PNAS RESEARCH ARTICLE | BIOCHEMISTRY

Reconstitution of the *S. aureus agr* quorum sensing pathway reveals a direct role for the integral membrane protease MroQ in pheromone biosynthesis

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Edited by Suzanne Walker, Harvard Medical School, Boston, MA; received February 14, 2022; accepted June 14, 2022

In *Staphylococcus aureus,* virulence is under the control of a quorum sensing (QS) circuit encoded in the accessory gene regulator (*agr*) genomic locus. Key to this pathogenic behavior is the production and signaling activity of a secreted pheromone, the autoinducing peptide (AIP), generated following the ribosomal synthesis and posttranslational modification of a precursor polypeptide, AgrD, through two discrete cleavage steps. The integral membrane protease AgrB is known to catalyze the first processing event, generating the AIP biosynthetic intermediate, AgrD (1–32) thiolactone. However, the identity of the second protease in this biosynthetic pathway, which removes an N-terminal leader sequence, has remained ambiguous. Here, we show that membrane protease regulator of *agr* QS (MroQ), an integral membrane protease recently implicated in the *agr* response, is directly involved in AIP production. Genetic complementation and biochemical experiments reveal that MroQ proteolytic activity is required for AIP biosynthesis in *agr* specificity group I and group II, but not group III. Notably, as part of this effort, the biosynthesis and AIP-sensing arms of the QS circuit were reconstituted together in vitro. Our experiments also reveal the molecular features guiding MroQ cleavage activity, a critical factor in defining *agr* specificity group identity. Collectively, our study adds to the molecular understanding of the *agr* response and *Staphylococcus aureus* virulence.

Staphylococcus aureus | quorum sensing | RiPP biosynthesis | bacterial pathogenesis | synthetic biology

Quorum sensing (QS) is a common form of chemical communication used by bacteria to coordinate behavior (1). Small molecules—acting as chemical signals—are continuously produced, secreted, and recognized by bacteria to dynamically monitor local population density and direct gene expression. In *Staphylococcus aureus*, a common pathogenic Gram-positive bacterium widely associated with community acquired and nosocomial infections, a QS system helps optimize bacterial behaviors throughout all stages of infection, from colonization of infected hosts to virulence factor expression and resultant pathogenicity (2–7). Pharmacological control of the *S. aureus* QS system would allow for the modulation of bacterial behavior, including attenuation of the detrimental effects of *S. aureus* infection, without imposing selective pressure to trigger the development of antibiotic resistance (8, 9). This makes the *S. aureus* QS circuit an attractive drug target and has motivated intense study of this system over the last three decades (2, 7, 10).

Much of the biochemical machinery necessary for *S. aureus* QS is encoded by the accessory gene regulator (*agr*) operon, which contains four open reading frames, *agrBDCA* (2). Biochemical studies have assigned specific functions to each of these gene products, leading to a detailed mechanistic understanding of much of the system (11–17). AgrC is a transmembrane histidine phosphokinase signal receptor which is acted upon by a peptide pheromone, the *agr* autoinducing peptide, AIP, of which AgrD is the precursor (18, 19). The AIP, a 5-membered macrocycle with a two- to four-amino acid N-terminal tail, is processed in two steps, of which the first is an AgrB-catalyzed cleavage of the C-terminal 18 amino acids concomitantly with the AgrB-induced formation of a thiolactone bond between the C-terminal carboxyl and an internal cysteine, generating a biosynthetic intermediate, the AgrD (1–32) thiolactone (Fig. 1*A*) (15). The N-terminal amphipathic helical domain of the thiolactone intermediate is cleaved in a second, separate proteolytic event, releasing the mature AIP into the extracellular milieu to serve as an index of local bacterial population density. When the AIP concentration exceeds a threshold, it is recognized by the sensor domain of the receptor histidine kinase, AgrC, leading to structural changes in the AgrC cytosolic domain that activates autophosphorylation and subsequent phosphoryl-transfer to the response regulator, AgrA (14, 16, 17). Together, AgrC and AgrA form a

Significance

Staphylococcus aureus (*S. aureus*) uses a short cyclic peptide known as the autoinducing peptide (AIP) for intercellular communication and regulation of pathogenic behavior. While the first step of AIP biosynthesis is well characterized, the identity of the protease(s) responsible for subsequent biosynthetic steps remained unknown. Here, we show that the recently discovered membrane protease regulator of *agr* QS (MroQ) is an essential component of AIP biosynthesis. Through biochemical characterization of AIP biosynthesis and signaling—representing the first reported full synthetic reconstitution of a prokaryotic quorum sensing pathway—combined with in vivo studies, we elucidate the mechanism of MroQ's substrate-specific recognition and cleavage. Together, these results significantly expand our understanding of *agr* QS in *S. aureus*.

Author contributions: A.Z., S.P.B., Q.X., and T.W.M. designed research; A.Z., S.P.B., and Q.X. performed research; A.Z., S.P.B., Q.X., B.W., G.R., and R.P.N. contributed new reagents/analytic tools; A.Z., S.P.B., Q.X., and T.W.M. analyzed data; and A.Z., S.P.B., and T.W.M. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2202661119/-/DCSupplemental.

Published August 8, 2022.

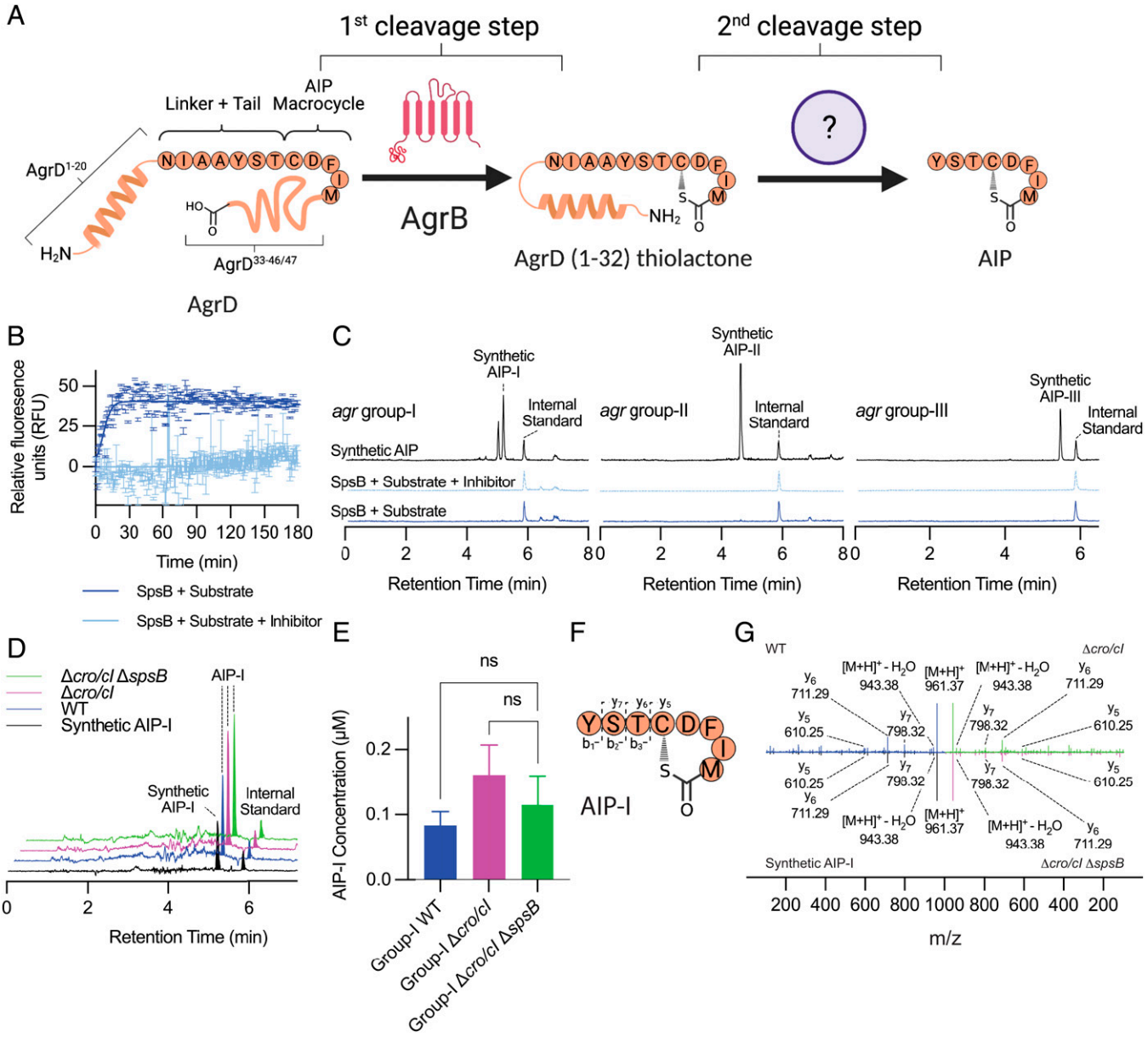

Fig. 1. SpsB does not directly participate in AIP biosynthesis. (*A*) Overview of the AIP biosynthetic pathway. AgrD (group-I sequence shown) is processed by AgrB to yield the biosynthetic intermediate, AgrD (1–32) thiolactone. This intermediate is converted into the mature AIP through the action of a second protease whose identity remains unclear. (*B*) SpsB cleavage of a validated fluorogenic substate, DABCYL-SceD-EDANS, in the presence and absence of a known SpsB inhibitor, M131. SpsB proteoliposomes were incubated with the substrate ± inhibitor and the cleavage reaction monitoring by fluorescence (510 nm) over time. Data presented as the mean ± SD (n = 3 replicates). (*C*) In vitro assay for AIP production. SpsB proteoliposomes were treated with indicated AgrD (1–32) thiolactone intermediates in the presence or absence of M131 inhibitor. Reaction mixtures were then subjected to a solid phase extraction (SPE) step, prior to which an internal standard (AIP derivative) was added, and analyzed by LC–MS. Shown are the extracted ion currents (EIC) traces for the expected AIP and the internal standard. A synthetic AIP treated with empty liposomes served as a positive control (top trace). (*D*) Cell-based assay for AIP production. Indicated *S. aureus* strains (group-I background) were grown for 8 h (the optimal timepoint for AIP-I production in the WT strain) at which point an internal standard was added and the media subjected to SPE and analyzed by LC–MS. Shown are the EIC traces for AIP-I and an internal standard. As a control, a synthetic AIP-I was added to media and subjected to the same purification protocol (black trace). (*E*) AIP-I produced by indicated *S. aureus* strains was quantified by LC–MS using a standard curve approach employing synthetic AIP-I. Data presented as the mean ± SD (n = 3–4 biological replicates). (*F* and *G*) Comparative LC–MS/MS analysis of AIP-I produced by WT, Δ *cro/cl*, and Δ *cro/cl*:Δ *spsB* variants of group-I *S. aureus* and a synthetic AIP-I standard. Color coding as in (*D*).

two-component signaling (TCS) pathway that up-regulates the transcription of the *agr* operon, leading to a positive feedback loop of AIP production and recognition (7, 18). Activated AgrA also promotes the transcription of the multifunctional RNA, RNAIII, which serves as the message for expression of the virulence factor δ-hemolysin and as a regulatory RNA suppressing the expression of adhesins and promoting the expression of various other virulence factors (20, 21). By this mechanism, *agr* QS functions as a master regulator of *S. aureus* biofilm dispersal and virulence (7, 22).

Allelic variation within the *agr* operon, occurring as a result of hypervariability in *agrB*, *agrD*, and *agrC*, results in four distinct *agr* specificity groups within *S. aureus* (19, 23, 24). Each allelic variant produces a unique AIP-AgrC pairing. While cognate AIP/AgrC interactions are agonistic, noncognate interactions are generally cross inhibitory to *agr* QS, with the exception of the closely related group-I and group-IV AIPs which cross activate (19, 23). Furthermore, biosynthesis of each unique AIP is performed through the proteolysis of a unique AgrD by a unique AgrB (15). While the size and hydrophobic nature of the

AIP macrocycle are conserved among the specificity groups, AIPs derived from group I/IV, group II, and group III are divergent in tail length and sequence (19, 23, 24). Structure-activity studies on AIPs reveal that shared structural motifs are necessary for AgrC binding, while divergent sequences determine if, upon binding, AIP acts as an agonist or an antagonist (10, 25, 26).

Despite decades of research into the *agr* response, the AIP biosynthetic pathway has not been fully elucidated (Fig. 1*A*). The second step of AIP maturation—cleavage of the N-terminal domain from the AgrD (1–32) thiolactone intermediate—is poorly understood relative to the rest of the system (7). Biochemical studies on model peptides derived from the group-I AgrD sequence indicate the signal peptidase I, SpsB, can perform this second cleavage step (27). While the involvement of SpsB in AIP maturation would be broadly consistent with the enzyme's known function in peptide secretion (27, 28), definitive in vitro and in vivo data establishing a role for this enzyme in AIP biosynthesis, including whether it is involved in the maturation of the group II–IV AIPs, is still lacking.

Recently, a putative integral membrane Abi-domain/M79 metalloprotease has been identified as an effector of *agr* QS in *S. aureus* (29, 30). Genetic studies indicate that this protein, designated membrane protease regulator of *agr* QS (MroQ), is required for *agr* activity and virulence production in a group-I *S. aureus* strain. These studies clearly implicate MroQ in the *agr-I* response, however, the precise function of the protein has not been definitively assigned. Indeed, Cosgriff et al. (29) propose a role for MroQ in AgrD processing and/or transport, while Marroquin et al. (30) conclude that MroQ is not directly involved in AgrD processing, but rather plays some other regulatory role.

In this study, we set out to characterize the second step in AIP maturation using a combination of biochemical and genetic approaches. Our studies argue against a major role for SpsB in AIP biosynthesis. By contrast, we provide evidence that MroQ catalyzes the second proteolytic cleavage step in the maturation of AIP-I/IV and AIP-II, but, surprisingly, not AIP-III. We also define the molecular features within the group-I and -II substrates that guide MroQ cleavage activity. As part of this effort, we successfully reconstitute much of the *agr* QS circuit using purified components, a first for a system of this type. Taken together, these results suggest a point of evolutionary divergence between the *S. aureus* specificity groups and underscore MroQ as a key component of the *agr* response.

Results

SpsB Does Not Efficiently Cleave AIP Biosynthetic Intermediates. Previous work has suggested a role for the membrane anchored protease, SpsB, in *agr* signaling (27). Notably, this study employed a truncated, soluble version of the enzyme and a short synthetic peptide substrate spanning the AgrD-I (1–32) thiolactone cleavage site. Thus, it remained to be determined whether the full-length, membrane-anchored enzyme can successfully process the native substrate, AgrD-I (1–32) thiolactone, into the mature AIP. It is also unclear if SpsB has any role to play in the maturation of AIPs from the other *S. aureus* specificity groups.

To address these questions, we overexpressed full-length *S. aureus* SpsB in *Escherichia coli* and then reconstituted the detergent solubilized purified protein into liposomes containing 1-palmitoyl-2-oleyl-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleyl-phosphatidylglyerol (POPG) (*SI Appendix*, Fig. S1 *A* and *B*). The bioactivity of these proteoliposomes was tested using a known synthetic SpsB substrate derived from the signal peptide sequence of *Staphylococcus epidermidis* pre-SceD, conjugated to an *N*- and C-terminal FRET pair DABCYL/EDANS (*SI Appendix*, Fig. S1*C*) (31). Efficient cleavage of this peptide was observed in the presence of the SpsB proteoliposomes, activity that was abolished in the presence of the known SpsB inhibitor, M131 (Fig. 1*B* and *SI Appendix*, Fig. S1 *D* and *E*) (32). We then incubated the SpsB-proteoliposomes with purified native AIP maturation substrate; AgrD (1–32) thiolactone from either *agr* group-I, group-II, or group-III *S. aureus* (*SI Appendix*, Fig. S2). AgrD-I (1–32) thiolactone and AgrD-III (1–32) thiolactone were generated recombinantly using an intein fusion strategy (15), whereas AgrD-II (1–32) thiolactone was chemically synthesized. In each case, the SceD substrate was also added as an internal control. Surprisingly, we did not observe the production of the native AIP in any of these reconstitution experiments, despite the fact that the SceD spike-in was successfully cleaved in each case (Fig. 1*C* and *SI Appendix*, Figs. S3 and S4*A*).

Given this unexpected biochemical result, we were eager to determine whether deletion of *spsB* gene has any impact on AIP maturation in *S. aureus* cells. This experiment is complicated by the key role of SpsB plays in *S. aureus* viability (33). Fortunately, this dependency can be circumvented by de-repression of a putative ABC transporter, achieved through knockout of the transcriptional repressor *cro/cI* (33). Thus, we generated a mutant *S. aureus* group-I strain lacking *cro/cI* and *spsB* and tested for the production of AIP-I using a sensitive LC-MS approach for detecting the peptide secreted into the growth media. Consistent with the biochemical data, wild-type (WT), Δ*cro/cI*, and Δ*cro/cI*:Δ*spsB* knockout strains exhibited similar levels of endogenous AIP-I production (Fig. 1 *D–G* and *SI Appendix*, Fig. S4 *B–D*). Together, these results argue against SpsB being the AIP maturation protease and point to the involvement of another membrane protease in the second biosynthetic step.

MroQ Activity Is Essential for AIP-I and AIP-II Production, But Not AIP-III. The recently identified *S. aureus* integral membrane protease, MroQ, presented itself as an attractive alternate candidate to SpsB in driving the second step in AIP maturation. This enzyme has been implicated in the group-I *agr* response, although the exact role it plays remains unclear (29, 30). We decided to expand investigation of MroQ to include AIP biosynthesis in *S. aureus agr* groups I, II, and III. Group IV was not examined in this study, as *agrB*, *agrD*, and *agrC* from group I and group IV are highly conserved and AIP-I and AIP-IV, which differ by only a single amino acid, are cross-activating for AgrC-I and AgrC-IV, respectively (23). Thus, we can assume that AIP biosynthesis in group I and group IV operate via the same pathway and mechanism.

We began by performing a series of genetic complementation experiments analogous to those carried out in the initial studies implicating MroQ in the group-I *agr* response (29, 30). In the current study, we generated Δ*mroQ* knockout strains for *S. aureus agr* group-I, -II, and -III genetic backgrounds. A Cd^{2+} inducible expression plasmid (34), containing *mroQ*, an inactive *mroQ* mutant, or no insert, was then transduced back into the various Δ*mroQ* and WT strains. We observed no major difference in growth rates for the three Δ*mroQ* knockout strains harboring these complementation plasmids relative to the corresponding WT strains (Fig. 2*A*). This is consistent with the proposed role of *mroQ* in the *agr* circuit, as *agr* QS is nonessential for *S. aureus* viability (35).

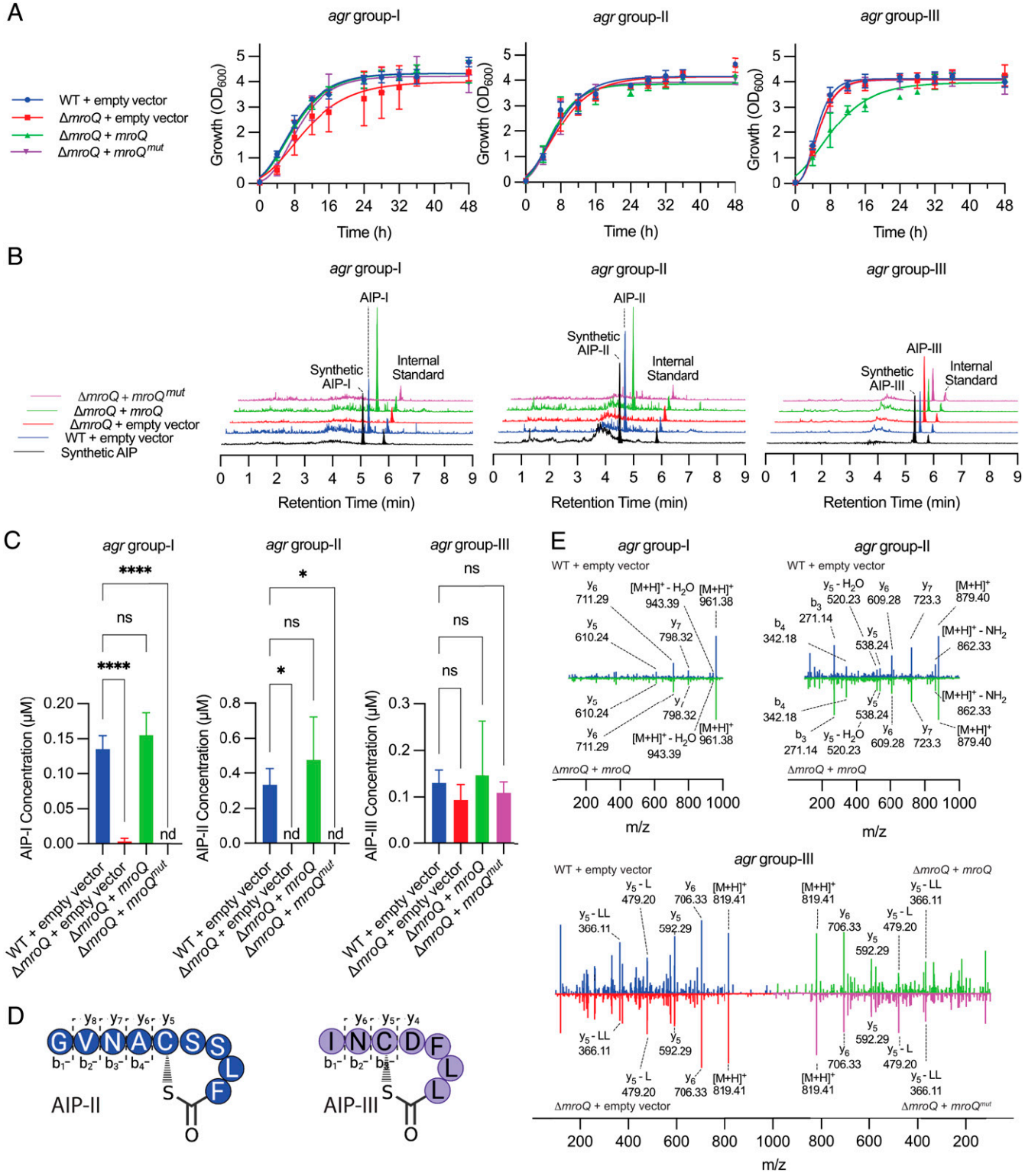

Fig. 2. Genetic complementation approach to explore the role of *mroQ* in AIP biosynthesis. (*A*) Relative growth of WT and Δ *mroQ* strains containing inducible plasmids expressing *mroQ*, *mroQ*^{mut}, or an empty plasmid, generated in *agr* group-I, -II, and -III background strains over 48 h, monitored by OD_{600}. Data presented as the mean ± SD (n = 3 biological replicates). (*B*) Cell-based assay for AIP production. Indicated *S. aureus* strains were grown for 8 h (group I) and 16 h (group II, group III) at which point an internal standard was added and the media subjected to SPE and analyzed by LC–MS. Shown are the EIC traces for the AIPs and the internal standard. As a control, a synthetic AIP was added to media and subjected to the same purification protocol (black traces). (*C*) AIP levels produced by indicated *S. aureus* strains were quantified by LC–MS using a standard curve approach employing synthetic AIPs. Data presented as the mean ± SD (n = 3–8 biological replicates). (*D* and *E*) Comparative LC–MS/MS analysis of AIPs generated by WT and mutant-complemented strains. Color coding as in (*C*).

To assess the impact of *mroQ* deletion on AIP production, growth media was taken from the various *S. aureus* strains at periodic time points and the levels of AIP quantified by liquid chromatography–mass spectrometry (LC–MS) using a standard curve approach (*SI Appendix*, Fig. S5 *A* and *B*). Building on previous findings (29, 30), we found that deletion of the

protease not only abolished AIP production in group-I strains relative to WT, but also profoundly reduced AIP levels in the group-II strain (Fig. 2 *B* and *C*). By contrast, deletion of *mroQ* had no significant effect on AIP production in the *S. aureus* group-III background. Use of a β-lactamase reporter cell assay also indicated that MroQ was needed for AIP production in group I and group II, but not group III (*SI Appendix*, Fig. S5*C*). Importantly, complementation with wild-type *mroQ*, but not an inactive mutant (29, 30), rescued AIP production in *agr* group-I and group-II Δ*mroQ* knockout strains to near-WT levels (Fig. 2 *B*–*E*). Taken together, these studies extend the previous work in this area by showing that MroQ activity is required for both AIP-I and AIP-II production, whereas the group-III *agr* response seems to be independent of this enzyme.

MroQ Efficiently Cleaves AgrD (1–32) Thiolactones In Vitro to Yield Mature AIPs. While the above genetic studies clearly link MroQ to AIP-I/II production, they do not reveal whether the protease is directly involved in the AgrD-I/II (1–32) thiolactone processing step, versus it playing an indirect role by regulating the activity of some other factor. To discriminate between these possibilities, we performed biochemical studies on the system using purified components. Full-length MroQ, as well as the inactive version of the enzyme MroQ^{mut} (29, 30), were successfully over-expressed as MBP-fusions in *E. coli* (*SI Appendix*, Fig. S6*A*). Following purification, the detergent-solubilized proteins were then reconstituted into proteoliposomes using the same lipid system employed for SpsB. We then incubated these MroQ-proteoliposomes with the purified AgrD (1–32) thiolactones from groups I–III and analyzed the reaction mixtures using our quantitative LC–MS assay (*SI Appendix*, Fig. S6*B*). Efficient and specific cleavage of AgrD (1–32) thiolactone from *agr* groups I and II was observed in the presence of MroQ, generating AIP-I and AIP-II respectively as determined by LC–MS and tandem mass spectrometry (MS/MS) (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S6 *C* and *D*). Notably, MroQ^{mut} failed to catalyze substrate cleavage under equivalent conditions (*SI Appendix*, Fig. S6*C*). Exposure of the group-III AgrD (1–32) thiolactone to the MroQ-proteoliposomes did not lead to efficient production of AIP-III, rather the major species observed in this reaction corresponded to a mis-cleavage product in which an additional Tyr is appended to the AIP-III N terminus (*SI Appendix*, Fig. S6 *E* and *F*). These biochemical data demonstrate that MroQ is able to efficiently catalyze the second proteolytic cleavage step in AIP-I and AIP-II biosynthesis. By contrast, and in keeping our genetic studies, the biochemical data argue against a major role for this enzyme in the AIP-III maturation process.

Next, we asked whether MroQ could work in conjugation with AgrB to produce mature AIP-I and AIP-II from the corresponding AgrD polypeptide precursors. A reconstituted system was again employed, in this case combining recombinant AgrD peptides with MroQ- and AgrB-proteoliposomes, the latter prepared as previously described (*SI Appendix*, Fig. S6*G*) (15). Employing our LC–MS readout, we observed the generation of mature AIP-I and AIP-II when the corresponding AgrD precursors were treated with the cognate AgrB protease in the presence of MroQ (Fig. 3*C* and *SI Appendix*, Fig. S6 *H* and *I*). Thus, AgrB and MroQ can work in tandem to produce a mature AIP. The success of this experiment represents the formal reconstitution of a complete AIP biosynthetic pathway.

Encouraged by these reconstitution experiments, we decided to explore the feasibility of establishing a one-pot in vitro system comprising both the biosynthetic and AIP-sensing arms of the *agr* response. For this, we employed our previously developed lipid nanodisc-embedded recombinant AgrC-I dimers (14), along with proteoliposomes containing the complete AIP biosynthetic pathway and precursor peptide (Fig. 3*D* and *SI Appendix*, Fig. S6 *G* and *J*). Remarkably, in the presence of ATP-γ-^{32}P, we observed robust AgrC-I autophosphorylation that was dependent upon the presence of all four protein components (Fig. 3 *E* and *F*). Moreover, AgrC-I activation was abolished in the presence of a known orthosteric inhibitor, QQ-3 (36), confirming that the AIP produced in situ was engaging the cognate AgrC-I sensing pocket. This represents the formal in vitro reconstitution of a full bacterial quorum sensing pathway, recapitulating ribosomally synthesized and posttranslationally modified peptide (RiPP) biosynthesis, receptor engagement, and receptor activation simultaneously under physiologically relevant conditions to achieve bioequivalent signal output in a single system.

MroQ Recognizes Specific Sequence Motifs in AgrD-I/II- (1–32) Thiolactone. We next turned to elucidating the molecular features dictating MroQ cleavage specificity, which we note must occur at different positions in the AgrD-I/II (1–32) thiolactone substrates in order to generate AIPs of the correct length—mature AIP-I is an 8-mer while AIP-II is one residue longer (*SI Appendix*, Fig. S7). Since the enzyme is fully conserved among the *S. aureus agr* specificity groups, we imagined that cleavage specificity must be driven by differences in the substrate. The AIP biosynthetic intermediate, the AgrD (1–32) thiolactone, contains three structural domains: the N-terminal amphipathic helix, the linker/AIP-tail region (which contains the MroQ cleavage site), and the AIP-macrocycle (*SI Appendix*, Fig. S7). The linker/AIP tail region is the most divergent between the different groups, making it the prime candidate as the specificity driver.

To test this possibility, we generated chimeric AgrD (1–32) thiolactone peptides containing the sequence of the linker domain from one specificity group, inserted into a peptide containing the N-terminal amphipathic helix and AIP-macrocycle sequences from a different specificity group (Fig. 4*A* and *SI Appendix*, Figs. S8 *A* and *B* and S9*A*). These peptides were generated recombinantly by a similar intein fusion method used to generate native AgrD (1–32) thiolactones. For example, the AgrD-I-II-I (1–32) thiolactone chimera has the group-II linker sequence flanked by group-I sequences. These chimeras were then incubated with MroQ-proteoliposomes and the cleavage products analyzed by LC–MS and MS/MS (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S9*B*). The linker sequence from *agr* group-II directed MroQ to cleave the I-II-I chimeric intermediate at a position four amino acids from the AIP macrocycle yielding the chimeric AIP product, AIP-II-I, bearing a tail identical to AIP-II (Fig. 4*B* and *SI Appendix*, Fig. S9*B*). Similarly, the linker sequence from group-I intermediate-directed cleavage of the chimeric substrate AgrD-II-I-II (1–32) thiolactone to yield AIP-I-II as the major product, indicating that the group-I linker sequence directs cleavage in AIP-I biosynthesis (*SI Appendix*, Fig. S9 *A*, *C*, and *D*).

To validate these biochemical findings, we generated *S. aureus* strains harboring equivalent chimeric *agrD* mutants. The desired chimeric *agrD* sequences were introduced into the *agr* null strain, RN7206, along with cognate *agrB*, *agrC*, and *agrA* sequences, paired based on the identity of the *agrD* AIP-macrocycle domain (*SI Appendix*, Fig. S9*E*). Importantly, the *agrC* sequences used in these experiments harbored a mutation (R238K) that leads to constitutive AgrC activity (37), ensuring continuous transcription of the mutant RNAII regardless of the agonist or antagonist

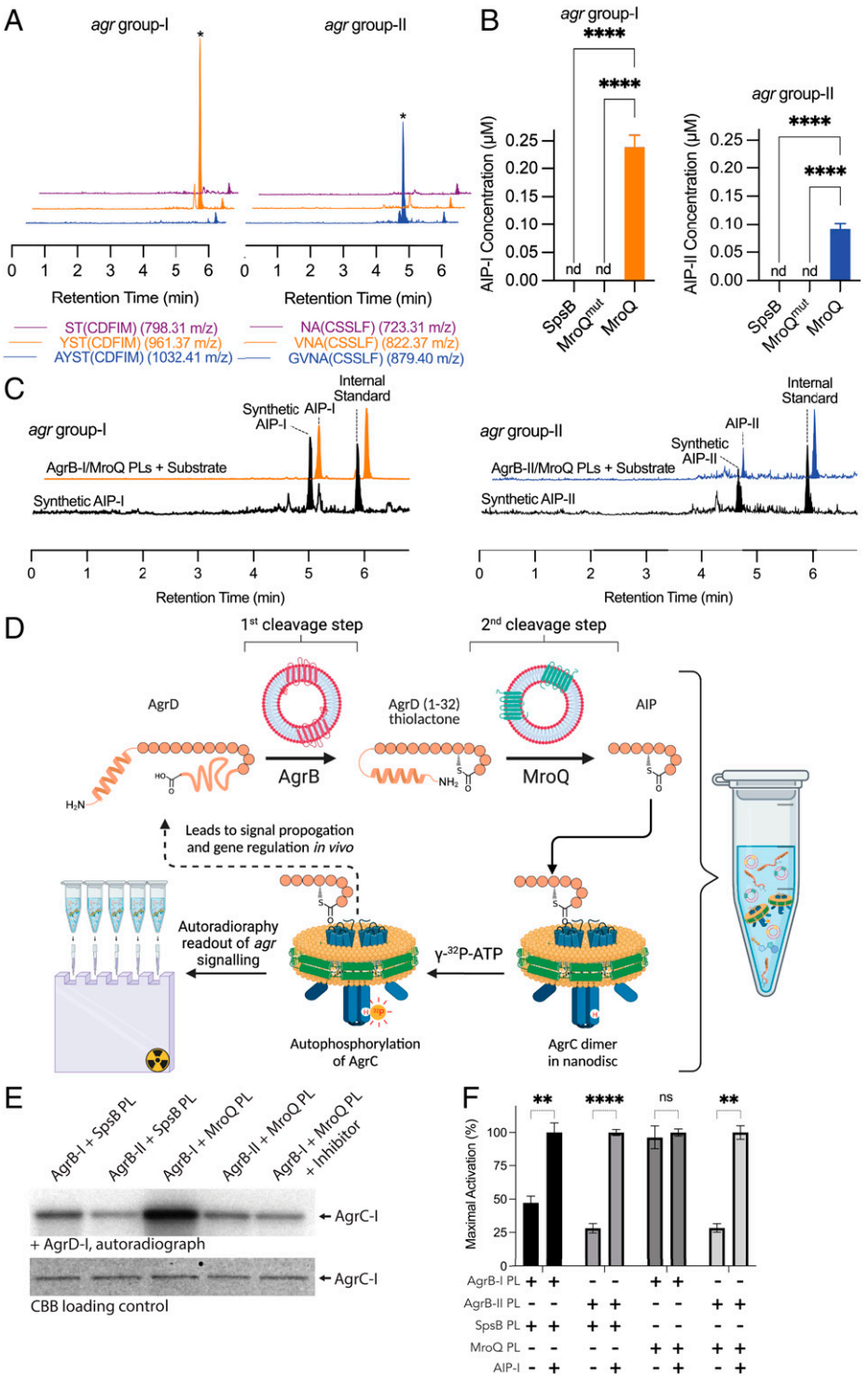

Fig. 3. In vitro reconstitution of AIP biosynthesis and sensing. (*A*) MroQ proteoliposomes were combined with indicated AgrD (1–32) thiolactone intermediates. Reaction mixtures containing a spike-in internal standard were then subjected to a SPE step and analyzed by LC–MS. Shown are the EIC traces for the possible cleavage products and the internal standard. *Indicates correct AIP product from each specificity group. (*B*) AIP-I and -II levels produced by MroQ, $MroQ^{mut}$, or SpsB proteoliposomes were quantified by LC–MS using a standard curve approach employing synthetic AIPs. Data presented as the mean ± SD ($n = 3$ biological replicates). (*C*) Reconstituted AIP biosynthesis. AgrD-I or AgrD-II was incubated with cognate AgrB and MroQ proteoliposomes. Reaction mixtures containing a spike-in internal standard were then subjected to a SPE step and analyzed by LC–MS. Shown are the EIC traces for the expected AIP and the internal standard. A synthetic AIP treated with empty liposomes served as a positive control (bottom trace). (*D*) Overview of one-pot in vitro AIP biosynthesis and sensing assay. Input AgrD is processed by AgrB and MroQ proteoliposomes into mature AIP which then binds and activates nanodisc-reconstituted AgrC. (*E*) Autoradiography of AgrC-I autophosphorylation employing ATP-γ-^{32}P. All samples contained AgrD and AgrC in addition to the indicated proteins. Inhibitor refers to a known tight binding antagonist of AgrC-AIP interaction. Note, AgrC-I is known have basal autokinase activity (14). (*F*) Quantification of AgrC-I autokinase activity as determined by densitometry analysis of the autoradiographs. All samples contained AgrD and AgrC in addition to the indicated proteins and peptides. Maximal activation of AgrC-I was determined by addition of synthetic AIP-I to the mixtures. Data presented as the mean ± SD ($n = 3$ biological replicates).

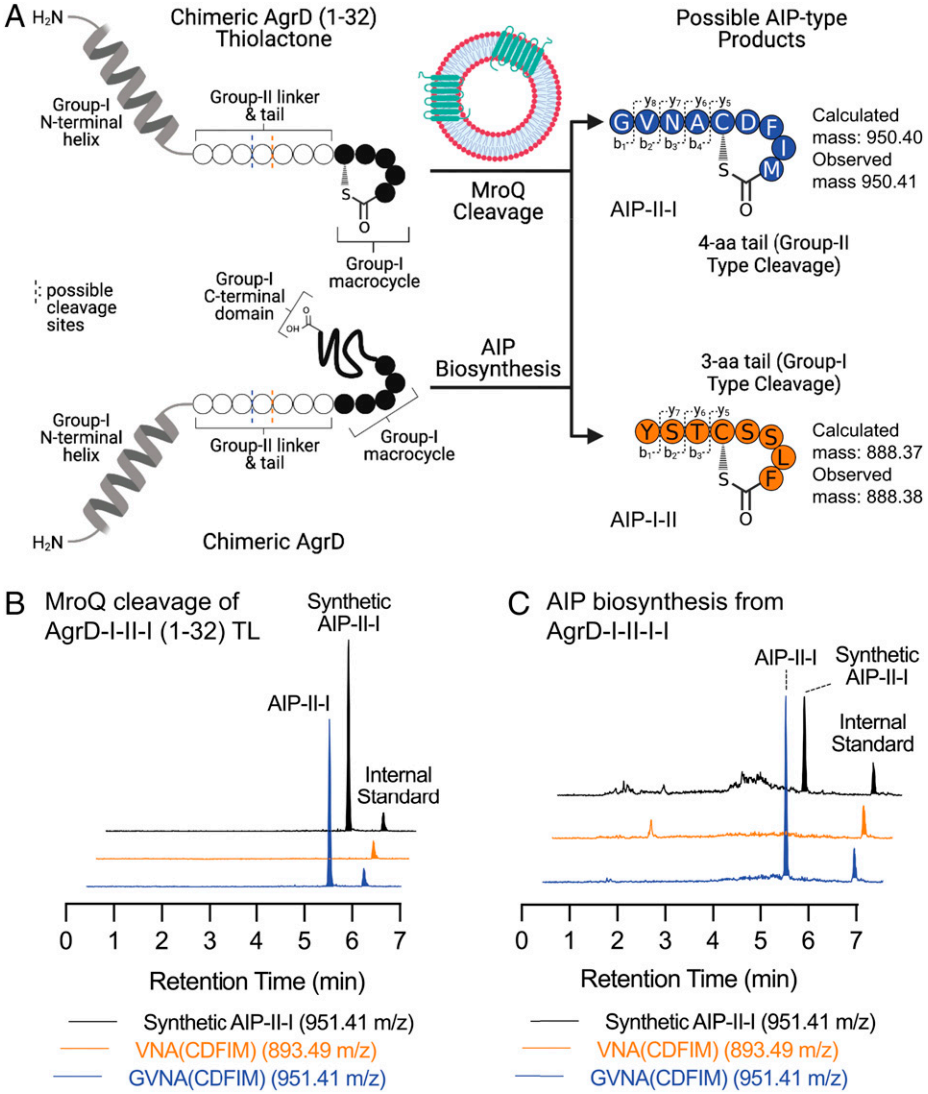

Fig. 4. The AgrD-I/II linker domain dictates MroQ specificity in AIP biosynthesis. (*A*) Overview of the in vitro MroQ cleavage assay and in vivo *agrD* mutant AIP production assays. AgrD-I/II thiolactone substrates, in which the linker-tail region of one specificity group is swapped for another, were coincubated with proteoliposome-reconstituted MroQ. In vivo studies utilized chimeric AgrD peptides containing the N-terminal helix domain, AIP macrocycle domain, and C-terminal domain from one *agr* specificity group, and a linker domain sequence from a different specificity group, expressed under P2 promoter control in an *agr*-null *S. aureus* background. Identity of cleaved or secreted chimeric AIPs was determined by LC–MS. (*B*) MroQ proteoliposomes were treated with AgrD-I-II-I (1–32) thiolactone. Reaction mixtures containing a spike-in internal standard were then subjected to a SPE step and analyzed by LC–MS. Shown are the EIC traces for the possible AIP cleavage products and the internal standard. Synthetic chimeric AIPs treated with empty liposomes served as positive controls (black traces). (*C*) EIC traces of chimeric AIP cleavage products and internal standard from LC–MS analysis of growth media from *S. aureus cells* expressing AgrD-I-II-I-I chimera. Synthetic chimeric AIP-II-I served as a positive control (black trace).

activity of secreted chimeric AIP analogs (*SI Appendix*, Fig. S9*E*). Consistent with the in vitro studies employing chimeric substrates, the identity of the linker region was observed to direct cleavage site position. Thus, expression of AgrD-I-II-I-I led to the secretion of the chimeric AIP-II-I, with an AIP-I macrocycle and a four-amino acid AIP-II tail consistent with group-II type cleavage (Fig. 4 *A* and *C* and *SI Appendix*, Fig. S9*B*). Similarly, expression of AgrD-II-I-II-II led to the secretion of the chimeric AIP-I-II, with a three-amino acid AIP-I tail consistent with group-I type cleavage (*SI Appendix*, Fig. S9 *A*, *C*, and *D*). Based on these data, we conclude that the AgrD linker motif sequence dictates specificity of MroQ mediated cleavage in AIP-I/II biosynthesis.

By extension, it is conceivable that the linker region within the group-III AgrD sequence, which we note contains a positively charged arginine in place of the Ile/Val residue in the other groups (*SI Appendix*, Fig. S7), may be refractory to MroQ cleavage. Indeed, a mutant version of the group-I AgrD (1–32) thiolactone containing an I22R substitution is not a substrate of the enzyme (*SI Appendix*, Fig. S10).

Discussion

The *S. aureus agr* quorum sensing circuit plays a central role in the regulation of virulence in this human pathogen, and as a consequence has been the subject of intense study for decades (2, 7). Despite this, our understanding of the biosynthetic pathway that leads to the generation of the secreted AIP signaling molecules has remained incomplete. In this study, we employed biochemical reconstitution and genetic complementation strategies to show that the integral membrane protease, MroQ, plays a direct role in the biosynthesis of the AIPs from *S. aureus agr* specificity groups I and II. Our data indicate that this enzyme can perform the second step in the maturation process, namely cleavage of the AgrD-I/II (1–32) thiolactone intermediates, and that the specificity of this processing step is dictated by the

linker region in the substrates. Given the high sequence homology between the group-I and group-IV *agr* systems, we propose that MroQ also plays a direct role in the maturation of the latter. By contrast, we find no evidence that MroQ is directly involved the biosynthesis of the group-III AIP. Our biochemical and genetic data also argue against the involvement of the signaling peptidase, SpsB, an enzyme previously implicated in AIP-I biosynthesis. Collectively, these data shed light on the mechanism of AIP biosynthesis and reveal an unexpected divergence in this maturation process between the *agr*-III system and the other specificity groups.

Several recent reports have highlighted the involvement of MroQ in *S. aureus* virulence. In a key pair of studies, Cosgriff et al. (29) and Marroquin et al. (30) used genetic approaches to show that MroQ is required for *agr* activity and virulence production in a group-I *S. aureus* strain. However, these investigators stopped short of assigning a specific biochemical role for the protein, leaving it unclear whether the protease is directly involved in AIP maturation, or plays some other function in the *agr* signaling circuit, for example by modulating AIP sensing. Adding to the uncertainty, a recent study has even suggested that MroQ might hydrolyze the AIP, leading to the downstream inactivation of AgrC and by extension *agr* QS (38). Our biochemical studies clarify this picture by firmly establishing a direct role for MroQ in the second step of AIP biosynthesis, at least for *agr* groups I and II. Moreover, we see no evidence that the enzyme metabolizes the mature AIP.

We also investigated whether the signal peptidase, SpsB, could convert the AIP (1–32) thiolactone intermediates from groups I–III into the corresponding AIPs. While we were able to reconstitute active full-length enzyme, as evidenced by robust cleavage of a known substrate, our efforts to generate AIPs using SpsB-proteoliposomes were unsuccessful. This result stands in contrast to the work of Kavanaugh et al. (27) who demonstrated that a soluble N-terminally truncated version of SpsB cleaves peptide mimics of the AgrD-I linker domain. Conceivably, the full-length, membrane-integrated protein might require some additional factor, not present in our reconstituted system, in order to cleave the membrane-associated AgrD (1–32) thiolactone. However, the fact that we observed cleavage of a spike-in control substrate in these experiments shows that the enzyme can at least access substrates in this proteoliposomal context. Furthermore, our use of a genetic rescue system that allows deletion of *spsB* in group-I *S. aureus* cells also failed to provide evidence supporting a role for this enzyme in AIP production. Taken together, our data suggest the SpsB is not required for AIP maturation.

While our biochemical and genetic experiments converge on a direct role for MroQ in AIP maturation in *S. aureus* groups I and II, they also argue against the involvement of this enzyme in the biosynthesis of AIP-III. Thus, we imagine that another, as yet unidentified, protease must be involved in the group-III system (i.e., it is divergent from the other specificity groups at the level of the biosynthetic pathway). Clues as to the origins of this divergence emerge from our structure-activity studies indicating that MroQ cleavage specificity is dictated by the linker region connecting the N-terminal amphipathic helical domain and the macrocyclic domain. In the case of the group-I and group-II systems, sequence variation within this region appears sufficient to direct MroQ cleavage to the correct site, resulting in AIPs of different length. This relationship appears not to hold true for the MroQ:AgrD-III pairing. We posit that the sequence of the linker region in AgrD-III is incompatible with efficient and specific MroQ activity, as substitution of a single group-III arginine residue in the linker region into group-I substrate is sufficient to prevent MroQ cleavage in vitro. Conceivably, other domains of AgrD may also contribute to MroQ substrate recognition as well, given that the N-terminal helical domain of AgrD-III differs at multiple positions from the other three groups (*SI Appendix*, Fig. S7). In particular, it contains substitutions that alter amino acid charge which might affect how this domain associates with the bacterial membrane. It is possible that either, or both, of these unique sequence characteristics direct AgrD-III (1–32) thiolactone intermediate to a different processing pathway in vivo.

Finally, our studies do not reveal where MroQ-mediated cleavage of the AgrD-I/II (1–32) thiolactone intermediates occurs relative to the plasma membrane. This has important implications for how the mature AIPs are secreted out of the cell. One can certainly imagine a scenario where the substrate enters the protease active site from the cytoplasmic side of the membrane, meaning that it is the mature AIP that subsequently crosses the membrane via a passive or active transport process. With respect to this, we note that MroQ does not sharehomology with known ABC transporters, however we cannot rule out that its proteolytic activity is somehow coupled to transport, bearing in mind that this is an integral membrane protein. Conversely, the substrate could enter the MroQ active site from the extracellular side. In this case, cleavage activity would release the mature AIP directly into the extracellular milieu. However, such a mechanism requires that the AgrD (1–32) thiolactone intermediate, or at least the linker region within in, somehow localizes to the extracellular side of the membrane in order to gain access to the enzyme active site. Again, both passive and active transport processes are possible (7). Ultimately, additional biochemical and likely structural studies will be required to fully address these outstanding questions. Regardless, our data reveal MroQ as a bona fide player in AIP biosynthesis and suggest a point of evolutionary divergence between the *S. aureus* specificity groups.

Materials and Methods

***S. aureus* Growth Conditions.** To initiate each growth experiment, *S. aureus* strains (*SI Appendix*, Table S1) were grown from glycerol stocks overnight in CYGP medium without glucose (39–41). For strains transduced with *pCN51* derivatives (*SI Appendix*, Table S2), media was supplemented with 10 μg/mL erythromycin and 2 μM $CdCl_2$. Subcultures of overnight growths were grown for 24–48 h at 37 °C in a shaker incubator and sampled every 1–4 h for growth monitoring by OD_{600} and AIP production measurement. AIP production was quantified by LC–MS and validated through a β-lactamase reporter cell assay when applicable (25, 42). Full experimental details for LC–MS analyses and reporter cell assays are provided in the *SI Appendix*.

Proteoliposome and Nanodisc Assembly. Proteoliposome assembly was performed by methods adapted from Wang et al. (15). Proteoliposomes were formed by coincubation of integral membrane proteases with polar lipids in detergent containing buffer, followed by removal of detergent through absorption by BioBeads SM-2 (BioRad) to force the formation of proteoliposomes. Reconstitution of purified recombinant AgrC-I into lipid nanodiscs was performed as previously described in Wang et al. (14), utilizing MSP1D1 membrane scaffold protein.

Biochemical Assays Using Proteoliposomes. For analysis of the second step of AIP maturation, SceD and AgrD (1–32) thiolactone substrates (0.5 μM) were coincubated with 2 μM of membrane protease in proteoliposomes in a reaction buffer (30 mM phosphate, 2.5 mM TCEP, pH 7.5). For reconstitution of the entire AIP biosynthetic pathway, full-length AgrD substrates (10 μM) were coincubated with 5 μM MroQ proteoliposomes and 10 μM cogenetic AgrB proteoliposomes in the same reaction buffer. M131 (2 μM) was added to relevant reactions. Reactions were performed at 37 °C for 3–24 h in a shaker incubator. Reactions were stopped by snap freezing or by acidification with neat TFA.

One-Pot AIP Biosynthesis and AgrC Autokinase Assay. Reactions were performed via a method adapted from previously reported AgrC autokinase assays (14, 16, 17, 36). Proteoliposomes (indicated combinations of SpsB, AgrB-I, AgrB-II, and MroQ at 1.7 μM) were added to AgrC-I nanodiscs at 0.7 μM in an assay buffer (50 mM Tris × HCl, 15 mM Hepes, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM TCEP). AgrD-I was added to 40 μM. Relevant reactions also contained AIP-I (10 μM) and/or the AgrC inhibitor QQ-3 (5 μM) (36). ATP was then added (10 μM cold ATP, 10 μM ATP-γ-^{32}P [10 Ci/mmol]) and the reaction mixture incubated for 40 min at 37 °C. The reactions were quenched and resolved by SDS-PAGE. Gels were processed by drying and exposed to film for analysis of ^{32}P phosphorylation of AgrC-I.

Data Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. We thank current members of the Novick and Muir laboratories for discussions and comments. We thank Connie Wang for assistance in development and synthesis of substrates and mutant *S. aureus* strains. We thank Dr. John Eng and Dr. Venu Vandavasi from the Princeton Chemistry Mass Spectrometry Facility, and Dr. Saw Kyin from the Princeton Molecular Biology Proteomics Facility for their assistance. We thank Merck for the gift of M131. Figures created with BioRender. This work was supported by NIH (AI042783 to T.W.M.). We note that our manuscript was recently posted on bioRxiv (10.1101/2021.12.29.473670).

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