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Growth-phase dependence of susceptibility to antimicrobial peptides in *Staphylococcus aureus*

Miki Matsuo,[1] Yuichi Oogai,[1] Fuminori Kato,[2] Motoyuki Sugai[2] and Hitoshi Komatsuzawa[1]

Correspondence
Hitoshi Komatsuzawa
hkomatsu@dent.kagoshima-u.ac.jp

[1]Department of Oral Microbiology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8544, Japan

[2]Department of Bacteriology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8553, Japan

Bacterial cell surface charge is responsible for susceptibility to cationic antimicrobial peptides. Previously, *Staphylococcus aureus dlt* and *mprF* were identified as factors conferring a positive charge upon cell surfaces. In this study, we investigated the regulation of cell surface charge during growth. Using a group of *S. aureus* MW2 mutants, which are gene-inactivated in 15 types of two-component systems (TCSs), we tested *dltC* and *mprF* expression and found that two TCSs, *aps* and *agr*, were associated with *dltC* and *mprF* expression in a growth phase-dependent manner. The first of these, *aps*, which had already been identified as a sensor of antimicrobial peptides and a positive regulator of *dlt* and *mprF* expression, was expressed strongly in the exponential phase, while its expression was significantly suppressed by *agr* in the stationary phase, resulting in higher expression of *dltC* and *mprF* in the exponential phase and lower expression in the stationary phase. Since both types of expression affected the cell surface charge, the susceptibility to antimicrobial peptides and cationic antibiotics was changed during growth. Furthermore, we found that the ability to sense antimicrobial peptides only functioned in the exponential phase. These results suggest that cell surface charge is tightly regulated during growth in *S. aureus*.

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INTRODUCTION

Staphylococcus aureus is a well-known major pathogen in humans. *S. aureus* produces many toxins and exoenzymes to cause various suppurative diseases, food poisoning and toxic shock syndrome (Foster, 2004; Lowy, 1998; Manders, 1998). Furthermore, strains isolated clinically, especially meticillin-resistant *S. aureus* (MRSA), exhibit multiple antibiotic resistance (Deurenberg *et al.*, 2007; Grundmann *et al.*, 2006), resulting in serious problems with regard to therapy of *S. aureus* infectious disease. Recently, besides these virulence factors, *S. aureus* has also been found to possess the ability to produce several factors that protect against host-derived innate immune factors, such as complement, antibodies and neutrophils, as well as recently recognized innate immune antimicrobial peptides, such as LL-37 and defensins (Foster, 2005; Rooijakkers *et al.*, 2005).

Human antimicrobial peptides are one of the innate immune factors and are produced in various tissues and organs, such as the skin, lung and intestines (Ganz *et al.*, 1985; Ganz & Lehrer, 1995; Lehrer & Ganz, 1999; Selsted & Ouellette, 2005; Zaiou & Gallo, 2002). The most well-known antimicrobial peptides are the defensins. Defensins are classified into two types: alpha-defensins from neutrophils and Paneth cells, and beta-defensins from epithelial cells (Cunliffe, 2003; Ganz *et al.*, 1985; Ganz & Lehrer, 1995). Another major peptide is CAP18/LL37, which is found in neutrophils and epithelial cells (Larrick *et al.*, 1995; Ramanathan *et al.*, 2002; Zaiou & Gallo, 2002). These cationic peptides are electrostatically attracted to bacterial cell surfaces. Then, the peptides interact with membrane lipids, causing membrane permeabilization and leading to the formation of pores or gaps in the membrane. Recently, some diseases such as Crohn's disease (Fellermann *et al.*, 2003a; Wehkamp *et al.*, 2002), atopic dermatitis (Fellermann *et al.*, 2003b; Ong *et al.*, 2002) and Kostmann's disease (Pütsep *et al.*, 2002) have been demonstrated to be associated with increased microbial infection because antimicrobial peptide production is decreased in these patients. Therefore, antimicrobial peptides are believed to be critical for host defence systems.

Previously, we showed that susceptibility to antimicrobial peptides, including beta-defensin-3 and LL37, differs substantially among 497 clinically isolated strains (Ouhara

Abbreviations: CP, chloramphenicol; hBD, human beta-defensin; MRSA, meticillin-resistant *S. aureus*; TC, tetracycline; TCS, two-component system.

et al., 2008). We also showed that bacterial cell surface charge is one of the factors that affects susceptibility to antimicrobial peptides. To date, in *S. aureus*, three types of mechanism that affect susceptibility to antimicrobial peptides have been identified. These are (1) trapping defensins by binding to staphylokinase (Braff *et al.*, 2007; Jin *et al.*, 2004), (2) digestion of peptides by proteinase (Sieprawska-Lupa *et al.*, 2004), and (3) changing the bacterial cell surface charge via *dlt* and *mprF* (Li *et al.*, 2007a; Peschel *et al.*, 1999, 2001). *mprF* was first identified as *fmtC*, which was shown to affect the meticillin-resistance level in MRSA (Komatsuzawa *et al.*, 2001). Among the above mechanisms, cell surface charge is considered to play a significant role in determining susceptibility to antimicrobial peptides, because *dlt* or *mprF* inactivation results in a high level of susceptibility to these peptides. Recently, Aps, one of the two-component systems (TCSs), has been shown to sense antimicrobial peptides and regulate the expression of *dlt* and *mprF* (Li *et al.*, 2007a, b).

In this study, we investigated the regulation of *dlt* and *mprF* expression mediated by TCSs during growth in *S. aureus*. Also, we investigated the interaction of *dlt* and *mprF* expression with bacterial surface charge and susceptibility to cationic antibacterial agents.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *S. aureus* was grown in Trypticase Soy Broth (TSB; Beckton Dickinson Microbiology Systems). Tetracycline (TC; 10 μg ml^{-1}) or chloramphenicol (CP; 10 μg ml^{-1}) for *S. aureus* was added when necessary. Fifteen sets of TCS mutants in *S. aureus* MW2, which had been constructed previously (Matsuo *et al.*, 2010), were used in this study.

Construction of insertion-inactivated mutants. In this study, we constructed the *agr*- and *aps*-inactivated mutants in *S. aureus* TY34 by a method described previously (Komatsuzawa *et al.*, 2004; Matsuo *et al.*, 2010). We also tried to construct an *agr-aps* double mutant in MW2, but this was not successful. For the complementation experiment, we constructed an *agrA*-inactivated mutant by the method described above. Since we had previously constructed the *agr* mutant, which inactivated *agrCA* (Matsuo *et al.*, 2010), we first constructed a plasmid harbouring all of the *agr* genes, although this failed. *agrA*, encoding a response regulator, is the last gene in an operon consisting of four genes (*agrBDCA*) (Novick, 2003). We checked that the *agrA* mutant showed a phenotype similar to that of the *agrCA* mutant. The DNA fragment of *apsR* or *agrA* amplified with specific primers was cloned into pCL15, an *E. coli–S. aureus* shuttle vector with a P*spac* promoter (Luong & Lee, 2006). The plasmid was electroporated into RN4220. Then, the plasmid was transduced to the *agr* or *aps* mutant by phage 80alpha by a method described previously (Komatsuzawa *et al.*, 2004). The primers used for the construction of plasmids are listed in Table 2.

Quantitative analysis of gene expression during *S. aureus* growth. A small sample of *S. aureus* cultured overnight was inoculated into fresh TSB. Then, *S. aureus* cells were grown at 37 °C with shaking, and bacterial cells in various phases were collected. Total RNA was extracted from bacterial cells with a FastRNA Pro Blue kit (MP Biomedicals) in accordance with the manufacturer's protocol. One microgram of total RNA was reverse-transcribed to cDNA using a First Strand cDNA Synthesis kit (Roche). Using cDNA as template DNA, quantitative PCR was performed using a LightCycler system (Roche). Primers for *dltC*, *mprF*, *apsR* and *agrA* were constructed and used to determine the optimal conditions for analysis of their expression. The amount of *gyrA* was used as an internal control. Primers for each TCS and *gyrA* are shown in Table 2.

Assay to determine the antibacterial activity of antimicrobial peptides. An antibacterial assay was performed following a protocol described previously (Midorikawa *et al.*, 2003). Briefly, *S. aureus* strains in the appropriate phase were harvested, washed with PBS and resuspended in 10 mM sodium phosphate buffer (PB). The bacterial suspension was diluted to 10^7–10^8 cells ml^{-1} with PB, and 10 μl of the bacterial suspension (10^5–10^6 cells) was inoculated into 500 μl PB with or without antimicrobial peptides [human beta-defensin (hBD)1, -2 and -3; Peptide Institute Inc.] and incubated aerobically for 2 h at 37 °C. Dilutions of the reaction mixture (100 μl) were plated on agar medium and incubated at 37 °C overnight. The c.f.u. was determined as the total number of colonies identified on each plate. The antibacterial effect was calculated as the ratio of the number of cells surviving after exposure to antimicrobial peptides (survival rate, %) to the total number of bacteria incubated in a control PB solution.

Cytochrome *c* binding assay. To evaluate the bacterial surface charge, we used a cytochrome *c* binding assay as described elsewhere (Peschel *et al.*, 1999). Bacterial cells collected in the appropriate phase were adjusted to a concentration of 10^{10} ml^{-1} in 10 mM PB (pH 6.8), and then cytochrome *c* (Sigma Aldrich) was added to the bacterial suspension at a final concentration of 250 μg ml^{-1}. After 10 min incubation at room temperature, centrifugation at 15 000 r.p.m. for 5 min was performed, and the supernatant absorbance was measured at 530 nm. The absorbance value obtained compared with that without bacterial cells was calculated as the absorption ratio, reflecting the bacterial surface charge.

Assay of susceptibility to cationic antibacterial agents. Positively charged antibacterial agents (gentamicin, nisin, polymyxin B, gramicidin S, indolicidin and vancomycin) were obtained from Sigma Aldrich. Bacterial cells collected in the exponential or stationary phase were suspended in 10 mM PB (pH 6.8) (10^5–10^6 cells 500 μl^{-1}) with or without antibacterial agent. Nisin (final concentration 1 μg ml^{-1}), polymyxin B (1 μg ml^{-1}), gramicidin S (1 μg ml^{-1}), indolicidin (0.1 μg ml^{-1}), gentamicin (4 μg ml^{-1}) and vancomycin (2 μg ml^{-1}) were added to the buffer. After 10 min (gentamicin and vancomycin) or 2 h (nisin, indolicidin, polymyxin and gramicidin S) of incubation at 37 °C, the reaction mixture was diluted and plated on trypticase soy agar (TSA). The number of c.f.u. was counted and used to calculate the proportion of bacteria that survived by comparing with the c.f.u. in the absence of antibacterial agents.

Analysis of antimicrobial peptide sensing in *S. aureus*. Bacterial cells collected in the appropriate phase were suspended in 10 mM PB (pH 6.8) at a final concentration of 10^9 ml^{-1}. Then, hBD1, -2 and -3 at a final concentration of 10 (hBD1) or 2.5 μg ml^{-1} (hBD2, hBD3) was added to the bacterial suspension, followed by incubation for 30 min at 37 °C. After centrifugation, total RNA was extracted using the method described above. Then, cDNA synthesis and quantitative PCR were performed using the methods described above. To check the viability of the bacterial cells exposed to antimicrobial peptides, the reaction mixtures were diluted and plated on TSA. After 24 h of incubation, the number of c.f.u. was recorded. The antibacterial effect was estimated by determining the rate of survival of cells in comparison with the total number of cells added.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Gene ID*	Gene name	Characteristics	Reference or source
Strains				
MW2	–	–	Clinical strain, meticillin-resistant (*mec*+)	Baba *et al.* (2002)
FK61	MW0198-99	Unassigned	MW0199::pCL52.1 in MW2, TC^r†	Matsuo *et al.* (2010)
FK62	MW0236-37	*lytSR*	*lytS*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK64	MW0621-22	*apsRS*/*graRS*	*apsR*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK65	MW0667-68	*saeRS*	*saeR*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK66	MW1208-09	Unassigned	MW1208::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK67	MW1304-05	*arlRS*	*arlR*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK68	MW1445-46	*srrAB*	*srrA*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK69	MW1636-37	*phoPR*	*phoP*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK71	MW1789-90	Unassigned	MW1790::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK72	MW1824-25	*vraSR*	*vraS*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK73	MW1962-63	*agrCA*	*agrC*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK74	MW2002-03	*kdpDE*	*kdpD*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK75	MW2282-83	*hssRS*	*hssR*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK76	MW2313-14	*nreBC*	*nreB*::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
FK77	MW2544-45	Unassigned	MW2545::pCL52.1 in MW2, TC^r	Matsuo *et al.* (2010)
MM11	MW1963	*agrA*	*agrA*::pCL52.1 in MW2, TC^r	This study
MM12	MW0621	*apsR*	pMM12 in FK64, TC^r, CP^r‡	This study
MM14	MW1963	*agrA*	pMM14 in MM11, TC^r, CP^r	This study
TY34	–	–	Clinically isolated strain, impetigo	This study
MM15	MW0621-22	*apsRS*/*graRS*	*apsR*::pCL52.1 in TY34, TC^r	This study
MM16	MW1962-63	*agrCA*	*agrC*::pCL52.1 in TY34, TC^r	This study
RN4220	–	–	Laboratory strain, meticillin-sensitive (*mec*−)	Kreiswirth *et al.* (1983)
TY1355	–	–	Clinically isolated strain, SSSS§	This study
TY1741	–	–	Clinically isolated strain, impetigo	This study
TF2890	–	–	Clinically isolated strain, atopy	This study
TF2988	–	–	Clinically isolated strain, sepsis	This study
TF3483	–	–	Clinically isolated strain, sepsis	This study
Plasmids				
pCL52.1		–	*S. aureus* integration vector, thermosensitive, TC^r in *S. aureus*	Sau *et al.* (1997)
pCL15		–	*E. coli*–*S. aureus* shuttle vector, AP^r‖ in *E. coli*, CP^r in *S. aureus*	Luong & Lee (2006)
pMM11		*agrA*	PCR fragment for the *agr* mutant/pCL52.1	
pMM12		*apsR*	PCR fragment for the *apsR* complementation/pCL15	
pMM14		*agrA*	PCR fragment for the *agrA* complementation/pCL15	

*Gene ID in *S. aureus* MW2.
†Tetracycline resistance.
‡Chloramphenicol resistance.
§Staphylococcal scalded skin syndrome.
‖Ampicillin resistance.

RESULTS

dlt expression in 15 sets of TCS mutants

Aps/Gra has been reported to be a factor controlling *dlt* and *mprF* expression (Kraus *et al.*, 2008; Li *et al.*, 2007a). To determine whether other factors are involved in the regulation of *dlt* and *mprF* expression, we investigated the expression of *dltC* and *mprF* in 15 sets of TCS mutants in *S. aureus* MW2 in the exponential and stationary phases (Fig. 1). Since the *dlt* operon is composed of four genes, *dltA–D*, we investigated *dltC* expression to determine *dlt* expression. The results showed that *dltC* expression was decreased in the *aps* mutant in the exponential phase compared with that in the wild-type and other mutants, but that the expression in the *aps* mutant in the stationary phase was similar to that of the other mutants, except for the *agr* mutant. In the *agr* mutant, *dltC* expression in the stationary phase was significantly higher than that of the wild-type and other mutants, but expression in the *agr* mutant in the exponential phase was similar to that of the others, except for the *aps* mutant. We also investigated *mprF* expression in TCS mutants, and obtained results that were similar to those for *dltC* expression (data not shown).

Table 2. Primers used in this study

Gene ID*	Gene name	Specific primer for quantitative PCR	
		Forward	Reverse
Quantitative PCR			
MW0006	*gyrA*	5′-aag gtg ttc gct taa ttc gc-3′	5′-att gca ttt cct ggt gtt tc-3′
MW0621	*apsR*	5′-gga tca agt gat gag tat gga a-3′	5′-aca gca tct tgc caa gtc a-3′
MM0816	*dltC*	5′-agc aga agt agc aga aaa tg-3′	5′-gcc cac tca tct cta tca a-3′
MW1247	*mprF*	5′-tca cag tgg cga cat tct t-3′	5′-tac tgg gcg ttt caa cct ac-3′
MW1963	*agrA*	5′-gca gtg aaa ttc gta agc at-3′	5′-cga gtt ctt aat tct gct gga-3′
Construction of the mutant			
MW1963	*agrA*	5′-gag gat cct tat gcg gtg ctt gag c-3′	5′-caa agc ttt ggc gat tga cga caa a-3′
Construction for complementation			
MW0621	*apsR*	5′-gat aag ctt cag gag aaa ata tga a-3′	5′-cca gga tcc cca gtt cat gcg aga ttt c-3′
MW1963	*agrA*	5′-aaa agc tta aat tat taa caa cta gcc-3′	5′-ttg gat ccc cag cta tac agt gca t-3′

*Gene ID in *S. aureus* MW2.

In the complementation experiment, we found that the phenotype of the *aps* mutant was restored by complementation with *apsR*, showing the decreased susceptibility to hBD3 and the increased expression of *dltC* compared with those of the *aps* mutant. Also, the phenotype of the *agrA* mutant was restored by complementation with *agrA* (Table 3). Furthermore, we found that complementation with *agrA* in the *agr* mutant decreased the expression of *aps*, while complementation with *apsR* in the *aps* mutant did not affect the expression of *agr* (data not shown).

Then, we constructed *aps*- and *agr*-inactivated mutants in another MRSA strain, TY34, to determine whether this result was specific to the MW2 strain. We obtained a result similar to that for MW2 (Table 3). The expression of *dltC* in the *aps*-inactivated mutant in the exponential phase was

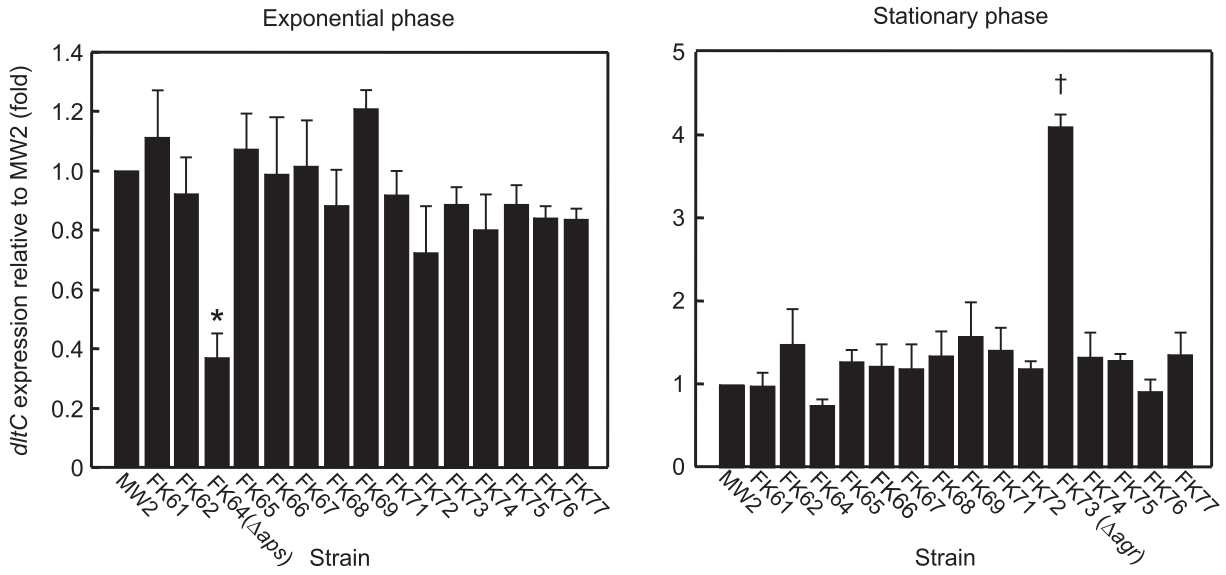

Fig. 1. *dltC* expression in the MW2 wild-type and its TCS mutants in the early exponential and stationary phases. Cells of MW2 and 15 TCS mutants were collected in the early exponential or stationary phase. *dltC* expression was measured by quantitative PCR, as described in Methods. Data shown represent mean ± SEM of triplicate measurements. *Significant decrease from the *dltC* expression of the wild-type strain MW2 as determined by Dunnett's test, $P<0.001$. †Significant increase from the *dltC* expression of the wild-type strain MW2 as determined by Dunnett's test, $P<0.001$.

Table 3. Susceptibility to hBD3 and *dltC* expression in the exponential and stationary phases

Strain	Characteristics	Survival rate (%) in the presence of hBD3*		*dltC* expression†	
		EXP‡	STA§	EXP	STA
MW2	Clinical	62.1	35.9	1.69	0.10
FK64	Δ*aps*	28.8	32.3	0.35	0.07
MM11	Δ*agr*	61.9	60.8	1.11	0.53
MM12	*apsR*/FK64	54.8	51.6	1.26	0.26
MM14	*agrA*/MM11	28.4	29.7	0.74	0.06
RN4220	Laboratory	87.1	49.2	1.20	0.25
TY1355	Clinical	30.3	12.0	0.003	0.0012
TY1741	Clinical	84.3	53.0	0.91	0.17
TF2890	Clinical	73.7	38.0	1.41	0.16
TF2988	Clinical	82.0	25.7	1.23	0.12
TF3483	Clinical	83.0	54.0	0.78	0.22
TY34	Clinical	52.8	43.6	0.95	0.30
MM15	Δ*aps*	34.2	45.0	0.26	0.24
MM16	Δ*agr*	61.6	69.2	1.11	2.57

*Survival rate was estimated as cell viability [c.f.u. (hBD3-treated)/c.f.u. (not treated)] expressed as a percentage. Data shown represent mean values of three independent experiments.
†*dltC* expression was evaluated as a ratio with respect to *gyrA* expression in three independent experiments.
‡EXP, exponential phase.
§STA, stationary phase.

lower than that of the wild-type, while its expression in the *agr*-inactivated mutant in the stationary phase was higher.

dltC and *mprF* expression during growth

S. aureus MW2 cells in early, mid- and late-exponential phases and in the stationary phase were collected (Fig. 2a), and then the mRNA expression of *dltC* and *mprF* was analysed by quantitative PCR (Fig. 2b, c). The patterns of *dltC* and *mprF* expression during growth were quite similar, with no significant changes in the expression levels of the two genes in the exponential phase but drastic decreases in both in the stationary phase. We then investigated *aps* and *agr* expression during growth (Fig. 2d, e). The peak in *aps* expression was in the mid-exponential phase; this expression then decreased, especially in the stationary phase. In contrast, *agr* expression was quite low in the early and mid-exponential phase, and high in the stationary phase.

To analyse *dltC* and *mprF* expression in more detail, we investigated their expression in both the *aps* mutant and the *agr* mutant (Fig. 2b, c). The growth rate of the two mutants was similar to that of the wild-type. The *aps* mutant exhibited decreased *dltC* and *mprF* expression in the exponential phase compared with the wild-type, and showed expression levels that were similar to those of the wild-type in the stationary phase. The *agr* mutant showed similar expression to that of the wild-type in the early to mid-exponential phase, and increased levels of expression of both *dltC* and *mprF* in the late-exponential phase and, especially, in the stationary phase.

Since *aps* and *agr* affected *dlt*/*mprF* expression during growth, we then investigated whether the two TCSs affected each other (Fig. 2d, e). In *the aps* mutant, the *agr* expression pattern during growth was quite similar to that of the wild-type. On the other hand, *aps* expression was increased in the *agr* mutant compared with that in the wild-type. In particular, the *aps* expression level in the *agr* mutant increased in the stationary phase.

Susceptibility to antimicrobial peptides during growth

Since we found that the levels of *dltC* and *mprF* expression changed during growth, we analysed susceptibility to hBD3 (0.5 μg ml^{-1}) in the wild-type (MW2 strain), *the aps* mutant and the *agr* mutant during growth (Fig. 3a). In the wild-type, susceptibility to hBD3 gradually increased during growth. In particular, the susceptibility to hBD3 in the stationary phase was higher (about half the level of survival compared with that in the early exponential phase) than that in the early exponential phase. The susceptibility to hBD3 of the *aps* mutant showed almost the same level of survival (40 %) throughout growth. This survival rate was significantly lower than that of the wild-type in the exponential phase, but was almost the same as that in the stationary phase. In contrast, the *agr* mutant showed almost the same survival rate as the wild-type in the early, mid- and late-exponential phases, while the survival rate in the stationary phase was higher than that of the wild-type. We also investigated the susceptibility to other beta-defensins (hBD1 and hBD2) in the early exponential and

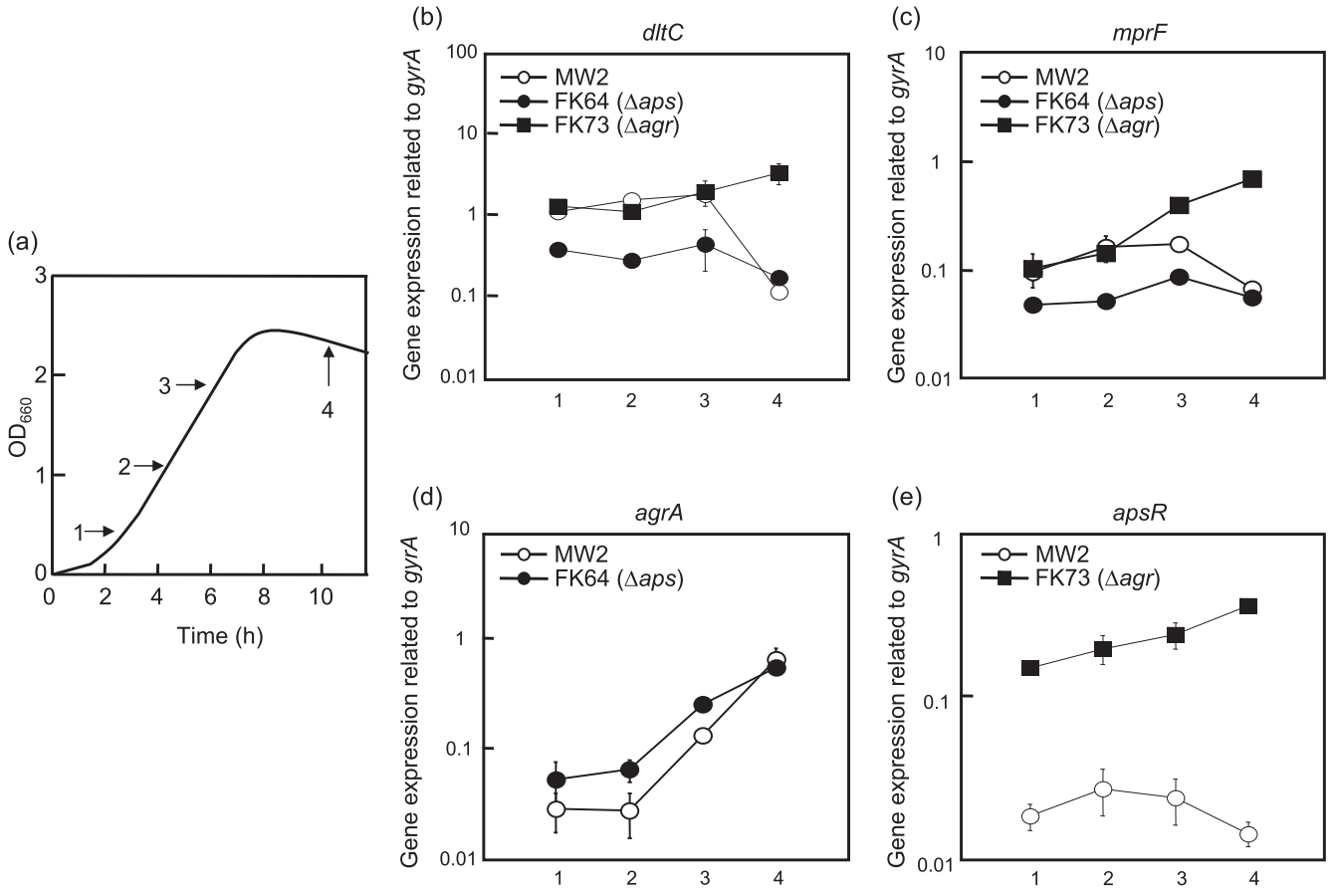

Fig. 2. *dlt*, *mprF*, *apsR* and *agrA* expression in *S. aureus* during growth. (a) Growth curve of MW2 wild-type and derived strains. Cells of MW2, FK64 (Δ*aps*) and FK73 (Δ*agr*) were collected at four time points, indicated by arrows (1, early exponential phase; 2, mid-exponential phase; 3, late-exponential phase; 4, stationary phase). *dltC* (b), *mprF* (c), *agrA* (d) and *apsR* (e) expression at the four points during growth was determined by quantitative PCR, as described in Methods. Data shown represent mean ± SEM of triplicate measurements.

stationary phases. The results showed the same trend as that of hBD3, with a high susceptibility in the *aps* mutant in the exponential phase and a low susceptibility in the *agr* mutant in the stationary phase compared with those of the wild-type (Table 4).

Cytochrome *c* binding assay

Bacterial cell surface charge is known to affect susceptibility to cationic antimicrobial peptides such as defensins. To evaluate the cell surface charge, we measured the cytochrome *c* binding affinity of the wild-type and mutant cells during growth (Fig. 3b). Cytochrome *c* is a cationic protein, and shows a high affinity for strongly negatively charged cells (Peschel *et al.*, 1999). In the wild-type strain, the binding affinity in the exponential phase (early, 40.8 %; mid, 45.2 %) was lower than that in the stationary phase (54.3 %), implying that the bacterial cell surface charge in the stationary phase is more negative than that in the exponential phase. The binding affinity of the *aps* mutant in early and mid-exponential phase was significantly higher than that of the wild-type, while the affinity of the two strains was almost the same in the stationary phase because the wild-type exhibited increased binding affinity in the stationary phase. The *agr* mutant in the stationary phase showed a drastically decreased binding affinity compared with the wild-type. In summary, the cell surface charge of the *aps* mutant in the exponential phase was highly negative, and the charge of the *agr* mutant in the stationary phase was significantly lower than that of the wild-type.

Susceptibility to cationic antibacterial agents during growth

In the wild-type strain, susceptibility to the cationic antibacterial agents nisin, indolicidin, polymyxin B, gramicidin S, gentamicin and vancomycin in the exponential phase was lower than that in the stationary phase (Table 4), showing similar results in terms of the susceptibility to antimicrobial peptides. In addition, in the exponential

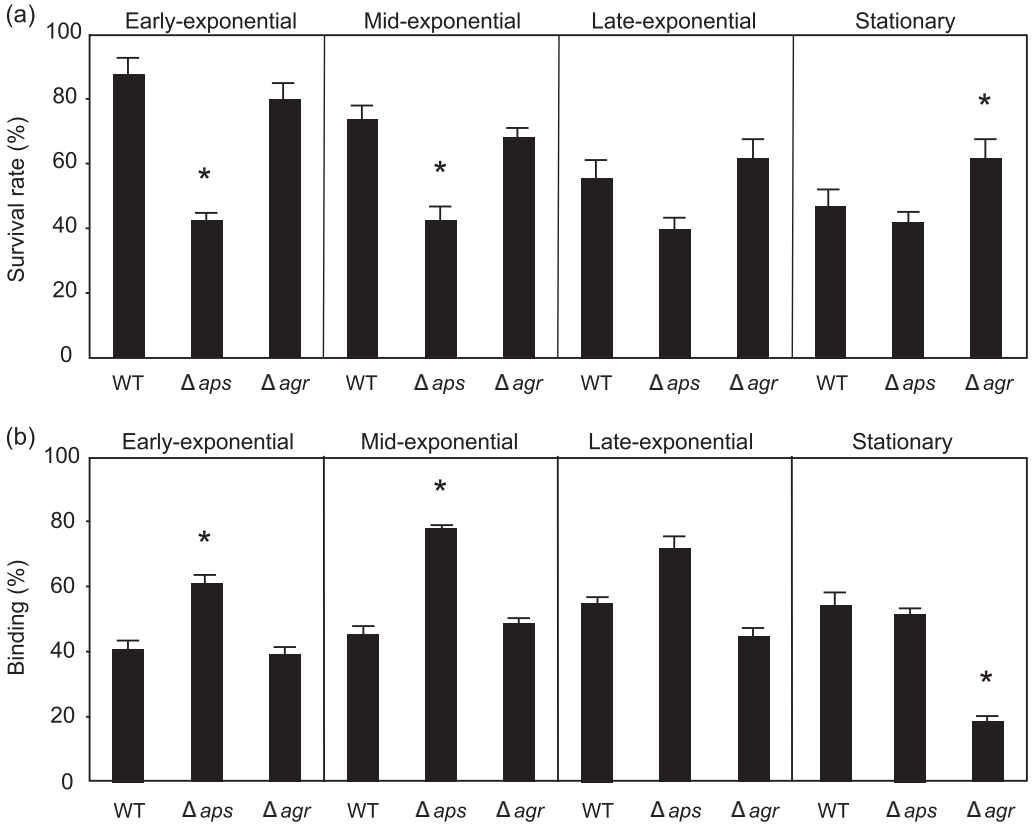

Fig. 3. Susceptibility to hBD3 and cytochrome *c* binding affinity of *S. aureus* during growth. (a) Susceptibility to hBD3 of strains MW2, FK64 (Δ*aps*) and FK73 (Δ*agr*) during growth. Bacterial cells (10^5–10^6) in various phases were inoculated into 500 μl 10 mM PB (pH 6.8) with or without 0.5 μg hBD3 ml^{-1} and incubated for 2 h at 37 °C. The antibacterial effect was calculated as the ratio of the number of surviving cells (survival rate, %) to the total number of bacteria incubated in buffer without hBD3. Data shown represent mean + SEM of triplicate measurements. (b) Evaluation of bacterial cell surface charge during growth. The graph shows percentage binding of 250 μg cytochrome *c* ml^{-1} after 10 min incubation with whole cells of strains MW2, FK64 (Δ*aps*) and FK73 (Δ*agr*) at room temperature. Data shown represent mean + SEM of triplicate measurements. $^*P<0.05$ (Dunnett's test). Calculations of statistical significance are for comparisons with the corresponding data for the MW2 wild-type.

phase the *aps* mutant showed increased susceptibility to cationic peptides compared with that of the wild-type; however, in the stationary phase, the susceptibility was almost the same in the wild-type and the mutant. The *agr* mutant in the stationary phase showed decreased susceptibility to cationic peptides compared with the wild-type.

Correlation between susceptibility to antimicrobial peptides and *dlt* expression in clinical isolates

Seven clinical isolates, including MW2 and TY34, and one laboratory strain were investigated (Table 3). All strains tested showed that susceptibility to hBD3 (0.5 μg ml^{-1}) in the exponential phase was lower than that in the stationary phase, although the ratio of the susceptibilities between the exponential and stationary phases differed among strains. In addition, in all strains, *dltC* expression in the exponential phase was higher than that in the stationary phase, indicating that susceptibility to hBD3 and *dltC* expression were correlated in all strains, as well as with the results for strain MW2. Strain TY1355, which exhibited a low level of *dlt* expression compared with the other strains, showed high susceptibility to hBD3.

Sensing antimicrobial peptides during growth

It has been reported that the *aps* sytem in *Staphylococcus epidermidis* senses antimicrobial peptides such as hBD3, nisin and histatin (Li *et al.*, 2007b). In *S. aureus*, the *aps* system also senses indolicidin, nisin and melitin and increases the expression of *dlt*, while the expression of *dlt* in the presence of hBD3 is weak compared with that of indolicidin, nisin or melitin (Li *et al.*, 2007a). Since we found that *dltC* expression changed during growth, we hypothesized that the sensing system in *S. aureus* changes during growth. Therefore, we analysed the ability to sense antimicrobial peptides in cells in the exponential and

Table 4. Susceptibility to various antibacterial agents in *S. aureus* TCS mutants

Agent	Dose (μg ml^{-1})	Survival rate (%)†					
		Exponential phase			Stationary phase		
		WT‡	Δ*aps*	Δ*agr*	WT	Δ*aps*	Δ*agr*
hBD1	2	37.9	13.3**	42.2	26.1	30.7	69.0**
hBD2	0.5	56.7	29.6**	57.8	26.1	23.7	45.8*
Nisin	1	38.1	13.1*	27.6	24.7	22.3	26.0
Indolicidin	0.1	55.1	15.6*	42.3	36.4	31.0	52.3
Polymyxin B	1	24.5	8.5*	32.6	15.3	15.7	28.5*
Gramicidin S	1	57.4	23.8*	51.6	28.6	26.3	54.4*
Gentamicin	4	43.8	6.7*	40.7	29.2	32.6	60.0**
Vancomycin	2	46.4	23.6*	41.9	26.4	35.0	70.5**

†Survival rate was estimated as cell viability [c.f.u. (antibacterial agent-treated)/c.f.u. (not treated)] expressed as a percentage. Data shown represent mean values of three independent experiments.
‡WT, wild-type.
*$P<0.05$, **$P<0.01$ (Dunnett's test). Calculations of statistical significance are for comparison with the corresponding data for the MW2 wild-type.

stationary phases. Before assaying, we confirmed that 2.5 μg hBD3 ml^{-1} had no significant effect on cell viability.

We investigated the *dltC* expression of the wild-type and *aps* mutant in the exponential and stationary phases (Fig. 4). We consider the difference from the earlier results (Li *et al.*, 2007b) (weak sensing of hBD3 in *S. aureus*) to be due to the phase of the bacterial cells and/or the conditions of the assay (10 mM phosphate buffer in this study, 10 mM phosphate buffer with 100 mM NaCl in the earlier study). We found that hBD3 induced *dltC* expression of the wild-type in the exponential phase, but found no induction in the *aps* mutant. Also, we found that *dltC* expression of the wild-type in buffer without hBD3 was decreased to almost half after 30 min incubation, while that of the *aps* mutant was not decreased (data not shown). In contrast, *dltC* expression of both strains in the stationary phase was not induced by hBD3. In addition, we used hBD1 and hBD2 as inducers, and obtained similar results to those for hBD3 (Fig. 4). The *aps* expression of the wild-type in the exponential phase was also induced by hBD3, but its expression in the stationary phase was not induced (data not shown).

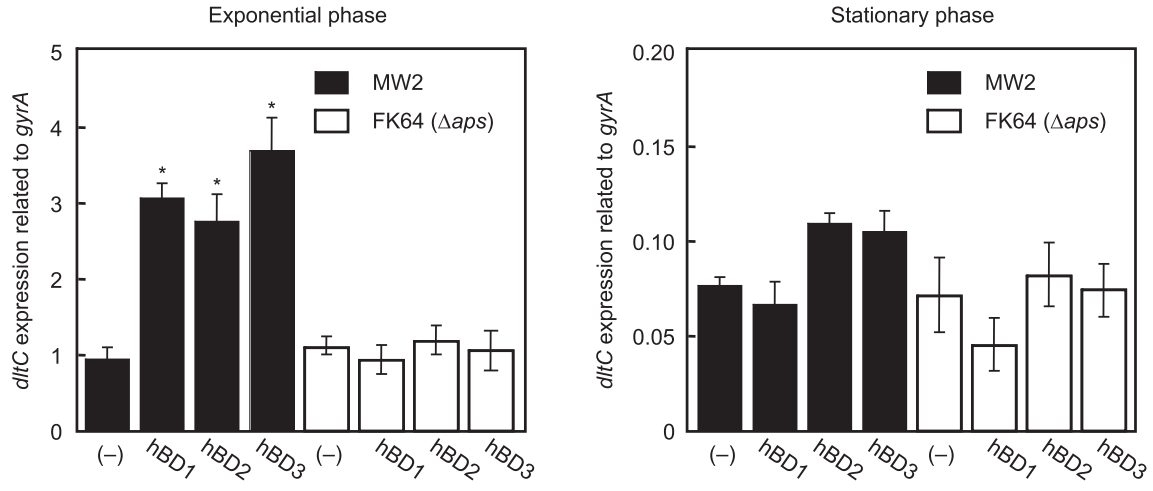

Fig. 4. Induction of *dlt* expression by sensing of hBD via *aps*. MW2 wild-type and *aps* mutant cells were collected in the early exponential and stationary phases. Cells were suspended in PB to give a concentration of 10^9 cells ml^{-1}. Concentrations of 10 μg hBD1 ml^{-1}, 2.5 μg hBD2 ml^{-1} and 2.5 μg hBD3 ml^{-1} were added separately to bacterial suspensions and incubated for 30 min at 37 °C. Calculations of statistical significance are for comparisons with the corresponding data for MW2. *$P<0.01$ (Dunnett's test). Data shown represent mean ± SEM of triplicate measurements. (–), No hBD added.

DISCUSSION

In this study, we initially showed that the charge of the bacterial cell surface in *S. aureus* changed during growth. Dlt and MprF have been reported to be major contributors to a decreased negative charge of cell surfaces because these factors are associated with the addition of amino acids (*dlt* for alanine and *mprF* for lysine) to teichoic acids and phosphatidylglycerol, respectively (Collins *et al.*, 2002; Peschel *et al.*, 1999, 2001). Alanylation of teichoic acids in cell walls and lysyl-phosphatidylglycerol in membranes confer a positive charge upon bacterial cell surfaces, which results in a shift to a weak negative charge on the cell surface (Peschel *et al.*, 1999, 2001). Earlier reports and our present findings demonstrate that *aps*/*gra*, a TCS in *S. aureus*, positively regulates the expression of *dlt* and *mprF* (Kraus *et al.*, 2008; Li *et al.*, 2007a). In addition, our new finding is that *aps*/*gra* expression is also regulated by *agr*, which has a central role in the quorum-sensing system (Novick & Geisinger, 2008); this implies that *agr* also regulates the expression of *dlt* and *mprF* by controlling *aps*. Since *agr* expression is dependent on cell density (Cheung *et al.*, 2004; Novick, 2003; Novick & Geisinger, 2008), *agr* expression gradually increases during growth, showing high expression in the stationary phase and low expression in the exponential phase. Our results showed that *aps* expression during growth decreases gradually in an inverse relationship with the level of *agr* expression. In addition, *aps* expression was significantly increased in the *agr* mutant, especially in the stationary phase. These results indicate that *aps* is negatively regulated by *agr*, and is mainly expressed in the exponential phase.

Agr, a quorum-sensing system, is involved in the expression of many factors, including virulence factors mediated by RNAIII. RNAIII has been demonstrated to directly upregulate *hla* (alpha-haemolysin) (Morfeldt *et al.*, 1995), and downregulate *spa* (protein A) (Huntzinger *et al.*, 2005) and the transcription factor *rot* responsible for the repression of toxins (Geisinger *et al.*, 2006). RNAIII binds to the target mRNA directly, resulting in the up- or down-regulation of gene expression. However, the precise mechanism of the expression of other virulence factors mediated by the *agr* system is still unknown. The mechanism of *aps* regulation by *agr* also needs further investigation. Since *aps* is a positive regulator of *dlt* and *mprF* (Kraus *et al.*, 2008; Li *et al.*, 2007a), *S. aureus* cells in the stationary phase are considered to be highly susceptible to antimicrobial peptides, owing to the strong negative charge of the cell surface caused by decreased *dlt* and *mprF* expression through the *agr–aps* pathway. Moreover, susceptibility to several positively charged agents has also been shown to be growth-dependent. Therefore, we conclude that, in *S. aureus*, susceptibility to some positively charged antibacterial agents changes during growth.

Since *aps* expression was higher in the exponential phase than in the stationary phase, we consider that the ability to sense antimicrobial peptides is also growth phase-dependent. Our results showed that the *aps* system to sense hBDs (hBD1–3) only functioned in the exponential phase, and not in the stationary phase. We also identified an ability to sense alpha-defensin and indolicidin in *S. aureus* cells in the exponential phase, but not in the stationary phase (data not shown). These results indicate

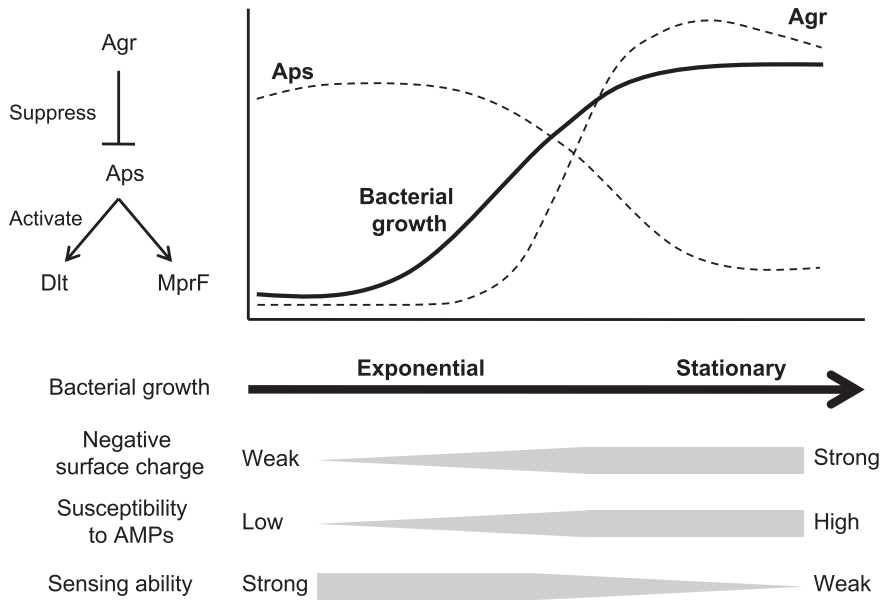

Fig. 5. Bacterial cell surface charge is regulated during growth. Gene products involved in the regulation of surface charge are indicated (upper left), and a model for *aps* and *agr* expression during growth is also shown (upper right). Cell surface charge, susceptibility to antimicrobial peptides (AMPs) and ability to sense antimicrobial peptides change during growth.

that the induction of *dlt* expression by antimicrobial peptides through the *aps* system is growth phase-dependent in *S. aureus*.

Besides affecting susceptibility to antimicrobial peptides, bacterial cell surface charge has been reported to affect several other biological activities, such as adhesion to biomaterials and mammalian cells, and phagocytic activity (Collins *et al.*, 2002; Gottenbos *et al.*, 2001; Gross *et al.*, 2001; Peschel *et al.*, 2001). In particular, *dlt* plays a central role in the regulation of cell surface charge. The absence of *dlt* in *S. aureus* results in a high susceptibility to being killed by neutrophils and also reduces the ability to colonize plastic or glass surfaces, a factor in catheter-associated infection (Collins *et al.*, 2002; Gross *et al.*, 2001). In addition, it has been found that a *dlt* mutant is highly susceptible to human phospholipase A_2, a cationic protein that exhibits antibacterial activity against *S. aureus*, owing to a strong surface negative charge (Koprivnjak *et al.*, 2002). Furthermore, *dlt* in group A streptococci has been found to promote survival in neutrophils and invasion of epithelial cells (Kristian *et al.*, 2005), and *dlt* in *Streptococcus agalactiae* and *Listeria monocytogenes* is associated with susceptibility to phagocytic cells (Abachin *et al.*, 2002; Poyart *et al.*, 2003). Taken together with our results in the present study, these findings suggest that in *S. aureus* not only susceptibility to antimicrobial peptides but also other biological activities change during growth.

As for antibiotic susceptibility, we demonstrated increased susceptibility to several cationic agents in the *aps* mutant, which implies that the charge of the bacterial cell surface affects susceptibility to antibiotics. Similarly, daptomycin susceptibility has been shown to be affected by cell surface charge mediated by *mprF* and *dlt* (Yang *et al.*, 2009a, b). Aps/Gra has been found to be related to susceptibility to glycopeptides in vancomycin-intermediate *S. aureus* (VISA), owing to transporters (*vraFG*) located immediately downstream of *aps*/*gra* and regulated by *aps*/*gra* (Howden *et al.*, 2008; Meehl *et al.*, 2007). Inactivation of *aps* in VISA has been demonstrated to increase susceptibility to vancomycin, because *vraFG* expression is suppressed. Although *vraFG* is strongly associated with vancomycin susceptibility, a more negatively charged cell surface in an *aps*/*gra* mutant also increased susceptibility to positively charged vancomycin (Table 4).

In conclusion, we have demonstrated that *S. aureus* cell surface charge is related to the expression of *dlt* and *mprF*, which are tightly regulated by *aps* and *agr* during growth (Fig. 5). It is interesting that not only many virulence factors but also cell surface charge depend on the growth phase and are mediated by the *agr* system. We have also shown that susceptibility to antimicrobial peptides and the ability to sense antimicrobial peptides change during growth. Our study indicates that bacterial cell surface charge is tightly regulated during growth, and that some features associated with surface charge are dependent on the growth phase.

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