Key Driving Forces in the Biosynthesis of Auto-Inducing Peptides Required for Staphylococcal Virulence

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Supporting Information

SI Figures

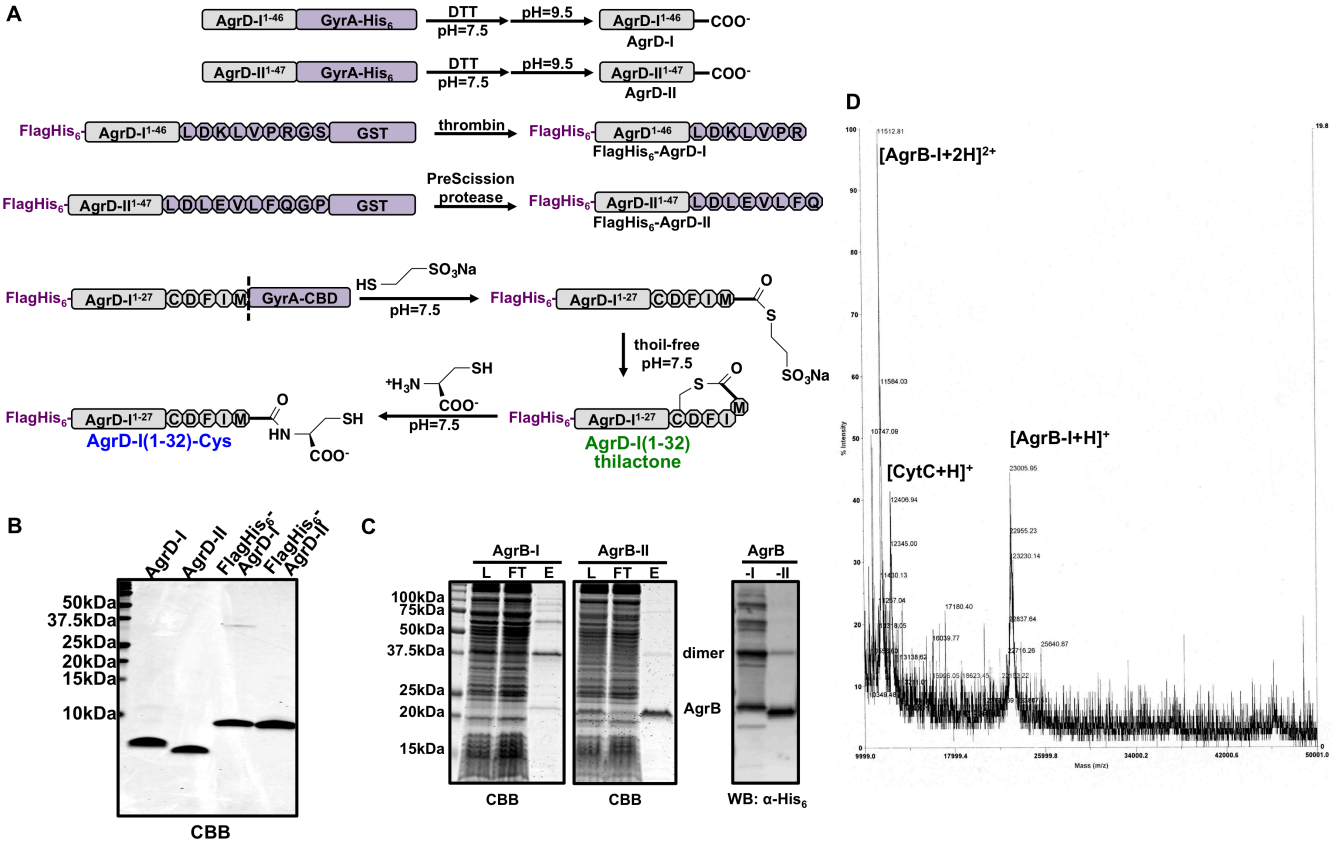


Fig. S1. Supplemental data for recombinant protein preparation.
(*A*) Schematic presentation of the recombinant approaches to prepare AgrD constructs. GyrA, the *Mxe* GyrA intein; GST, glutathione S-transferase; DTT, dithiothreitol; MESNa, 2-mercaptoethanesulfonate (MESNa). The FlagHis$_6$-AgrD(1-32)-thiolactone product was treated with L-cysteine to give the linear FlagHis$_6$-AgrD(1-32)-Cys, which was used as the surrogate of FlagHis$_6$-AgrD(1-32) in certain biophysical experiments. (*B* and *C*) SDS-PAGE analysis of all four AgrD constructs prepared herein (*B*) and samples from the affinity purification of AgrB proteins (*C*). L: bacterial membrane extract; FT: flow through; E: elution. An anti-His$_6$ western blot of purified AgrB proteins is shown to the right. (*D*) MALDI mass spectrum of AgrB-I (calculated molecular weight = 22.7kDa) showing the absence of covalent dimer. Bovine cytochrome C (molecular weight = 12,384 Da, showed up at m/z = 12,407Da) was used as an internal standard for m/z calibration.

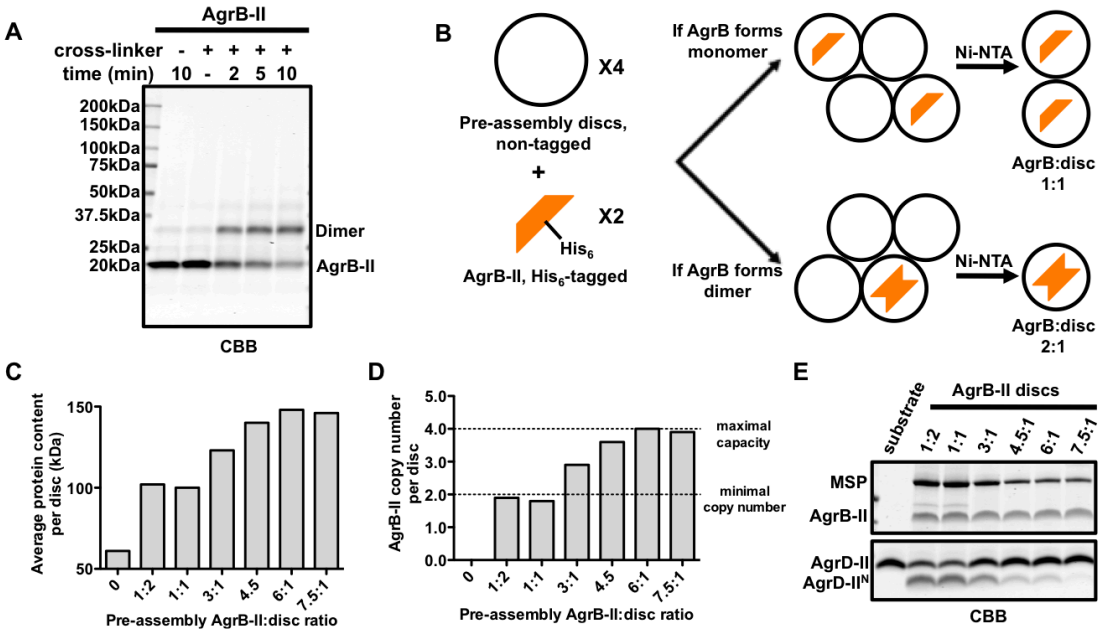


Fig. S2. AgrB-II dimerizes under non-denaturing conditions.
(*A*) SDS-PAGE analysis of detergent-solubilized AgrB-II before or after glutaraldehyde treatment. (*B*) Distribution of AgrB structural units in nanodiscs: diagram showing the scenarios of reconstituting AgrB into excess nanodiscs assuming AgrB preferably forms monomers or dimers. (*C* and *D*) SEC-MALS analysis of Ni-NTA-purified AgrB-II nanodiscs: protein content per disc (*C*) and AgrB-II copy number per disc (*D*) are depicted for nanodiscs assembled from pre-assembly mixtures containing AgrB-II and nanodisc at indicated ratios. (*E*) Proteolysis of FlagHis$_6$-AgrD-II after treatment with AgrB-II nanodiscs: reactions were analyzed by SDS-PAGE. Average AgrB-II copy number per disc is indicated. The products, AgrD-II(1-32) fragments (linear and/or thiolactone) are collectively indicated as AgrD-II^N. MSP, membrane scaffold protein.

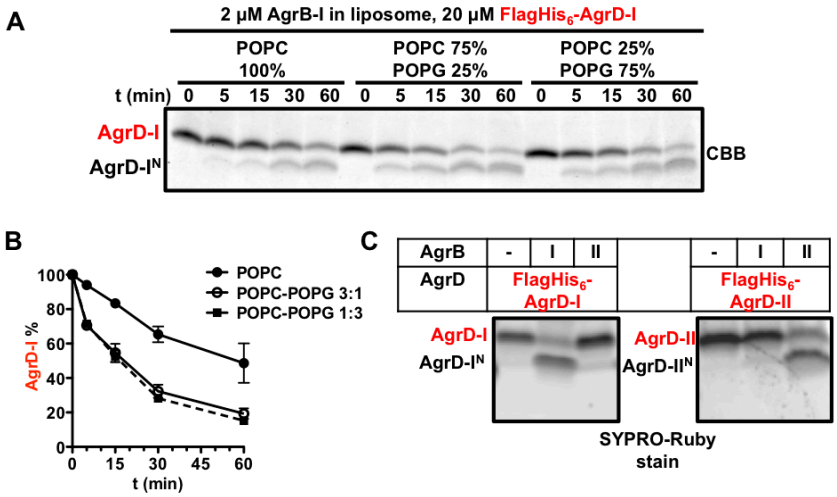


Fig. S3. Supplemental data for the reconstitution of AgrB-catalyzed proteolysis of AgrD. (*A* and *B*) Lipid-composition dependence of AgrB-I activity. FlagHis$_6$-AgrD-I was treated with AgrB-I proteoliposomes consisting of phospholipids at indicated compositions. Samples were withdrawn at indicated time points for SDS-PAGE analysis. (*A*) SDS-PAGE analysis of one representative time course and (*B*) kinetic plot of AgrD-I consumption from densitometric quantification of the gel were shown. Error bars = range (n = 2). (*C*) Proteolysis specificity between AgrB-AgrD pairs from *agr*-I and *agr*-II alleles. FlagHis$_6$-tagged AgrDs were treated with indicated liposomal reagents at 37^{o}C for 6hrs. The reactions were resolved on Tris-Tricine SDS-PAGE and proteins visualized with SYPRO-Ruby staining (Invitrogen). In panels (*A*) and (*C*), the AgrD(1-32) fragments (linear and/or thiolactone) are collectively indicated as AgrD-I^N or AgrD-II^N.

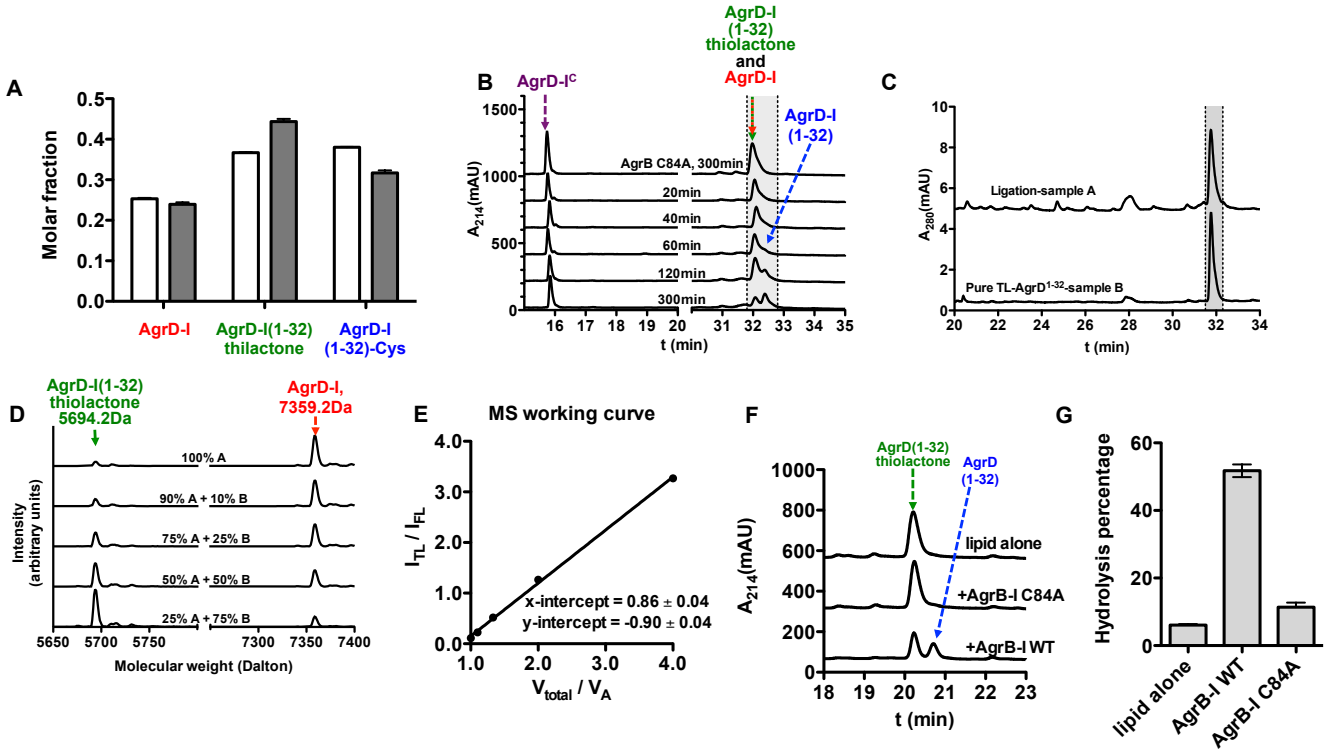


Fig. S4. Supplemental data for the reversibility of the proteolytic cyclization of AgrD. (*A*) The relative Sep-Pak recovery among FlagHis$_6$-AgrD-I, FlagHis$_6$-AgrD-I(1-32)-thiolactone, and FlagHis$_6$-AgrD-I$^{1\text{-}32}$-Cys: bar graph shows the molar fraction of each species in a mixture of three before (open bars) or after (closed bars) solid-phase extraction (SPE) using Sep-Pak. Pre- and post-SPE samples were quantified using HPLC. Error bars = range (n = 2). (*B*) Time course of the ligation between AgrD-I(1-32)-thiolactone and AgrD-I^C as analyzed by RP-HPLC: The shade indicates the segment of the elution from which samples were collected for mass spectroscopic analysis (see spectra in Fig. 3C). (*C* to *E*) The relative ionization efficiency of FlagHis$_6$-AgrD-I with respect to FlagHis$_6$-AgrD-I(1-32)-thiolactone: see SI Materials and Methods, section **9-6** for the principle of data processing. (*C*) RP-HPLC preparation of standard samples A and B: 20μM of FlagHis$_6$-AgrD-I(1-32)-thiolactone was treated with 300μM AgrD^C in the presence of AgrB-I proteoliposomes or mock-treated and then subject to RP-HPLC. Shaded segments of elution were collected from the ligation (as sample A) and the mock treatment (as sample B). Both samples were diluted to the same molarity; (*D*) Deconvoluted mass spectra for mixtures of samples A and B at indicated ratios. (*E*) The working curve: plot shows the MS peak intensity ratio between FlagHis$_6$-AgrD-I(1-32) and FlagHis$_6$-AgrD-I as a function of the reciprocal of the weight of sample A in the mixture. The inverse reciprocal of the y-intercept, *k*, converts MS peak intensity ratio to the molar ratio by simple multiplication. Error bars (range between two parallels) are not visible due to the size of data symbols. (*F* and *G*) Hydrolysis of AgrD(1-32)-thiolactone requires the catalysis of AgrB: the thiolactone was treated with indicated liposomal reagent at 37^{o}C for 1hr and then Sep-Pak processed and resolved on RP-HPLC. A representative set of chromatograms (panel *F*) and a bar graph showing the percentage of hydrolysis (corrected for SPE recovery) (panel *G*) are shown. Error bars = range (n=2).

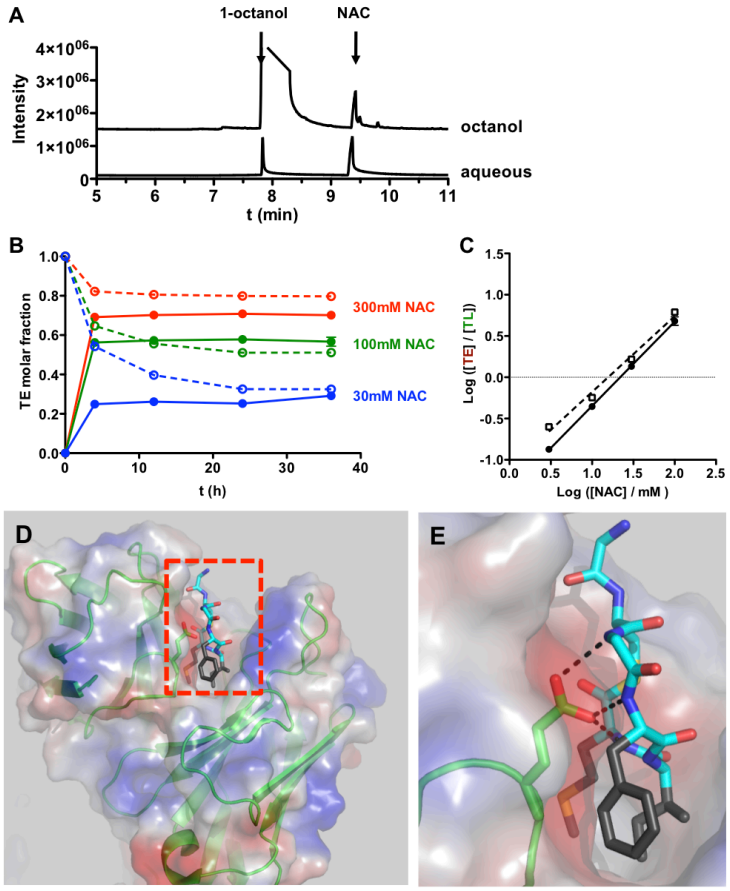


Fig. S5. Supporting data for the ring-opening equilibrium of thiolactone macrocycles. (*A*) The octanol-water partitioning equilibrium of NAC: 100 μL NAC was added to 2 mL aqueous PBS buffer (pH = 7.5) and 2 mL 1-octanol and the system was allowed to equilibrate at RT. The panel shows GC-MS chromatograms of equivolume samples from the aqueous and 1-octanol phases. (*B*) Time course of FlagHis$_6$-AgrD-I(1-32)-NAC ring closure in HBS buffer, pH = 7.0 containing 1.5 mM POPC and 0.5 mM POPG in the presence of indicated level of NAC. Plot shows the molar fraction of the thioester (TE) as a function of time (dashed lines). The ring-opening time courses (solid lines) under each NAC concentration are overlaid. (*C*) Ring-opening equilibrium positions of AIP-I (closed circles, solid line) and FlagHis$_6$-AgrD-I(1-32)-thiolactone (open squares, dashed line) in 6M GuHCl buffered at pH = 7.0. For (*B*) and (*C*), Error bars = range (n = 2) and are not visible due to the size of data symbols for most data points. TL, thiolactone. (*D* and *E*) AIP-IV-antibody interface in a crystal structure (PDBID: 3QG6): An overview (panel *D*) and a zoom-in view at the interface (red rectangle in *D*, panel *E*) are shown. AIP-IV is presented as a stick model with the backbone carbons in cyan and side-chain carbons of Phe6, Ile7 and Met8 in gray. Side chains of exocyclic residues are not shown for clarity. The ribbon diagram and electrostatic surface of the antibody are displayed. In (*E*), Hydrogen bonds between a glutamate residue in the antibody (highlighted in green) and the AIP backbone are highlighted by black dashes.

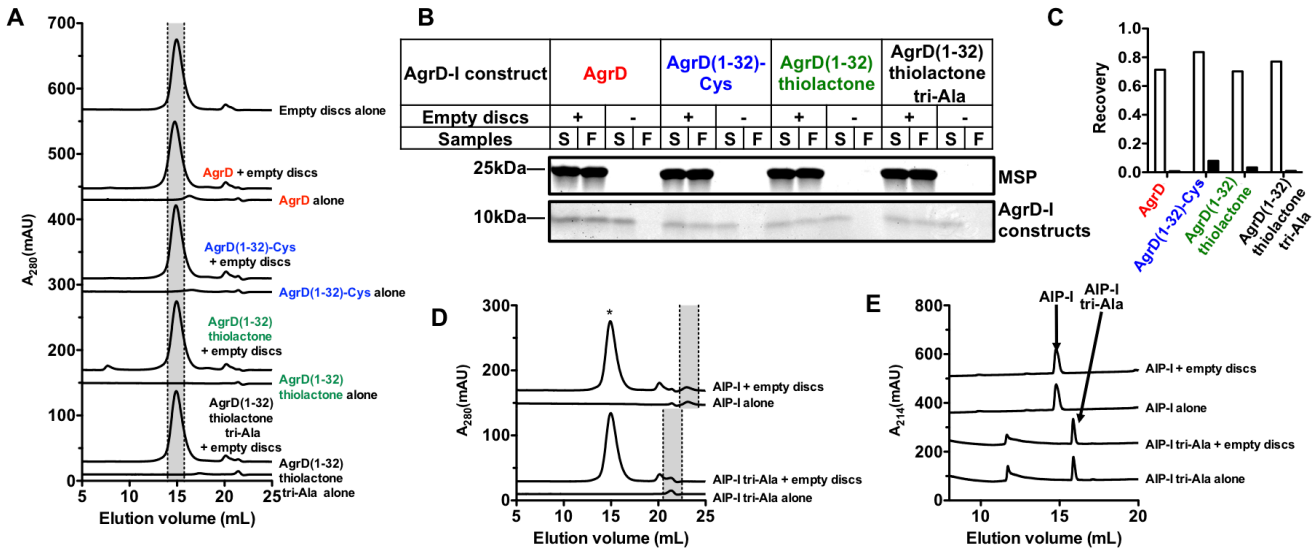


Fig. S6. Nanodisc co-migration assays for AIP-I and AgrD-I constructs.
(*A*) SEC chromatograms: SEC sample (250 μL) contained 12 μM appropriate AgrD peptide and/or equimolar bilayer nanodiscs as indicated. The bilayer discs consisted of POPC-POPG in a molar ratio of 3:1. Shadow indicates the position at which nanodiscs (unbound or bound to AgrD) are eluted. (*B*) Recovery of AgrD peptides within eluent containing nanodiscs: for each sample containing AgrD peptide, the pre-SEC sample (denoted as **S**) and the combined fractions from the shadowed segment in (*A*) (denoted as **F**) were analyzed by SDS-PAGE. MSP, membrane scaffold protein. (*C*) Quantification of the gel in (*B*). Bar graph depicts the recovery of indicated AgrD peptide from the shadowed segment of elution after resolved on SEC alone (close bars) or in the presence of bilayer nanodiscs (open bars). (*D*) SEC chromatograms of the co-migration assay of indicated AIP-I peptide. Positions where nanodiscs and AIP-I were eluted are indicated by asterisk and shadow, respectively. (*E*) RP-HPLC traces showing the recovery of either AIP-I peptide from the shadowed segment of elution in (*D*) to be unaffected by the presence of nanodiscs in the SEC samples.

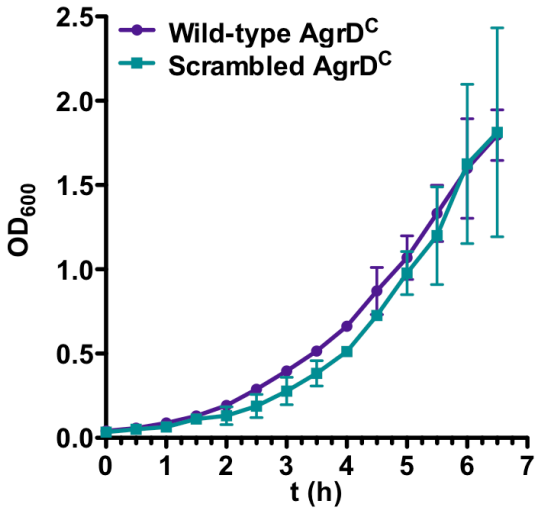


Fig. S7. Growth curves for RN6390 (*agr*-I wild type) cells producing either wild-type or scrambled AgrD-I^C peptide at 30^{o}C. Error bar = range (n = 2).

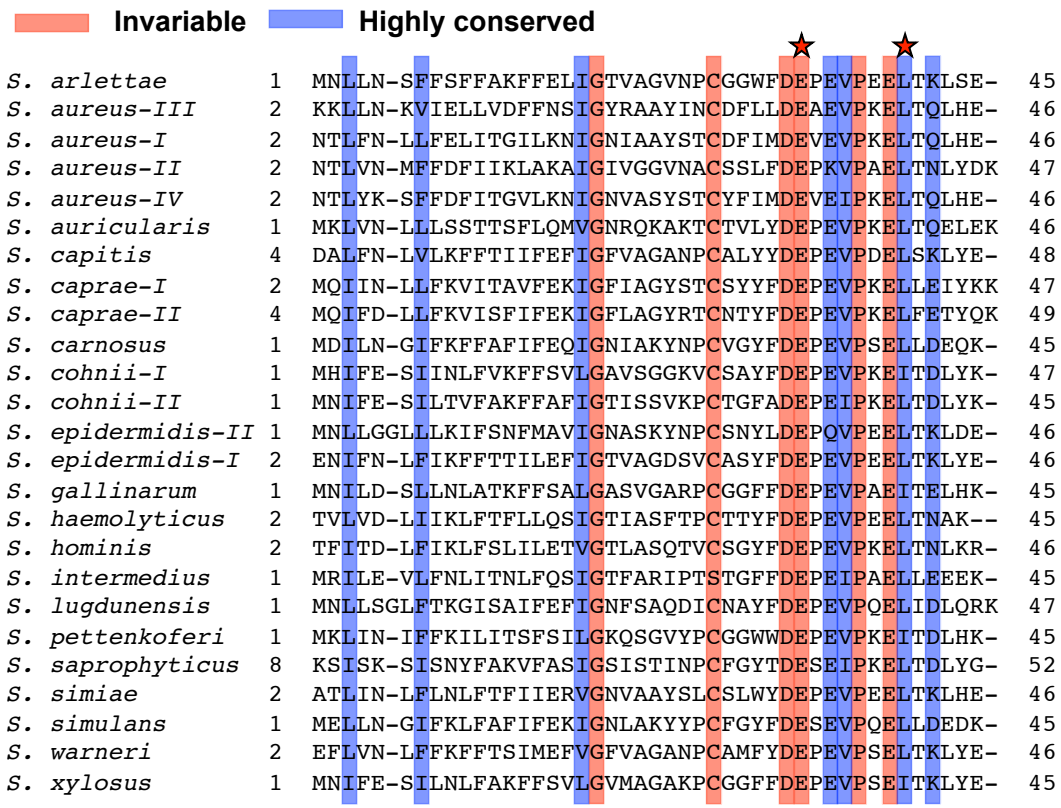
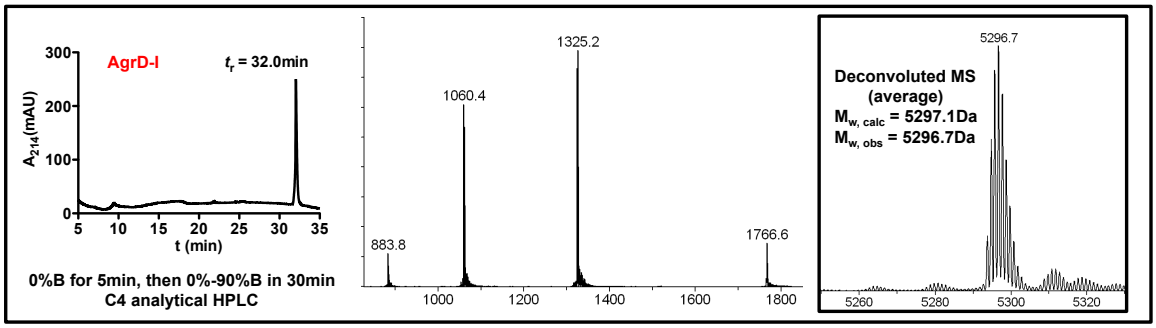
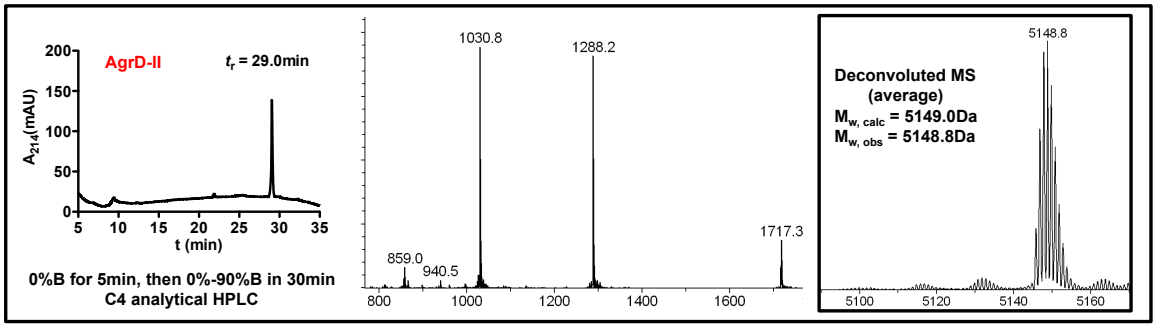
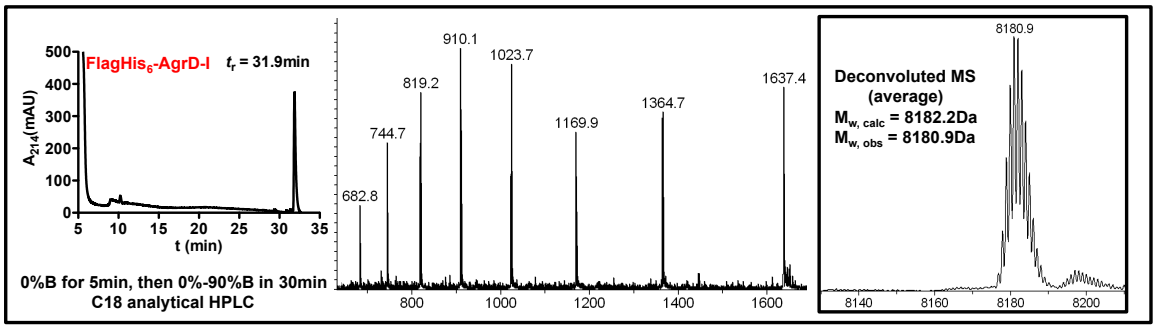
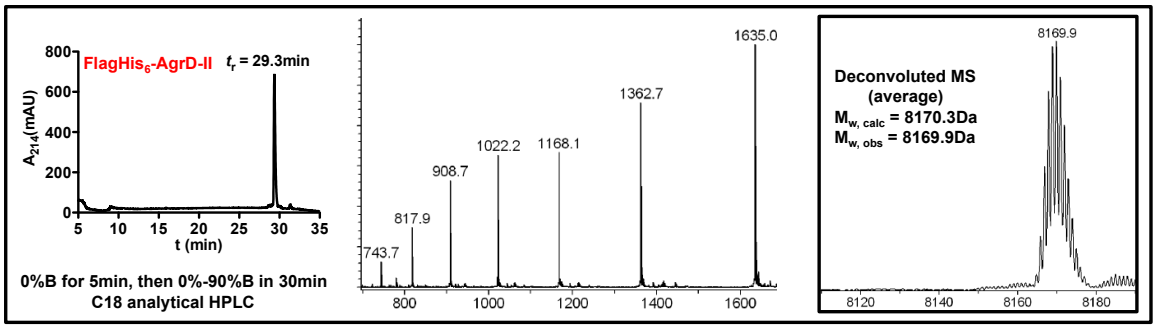


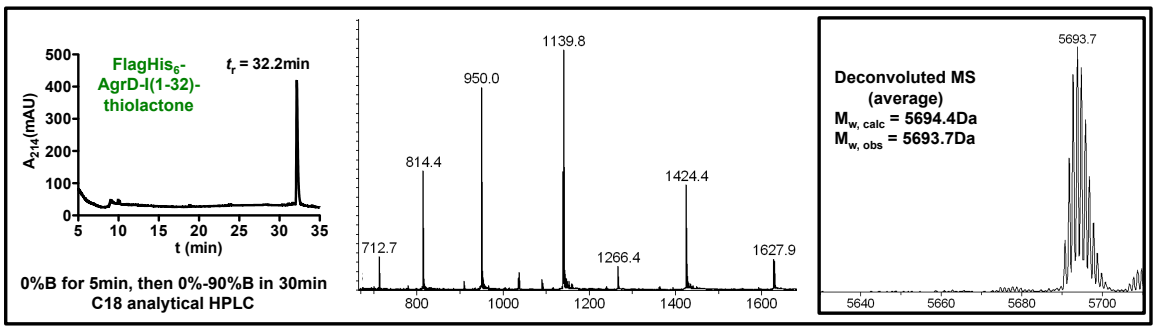
Fig. S8. Multiple sequence alignment of Staphylococcal AgrDs.
All AgrD sequences from the genus *Staphylococcus* in the pfam database (05931) are subject to an identity cutoff at 80%. The resulting 25 non-redundant sequences were aligned to the hidden-Markov model of the AgrD family. Invariable and highly conserved positions are highlighted in red and blue, respectively. Two red stars indicate positions at which mutation to alanine abolishes AgrB-catalyzed processing *in vivo*.


AgrD-I
t_r = 32.0min
A_{214}(mAU)
t (min)
0%B for 5min, then 0%-90%B in 30min
C4 analytical HPLC
1325.2
1060.4
883.8
1766.6
Deconvoluted MS
(average)
$M_{w, calc}$ = 5297.1Da
$M_{w, obs}$ = 5296.7Da
5296.7


AgrD-II
t_r = 29.0min
A_{214}(mAU)
t (min)
0%B for 5min, then 0%-90%B in 30min
C4 analytical HPLC
1030.8
1288.2
1717.3
859.0
940.5
Deconvoluted MS
(average)
$M_{w, calc}$ = 5149.0Da
$M_{w, obs}$ = 5148.8Da
5148.8


FlagHis$_6$-AgrD-I
t_r = 31.9min
A_{214}(mAU)
t (min)
0%B for 5min, then 0%-90%B in 30min
C18 analytical HPLC
910.1
1023.7
819.2
1637.4
1364.7
1169.9
744.7
682.8
Deconvoluted MS
(average)
$M_{w, calc}$ = 8182.2Da
$M_{w, obs}$ = 8180.9Da
8180.9


FlagHis$_6$-AgrD-II
t_r = 29.3min
A_{214}(mAU)
t (min)
0%B for 5min, then 0%-90%B in 30min
C18 analytical HPLC
1635.0
1362.7
1022.2
1168.1
908.7
817.9
743.7
Deconvoluted MS
(average)
$M_{w, calc}$ = 8170.3Da
$M_{w, obs}$ = 8169.9Da
8169.9


FlagHis$_6$-
AgrD-I(1-32)-
thiolactone
t_r = 32.2min
A_{214}(mAU)
t (min)
0%B for 5min, then 0%-90%B in 30min
C18 analytical HPLC
1139.8
950.0
814.4
1424.4
712.7
1266.4
1627.9
Deconvoluted MS
(average)
$M_{w, calc}$ = 5694.4Da
$M_{w, obs}$ = 5693.7Da
5693.7

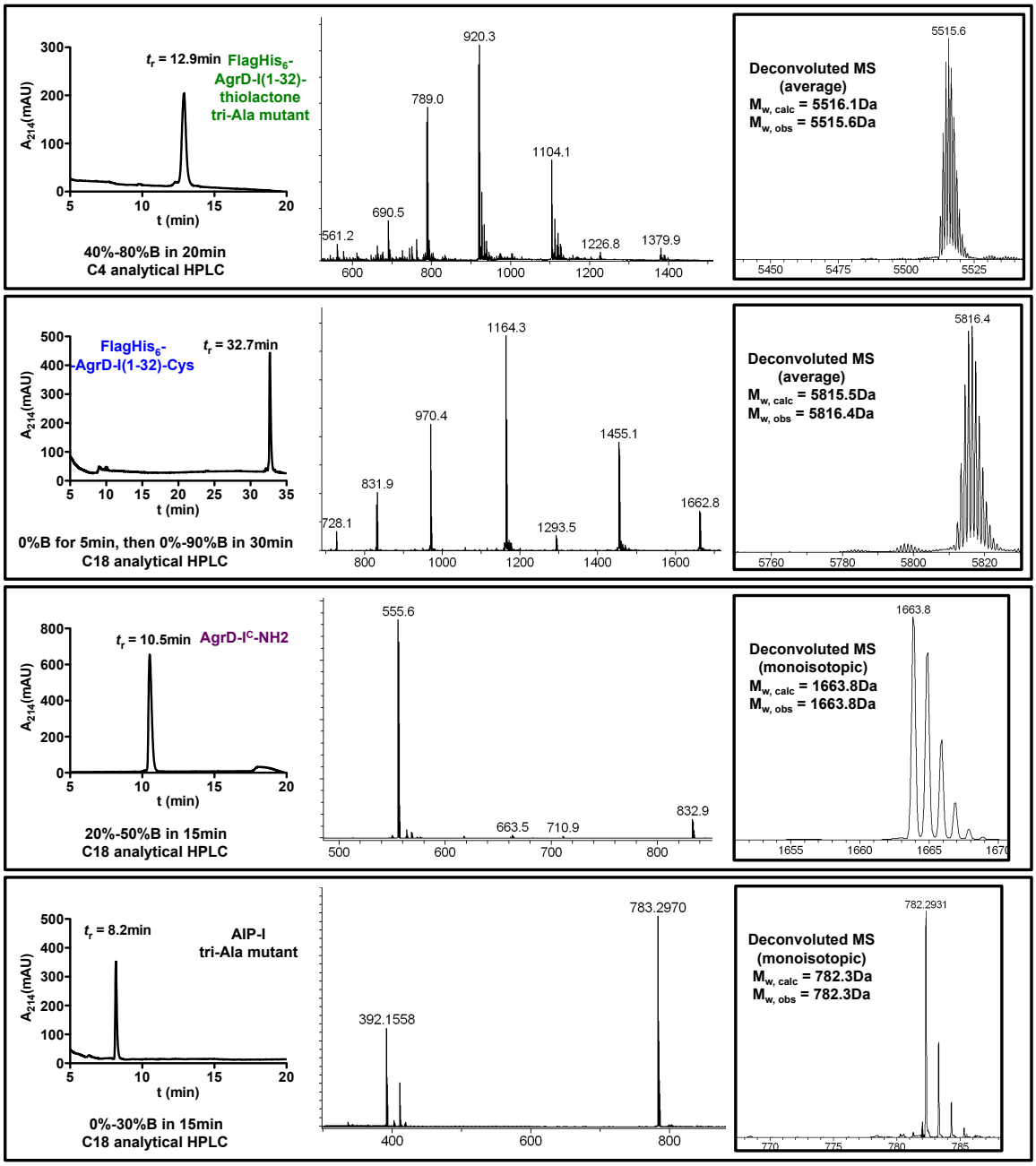


Fig. S9. RP-HPLC-MS analysis of all recombinantly prepared or chemically synthesized AgrD constructs. For each peptide, an RP-HPLC chromatogram, an ESI-mass spectrum and the peak region of a deconvoluted mass spectrum (from left to right) are shown.

SI Table

Table S1. AgrD constructs prepared as starting materials or detected as products

Entry	Construct Name	Tags/Terminal Groups
R_1-AgrD-R_2		
1*	AgrD-I	**R_1**= H-, **R_2** = -OH
2*	AgrD-II	**R_1**= H-, **R_2** = -OH
3*	FlagHis$_6$-AgrD-I	**R_1**= H-MDYKDDDDKHHHHHHGG-; **R_2** = -LDKLVPR-OH
4*	FlagHis$_6$-AgrD-II	**R_1**= H-MDYKDDDDKHHHHHHGG-; **R_2** = -LDLELVFQ-OH
5	FlagHis$_6$-AgrD-I-NH_2	**R_1**= H-MDYKDDDDKHHHHHHGG-; **R_2** = -NH_2
AgrD^C-**R_2**		
6	AgrD-I^C	**R_2** = -OH
7	AgrD-II^C	**R_2** = -OH
8	AgrD-I^C-LDKLVPR	**R_2** = -LDKLVPR-OH
9**	AgrD-I^C-NH_2	**R_2** = -NH_2
R_1-AgrD(1-32)-thiolactone		
10	AgrD-I(1-32)-thiolactone	**R_1**= H-,
11	AgrD-I(1-32)-thiolactone	**R_1**= H-,
12*	FlagHis$_6$-(TL)-AgrD-I-(1-32)-thiolactone	**R_1**= H-MDYKDDDDKHHHHHHGG-;
R_1-AgrD(1-32)		
13	AgrD-I(1-32)	**R_1**= H-,
14	AgrD-II(1-32)	**R_1**= H-,
15	FlagHis$_6$-AgrD-I(1-32)	**R_1**= H-MDYKDDDDKHHHHHHGG-;
Others		
16**	AIP-I	H-YST(CDFIM)***
17**	AIP-I tri-Ala mutant	H-YST(CDAAA)***
18*	FlagHis$_6$-(TL)-AgrD-I-(1-32)-thiolactone tri-Ala mutant	H-MDYKDDDDKHHHHHHGGMNTLFNLLFELITGILKNIGNIAAYST(CDAAA)***

Sequence of full-length AgrD-I (residues 1-46) is
MNTLFNLFFDFITGILKNIGNIAAYSTCDFIMDEVEVPKELTQLHE
Sequence of full-length AgrD-II (residues 1-47) is
MNTLVNMFFDFIIKLAKAIGIVGGVNACSSLFDEPKVPAELTNLYDK

*: constructs processed from recombinant protein;
**: constructs synthesized chemically.
***: parenthesis indicates the participating residues of the thiolactone macrocycle.

SI Material and Methods

Section 1. General Reagents and Equipment

1-1: Reagents

All buffering salts, isopropyl-β-D-thiogalactopyranoside (IPTG), urea, tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), Coomassie brilliant blue, phenylmethylsulfonyl fluoride (PMSF) were purchased from Fisher Scientific (Pittsburgh, PA). All medium ingredients for bacterial cell cultures were from Difco Biosciences, Inc. (San Diego, CA). All antibiotics, lysostaphin, *N, N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), *N, N*-diisopropylethylamine (DIEA), piperidine, triisopropylsilane (TIS), β-mercaptoethanol (BME), N-Acetylcysteamine (NAC), gluaraldehyde solution, guanidinium chloride (GuHCl) and sodium 2-sulfanylethanesulfonate (MESNa) were purchased from Sigma-Aldrich (St. Louis, MO). All protected amino acids, coupling reagents and Rink-amide MBHA resin were purchased from Novabiochem (Läufelfingen, Switzerland). All lipids were purchased from Avanti Polar lipids (Alabaster, AL) and all detergents were obtained from Anatrace (Maumee, OH). Trifluoroacetic acid (TFA) was from Halocarbon (North Augusta, SC). Anhydrous HF was from Matheson. Nickel-nitrilotriacetic acid (Ni-NTA) resin and KOD polymerase were from Novagen (Gibbstown, NJ). Talon cobalt resin was from Clontech (Mountain View, CA). Glutathione Sepharose 4 Fast Flow (GS4FF) resin was from GE Healthcare (Piscataway, NJ). The QuikChange Site-Directed Mutagenesis kit was from Agilent (La Jolla, CA). tC2 Sep-Pak cartridges were from Waters (Milford, MA). T4 DNA ligase, restriction enzymes and the Phusion High-Fidelity PCR kit were from New England Biolabs (Ipswich, MA). DNA purification kits (QIAprep spin minikit, QIAquick gel extraction kit, QIAquick PCR purification kit) were from Qiagen (Valencia, CA). DH5α and BL21(DE3) *E. coli* strains were purchased from Invitrogen (Carlsbad, CA) and used to generate "in-house" high-competency cell lines. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Codon-optimized cDNAs were generated synthetically and purchased from GENEWIZ (South Plainfield, NJ). All plasmids used in this study were sequenced by GENEWIZ. Centrifugal filtration units were from Sartorius (Goettingen, Germany). All denaturing gels, PVDF membrane (0.2 μm), Bio-Beads SM2 was from Bio-Rad (Hercules, CA).

1-2: Equipment

Solid-phase peptide synthesis (SPPS) was carried out on a Liberty Synthesizer (CEM, Matthews, NC). Size exclusion chromatography was carried out on an ÄKTA FPLC system from GE Healthcare using a Superdex200 10/300 column or a Superose6 10/30 HR column. For all runs, proteins were eluted over one column volume of buffer (flow rate: 0.5 mL/min). For SEC-MALS, the ÄKTA FPLC system was connected in tandem to a DAWN HELEOS-II multi-angle light scattering instrument and OptiLab TrEX differential refractometer. RP-HPLC was performed on Hewlett-Packard 1100 and 1200 series instruments equipped with an analytical C_{18} or C_4 Vydac column (5µm, 4.6 x 150 mm) at a flow rate of 1 mL/min or a semiprep C_{18} or C_4 Vydac column (5µm, 10 x 250 mm) at a flow rate of 4 mL/min. All runs used 0.1 % TFA (trifluoroacetic acid) in water (solvent A) and 90 % acetonitrile in water with 0.1% TFA (solvent B). Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) mass spectrometric analysis was performed on a Bruker Daltonics MicrOTOF-Q II and a PerSeptive Voyager DE STR MALDI-TOF spectrometer, respectively. Coomassie stained gels were imaged on a LI-COR Odyssey Infrared Imager. Concentration of DNA, peptide and protein samples was measured on nanodrop2000 spectrometer from Thermo scientific or UV-Vis 8453 from Agilent.

Section 2. Molecular cloning strategies

2-1: cDNA sequence of Agr proteins

Amino acid sequences of full-length AgrB-I (1-189), AgrD-I (1-46), AgrB-II (1-187) and AgrD-II (1-47) were obtained from the NCBI protein database (Refseq accession: YP_001332977.1, YP_001332978.1, NP_372560.1 and NP_372561.1). The cDNA sequences were back-translated from the amino acid sequences with optimized codons according to the default *E. coli* codon usage table (1). cDNA was purchased from Genewiz. The cDNA were received in pUC57 vectors.

2-2: Expression constructs of AgrB-I-His_6 and AgrB-II-His_6

Coding sequences corresponding to full-length AgrB-I or AgrB-II were cloned between NdeI and XhoI sites of a pET24b vector (Novagen).

2-3: Expression constructs for the preparation of non-tagged AgrD-I and AgrD-II

A 24-nucleotide (nt) sequence encoding seven histidines and a stop codon was inserted in-frame immediately downstream to the coding sequence of the *Mxe* GyrA intein in the pTXB1 vector (NEB) to generate pTXB1-His_7. Coding sequences corresponding to full-length AgrD-I or AgrD-II were cloned between NdeI and SapI sites in pTXB1-His_7.

2-4: Expression constructs for the preparation of FlagHis_6-AgrD-I(1-32)-thiolactone

Coding sequence of FlagHis_6-AgrD-I(1-32) was inserted into the pTXB1 vector. The amino acid sequence of the affinity tag is MDYKDDDDKHHHHHHGG.

2-5: Expression constructs for the preparation of FlagHis_6-AgrD-I and FlagHis_6-AgrD-II

FlagHis_6-AgrD-I and FlagHis_6-AgrD-II was fused to GST within an intervening sequence for thrombin (LDKLVPRGSS) or PreScission protease (LDLEVLFQGPGS) recognition, respectively. Coding sequences of these two constructs was inserted between NdeI and XhoI sites in pET24b, with a stop codon immediately downstream to the XhoI site. Coding sequence of GST was from pGEX4T1 (GE Healthcare).

2-6: Plasmids that produce wild-type or scrambled AgrD-I^C-Ub-$Flag_2$ in *S. aureus*

Production of AgrD-I^C-Ub-$Flag_2$ was achieved by co-expression of two fusion proteins, Sumo-AgrD-I^C-Ub-$Flag_2$ and His_6-Ulp1 from the pCN51 shuttle plasmid (2). The insert sequence begins with the strong, constitutive *pblaZ*^{C} promoter (3), followed by coding sequences of AgrD-I^C-Ub-$Flag_2$ and His_6-Ulp1, both preceded by a consensus Shine-Dalgarno (SD) sequence and a short spacer. This insert sequence was synthesized by Genewiz and cloned between PstI and EcoRI sites of the vector. In a control plasmid, coding sequence of AgrD-I^{33-46} (DEVEVPKELTQLHE) was substituted with that of a scrambled peptide, TLEVKEVQELPEHD. The above substitution and insertion were both achieved using an overlap extension PCR technique (4).

2-7. Site-directed mutagenesis

Expression plasmids for the preparation of $AgrB^{C84A}$ and the triple-Ala mutant of FlagHis_6-AgrD-I(1-32)-thiolactone were generated from their respective parental, wild-type plasmids using a QuikChange Site-Directed Mutagenesis kit.

Section 3. Expression and purification of recombinant proteins

3-1: AgrB-I and AgrB-II

E. coli C43(DE3) cells transformed with the appropriate AgrB expression plasmids were grown at 37 °C in one liter of LB medium containing 50 μg/mL kanamycin. When the optical density at 600 nm (OD_{600}) reached 0.8, the medium was cooled down to 22°C and overnight expression was induced by addition of 0.4 mM IPTG. Cells were harvested at 6000g for 20 min. and the cell pellets were resuspended in 18 mL of lysis buffer (20 mM sodium phosphate, 100 mM NaCl and 1mM PMSF, pH 7.5). Cells were lysed by four passages through a French-press homogenizer. Cell-wall debris was spun down at 15000g for 10 min. and removed. Cell-membrane vesicles were then pelleted by ultracentrifugation at 200,000g for 1 h. The membrane fraction was extracted using 5 mL of buffer containing 20 mM phosphate pH 7.5, 100 mM NaCl, 2% (w/v) n-dodecyl-β-D-maltopyranoside (DDM) for 3 h at 4 °C. After another ultracentrifugation step at 100,000g for 20 min, supernatant was loaded to 2.5 mL of Talon cobalt resin. After incubation at 4 °C for 60 minutes, the resin was repacked in a 25-mL Bio-Rad disposable plastic column. The flow-through was discarded and the column was washed with 20 column volumes (CV) of wash buffer (20 mM sodium phosphate, 100 mM NaCl, 5mM β-mercaptoethanol, 0.1% (w/v) fos-choline-12 (FC-12) and 25 mM imidazole, pH 7.5). Bound protein was eluted with 3CV of elution buffer (wash buffer with 500 mM imidazole). The elution was concentrated to 1 mL and further purified on Superdex200 size-exclusion chromatography with running buffer (20 mM phosphate, 100 mM NaCl, 0.14% (w/v) FC-12, 1mM TECP, pH 7.0), from which the desired peak fractions were collected.

3-2: Non-tagged AgrD-I and AgrD-II

Both constructs were expressed as C-terminal GyrA-His_7 fusion proteins in *E. coli* BL21(DE3) cells. Transformed cells harboring the appropriate expression vector were grown at 37^{o}C to an OD_{600} = 0.6 and then induced by addition of 1.0mM IPTG. The culture was shaken at 37^{o}C for 4 additional hours post-induction and cells were collected through centrifugation. Cells were resuspended in PBS (20 mM Phosphate pH=7.5, 100 mM NaCl). PMSF (100 mM stock in ethanol) was added to the cell suspension immediately prior to lysis at a final concentration of 1 mM. Cells were disrupted by four

passages through a French-Press. The lysate was centrifuged at 30000g for 1hr and the pellet resuspended in a buffer containing 7.5 M GuHCl, 50 mM phosphate buffer, pH = 7.5 and 2 mM TCEP. The suspension was homogenized, gently shaken at 4^{o}C for 1hr and then spun at 30000g for 1hr. The supernatant was loaded to a column packed with Ni-NTA resin (Qiagen), and the resin was washed with buffer containing 25 mM imidazole and 6 M GuHCl and eluted using a buffer containing 300 mM imidazole, 7.2 M urea and 0.05% (w/v) DDM. The eluted protein was immediately chilled in an ice-water bath and refolded through dialysis against a series of phosphate buffer with decreasing urea concentrations at 4^{o}C. Upon dialysis, refolded AgrD-intein-His$_7$ proteins were cleaved by treatment with 50 mM DTT and 5 mM TCEP in the presence of 0.1% (w/v) DDM under an argon atmosphere at RT for 12 hrs. Free intein released from DTT treatment as well as uncleaved fusion protein was removed in a reverse-Ni-NTA affinity process. The combined flow-through and wash fractions containing predominantly AgrD-DTT esters were then supplemented by 0.1% FC-12. Residual urea in this solution was removed through two concentration-dilution cycles. In each cycle, the solution was concentrated to 10% of its original volume and then diluted back using a buffer containing 50 mM phosphate pH=7.5, 50 mM DTT and 0.07% FC-12. The solution after the second dilution was concentrated to <5mL, to which GuHCl was added to a give a final concentration of 6 M. EDTA was added at 10 mM and NaOH was used to basify the solution to pH=9.5. The solution was incubated under argon atmosphere at 37^{o}C for 4hrs for complete saponification of AgrD^{FL}-DTT esters, acidified to pH < 2 using neat TFA, filtered, and purified by semi-prep RP-HPLC using a C4 column.

3-3: FlagHis$_6$-AgrD-I and FlagHis$_6$-AgrD-II

Both constructs were expressed as C-terminal GST fusion proteins in *E. coli* BL21(DE3) host cells. The expression culture was grown at 37^{o}C to an OD_{600} = 0.6 and then was cooled down to 16°C prior to induction by addition of 0.4 mM IPTG. After overnight incubation, cells were isolated from the culture medium, resuspended in lysis buffer (20 mM sodium phosphate, 100 mM NaCl, pH 7.5), and lysed by four passages through a French-press homogenizer. After centrifugation at 30000g for 30min, the cleared lysate was supplemented with 0.1% (v/v) DDM and then incubated with GS4FF resin in a plastic column at 4 °C for 60 minutes. The lysate was then drained, and the resin was

washed by 20 CV of lysis buffer, resuspended in 2 CV of lysis buffer, and treated with 10 units of thrombin for AgrD-I or 0.2 mg/mL PreScission protease for AgrD-II at room temperature with gentle shaking for 3hrs. The released AgrD product was collected and allowed to flow through a 4-mL bed packed with Ni-NTA resin. The Ni-NTA bed was washed with 10 CV of lysis buffer with 15 mM imidazole, 10 CV of 6 M GuHCl buffered by Na-phosphate at pH = 7.5 and eluted with 3 CV of elution buffer (6 M Guanidinium chloride, 0.2 M acetic acid). Elution was treated with TCEP (5 mM, final concentration) and then purified over a semiprep-scale C18 RP-HPLC column.

3-4: FlagHis$_6$-AgrD-I(1-32)-thiolactone and its tri-Ala (F30A/I31A/M32A) mutant

Both constructs were expressed as C-terminal GyrA-CBD fusion proteins in *E. coli* BL21(DE3) host cells. Protein expression and lysis procedures were identical to that used for FlagHis$_6$-AgrD preparation. Cleared cell lysate was supplemented with 0.05% (w/v) DDM and subject to Ni-NTA purification. Upon flowing the lysate through an Ni-NTA column, the column was washed with 5 CV of wash buffer 1 (20 mM phosphate, 500 mM NaCl, 1 mM TECP 15 mM imidazole and 0.05% (w/v) DDM, pH 7.5) and 10 CV of wash buffer 2 (20 mM phosphate, 100 mM NaCl, 1 mM TECP 25 mM imidazole and 0.05% (w/v) DDM, pH 7.5). Bound protein was eluted with 4 CV of elution buffer (wash buffer 2 with 300 mM imidazole). The elution was dialyzed against a buffer containing (20 mM phosphate, 1 mM TECP and 0.01% (w/v) DDM, pH 7.5), and then cleaved after the addition of 100 mM MESNa and 5 mM TCEP. After an overnight incubation, a Ni-NTA affinity step was employed to remove the GyrA-CBD generated during cleavage. Upon washing with MESNa-free buffers, the bound FlagHis$_6$-AgrD-I(1-32)-MESNa thioester spontaneously cyclized into the thiolactone. All His-tag-containing constructs, primarily the thiolactone and the uncleaved fusion protein, were eluted in 100 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)-Na, 50mM EDTA, 1mM TECP and 0.1% SDS, pH = 7.0. Concentrated elution was submitted, in 1-mL aliquots, to Superdex200 SEC purification with running buffer (20 mM 2-(N-morpholino)ethanesulfonic acid (MES)-Na, 100 mM NaCl, 0.1%SDS, 5mM L-methionine, pH = 6.1). Fractions free from uncleaved fusion protein were pooled and dialyzed extensively against a buffer containing 0.1% (v/v) TFA and 5mM L-methionine

until precipitate emerged. GuHCl was added to a final molarity of 6 M, and the solution was filtered and purified on a semiprep-scale C18 RP-HPLC column.

3-5: FlagHis$_6$-AgrD-I(1-32)-Cys and FlagHis$_6$-AgrD-I(1-32)-NAC

FlagHis$_6$-AgrD-I(1-32)-thioactone (1 mg) was dissolved in 1mL 7.5 M GuHCl buffered at pH = 7.5 with sodium phosphate. To the solution was added 50 μL 1 M TCEP together with 100 μL 1 M L-cysteine or N-acetylcysteamine (NAC). The reactions reached maximal conversion after 1-hr incubation at RT as analyzed by analytical RP-HPLC (not shown). The products were then purified using semi-prep C18 RP-HPLC.

Section 4. Production and detection of AgrD^C-Ub-Flag$_2$ in *S. aureus*

4-1: Construction of *S. aureus* strains and western blotting analysis thereof

Shuttle plasmids for the *pblaZ*^{C}-driven co-expression of Sumo-AgrD^C-Ub-Flag$_2$ and His$_6$-Ulp1 were first electroporated into strain RN4220 (a restriction-deficient host) and then transduced to strain RN6390 (agr-I wild type) or RN7206 (RN6390 carrying an *agr* locus deletion) *via* phage 80 using standard protocols (5). Transductants were grown in CYGP medium containing 10 μg/mL erythromycin at 37^{o}C. Upon reaching the stationary phase, cells from a 1 mL culture were collected, washed twice with ice-cold water and finely resuspended in fresh CYGP medium on ice. 20 mL CYGP medium containing 10 ug/mL erythromycin was inoculated with 40 μL cell suspension (c.a. 2x10^8 cfu) and then shaken at 250 rpm at 30^{o}C. Cell density was measured every 30 min. For the accuracy of OD_{600} measurement, cell cultures at OD_{600} above 0.60 was diluted using fresh CYGP medium to give an OD_{600} of between 0.20 and 0.60 prior to measurement, and the readout was then corrected for the dilution.

For the detection of AgrD^C-Ub-Flag$_2$ and AgrD-Ub-Flag$_2$ using anti-Flag western blot, 1 mL cell culture was sampled when the culture OD_{600} reached 0.50, 1.00, 1.50 and 2.00. Cells were pelleted and resuspended in lysis buffer (10mM Tris-HCl 8.0, 1mM TCEP, 100μM $ZnCl_2$, supplemented with 50 μg/mL lysostaphin and 1X protease inhibitor cocktail). The volume of suspension was proportional to the cell density of the original sample (50 μL per 1.0 OD_{600}). The suspension was incubated at 37^{o}C for 30 min, mixed with appropriate amount of 3X SDS-PAGE loading dye and boiled at 95^{o}C for 10min.

Samples were then resolved on 15% Tris-HCl SDS-PAGE. To avoid interference of western blotting by endogenous protein A, the PAGE gel was cut at the level of the 37.5kDa standard band and the upper half was stained with Coomassie blue for loading control. Proteins in the lower half of the gel were transferred to a PVDF membrane and blotted with Flag-specific mouse monoclonal antibody (Sigma-Aldrich) using standard protocols. After blotting the membrane with goat anti-mouse IgG HRP conjugate (Bio-Rad), bands were visualized through chemiluminescence.

4-2: β-lactamase reporter gene assay

Extracellular AIP-I activity in plasmid-transformed RN6390 culture was determined using a reporter gene assay (6). Briefly, RN9222 (an *agr*-I reporter strain harboring a β-lactamase gene driven by a P3 promoter) was grown in CYGP medium at 37°C to OD_{600} = 0.60. A 50-μL aliquot of the culture was mixed with 50μL fresh CYGP (for background) or cell-free medium isolated from RN6390 samples. The mixture was cultured at 37°C for 1hr and cell density (OD_{600}) was measured. Then, 50 μL of the mixture was transferred to a 96-well plate and mixed with 50 μL 200 μM nitrocephin in assay buffer (100 mM phosphate-Na pH = 5.9, 27% (v/v) propylene glycol, 5 mM sodium azide). Progress of the substrate hydrolysis was monitored at 37°C in a plate reader. The slope of the linear increase of absorbance at 490 nm during the initial period of the reaction was designated as the β-lactamase activity in (0.001 absorbance unit/min). The final result was background-subtracted and normalized by the pre-reaction cell density of RN9222.

Section 5. Solid-phase peptide synthesis

5-1: AIP-I and the tri-Ala (F6A/I7A/M8A) mutant

Wild-type and tri-Ala mutant AIPs were synthesized using Boc-based solid phase peptide chemistry. The 3-mercaptopropionic acid linker was coupled to MBHA resin as is described previously (7). Chain assembly was carried out using a 5-fold excess of standard N^{α}-Boc protected amino acids with HBTU (4.9 eq.) activation over the resin in DMF with DIEA (8 eq.). The Boc protecting group was removed with TFA. Thioesters were cleaved from the resin with HF using *p*-cresol as a scavenger. Crude peptide products were precipitated and washed with cold diethyl ether, dissolved in 50% HPLC

solvent B and lyophilized. Cyclization of the thioesters was performed in cyclization buffer (100mM HEPES, 22.5% CH_3CN, pH 7.3) at room temperature for 2 hours. The thiolactone products were purified by RP-HPLC.

5-2. $AgrD^C$-I-NH_2:

The peptide was synthesized using Fmoc-based solid phase peptide chemistry on a Rink-amide MBHA resin (0.59 mmol/g). Synthesis was carried on a Liberty Synthesizer. Chain assembly was carried out with HBTU and HOBT (4.9 eq.) activation using a 5-fold excess of standard N^{α}-Fmoc protected amino acid over the resin in DMF with DIEA (8 eq.). The Fmoc protecting group was removed with 20% piperidine in DMF. Peptides were cleaved from the resin with a cocktail containing 95% TFA (v/v), 2.5% TIS (v/v) and 2.5% water (v/v). Crude peptide products were precipitated and washed with cold diethyl ether, dissolved in HPLC solvent A and then purified by RP-HPLC.

Section 6. Glutaraldehyde crosslinking of AgrB-II

Crosslinking was performed on SEC-purified AgrB-II protein solubilized by FC-12. The protein was diluted in PBST buffer (20 mM sodium phosphate, 100 mM NaCl and 2 mM TCEP pH 7.5) supplemented with 0.14% FC-12 to 2.5 μM. 20 mM glutaraldehyde was prepared in 100 mM phosphate buffer (pH = 7.5) immediately prior to use. The crosslinking was performed at room temperature. 90μL of protein and 10μL crosslinker were mixed at t = 0, and 20μL aliquots were withdrawn at indicated time points and quenched by mixing with 2μL 1M Tris buffered at pH=8.7. The t = 0 control was prepared by adding first 2μL 1M Tris pH=8.7 and then 2μL crosslinker to 18μL protein stock. All quenched reactions were mixed with 4X SDS sample buffer and resolved on 4-20% TGX SDS-PAGE.

Section 7. Reconstitution of the AgrB peptidase and activity assays

7-1: Reconstitution of AgrB-II to nanodiscs

The membrane scaffold protein, MSP1E3D1, was prepared as reported (8). The His_7-tag on this recombinant protein was removed by TEV-protease digestion followed by anion exchange chromatography with a Hiprep Q FF 16/10 column (GE Healthcare). DLPC (1,2-*di*l*l*auryl-sn-*g*lycero-3-*p*hosphocholine) and DLPG (1,2-*di*l*l*auryl-sn-glycero-3-

*p*hospho-(1'-rac-*g*lycerol)) were dried from organic solvents and prepared in 50 mM-stocks solubilized in a buffer containing 20 mM Tris pH = 8.0, 100 mM NaCl, 5 mM TCEP and 100 mM sodium cholate. Lipid stocks were mixed such that the molar ratio between DLPC and DLPG was 1:3. The optimal lipid-MSPE3D1 ratio for empty disc assembly was found to be 200:1, while incorporation of each AgrB-II subunit was expected to displace 30 lipid molecules. Pre-assembly mixtures were prepared with appropriate AgrB-II-to-disc ratios. Upon detergent removal using SM-2 Adsorbent Bio-Beads (Bio-Rad), the post-assembly mixture was subjected to Ni-NTA affinity purification. Elutions containing AgrB-II-incorporated nanodiscs were resolved on Superose6 size exclusion chromatography using nanodisc buffer (20 mM HEPES, 100 mM NaCl, 1 mM TCEP, pH 7.0) from which peak fractions were pooled as product.

7-2: SEC-MALS analysis of nanodisc particle size and protein content

Nanodisc samples were resolved on a Superose6 size-exclusion column equilibrated with the nanodisc buffer. The eluate was directed to a DAWN HELEOS-II multi-angle light scattering instrument in tandem with OptiLab TrEX differential refractometer for real-time analysis. Flow-cells of all instruments were set at 4^{o}C. Fitting was accomplished using the "protein conjugate" method in the ASTRA 6.0 software package, which calculates both the averaged particle size and the protein content utilizing UV, MALS and dRI signals simultaneously (9). Input for refractive index increment, dn/dc, was 0.185 mL/g for protein and 0.135 mL/g for the modifier (treating lipids as the modifier). Input for extinction coefficient of protein was 0.898 for empty discs (corresponding to MSPE3D1) and 0.840 as in an equimolar mixture of MSP1E3D1 and AgrB-II for AgrB-II discs, both in the unit of mL/(mg·cm).

7-3: Proteolysis assays using AgrB-II nanodiscs

SEC-purified AgrB-II nanodiscs were diluted in PBST such that each nanodisc sample contains 2 μM AgrB-II (counted as monomers). FlagHis$_6$-AgrD-II was dissolved in DMSO at 0.5 mM, from which 1 μL was mixed with 25μL AgrB-II nanodisc sample. Reactions were incubated at 37^{o}C for 2hr before mixing with 4 X SDS sample buffer and resolved on Criterion 16.5% Tris-Tricine SDS-polyacrylamide gel. The gel was stained with Coomassie blue.

7-4: Lipid stocks and AgrB-I proteoliposome assembly

A 3:1 mixture of POPC (1-*p*almitoyl-2-*o*leoyl-sn-*g*lycero-3-*p*hosphocholine) and POPG (1-*p*almitoyl-2-*o*leoyl-sn-glycero-3-*p*hospho-(1'-rac-*g*lycerol)) was dried from organic solvents and then hydrated with PBST buffer containing 20 mM sodium phosphate, 100mM NaCl and 2mM TCEP pH 7.5. The liposome suspension (1.0% w/v) was stored at -20^{o}C, and thawed and ultrasound-treated in a sonicator water bath immediately prior to use.

For proteoliposome reconstitution, liposome suspension was mixed with an equal volume of 3.0% (w/v) 7-cyclohexylheptyl-β-D-maltopyranoside (Cymal-7) in PBST buffer and incubated at RT until the mixture turns clear. A typical reconstitution reaction was started by mixing 400 μL Cyaml-7-solubilized lipid with 200 μL of a 15 μM solution of SEC-purified AgrB-I-His$_{6}$. The mixture was incubated with 250 mg of Bio-Beads SM2 at RT with gentle shaking for 1hr. The cloudy suspension was withdrawn from Bio-Beads and the proteolipsomes were spun down at 17000g at 4^{o}C for 30min. Upon removal of supernatant, the proteoliposomes were washed with 2 x 200 μL PBST buffer, re-suspended in 450μL PBST buffer and gently sonicated before being used for biochemical assays.

7-5: Proteolysis/hydrolysis assays using AgrB-I proteoliposomes

For a typical 100 μL reaction, to 45 μL PBST buffer was added 5μL of a 400 μM solution of AgrD-I or FlagHis$_{6}$-AgrD(1-32)-thiolactone substrate in DMSO. At t = 0, 50 μL AgrB-I proteoliposomes (containing 2 μM AgrB-I monomer as quantified by SDS-PAGE) were added with rigorous mixing and the reaction tube was immediately transferred into a 37^{o}C water bath. 20μL aliquots were withdrawn at indicated time points and mixed with 4 X SDS sample buffer before resolved on 16.5% Tris-Tricine SDS-PAGE. For HPLC-MS analysis, the reaction could be linearly scaled up. At the indicated time points, 100 μL of the reconstitution system was mixed with 400 μL of a solution containing 7.5 M GuHCl, 0.1 M TFA and then subject to Sep-Pak solid phase extraction (see section **7-7**). The Sep-Pak eluate was lyophilized, dissolved in the GuHCl-TFA solution and resolved on a C_{4} (for non-tagged constructs) or C_{18} (for FlagHis$_{6}$-tagged

constructs) analytical RP-HPLC column. For proteolysis reactions, a linear gradient with increasing buffer B percentage from 0 to 90% between t = 5 min and 35 min was used. For hydrolysis reactions, a two-segment gradient was employed with buffer B percentage increasing from 0 to 50% in the first 5 min and from 50 to 90% between t = 5 min and 25 min. Peaks with significant absorbance at 214 nm were collected for ESI-MS analysis.

7-6: Ligation assays using AgrB-I proteoliposomes

Immediately prior to ligation assays, AgrB-I proteolipsomes were spun down and re-suspended in PBST buffer containing AgrD-I^{C}-NH_2 peptide at 2X final concentration. This suspension was mixed with PBST buffer and 400 μM DMSO stock of $FlagHis_6$-AgrD-I(1-32)-thiolactone at a volume ratio of 50:45:5 to initiate the reaction. At indicated time points, 100 μL aliquots from the reaction were acidified, Sep-Pak-processed and analyzed using RP-HPLC-MS as described in the previous section.

7-7: Sep-Pak solid phase extraction

The tC2 Sep-Pak cartridge (100 mg adsorbant, Waters) was activated with 3 x 1 mL of HPLC solvent B and then equilibrated with 2 x 1 mL of HPLC solvent A. Samples acidified with the GuHCl-TFA solution (see section **7-5**) were then load on to the cartridge. The cartridge was subsequently washed with 2 x 1 mL of HPLC solvent A and bound protein was eluted consecutively with 0.5 mL 50% (v/v) acetonitrile in water, 0.5 mL 67% (v/v) acetonitrile in water and 1 mL of 75% (v/v) acetonitrile in water. All solvents used for elution were buffered by 10 mM triethylammonium-acetate at pH = 8.0. The combined eluate was lyophilized.

Section 8. Ring-opening of $FlagHis_6$-AgrD-I(1-32)-thiolactone and AIP-I

A 3 M aqueous solution of N-acetylcysteamine (NAC) was prepared and buffered at pH = 7.0 using 100mM HEPES-Na. For each reaction, a 10X NAC solution was prepared from this concentrated stock through serial dilution using 100 mM HEPES-Na pH = 7.0. The following three buffers were used in the reaction:

HBST buffer: 100 mM HEPES-Na, 100 mM NaCl, 2 mM TCEP, pH=7.0;

Liposome buffer: 1 volume of the 1% (w/v) liposomal suspension diluted in 4 volumes of HBST buffer;

GuHCl buffer: 100 mM HEPES-Na, 7.5 M GuHCl, 2 mM TCEP, pH=7.0;

Each reaction was initiated by combining 50 μL 1M TCEP-Na (buffered at pH = 7.0), 100μL 10X NAC solution and 50 μL of a 400 μM DMSO stock of $FlagHis_6$-AgrD-I(1-32)-thiolactone or AIP-I with 800 μL of one of the above three buffers. For reactions performed in HBST or GuHCl buffers, aliquots (100 μL for AIP-I or 20 μL for $FlagHis_6$-AgrD-I(1-32)-thiolactone) were removed and analyzed by C_{18} analytical RP-HPLC at indicated time points. For reactions containing liposomes, aliquots were added to 0.5 mL 0.1 M aqueous TFA and freeze-dried. The lyophilized sample was triturated with 150 μL GuHCl-TFA solution. The extract was then cleared through centrifugation and 100 μL of the supernatant was analyzed on RP-HPLC. Ring-opening reactions of the tri-Ala mutants of $FlagHis_6$-AgrD-I(1-32)-thiolactone and AIP-I were initiated, processed and analyzed using the same procedures.

Analytical HPLC columns and gradients:

Samples from reactions involving AIP-I: solvent B percentage 20% to 50% over 15min;

Samples from reactions involving tri-Ala mutant of AIP-I: solvent B percentage 0% to 30% with 15min;

Samples from reactions involving wild-type or tri-Ala mutant of $FlagHis_6$-AgrD-I(1-32)-thiolactone: two-segment gradient such that solvent B percentage increases from 0% to 50% in 5 min and then to 80% in 15min.

Section 9. Data processing

9-1: $\Delta G^{0\prime}$ estimation of NAC thiolysis of a generic peptide bond (see also Fig. 1B)

NAC thiolysis of a generic peptide bond can be accomplished in a two-step process involving first the hydrolysis of the scissile peptide bond and subsequent thioesterification of the N-fragment by NAC. For the first step we used the hydrolysis of a Gly-Gly bond; $\Delta G^{0\prime}$ = 2.2kcal/mol. For the second step, the $\Delta G^{0\prime}$ for thioesterification of acetic acid by coenzyme A (CoA), i.e., 7.5 kcal/mol, needs to be adjusted for the difference in acidity between the C-terminal carboxylic group of a generic peptide (pK_a = 3.3) and acetic acid (pK_a = 4.8) (10). pK_a value of acids have been found to linearly correlate with the pK_{eq} (negative logarithm of the equilibrium constant) of the hydrolysis of their methyl esters into their deprotonated anions and methanol (11). The slope is 1.02. Assuming this linear relationship holds for thioesters, thioesterification of a C-terminal

carboxylate on a peptide should be 2.1 kcal/mol less favorable than that of acetate (12). Therefore, the estimated overall $\Delta G_1^{0'}$ for the thiolysis of a generic peptide bond should be:

$\Delta G_1^{0'}$ = -2.2 + 7.5 + 2.1 = 7.4 kcal/mol.

9-2: $\Delta G^{0'}$ estimation of the cyclization of a mercapto-thioester through trans-thioesterification (see also Fig. 1B)

$\Delta G^{0'}$ of any ring closure reactions is strongly dependent on the ring being formed. As a moderate estimation, we assume that the ring formed upon trans-thioesterification to be a typical large ring such that it is neither strongly stabilized, like a five- or six-membered ring, nor destabilized like those in mid-sized rings. We also assume that the trans-thioesterification is isoenthalpic, primarily because the pK_a of the thiol group in a cysteine side chain is close to that in NAC.

We calculate the $\Delta G_2^{0'}$ of the cyclization from an estimated equilibrium constant K_{eq}, which equals the apparent first-order kinetic constant of the forward reaction divided by the apparent second-order kinetic constant of the reverse (thiolysis) reaction. The denominator should be equivalent to the secondary kinetic constant of a thiol-exchange process involving a linear peptidyl-NAC thioester, in that the rotational movements around the bonds adjacent to the thioester motif are not restrained in the large ring. The equilibrium constant therefore equals to the ratio between the first-order kinetic constant of the intramolecular (cyclization) reaction and the second kinetic constant of an analogous intermolecular (thiol-exchange) reaction between a thioester and a thiol group. This ratio, by definition, represents the effective molarity (EM) of the cyclization. Empirically, the EM of large, unstrained ring formation from bifunctional chain molecules falls predominantly within the range 0.01M - 0.05M independent of the functional groups involved, as was summarized by Illuminati and Mandonlini (reference 15 in the main text). We therefore used the mid-point of the range, i.e., K_{eq} = 0.024 to calculate the $\Delta G_2^{0'}$ in our model.

9-3: Quantification of SDS-PAGE bands

The gel was stained with Coomassie brilliant blue and imaged on a LI-COR Odyssey Infrared Imager and the intensity of each band was calculated using the built-in densitometry software.

9-4: Quantification of all species in the proteolysis reaction from HPLC analysis

The proteolysis reaction begins with 20 μM pure FlagHis$_6$-AgrD-I. This AgrD-I construct has two tyrosines, both located in the N-fragment that results from AgrB-I cleavage. Therefore, the full-length AgrD-I, the linear and the thiolactone N-fragment should have identical extinction coefficients at 280 nm. Peak areas of these species in the 280-nm HPLC chromatogram are hence proportional to their respective molarities. The molar fraction of each species (out of the sum of three) was calculated accordingly after factoring in their respective Sep-Pak recovery. The molarity of AgrD-I^C is given by the sum of the molarity of both linear and thiolactone N-fragments as a consequence of mass balance.

9-5: Quantification of the ligation reaction based on HPLC-MS analysis

The ligation reaction starts with 20 μM FlagHis$_6$-AgrD-I(1-32)-thiolactone and a variable concentration of AgrD-I^C-NH_2. The starting thiolactone overlaps, on the RP-HPLC chromatogram, with the ligation product, but is separable from the linear FlagHis$_6$-AgrD-I(1-32). Again, due to the lack of a chromogenic residue in the AgrD-I^C segment, the extinction coefficient at 280 nm is expected to be the same for the starting thiolactone, the linear AgrD(1-32) and the ligation product AgrD. Accordingly, the linear AgrD(1-32) was quantified based on RP-HPLC peak areas as described in the previous section. The molar ratio between the starting thiolactone and the ligation product was determined using MS. Briefly, MS spectra were recorded for the eluate containing AgrD and both AgrD(1-32) fragments and the profile between m/z = 600 and 2000 was de-convoluted using the "maximum entropy" method (13). Target MS range and instrument resolution were set at 3000-8000 Da and 10000, respectively. The peaks corresponding to the starting thiolactone (5694.2Da) and ligation product (7359.2Da) in the de-convoluted profile were integrated. The molar ratio between the two species was calculated from

their MS intensity after factoring in a coeffecient that accounts for their difference in ionization efficiency (see section **9-6**).

9-6: Relative MS ionization efficiency of FlagHis$_6$-AgrD-I with respect to FlagHis$_6$-AgrD-I(1-32)-thiolactone, see also Fig. S4C-S4E

This procedure was used to empirically determine a coeffecient, *k*, that converts the MS peak intensity ratio between the starting thiolactone (**TL**) and the full-length ligation product (**FL**) ($I_{\mathbf{TL}}/I_{\mathbf{FL}}$) into the molarity ratio ($c_{\mathbf{TL}}/c_{\mathbf{FL}}$).

$$k\frac{I_{\mathrm{TL}}}{I_{\mathrm{FL}}}=\frac{c_{\mathrm{TL}}}{c_{\mathrm{FL}}} \qquad \text{(equation S1)}$$

For simplicity, we prepared sample B containing pure **TL** and sample A as a mixture containing predominantly **FL**. Based on their peak areas on the 280-nm RP-HPLC chromatogram, both samples were diluted to the same molarity. Assuming the molar fraction of **FL** in sample A to be x (x<1), the **TL**-to-**FL** molarity ratio in a A-B mixture is given by equation S2:

$$\frac{c_{\mathrm{TL}}}{c_{\mathrm{FL}}}=\frac{V_{\mathrm{B}}+(1-x)V_{\mathrm{A}}}{V_{\mathrm{A}}x} \qquad \text{(equation S2)}$$

where $V_{\mathbf{A}}$ and $V_{\mathbf{B}}$ are the volume of A and B in the mixture. Equations S1 and S2 could be transformed into:

$$\frac{I_{\mathrm{TL}}}{I_{\mathrm{FL}}}=\frac{1}{kx}\frac{V_{\mathrm{B}}+V_{\mathrm{A}}}{V_{\mathrm{A}}}-\frac{1}{k} \qquad \text{(equation S3)}$$

Therefore, after plotting the MS intensity ratio between **TL** and **FL** versus the overall volume of the A-B mixture divided by the participating sample A volume, linear regression of the plot returns *k* as the inverse reciprocal of the y-intercept.

9-7. Quantification of the thioester/thiolactone in the ring-opening assays

Molar ratios of thioester versus thiolactone were quantified based on integration of 280-nm RP-HPLC chromatograms, as is described in section **9-4**.

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