Rearrangement of Thiodepsipeptides by S→N Acyl Shift Delivers Homodetic Autoinducing Peptides

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ABSTRACT: Group behavior in many bacteria relies on chemically induced communication called quorum sensing (QS), which plays important roles in regulation of colonization, biofilm formation, and virulence. In Gram-positive bacteria, QS is often mediated by cyclic ribosomally synthesized and posttranslationally modified peptides (RiPPs). In staphylococci for example, most of these so-called autoinducing peptides (AIPs) contain a conserved thiolactone functionality, which has been predicted to constitute a structural feature of AIPs from other genera as well. Here, we show that pentameric AIPs from *Lactiplantibacillus plantarum, Clostridium perfringens,* and *Listeria monocytogenes* that were previously presumed to be thiolactone-containing structures readily rearrange to become homodetic cyclopeptides. This finding has implications for the developing understanding of the cross-species and cross-genus communication of bacteria and may help guide the discovery of peptide ligands to perturb their function.

In bacteria, quorum sensing (QS) is a mechanism that relies on secretion and detection of signaling molecules, which modulate synchronized changes in population-wide behavior in a cell density-dependent manner. This cell-to-cell communication has been shown to regulate important functions, such as colonization, biofilm formation, and virulence.[1] The accessory gene regulator *(agr)* locus is a group of four genes (*agrBDCA*) encoding the components of QS systems found in several Gram-positive bacteria,[2] including *Listeria monocytogenes* (Figure 1A).[3] *agr* Systems utilize short cyclic peptides, usually containing a thiolactione functionality, as QS signals. These so-called autoinducing peptides (AIPs) originate from the ribosomally synthesized AIP precursor peptide AgrD, which is intracellularly processed by the membrane-embedded protein AgrB. Outside the cell, the mature AIP binds the receptor protein AgrC, which induces activation of the response regulator, AgrA. In turn, activated AgrA binds to the P2 promoter, resulting in upregulated expression of all *agr* genes, creating a positive-feedback loop.[1,2] QS interference through inhibition of the AgrC receptor by foreign AIPs has been well established among staphylococci.[4-7] Understanding this bacterial crosstalk may help elucidate complex social interactions that occur in mixed bacterial communities. Moreover, QS is linked to virulence in opportunistic pathogens like *Staphylococcus aureus*[8] and *Listeria monocytogenes*[9] and, consequently, inhibition of QS may offer an interesting therapeutic alternative to antibiotics for the treatment of infectious diseases caused by pathogenic bacteria.[10] Thus, knowledge about AIP structures is crucial for probing the molecular mechanisms of QS and pharmacological aspects of QS inhibition.[11] We recently developed a simple and robust procedure for enrichment of thiolactone-containing AIPs from complex bacterial supernatant, which facilitated the structural elucidation of a number of staphylococcal AIPs.[12] The method is predicated on the chemoselective trapping of thiolactones by resin-bound cysteine residues through a native chemical ligation (NCL)[13] like transformation. Using this method, we were unable to identify a native AIP for *L. monocytogenes*, which had been reported as both a hexamer (**P1**),[14] also found in *Listeria innocua*,[15] and a pentamer (**P2**).[16] This led us to speculate that a cyclic homodetic pentamer (**P3**), arising from spontaneous S→N acyl shift of the thiolactone in **P2**, might be the native AIP (Figure 1B and Figure 2A).[12] The resulting AIP (**P3**) would be unreactive towards our cysteine resin, explaining why no peptide could be identified from the supernatant (Figure S1).[12,17] Two additional exotail-free thiolactone-containing AIPs have been reported in the literature, peptide **P4** from *Lactiplantibacillus plantarum*[18] and peptide **P6** from *Clostridium perfringens*[19] (Figure 1C), which we also predicted to undergo the same rearrangement. To investigate this hypothesis, we first synthesized the trifluoroacetic acid salts of exotail-free thiolactone-containing peptides **P2**, **P4**, and **P6** by solid-phase peptide synthesis (SPPS) (Scheme S1) as well as their corresponding homodetic peptides **P3**, **P5**, and **P7** applying our previously reported cleavage-inducing cyclization method (Scheme S2).[20]

To investigate the predicted S→N acyl shift of exotail-free thiolactone-containing pentamers, we first examined the influence of pH on the rearrangement of **P2** to **P3** at a single time point (Figure S2A).

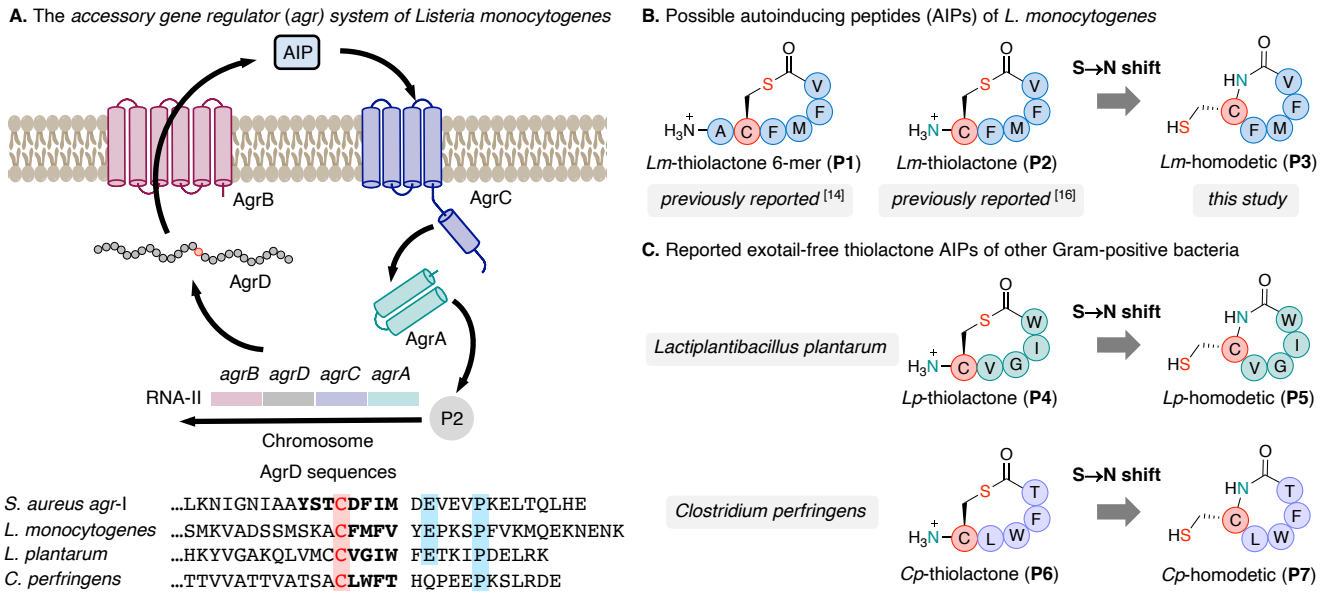
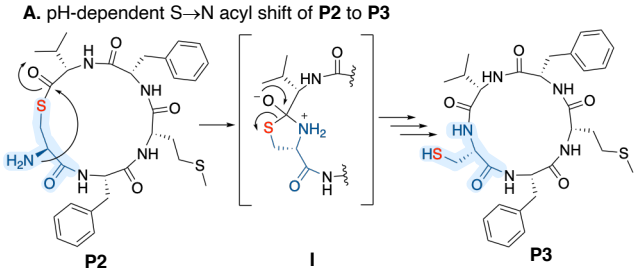


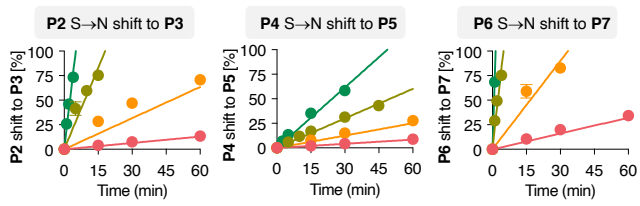
Figure 1. **(A)** The *agr* QS system of *L. monocytogenes* and aligned AIP-precursor peptide sequences (AgrD) of Gram-positive bacteria with *agr* genes. Structures of (**B**) the proposed AIPs of *L. monocytogenes and* (**C**) the possible AIPs of *L. plantarum* and *C. perfringens*.

After 15 min, we observed no formation of **P3** at low pH (pH 2 and 3), while at higher pH (pH ≥ 7) **P2** was fully converted to **P3**. The formation of **P3** was confirmed by comparison to synthesized **P3** (Figure S9–10). Next, we followed the S→N acyl shift of **P2** over time at pH 4–7 to assess the half-life of **P2** at different pH values (Figure 2B and Figure S3–4). The rearrangement at pH 4 proceeds with a rate constant of 0.22 M min^{-1} and a half-life time of 227 min, if the S→N shift proceeds under zero order kinetics without any rate-determining proton transfer steps. The rate increases significantly with each pH unit, resulting in a ~90-fold faster reaction rate at pH 7 compared to pH 4. As the assay conditions require MeCN to keep the peptide solubilized, we evaluated the effect of the co-solvent on the reaction rate and found that the acyl shift occurs slightly faster with decreasing amount of MeCN (Figure S2B). Following the same procedure as for **P2**, we then determined the rate constants for the S→N shift of **P4** to **P5** and **P6** to **P7** at pH 4–7 (Figure 2B and Figure S5–8). The rearrangement of the exotail-free thiolactone **P4** was substantially slower and significantly less pH-dependent (only 15-fold faster at pH 7 vs pH 4) than the other two peptides, which is highlighted by a half-life of 24 min at pH 7. In contrast, peptide **P6** exhibited the fastest S→N shift rates with a half-life of less than 1 minute at pH 7. It is noteworthy that differences of up to 30-fold for the rate of intramolecular S→N shift is observed despite identical macrocycle size of **P2**, **P4**, and **P6**.

The longer half-life of **P4** together with fact that *L. plantarum* is capable of inhabiting acidic environments leave the possibility open that either **P4** or **P5** may act as its QS signaling molecule. We were not able to identify the exotail-free thiolactone **P4**, using our NCL trapping method despite its higher stability at pH 7 (Figure S1D), which may indicate that the rearranged compound is the AIP. However, further investigation into the *agr* system of *L. plantarum* is required to unambiguously determine the identity of its AIP.

The rates by which synthetic **P6** rearranges in neutral media suggest that the homodetic peptide **P7** is likely to be an AIP, stimulating the *agr* related downstream effects reported.[19] The two peptides, **P1** and **P2**, have been identified from bacterial cultures as AIPs of *L. monocytogenes* by mass spectrometry methods. In the study identifying **P2** by Zetzmann *et al.,* a variety of synthetic peptides based on the AgrD sequence, including **P1** and **P2**, were tested against luciferase-expressing *L. monocytogenes* reporter strains. One strain was capable of producing AIPs (WT::P2-lux) while the other one had the *agrD* gene deleted (*ΔagrD*::P2-lux) and was therefore incapable of AIP biosynthesis (Figure 3A).[16] Strong activation of the reporter strains were reported for **P2**.





	S→N acyl shift rate constant *k* [M min^{-1}] ($t_{1/2}$)		
	P2 to **P3**	**P4** to **P5**	**P6** to **P7**
pH 4	0.22 (227 min)	0.14 (358 min)	0.54 (93 min)
pH 5	1.1 (45 min)	0.42 (119 min)	3.0 (17 min)
pH 6	5.5 (9.1 min)	1.0 (50 min)	20.8 (2.4 min)
pH 7	19.6 (2.6 min)	2.1 (24 min)	68.5 (0.7 min)

Figure 2. (**A**) Simplified mechanism of the pH-dependent S→N acyl shift of **P2** via the tetrahedral intermediate **I**. (**B**) Estimated S→N acyl shift rates of **P2**, **P4**, and **P6** at 30 °C in buffer (pH 4–7)–MeCN (1:1), measured by UPLC analysis and based on the relative areas under the peak corresponding to each peptide.

However, based on the half-life of **P2** at 30 °C (2.7 min; pH 7) we anticipate that **P2** would quickly convert to **P3** in the performed assays at pH 7.4, rendering **P3** likely to be responsible for the observed effect. To test this hypothesis, we treated both WT::P2-lux and *ΔagrD*::P2-lux reporter strains with synthetic **P1** and **P3** using brain heart infusion (BHI) growth medium (Figure 3B and Figure S11–12). Under these conditions, we observed significant early induction of the *agr* system by **P3** in both strains. For **P1**, inhibition of the WT::P2-lux was observed, while for *ΔagrD*::P2-lux a later induced signal was visible. The bimodal shape of the luminesce recording in these experiments led us to speculate whether there could be a nutrient dependence on the readout, and the effect of performing the assays in tryptic soy broth (TSB) growth medium was investigated (Figure 3C–D and Figure S13–S14).

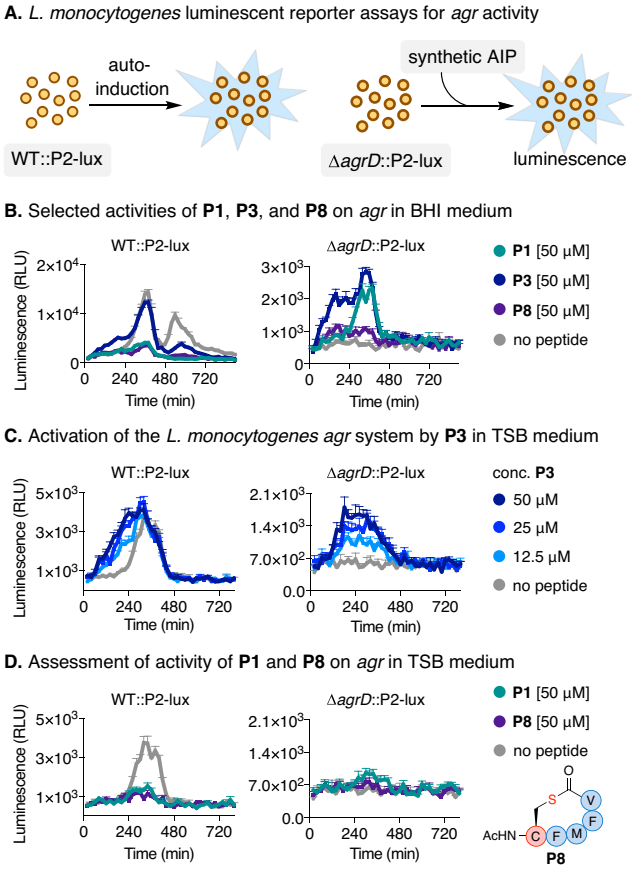


Figure 3. (**A**) Luciferase-expressing reporter strains of *L. monocytogenes*. (**B**) Assay data for **P1**, **P3**, and **P8** in brain heart infusion (BHI) medium. (**C**) Activation of luciferase reporter strains grown in tryptic soy broth (TSB) in the presence of **P3**. (**D**) Assessment of *agr* activity of **P1** and **P8**. Error bars represent the standard error of the mean (SEM).

Under these conditions, the bimodal curve disappeared and both strains remained activated by **P3**. Activation of the *ΔagrD*::P2-lux reporter by **P1** nearly disappeared while the WT::P2-lux strain remained inhibited by this peptide (Figure 3D). We then performed assays with lower concentrations of the compounds with both reporter strains (Figure S13–S15). These experiments revealed that the activation threshold of **P3** for the early activation at 180 min is in the micromolar range (EC_{50} = 9±3.6 μM; Figure S13C). Determining the potency of **P1** inhibition of QS in the WT strain at maximum luciferase activity (t = 360 min) provided an IC_{50} = 0.11±0.02 μM (Figure S15A). The determined EC_{50} value for **P3** is relatively high compared to for example AIPs of staphylococcal *agr* systems and the kinetics of QS activation appear to be different, with QS being activated during exponential growth phase and dropping rapidly once entering stationary phase. We therefore tested the effect of delayed addition of the potential AIPs to the *ΔagrD*::P2-lux strain (**P1** and **P3** added at t = 180 min). These assays showed loss of the early activation in the absence of **P3** as expected and still produced a rapid decrease in luciferase activity when entering stationary phase (Figure S15B). Finally, we tested the *agr* activity of the *N*-acetylated version of thiolactone **P2**, Ac-*Lm*-thiolactone (**P8**), which cannot undergo S→N acyl shift (Figure 3D). The peptide acted as an inhibitor of WT::P2-lux reporter and had no effect on the Δ*agrD*::P2-lux reporter.

The significant early QS induction by **P3** in the *ΔagrD* strain and its lack of inhibition of QS in the wild type provide compelling evidence for its role as QS signaling peptide but **P1** was previously identified from bacterial culture. Thus, we attempted to confirm the presence of **P3** in the supernatant of wild type *L. monocytogenes* (EGDe), which was the strain used as background to construct the applied luciferase reporter strains.

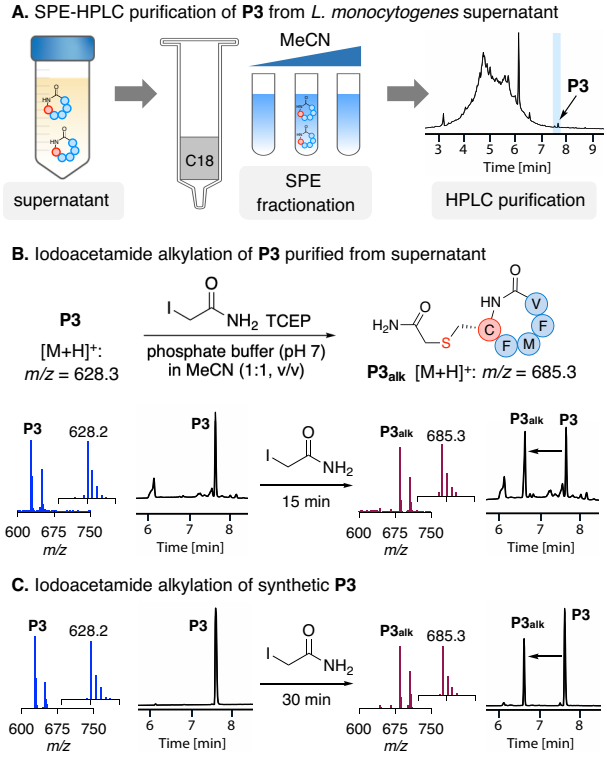


Figure 4. (**A**) Overview of the isolation of **P3** from bacterial supernatant through solid-phase extraction (SPE) followed by HPLC purification. (**B**) Iodoacetamide alkylation of **P3** isolated from supernatant. (**C**) Iodoacetamide alkylation of synthetic **P3**.

We envisioned that fractionation of the supernatant by solid-phase extraction (SPE) could provide a less complex AIP-containing mixture, which would facilitate identification of **P3** (Figure 4A). To test the SPE protocol, we spiked 50 mL of supernatant with synthetic **P3** and fractionated the sample using water–MeCN mixtures. The peptide eluted primarily in the 50% MeCN fraction (Figure S16A), which encouraged us to perform

SPE fractionation of supernatant and lyophilize the fractions collected at water–MeCN (1:1). Gratifyingly, LC-MS analysis of the concentrated sample revealed the presence of **P3** (Figure S16B). We then performed SPE fractionation on 250 mL supernatant, pooled and lyophilized the fractions containing **P3**, and subjected the resulting residue to preparative HPLC purification. This provided a semi pure sample of **P3**, which was confirmed by HRMS analysis (Figure S17). In addition, cysteine-selective alkylation with iodoacetamide provided evidence for the presence of a free cysteine residue in the isolated peptide **P3** (Figure 4B), which behaved identically to a synthetic sample of **P3** (Figure 4C and Figure S18). When applying the same SPE strategy for **P1**, we were not able to identify the peptide in bacterial supernatant. We further analyzed supernatants from bacteria grown in either TSB or BHI medium at both 30 °C and 37 °C where **P3** could be identified under all four conditions, while **P1** was not identified (Figures S16B and S19B,C). Furthermore, neither peptide was identified from the *ΔagrD*::P2-lux strain, applying the same protocol, showing that the identified **P3** is encoded by *agr* (Figure S19B).

In summary, we provide compelling evidence to suggest that the structures of AIPs previously believed to exist as thiolactones may be cysteine-containing homodetic peptides in their functional state. The rates of the rearrangement of thiolactone-containing peptides without an exotail to give their homodetic counterparts are highly pH-dependent and differ significantly between the tested peptides. For *L. monocytogenes*, the synthetically prepared homodetic pentamer peptide (**P3**) furnished rapid induction of quorum sensing in luciferase reporter strains with either wild type *agr* system or *ΔagrD*. In addition, the peptide **P3** was isolated from supernatant and chemically modified in a predictable fashion, to confirm that the used isolate of *L. monocytogenes* contains **P3**.

Our data also raises the question whether other bacteria, such as *L. plantarum* and *C. perfringens*, rely on thiolactones and/or homodetic peptides as AIPs. Finally, the discovery of this mechanism in the bacterial synthesis of homodetic peptides is independently corroborated by Hertweck and coworkers, who show that hexameric thiolactones produced in clostridia undergo the same transformation by S→N acyl shift chemistry.[21]

This research provides fundamental insight into bacterial peptide biosynthesis and has implications for the future investigation of QS modulation and development of inhibitors of QS in several bacteria.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Supplementary methods, schemes, figures, and tables as well as copies of HPLC traces, ^{1}H and ^{13}C NMR spectra (PDF)

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Cyclic autoinducing peptides, produced by Gram-positive bacteria for chemical communication through quorum sensing, were previously believed to contain thiolactone- or lactone functionalities. It has now been shown that this prediction is not always accurate. In examples where the biosynthesized thiolactone-containing peptide is void of an exotail, spontaneous rearrangement produces its homodetic cyclopeptide counterpart.