T. W. & Novick, R. P. Key determinants of receptor activation in the *agr* autoinducing peptides of *Staphylococcus aureus*. *Biochemistry* **41**, 10095–10104 (2002).
11. George, E. A., Novick, R. P. & Muir, T. W. Cyclic peptide inhibitors of staphylococcal virulence prepared by Fmoc-based thiolactone peptide synthesis. *J. Am. Chem. Soc.* **130**, 4914–4924 (2008).
12. Tal-Gan, Y., Stacy, D. M., Foegen, M. K., Koenig, D. W. & Blackwell, H. E. Highly potent inhibitors of quorum sensing in *Staphylococcus aureus* revealed through a systematic synthetic study of the group-III autoinducing peptide. *J. Am. Chem. Soc.* **135**, 7869–7882 (2013).
13. Tal-Gan, Y., Stacy, D. M. & Blackwell, H. E. *N*-Methyl and peptoid scans of an autoinducing peptide reveal new structural features required for inhibition and activation of AgrC quorum sensing receptors in *Staphylococcus aureus*. *Chem. Commun.* **50**, 3000–3003 (2014).
14. Johnson, J. G., Wang, B., Debelouchina, G. T., Novick, R. P. & Muir, T. W. Increasing AIP macrocycle size reveals key features of *agr* activation in *Staphylococcus aureus*. *ChemBioChem* **16**, 1093–1100 (2015).
15. Tal-Gan, Y., Ivancic, M., Cornilescu, G., Yang, T. & Blackwell, H. E. Highly stable, amide-bridged autoinducing peptide analogues that strongly inhibit the AgrC quorum sensing receptor in *Staphylococcus aureus*. *Angew. Chem. Int. Ed.* **55**, 8913–8917 (2016).
16. Hansen, A. M. et al. Lactam hybrid analogues of solonamide B and autoinducing peptides as potent *S. aureus* AgrC antagonists. *Eur. J. Med. Chem.* **152**, 370–376 (2018).
17. Yang, T., Tal-Gan, Y., Paharik, A. E., Horswill, A. R. & Blackwell, H. E. Structure–function analyses of a *Staphylococcus epidermidis* autoinducing peptide reveals motifs critical for AgrC-type receptor modulation. *ACS Chem. Biol.* **11**, 1982–1991 (2016).
18. Canovas, J. et al. Cross-talk between *Staphylococcus aureus* and other staphylococcal species via the *agr* quorum sensing system. *Front. Microbiol.* **7**, 1733 (2016).
19. Gless, B. H. et al. Structure–activity relationship study based on autoinducing peptide (AIP) from dog pathogen *S. schleiferi*. *Org. Lett.* **19**, 5276–5279 (2017).
20. Paharik, A. E. et al. Coagulase-negative staphylococcal strain prevents *Staphylococcus aureus* colonization and skin infection by blocking quorum sensing. *Cell Host Microbe* **22**, 746–756 (2017).
21. Gordon, C. P., Olson, S. D., Lister, J. L., Kavanaugh, J. S. & Horswill, A. R. Truncated autoinducing peptides as antagonists of *Staphylococcus lugdunensis* quorum sensing. *J. Med. Chem.* **59**, 8879–8888 (2016).
22. Otto, M., Süßmuth, R., Jung, G. & Götz, F. Structure of the pheromone peptide of the *Staphylococcus epidermidis agr* system. *FEBS Lett.* **424**, 89–94 (1998).
23. Jarraud, S. et al. Exfoliatin-producing strains define a fourth *agr* specificity group in *Staphylococcus aureus*. *J. Bacteriol.* **182**, 6517–6522 (2000).
24. Kalkum, M., Lyon, G. J. & Chait, B. T. Detection of secreted peptides by using hypothesis-driven multistage mass spectrometry. *Proc. Natl Acad. Sci. USA* **100**, 2795–2800 (2003).
25. Olson, M. E. et al. *Staphylococcus epidermidis agr* quorum-sensing system: signal identification, cross talk, and importance in colonization. *J. Bacteriol.* **196**, 3482–3493 (2014).
26. Todd, D. A. et al. Signal Biosynthesis Inhibition with Ambuic Acid as a Strategy To Target Antibiotic-Resistant Infections. *Antimicrob. Agents Chemother.* **61**, e00263-17 (2017).
27. Tsuda, S., Yoshiya, T., Mochizuki, M. & Nishiuchi, Y. Synthesis of cysteine-rich peptides by native chemical ligation without use of exogenous thiols. *Org. Lett.* **17**, 1806–1809 (2015).
28. Wang, B., Zhao, A., Novick, R. P. & Muir, T. W. Key driving forces in the biosynthesis of autoinducing peptides required for staphylococcal virulence. *Proc. Natl Acad. Sci. USA* **112**, 10679–10684 (2015).
29. Rink, H. Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin. *Tetrahedron Lett.* **28**, 3787–3790 (1987).
30. Dufour, P. et al. High genetic variability of the *agr* locus in *Staphylococcus* species. *J. Bacteriol.* **184**, 1180–1186 (2002).
31. Pyörälä, S. & Taponen, S. Coagulase-negative staphylococci—emerging mastitis pathogens. *Vet. Microbiol.* **134**, 3–8 (2009).
32. Devriese, L. A., Hájek, V., Oeding, P., Meyer, S. A. & Schleifer, K. H. *Staphylococcus hyicus* (Sompolinsky 1953) comb. nov. and *Staphylococcus hyicus* subsp. *chromogenes* subsp. nov. *Int. J. Syst. Evol. Microbiol.* **28**, 482–490 (1978).
33. Tong, S. Y. et al. Novel staphylococcal species that form part of a *Staphylococcus aureus*-related complex: the non-pigmented *Staphylococcus argenteus* sp. nov. and the non-human primate-associated *Staphylococcus schweitzeri* sp. nov. *Int. J. Syst. Evol. Microbiol.* **65**, 15–22 (2015).
34. Novick, R. P., Ross, H. F., Figueiredo, A. M. S., Abramochkin, G. & Muir, T. W. Activation and inhibition of the staphylococcal *AGR* system. *Science* **287**, 391 (2000).
35. Kamath, U., Singer, C. & Isenberg, H. D. Clinical significance of *Staphylococcus warneri* bacteremia. *J. Clin. Microbiol.* **30**, 261–264 (1992).
36. Webster, J. A. et al. Identification of the *Staphylococcus sciuri* species group with *Eco*RI fragments containing rRNA sequences and description of *Staphylococcus vitulus* sp. nov. *Int. J. Syst. Evol. Microbiol.* **44**, 454–460 (1994).
37. Nakatsuji, T. et al. Antimicrobials from human skin commensal bacteria protect against *Staphylococcus aureus* and are deficient in atopic dermatitis. *Sci. Transl. Med.* **9**, eaah4680 (2017).
38. Barros, E. M., Ceotto, H., Bastos, M. C. F., dos Santos, K. R. N. & Giambiagi-deMarval, M. *Staphylococcus haemolyticus* as an important hospital pathogen and carrier of methicillin resistance genes. *J. Clin. Microbiol.* **50**, 166–168 (2012).
39. Robinson, D. A., Monk, A. B., Cooper, J. E., Feil, E. J. & Enright, M. C. Evolutionary genetics of the accessory gene regulator (*agr*) locus in *Staphylococcus aureus*. *J. Bacteriol.* **187**, 8312–8321 (2005).
40. Thoendel, M. & Horswill, A. R. Biosynthesis of peptide signals in Gram-positive bacteria. *Adv. Appl. Microbiol.* **71**, 91–112 (2010).
41. Autret, N., Raynaud, C., Dubail, I., Berche, P. & Charbit, A. Identification of the *agr* locus of *Listeria monocytogenes*: role in bacterial virulence. *Infect. Immun.* **71**, 4463–4471 (2003).
42. Riedel, C. U. et al. AgrD-dependent quorum sensing affects biofilm formation, invasion, virulence and global gene expression profiles in *Listeria monocytogenes*. *Mol. Microbiol.* **71**, 1177–1189 (2009).
43. Vivant, A.-L., Garmyn, D., Gal, L. & Piveteau, P. The Agr communication system provides a benefit to the populations of *Listeria monocytogenes* in soil. *Front. Cell. Infect. Microbiol.* **4**, 160 (2014).
44. Zetzmann, M., Sánchez-Kopper, A., Waidmann, M. S., Blombach, B. & Riedel, C. U. Identification of the *agr* peptide of *Listeria monocytogenes*. *Front. Microbiol.* **7**, 989 (2016).
45. Piewngam, P. et al. Pathogen elimination by probiotic *Bacillus* via signalling interference. *Nature* **562**, 532–537 (2018).
46. Lee, A. S. et al. Methicillin-resistant *Staphylococcus aureus*. *Nat. Rev. Dis. Primers* **4**, 18033 (2018).

Acknowledgements

We thank P. Martín-Gago for fruitful input and T.W. Muir for encouraging comments. P. S. Andersen is acknowledged for providing bacterial strains. This work was supported by the Carlsberg Foundation (2013-01-0333 to C.A.O.) and University of Copenhagen (PhD fellowship to B.H.G.).

Author contributions

B.H.G. and C.A.O. conceptualized the study. B.H.G., M.S.B., P.P. and M.B. performed the experiments. B.H.G. and C.A.O. wrote the original draft of the manuscript. B.H.G., M.S.B., H.I. and C.A.O. reviewed and edited the final manuscript. C.A.O. acquired funding. H.I. and C.A.O. provided resources and materials. H.I. and C.A.O. supervised the study.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41557-019-0256-3.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to C.A.O.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

nature research

Corresponding author(s): Christian Adam Olsen

Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☒	☐	A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated
☐	☒	Clearly defined error bars *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection	Liquid chromatography–mass spectrometry data was collected on MassLynx V4.1 SCN849 Waters Inc.
Data analysis	Assay data was anaylised with GraphPad Prism Version 7.0c. Liquid chromatography–mass spectrometry data was anaylised with MassLynx V4.1 SCN849 Waters Inc.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Primary sequencing data is deposited at NCBI and will be released upon acceptance of this manuscript for publication. All other data is available from the corresponding author on reasonable request.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The study included the trapping and identification of 16 autoinducing peptides from staphylococci supernatants, of which 5 were previously identified and 11 were unknown. This represents a respectable sample size in comparison to the number of previously identified autoinducing peptides. 16 synthetic autoinducing peptides were assayed for the ability to modulate quorum sensing in Staphylococcus aureus agr-I-IV.
Data exclusions	No data was excluded from the analyses.
Replication	All attempts of replication were successful using bacterial supernatant from the same bacterial strain.
Randomization	n/a
Blinding	n/a

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a	Involved in the study
☐	☒ Unique biological materials
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology
☒	☐ Animals and other organisms
☒	☐ Human research participants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials	All bacterial strains are available from the corresponding author upon request.