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Global Virulence Regulation in *Staphylococcus aureus*: Pinpointing the Roles of ClpP and ClpX in the *sar*/*agr* Regulatory Network

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Staphylococcus aureus **causes infections ranging from superficial wound infections to life-threatening systemic infections. Essential for *S. aureus* pathogenicity are a number of cell-wall-associated and secreted proteins that are controlled by a complex regulatory network involving the quorum-sensing *agr* locus and a large set of transcription factors belonging to the Sar family. Recently, we revealed a new layer of regulation by showing that mutants lacking the ClpXP protease produce reduced amounts of several extracellular virulence factors and that, independently of ClpP, ClpX is required for transcription of *spa*, encoding Protein A. Here we find that the independent effect of ClpX is not general for other cell wall proteins, as expression of fibronectin- and fibrinogen-binding proteins was increased in the absence of either ClpX or ClpP. To assess the roles of ClpX and ClpP within the *sar*/*agr* regulatory network, deletions in *clpX* and *clpP* were combined with mutations in these genes. Interestingly, the derepression of *spa* transcription normally observed in an *agr*-negative strain was abolished in cells devoid of ClpX, and apparently ClpX modulates both SarS-dependent and SarS-independent control of *spa* expression, perhaps through the Sar family member Rot. Examination of expression of a single secreted protein, the SspA serine protease, revealed that ClpXP, similar to *agr*, is required for growth phase-dependent transcriptional induction of *sspa*. Intriguingly, induction was restored by the concomitant inactivation of Rot. We hypothesize that RNAIII accumulating in the postexponential phase may target Rot for degradation by ClpXP, leading to derepression of *sspA*.**

Staphylococcus aureus is a member of the normal skin and nasal flora in at least 25 to 30% of healthy humans but is also a major opportunistic pathogen capable of causing a wide spectrum of infections, ranging from superficial wound infections to life-threatening deep infections such as septicemia, endocarditis, and toxic shock syndrome. Hospitalized patients are at particular risk, and *S. aureus* is one of the major causes of hospital-acquired infections. Essential for *S. aureus* pathogenicity are a large number of cell-surface-associated proteins and secreted proteins. The surface-associated proteins allow *S. aureus* to bind to host fibrinogen, fibronectin, collagen, and von Willebrand factor, thus enabling the bacteria to colonize and establish a focus of infection (15, 32, 33). The secreted proteins include tissue-degrading enzymes and toxins (hemolysins, enterotoxins, proteases, lipases, and coagulases) (28). The secreted and cell surface proteins are produced coordinately in a growth-phase-dependent manner so that the cell surface-anchored proteins are synthesized mainly in the beginning of infection, whereas the toxins and tissue-degrading enzymes are preferentially synthesized when infection is well established (26, 35). Essential for this coordinated regulation is the *agr* locus carrying two divergently transcribed transcripts, RNAII and RNAIII (22). The RNAII transcript encodes a two-component signal transduction system which responds to the extracellular concentration of a secreted octapeptide also encoded by RNAII (19, 30). Induction of this quorum-sensing mechanism results in production of the 514-nucleotide RNAIII transcript that is the actual effector of virulence gene expression (18, 31). RNAIII acts primarily on target gene transcription; however, the molecular details of how RNAIII stimulates transcription of exoproteins such as α-toxin and represses transcription of surface proteins like Protein A remain obscure (reviewed in reference 29). Another global regulator, the DNA binding protein SarA, is required for maximal expression of RNAIII (7, 14). Furthermore, SarA independently of *agr* regulates transcription of selected target genes by a mechanism that apparently involves direct binding of SarA to the promoter region (10, 39, 44). Genes encoding extracellular proteases are generally repressed by SarA and positively regulated by *agr*, while Protein A (encoded by *spa*) is an example of a cell-wall-anchored protein whose synthesis is negatively regulated independently by both *agr* and SarA. Genome sequencing has revealed that the *S. aureus* genome encodes at least 13 proteins that have homology to SarA, and presently a regulatory role in virulence gene expression has been verified for 7 of these (SarS, SarT, SarU, SarV, Rot, MgrA, and TcaR) (reviewed in reference 9). Current knowledge supports that these regulators can control expression of target genes either directly (by binding to the promoter sequence of target genes) or indirectly (by modulating the level of other regulatory proteins), thereby forming a complicated regulatory network (8, 17, 25, 38, 39). As an example, SarS was identified as a direct activator of *spa* transcription, and it was verified that the strong induction of *spa* transcription, observed in the absence of the *agr* locus, was partly due to enhanced transcription of *sarS* (8, 39). The *agr*-mediated down-regulation of *sarS* transcription involves another Sar homologue, namely SarT. Apparently SarT functions as a positive activator of *sarS* transcription by directly binding to the *sarS* promoter, thereby stimulating tran-

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristic(s)	Strain background	Source or reference
8325-4	Wild-type strain (*rsbU* mutant)	8325-4	27
8325-4 Δ*clpP*	The entire *clpP* gene deleted	8325-4	11
8325-4 Δ*clpX*	651 in-frame deletion in *clpX*	8325-4	11
RN6911	*agr*::Δ*tetM* (Tc^r)	RN6390	31
DF2268	Δ*clpP agr*::Δ*tetM* (Tc^r)	8325-4	This study
DF2269	Δ*clpX agr*::Δ*tetM* (Tc^r)	8325-4	This study
PC1839	*sarA*::*km* (Km^r)	8325-4	5
DF2271	Δ*clpP sarA*::*km* (Km^r)	8325-4	This study
DF2308	*agr*::Δ*tetM* (Tc^r) *sarA*::*km* (Tc^r Km^r)	8325-4	This study
KT201	*sarHI*::pKT200 (Em^r)	8325-4	39
DF2296	Δ*clpP sarHI*::pKT200 (Em^r)	8325-4	This study
DF2297	Δ*clpX sarHI*::pKT200 (Em^r)	8325-4	This study
KT202	*agr*::Δ*tetM sarHI*::pKT200 (Tc^r Em^r)	8325-4	39
DF2298	Δ*clpP agr*::Δ*tetM sarHI*::pKT200 (Tc^r Em^r)	8325-4	This study
DF2299	Δ*clpX agr*::Δ*tetM sarHI*::pKT200 (Tc^r Em^r)	8325-4	This study
PM614	*agr* null; *rot*::TN*917*	RN6390	25
DF2332	Δ*clpP rot*::TN*917*	8325-4	This study
DF2333	Δ*clpX rot*::TN*917*	8325-4	This study
DF2405	*rot*::TN*917 sarA*::*km* (Km^r)	8325-4	This study
DF2432	*rot*::TN*917 agr*::Δ*tetM* (Tc^r)	RN6390	This study

scription (38). Additionally, Rot, originally identified in a transposon mutagenesis search as a repressor of toxins, was shown to be a positive regulator of *spa* transcription (25, 36). Preliminary data, moreover, showed that Rot was required for transcription of *sarS*, indicating that the positive effect of Rot on *spa* transcription is also mediated through SarS (36). Finally, the complexity of the regulatory circuit controlling *spa* transcription was strengthened by the recent finding that MgrA, yet another Sar homologue, impacts negatively on *spa* transcription by independently of SarT, controlling SarS expression (17).

Energy-dependent proteolysis plays an important role in the general turnover of damaged protein and in regulated degradation of short-lived regulatory proteins in both prokaryotic and eukaryotic cells (13). In the past decade extensive research has focused on the well-conserved ClpP proteolytic complexes that bear structural resemblance to the eukaryotic 26S proteasome (21, 41). Two heptameric rings of the ClpP peptidase form a central proteolytic barrel, and an attached Clp ATPase determines access to this proteolytic chamber. Independently of ClpP, the Clp ATPase subunit has protein reactivation and remodeling activities characteristic of molecular chaperones (42, 43). Recent work in our laboratory has demonstrated that inactivation of *clpP* or *clpX*, encoding a Clp ATPase that in *Escherichia coli* and *Bacillus subtilis* combines with ClpP (12, 42), severely reduced virulence of *S. aureus* when tested in a murine skin abscess model (11). Furthermore, our data showed that the activity of α-hemolysin and extracellular proteases was greatly reduced in the mutants, and that at least for α-hemolysin, the reduction occurred at the transcriptional level. The finding that both transcription of RNAIII and the activity of the autoinducing peptide were reduced in the *clp* mutants led us to propose that ClpXP regulates synthesis of virulence genes through *agr* (11). Additionally, we observed that while transcription of *spa*, encoding Protein A, was only slightly reduced in the *clpP* mutant strain, it was nearly abolished in the *clpX* mutant, suggesting that ClpX independently of ClpP is required for *spa* transcription.

In the present study we aimed to assess the roles of ClpX and ClpP within the Sar/*agr* regulatory network by combining the *clpX* and *clpP* deletions with mutations in *agr*, *sarA*, and other relevant genes encoding Sar homologues and looking at expression of the cell-wall-associated protein Protein A and an extracellular protein, SspA. Moreover, we have examined whether ClpP or ClpX influences expression of other adhesion proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study are listed in Table 1. *S. aureus* strains were maintained in tryptic soy broth (TSB) medium (Oxoid). For solid medium, 1.5% agar was added to give tryptic soy agar (TSA) plates. To select for antibiotic-resistant *S. aureus* strains, tetracycline (5 μg ml^{-1}), erythromycin (5 μg ml^{-1}), lincomycin (25 μg ml^{-1}), or kanamycin (50 μg ml^{-1}) was added as required.

RNA extraction and Northern blot analysis. Cultures were grown at 37°C with vigorous shaking and at an optical density at 600 nm (OD_{600}) of 0.8 ± 0.1, and at an OD_{600} of 2.0 ± 0.1 samples were withdrawn for the isolation of RNA. Cells were quickly cooled on a ethanol-dry ice bath and frozen at −80°C until extraction of RNA. Cells were lysed mechanically using the FastPrep machine (Bio101; Q-biogene), and RNA was isolated by the RNeasy mini kit (QIAGEN, Valencia, Calif.) according to the manufacturer's instructions. Total RNA was quantified by spectrophotometric analysis (λ = 260 nm), and 5 μg of RNA of each preparation was loaded onto a 1% agarose gel and separated in 10 mM sodium phosphate buffer as described previously (34). RNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) by capillary blotting as described by Sambrook et al. (37). Hybridization was performed according to Arnau et al. (1) using gene-specific probes that had been labeled with [^{32}P]dCTP using the Ready-to-Go DNA-labeling beads from Amersham Biosciences. Internal fragments of the genes below (amplified with the primers given in parenthesis) were used as templates in the labeling reactions: *fnbA* (5′-CACAATCTCA AGACAATAGCG and 5′-CGTATTTGCATATACACTC), *clfB* (5′-GAGTCG CTGTCTGAATCTG and 5′-GGTGTAGATACAGCTTCAG), *spa* (5′-GGTG TAGGTATTGGATCTG and 5′-GCTCCTGAAGGATCGTC), and *sspA* (5′-C ACTTGTGAGTTCTCCAGC and 5′-CCCAATGAATTCCGATCAG). All steps were repeated in two (*clfB* and *fnbA*) or three (*spa* and *sspA*) independent experiments giving similar results.

Preparation and analysis of extracellular and cell surface proteins. Western blotting for detection of Protein A, ClfA, and ClfB. The strains were streaked on TSA plates containing appropriate antibiotics and were incubated overnight at 37°C. The next day a streak of small colonies was used to inoculate 25 ml of prewarmed TSB in a 250-ml Erlenmeyer flask (no antibiotics added) to an OD_{600}

of <0.05. The cultures were incubated with vigorous shaking at 37°C overnight (~17 h). The next morning the OD_{600} of the cultures was measured, and 15 ml of culture was centrifuged to precipitate the cells. The supernatant was transferred to a 50-ml blue cap bottle (placed in an ice-water bath), and the extracellular proteins were precipitated by adding 1 volume of ice-cold 96% ethanol and left in the refrigerator overnight for proteins to precipitate. Precipitated proteins were collected by centrifugation (15,000 × *g*; 30 min; 0°C). Protein pellets were suspended in a volume of 50 mM Tris-HCl adjusted to the original OD_{600} of the overnight culture so that 15 ml of overnight culture with an OD_{600} of 5.0 was suspended in 0.8 ml of 50 mM Tris-HCl. Fifteen microliters of the protein extracts was analyzed on NuPAGE Bis-Tris gels (Invitrogen) using the X Cell SureLock Mini-Cell system (Invitrogen) as recommended by the supplier. To visualize the proteins the gels were Coomassie stained using Safestain (Invitrogen).

Cell-wall-associated proteins were extracted from 25 ml of culture (OD_{600} = 1 ± 0.1) as previously described (6). The proteins were separated on NuPAGE Bis-Tris gels (Invitrogen). To immunologically detect selected proteins, the cell-wall-associated proteins were blotted onto polyvinylidene difluoride membranes (Invitrogen) using the XCell II Blot Module (Invitrogen) as recommended by the supplier. Protein A was probed using rabbit anti-staphylococcal Protein A antibody (Sigma) at a 1:10,000 dilution. ClfA and ClfB was detected using specific rabbit antibodies recognizing the A domains of each protein at a 1:5,000 dilution (a generous gift from Timothy J. Foster, Triniti College, Dublin, Ireland). Bound antibody was detected with the WesternBreeze Chemiluminescent Anti-Rabbit kit (Invitrogen). All Western blots were repeated three times with similar results.

RESULTS

ClpX is required to relieve the negative regulatory effect of *agr* on *spa* transcription. Previously we showed that transcription of *spa*, encoding Protein A, was severely reduced in cells lacking the Clp ATPase ClpX but was largely unaffected in cells lacking the proteolytic component ClpP (11). To examine where in the regulatory cascade ClpX mediates its function, mutations in regulatory genes (*agr*, *sarS*, and *rot*) known to affect expression of *spa* were transduced into the *clpX* and *clpP* deletion strains. In a similar way, we attempted to transduce the *sarA* mutation from PC1839 (5) into the *clpX* mutant; however, despite several attempts we did not obtain a *clpX sarA* double mutant. In contrast, the *sarA* mutation was successfully transduced into the *clpP* mutant strain and into RN6911, but growth of these double mutants was markedly reduced (data not shown).

spa transcription was examined by Northern blot analysis, and RNA was extracted from cells either in mid-exponential growth phase (OD_{600} = 0.8 ± 0.1) or from cells in transition to stationary phase (OD_{600} = 2.0 ± 0.1). The Northern blot revealed that the level of *spa* transcript was similar in the *clpP* mutant and wild-type cells (Fig. 1A, lanes 1 and 2) and confirmed that the level was very low in cells lacking ClpX (Fig. 1A, lane 3). As expected, *spa* transcription was induced in *agr* and *sarA* mutant cells, confirming that both *agr* and SarA act as negative regulators of *spa* transcription. Remarkably, the *spa* transcript was barely detectable in the *agr clpX* double mutant (Fig. 1A, compare lane 1 to lanes 4 and 6). Thus, the strong derepression of *spa* transcription, normally observed in cells with impaired *agr*, is abolished in the absence of ClpX. Therefore, we conclude that ClpX is required to relieve the negative regulatory effect of *agr* on *spa* transcription.

SarS was recently found to be a positive regulator of *spa* transcription, and it was shown that part of the *agr*-controlled repression of *spa* transcription occurs indirectly by repression of *sarS* transcription (8, 39). In accordance with previous findings, we saw that inactivation of *agr* still increased *spa* transcription significantly in the absence of SarS (Fig. 1A, compare lanes 9 and 12), demonstrating that *agr*-mediated derepression of *spa* transcription also occurs in a SarS-independent manner (8, 39). Notably, this additional induction was abolished in the *sarS clpX* double mutant (lane 14). Thus, our results suggest that ClpX modulates the SarS-independent pathway of *agr*-mediated induction of *spa* transcription, although we cannot exclude that ClpX may also affect the SarS-dependent pathway. In the *clpP sarS* mutant, *spa* mRNA levels equaled the levels observed in the *sarS* mutant, in accordance with the notion that ClpP does not impact on *spa* transcription.

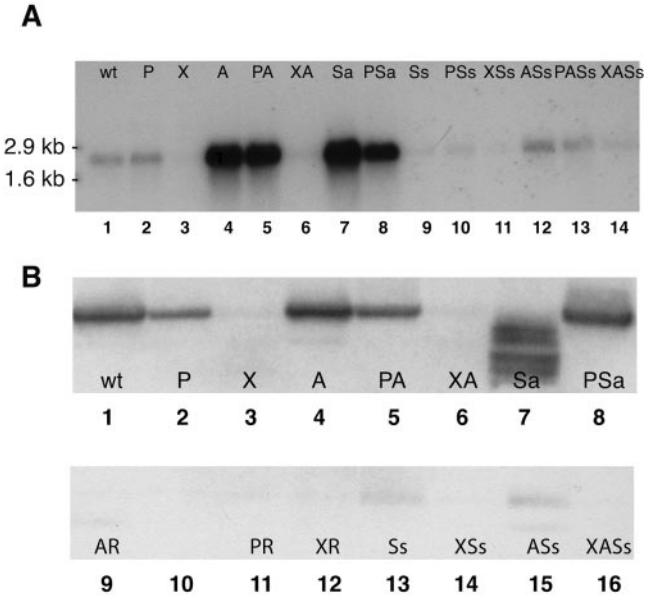

FIG. 1. ClpX is required to relieve the negative regulatory effect of *agr* on *spa* transcription. A) Northern blot detection of the *spa* transcript. RNA was isolated from cells in the transition to stationary phase (OD_{600} = 2.0 ± 0.1). Lane 1 (wt), 8325-4 (wild-type); lane 2 (P), 8325-4 Δ*clpP*; lane 3 (X), 8325-4 Δ*clpX*; lane 4 (A), RN6911 (Δ*agr*); lane 5 (AP), DF2268 (Δ*clpP* Δ*agr*); lane 6 (AX), DF2269 (Δ*clpX* Δ*agr*); lane 7 (Sa), PC1839 (*sarA*); lane 8 (PSa), (Δ*clpP sarA*); lane 9 (Ss), KT201 (*sarS*); lane 10 (PSs), DF2296 (Δ*clpP sarS*); lane 11 (XSx), DF2297 (Δ*clpX sarS*); lane 12 (ASs), KT202 (Δ*agr sarS*); lane 13 (PASs), DF2298 (Δ*clpP* Δ*agr sarS*); lane 14 (XASs), DF2299 (Δ*clpX* Δ*agr sarS*). The arrows indicate the migration of the 16S and 23S rRNA. B) Protein A levels measured by Western blot analysis. Cell wall proteins were extracted from an equal number of cells in late-exponential growth phase (OD_{600} = 1.0 ± 0.1). Extracted proteins were analyzed by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and probed with anti-Protein A antibody. The two gels were analyzed in parallel. Lane 1, 8325-4 (wild type); lane 2, 8325-4 Δ*clpP*; lane 3, 8325-4 Δ*clpX*; lane 4, RN6911 (Δ*agr*); lane 5, DF2268 (Δ*clpP* Δ*agr*); lane 6, DF2269 (Δ*clpX* Δ*agr*); lane 7, PC1839 (*sarA*); lane 8, DF2271 (Δ*clpP sarA*); lane 9 (AR), DF2432 (Δ*agr* Δ*rot*); lane 10, protein size marker; lane 11 (PR), DF2332 (Δ*clpP* Δ*rot*); lane 12 (XR), DF2333 (Δ*clpX* Δ*rot*); lane 13, KT201 (*sarS*); lane 14, DF2297 (Δ*clpX sarS*); lane 15, KT202 (Δ*agr sarS*); lane 16, 2299 (Δ*clpX* Δ*agr sarS*).

Another Sar homologue, Rot, was recently shown to be a positive regulator of *spa* transcription (36). In accordance with this finding, we did not see induction of *spa* transcription in the *rot agr* double mutant, showing that Rot, like ClpX, is absolutely required to alleviate the repression of *spa* transcription by *agr* (data not shown). Furthermore, the additional inactivation of *rot* in the *clpP* mutant strain, which in this context

resembles a wild-type strain, reduced the amount of *spa* transcript to a nondetectable level, emphasizing that Rot is required for *spa* transcription also in an *agr*-positive background (data not shown).

To substantiate our findings, we additionally monitored Protein A expression by Western blot analysis and observed that the level of cell-wall-associated Protein A reflected the level of *spa* transcript (Fig. 1B). The Western blot confirmed that Protein A was absent in cells devoid of ClpX both in a wild-type background (lane 3) and in the *agr clpX* double mutant (lane 6). The level of Protein A produced by cells lacking ClpX was even lower than in cells lacking SarS, the activator of *spa* transcription (compare lanes 3 and 6 to lane 13). Moreover, the concomitant inactivation of *clpX* and *sarS* reduced the level of Protein A below the level of detection, suggesting that the effects of ClpX and SarS are additive (compare lanes 13 and 14 and also 15 and 16). Protein A appeared as a very faint band in all strains having the *rot* disruption, supporting that Rot is more essential for expression of Protein A than is SarS. Interestingly, the Protein A levels in the *agr rot* and *agr clpX* double mutants were comparably low. Moreover, the level of Protein A was similar in *clpP rot* and *clpX rot* mutant cells, indicating that the effects of Rot and ClpX on Protein A expression are not additive.

In cells lacking SarA, the Protein A-specific antibody recognized several smaller proteins (Fig. 1B, lane 7). Since it is well documented that the significant up-regulation of extracellular proteases in the *sarA* mutant results in increased degradation of Protein A, these proteins presumably represent Protein A degradation products (20, 39). Interestingly, Protein A is observed only as the full-length protein in the *sarA clpP* double mutant (lane 8), indicating that in the absence of ClpP proteolytic degradation of Protein A is abolished in the *sarA* mutant.

ClpX and ClpP do not affect expression of cell wall proteins uniformly. To examine if the control of *spa* expression by ClpX is representative of other cell-wall-associated proteins, we extracted total cell wall proteins from various mutant strains. When the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the overall protein profiles appeared similar (data not shown). Instead, we specifically monitored expression of selected cell wall proteins (fibronectin binding protein A and clumping factors A and B) by Western and/or Northern blot analysis. The anti-ClfA antibodies reacted with two proteins of 170 and 130 kDa in wild-type cells (Fig. 2A, lane 1). Presumably, the 170-kDa protein corresponds to full-length ClfA while the 130-kDa protein represents ClfA that has been cleaved at the motif SLAAVA by the staphylococcal metalloprotease, as described by O'Brien et al. (32). Similar to ClfA, ClfB appears in a full-length intact form (140 kDa) and a metalloprotease-processed form of 110 kDa (Fig. 2B) (24).

In wild-type cells the truncated forms of ClfA and ClfB proteins are the most abundant forms, while the full-length proteins dominate in the absence of either *clpX* or *clpP* as well as in the absence of *agr*. In contrast, only the processed forms of ClfA and ClfB are present in the *sarA* mutant. The variation in the abundances of the two forms of Clfs may reflect the relative expression of metalloprotease among the strains; however, this issue has not been examined. Curiously, the full-length ClfA and ClfB proteins appear slightly bigger in the *clpX* and *clpX agr* mutant strains, suggesting that ClpX plays an additional role in processing of clumping factors A and B.

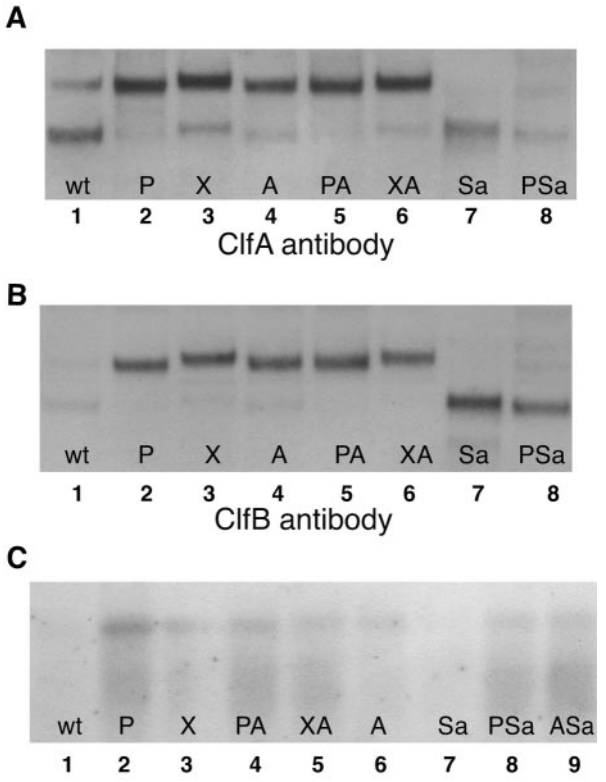

FIG. 2. The level of ClfB is enhanced in the absence of ClpP or ClpX while the level of ClfA is unaffected. (A and B) Western blot analysis was performed to detect expression of clumping factors A and B. Cell wall proteins were extracted from an equal number of cells in late-exponential growth phase (OD_{600} = 1. 0 ± 0.1). Extracted proteins were analyzed by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and probed with anti-Clf antibody. The two gels were analyzed in parallel. Lane 1, 8325-4 (wild type); lane 2, 8325-4 Δ*clpP*; lane 3, 8325-4 Δ*clpX*; lane 4, RN6911 (Δ*agr*); lane 5, DF2268 (Δ*clpP* Δ*agr*); lane 6, DF2269 (Δ*clpX* Δ*agr*); lane 7, PC1839 (*sarA*); lane 8, DF2271 (Δ*clpP sarA*). To the left, the migration of the protein size marker of 100 kDa and 150 kDa, respectively, has been indicated. (C) *clfB* transcription measured by Northern blot analysis. RNA was isolated from cells in the transition to stationary phase (OD_{600} = 2.0 ± 0.1). Lane 1, 8325-4 (wild-type); lane 2, 8325-4 Δ*clpP*; lane 3, 8325-4 Δ*clpX*; lane 4, RN6911 (Δ*agr*); lane 5, DF2268 (Δ*clpP* Δ*agr*); lane 6, DF2269 (Δ*clpX* Δ*agr*); lane 7, PC1839 (*sarA*); lane 8, DF2271 (Δ*clpP sarA*); lane 9, DF2308 (*sarA* Δ*agr*). Abbreviations above lane numbers are as defined in the legend to Fig. 1.

The amount of ClfA appeared similar in all strains, implying that *clfA* expression is unaffected by *agr*, ClpXP, and SarA, in agreement with published data (29). However, from the ClfB Western blot it is clear that much more ClfB is present in the *clpX*, *clpP*, *agr*, and *sarA* mutants than in wild-type cells, indicating that ClfB expression is negatively regulated by ClpXP, *agr*, and SarA. Northern blot analysis revealed that *agr* and ClpXP affect *clfB* expression at the level of transcription, as the amount of *clfB* mRNA was significantly induced in *clpXP* and *agr* mutant cells (Fig. 2C). The derepression was most clearly observed in the postexponential growth phase (OD_{600} = 2.0), but a minor derepression was also observed in cells in expo-

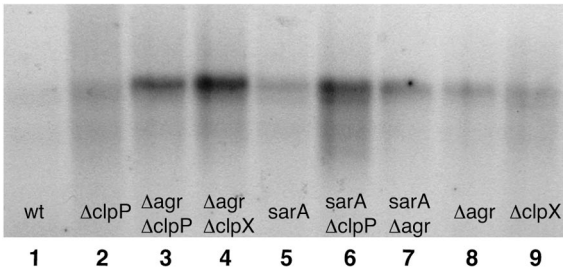

FIG. 3. Transcription of *fnbA* is enhanced in mutants lacking *agr*, ClpX, or ClpP. RNA was isolated from cells in the mid-exponential growth phase (OD_{600} = 0.8 ± 0.1). Lane 1, 8325-4 (wild-type); lane 2, 8325-4 Δ*clpP*; lane 3, DF2268 (Δ*clpP* Δ*agr*); lane 4, DF2269 (Δ*clpX* Δ*agr*); lane 5, PC1839 (*sarA*); lane 6, DF2271 (Δ*clpP sarA*); lane 7, DF2308 (Δ*agr sarA*); lane 8, RN6911 (Δ*agr*); lane 9, 8325-4 Δ*clpX*. wt, wild type.

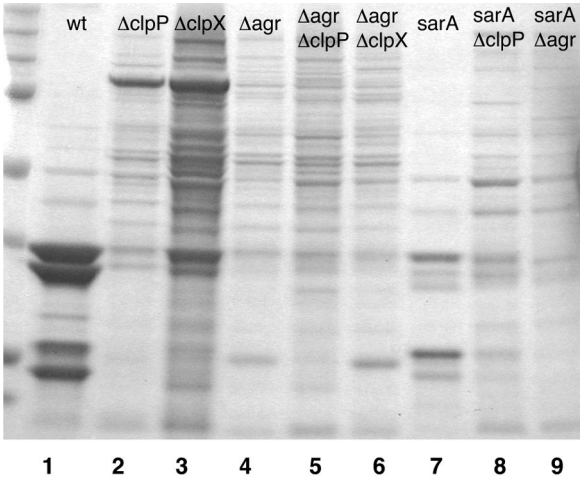

FIG. 4. SDS-PAGE analysis of total extracellular proteins extracted from the growth medium of stationary-phase cultures. Lane 1, 8325-4 (wild-type); lane 2, 8325-4 Δ*clpP*; lane 3, 8325-4 Δ*clpX*; lane 4, RN6911 (Δ*agr*); lane 5, DF2268 (Δ*clpP* Δ*agr*); lane 6, DF2269 (Δ*clpX* Δ*agr*); lane 7, PC1839 (*sarA*); lane 8, DF2271 (Δ*clpP sarA*); lane 9, DF2308 (*sarA* Δ*agr*). wt, wild type.

nential phase (data not shown). Furthermore, we found that *clfB* transcription was not induced in the *sarA* mutant in the postexponential cells (Fig. 2C, lane 7), suggesting that the increase in the amount of ClfB observed in the absence of *sarA* occurs at the posttranscriptional level. Previously, it has been published that mutation in neither *sarA* nor *agr* affected *clfB* transcription when measured by *lacZ* transcriptional fusions (24). However, this analysis was performed in strain Newman, thus, the impact of *agr* on *clfB* transcription may vary between strains.

Finally, we examined the amount of *fnbA* transcript-encoding fibronectin binding protein in the mutants and saw that the amount of *fnbA* transcript increased significantly in cells devoid of *agr*, *clpXP*, or *sarA*, implying that *fnbA* transcription is negatively regulated by ClpXP in addition to SarA and *agr* (Fig. 3). Interestingly, *fnbA* transcription was more derepressed in the double mutants than in the corresponding single mutants, implying that the regulatory effects are additive. In general, *fnbA* transcription decreased when the wild-type cells entered the transition phase, and this decrease in *fnbA* transcription was also observed in the *clp*, *agr*, and *sarA* mutant cells (data not shown).

To summarize, the amount of Protein A is severely reduced by the absence of ClpX. In contrast, the absence of either ClpP or ClpX derepressed expression of ClfB and FnbA while expression of ClfA was unaffected. We conclude that expression of cell wall proteins is not uniformly affected by ClpX or ClpP.

Exoprotein profiles are indicative of ClpXP working epistatic to *agr* in global regulation of extracellular proteins. *S. aureus* produces a large number of extracellular virulence factors that, under laboratory conditions, are induced in the postexponential growth phase in an *agr*-dependent manner. To obtain an overview of how the combined mutations affected synthesis of extracellular proteins, excreted proteins from overnight cultures of the mutant strains were analyzed by SDS-PAGE. As previously published, the *clpX* mutant strain excretes large amounts of extracellular proteins (11), but the qualitative profiles of exoproteins secreted by the *clpP* and *clpX* mutants are almost identical to the exoprotein profile of the *agr* mutant strain (Fig. 4, compare lanes 2 to 4). Therefore, it was not surprising that the profiles of the *agr clp* double mutants turned out to be very similar to the profile of the *agr* mutant (compare lanes 4 to 6). The exoprotein profile of the *sarA clpP* double mutant deviated from the profiles of both the *clpP* and the *sarA* single mutants but, interestingly, appeared very similar to the profile of the *sarA agr* double mutant (compare lanes 8 and 9). On the basis of these observations, we speculate that exoprotein regulation mediated by ClpXP and *agr* occurs at the same level in the regulatory cascade.

ClpXP is required for transcriptional induction of *sspA* in the postexponential growth phase. We next monitored transcriptional regulation of a single excreted protein known to be regulated by *agr* and SarA. *sspA*, encoding the serine protease, was chosen as a model gene, as we have previously reported that inactivation of *clpX* or *clpP* significantly reduced extracellular proteolytic activity (11). Northern blot analysis confirmed that *sspA* expression was very low in both wild-type and mutant strains in early exponential phase (Fig. 5A). The amount of *sspA* transcript increased when wild-type cells were in transition to stationary phase (OD_{600} = 2), and, as expected, this induction was dependent on the presence of the *agr* locus (Fig. 5B, compare lanes 1B and 4B). Interestingly, the *sspA* transcript was not detected in RNA samples from *clpX* and *clpP* mutant cells (Fig. 5B, lane 2B and 3B), indicating that ClpX and ClpP, like *agr*, are required for induction of *sspA* transcription in the postexponential growth phase. In contrast, the absence of SarA resulted in a dramatic derepression of *sspA* transcription in the postexponential growth phase (lane 7B), as previously reported (39). In the *sarA clpP* double mutant, transcription of *sspA* exceeded the wild-type level; however, it was significantly reduced compared to the level in the *sarA* mutant (Fig. 5B, lane 8B). Similar results were obtained using the *sarA agr* double mutant (data not shown). Thus, in the absence of *agr* or ClpP, inactivation of SarA still results in a significant derepression of *sspA* expression.

Notably, the absence of SarA resulted only in a slight derepression of *sspA* transcription in the exponential growth phase (Fig. 5A, lane 7A), and this slight increase was also observed in

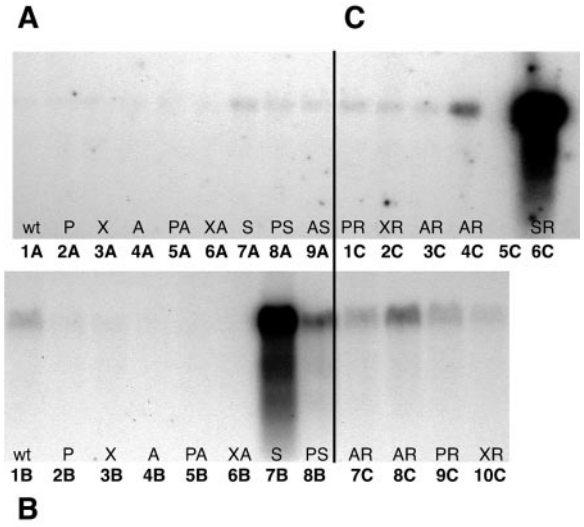

FIG. 5. ClpXP is required for transcriptional induction of *sspA* in the postexponential growth phase (A and B), but *sspA* transcription in the *clp* mutants can be restored by the concomitant inactivation of the Rot repressor (C). RNA was isolated from cells either in the mid-exponential growth phase (OD_{600} = 0.8 ± 0.1) (A and lanes 1C to 6C) or in the transition to stationary phase (OD_{600} = 2.0 ± 0.1) (B and lanes 7C to 10C). The two blots were hybridized to the labeled *sspA* probe in parallel. Lanes 1A and 1B, 8325-4 (wild type); lanes 2A and 2B, 8325-4 Δ*clpP*; lanes 3A and 3B, 8325-4 Δ*clpX*; lanes 4A and 4B, RN6911 (Δ*agr*); lanes 5A and 5B, DF2268 (Δ*clpP* Δ*agr*); lanes 6A and 6B, DF2269 (Δ*clpX* Δ*agr*); lanes 7A and 7B, PC1839 (*sarA*); lanes 8A and 8B, DF2271 (Δ*clpP sarA*); lane 9A, DF2308 (*sarA* Δ*agr*); lanes 1C and 9C, DF2332 (Δ*clpP* Δ*rot*); lanes 2C and 10C, DF2333 (Δ*clpX* Δ*rot*); lanes 3C and 7C, DF2432 (Δ*agr* Δ*rot*); lanes 4C and 8C, PM614 (Δ*agr* Δ*rot*); lane 5C, empty; lane 6C, DF2405 (*sarA* Δ*rot*). Abbreviations above lane numbers are as defined in the legend to Fig. 1.

the *agr sarA* and *clpP sarA* double mutants (Fig. 5A, lane 8A and 9A).

Inactivation of Rot restores *sspA* transcription in the *clpX* and *clpP* mutants. The obtained results support that *agr* and ClpXP work epistatic in the regulation of *sspA* transcription. It was previously hypothesized that RNAIII mediates its effect on virulence gene transcription through the interaction with short-lived regulatory proteins (29, 31). Rot is a candidate to be such a protein, since it was originally identified in a screen for mutations that restored the protease and α-hemolysin production of an *agr*-null mutant (25). Furthermore, Rot was shown to negatively affect *sspA* transcription (36). We wished to examine if the disruption of *rot* likewise could restore transcription of *sspA* in the *clpX* and *clpP* mutants. Intriguingly, the additional inactivation of *rot* in the *clp* mutant strains increased the level of *sspA* transcription to a level similar to the induced, postexponential level observed in the wild-type cells (Fig. 5C). Importantly, the derepression of *sspA* was observed both in exponential (Fig. 5C, lanes 1C and 2C) and postexponential (Fig. 5C, lanes 9C and 10C) cells and was quantitatively comparable to the derepression observed for the *agr rot* double mutant (lanes 3C and 7C) made by transducing the original *rot* transposon disruption into the RN6911 background but was slightly less than that in PM614 (lanes 4C and 8C), the original published *agr rot* double mutant (23). Thus, in the absence of Rot, ClpXP activity (like *agr* activity) is not required for induction of *sspA* transcription. In accordance with the transcriptional data, we observed that the *rot clp* and the *rot agr* double mutants, in contrast to the *agr* and *clp* single mutants, exhibited proteolytic activity on agar plates containing gelantine or skim milk (data not shown). From this experiment, we tentatively conclude that ClpXP, similar to *agr*, controls *sspA* transcription by controlling the repressor activity of Rot.

Since SarA is also known to repress *sspA* transcription, we finally examined how *sspA* transcription was influenced by the absence of both SarA and Rot. Interestingly, the dual inactivation of Rot and SarA led to a very dramatic derepression of *sspA* transcription in the exponential growth phase. This strong derepression of *ssp*A transcription was also observed in the postexponential cells; however, the data have not been presented, as the *sarA rot* double mutant aggregated in the late exponential cells, thus making it impossible to accurately monitor growth.

DISCUSSION

The complex regulation of virulence gene expression in *S. aureus* involves at least four two-component systems, the alternative sigma factor σ^B, and a large set of transcription factors belonging to the Sar family (reviewed in references 2 and 29). Recently, we revealed a new layer of regulation by showing that mutants lacking either ClpX or ClpP produced reduced amounts of several extracellular virulence factors and that, at least in the case of α-hemolysin, synthesis was reduced at the transcriptional level (11). Mutations in *clpP* and *clpX* had similar effects on exoprotein synthesis, indicating that the effect is mediated by the ClpXP proteolytic complex. Additionally, ClpX by itself appeared to be required for transcription of *spa*.

The quorum-sensing *agr* locus is the best characterized of the many regulators identified in *S. aureus*. Here we combined deletions in *clpX* or *clpP* with mutations in *agr* and genes encoding relevant Sar transcriptional regulators in order to determine the place of ClpX and ClpP within the *sar*/*agr* regulatory network. As our data indicate that ClpX and ClpXP have separate effects on surface-associated and extracellular factors, respectively (11), we assessed the impact of the combined mutations on both expression of a cell wall protein (Protein A) and a secreted protein (serine protease [SspA]).

We showed that the strong derepression of *spa* transcription normally observed in an *agr*-negative background was abolished in the absence of ClpX, emphasizing the requirement of ClpX for *spa* transcription. In other organisms, ClpX independently of ClpP has been shown to possess chaperone activity (42, 43). Thus, one possible scenario is that ClpX is required to fold a positive regulator of *spa* transcription into its active conformation. One such factor could be SarS, which recently was identified as a positive regulator of *spa* transcription, and it was shown that *agr* partly represses *spa* transcription indirectly by repressing transcription of *sarS* (8, 39). SarS presumably functions by direct binding to the *spa* promoter (8, 39) and, thus, ClpX could be required to fold SarS into its active DNA-binding conformation. However, the level of *spa* transcript and the level of Protein A were lower in *clpX sarS* double mutants than in the *sarS* mutant, indicating that the effects of SarS and ClpX on *spa* transcription are additive. Moreover, we, similar to others, observed that a significant derepression

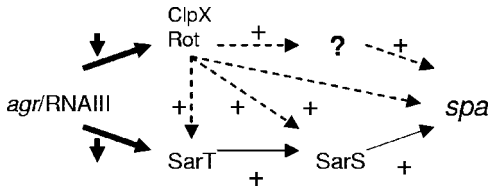

FIG. 6. *agr*/RNAIII negatively regulates *spa* transcription by two pathways that both depend on Rot and ClpX. In one pathway, *agr*/RNAIII reduces *spa* transcription by reducing transcription of *sarT*, resulting in decreased transcription of *sarS* encoding a transcriptional activator of *spa* (see the text for details). In the second pathway, *agr*/RNAIII reduces *spa* transcription by inactivating the function of Rot. Rot is an activator of *spa* transcription that, in addition to stimulating transcription of *sarS* (perhaps through SarT) can enhance *spa* transcription by an alternative pathway. At present, the molecular basis of this alternative pathway is unknown (denoted with a question mark); however, Rot-mediated activation of *spa* transcription may occur directly or indirectly. We propose that ClpX is required to fold Rot in an active conformation that will facilitate interaction with other positive regulators or operator regions. The downward-pointing arrows denote downregulation (the molecular basis is unknown). The plus sign denotes induction on the transcriptional level. The solid arrows depict verified interactions, and the dashed arrows depict putative interactions.

of *spa* transcription was still achieved by inactivating *agr* in cells lacking SarS (8, 39), and, interestingly, this derepression of *spa* transcription was eliminated in the *agr clpX* double mutant. On the basis of our findings, we conclude that ClpX may modulate the SarS-dependent pathway of *agr*-controlled *spa* expression but is also required for the SarS-independent pathway by which *agr* represses *spa* transcription. The regulators of this pathway are currently unrecognized.

Recently, *spa* and *sarS* transcription were shown to be positively regulated by Rot, another SarA homologue (36). Interestingly, we showed here that inactivation of *rot* reduced the Protein A level to the same low level observed in the *clpX* mutant. Additionally, the absence of Rot, similar to the absence of ClpX, completely eliminated the *agr*-mediated derepression of *spa* transcription. Therefore, ClpX could alternatively be required for folding Rot into an active conformation that, directly or indirectly, stimulates *spa* transcription. Preliminary data has shown that Rot is required for transcription of *sarS*, indicating that the positive effect of Rot on *spa* transcription is also mediated in part through SarS (36). Our data point to a stimulatory role of Rot on *spa* transcription that works independently of SarS and in collaboration with ClpX. A tentative model is depicted in Fig. 6.

Finally, SarA is a negative regulator of *spa*. Unfortunately, we could not assess the requirement of ClpX for *spa* transcription in a *sarA* background, as we did not succeed in constructing a *sarA clpX* double mutant. The difficulties in obtaining a *clpX sarA* double mutant could indicate that the double mutant is not viable. Both ClpX and SarA influence global virulence regulation at multiple levels, and it is possible that the absence of both regulators will imbalance the levels of other intermediary regulators or proteins in a way that will be lethal to the cell.

The synthesis of many cell wall proteins is coordinately regulated; however, the requirement for ClpX seems to be specific for *spa* transcription. In contrast, transcription of *clfB* and *fnbA* was stimulated by the absence of ClpX as well as by the absence of ClpP, hinting that the regulatory effect on *clfB* and *fnbA* transcription is mediated by the ClpXP proteolytic complex. Curiously, derepression of *clfB* expression in the *clp* mutants occurs primarily in the postexponential phase while derepression of *fnbA* occurs in the exponential phase. Additionally, ClpXP and *agr* have additive effects on transcription in the case of *fnbA* but not in the case of *clfB* transcription. Thus, presumably, ClpXP-mediated regulation of cell-wall-associated adhesins involves both *agr*-dependent and -independent pathways that respond to various signals.

In wild-type cells synthesis of extracellular virulence factors is generally induced in the postexponential phase by an *agr*-dependent mechanism. Mutants lacking either ClpX or ClpP fail to induce transcription of *hla*-encoding α-hemolysin and produce reduced amounts of several extracellular virulence factors (11). The finding that both transcription of RNAIII and the activity of the autoinducing peptide were reduced in the *clp* mutants led us to propose that ClpXP regulates synthesis of virulence genes through *agr* (11). In support of this hypothesis, we show here that the patterns of extracellular proteins synthesized by the *clp* and *agr* single and double mutants are very similar. Moreover, combining the *sarA* mutation with mutations in either *clpP* or *agr* resulted in similar changes in the profiles of extracellular proteins, indicating that ClpXP and *agr* function at the same level in the regulatory hierarchy relative to SarA. When we examined expression of a single secreted protein, SspA, we found that postexponential induction was eliminated at the transcriptional level by the absence of either ClpX, ClpP, or *agr*. These observations all support that *agr* and ClpXP work epistatic in the regulation of exoprotein synthesis. In other bacteria, the combination of a two-component system and a Clp proteolytic complex regulates important developmental pathways. In *E. coli*, the two-component response regulator, RssB, functions as an adaptor protein of the ClpXP proteolytic complex (45). In its phosphorylated form, RssB binds the stationary sigma factor σ^s and thereby targets it for degradation by ClpXP. A more complex mechanism involving both a quorum-sensing system and the ClpCP proteolytic complex controls development of genetic competence in *B. subtilis* (40). It could be speculated that related mechanisms link the function of *agr* and ClpXP to control growth-phase-dependent expression of extracellular virulence factors in *S. aureus*. The molecular basis of how RNAIII regulates transcription of target genes remains unknown. The proposed structure of RNAIII shows that it is able to form 14 different hairpin structures that may create protein binding sites (4), and preliminary data indicate that the regulatory role of RNAIII is mediated through interaction with short-lived regulatory proteins (2, 3). Rot is a candidate to be an *agr*-interacting protein, since it was originally identified in a screen for mutations that restored the protease and α-hemolysin activity of an *agr*-null mutant (25). This led to the hypothesis that RNAIII induces transcription of *hla* and *sspA* in the postexponential phase by inhibiting the repressor activity of Rot (25). Intriguingly, we showed here that the concomitant disruption of *rot* restored *sspA* transcription in the *clpXP* mutants. We hypothesize that the regulatory link between ClpXP and *agr* could be mediated by transcriptional regulators that upon interaction with RNAIII will be tagged for degradation by ClpXP. According to

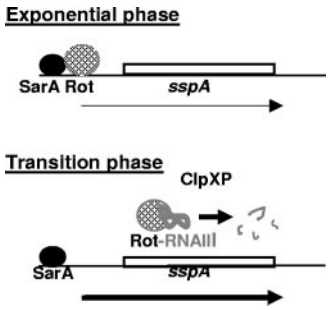

FIG. 7. Model linking the activity of ClpXP, RNAIII, Rot, and SarA in controlling transcription of *sspA*. In the exponential phase, *sspA* transcription is very low due to the dual repression of *sspA* transcription mediated by Rot and SarA. When the cells transit to stationary phase, the quorum-sensing *agr* locus is induced, resulting in high levels of RNAIII in the cell. RNAIII binds to Rot and thereby targets it for degradation by the ClpXP protease, resulting in partial derepression of *sspA* transcription.

our model, *sspA* transcription is repressed by Rot during the exponential growth phase. In the postexponential growth phase, accumulating RNAIII binds to Rot, thereby targeting Rot for degradation by ClpXP, leading to derepression of *sspA* (Fig. 7). In support of this model, we saw that in all strains carrying the *rot* disruption, expression of *sspA* in exponential phase was derepressed to a level comparable to what is observed in postexponential wild-type cells. Thus, in the absence of Rot, *agr* and ClpXP are no longer required for inducing *sspA* transcription in the transition to stationary phase. As *sspA* is additionally repressed by SarA, the model implies that *sspA* transcription is repressed by both SarA and Rot in the exponential phase and by SarA alone in the postexponential phase. Accordingly, maximal induction of *sspA* is observed in a *sarA*-negative strain only in the postexponential growth phase and only in the presence of functional *agr* or ClpXP. From the model we expect that maximal expression of *sspA* can be achieved also in a *rot sarA* double mutant, and this was confirmed experimentally (Fig. 5, lane 6C). In accordance with the model, the maximal expression of *sspA* was seen both in the exponential and postexponential growth phases. Direct evidence of the proposed model has not been obtained in this study. Moreover, we have not examined the contribution of σ^B to this model, as all the used strains are derivatives of 8325-4, which has reduced levels of σ^B due to a small deletion in *rsbU*, encoding an activator of σ^B (14). However, we do not expect σ^B to influence the regulatory events downstream of *agr* that were the focus of this study, since σ^B appears to affect regulation of virulence genes solely by reducing transcription of RNAIII (16). However, this assumption has to be verified using σ^B-proficient strains. Future studies will be designed to examine, in vitro and in vivo, if stability of Rot is affected by the absence of ClpX, ClpP, or *agr*. DNA arrays have established that Rot and *agr* have opposing effects on the expression of virulence genes (36). Generally, secreted proteins (like hemolysins, proteases, and lipases) are negatively regulated while cell surface adhesins are positively regulated by Rot (and vice versa by *agr*). Our data indicated that *agr* and ClpXP work epistatic in the overall regulation of exoprotein synthesis, and we are currently assessing if Rot is the cofactor linking ClpXP and *agr* in global regulation of exoproteins. Notably, we have shown that ClpXP-mediated regulation of cell-wall-associated adhesins must involve several pathways that respond to different signals.

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