INFECTION AND IMMUNITY, Aug. 1992, p. 3381–3388
0019-9567/92/083381-08$02.00/0

Vol. 60, No. 8

Glucose and Nonmaintained pH Decrease Expression of the Accessory Gene Regulator (*agr*) in *Staphylococcus aureus*

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Received 31 January 1992/Accepted 18 May 1992

The effect of glucose on accessory gene regulator (*agr*) expression in *Staphylococcus aureus* was examined. *agr* is a global regulator that affects the expression of numerous genes, including those for some factors implicated in virulence, such as toxic shock syndrome toxin 1, alpha-hemolysin, and protein A. The *agr* locus determines two divergent transcripts, designated RNAII and RNAIII. RNAII contains four open reading frames (*agrABCD*), and RNAIII encodes delta-hemolysin. The mechanisms responsible for *agr*-mediated regulation are not well understood, but it appears that the RNAIII transcript plays a central role in the regulation of a number of target genes, including those for alpha-hemolysin (*hla*), beta-hemolysin (*hlb*), protein A (*spa*), and staphylococcal enterotoxin B (*seb*$^+$). In this study, *S. aureus* cultures were grown either in a shake flask system with a complex medium or in a fermentor system with a completely defined medium in which the pH and glucose concentration were maintained. Northern (RNA) blot analysis revealed that a dramatic reduction in *agr* expression was apparent only when the cultures contained glucose and when the pH was 5.5 or was not maintained. The effect of glucose on two *agr* target genes, *sec*$^+$ and *hla*, was also studied. Glucose-containing cultures produced less *sec*$^+$ and *hla* mRNAs at maintained pH (6.5). In addition, the glucose effect on *sec*$^+$ and *hla* was enhanced under conditions that inhibited *agr* expression (i.e., pH 5.5 or a nonmaintained pH).

Staphylococcus aureus is a human pathogen capable of producing a variety of extracellular and cell surface-associated proteins, some of which are virulence factors (1). Global regulation of a number of these proteins, including alpha-hemolysin, beta-hemolysin, toxic shock syndrome toxin 1 (TSST-1), coagulase, protein A, and staphylococcal enterotoxins B and C (SEB and SEC, respectively), is mediated by the accessory gene regulator (*agr*; 9, 11, 23, 25). The *agr* locus determines two divergent transcripts, RNAII and RNAIII. RNAIII (0.5 kb) encodes delta-hemolysin (13). RNAII is a 3.5-kb transcript that contains four open reading frames, including *agrA* and *agrB* (16, 19, 22). The derived amino acid sequences of AgrA and AgrB show homology to those of members of two-component signal-transducing systems (16), suggesting the possibility that *agr* may respond to environmental stimuli. Environmental signals that affect *agr* expression would also be expected to result in the altered expression of *agr* target genes.

The mechanisms involved in *agr*-mediated regulation are beginning to be elucidated. A number of observations concerning *agr* expression and regulation have been made (reviewed in references 2, 3, 16, and 20). *agr* is temporally regulated, with maximal expression occurring postexponentially (13, 16, 22). Expression of the *agr* locus appears to be autoregulatory; *agr* RNAII is required for the expression of RNAIII (13, 19, 22). RNAIII plays a central role in the regulation of some target genes (11, 16). In an Agr$^-$ background, in which there is no detectable RNAIII, the introduction of a recombinant plasmid encoding RNAIII complements the Agr$^-$ phenotype for several target genes, including those for alpha-hemolysin, beta-hemolysin, protein A, and SEC (*hla*, *hlb*, *spa*, and *sec*$^+$, respectively; 11, 16, 24). It has also been observed that there is a correlation between the levels of extracellular SEB and RNAIII in several strains; the strains with the highest levels of RNAIII also produce the highest concentrations of extracellular SEB (5).

The addition of glucose to the growth medium of *S. aureus* cultures results in the decreased production of several exoproteins, including TSST-1, alpha-hemolysin, staphylococcal enterotoxin A (SEA), SEB, and SEC (8, 10, 14, 27). The glucose effect on SEA, SEB, and SEC production occurs independently of the decrease in pH that normally results from glucose metabolism (14, 25). The molecular mechanisms involved in this glucose effect are not understood; however, the glucose-mediated regulation of *sec*$^+$ occurs at the level of mRNA (25). The glucose effect in *S. aureus* is different from the well-characterized catabolite repression in *Escherichia coli*. In *E. coli*, catabolite repression can be overcome by the addition of exogenous cyclic AMP (reviewed in reference 29); however, the addition of cyclic AMP to glucose-grown *S. aureus* cultures does not relieve the glucose effect on SEA or SEB (10, 28).

In this study, we examined the effect of glucose and the accompanying decrease in pH on *agr* expression. A reduction in *agr* expression was observed for cultures that contained glucose and that had a pH of 5.5 or a nonmaintained pH. *sec*$^+$ and *hla* mRNAs were included in the Northern (RNA) blot analysis as examples of genes subject to both glucose- and *agr*-mediated regulation (8, 12, 25). *sec*$^+$ and *hla* mRNAs were affected by glucose independently of either changes in pH or a glucose effect on *agr*. In addition, the glucose effect on *sec*$^+$ and *hla* was enhanced under conditions that led to reduced *agr* expression (i.e., a decreased pH in the presence of glucose), consistent with the existence of *agr*-dependent and *agr*-independent mechanisms that affect *sec*$^+$ and *hla* expression in the presence of glucose.

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TABLE 1. Bacterial strains and plasmids used in this study

S. aureus strain or *E. coli* plasmid	Relevant characteristic(s)	Source or reference
Strains		
Col	*seb*$^+$	M. S. Bergdoll[a]
DU4916	*seb*$^+$	M. S. Bergdoll
FRI1400	*sec*$^+$	M. S. Bergdoll
FRI429	*sec*$^+$	M. S. Bergdoll
FRI913	*sec*$^+$ *tst*	M. S. Bergdoll
FRI1038	*sec*$^+$	M. S. Bergdoll
FRI1139	*sec*$^+$	M. S. Bergdoll
FRI1230	*sec*$^+$ *hla*	M. S. Bergdoll
FRIS6	*seb*$^+$	M. S. Bergdoll
RN450	NCTC 8325 derivative cured of prophages ϕ11, ϕ12, and ϕ13	R. P. Novick[b] (21)
RN4282	*tst*	R. P. Novick (17)
Plasmids		
pDU1150	pBR322 with a 3.3-kbp *Hin*dIII-*Eco*RI fragment that contains *hla*	T. J. Foster[c] (15)
pMJB124	pGEM7Zf(+) with a 1.8-kbp insert fragment that contains *sec*$^+$	6
pRN6650	pUC18 with a 6.1-kbp insert fragment that contains *agr*	R. P. Novick (22)
pRN6656	pUC18 with a 1.2-kbp *Hin*cII-*Rsa*I fragment that contains *agrA*	R. P. Novick (22)

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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 shake flasks were described previously (25), and these conditions are referred to as the shake flask system. The cultures were grown in 2-liter Erlenmeyer flasks that contained 500 ml 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.). When NaCl, galactose, and glucose were added to the culture medium, their initial concentrations were 0.05, 0.1, and 0.1 M, respectively.

Bioflo II controlled bench-top fermentors (New Brunswick Scientific) were used to grow *S. aureus* strains under conditions of controlled pH and constant carbohydrate concentrations with a completely defined amino acid-containing culture medium. The culture conditions were described previously (25). When glucose was added, its concentration was maintained between 0.08 and 0.11 M by manually adding 50% glucose solution (25). The concentration of galactose was maintained between 0.08 and 0.11 M by adding 1.2 ml of a 25% galactose solution for each milliliter of 2 N NaOH added to maintain the pH. NaCl was added to the culture medium at a final concentration of 0.05 M.

Glucose and galactose assays. Glucose concentrations were determined with a 16-UV glucose assay kit (Sigma). Galactose concentrations were determined with galactose oxidase in a peroxidase–*o*-tolidine system as described by the supplier (Sigma).

Extraction of RNA and DNA-RNA hybridization. RNA was prepared as described by Sandler and Weisblum (26). For a given gel, samples with the same dilution factor contained the same amount of total cellular RNA, as determined by measuring the A_{260} and as verified by staining the filters with methylene blue and comparing the intensities of the signals of the 23S and 16S rRNA species (18). For Northern blot analysis, samples were electrophoresed through a 1% agarose gel and transferred to Magna NT filters (Micron Separations Inc., Westborough, Mass.) (25). For dot blot analysis, total cellular RNA was transferred to Magna NT filters by use of a Manifold I dot blot apparatus (Schleicher and Schuell, Keene, N.H.). Filters from both Northern blot and dot blot analyses were hybridized to ^{32}P-labeled probes as described previously (25), and radiation on the filters was quantified with an Ambis radioanalytic imaging system (AMBIS Systems, San Diego, Calif.).

Labeled probes. The protocols used for the isolation of *E. coli* plasmid DNA, DNA endonuclease restriction digestions, the isolation of DNA fragments from agarose gels, and labeling of double-stranded DNA with ^{32}P were described elsewhere (4).

The probe used for the detection of *sec*$^+$ mRNA in the Northern blot analysis was a 562-bp *Ssp*I fragment derived from pMJB124 (C-562); it consisted solely of *sec*$^+$ sequences (6). Northern blot analysis of RNA from *sec*$^+$ strains with C-562 revealed one signal. C-562 did not detectably hybridize to any RNA isolated from non-enterotoxin-producing strain RN450 (25). The RNAII-specific probe used for the Northern blot analysis was a 1.2-kbp *Eco*RI-*Bam*HI fragment derived from pRN6656 (Agr-1200 [previously referred to as *agr*-a14]) (22); Agr-1200 contained only RNAII-encoding sequences. RNAIII was detected by Northern blot analysis with a 962-bp *Cla*I-*Pst*I fragment (20) derived from pRN6650 (Agr-962) (22); this probe contained sequences corresponding to both RNAII and RNAIII. Agr-962 revealed signals of the expected sizes for RNAII (3.5 kbp) and RNAIII (0.5 kbp) when hybridized to Northern blots containing RNA isolated from Agr$^+$ strains. No signals were seen when Northern blots containing RNA isolated from Agr$^-$ strains were hybridized to either Agr-962 or Agr-1200 (data not shown). *hla* mRNA was detected by Northern blot analysis with a 722-bp *Cla*I fragment derived from pDU1150 (Hly-722); this probe contained only *hla* sequences and resulted in a signal of the expected size for *hla* mRNA (approximately 1.2 kb) (15).

Western blot (immunoblot) analysis. Twofold serial dilutions of supernatant samples were prepared in sterile growth medium. Samples were electrophoresed through a 12.5% acrylamide gel, transferred to a nitrocellulose filter (Schleicher and Schuell), and treated with rabbit antiserum prepared against SEC (kindly provided by Merlin S. Bergdoll, University of Wisconsin, Madison). Signals were visualized with the ProtoBlot AP system (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 non-enterotoxin-producing strain RN450 were used as negative controls as described previously (25). Different amounts of culture supernatant were analyzed to adjust for differences in turbidity (optical density at 540 nm [OD_{540}]) values among the cultures being compared.

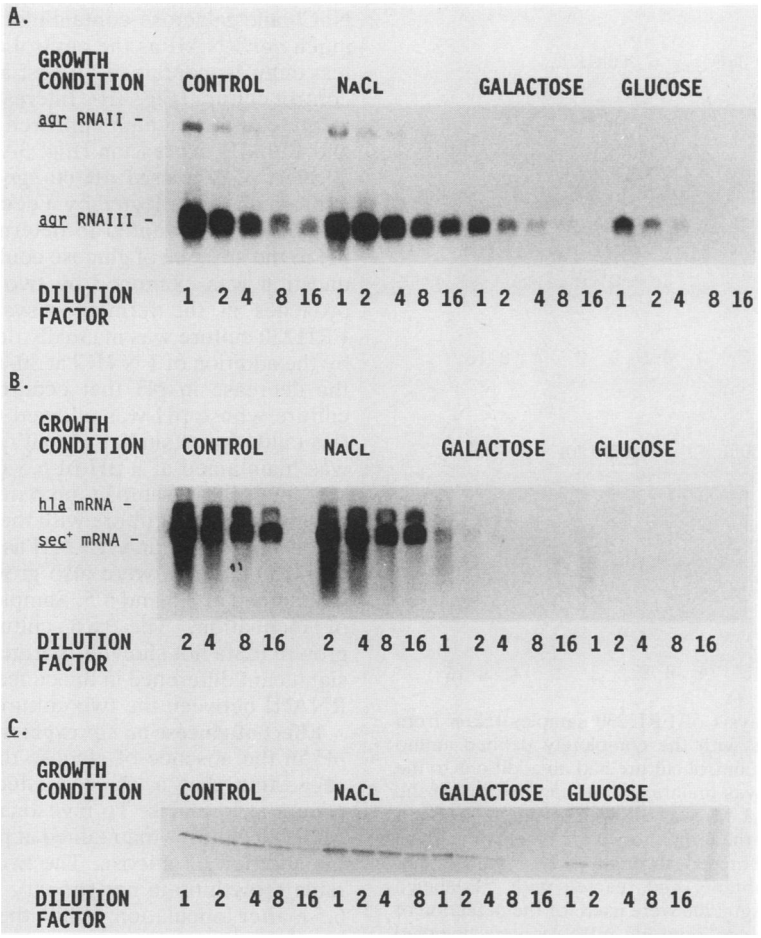

FIG. 1. Northern (A and B) and Western (C) blot analyses of FRI1230 samples taken from cultures grown in shake flasks with medium consisting of 3% N-Z amine type A and 1% yeast extract and with or without the addition of 0.05 M NaCl, 0.1 M galactose, or 0.1 M glucose. Samples were prepared after 6 h of incubation. (A and B) Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a nylon membrane, and reacted with ^{32}P-labeled probes with homology to either RNAII (Agr-1200) and RNAIII (Agr-962) (A) or *sec*$^+$ (C-562) and *hla* (Hly-722) mRNAs (B). Different samples with the same dilution factor contained the same amount of total cellular RNA. (C) Samples of culture supernatant were electrophoresed through a 12.5% acrylamide gel, electrophoretically transferred to a nitrocellulose membrane, reacted with antiserum prepared against SEC, and visualized with the ProtoBlot AP system. Different amounts of culture supernatant were analyzed to adjust for differences in OD_{540} values among the cultures. The respective OD_{540} values for the control and NaCl-, galactose-, and glucose-containing cultures were 5.1, 4.6, 4.9, and 6.0. The undiluted sample for the control culture contained 35 μl of supernatant, while the undiluted samples for the NaCl-, galactose-, and glucose-containing cultures contained 40, 37, and 31 μl, respectively.

RESULTS

Effect of glucose and galactose on *agr* expression in the shake flask system. *S. aureus* FRI1230 was grown in the shake flask system to determine whether the addition of glucose or galactose to the growth medium would affect *agr* expression. In addition to glucose- and galactose-containing cultures, a control culture that lacked a carbohydrate and an NaCl-containing culture were also examined. The NaCl-containing culture was included to determine whether the increased osmotic pressure per se would alter *agr* expression; this culture was approximately iso-osmotic in relation to the glucose- and galactose-containing cultures (7). Samples were prepared after 6 h of incubation, corresponding to postexponential growth. It was previously determined that maximal *agr* expression occurs postexponentially (13, 16, 22). Northern blot analysis of total cellular RNA revealed that the cultures grown in either glucose- or galactose-containing medium had approximately eightfold less *agr* RNAII and RNAIII than the control culture (Fig. 1A). Samples prepared from the NaCl-containing culture had at least as much *agr* RNAII and RNAIII as the control culture (Fig. 1A).

FRI1230 exhibited comparable growth under the four different growth conditions (data not shown). However, the pHs of the cultures varied during the course of the experiment. The pHs for the control and NaCl-containing cultures increased from 6.5 to 7.0, while the pHs for the glucose- and galactose-containing cultures decreased from 6.5 to 5.0. To examine *agr* expression at maintained pHs, we conducted a set of experiments with bench-top fermentors.

Effect of glucose on *agr* expression in the fermentor system when the pH was allowed to decrease. Before attempting to use the fermentor system to distinguish a carbohydrate effect from a possible pH effect on *agr* expression, we wanted to ensure that at least one of the carbohydrates (glucose) caused a decrease in *agr* expression when the pH was not

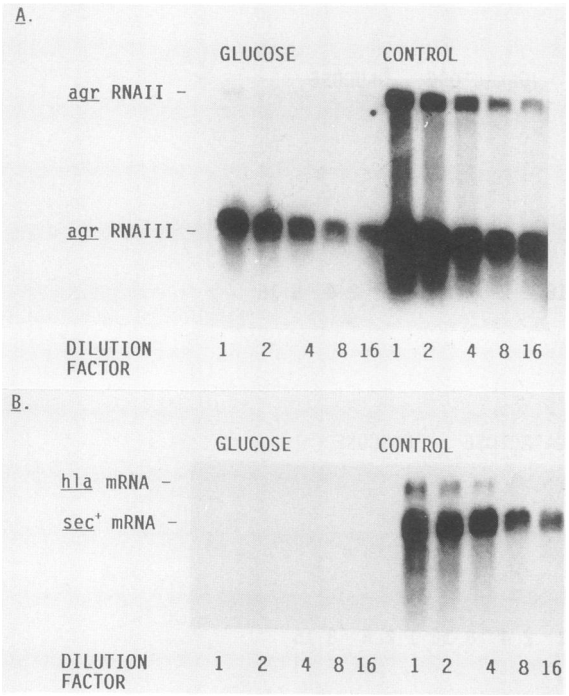

FIG. 2. Northern blot analysis of FRI1230 samples taken from cultures grown in fermentors with the completely defined amino acid-containing medium. The control culture had no additions to the growth medium, and its pH was maintained at 6.5. The pH of the glucose-containing culture (0.1 M) was allowed to fluctuate. Samples were prepared 6.5 h after the fermentors were inoculated. Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a nylon membrane, and reacted with ^{32}P-labeled probes. Probes Agr-962 and Agr-1200 were used for the detection of *agr* RNAII and RNAIII (A). *sec*$^+$ and *hla* mRNAs were detected with probes C-562 and Hly-722 (B). Different samples with the same dilution factor contained the same amount of total cellular RNA.

maintained. FRI1230 cultures were grown in the fermentor system by using a defined amino acid-containing medium either with or without the addition of glucose. The pH of the control culture was maintained at 6.5, but the pH of the glucose-containing culture was allowed to decrease. The two cultures exhibited roughly comparable growth (data not shown). The glucose-containing culture had a pH of 5.3 when samples were taken after 6.5 h of incubation. Samples prepared from the glucose-containing culture had at least eightfold less *agr* RNAII and at least fourfold less *agr* RNAIII than the control culture (Fig. 2).

Effect of glucose and galactose on *agr* expression in the fermentor system at a constant pH. After determining that glucose affected *agr* expression in the fermentor system when the pH was allowed to fluctuate, we examined FRI1230 cultures in the fermentor system with the pHs of all cultures maintained at 6.5. FRI1230 was grown in the fermentor system with the addition of glucose, galactose, or NaCl to the defined amino acid-containing medium. The control culture contained no additional components. The four cultures exhibited roughly comparable growth phases (data not shown). Samples were prepared after 6.5 h of incubation, and Northern blot analysis was performed on total cellular RNA (Fig. 3). In four separate experiments, twofold less *agr* RNAII was produced by the glucose-containing culture than the control culture (Fig. 3A). The NaCl- and galactose-containing cultures contained at least as much *agr* RNAII as the control culture (Fig. 3A). All of the test cultures contained at least as much *agr* RNAIII as the control culture (Fig. 3B). Interestingly, the addition of NaCl to the culture medium appeared to slightly enhance RNAII and RNAIII expression (Fig. 3A and B).

Effect of decreased pH on *agr* expression. Given that *agr* expression was affected by a decrease in pH in the presence of glucose, we wanted to determine whether a decrease in pH in the absence of glucose could alter *agr* expression. This question was examined by two different experimental approaches in the fermentor system. First, the pH of an FRI1230 culture was manually decreased throughout growth by the addition of 1 N HCl at 30- to 60-min intervals to mimic the decrease in pH that occurred in a glucose-containing culture whose pH was allowed to fluctuate. The growth of this culture was similar to that of the control culture, which was maintained at a pH of 6.5 (data not shown). Northern blot analysis of samples prepared after 6.5 h of incubation revealed that the culture with the decreased pH contained as much *agr* RNAII and RNAIII as the control culture (Fig. 4). FRI1230 cultures were also grown in fermentors with pHs maintained at 5.5 and 6.5. Samples were prepared after 6.5 h of incubation. The two cultures exhibited comparable growth (data not shown). Northern blot analysis revealed no significant difference in the concentrations of *agr* RNAII and RNAIII between the two cultures (Fig. 5).

Effect of glucose on *agr* expression at pH 5.5. A decrease in pH in the absence of glucose did not alter *agr* expression, suggesting that a glucose effect on *agr* expression only occurs at low pHs. To investigate this possibility, we grew FRI1230 cultures maintained at pH 5.5 either with or without the addition of glucose. The two cultures exhibited comparable growth (data not shown), and samples were prepared 6.5 h after inoculation. When the samples were examined by Northern blot analysis, the glucose-containing culture contained twofold less *agr* RNAII and between four and eightfold less *agr* RNAIII than the culture lacking glucose (Fig. 6).

Effect of glucose on *agr* expression in many *S. aureus* strains. To determine whether other strains responded to a decrease in pH in the presence of glucose in a manner similar to that of FRI1230, we screened 11 additional *S. aureus* strains in the shake flask system. Each strain was grown in the presence or absence of glucose, and samples were prepared after 6 h of incubation, during postexponential growth. All of the strains exhibited roughly comparable growth (data not shown). Dot blot analysis of the RNA samples revealed that all but one of the strains showed decreased levels of *agr* RNAIII when grown in the presence of glucose (Fig. 7). FRIS6 was the only strain examined that produced equal concentrations of RNAIII whether grown with or without the addition of glucose (Fig. 7). The insensitivity of *agr* to the presence of glucose in strain FRIS6 was further confirmed by Northern blot analysis of total cellular RNA (data not shown).

Glucose effect on *agr*-regulated genes (*sec*$^+$ and *hla*). We previously demonstrated that the glucose effect on SEC expression occurs at the level of steady-state *sec*$^+$ mRNA (25). *sec*$^+$ mRNA was included in the Northern blot analysis as an example of a gene subject to both glucose- and *agr*-mediated regulation and to extend our observations concerning the glucose effect on *sec*$^+$ (25). The decrease in the *sec*$^+$ mRNA level correlated with a decrease in the extracellular SEC level for the glucose-containing cultures in both the fermentor and the shake flask systems (Fig. 1B and

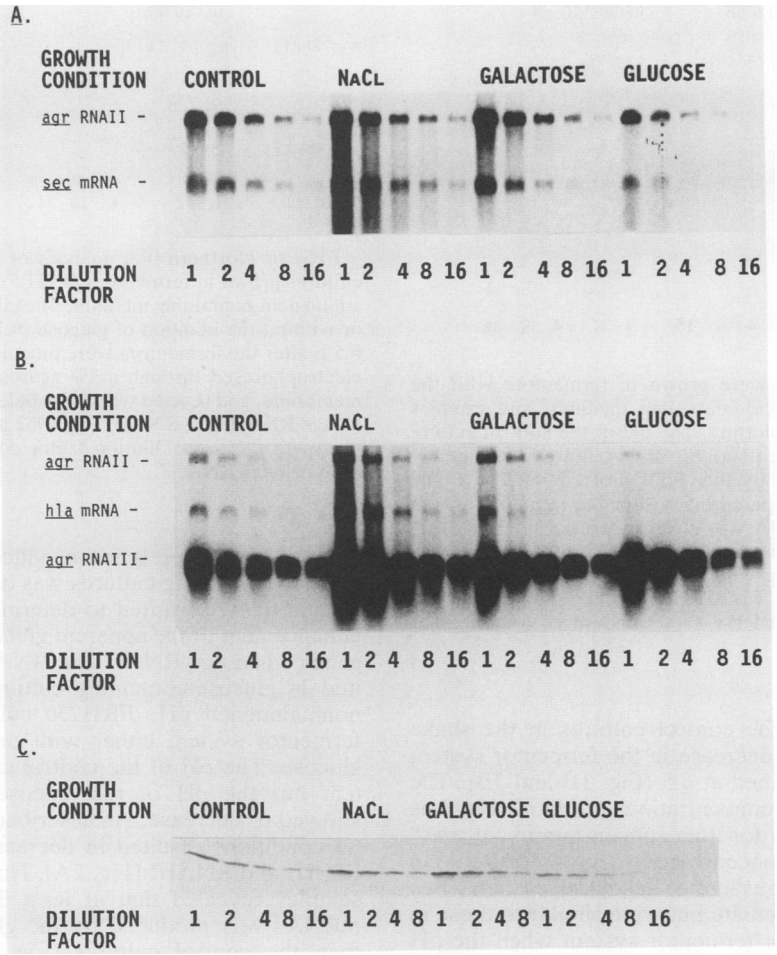

FIG. 3. Northern (A and B) and Western (C) blot analyses of FRI1230 samples taken from cultures grown in fermentors with the completely defined amino acid-containing medium with the pHs of the cultures maintained at 6.5. The control culture had no additions to the growth medium. The remaining cultures contained 0.05 M NaCl, 0.1 M galactose, or 0.1 M glucose. Samples were prepared 6.5 h after the fermentors were inoculated. (A and B) Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a nylon membrane, and reacted with ^{32}P-labeled probes. *agr* RNAII- and *sec*$^+$-specific probes (Agr-1200 and C-562; A) were used in addition to an *hla*-specific probe (Hly-722) and a probe with homology to both *agr* RNAII and RNAIII (Agr-962; B). Different samples with the same dilution factor contained the same amount of total cellular RNA. (C) Samples of culture supernatant were electrophoresed through a 12.5% acrylamide gel, electrophoretically transferred to a nitrocellulose membrane, reacted with antiserum prepared against SEC, and visualized with the ProtoBlot AP system. Different amounts of culture supernatant were analyzed to adjust for differences in OD_{540} values among the cultures. The respective OD_{540} values for the control and NaCl-, galactose-, and glucose-containing cultures were 5.4, 6.6, 9.4, and 11.4. The undiluted sample for the control culture contained 40 μl of supernatant, while the undiluted samples for the NaCl-, galactose-, and glucose-containing cultures contained 33, 23, and 19 μl, respectively.

C and 3A and C); these results were consistent with previous observations (25). There was a 16-fold decrease in the *sec*$^+$ mRNA level in the shake flask system (Fig. 1B) and a 4-fold decrease in the fermentor system (Fig. 3A) in comparison with the control cultures. NaCl-containing cultures that were approximately iso-osmotic in relation to the glucose-containing cultures were included to determine whether the observed decreases in *sec*$^+$ mRNA concentrations were due to increased osmolarity of the growth medium; the NaCl-containing cultures produced at least as much *sec*$^+$ mRNA as the control cultures (Fig. 1B and 3A). The galactose-containing cultures were examined to determine whether the glucose effect on *sec*$^+$ expression could be a general carbohydrate effect. Although *sec*$^+$ mRNA concentrations decreased 16-fold in the galactose-containing cultures in the shake flask system when the pH was decreased to 5.0 (Fig. 1B), there was no decrease in *sec*$^+$ mRNA concentrations in the galactose-containing cultures in the fermentor system when the pH was maintained at 6.5 (Fig. 3A).

Other groups have shown that the presence of glucose in the culture medium results in a decrease in the level of extracellular alpha-hemolysin (8). *hla* mRNA was included in the Northern blot analysis to determine whether the glucose effect on *hla* expression was similar to that observed for *sec*$^+$ expression. Although both *sec*$^+$ and *hla* exhibit *agr*- and glucose-mediated regulation, *hla* expression is more sensitive to regulation by *agr* than is *sec*$^+$ expression (12, 16, 25). Here, we demonstrated that the presence of glucose in the culture medium resulted in a decrease in the concentrations of *hla* mRNA in both the shake flask and the fermentor systems (Fig. 1B and 3B). There was a 16-fold decrease in the *hla* mRNA concentrations in the glucose-containing

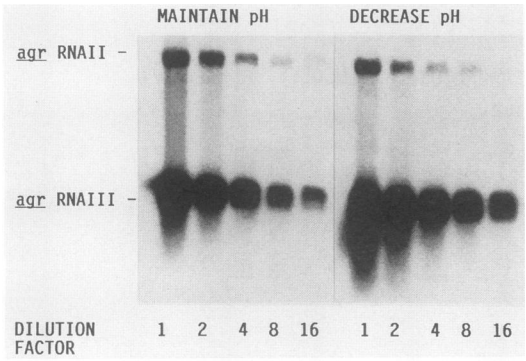

FIG. 4. FRI1230 cultures were grown in fermentors with the completely defined amino acid-containing medium, and samples were analyzed by Northern blotting. The pH of the control culture was maintained at 6.5, and the pH of the other culture was manually decreased throughout growth by the addition of 1 N HCl to a final pH of 5.3. Samples were prepared 6.5 h after the fermentors were inoculated. Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a nylon membrane, and reacted with ^{32}P-labeled probes. Probes Agr-962 and Agr-1200 were used for the detection of *agr* RNAII and RNAIII. Different samples with the same dilution factor contained the same amount of total cellular RNA.

cultures compared with the control cultures in the shake flask system and a 4-fold decrease in the fermentor system when the pH was maintained at 6.5 (Fig. 1B and 3B). The effect of galactose on the concentrations of *hla* mRNA was similar to that observed for the concentrations of *sec*$^+$ mRNA. The *hla* mRNA concentrations decreased 16-fold in galactose-containing cultures in the shake flask system when the pH was allowed to fluctuate but were similar to those in the control cultures in the fermentor system when the pH was maintained at 6.5 (Fig. 1B and 3B). The addition of NaCl to the culture medium did not decrease *hla* expression (Fig. 1B and 3B).

Both *sec*$^+$ and *hla* exhibited glucose-mediated regulation independently of pH changes (Fig. 3A and B). In contrast, the levels of *agr* RNAII and RNAIII were not dramatically

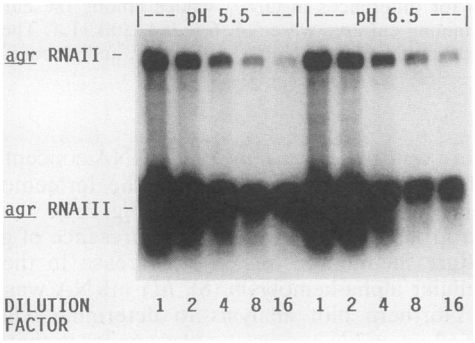

FIG. 5. FRI1230 cultures were grown in fermentors with the completely defined amino acid-containing medium. Samples were prepared 6.5 h after the fermentors were inoculated and analyzed by Northern blotting. The pHs of the cultures were maintained at 5.5 or 6.5. Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a nylon membrane, and reacted with ^{32}P-labeled probes with homology to *agr* RNAII and RNAIII (Agr-962 and Agr-1200). Different samples with the same dilution factor contained the same amount of total cellular RNA.

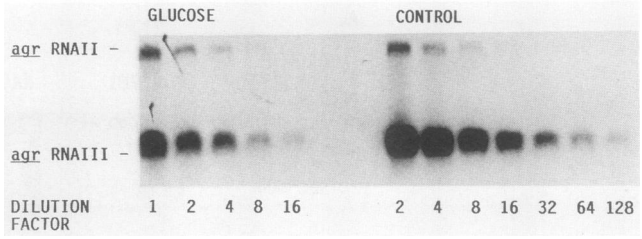

FIG. 6. Northern blot analysis of FRI1230 samples taken from cultures grown in fermentors at pH 5.5 with the completely defined amino acid-containing medium. The cultures were grown either with or without the addition of glucose (0.1 M). Samples were prepared 6.5 h after the fermentors were inoculated. Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a nylon membrane, and reacted with ^{32}P-labeled probes that show homology to *agr* RNAII and RNAIII (Agr-962 and Agr-1200). Different samples with the same dilution factor contained the same amount of total cellular RNA.

affected by the presence of glucose unless the pH of the glucose-containing cultures was decreased (Fig. 1A, 3A and B, and 6). We wanted to determine whether there was an enhancement of the apparent glucose effect on *agr*-regulated genes when *agr* RNAII and RNAIII expression was inhibited in glucose-containing cultures under conditions of a nonmaintained pH. FRI1230 cultures were grown in the fermentor system either with or without the addition of glucose. The pH of the control cultures was maintained at 6.5, but the pH of the glucose-containing cultures was allowed to decrease. As described above, these experimental conditions resulted in decreased concentrations of *agr* RNAII and RNAIII (Fig. 2A). Northern blot analysis of the samples revealed that at least 32-fold less *sec*$^+$ and *hla* mRNAs was produced by the glucose-containing cultures than the control cultures (Fig. 2B). This result was in contrast to the fourfold difference observed in the same system when the pH of both the glucose-containing and the control cultures was kept at 6.5 (Fig. 3A and B).

DISCUSSION

The addition of glucose to the growth medium of *S. aureus* FRI1230 in the shake flask and fermentor systems resulted in a dramatic decrease in the concentrations of *agr* RNAII and

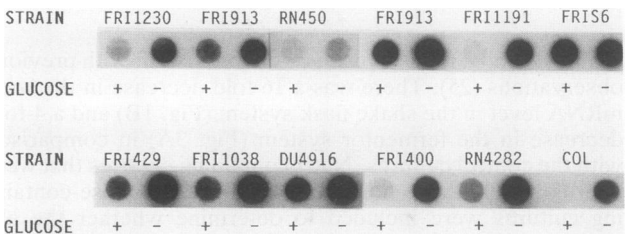

FIG. 7. RNA dot blot analysis of samples isolated from *S. aureus* strains grown in the shake flask system with medium containing 3% N-Z amine type A and 1% yeast extract and either with or without the addition of glucose. Samples were prepared after 6 h of incubation. Total cellular RNA was loaded onto a nylon filter with a dot blot apparatus and reacted with a ^{32}P-labeled probe (Agr-962) that shows homology to *agr* RNAIII. For a given strain, samples from cultures with and without glucose contained the same amount of total cellular RNA.

RNAIII when the pH of the cultures was allowed to decrease (Fig. 1A and 2A). This effect on *agr* was not limited to glucose. The addition of galactose to the growth medium resulted in a similar decrease in *agr* expression (Fig. 1A). The altered *agr* expression in the glucose- and galactose-containing cultures in the shake flask system was not due to increased osmolarity; an approximately iso-osmotic NaCl-containing culture produced at least as much RNAII and RNAIII as the control culture (Fig. 1A). The reduction in RNAII and RNAIII concentrations could have been due to the presence of the carbohydrates. Alternatively, the decreased *agr* expression may have resulted from some change in cellular metabolism or the environment due to carbohydrate utilization, such as the decrease in pH that presumably resulted from the accumulation of fermentative end products.

The dramatic decrease in the levels of both *agr*-associated transcripts when the pH of the glucose-containing culture was allowed to decrease (Fig. 1A and 2A) did not occur at a constant pH of 6.5 (Fig. 3A and B). At a constant pH of 6.5, RNAIII concentrations were unaffected by the addition of glucose (Fig. 3B). A slight glucose effect on RNAII expression was observed (Fig. 3A); however, the significance of this twofold decrease in RNAII expression is unclear.

agr RNAIII was subject to a pH-dependent glucose effect. This conclusion is based on the observations that there was no apparent decrease in RNAIII levels in the glucose-containing culture maintained at pH 6.5 (Fig. 3) but that RNAIII levels decreased between four and eightfold in the glucose-containing culture maintained at pH 5.5 compared with the control culture maintained at pH 5.5 (Fig. 6). There was no evidence that a decrease in pH from 6.5 to 5.3 (without glucose) contributed to the decrease in RNAIII levels (Fig. 4).

The decrease in *agr* expression occurring in medium containing glucose under conditions of a nonmaintained pH would be expected to affect the expression of *agr* target genes, such as *sec*$^+$ and *hla*. Both of these *agr* target genes were also subject to glucose-mediated regulation (Fig. 1B, 2B, and 3A and B) (8, 25). The glucose effect on *sec*$^+$ and *hla* expression appeared to be the result of two cumulative effects; one effect was correlated with a decrease in *agr* RNAIII levels, and the other effect was independent of *agr*.

Consistent with there being an *agr*-independent glucose effect on *sec*$^+$ expression is the observation that a glucose effect on *sec*$^+$ expression is still observed in an Agr$^-$ strain (MJB407 *agr*::Tn*551*) (25). In this report, a glucose effect on *sec*$^+$ and *hla* expression was also observed independently of a glucose effect on *agr* RNAIII expression when cultures were maintained at pH 6.5. Under these conditions, *sec*$^+$ and *hla* mRNA concentrations were decreased approximately fourfold in the glucose-containing culture compared with the control culture; *agr* RNAIII expression was unaffected (Fig. 3A and B).

It would be expected that the *agr*-independent effect of glucose on the expression of *sec*$^+$ and *hla* would be in addition to the glucose-pH effect on these genes via *agr*, and experimental data support this prediction. When the pH of the glucose-containing culture was allowed to fluctuate in the fermentor system, the glucose-containing culture produced about 8-fold less *agr* RNAIII and at least 32-fold less *sec*$^+$ and *hla* mRNAs than the control culture (Fig. 2A and B). This 32-fold decrease in *sec*$^+$ and *hla* mRNA levels when RNAIII concentrations were decreased was in contrast to the 4-fold decrease in *sec*$^+$ and *hla* mRNA levels when RNAIII concentrations were unchanged (Fig. 3A and B). This correlation between the reduced expression of RNAIII and *agr* target genes suggested the existence of an *agr*-dependent glucose-pH effect on *sec*$^+$ and *hla* expression and was consistent with our understanding of the *agr* regulation of these genes being mediated through RNAIII or a product of RNAIII. The Agr$^-$ phenotype for both *sec*$^+$ and *hla* could be complemented by the introduction of a recombinant plasmid encoding RNAIII (11, 24). Also, there was a correlation between the levels of extracellular SEC and RNAIII in several *S. aureus* strains; the strain with the highest concentration of RNAIII produced the most SEC (24). These observations suggested that RNAIII levels were important for the regulation of *sec*$^+$ and *hla* expression, in agreement with observations made by Compagnone-Post et al. (5). These authors reported that among wild-type *S. aureus* strains, there was a correlation between steady-state levels of *seb*$^+$ mRNA and RNAIII (5).

agr is a global regulator that affects the expression of a large number of genes in *S. aureus*. An Agr$^+$ strain produces more alpha-hemolysin, beta-hemolysin, TSST-1, SEB, and SEC and less protein A than its Agr$^-$ derivative (reviewed in references 2 and 16). Growth conditions that alter *agr* expression would be expected to indirectly modulate the expression of numerous *agr* target genes. The results presented in this paper suggest that glucose under conditions of a nonmaintained pH (or at pH 5.5) may be one such factor that can alter *agr* expression.

ACKNOWLEDGMENTS

This work was supported in part by funds from the Hatch Act administered through the Agricultural Experiment Station, University of Wisconsin—Madison. L. B. Regassa was supported with funds from Cellular and Molecular Biology training grant 5 T32 GM07215.

We thank Scott Cayley for helpful discussions concerning osmotic pressure, Linda Hilsenhoff for the photographic work, and Sue Reis for technical assistance in preparing the manuscript.

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