Inhibition of Colony-spreadign Activity of *Staphylococcus aureus* by Secretion of δ-Hemolysin

Yosuke Omae, Kazuhisa Sekimizu, and Chikara Kaito

From the Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, University of Tokyo, 3-1, 7-Chome, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

**Background:** *Staphylococcus aureus* spreads on soft agar surfaces, a phenomenon called “colony spreading.” Here, we found that *S. aureus* culture supernatant inhibited colony spreading. We purified δ-hemolysin (Hld, δ-toxin), a major protein secreted from *S. aureus*, as a compound that inhibits colony spreading. The culture supernatants of *hld*-disrupted mutants had 30-fold lower colony-spreadign inhibitory activity than those of the parent strain. Furthermore, *hld*-disrupted mutants had higher colony-spreadign ability than the parent strain. These results suggest that *S. aureus* negatively regulates colony spreading by secreting δ-hemolysin.

**Results:** We purified δ-hemolysin from *S. aureus* culture supernatant as an inhibitor of colony spreading, and its disrupted mutant had high colony-spreadign ability.

**Conclusion:** *S. aureus* negatively regulates colony spreading by secreting δ-hemolysin.

**Significance:** This is the first example of an endogenous molecule that inhibits bacterial self-motiliy.

---

Hospital-associated methicillin-resistant *S. aureus* (HAMSRA)² strains have lower colony-spreadign ability than community-acquired MRSA (CA-MRSA) strains that cause severe diseases (10). The correlation between high colony-spreadign activity and high virulence of *S. aureus* strains suggests that colony spreading has a role in the infectious processes of *S. aureus* (10). The difference in colony-spreadign activity and virulence between HA-MRSA and CA-MRSA is attributed to the virulence regulatory RNA harbored specifically in the SCC-mec region of the HA-MRSA strain (11). Determining the regulatory mechanism of *S. aureus* colony spreading is important for understanding the virulence properties of *S. aureus*. In this study, we searched for a factor secreted from *S. aureus* that modulates colony spreading, and we identified δ-hemolysin as a compound that inhibits colony spreading. Our results demonstrate a novel function of δ-hemolysin as a negative regulator of colony spreading besides the hemolytic activity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The JM109 strain of *Escherichia coli* was used as a host for pUC119, pINT, pND50K, and their derivatives. *E. coli* strains transformed with the plasmids were cultured at 37 °C in Luria-Bertani broth containing 100 µg/ml ampicillin, 25 µg/ml chloramphenicol, or 50 µg/ml kanamycin. *S. aureus* strains were aerobically cultured in tryptic soy broth at 37 °C, and 12.5 µg/ml chloramphenicol, 50 µg/ml kanamycin, or 5 µg/ml tetracycline was added if required. The details of the bacterial strains and plasmids used in this study are shown in Table 1.

**Reagent**—Chemically synthesized δ-hemolysin (Hld) with purity greater than 95% was obtained from ANASPEC (Fremont, CA). Chemically synthesized Hld-M with 90% purity was obtained from Operon Biotechnologies (Tokyo, Japan).

**Colony-spreadign Assay**—Tryptic soy broth (BD Biosciences) supplemented with 0.24% agar (Nacalai Tesque Inc., Kyoto, Japan) was autoclaved. Sterile medium (50 ml) was poured onto a plate (150 mm diameter, FALCON 351058, BD Labware,

---

2 The abbreviations used are: HA-MRSA, hospital-associated methicillin-resistan *S. aureus*; CA-MRSA, community-acquired MRSA; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; PSM, phenol soluble modulin.
Franklin Lakes, NJ). Plates were dried in a safety cabinet for 20 min before inoculation with bacteria. Overnight cultures of S. aureus (2 μl) were spotted onto the center of the plates using a P-20 Pipetman (Gilson S. A. S., Villiers-le-Bei, France). After inoculation, the plates were dried in a safety cabinet for 15 min before inoculation with bacteria. Overnight cultures of E. coli strain JM109 were used as the general purpose host strain for cloning (Takara Bio).

**Measurement of Inhibitory Activity against Colony Spreading**—Tryptic soy broth supplemented with 0.24% agar was autoclaved. Sterile medium (20 ml) was mixed with the sample (200 μl) and poured onto a plate (80 mm diameter). Incubation of bacteria and drying procedures of plates were the same as those described above. Plates were incubated at 37 °C for 8 h. Samples were serially diluted 2-fold with buffer (10 mM sodium acetate (pH 5.0), 200 mM NaCl), and the dilution that inhibited 50% of colony spreading was determined. The amounts of inhibitory activity against colony spreading in the samples were calculated based on our definition that 1 unit is the activity required to inhibit colony spreading by 50%. The addition of 1 ml of buffer to soft agar did not affect colony-spreading activity.

**Purification of a Colony-spreading Inhibitor from Culture Supernatants**—The Newman strain was aerobically cultured in 100 ml of tryptic soy broth in a 500-ml flask at 37 °C for 48 h. The culture was centrifuged at 10,000 × g for 5 min, and the supernatant was filtered with a 0.22-μm PVDF membrane (Millipore). The sample was boiled for 10 min and the supernatant was filtered with a 0.22-μm PVDF membrane (Millipore). Quantitative densitometric analysis was performed using the ImageJ band quantification software (National Institutes of Health, Bethesda). The protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

**Determination of Amino Acid Sequence of the Colony-spreading Inhibitor**—The 4-kDa protein band in Fraction II stained with Coomassie Brilliant Blue was excised and subjected to in-gel digestion with trypsin (Promega). The resulting peptides were analyzed by LC-MS/MS using an Orbitrap Velos (Thermo Scientific) coupled to an Acquity UPLC system. The protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

---

**TABLE 1**

| Strain or plasmid | Genotypes or characteristics | Source or Ref. |
|-------------------|-----------------------------|---------------|
| **S. aureus strains** | Laboratory strain, high level of clumping factor | (42) |
| Newman | | Takara Bio |
| MN1848 | Newman Δagr::tetM (transduction from RN6911) | 31 |
| RN4220 | 8325-4, restriction mutant, partially agr suppressed | 18 |
| CK3 | RN4220 Δagr::tetM (transduction from RN6911) | 19 |
| RN6911 | RN6390 Δagr::tetM | 29 |
| NCT8325 | Clinical isolate, wild-type strain | NCTC |
| NCT8325-4 | NCT8325 cured of phl, φ12, and φ13 | 37 |
| Smith | Laboratory strain | (38) |
| Cowan I | NCT8350 (isolated from septic arthritis) | ATCC 12598 |
| 209P | Laboratory strain | IID 671 |
| NI-1–NI-18, NI-20–NI-31, NI-33–NI-42 | HA-MRSA strains, isolated in Tokyo, Japan | 10 |
| FRP3757 | CA-MRSA, USA300, isolated in San Francisco | 39 |
| MW2 | CA-MRSA, USA400, isolated in North Dakota | 40 |
| CA04 | CA-MRSA, isolated in Chicago | 41 |
| CA05 | CA-MRSA, isolated in Chicago | 41 |
| CA07 | CA-MRSA, isolated in Chicago | 41 |
| CA10 | CA-MRSA, isolated in Chicago | 41 |
| CA11 | CA-MRSA, isolated in Chicago | 41 |
| CA12 | CA-MRSA, isolated in Chicago | 41 |
| 4/16-6N | CA-MRSA, isolated in Chicago | 41 |
| 4/16-11A | CA-MRSA, isolated in Chicago | 41 |
| 5/6-8N | CA-MRSA, isolated in Chicago | 41 |
| 6/11-1N | CA-MRSA, isolated in Chicago | 41 |
| 6/20-1N | CA-MRSA, isolated in Chicago | 41 |
| 8/6-3P | CA-MRSA, isolated in Chicago | 41 |
| **E. coli strain** | General purpose host strain for cloning | Takara Bio |
| JM109 | | |
| **Plasmids** | | |
| pCK20 | S. aureus integration vector; Cm’ | (42) |
| pUC119 | E. coli vector; Amp’ | Takara Bio |
| plnt | pCK20 with partial genomic region from RN4220 | This study |
| pW | plnt with agr region from Newman | This study |
| pH1 | plnt with agr with frameshift mutation in hld | This study |
| pH2 | plnt with agr with deletion mutation in hld | This study |
| pND50K | E. coli-S. aureus shuttle vector; Kan’ | 11 |
| pCK5000K | pND50K with luc’ gene from pGL3 | This study |
| pCK5003K | pCK5000K with ada promoter from RN4220 | This study |
| pCK5004K | pCK5000K with spa promoter from RN4220 | This study |
mained by Edman degradation using a protein sequencer (Shimadzu Co., Kyoto, Japan). A data base search was performed by a FASTA3 search of the \textit{S. aureus} Newman genome data base.

**DNA Manipulation**—Transformation of \textit{E. coli}, extraction of plasmid DNA from \textit{E. coli}, PCR, and Southern blot analyses were performed as reported previously (12). Extraction of genomic DNA from \textit{S. aureus} was performed using a QIAamp DNA blood kit (Qiagen Sciences, Germantown, MD) after cell lysis by lysostaphin. \textit{S. aureus} was transformed with plasmid DNA by electroporation (25 microfarads, 2.3 kV, 100 ohms) (13, 14). The primers used in this study are shown in Table 2.

**Construction of the hld-disrupted Mutants**—The 815-bp genomic region of RN4220 (position 41,284–42,099 in the \textit{NCTC8325} genome data base) was amplified by PCR using primers FInt and RInt and cloned into the XbaI and HindIII sites of pCK20, resulting in pInt, which can integrate into the \textit{S. aureus} chromosome by homologous recombination without disrupting open reading frames.

Mutations were introduced into the hld gene as described previously (15–17). In brief, a DNA fragment containing the \textit{agr} locus was amplified by PCR using primers Fagr and Ragr from \textit{Newman} genomic DNA as a template. The DNA fragment was cloned into the Smal site of pUC119, resulting in pUC-agr. To introduce a frameshift into the \textit{hld} gene in pUC-agr, mutant strands were synthesized by thermal cycling using primers for the frameshift (Table 2) and pUC-agr as a template, and the template plasmid was digested with DpnI and amplified in \textit{E. coli} JM109, resulting in a pUC-agr-hld frameshift. To delete the \textit{hld} gene in pUC-agr, a DNA fragment was amplified by PCR using primers for deletion (Table 2) and pUC-agr as a template, self-ligated, and then amplified in \textit{E. coli} JM109, resulting in a pUC-agr-hld deletion. These plasmids were sequenced to confirm the desired mutations in the \textit{hld} gene. DNA fragments containing the \textit{agr} locus harboring the mutated \textit{hld} genes were amplified by PCR using primer pairs Fagr2 and Ragr2, and pUC-agr, pUC-agr-hld frameshift, and pUC-agr-hld deletion as templates. The amplified DNA fragments were cloned into the SmaI site of pUC119, resulting in pUC-agr. To clone the desired mutations in the pUC-agr-hld deletion. These plasmids were sequenced to confirm the desired mutations in the \textit{hld} gene. DNA fragments containing the \textit{agr} locus harboring the mutated \textit{hld} genes were amplified by PCR using primer pairs Fagr2 and Ragr2, and pUC-agr, pUC-agr-hld frameshift, and pUC-agr-hld deletion as templates. The amplified DNA fragments were cloned into the Smal site of pInt, resulting in pW, pH1, and pH2.

The \textit{agr}-null mutants of RN4220 and \textit{Newman} strains (CK3 and MN1844) do not harbor the \textit{agr} locus containing \textit{agrBDCA} and RNAIII, a deletion originally constructed by Peng et al. (18). CK3 (19) was transformed with pInt, pW, pH1, and pH2, and colonies resistant to chloramphenicol were isolated. These plasmids were integrated into the chromosome by a single crossover recombination without disrupting the open reading frames and conferred resistance against chloramphenicol. The chromosomal regions harboring the plasmids were transferred to MN1844 by transduction using phage 80\textalpha{} (20). Integration of the \textit{agr} locus into the desired chromosomal locus was confirmed by Southern blot analysis using DNA fragments containing \textit{hld} and pInt as probes.

**Determination of Viable Cell Numbers in the Giant Colony**—Bacterial cells were sampled from the center of giant colonies by picking up soft agar using a 1-ml disposable tip for a P-1000 Pipettman (Gilion S. A. S.) and suspended in PBS. After appropriate dilution, the samples were spread on tryptic soy agar plates and incubated overnight, and the number of colonies was counted.

**Reporter Assay**—The DNA fragment containing either the \textit{spa} or \textit{hla} promoter and the \textit{luc} gene were amplified from pCK5004 or pCK5003, respectively (21), and cloned into pND50K, resulting in pCK5004K and pCK5003K. The RN4220 strain was transformed with pCK5004K and pCK5003K. The plasmids were transferred to plnt-, pW-, pH1-, and pH2-integrated \textit{agr} mutants by phage transduction. The \textit{S. aureus} strains were cultured, and the cells were harvested. The cells were lysed in buffer (20 mM KH\textsubscript{2}PO\textsubscript{4} (pH 7.8), 0.04% Triton X-100, 0.1 mM DTT, 10 \mu{}g of lysostaphin/ml, 1 tablet of protease inhibitor (Complete, Roche Applied Science)/50 ml). The cell lysates were centrifuged at 20,000 \times g for 3 min, and the supernatants were mixed with the luciferase substrate (Roche Applied Science). Fluorescence was measured using a luminometer (Lumat LB 9507, Berthold Technologies, Bad Wildbad, Germany). The promoter activity was calculated as the luminescence units/mg of protein.

**Drop-collapse Assay**—A drop-collapse assay was performed using the previously described method with slight modifications (22). Briefly, 200 \mu{}l of samples serially diluted 2-fold were spotted inside each circle of the lids of polystyrene microplates (11 \times 8 cm, Code 3860–996, Iwaki, Asahi Glass Co., Tokyo, Japan). Drop shapes were inspected, and the time required to induce drop-collapse was recorded.

**Cell Surface Hydrophobicity Test**—Microbial surface hydrophobicity was assessed based on microbial adhesion to the hydrocarbon method with slight modifications (23). Overnight culture of \textit{S. aureus} Newman was centrifuged, and the cells were washed with 50 mM Tris-HCl buffer (pH 7.5). Cell suspension was adjusted to A\textsubscript{600} = 1.0 using the same buffer. Hexadecane (50 \mu{}l), protein sample (100 \mu{}l), and cell suspension (900 \mu{}l) were vortexed in a 2-ml polypropylene tube (Eppendorf,
Hamburg, Germany) for 2 min and equilibrated for 15 min. The bottom aqueous phase was carefully removed to measure the absorbance at 600 nm. The amount of \textit{S. aureus} cells adsorbed onto hexadecane relative to that without protein sample was calculated using the following formula: \( \frac{100}{A_{600 \text{ before vortex without protein sample}}} \times \frac{A_{600 \text{ after vortex with protein sample}}}{A_{600 \text{ after vortex without protein sample}}} \).

### RESULTS

**S. aureus Secretes Inhibitors against Colony Spreading**

To evaluate the presence of modulators against colony spreading, we tested the effect of liquid culture supernatants on the colony-spreading activity of Newman strain on soft agar. When soft agar media were supplemented with the liquid culture supernatant, colony spreading was inhibited (Fig. 1A). Based on this finding, we assumed that the supernatant contained colony-spreading inhibitors. The inhibitory activity was not detected in culture supernatant of the null mutant of the \textit{agr} locus, which regulates the expression of various extracellular proteins (Fig. 1A). In addition, the inhibitory activity in the culture supernatant was abolished by preincubation with proteinase K (data not shown). These results suggested that \textit{S. aureus}, depending on the function of the \textit{agr} locus, secretes inhibitory exoprotein(s) against colony spreading.

The addition of excess amounts of culture supernatant to soft agar inhibited 90\% of colony spreading (Fig. 1B). We defined 1 unit of colony-spreading inhibitory activity as the fraction that inhibits colony spreading by 50\%. The inhibitory activity against colony spreading was low when cells were in the exponential phase and increased during the stationary phase (Fig. 1C). The inhibitory activities in culture supernatants differed among different \textit{S. aureus} strains (Fig. 1D). To further understand the molecular mechanisms of the inhibitor, we purified the inhibitory factor from the culture supernatant of the \textit{S. aureus} Newman strain.

**Purification of the Inhibitory Factor against Colony Spreading from \textit{S. aureus} Culture Supernatants**

We attempted to purify the inhibitory molecule from culture supernatants of \textit{S. aureus} Newman strain based on the increase in the specific activity as defined above. The specific activity was increased 2.5-fold by precipitation with ammonium sulfate, boiling, and phosphocellulose column chromatography. The overall recovery of activity was 63\% (Table 3). SDS-PAGE of the final fraction (Fraction III) revealed that the fraction was almost homogeneous with a single 4-kDa protein band (Fig. 2A). Quantitative densitometric analysis revealed that the purity of the protein was 95\%. In phosphocellulose column chromatography, the inhibitory activity against colony spreading coincided with the presence of...
Role of δ-Hemolysin in Colony Spreading

The 4-kDa protein (Fig. 2, B and C). These results indicate that the purified 4-kDa protein acts as an inhibitor against colony spreading. Amino acid sequencing revealed that the N terminus of the 4-kDa protein was MAQDIISTG. The sequence was matched with amino acid residues of δ-hemolysin (Hld, δ-toxin), which is encoded by the hld gene in the agr locus (24). δ-Hemolysin is reported to have hemolytic activity on horse erythrocytes (25). We demonstrated that our purified fraction (Fraction III) possessed hemolytic activity on horse erythrocytes. The hemolytic activity was 790 units/mg protein, which is consistent with that in a previous report (310 units/mg protein) (25). To confirm the inhibitory activity of δ-hemolysin against colony spreading, we examined whether chemically synthesized δ-hemolysin inhibits colony spreading. Chemically synthesized δ-hemolysin inhibited colony spreading in the same manner as the purified δ-hemolysin (Fig. 2D). Based on these findings, we concluded that the colony-spreading inhibitor identified in the culture supernatant of the Newman strain was δ-hemolysin.

TABLE 3
Purification of the colony-spreading inhibitor from the culture supernatant of the Newman strain

| Fraction | Protein | Total activity | Specific activity | Yield |
|----------|--------|---------------|-----------------|-------|
| I. Culture supernatant | 34 | 4300 | 130 | 100 |
| II. Boil supernatant | 20 | 3500 | 180 | 81 |
| III. Phosphocellulose column | 8.3 | 2700 | 330 | 63 |

![FIGURE 2. Purification of colony-spreading inhibitor from the culture supernatant of S. aureus. A, 1 μg of protein from each purification step was electrophoresed on a Tricine-buffered SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. B, elution profile of phosphocellulose column chromatography. Open circles indicate inhibitory activity against colony spreading. Filled circles indicate protein concentration. The dashed line shows the NaCl concentration gradient. C, SDS-PAGE analysis of phosphocellulose fractions (22–32). The gel was stained with Coomassie Brilliant Blue. The 4-kDa protein indicated by the arrowhead was determined to be δ-hemolysin by amino acid sequencing. Lane M, marker proteins. D, final fraction (Fraction III, closed circle) or chemically synthesized δ-hemolysin (open circle) was serially diluted 2-fold, and the inhibitory activity against colony spreading was measured. E, growth curve of Newman in tryptic soy broth supplemented with an amount of δ-hemolysin 300-fold in excess (15 μM) of the amount that inhibits 50% of colony spreading.]

Detergent Activity of δ-Hemolysin Is Required for the Inhibition of Colony Spreading—We then examined whether the inhibitory effect of δ-hemolysin on colony spreading could be explained by the inhibition of cell growth. The addition of amounts of δ-hemolysin 300-fold greater than that needed to inhibit colony spreading did not inhibit S. aureus Newman growth in liquid medium (Fig. 2E). Therefore, the inhibition of colony spreading by δ-hemolysin is not due to growth inhibition.

Detergent activity of δ-hemolysin has been reported (25). Our final fraction (Fraction III) showed drop-collapse within 200 s at 4 μM (Fig. 3C, open circle), which is an indication of the detergent activity that inhibits hydrophobic interactions. S. aureus colony spreading is the phenomenon of the rapid spread of the bacteria on an aqueous surface of a soft agar plate. We hypothesized that the detergent activity of δ-hemolysin interferes with the hydrophobic interactions between S. aureus cell surfaces, which inhibits the colony-spreading activity. Because S. aureus cell surfaces show hydrophobic characteristics, cells adsorb onto hexadecane, an organic solvent (23). We examined whether δ-hemolysin inhibits the adsorption of S. aureus onto hexadecane. The results demonstrated that the addition of δ-hemolysin decreased the amount of S. aureus adsorbed onto hexadecane (Fig. 3D, open circle), indicating that δ-hemolysin inhibits the hydrophobic interactions between S. aureus cells in an aqueous environment.

To test whether the inhibition of hydrophobic interactions between S. aureus cell surfaces by δ-hemolysin is necessary for the inhibition of colony spreading, we constructed a mutated form of δ-hemolysin (Fig. 3A). The detergent activity of δ-he-
molysin requires an amphiphilic α-helical structure comprising 5–20 amino acid residues (Fig. 3B) (26, 27). We designed a mutated δ-hemolysin (Hld-M) in which threonine 8 and lysine 14 were replaced with proline to disrupt the α-helical structure, and isoleucine 5, valine 13, and valine 20 were replaced with glutamate to decrease the hydrophobicity (Fig. 3A). We used Heliquest to predict the presence of the amphiphilic α-helical structure (28) and determined that the hydrophobic moment value was decreased in Hld-M, suggesting that the amphiphilic α-helical structure of Hld-M had been removed (Fig. 3B). We used the drop-collapse assay to examine whether this mutated δ-hemolysin loses its detergent activity. The results demonstrated that Hld-M did not cause drop-collapse (Fig. 3C, closed triangle). Therefore, detergent activity of this mutated δ-hemolysin was lost. Furthermore, Hld-M lost both activities, inhibition of the adsorption of S. aureus onto hexadecane and inhibition of colony-spreading activity (Fig. 3, D and E, closed triangle). These results suggest that the inhibition of hydrophobic interactions between S. aureus cell surfaces by the detergent activity of δ-hemolysin is required for the inhibition of colony spreading.

**Increased Colony Spreading Induced by an hld-disrupted Mutant**—We constructed gene-disrupted mutants for the hld gene that encodes δ-hemolysin, and we examined the culture supernatant inhibitory activity against colony spreading. The hld gene locates inside of the agr locus, and its transcript is a regulatory RNA called RNAIII. RNAIII has a virulence regulatory function, such as inhibition of the expression of the spa gene encoding protein A and activation of the expression of the hla gene encoding δ-hemolysin (29, 30). We determined that the agr locus is required for S. aureus colony-spreading activity (31). Therefore, we attempted to disrupt the hld gene without affecting the function of the agr locus. We constructed plasmids containing the hld gene with nucleotide insertion (pH1) and nucleotide deletion (pH2) that did not inactivate the function of RNAIII as a regulatory RNA but did inactivate the expression of the hla gene encoding δ-hemolysin (29, 30). We determined that the agr locus is required for S. aureus colony-spreading activity (31). Therefore, we attempted to disrupt the hld gene without affecting the function of the agr locus. We constructed plasmids containing the hld gene with nucleotide insertion (pH1) and nucleotide deletion (pH2) that did not inactivate the function of RNAIII as a regulatory RNA but did inactivate the expression of the hla gene encoding δ-hemolysin (29, 30). We determined that the agr locus is required for S. aureus colony-spreading activity (31). Therefore, we attempted to disrupt the hld gene without affecting the function of the agr locus. We constructed plasmids containing the hld gene with nucleotide insertion (pH1) and nucleotide deletion (pH2) that did not inactivate the function of RNAIII as a regulatory RNA but did inactivate the expression of the hla gene encoding δ-hemolysin (29, 30). We determined that the agr locus is required for S. aureus colony-spreading activity (31). Therefore, we attempted to disrupt the hld gene without affecting the function of the agr locus. We constructed plasmids containing the hld gene with nucleotide insertion (pH1) and nucleotide deletion (pH2) that did not inactivate the function of RNAIII as a regulatory RNA but did inactivate the expression of the hla gene encoding δ-hemolysin (29, 30). We determined that the agr locus is required for S. aureus colony-spreading activity (31). Therefore, we attempted to disrupt the hld gene without affecting the function of the agr locus. We constructed plasmids containing the hld gene with nucleotide insertion (pH1) and nucleotide deletion (pH2) that did not inactivate the function of RNAIII as a regulatory RNA but did inactivate the expression of the hla gene encoding δ-hemolysin (29, 30). We determined that the agr locus is required for S. aureus colony-spreading activity (31). Therefore, we attempted to disrupt the hld gene without affecting the function of the agr locus. We constructed plasmids containing the hld gene with nucleotide insertion (pH1) and nucleotide deletion (pH2) that did not inactivate the function of RNAIII as a regulatory RNA but did inactivate the expression of the hla gene encoding δ-hemolysin (29, 30). We determined that the agr locus is required for S. aureus colony-spreading activity (31). Therefore, we attempted to disrupt the hld gene without affecting the function of the agr locus. We constructed plasmids containing the hld gene with nucleotide insertion (pH1) and nucleotide deletion (pH2) that di...
Introduction of pH1 harboring the agr locus that included the nucleotide-inserted hld gene or pH2 harboring the agr locus that included the nucleotide-deleted hld gene into the agr-null mutant also inhibited the promoter activity of the spa gene as well as the introduction of pW (Fig. 4B). In contrast, introduction of pW into the agr-null mutant activated the hla gene promoter (Fig. 4C). Introduction of pH1, pH2, and pW into the agr-null mutant also activated the hla gene promoter (Fig. 4C). Furthermore, exoprotein profiles other than δ-hemolysin were similar between pW, pH1, and pH2, whereas the exoprotein profile of pInt was different compared with pW, pH1, and pH2 (Fig. 4D). Therefore, we concluded that pH1 and pH2 did not lose their RNAIII regulatory function. SDS-PAGE of exoproteins in culture supernatant revealed that the pW-introduced strain produced δ-hemolysin but the pH1- and pH2-introduced strains did not (Fig. 4D), confirming disruption of the hld gene. The inhibitory activities against colony spreading in the culture supernatants of pH1- and pH2-intro-
duced strains were decreased to 30-fold less than that of pW-introduced strain (Fig. 4E). Inhibitory activity against colony spreading was also detected in soft agar plates that were spotted with the pW-introduced strain, but it was decreased in the plates spotted with the pH1- and pH2-introduced strains (Fig. 4F). These results provide genetic evidence that the H9254-hemolysin encoded by the hld gene inhibits colony spreading and is contained in the culture supernatant of S. aureus and the soft agar milieu around the S. aureus giant colony.

We then compared the colony spreading of the pW-, pH1-, and pH2-introduced strains. The result demonstrated that the pH1- and pH2-introduced strains had higher colony-spreading abilities than the pW-introduced strain (Fig. 5, A and B). In contrast, the pH1- and pH2-introduced strains had a growth level indistinguishable from that of the pW-introduced strain in liquid medium (Fig. 5C). These results suggest that δ-hemolysin is secreted from S. aureus into soft agar and inhibits colony spreading without affecting the growth rate. In addition, macroscopic observations revealed that the cell layers of the giant colonies of pH1- and pH2-introduced strains were thinner than those of the pW-introduced strain. The number of viable cells in 1 cm² of the giant colony of the pH1- or pH2-introduced strain was half that in pW-introduced strain (Fig. 5D). This finding suggests that the secretion of δ-hemolysin inhibits colony-spreading activity, resulting in increased cell density.

**Amount of δ-Hemolysin Does Not Parallel the Colony-spread- ing Activity**—In our previous study, we showed that HA-MRSA strains showed less colony spreading than CA-MRSA strains (10). We examined whether the decreased colony spreading of HA-MRSA or the increased colony spreading of CA-MRSA can be explained by the amount of δ-hemolysin, which inhibits colony spreading. Both HA-MRSA and CA-MRSA strains express δ-hemolysin, and there was no correlation between the colony-spreading ability and the amount of δ-hemolysin ($R^2 = 0.1293$) (Fig. 6). Therefore, the amount of δ-hemolysin does not explain the total colony-spreading activity in each S. aureus strain.

**DISCUSSION**

The findings of this study demonstrated that δ-hemolysin possesses inhibitory activity against colony spreading in addition to the previously reported hemolytic activity. To our knowledge, δ-hemolysin is the first example of an endogenous molecule that inhibits bacterial self-motility. Furthermore, we revealed that inhibition of colony-spreading activity by δ-hemolysin is due to its detergent effects, which interfere with the hydrophobic nature of S. aureus cell surfaces. The concentration of δ-hemolysin required to inhibit interactions between S. aureus and hexadecane was 5-fold higher than that required to inhibit colony spreading (Fig. 3, D and E). Because the force of hydrophobic interactions depends on the hydrophobicity of the interacting molecules, we assumed that the hydrophobic interaction between S. aureus cells necessary for colony spreading is weaker than that between S. aureus and hexadecane and therefore is easily inhibited by δ-hemolysin.

δ-Hemolysin is encoded by the hld gene, which locates inside of RNAIII that encodes a regulatory RNA. RNAIII plays a key role in S. aureus to regulate the expression of various virulence genes via a quorum-sensing system (29, 32). The amount of...
extracellular δ-hemolysin is increased, accompanied by the increased expression of RNAIII, and at the stationary phase the amount of δ-hemolysin reaches almost half the amount of total exoproteins (33). A δ-hemolysin mutant in the S. aureus MW2 strain, which is a community-acquired methicillin-resistant strain, has attenuated virulence in a mouse bacteremia model (34), suggesting that δ-hemolysin is required for S. aureus virulence. This study revealed that δ-hemolysin increases the cell number per unit area of colony by inhibiting colony spreading, resulting in a thicker giant colony (Fig. 5D). This might promote efficient colonization in S. aureus infectious processes. We also assume that δ-hemolysin inhibits the access of other colonies by inhibiting colony spreading, which might be beneficial for the compartmentalization of S. aureus colonies. Thus, δ-hemolysin has novel functions to modulate the physical state of S. aureus colonies.

In this study, we showed that δ-hemolysin, which is encoded by hld located in the agr locus, inhibited S. aureus colony spreading. δ-Hemolysin is one of the small hydrophobic polypeptides called phenol-soluble modulins (PSMs). In addition to δ-hemolysin, other PSMs include PSMα1, -α2, -α3, -α4, -β1, and -β2 (34). We and other researchers demonstrated that S. aureus colony-spreading activity requires the psmα operon, which encodes PSMα1, -α2, -α3, and -α4, and whose expression depends on the agr locus (11, 35). We and others also reported that the agr locus is required for S. aureus colony spreading (31, 35). Therefore, the agr locus up-regulates psmα to stimulate colony spreading, although it also encodes δ-hemolysin as an inhibitor against colony-spreading activity. The ratio of δ-hemolysin to PSMαs decreases from the exponential phase to the stationary phase in liquid culture (34). The balance between the stimulator and the inhibitor might be altered by the position or growth phase of bacterial cells in the giant colony and contribute to the regulation of S. aureus colony spreading.

REFERENCES
1. Cossart, P., and Sansonetti, P. J. (2004) Bacterial invasion. The paradigms of enteroinvasive pathogens. Science 304, 242–248
2. Josenhans, C., and Suerbaum, S. (2002) The role of motility as a virulence factor in bacteria. Int. J. Med. Microbiol. 291, 605–614
3. Krukonis, E. S., and DiRita, V. J. (2003) From motility to virulence. Sensing and responding to environmental signals in Vibrio cholerae. Curr. Opin. Microbiol. 6, 186–190
4. Brown, I. L., and Häse, C. C. (2001) Flagellum-independent surface migration of Vibrio cholerae and Escherichia coli. J. Bacteriol. 183, 3784–3790
5. Kinsinger, R. F., Shirk, M. C., and Fall, R. (2003) Rapid surface motility in Bacillus subtilis is dependent on extracellular surfactin and potassium ion. J. Bacteriol. 185, 5627–5631
6. Kinsinger, R. F., Kearns, D. B., Hale, M., and Fall, R. (2005) Genetic requirements for potassium ion-dependent colony spreading in Bacillus subtilis. J. Bacteriol. 187, 8462–8469
7. Recht, J., and Kolter, R. (2001) Glycopeptidolipid acetylation affects sliding motility and biofilm formation in Mycobacterium smegmatis. J. Bacteriol. 183, 5718–5724
8. Recht, J., Martínez, A., Torello, S., and Kolter, R. (2000) Genetic analysis of sliding motility in Mycobacterium smegmatis. J. Bacteriol. 182, 4348–4351
9. Kaito, C., and Sekimizu, K. (2007) Colony spreading in Staphylococcus aureus. J. Bacteriol. 189, 2553–2557
10. Kaito, C., Omae, Y., Matsumoto, Y., Nagata, M., Yamaguchi, H., Aoto, T., Ito, T., Hiramatsu, K., and Sekimizu, K. (2008) A novel gene, fudoh, in the SCCmec region suppresses the colony spreading ability and virulence of Staphylococcus aureus. PLoS ONE 3, e3921
11. Kaito, C., Saito, Y., Nagano, G., Ikuo, M., Omae, Y., Hanada, Y., Han, X., Kuwahara-Arai, K., Hishinuma, T., Baba, T., Ito, T., Hiramatsu, K., and Sekimizu, K. (2011) Transcription and translation products of the cytosol protein psm-mec on the mobile genetic element SCCmec regulate Staphylococcus aureus virulence. PLoS Pathog. 7, e1001267
12. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13. Inoue, R., Kaito, C., Tanabe, M., Kamura, K., Akimitsu, N., and Sekimizu, K. (2001) Genetic identification of two distinct DNA polymerases, DNAe and PoIC, that are essential for chromosomal DNA replication in Staphylococcus aureus. Mol. Genet. Genomics 266, 564–571
14. Schenk, S., and Laddaga, R. A. (1992) Improved method for electroporation of Staphylococcus aureus. FEMS Microbiol. Lett. 73, 133–138
15. Kaito, C., Morishita, D., Matsumoto, Y., Kurokawa, K., and Sekimizu, K. (2006) Novel DNA-binding protein SarZ contributes to virulence in Staphylococcus aureus. Mol. Microbiol. 62, 1601–1617
16. Nagata, M., Kaito, C., and Sekimizu, K. (2008) Phosphodiesterase activity of CvF is required for virulence in Staphylococcus aureus. J. Biol. Chem. 283, 2176–2184
17. Li, S., and Wilkinson, M. F. (1997) Site-directed mutagenesis. A two-step method using PCR and Dmpl. BioTechniques 23, 588–590
18. Peng, H. L., Novick, R. P., Kreiswirth, B., Kornblum, J., and Schleupert, P. (1988) Cloning, characterization, and sequencing of an accessory gene regulator (agr) in Staphylococcus aureus. J. Bacteriol. 170, 4365–4372
19. Kaito, C., Kurokawa, K., Matsumoto, Y., Terao, Y., Kawabata, S., and Sekimizu, K. (2005) Silkworm pathogenic bacteria infection model for identification of novel virulence genes. Mol. Microbiol. 56, 934–944
20. Novick, R. P. (1991) Genetic systems in staphylococci. Methods Enzymol. 204, 587–636
21. Matsumoto, Y., Kaito, C., Morishita, D., Kurokawa, K., and Sekimizu, K. (2007) Regulation of exoprotein gene expression by the Staphylococcus aureus cvfB gene. Infect. Immun. 75, 1964–1972
22. Jain, D. K., Collinsthompson, D. L., Lee, H., and Trevors, J. T. (1991) A drop-collapsing test for screening surfactant-producing microorganisms.
23. Rosenberg, M., Gutnick, D., and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons; a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* 9, 29–33

24. Fitton, J. E., Dell, A., and Shaw, W. V. (1980) The amino acid sequence of the haemolysin of *Staphylococcus aureus*. *FEBS Lett.* 115, 209–212

25. Birkbeck, T. H., and Freer, J. H. (1988) Purification and assay of staphylococcal δ-lysin. *Methods Enzymol.* 165, 16–22

26. Tappin, M. J., Pastore, A., Norton, R. S., Freer, J. H., and Campbell, I. D. (1988) High resolution 1H NMR study of the solution structure of δ-heamolysin. *Biochemistry* 27, 1643–1647

27. Lee, K. H., Fitton, J. E., and Wüthrich, K. (1987) Nuclear magnetic resonance investigation of the conformation of δ-heamolysin bound to dodecylphosphocholine micelles. *Biochim. Biophys. Acta* 911, 144–153

28. Novick, R. P., Ross, H. F., Projan, S. J., Kornblum, J., Kreiswirth, B., and Moghazeh, S. (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.* 12, 3967–3975

29. Janzon, L., and Arvidson, S. (1990) The role of the δ-lysin gene (hld) in the regulation of virulence genes by the accessory gene regulator (agr) in *Staphylococcus aureus*. *EMBO J.* 9, 1391–1399

30. Ueda, T., Kaito, C., Omae, Y., and Sekimizu, K. (2011) Sugar-responsive gene expression and the agr system are required for colony spreading in *Staphylococcus aureus*. *Microb. Pathog.* 51, 178–185

31. Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K., and Hiramatsu, K. (2002) Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* 359, 1819–1827

32. Daum, R. S., Ito, T., Hiramatsu, K., Hussain, F., Mongkolrattanothai, K., Jamklang, M., and Boyle-Vavra, S. (2002) A novel methicillin-resistance cassette in community-acquired methicillin-resistant *Staphylococcus aureus* isolates of diverse genetic backgrounds. *J. Infect. Dis.* 186, 1344–1347

33. Ichihashi, N., Kurokawa, K., Matsuo, M., Kaito, C., and Sekimizu, K. (2003) Inhibitory effects of basic or neutral phospholipid on acidic phospholipid-mediated dissociation of adenine nucleotide bound to DnaA protein, the initiator of chromosomal DNA replication. *J. Biol. Chem.* 278, 28778–28786