SUPPLEMENTAL INFORMATION

Functional Properties of N-Terminal ArgD Peptides from the Classical *Staphylococcus aureus* Agr System

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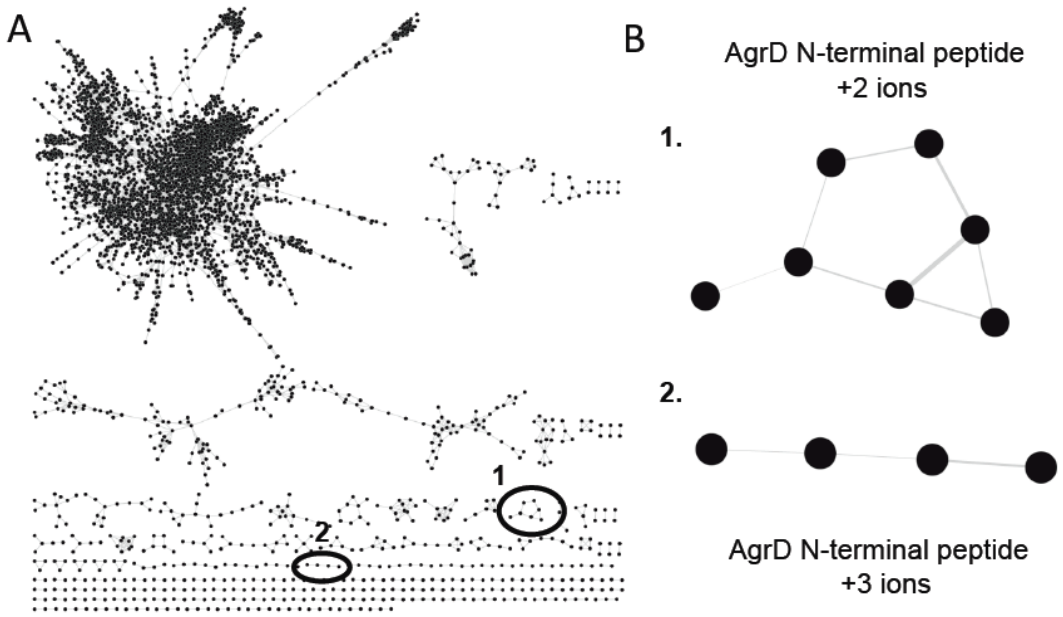
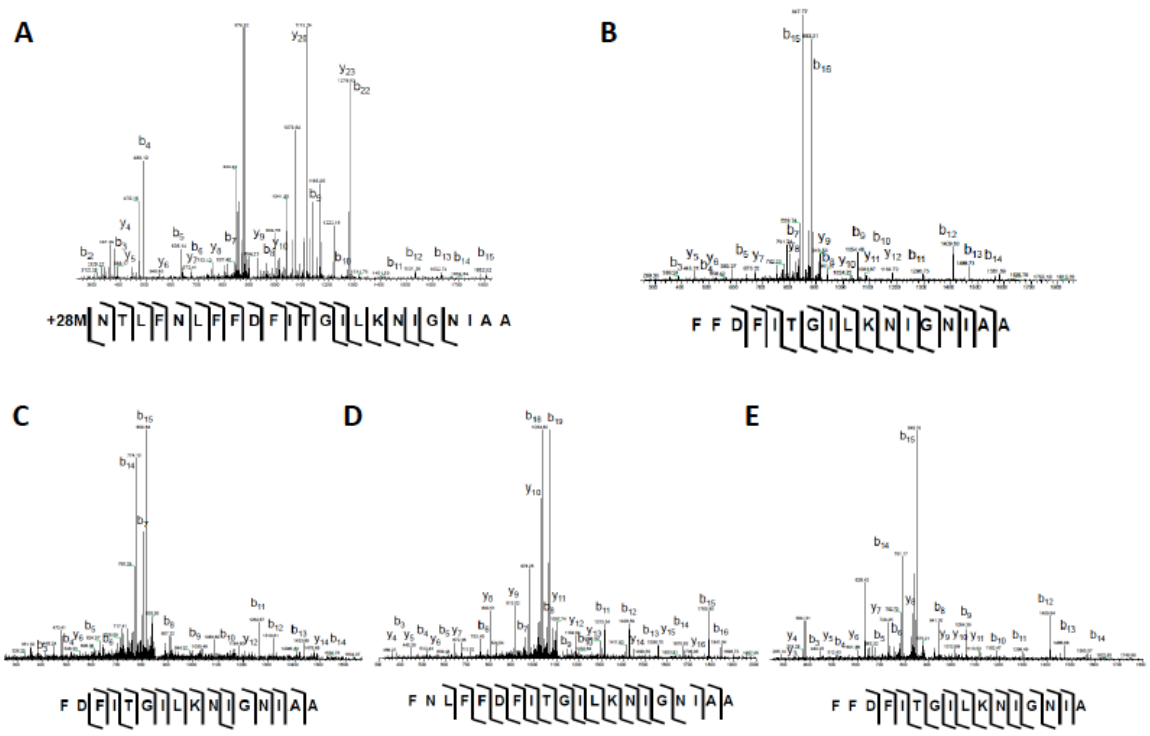
Staphylococcus aureus Strain	Agr Type	Predicted Clonal Colony Type	AgrD MS ID
Clinical Isolate 03	1	8	Yes
Clinical Isolate 08	1	8	Yes
Clinical Isolate 42	1	8	No
Clinical Isolate 44	1	45	No
Clinical Isolate 61	1	59	Yes
Clinical Isolate 79	1	45	Yes
JH1	2	5	No
RN9120 (Agr null)	2	na	No
Mu50	na	5	No
Sanger 252	3	36	No
Clinical Isolate 59	1	59	Yes
TCH1516	1	8	Yes

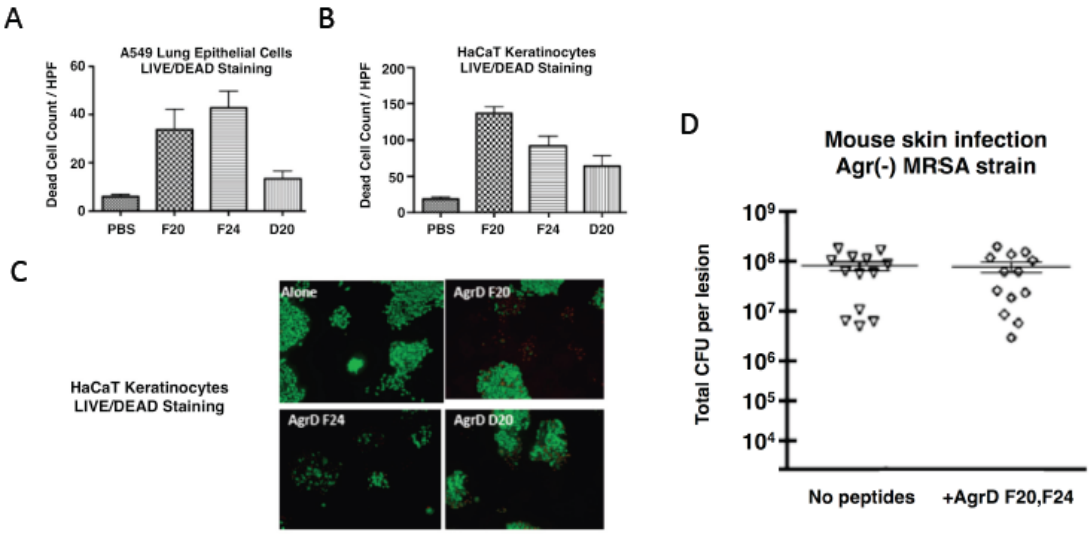
Supplemental Table S1, related to Figure 1. *Staphylococcus aureus* strains in AgrD peptide screen. A search for *Staphylococcus aureus* strains that released detectable quantities of N-terminal AgrD peptides was expanded to 12 isolates including several clinically derived strains. Among these 12 isolates AgrD peptides were detectable in five strains in three USA300 lineage MRSA, TCH1516 and two designated sequence type (ST) 8, a ST59 MRSA or commonly known as the Taiwan clone, and clonal colony ST45 of USA600 MRSA lineage.



Supplemental Figure S1, related to Figure 1. *S. aureus* TCH1516 molecular network map. **(a)** Complete Molecular Network map constructed from *Staphylococcus aureus* USA300 strain TCH1516 peptides and small molecules. Media components and mass spectrometry solvent-derived ions were subtracted from the network. **(b)** Cluster 1 shows a node cluster identified through sequencing to originate from the N-terminal region of the AgrD peptide substrate. Cluster 1 was composed of +2 charged ions. It was also determined that Cluster 2 originated from the N-terminal region of AgrD and was composed of +3 ion pairs.



Supplemental Figure S2, related to Figure 1. Tandem MS for identified AgrD N-terminal variants. The parent ion for each displayed peptide in was isolated within the mass spectrometer and fragmented using collision-induced dissociation. Ions corresponding to fragmentations at the peptide bond are indicated as b (N-terminal coverage) or y (C-terminal coverage) and so labeled. **A.** Tandem mass spectrum for the peptide formylated AgrD F24. **B.** Tandem mass spectrum for non-formylated AgrD D17. **C.** Tandem mass spectrum for the peptide non-formylated AgrD D16. **D.** Tandem mass spectrum for the peptide non-formylated AgrD D20. **E.** Tandem mass spectrum for the peptide non-formylated AgrD D16B.



Supplemental Figure S3, related to Figure 3. Cytotoxicity, hemolysis and murine skin lesion colony forming unit counts. A549 and HaCaT LIVE/DEAD cell counts after incubation with the AgrD peptides. **(a)** A549 cells were incubated with 10 µg/µl of the indicated AgrD peptides. Thereafter, cells were stained using the Invitrogen LIVE/DEAD cell viability kit and counted under microscopy through four random fields of view. **(b)** HaCaT cells were incubated with 10 µg/µl of the indicated AgrD peptides. Thereafter, cells were stained with the Invitrogen LIVE/DEAD cell viability kit and counted under microscopy through four random fields of view. **(c)** HaCaT cells stained with LIVE/DEAD fluorescent dyes. Red dye is indicative of damaged cells and green stain is indicative of viable cells. A larger number of non-viable cells were observed post-incubation with the AgrD peptides compared to the untreated control. **(d)** Addition of AgrD F20 and AgrD F24 peptides do not alter *Staphylococcus aureus* survival in a skin lesion model. Subcutaneous infection of C57Bl/6 mice with an *S. aureus agr* null strain was tested +/- addition of a 1:1 mixture of AgrD F20 and AgrD F24 (10 µg/ml) on contralateral flanks; each mouse served as its own control. Statistical analysis was performed by Student's T-test; nonsignificant. This contrasts with increased lesion size associated with AgrD peptide treatment.

Supplemental Materials and Methods

Neutrophil chemotaxis

Human neutrophils were isolated by Polymorph Prep kit per manufacturer's instructions. Neutrophil migration was quantified by a previously reported transwell assay[1]. Briefly, either fMLP (Sigma Aldrich, MO) or formylated peptides in HBSS were placed in the lower wells (650 µl total volume, 385 nM final peptide concentration) of a 24-well Transwell plate with 3 µm pore size (Corning). A total of 1 x 10^7 cells in 150 µl of Hank's buffered saline solution (HBSS) were placed in the upper chambers, and plates incubated at 37˚C for 30 min with 5% CO2 to allow cell migration. Cells entering the lower chambers were lysed by adding 130 µl 0.5% Triton X-100 (pH 7.4) for 10 min. Elastase activity, a measure of relative cell migration to the lower wells, was quantified by adding 1 mM (final concentration) p-nitroanilide (Sigma Aldrich, MO) for 30 min and absorbance at 405 nm using a SpectraMax M3 plate reader (Molecular Devices, CA).

Neutrophil calcium flux

Calcium fluxes in human neutrophil were measured as described [2], with minor modifications. Briefly, neutrophils were incubated with 2 µM Fluo-3 in PBS at 37˚C for 30 min. After a 5 min spin at 300 g, cells were resuspended in PBS at 1 x 10^6 cells/ml. Prior to the assay, cell preparations were either left untreated or pre-incubated with 10 µM final concentration of BOC-MLF or PBP10 (both obtained from Tocris, UK), antagonists of FPR1 and FPR2, respectively. Samples (500 µl) were transferred to 5 ml polystyrene round bottom tubes and analyzed by flow cytometry before and after addition

of formylated peptides (385 nM final concentration) while vortexing. A total of 3000 events were recorded, and resulting data analyzed by gating for the neutrophil population and measuring geometric mean fluorescence intensity (gMFI) using FloJo (Tree Star, OR) software. Data are expressed as gMFI relative to untreated cells.

Neutrophil extracellular traps (NETs)

Neutrophils were added at 2 x 105 cells/well in RPMI media a 48-well tissue culture plate. Phorbol 12-myristate 13-acetate (PMA) stimulation (25 nM) was used as a positive control. AgrD peptides were incubated for 180 min at 10 μg/ml. Thereafter, 500 mU of micrococcal nuclease were added for 10 min and the reaction halted with 5 mM EDTA. The plate was centrifuged and supernatants analyzed by the Quant-iT Picogreen (Invitrogen) assay per manufacturer's instructions. For microscopic confirmation, neutrophils were placed on poly-L-lysine-coated glass slides, treated with AgrD peptides or vehicle controls, and NETs visualized using a rabbit antimyeloperoxidase antibody followed by a secondary goat anti-rabbit Alexa 488 antibody; samples were embedded in 4,6-diamidino-2-phenylindole (DAPI) to counterstain DNA in blue. Mounted samples were examined using an inverted confocal laser-scanning two-photon microscope, Olympus Fluoview FV1000.

AgrD peptide cytotoxicity assays

A549 adenocarcinomic human alveolar epithelial cells or HaCaT keratinocytes were maintained in a 5% CO2, water-saturated atmosphere at 37°C in DMEM (Gibco). Cells were grown to 70–90% confluence in 24-well microtiter plates, washed and treated with

10 μg/mL of AgrD F20, AgrD F24 and AgrD D20 for 4 h. Culture supernatants were prepared for the Promega CytoTox 96 non-radioactive cytotoxicity assay kit per manufacturer's instructions and absorbance at 490 nm recorded.

AgrD peptide hemolysis assay

Defibrinated sheep blood or human blood was washed in PBS and diluted to a final concentration of 1:25 (v/v). Washed blood was placed in individual wells of a flat-bottom 96-well microtiter plate (Costar, Corning). AgrD peptides at 10 μg/ml were added directly to the wells, and the mixture was incubated for 4 h at 37°C. After incubation, plates were centrifuged at 500 x *g* for 5 min, and an aliquot of supernatant placed in a separate microtiter plate to measure hemoglobin absorbance at 450 nm on a microplate reader.

AgrD murine skin infection model

Permission to undertake experiments was obtained from the Animal Subject Ethics Committee of the University of California, San Diego. Flanks of 9 week old female C57 mice (n = 12) were shaved and naired. The following day, mice were anaesthetized with ketamine/xylazine and contralateral flanks injected with 1 x 10^8 CFU of S. aureus RN691 (agr I null) strain in 100 μl saline in the presence or absence of a 1:1 mixture of AgrD F20 and AgrD F24100 (10 μg/mL) – each mouse serving as its own control. After 3 days, lesions were harvested using sterile surgical scissors/forceps at the lesion margin; if no lesion was present, the area at the site of injection was excised. The program ImageJ was used to calculate lesion area and expressed in millimeters squared. Statistical

analysis of the lesion areas was performed by paired T-test using the Prism software. Tissue was homogenized using a BeadBeater (Biospec) and serial dilutions plated on THB agar for enumeration of colony forming units and calculation of bacterial burden (Supplemental Figure 4).

References

1. Corriden, R. et al. Ecto-nucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1/CD39) regulates neutrophil chemotaxis by hydrolyzing released ATP to adenosine. *J Biol Chem* **283**, 28480-6 (2008).
2. Veldkamp, K.E., Heezius, H.C., Verhoef, J., van Strijp, J.A. & van Kessel, K.P. Modulation of neutrophil chemokine receptors by *Staphylococcus aureus* supernate. *Infect Immun* **68**, 5908-13 (2000).