Alkaline pH Decreases Expression of the Accessory Gene Regulator (*agr*) in *Staphylococcus aureus*

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The effect of alkaline pH on expression of the accessory gene regulator (*agr*) in *Staphylococcus aureus* was examined. *agr*, a global regulator, affects the expression of numerous exoproteins, including α -hemolysin, toxic shock syndrome toxin 1, protein A, and staphylococcal enterotoxins types B, C, and D. *agr* contains two major, divergent transcripts, designated RNAII and RNAIII. In this study, the level of RNAIII was used to monitor *agr* expression because this transcript and/or its protein product(s) appears to be responsible for altering target gene expression. *S. aureus* FRI1230 and its Agr⁻ derivative were examined in a fermentor system which allowed batch cultures to be maintained at a constant pH. FRI1230 cultures were grown at pH 6.5, 7.0, 7.5, and 8.0. Northern (RNA blot) analysis of samples revealed that maximal *agr* expression occurred at pH 7.0, with virtually no RNAIII observed at pH 8.0. The effect of alkaline pH on an *agr* target gene, *sec*, was also evaluated. *sec* expression was reduced at alkaline pH in strain FRI1230 (Agr⁺) but not in its Agr⁻ derivative, indicating that an intact *agr* allele is required for the pH effect on *sec*. Examination of batch cultures under conditions of nonmaintained pH gave results that were also consistent with a role for alkaline pH in repressing *agr* expression.

The *Staphylococcus aureus* accessory gene regulator (*agr*) was identified initially as a locus which, when inactivated, results in altered production of several exoproteins and cell surface-associated proteins (7, 25). An Agr⁺ strain produces more α -hemolysin, β -hemolysin, toxic shock syndrome toxin 1 (TSST-1), and staphylococcal enterotoxin types B, C, and D (SEB, SEC, and SED, respectively) and less coagulase and protein A (2, 3, 12, 13, 25, 27) than its Agr⁻ derivative. The ability of *agr* to function as both an activator and a repressor suggests the existence of two mechanisms for *agr*-mediated regulation.

Nucleotide and mutational analysis of the *agr* locus revealed that it is a divergent operon containing two major transcripts that are referred to as RNAII and RNAIII (3.5 and 0.5 kb in length, respectively [1, 15, 16, 20, 23, 24]). RNAIII includes the gene for δ -hemolysin (*hld*), and RNAII has four open reading frames, *agrABCD* (1, 15, 16, 20, 23, 24). That *agrA* is required for an Agr⁺ phenotype has been clearly demonstrated by complementation analysis (16), and a possible role for AgrA has been suggested. The deduced amino acid sequence of *agrA* is homologous to that of other proteins that serve as the response regulator in signal-transducing systems (16). *agrB* may also play a role in signal transduction; the derived amino acid sequence of *agrB* has homology to that of signal receptor proteins (16).

The mechanisms involved in *agr* regulation are beginning to be elucidated. RNAII and RNAIII are first detected during mid- to late exponential growth, but maximal *agr* expression is observed postexponentially (15, 16, 24, 34). Under wild-type conditions, RNAII (or its putative protein product[s]) is required for RNAIII expression (15, 20, 24). Kornblum et al. have reported that introduction of a recombinant plasmid containing RNAIII under the control of the β -lactamase (*blaZ*) promoter is able to complement the Agr⁻ phenotype with respect to *agr* target genes (16), including *sec* (26), demonstrating that supplying RNAIII bypassed the need for RNAII.

Although RNAIII contains *hld* and a second, small open reading frame of 21 codons, apparently the RNAIII transcript and not a protein product is responsible for regulation of some *agr* target genes (1, 13, 16). Mutants that have either *hld* or the 21-codon open reading frame disrupted by site-directed mutagenesis still have an Agr⁺ phenotype with respect to most *agr* target genes (1, 13, 16). Consistent with the crucial role of RNAIII for the Agr⁺ phenotype is the correlation between the level of RNAIII and the production of SEB (9). *S. aureus* strains that produce high levels of SEB also contain high concentrations of RNAIII, compared with strains that produce low levels of SEB, which have less RNAIII (9).

Little is known about the modulation of *agr* expression. However, the observation that *agrA* and *agrB* have homology to components of signal transduction systems suggests that *agr* expression may be altered in response to environmental signals (16). We have previously reported a potential environmental signal that results in a change in *agr* expression; addition of glucose to the medium under conditions of nonmaintained pH results in an eightfold reduction in RNAII and RNAIII concentrations compared with the levels in medium lacking glucose (28). This reduction in RNAII and RNAIII levels correlates with repressed expression of two *agr* target genes, *hla* (α -hemolysin) and *sec* (28).

Cultural conditions that affect the expression of *agr* target genes may be helpful in identifying other possible modulators of *agr* expression. Several groups have shown that the pH of the culture medium alters the amount of extracellular SEB and SEC produced (8, 19, 30). Under conditions of nonmaintained pH, SEB and SEC production is enhanced by using an initial pH of 6.8 rather than 6.0 or 5.3 (30). In two independent studies with cultures grown at maintained pH, SEB was maximally produced by cultures maintained at pH 7.0 versus pH 6.0, 6.5, 7.5, and 8.0 (8, 19). Metzger et al., using an *S. aureus* S6 mutant, reported an 18-fold reduction in extracellular SEB when cultures were grown at pH 8.0 compared with pH 7.0 (19). The enterotoxins are extremely stable proteins at pH 5 to 10, and therefore the differences in

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>S. aureus</i> strains		
FRI1230	<i>sec</i> ⁺ Hem ⁺ Em ⁺	M. S. Bergdoll, University of Wisconsin, Madison (29)
ISP546	<i>Sec</i> ⁻ <i>agrA</i> ::Tn551 Hem ⁻	P. A. Pattee, Iowa State University, Ames (17)
MJB710	<i>sec</i> ⁺ <i>agrA</i> ::Tn551 Hem ⁻	FRI1230 × 80α/ISP546, select for Em ^r (this study) ^b
RN450	NCTC8325 derivative cured of prophages φ11, φ12, and φ13	R. P. Novick, Public Health Research Institute, New York, N.Y. (21)
<i>E. coli</i> plasmids		
pMJB124	pGEM7Zf(+) containing a 1.8-kbp insert fragment that has <i>sec</i>	10
pRN6650	pUC18 with a 6.1-kbp insert fragment containing <i>agr</i>	R. P. Novick (24)

^a Hem⁺ signifies that there was a zone of hemolysis surrounding the colonies following incubation on medium containing sheep erythrocytes. Tn551 encodes a determinant for erythromycin resistance (Em^r) (22).

^b FRI1230 was transduced with a lysate of phage 80α that had been prepared on strain ISP546.

the amount of accumulated, extracellular enterotoxins at various pHs are not likely due to differential degradation (4).

In this report, we have shown that the pH of the culture medium affected the expression of *agr* RNAIII and, in turn, expression of an *agr* target gene, *sec*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1.

The conditions used for growing batch cultures of *S. aureus* strains in Bioflo II controlled benchtop fermentors (New Brunswick Scientific) were as described previously (27) with two exceptions. In these studies, the cultures were grown in 1 liter of medium consisting of 3% (wt/vol) N-Z amine type A (Sheffield Products, Norwich, N.Y.) and 1% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.), and the aeration was maintained at 1.5 liters of air per min.

Phage 80α (obtained from P. A. Pattee) was propagated and assayed on Trypticase soy agar (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md.) supplemented with 5 μM CaCl₂ by using a soft-agar overlay of Trypticase soy broth plus 0.5% (wt/vol) agar (Difco). Erythromycin-resistant (Em^r) transductants were selected on brain heart infusion agar (Difco Laboratories) that contained 5 μg of sodium citrate and 5 μg of erythromycin per ml.

Transduction. Strain MJB710 (Agr⁻) was constructed by transducing the *agr*::Tn551 marker from ISP546 into FRI1230 (Agr⁺). Phage 80α transducing lysates were propagated on strain ISP546 as described previously (33). The transduction was performed essentially by the method of Schroeder and Pattee (33) except for two modifications. The multiplicity of infection was increased to 5, and the recipient cells (FRI1230) that had been resuspended in Trypticase soy broth (10⁹ CFU/ml; BBL Microbiology Systems) were incubated at 56°C for 3 min prior to the addition of the transducing lysate. The isolated Em^r transductants were screened for hemolysin production on Trypticase soy agar with 5% (vol/vol) sheep erythrocytes; all transductants exhibited reduced hemolytic activity compared with FRI1230. This reduction in hemolysin production is consistent with an Agr⁻ phenotype (14). Total cellular RNA samples prepared from MJB710 were analyzed by Northern (RNA) blot, and no *agr* RNAII or RNAIII was detected (data not shown).

Extraction of RNA and Northern blot analysis. RNA was prepared by the method described by Sandler and Weisblum

(31). Methods for RNA quantification have been described previously (27). For a given gel, samples with the same dilution factor contain the same amount of total cellular RNA, as determined by the A₂₆₀. Samples were electrophoresed through a 1% agarose gel, transferred to Magna NT filters (Micron Separations Inc., Westborough, Mass.), and hybridized to ³²P-labeled probes as described previously (27). Radiation on the filter was quantified with the Ambis Radioanalytic Imaging System (AMBIS Systems, San Diego, Calif.). At the end of each experiment, methylene blue stain was used to verify that comparable dilutions of different samples contained the same amount of total cellular RNA, based on the intensities of the signals of the 23S and 16S rRNA species.

Labeled probes. The protocols used for isolation of *Escherichia coli* plasmid DNA, DNA endonuclease restriction digestions, isolation of DNA fragments from agarose gels, and labeling of double-stranded DNA with ³²P have been described elsewhere (6, 18).

The probe used for detection of *sec* mRNA in Northern blot analysis was a 562-bp *Ssp*I fragment derived from pMJB124 (C-562); it consisted solely of *sec* sequence (10). RNAIII was detected by Northern blot analysis with a 962-bp *Cl*aI-*Pst*I fragment derived from pRN6397 (Agr-962); this probe contains both RNAII and RNAIII sequence. The use of these probes for Northern blot analysis of *sec* mRNA and RNAIII has been described before (28).

Western immunoblot analysis. Twofold serial dilutions of supernatant samples were prepared in uninoculated medium. For supernatant samples prepared from MJB710 (Agr⁻) cultures, it was necessary to concentrate the samples threefold with a Speed Vac concentrator (Savant Instruments, Inc., Farmingdale, N.Y.). Samples were electrophoresed through a 12.5% acrylamide gel, transferred to a nitrocellulose filter, and treated with rabbit antiserum prepared against SEC (Toxin Technology, Sarasota, Fla.). Signals were visualized with the ProtoBlot System AP (Promega Corp., Madison, Wis.). The intensity of the signals was quantified by densitometry (GS 300 Densitometer; Hoefer Scientific Instruments, San Francisco, Calif.). Supernatant samples from RN450, a non-enterotoxin-producing strain, were used as negative controls as described previously (27) (data not shown). Differential amounts of culture supernatant were analyzed to adjust for differences in optical density at 540 nm (OD₅₄₀) between the cultures being compared.

FIG. 2. MJB710 cultures were grown in fermentors with 3% (wt/vol) N-Z amine type A and 1% (wt/vol) yeast extract medium, with the pH maintained at 6.5 or 8.0. Samples were prepared 5.5 h after inoculation. (A) Growth curves were generated by plotting the logarithm of the OD₅₄₀ for the pH 6.5 and 8.0 cultures against the time that had elapsed since inoculation. (B) For Northern blot analysis of the samples, total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a nylon membrane, and reacted with a ³²P-labeled, *sec*-specific probe (C-562). Different samples with the same dilution factor contain the same amount of total cellular RNA. (C) Culture supernatant samples were analyzed by Western blot after being concentrated threefold. The samples were electrophoresed through a 12.5% acrylamide gel, electrophoretically transferred to a nitrocellulose membrane, reacted with antiserum prepared against SEC, and visualized by using the Pro-Blot Western Blot AP System. Differential amounts of culture supernatant were analyzed to adjust for differences in OD₅₄₀ values between the cultures. The OD₅₄₀ values for the pH 6.5 and 8.0 cultures were 10.4 and 9.4, respectively. The undiluted sample for the pH 8.0 culture contained 35 μ l of supernatant, while the undiluted sample for the pH 6.5 culture contained 32 μ l.

initial pH of 6.5 will reach pH 8.0 during the post-exponential growth phase (Fig. 3). Given that *agr* expression was altered by growth at different maintained pHs, we wanted to determine whether the increase in pH of cultures grown in complex medium was at least in part responsible for the variation in *agr* expression during growth. Two FRI1230 cultures were grown in medium containing 3% N-Z amine type A and 1% yeast extract. Both cultures had an initial pH of 6.5; the pH of the test culture was allowed to fluctuate, and the pH of the control culture was maintained at 6.5. Growth of these cultures was nearly identical, as judged by OD₅₄₀ measurements (Fig. 3A).

FIG. 3. FRI1230 cultures were grown in fermentors with 3% (wt/vol) N-Z amine type A and 1% (wt/vol) yeast extract medium. Both cultures had an initial pH of 6.5; the pH of the control culture was maintained at 6.5, and the pH of the test culture was allowed to fluctuate. Samples were prepared throughout growth at 2, 3.25, 4.5, 6.5, 8, 12, and 24 h, corresponding to samples a, b, c, d, e, f, and g, respectively. The b, c, and d samples were prepared as soon as the test culture reached the indicated pH. (A) Growth curves were generated for the control and test cultures by plotting the logarithm of the OD₅₄₀ against the time that had elapsed since inoculation. (B and C) Samples prepared from the test (B) and control (C) cultures were analyzed by Northern blotting. Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a nylon membrane, and reacted with ³²P-labeled DNA that corresponded to a portion of RNAIII (Agr-962) or *sec* mRNA (C-562). Different samples with the same dilution factor contain the same amount of total cellular RNA.

The level of RNAIII expression was examined by Northern blot analysis. For both cultures, there was an increase in RNAIII expression post-exponentially (Fig. 3). Samples taken 3.25 h after inoculation (point b, Fig. 3) contained two to threefold more RNAIII than samples taken 2 h after inoculation (point a, Fig. 3). A difference in RNAIII expression between the test and control cultures was observed for samples taken 6.5 h after inoculation (point d, Fig. 3), which corresponded to a pH of 8.0 for the test culture. At this sample time, the test culture had no visible RNAIII signal

(point d, Fig. 3B); analysis of the radiation on the filter with the Ambis Radioanalytic Imaging System revealed that the test culture contained at least 16-fold less RNAIII than the control culture. Samples from the control culture taken 6.5, 8, 12, and 24 h after inoculation revealed a decrease in the amount of RNAIII over this time period (points d to g, Fig. 3C). In contrast, there was no detectable RNAIII in any samples prepared from the test culture after 6.5 h of incubation, corresponding to pHs of 8.0 or greater (points d to g, Fig. 3B).

We wanted to determine whether there was a decrease in *sec*, an *agr* target gene, at alkaline pH that correlated with the decrease in RNAIII. Northern blot analysis of samples prepared 3.25 and 4.5 h after inoculation (points b and c, Fig. 3) indicated that *sec* mRNA levels were similar in the control and test cultures. However, at 6.5 h after inoculation, *sec* mRNA was detected only in the control culture which was maintained at pH 6.5 and not in the test culture, which had a pH of 8.0 (point d, Fig. 3).

DISCUSSION

agr expression, as determined by RNAIII levels, was shown to be affected by the pH of the culture medium in this report. Specifically, strain FRI1230 grown at pH 6.5 and 7.0 had at least 16- and 32-fold more RNAIII, respectively, than a culture maintained at pH 8.0 (Fig. 1B).

In a typical batch culture, both pH and *agr* expression vary with the growth of a staphylococcal culture. To evaluate the effect of pH on *agr* expression under such conditions, RNAIII levels were compared for a culture maintained at pH 6.5 and a test culture grown with nonmaintained pH. Consistent with previous reports (15, 16, 24, 34), we observed increased RNAIII expression as the cultures entered the transition from the exponential to the stationary phase (Fig. 3). This increase in RNAIII levels occurred independently of the pH; a culture maintained at pH 6.5 and a culture with nonmaintained pH exhibited identical patterns of increased *agr* expression (Fig. 3B and C). In contrast, pH appeared to play a role in repressing *agr* expression as the cultures entered the stationary phase. After 6.5 h of incubation (point d), a culture with nonmaintained pH was at pH 8.0 and had no detectable RNAIII (Fig. 3B), whereas the control culture maintained at pH 6.5 still had high levels of RNAIII after 6.5 h of incubation (point d, Fig. 3C). Although the concentration of RNAIII in the pH 6.5 culture continued to be higher than that in the nonmaintained pH culture throughout the stationary phase, the amount of RNAIII in the pH 6.5 culture gradually decreased with time (Fig. 3C). This decrease in RNAIII levels in the pH 6.5 culture suggests that a second factor, in addition to pH, modulates *agr* expression during the stationary phase.

Because this tremendous decrease in *agr* in response to pH changes was observed, it seemed that there would have to be a concomitant change in *agr* target genes. We chose to examine the *agr* target gene *sec*. Reiser and Weiss have reported that SEC production is affected by the initial pH of the culture medium (30). First, we demonstrated that pH did affect *sec* expression in our system under conditions of maintained pH. FRI1230 cultures were grown at pHs of 6.5, 7.0, 7.5, and 8.0. Northern blot analysis revealed that the pH 6.5 culture produced approximately fourfold more SEC and *sec* mRNA than the pH 8.0 culture, indicating that pH-mediated regulation of *sec* expression was occurring at the level of transcription or mRNA stability (Fig. 1B and C).

The experimental data presented in this report are consis-

tent with *agr* being involved in the pH-mediated regulation of *sec*. When strain FRI1230 was grown at pH 6.5 and 8.0, there was a reduction in the level of *sec* mRNA for the pH 8.0 culture compared with that in the culture grown at pH 6.5, which correlated with a decrease in RNAIII (Fig. 1B). In contrast, when an *Agr*⁻ derivative of FRI1230 (*agrA*::Tn551) was grown at pH 6.5 and 8.0, there was no reduction in *sec* expression at pH 8.0 compared with pH 6.5 (Fig. 2B). These results demonstrate that a functional *agr* system is required for the pH effect on *sec* expression; for FRI1230 (*Agr*⁺), the correlation observed between *sec* mRNA and RNAIII levels at pH 6.5 and 8.0 suggests that the pH effect could be mediated either directly or indirectly by RNAIII. RNAIII appears to be the *agr* component responsible for altering target gene expression (1, 13, 16). A correlation has also been observed between the amount of SEB or SEC produced and RNAIII levels; the strains that produce the most enterotoxin also have the highest concentrations of RNAIII (9, 26).

Growth conditions that inhibit *agr* expression would be expected to alter expression of *agr* target genes. In this report, we have demonstrated that alkaline pH results in a decrease in RNAIII expression and consequently in *sec* expression; therefore, it is reasonable to assume that other *agr* target genes would also show changed expression under these growth conditions. Consistent with a global effect due to pH-mediated regulation of *agr* are observations concerning the pH sensitivity of three additional *agr* target genes, *seb*, *hla*, and *tst*. Several groups have shown that less SEB is produced under conditions of alkaline pH (8, 19, 30). Duncan and Cho examined the effect of alkaline pH on α -hemolysin production by dividing a 4-h culture; the pH of one portion was adjusted to 8.0, while that of another portion was not altered, to serve as the control (11). After an additional 5 h of incubation, the pH 8.0 culture contained fourfold less α -hemolysin than the control culture. However, it is not clear whether the reduction in α -hemolysin at pH 8.0 was due solely to the pH or whether differential culture growth contributed to the decrease in α -hemolysin levels. The effect of pH on TSST-1 production has been studied more extensively. If the medium does not contain substantial concentrations of a fermentable carbon source, maximum toxin production occurs when the initial pH of the medium is between 6.5 and 7.0, with essentially no TSST-1 produced with an initial pH of 8.0 (32, 35). Under conditions of maintained pH, the maximum amount of TSST-1 is produced at pH 6.5 and 7.0 (35). Approximately fourfold less TSST-1 is produced at pH 7.5 than at pH 7.0 (35).

The mechanism for pH-mediated regulation of *agr* is not understood. It is not clear whether pH directly affects *agr* and possibly other regulatory pathways or whether there is a master pH regulon that in turn alters the expression of many systems, including *agr*. The experiments presented here demonstrate that pH has a dramatic effect on *agr* expression; 32-fold less RNAIII was produced at pH 8.0 than at pH 7.0 (Fig. 1). This alkaline pH effect on *agr* appears to be responsible for the decreased expression of *sec*, an *agr* target gene. FRI1230 (*Agr*⁺) produced fourfold more *sec* mRNA at pH 6.5 than at pH 8.0 (Fig. 2), whereas MJB710 (*Agr*⁻ derivative of FRI1230) had twofold less *sec* mRNA at pH 6.5 than at pH 8.0 (Fig. 2). These experiments clearly demonstrate that alkaline pH alone (independent of *agr*) was not sufficient to decrease expression of *sec*.

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