NOTES

Colony Spreading in *Staphylococcus aureus*[▽]

Chikara Kaito and Kazuhisa Sekimizu*

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

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Wild-type *Staphylococcus aureus* rapidly expands on the surface of soft agar plates. The rates of expansion and the shapes of the resultant giant colonies were distinct for different strains of laboratory stocks and clinical isolates. The colony spreading abilities did not correlate with the biofilm-forming abilities in these strains. Insertional disruption of the *dltABCD* operon, which functions at the step of D-alanine addition to teichoic acids, and of the *tagO* gene, which is responsible for the synthesis of wall teichoic acids, decreased the colony spreading ability. The results indicate that wall teichoic acids and D-alanylation of teichoic acids are required for colony spreading.

In bacteria, motility is advantageous for the acquisition of nutrients. Many bacteria translocate by the propeller function of flagella (16, 20). Although mycobacteria and streptococci do not have flagella, they can spread on solid surfaces via a mechanism called sliding (2, 7, 8, 14). The sliding ability of these bacteria is provided by the expansive forces of a growing culture in combination with special surface properties of the cells resulting in reduced friction between the cell and its substrate (6, 7). In *Mycobacterium smegmatis*, glycopeptide lipids in the cell envelope are required for sliding (21, 22). *Bacillus subtilis* and a nonflagellated mutant of this species have the sliding type of surface motility (11). In *B. subtilis*, surfactin secreted by the bacterium and extracellular potassium ions are required for the spreading (10). In addition, the gram-negative bacteria *Escherichia coli*, *Vibrio cholerae*, and *Serratia marcescens* have sliding ability that is independent of their flagella (3, 15). The biologic significance of the ability to slide and the sliding mechanism remains to be elucidated.

Staphylococcus aureus is a nonflagellated gram-positive pathogen that causes various diseases, such as suppurative wound infections, meningitis, and sepsis. The recent emergence of methicillin-resistant *S. aureus*, which is resistant to a broad range of antibiotics, is the source of serious clinical problems. *S. aureus* forms a biofilm on medical devices, such as catheters left in the body. Biofilm formation is caused by the attachment of *S. aureus* to artificial surfaces and attenuates the effectiveness of antibiotics and antimicrobial peptides (4). Whether this bacterium can spread on a solid surface, however, is unknown. *Staphylococcus epidermidis*, a species closely related to *S. aureus*, has a spreading ability called darting, which has a speed of 6 $\mu\text{m}/\text{min}$ (7). In this report, we describe the ability of *S. aureus* to spread on soft agar surfaces at a speed of 100 $\mu\text{m}/\text{min}$. The results of the present

study indicate that wall teichoic acid and D-alanylation of teichoic acids are required for the colony spreading ability of *S. aureus*.

***S. aureus* spreads rapidly on the surface of soft agar plates.** Tryptic soy broth (Becton, Sparks, MD) supplemented with 0.24% agar (Nacalai Tesque, Inc., Kyoto, Japan) was autoclaved and poured into plates (diameter, 12 cm). Plates were dried in a safety cabinet for 20 min before inoculation with bacteria. Overnight cultures of *S. aureus* RN4220 (Table 1) (2 μl) were spotted onto the center of the plates using a P-20 Pipetteman (Gilson S. A. S., Villiers-le-Bel, France). After inoculation, the plates were dried in a safety cabinet for 15 min and incubated at 37°C for 10 h. Photographs were taken using a digital camera (FAS-III; TOYOBO Co. Ltd., Osaka, Japan). After 10 h of incubation, the bacteria had spread to form colonies 60 mm in diameter (Fig. 1A). When a culture was spotted onto hard agar plates (1.5% agar), the colonies were 6 mm in diameter, which was the size of the spotted area before incubation. These results indicate that *S. aureus* has the ability to spread on soft agar surfaces. In this report, we call this phenomenon colony spreading. Microscopic observation of the edges of the spreading colonies on the soft agar was performed using a BH-2 microscope (Olympus Co., Tokyo, Japan). The images were captured with a Leica DC camera (Leica Microsystems Ltd., Heerbrugg, Germany) integrated with a personal computer running Microsoft Windows. The cells were closely packed, and multiple layers of cells constituted the growth area (Fig. 1B). Colony spreading was enhanced when the period of plate drying was shortened, whereas colony spreading was inhibited when the period was prolonged (data not shown). This result indicates that water in plates is an important factor for the spreading. Colony spreading was observed when tryptic soy broth or brain heart infusion broth was used but not when Luria-Bertani broth or Sabouraud dextrose broth was used, indicating that some specific factors in broth media are required for the spreading. The maximum diameter of the colonies occurred when the plates were incubated at 40°C. Spreading did not occur at temperatures below 30°C or over 43°C (data not shown).

Rate of spreading and shape of the resultant giant colonies were distinct for different strains of *S. aureus*. We evaluated whether *S. aureus* strains other than RN4220 have colony

* Corresponding author. Mailing address: Graduate School of Pharmaceutical Sciences, The University of Tokyo, 3-1, 7-Chome, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Phone: 81 3 (5841) 4820. Fax: 81 3 (5684) 2973. E-mail: sekimizu@mol.f.u-tokyo.ac.jp.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or characteristics ^a	Source or reference
<i>S. aureus</i> strains		
RN4220	NCTC8325-4, restriction mutant	18a ^b
NCTC8325	Clinical isolate, wild-type strain	NCTC
NCTC8325-4	NCTC8325 cured of ϕ 11, ϕ 12, and ϕ 13	17 ^c
Newman	Laboratory strain, high level of clumping factor	3a ^b
Smith	Laboratory strain	22a ^d
Cowan I	NCTC8530 (isolated from septic arthritis)	ATCC 12598
MSSA1	Clinical isolate	1
MRSA8	Clinical isolate, methicillin resistant	1
209P	Laboratory strain	IID 671 ^e
M0793	RN4220 <i>dltABCD</i> ::pT0793	This study
M0702	RN4220 <i>tagO</i> ::pT0702	This study
M0875	RN4220 <i>ypfP</i> ::pT0875	This study
N0793	Newman <i>dltABCD</i> ::pT0793	This study
N0702	Newman <i>tagO</i> ::pT0702	This study
N0875	Newman <i>ypfP</i> ::pT0875	This study
<i>E. coli</i> JM109	General-purpose host strain for cloning	Takara Bio
Plasmids		
pHY300	<i>E. coli</i> - <i>S. aureus</i> shuttle vector; Amp ^r Tet ^r	Takara Bio
p0793	pHY300 with intact <i>dltABCD</i> from RN4220	This study
p0702	pHY300 with intact <i>tagO</i> from RN4220	This study
p0875	pHY300 with intact <i>ypfP</i> from RN4220	This study
pMutinT3	<i>S. aureus</i> integration vector; Amp ^r Erm ^r	25 ^f
pT0793	pMutinT3 with partial <i>dltA</i> gene from RN4220	This study
pT0702	pMutinT3 with partial <i>tagO</i> gene from RN4220	This study
pT0875	pMutinT3 with partial <i>ypfP</i> gene from RN4220	This study

^a Erm, erythromycin; Amp, ampicillin; Tet, tetracycline.

^b Provided by Keiichi Hiramatsu (Juntendo University, Tokyo, Japan).

^c Provided by Timothy J. Foster (Moyne Institute of Preventive Medicine, Trinity College Dublin, Dublin, Ireland).

^d Provided by Masa Hamada (Microbial Chemistry Research Foundation, Shinagawa, Japan).

^e Provided by Motoyuki Sugai (Hiroshima University, Hiroshima, Japan).

^f Provided by Naotake Ogasawara (Nara Institute of Science and Technology, Nara, Japan).

spreading ability. Photographs were taken using a digital camera (FinePix S9000; Fuji Photo Film Co. Ltd., Tokyo, Japan). Strain Newman and clinical isolate MRSA8 had colony spreading ability that resulted in giant colonies with a number of branched arms (Fig. 2). Strains NCTC8325, Smith, and 209P formed giant colonies with few branched arms. Therefore, colony spreading was observed in strains other than RN4220, and the motility rate and morphology of the resultant giant colonies were distinct for each strain.

Colony spreading ability did not correlate with biofilm-

forming ability. To investigate whether the mechanism underlying colony spreading is the same as the mechanism for biofilm formation, we studied biofilm formation by *S. aureus* strains with different colony spreading abilities. Quantification of the biofilms was performed by using the methods described previously (23). Overnight cultures of *S. aureus* were diluted 250-fold with tryptic soy broth supplemented with 0.25% glucose and incubated in 96-well polystyrene microtiter plates at 37°C for 35 h. The plates were stained with 0.1% safranin, and the optical density at 490 nm was determined. Of colony

FIG. 1. Colony spreading of *S. aureus* on soft agar plates. (A) Overnight culture of *S. aureus* RN4220 spotted onto 0.24% and 1.5% agar plates and incubated at 37°C for 10 h. (B) Microscope image of the edge of a giant colony of RN4220 formed on 0.24% agar.

FIG. 2. Shapes of giant colonies of various strains of *S. aureus*. Overnight cultures of *S. aureus* strains were spotted onto soft agar plates (0.24% agar) and incubated at 37°C for 10 h.

spreading strains RN4220, Newman, Smith, MRSA8, and 209P, only 209P formed large amounts of biofilm (data not shown). Moreover, NCTC8325-4 and Cowan I had low colony spreading abilities, although they formed large amounts of biofilm (data not shown). Therefore, colony spreading and biofilm formation have different mechanisms.

Mutations in the *dltABCD* operon, the *tagO* gene, and the *ypfP* gene decreased colony spreading ability. To identify the molecular mechanism required for colony spreading, we searched for mutants with colony spreading ability. We hypothesized that the surface structure of *S. aureus* has a role in spreading and examined a mutant with a mutation in the *mprF* gene, which functions in the synthesis of the membrane phospholipid lysylphosphatidylglycerol, and a mutant with a mutation in the *dltABCD* operon, which is involved in the addition of D-alanine to teichoic acids. The *mprF* mutant constructed in our previous study (9) did not decrease the spreading ability (data not shown). The *dltABCD* mutant (M0793) was constructed by insertional disruption of the *dltA* gene. The internal region of the *dltA* gene (positions 46 to 694; position 1 is the translation initiation site) was amplified by PCR and was inserted into pMutinT3, resulting in plasmid pT0793. Strains resistant to erythromycin were obtained by transformation of the RN4220 strain with pT0793, which resulted in M0793. Disruption of the *dltA* gene and integration of the targeting vector into the desired chromosomal locus were confirmed by Southern blot analysis. Strain M0793 had decreased colony spreading ability (Fig. 3A and B). To perform a complementation analysis, the *dltABCD* operon (positions –498 to 4201; position 1 is the translation initiation site of the *dltA* gene) was amplified by PCR and was inserted into pHY300 at the BamHI and HindIII sites, resulting in p0793. Introduction of p0793 restored the colony spreading ability of M0793. The growth rates of strain M0793 were indistinguishable from those of the parent strain (data not shown), indicating that decreased colony spreading ability is not due to slow growth. Thus, we concluded that D-alanylation of teichoic acids is required for colony spreading.

The results described above suggested that teichoic acids on the cell surface of *S. aureus* have a role in the spreading ability of this bacterium. Two distinct types of teichoic acids, wall

teichoic acids and lipoteichoic acids, are present on the *S. aureus* cell surface. To evaluate whether teichoic acids are required for colony spreading, we constructed wall teichoic acid biosynthesis mutants and evaluated their colony spreading abilities. TagO is required for the first step of wall teichoic acid synthesis, the synthesis of lipid-PP-GlcNAc. The cell walls of a *tagO* mutant do not contain wall teichoic acids (26). The internal region of the *tagO* gene (positions 36 to 645) was amplified by PCR and was inserted into pMutinT3, resulting in plasmid pT0702. We transformed RN4220 with pT0702 and obtained a *tagO*-disrupted mutant (M0702). The growth rate of strain M0702 was indistinguishable from that of the parent strain. We confirmed the absence of wall teichoic acids in strain M0702 (data not shown). Strain M0702 had decreased colony spreading ability (Fig. 3A and B). Introduction of p0702 harboring the *tagO* gene (positions –580 to 1268) restored the spreading ability, suggesting that the *tagO* gene is required for colony spreading. Based on these results, we concluded that wall teichoic acids are required for colony spreading.

Lipoteichoic acids are anchored in the cell membrane by a diglucosyldiacylglycerol moiety. YpfP is an enzyme responsible for synthesis of diglucosyldiacylglycerol. Lipoteichoic acids of a *ypfP* deletion mutant are anchored to diacylglycerol in the phospholipid bilayer, and free glycolipids are not present in the cell membrane (12). The internal region of the *ypfP* gene (positions 38 to 695) was amplified by PCR and inserted into pMutinT3, resulting in pT0875. Strain RN4220 was transformed with pT0875, resulting in a *ypfP*-disrupted mutant (M0875). The growth rate of strain M0875 was indistinguishable from that of the parent strain (data not shown), as reported previously. Strain M0875 had decreased colony spreading ability on soft agar plates (Fig. 3A and B). The phenotype was restored by introducing p0875 harboring the intact *ypfP* gene (positions –431 to 1395). Thus, the *ypfP* gene is required for colony spreading ability. These results suggest that the anchoring motif of lipoteichoic acids and/or free glycolipids in the membrane is necessary for colony spreading ability.

The *S. aureus* RN4220 strain is a strain mutagenized by nitrosoguanidine (24). There are uncharacterized mutations in the genome of this strain that might affect the colony spreading abilities of the *dltABCD*, *tagO*, and *ypfP* mutants. To determine

FIG. 3. Involvement of teichoic acids in colony spreading. (A) Overnight cultures of *S. aureus* RN4220 transformed with an empty vector (pHY300), a *dltABCD* mutant (M0793/pHY300), a *dltABCD* mutant transformed with p0793 harboring the intact *dltABCD* operon (M0793/p0793), a *tagO* mutant (M0702/pHY300), a *tagO* mutant transformed with p0702 harboring the intact *tagO* gene (M0702/p0702), a *ypfP* mutant (M0875/pHY300), and a *ypfP* mutant transformed with p0875 harboring the intact *ypfP* gene were spotted onto soft agar plates and incubated at 37°C for 10 h. (B) Diameters of the halos in panel A. The data are means $\pm$ standard deviations from three independent experiments. (C) Overnight cultures of the Newman strain and *dltABCD* (N0793), *tagO* (N0702), and *ypfP* (N0875) mutants of this strain were spotted onto soft agar plates and incubated at 37°C for 8 h. The data are means $\pm$ standard deviations from three independent experiments.

whether these genes are required for colony spreading in other *S. aureus* strains, we examined whether disruptions of these genes in the Newman strain decreased colony spreading. To construct *dltABCD*-, *tagO*-, and *ypfP*-disrupted mutants of the Newman strain, phage transduction was performed as described previously (18). Phage 80 α lysates of the M0793, M0702, and M0875 strains were used to infect strain Newman. Deletion of the *dltABCD* operon, the *tagO* gene, and the *ypfP* gene prevented colony spreading, even in the Newman strain (Fig. 3C). Thus, we concluded that the *dltABCD* operon, the *tagO* gene, and the *ypfP* gene are ubiquitously required for the colony spreading ability of *S. aureus*.

Concluding comments. The findings of the present study indicated that *S. aureus* can rapidly expand on soft agar surfaces. The colony spreading of *S. aureus* is different from the

darting of *S. epidermidis* described by Henrichsen (7). First, in darting, there is an empty area between cells, whereas there is no empty area between cells in colony spreading. Second, darting is observed on 1% agar plates, whereas colony spreading was not observed on 1% agar plates but was observed on 0.24% agar plates (data for 1% agar are not shown). Third, the darting speed is 6 $\mu\text{m}/\text{min}$, whereas the colony spreading speed is 100 $\mu\text{m}/\text{min}$. Therefore, we considered colony spreading to be different from darting. We examined whether *S. epidermidis* has colony spreading ability and found that the spreading ability of two *S. epidermidis* isolates from nares is much less than that of *S. aureus* (data not shown), indicating that the colony spreading ability of *S. aureus* is superior to the colony spreading ability of *S. epidermidis*. Colony spreading is apparently similar to sliding, which is one of the six classifications of

bacterial translocation described by Henrichsen (7). The definition of sliding includes a uniform sheet of closely packed cells in a single layer. Because the colony spreading of *S. aureus* is due to multiple layers of growing cells, similar to the spreading of *B. subtilis* (11), we concluded that colony spreading is similar to, but distinct from, sliding.

Genetic studies revealed that wall teichoic acid and D-alanylation of teichoic acid are required for colony spreading. In addition, colony spreading requires water, because it was observed on soft agar plates containing a lot of water on the surface and drying soft agar plates inhibited the spreading. Surfactants, including glycopeptide lipids, glycolipids, and lipopolysaccharides, have been suggested to contribute to the sliding or spreading of *M. smegmatis*, *S. marcescens*, and *V. cholerae*, respectively (3, 6, 15, 21). Teichoic acids might function as surfactants in *S. aureus* colony spreading. Teichoic acids might reduce the friction between cells and the agar surface in cooperation with water. The absence of D-alanylated teichoic acids in the *dltABCD* mutant induces a structural change in the teichoic acids due to an increased negative charge, which might result in decreased surfactant activity of the teichoic acids. A change in the lipoteichoic acid structure or the absence of free glycolipids in the *ypfP* mutant could also change the friction between the cell surface and the agar surface. Further analyses are needed to examine how teichoic acids influence *S. aureus* colony spreading ability.

Teichoic acids contribute to the pathogenicity of *S. aureus* by functioning in the attachment of *S. aureus* to the host cell surface. Previous studies revealed that *tagO* and *dltA* mutants have decreased abilities to colonize the noses of mice and to attach to human epithelial cells (26, 27). In addition, the *dltA* mutant has decreased biofilm-forming ability and is susceptible to antimicrobial peptides and phospholipase A2, both of which are involved in the host defense system (5, 13, 19). Based on the present results, teichoic acids have a new role in colony spreading of *S. aureus*. Colony spreading might allow *S. aureus* to effectively enlarge colonized areas on host tissue surfaces and catheters. The diversity of colony spreading speeds and morphologies in *S. aureus* strains might reflect the adaptation of each strain to the host microenvironment. Further investigation of the underlying mechanism of colony spreading diversity and its role in the pathogenesis of *S. aureus* might provide a better understanding of the *S. aureus* infectious process.

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