Development of Stabilized Peptide Analogues to Study a Short-lived Peptide Signal that Regulates Cell-to-Cell Communication in *Listeria monocytogenes*

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Abstract

Quorum sensing (QS) is a mechanism that regulates group behavior in bacteria, and in Gram-positive bacteria, the communication molecules are often cyclic peptides, called autoinducing peptides (AIPs). We recently showed that pentameric thiolactone-containing AIPs from *Listeria monocytogenes*, and from other species, spontaneously undergo rapid rearrangement to homodetic cyclopeptides, which hampers our ability to study the activity of these short-lived compounds. Here, we developed chemically modified analogues that closely mimic the native AIPs while remaining structurally intact, by introducing N-methylation or thioester-to-thioether substitutions. The stablilized AIP analogues exhibit strong QS agonism in *L. monocytogenes* and allow structure–activity relationships to be studied. Our data provide evidence that loudly suggest that the most potent AIP is in fact the very short-lived thiolactone-containing pentamer. Further, we find that the QS system in *L. monocytogenes* is more promiscuous with respect to the structural diversity allowed for agonistic AIPs than reported for the more extensively studied QS systems in *Staphylococcus aureus* and *Staphylococcus epidermidis*. The developed compounds will be important for uncovering the biology of *L. monocytogenes*, and the design principles should be broadly applicable to the study of AIPs in other species.

Introduction

Listeria monocytogenes is a notorious foodborne pathogenic Gram-positive bacterium, causing intracellular infections in humans and animals. It is found in soil and wastewater, and can persist in food-processing facilities, even after sanitization. When ingested, *L. monocytogenes* can cause disease ranging from gastroenteritis to life-threatening conditions in immunocompromised, pregnant, or elderly individuals. Being able to survive a wide range of environmental conditions requires tightly regulated gene expression.(1, 2) This is in part accomplished by quorum sensing (QS)(3-9) that in *L. monocytogenes* affects protein secretion, cell invasion, biofilm formation, and virulence gene expression. QS constitutes regulatory systems for sensing population density, through excretion and recognition of autoinducer molecules, causing changes in population behavior in different bacteria.(10) In Gram-postive bacteria, the best characterized QS system is that enoded by the accessory gene regulator (*agr*) locus in *Staphylococcus aureus*.(11) In this system, the signaling molecules are cyclic, autoinducing peptides (AIPs), that commonly contain a thiolactone formed between the C-terminal carboxylate and the thiol side chain of a cysteine residue in the *i*–5 position from the C-terminus, together with a linear N-terminal exotail of varying length.(12-16) The *agr* locus encodes the AIP precursor peptide (AgrD) and a membrane-bound peptidase (AgrB) needed for the closing of the thiolactone ring. Additionally, it encodes a two-component system, consisting of the receptor-histidine kinase (AgrC) and response regulator (AgrA) that together comprise the AIP-sensing and signal transduction components.(17) The *agr* system of *L. monocytogenes*, like that of *S. aureus*, contains the four genes, *agrBDCA*, that are autoregulated and expressed from the same promoter.(3, 4) The system has been known for 20 years has been shown to affect the expression of hundreds of genes,(6, 18) but its regulatory function is not yet well understood.

The identity of the *L. monocytogenes* AIP is debated. Previously, a hexameric AIP with a single amino acid exotail (**P1**, Figure 1) has been identified using LC-MS/MS,(19) and Zetzmann *et al.* provided evidence to suggest a pentameric AIP (**P2**, Figure 1).(8) Previously, we failed to identify **P1** by using a thiolactone-trapping methodology,(16, 20) and

we went on to show that AIPs without an exotail will undergo spontaneous S→N acyl shift to give homodetic peptides, like **P3** (Figure 1).(20) Further, we were able to identify **P3** in supernatant from *L. monocytogenes* bacterial culture and showed that it can induce QS in a bioluminescent reporter strain of *L. monocytogenes*; albeit, at high concentrations compared to those needed for autoinduction in Staphylococci.(16) Concurrently, a similar S→N acyl shift phenomenon was described in the bacterium *Ruminiclostridium cellulolyticum*.(21) Although, our work demonstrated the rapid rearrangement of **P2** to give **P3** at pH 7 ($t_{1/2}$ = 2.6 min), the high concentration needed for **P3** to activate QS still led us to speculate whether the **P2** thiolactone could play a role in the activation of QS in *L. monocytogenes*.

Potentially, fast-on–tight-binding kinetics on the receptor could explain how such a short-lived molecule could function as an AIP. Here, we report the development of stabilized analogues, mimicking the structure of **P2**, to investigate whether **P2** could be a *bona fide* AIP.

Results

We initially compared the effects of **P1**–**P3**, using *L. monocytogenes* EGDe WT and a Δ*agrD* mutant strain, both carrying a chromosomal integration of the *agr* promoter fused to the *lux* operon, enabling a bioluminescent readout as measure of *agr* activity (referred to as WT::P2-lux and Δ*agrD*::P2-lux, respectively).(8) Thus, we first optimized the assay conditions inspired by a study on luciferase-based reporter strains of *Lactococcus lactis*, in which the luciferase substrate flavin mononucleotide was added.(22) We found that addition of flavin mononucleotide (10 mg/L, ~26 µM) amplified maximal luminescence readout of the WT::P2-lux reporter strain by ~80% and total area under the curve by ~90% when grown in tryptic soy broth (TSB), without affecting the growth (Figure S1). We then tested synthetic peptides **P1**–**P3** against the WT::P2-lux and Δ*agrD*::P2-lux reporter strains in TSB and brain heart infusion (BHI) medium at 37 °C. Importantly, **P2** was added directly from DMSO stock solution, to preserve the integrity of the thiolactone for as long as possible during the

experiment (Figure 2 and Figure S2–5). The trends previously observed for **P1** and **P3** at 30 °C were recapitulated under the optimized conditions. Treating WT::P2-lux with **P1** resulted in inhibition of the signal in both BHI and TSB media, while giving rise to a slight induction in the □*agrD*::P2-lux reporter strain after 240 min, which was most pronounced in BHI medium (Figure 2A and Figure S2–5). The homodetic peptide **P3** did not cause inhibition of WT::P2-lux and produced early induction of *agr* in both TSB and BHI media (Figure 2C, Figure S2 and S4). More strikingly, **P2** was able to increase *agr* activity 3-fold at 50 µM and 2-fold at 5 µM for WT::P2-lux in TSB, compared to the untreated WT::P2-lux (Figure 2, Figure S2 and S4).

A less pronounced increase in peak intensity was also observed for **P2** at 5 µM in BHI (Figure S4) and early induction of *agr* was observed in both TSB and BHI (Figure 2, Figure S2 and S4). For the activation of Δ*agrD*::P2-lux by **P2***,* we observed full restoring of WT levels in BHI and surpassing the WT level in TSB, even at 5 µM compound concentration (Figure 2, Figure S3 and S5; for further discussion, see the Supporting Information page S7). The ability of **P2** to activate *agr* at lower concentrations than **P1** and **P3**, even though it is inherently unstable due to its spontaneous S→N acyl shift at near-neutral pH, strongly supports **P2** as an active AIP in *L. monocytogenes*.

However, the short-lived nature of **P2** makes it difficult to study, calling for the development of synthetic analogues to fill this gap. Previously, we synthesized an *N*-acetylated version of **P2**, to obtain a stable thiolactone mimic of **P2**, but this peptide showed no activation of Δ*agrD*::P2-lux and inhibited the *agr* activity of WT::P2-lux,(20) which we confirmed with the optimized assay protocol (Figure S10). Since the N-acetylated mimic cannot be protonated, we speculated if a positively charged amino group at the N-terminus, as present in **P2**, could be important for activity. We therefore designed a stabilized mimic of the compound by introducing two methyl groups instead of acetyl **(1**, Figure 1 and Scheme S1). The two methyl groups prevented S□N acyl shift as intended, and we observed >90%

purity of compound **1** after incubation for 22 hrs in phosphate buffer (pH 7.0) at 37 °C (Figure S11).

Remarkably, the thiolactone **1** caused early activation of *agr* in the WT::P2-lux strain and significant induction of *agr* in Δ*agrD*::P2-lux at just 80 nM compound concentration, displaying superior potency to any of the previously tested peptides (Figure 3A, Table 1, Figure S6 and S7).

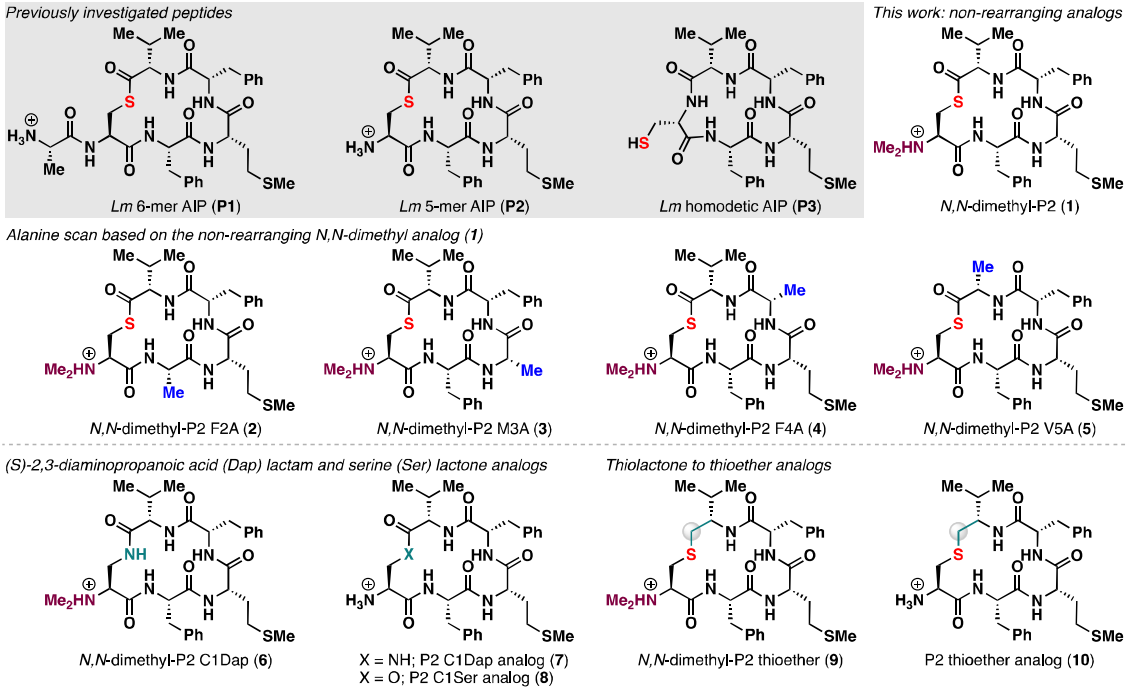

Figure 1. Proposed autoinducing peptides from *L. monocytogenes* (**P1**–**P3**) and synthetic analogues designed in this study (**1**–**10**).

However, thiolactone **1** was unable to increase *agr* activity above the WT levels, unlike the effect observed with **P2** and WT::P2-lux. Similarly, against Δ*agrD*::P2-lux we saw an upper limit of activation (~6500 RLU) with this **P2** analogue, which was lower than the maximum luminescence of the WT::P2-lux strain (Figure 3A and Figure S7). Nevertheless, the potency of **1** supports the hypothesis that *N*-acetylation is inappropriate for studying the biological

activity of exotail-free thiolactone-containing AIPs. Dimethylation of the N-terminus therefore provides a novel architecture to achieve stabilization of these AIPs.

Next, we performed an alanine scan based on **1** (peptides **2**–**5**, Figure 1), to study the effect of each position in **P2** on *agr* activity. We observed the most pronounced effect when substituting either phenylalanine (**2** and **4**), which resulted in a complete loss of activation of the Δ*agrD*::P2-lux reporter (Figure S6 and S7). Interestingly, compound **2** was able to fully inhibit *agr* activity in the WT::P2-lux strain at 50 μM concentration. In contrast to **2** and **4**, the alanine analogues **3** and **5** were partial agonists unable to reach the same maximum activation of Δ*agrD*::P2-lux as **1**. Compounds **3** and **5** also displayed inhibition of peak luminescence in the WT::P2-lux, with analog **5** being able to inhibit the activity by 20% at just 80 nM concentration (Figure S6 and S7). Although all alanine substitutions affected the agonistic effect, only modest inhibitors were discovered.

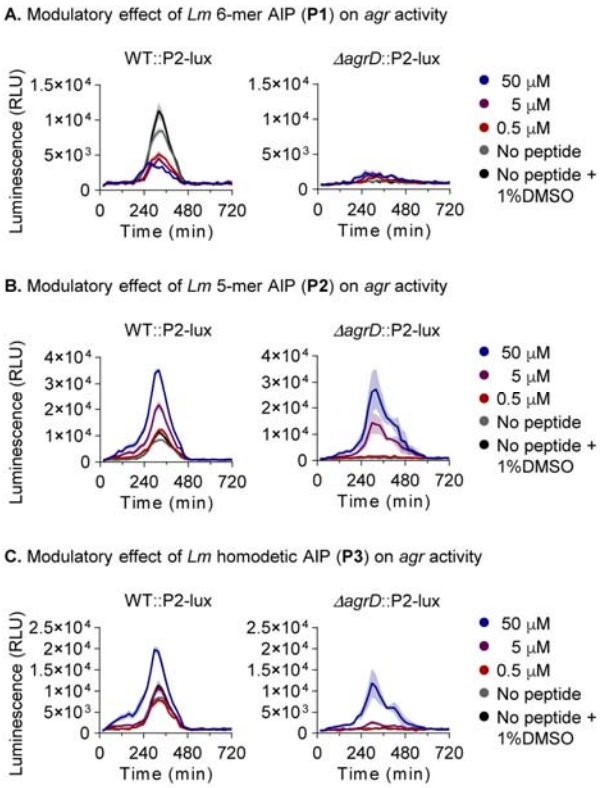

Figure 2. Effect of **P1**–**P3** on the *agr* activity in luminescence-based reporter strains of *L. monocytogenes* grown at 37 °C in tryptic soy broth (TSB) medium. (**A**) Selected

concentrations of **P1**. (**B**) Selected concentrations of **P2**. (**C**) Selected concentrations of **P3**. Shadings on the graphs represent the standard error of the mean (SEM). All data are based on at least three individual assays performed in at least technical duplicate.

To explore alternative ways of stabilizing the AIP structures, we synthesized lactam analogues of **1** and **P2** (**6** and **7**; Figure 1 and Scheme S2). Based on structure-activity relationship (SAR) studies performed on AIPs from *S. aureus*, we expected the lactam substitution to cause a decrease in activity.(23-27) However, the lactam analog **7** proved to be an effective agonist that increased *agr* activity of WT::P2-lux above controls at concentrations down to 80 nM (Figure 3C, Table 1, and Figure S8). At the highest concentration tested for **7** (50 µM), the culture reached same maximal level of *agr* activity in WT::P2-lux and Δ*agrD*::P2-lux, around 2.5-fold higher than the untreated WT control (Figure 3C and Figure S8), which was slightly lower than the maximal activation achieved with the native, short-lived compound **P2**. With compound **6** we observed an upper maximal *agr* activation in Δ*agrD*::P2-lux similar to the effect observed for **1** and, contrary to compound **7**, this analog did not induce *agr* at 80 nM concentration (Figure 3B, Table 1, and Figure S8).

Table 1. Activation of *agr* in *L. monocytogenes*.[a]

compound	%-activation [2 µM]	%-activation [80 nM]
P1	11 ± 6[b]	inactive[c,d]
P2	**160 ± 51**[b]	inactive[c,d]
P3	15 ± 6[b]	inactive[c,d]
1	63 ± 5	35 ± 3
6	27 ± 3	Inactive[c]
7	**210 ± 10**	38 ± 2
8	**270 ± 10**	43 ± 8
9	80 ± 5	74 ± 8
10	**350 ± 12**	**274 ± 7**

[a] Values are extracted from assays with Δ*agrD*::P2-lux and normalized to average maximum luminescence of untreated Δ*agrD*::P2-lux (0%) and average maximum luminescence of untreated

WT::P2-lux (100%). [b] Tested at 5 μM. [c] Inactive defined as less than 10% normalized activation. [d] Tested at 500 nM.

We also synthesized a lactone analog of **P2** (compound **8**; Figure 1 and Scheme S4), which showed a similar degree of induction and potency as the lactam **7**, reaching roughly the same maximal luminescence levels in Δ*agrD*::P2-lux and half-maximal activation at 400 nM (Figure S8 and Figure S12C). However, the lactone proved inherently unstable, which causes an underestimation of its activity, as discussed for compound **P2**. Thus, after 22 hrs at 37 °C and pH 7.0, 36% of **8** was hydrolyzed and 48% had undergone rearrangement through O→N acyl shift, to form its corresponding homodetic peptide (Figure S12A and S12B).

Finally, we further extended our series to include thioether analogues **9** and **10** (Figure 1 and Scheme S5 and S6). When treating WT::P2-lux with the structurally closest analog of **P2**–compound **10**–we observed a striking 8-fold increase in *agr* activation compared to untreated WT. Compound **10** provided early induction in WT::P2-lux and increased *agr* activity of Δ*agrD*::P2-lux to ~50% of the untreated WT levels at concentrations as low as 640 pM (Figure 3E and Figure S9). Furthermore, at just 3.2 nM compound concentration, the luminescence levels observed for Δ*agrD*::P2-lux paralleled those of WT::P2-lux (Figure 3E and Figure S9). For Δ*agrD*::P2-lux treated with compound **9**, we observed attenuated maximal activation, as for *N,N*-dimethylated analogues **1** and **6** (Figure 3D and Figure S9). However, maximal induction of *agr* was observed at 80 nM concentration of **9**, rendering this compound a more potent mimic of the native AIP than the lactam **6** (Table 1).

In summary, we first investigated the mimicking of exotail-free thiolactone-containing AIPs by incorporating methyl groups at the N-terminus of **P2** to circumvent its S→N acyl shift to give **P3**. This new strategy for studying exotail-free AIPs enabled us to investigate the importance of each amino acid in the peptide through an alanine scan. While all positions are indispensable for full activity, the phenylalanine positions proved most important for

retaining *agr* activation. The F2A (**2**) and F4A (**4**) analogues inhibited *agr*, but further efforts are necessary for developing potent antagonists of the *L. monocytogenes agr* system that can function as tool compounds.

Unlike what is known from studies of the paradigm *agr* systems in *S. aureus*, agonistic activity was retained when the native thiolactone functionality was substituted for a lactam or a lactone. Moreover, an unprecedented thiolactone-to-thioether substitution, to give compound **10**, yielded the most potent inducer of the *L. monocytogenes agr* system reported so far (Table 1 and Figure 3 and Figure S9), which provides yet another novel architecture for studying exotail-free and other AIPs. Comparison of lactam and thioether analogues with and without dimethylated N-termini (**6** vs **7** and **9** vs **10**), revealed that N-methylation reduces *agr* activation, which should be considered when using this strategy to stabilize thiolactone AIPs.

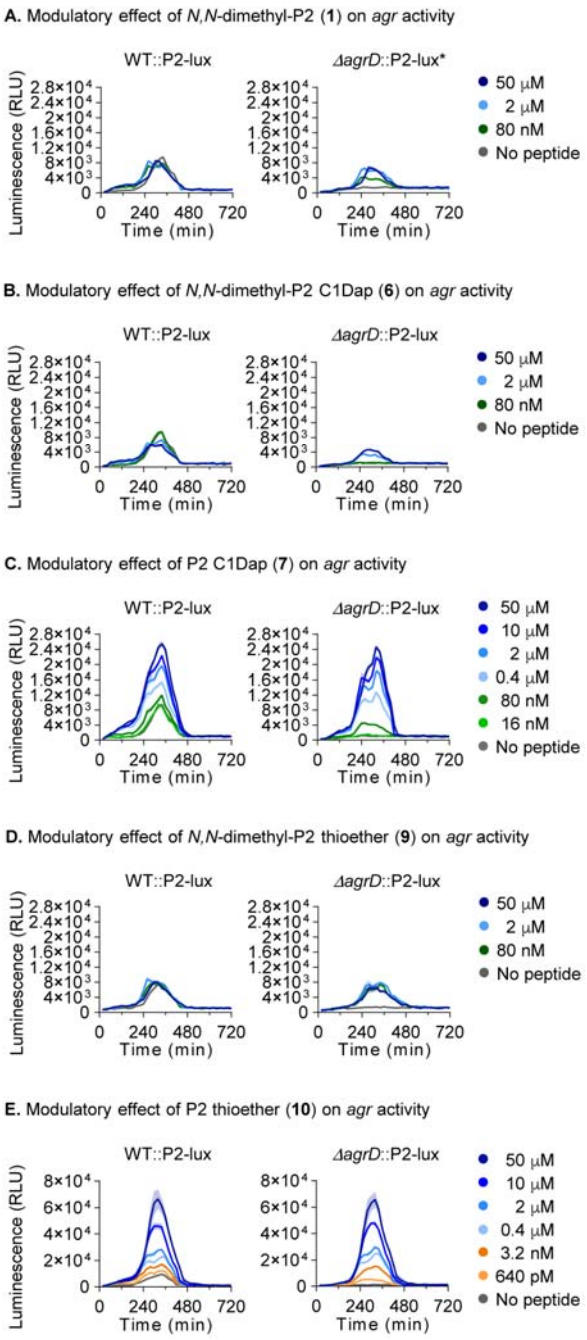

Figure 3. (**A**–**E**) Activation of *agr* in *L. monocytogenes* luminescence-based reporter strains grown at 37 °C in tryptic soy broth (TSB) medium, by selected concentrations of **1**, **6**, **7**, **9**, and **10**. Shadings on the graphs represent the standard error of the mean (SEM). Data are based on at least three individual assays performed in at least duplicate unless otherwise is noted. *Based on two individual assays performed in triplicate.

Discussion

Collectively, our data show that the AgrC receptor in *L. monocytogenes* is more promiscuous with respect to activation than previously found for *S. aureus* systems. Further, **P2** is more active than **P1** and **P3**, despite its very short-lived nature, and development of stabilized analogues of **P2** further supported a role of this exotail-free thiolactone as a tight-binding native AIP.

Our findings thus raise questions about the biological significance of this transient nature of **P2**. It has been proposed that *agr* in *L. monocytogenes* only serves the purpose of sensing cell density,(5) and differential regulation of *agr* has been shown to depend on temperature and overlap with other regulons.(18) Further, heterogeneous *agr* activity at population level was observed for several strains of *L. monocytogenes*, and it is possible that this short-lived signal may function as a single-cell autoinducer under certain conditions.(28)

This work adds to the potential effects of outside stimuli on *agr* regulation in *L. monocytogenes*, as the pH-dependent nature of the peptide signal (**P2**) could be speculated to provide a way to fine tune *agr* activity in response to changing pH in the environment. A compartmentalized regulatory role for **P2** could be imagined, possibly related to acidic host cell compartments such as the phagolysosome. The optimum activity of the important hemolysin, listeriolysin O (LLO), is similarly regulated. Inside the acidifying phagolysosome, LLO helps *L. monocytogenes* escape and upon release into the neutral environment of the host cell cytosol, it rapidly denatures.(29, 30)

L. monocytogenes infection relies on traversing the environments of the gastro-intestinal tract and its varying pH values.(1) It is therefore intriguing to start thinking about how such an adaptive peptide signal might play a role during this process, possibly even in concert with the more chemically stable peptides **P1** and **P3**.

This research provides essential insight into the peptide-mediated cell-to-cell communication of *L. monocytogenes* and provides tool compounds for future studies of the biological importance of *agr* regulation in this bacterium. More generally, several other bacteria also produce exotail-free AIPs, and we hope that the chemical framework provided

in this study can help guide further research in their respective physiological regulation through cell-to-cell communication.(21, 31, 32)

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Supporting Information

Supplementary figures, schemes depicting the synthesis of new compounds; experimental methods; chemical synthesis and compound characterization data; as well as copies of HPLC traces, ^{1}H and ^{13}C NMR spectra.

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