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Steady-State Staphylococcal Enterotoxin Type C mRNA Is Affected by a Product of the Accessory Gene Regulator (*agr*) and by Glucose

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The effects of the accessory gene regulator (*agr*) and glucose on staphylococcal enterotoxin type C (SEC) gene (*sec*$^+$) expression were examined. For the *agr* studies, a Tn*551* insertionally inactivated *agr* was transferred into two different *sec*$^+$ *Staphylococcus aureus* strains. Western blot (immunoblot) analysis showed that each of the *sec*$^+$ Agr$^-$ derivatives produced less extracellular SEC than their Agr$^+$ parent strains. Analysis of Northern (RNA) blots was consistent with at least part of the *agr* effect being at the level of steady-state *sec*$^+$ mRNA. We examined the glucose effect on *sec*$^+$ expression by utilizing both a fermentor system with a completely defined amino acid-containing medium in which the pH of the medium was maintained at 6.5 and a shake flask system with a complex medium in which the pH was allowed to fluctuate during bacterial growth. In both systems, samples from the cultures containing glucose had less extracellular SEC and less steady-state *sec*$^+$ mRNA compared with the control cultures which lacked glucose. An intact *agr* was not required for the glucose effect on *sec*$^+$ expression; MJB407, an Agr$^-$ *sec*$^+$ strain, produced more SEC and had more steady-state *sec*$^+$ mRNA when grown in medium that lacked glucose compared with medium that contained glucose.

Staphylococcal enterotoxins are well known as the cause of the intoxication staphylococcal food poisoning, a common cause of confirmed foodborne disease in the United States (24). In addition, epidemiological and experimental data have implicated the enterotoxins as important virulence factors in some toxic shock syndrome-like cases (9, 12, 13, 19, 31, 38).

The staphylococcal enterotoxins are classified into five major serological groups (A through E, referred to as SEA through SEE) (2). Each is a simple, single-chain extracellular protein (2). The nucleotide sequences have been determined for each staphylococcal enterotoxin type (1, 3, 5–8, 17, 23). Among these genes, there is 43 to 85% nucleotide sequence identity; the genes for SEA (*sea*$^+$) and SEE (*see*$^+$) are the most closely related (85% nucleotide sequence identity) followed by the SEB and SEC gene pair (*seb*$^+$ and *sec*$^+$) which share 75% nucleotide sequence identity with one another.

The phenomenon of staphylococcal enterotoxin regulation by glucose has been studied under several different culture conditions. In addition to glucose and glucose analogs, glycerol, maltose, and pyruvate also result in a decrease in extracellular enterotoxin concentration (18, 22, 40, 41). The glucose effect on staphylococcal enterotoxin production is not simply due to a decrease in the pH of the glucose-containing cultures. Jarvis and co-workers demonstrated that glucose affects SEA, SEB, and SEC production in batch cultures with the pH maintained at 6.5 (22). Iandolo and Shafer demonstrated a glucose effect on SEB production with a complex medium buffered with 1% K_2HPO_4; in this medium, the pH of the cultures never dropped below 6.8 (18). The glucose effect on staphylococcal enterotoxin production in *Staphylococcus aureus* is different from the catabolite repression system of *Escherichia coli* that depends on cyclic AMP and the catabolite gene activator protein. *S. aureus* cells take up cyclic AMP, but the glucose effect on staphylococcal enterotoxin production is not altered by cyclic AMP (18).

agr is an accessory gene regulator that affects the production of several exoproteins including α-hemolysin, β-hemolysin, toxic shock syndrome toxin-1, coagulase, and protein A (20, 34). There is also evidence that *agr* affects the expression of some types of enterotoxins. More SEB and SED are produced in strains that have active *agr*$^+$ alleles than by derivatives that have an insertionally inactivated *agr* (1, 14). For *seb*$^+$, the *agr* effect is at the level of steady-state *seb*$^+$ mRNA (14).

In this study, *sec*$^+$ expression was examined. For FRI1230, there was a dramatic increase in the extracellular SEC concentration following exponential growth. This increase correlated with a peak in the level of steady-state *sec*$^+$ mRNA. Two *sec*$^+$ strains with insertionally inactivated *agr* alleles produced less extracellular SEC and less *sec*$^+$ mRNA than their *agr*$^+$ parent strains. Glucose resulted in decreased amounts of both extracellular SEC and steady-state *sec*$^+$ mRNA. The glucose effect on *sec*$^+$ expression does not require an intact *agr* because an Agr$^-$ strain, MJB407, still exhibited a glucose effect on *sec*$^+$ expression.

MATERIALS AND METHODS

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

The following growth conditions were referred to as the "shake flask system." 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.). This medium was inoculated with an overnight culture to an optical density at 540 nm (OD_{540}) of 0.2 (Spec 20 [Bausch and Lomb]; rebuilt by Update Instrument, Madison, Wis.) and incubated in a Controlled Environmental Incubator Shaker (New Brunswick Scientific, Edison, N.J.) at 37°C with agitation (200 rpm). The pH of the cultures

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or origin
S. aureus		
FRI400	*sec*$^+$ Tmn^r Hem$^+$[a] Em^s	M. S. Bergdoll, University of Wisconsin-Madison
FRI1230	*sec*$^+$ Tmn^s Hem$^+$ Em^s	M. S. Bergdoll (35)
ISP484	Tmn^r	P. A. Pattee, Iowa State, Ames (32)
ISP546	Sec$^-$ Tmn^s *agr*::Tn*551*[b] Hem$^-$	P. A. Pattee (25)
MJB392	*sec*$^+$ Tmn^r *agr*::Tn*551* Hem$^-$	FRI400 × 80α/ISP546 select for Em^r (this study)[c]
MJB466	*sec*$^+$ Tmn^r Hem$^+$ Em^s	FRI1230 × 80α/ISP484 select for Tmn^r (this study)
MJB407	*sec*$^+$ Tmn^r *agr*::Tn*551* Hem$^-$	MJB466 × 80α/ISP546 select for Em^r (this study)
RN450	Sec$^-$	R. P. Novick, Public Health Research Institute, New York, N.Y. (28)
E. coli plasmid		
pMJB124	pGEM7Zf(+) containing a 1.8-kbp insert fragment that has *sec*$^+$	Reference 8
pRN6656	pUC18 containing a 1.2-kbp *Hin*cII/*Rsa*I *agr*-containing fragment	R. P. Novick, Public Health Research Institute, New York, N.Y. (33)

[a] Hem$^+$ signifies that there was a zone of hemolysis surrounding the colonies following incubation on medium containing sheep erythrocytes.
[b] Tn*551* encodes a determinant for erythromycin resistance (Em^r) (29).
[c] FRI400 was transduced with a lysate of phage 80α that had been prepared on strain ISP546.

was monitored by using ColorpHast pH paper (VWR, Chicago, Ill.).

Bioflo II Controlled Bench Top Fermentors (New Brunswick Scientific) were used to maintain the pH and the glucose concentration of the cultures during incubation; these culture conditions were referred to as the "fermentor system." The completely defined medium described by Wu and Bergdoll as medium 4 with a total amino acid concentration of 2.08% (wt/vol) was used (42). Each fermentor was inoculated with 50 ml of an overnight culture to an initial OD_{540} of 0.2 to 0.3. The inoculum had been washed and resuspended in the defined amino acid-containing medium. The cultures were incubated at 37°C with the air flow maintained at 0.4 liter/min, unless stated otherwise. Agitation of the cultures was provided by a dual blade agitator (300 rpm). Equal volumes of Antifoam A emulsion (10% [vol/vol]; Sigma Chemical Co., St. Louis, Mo.) were added manually to both the control culture which lacked glucose and the test culture which contained glucose to reduce foaming. The pH of the culture was monitored with a pH electrode (Ingold Electrodes Inc., Wilmington, Mass.), and the pHs of the cultures were automatically maintained at pH 6.5 by the addition of 1 N HCl or 2 N NaOH for experiments done at a constant pH. Glucose concentration for the test cultures was maintained between 14 and 20 mg/ml by manually adding 588 μl of 50% glucose solution for each milliliter of 2 N NaOH used (22, 34a). At 30- or 60-min intervals, glucose was added to the cultures, with samples for analysis being taken just prior to the addition of glucose. The glucose concentrations of the culture samples were confirmed with the 16-UV glucose assay kit (Sigma).

Bacteriophage 80α (obtained from P. A. Pattee [27]) was propagated and assayed on Trypticase soy agar (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md.) containing 5×10^{-3} $CaCl_2$ mM by using a soft-agar overlay of Trypticase soy broth plus 0.5% (wt/vol) agar (Difco). Tetracycline- and erythromycin-resistant transductants were selected with brain heart infusion agar (Difco) containing 5 μg of sodium citrate, 3 μg of tetracycline, and 5 μg of erythromycin per ml.

Medium and conditions used to store and grow *E. coli* have been described (3).

Glucose assay. The glucose concentrations of the culture samples were determined with the 16-UV glucose assay kit (Sigma).

Transduction. Phage 80α transducing lysates were propagated on donor strains as described previously (39). Transductions were performed essentially by the method of Schroeder and Pattee, except for two modifications (39). The multiplicity of infection was increased to between 2 and 5 and the recipient cells that had been resuspended in Trypticase soy broth (10^9 CFU/ml; BBL Microbiology Systems) were incubated at 56°C for 3 min prior to the addition of the transducing lysate. These modifications were required for successful transduction of both FRI400 and FRI1230, using transducing lysates prepared on either ISP484 or ISP546.

Extraction of RNA and Northern (RNA) blot analysis. RNA was prepared by the method described by Sandler and Weisblum (37). OD readings were used to determine the quality and concentration of RNA (26). Visualization of the RNA samples on a 1% ethidium bromide-stained agarose gel did not reveal any DNA contamination. For the RNA preparations, the A_{260}/A_{280} ratio was between 1.9 and 2.0. For a given gel, the undiluted samples contained the same quantity of RNA as determined by the A_{260}. Samples were electrophoresed through a gel of 1.0% agarose–2.2 M formaldehyde, and the gel running buffer was 0.2 M morpholinepropanesulfonic acid–50 mM sodium acetate–5 mM EDTA (pH 7). RNA was transferred from the gel onto Nytran filters (Schleicher & Schuell, Keene, N.H.) and hybridized to a ^{32}P-labeled *sec*$^+$-specific (C-562) probe, using a procedure described previously (26). Northern blot analysis of RNA from *sec*$^+$ strains, using C-562 as the probe, revealed one signal. C-562 did not detectably hybridize to any RNA isolated from the non-enterotoxin-producing strain RN450 (data not shown). Radiation on the filter was quantified by the Ambis Radioanalytic Imaging System (AMBIS Systems, San Diego, Calif.). The relative amounts of RNA among samples were determined by using data corresponding to sample dilutions that produced signals which were on the linear portion of the dose (dilution factor)-response (signal intensity) curve. Finally, the intensities of the signals of the 23S and 16S rRNA species as visualized by the methylene blue stain were compared to verify that comparable dilutions of different samples contained the same amount of total cellular RNA.

All experiments that involved determination of relative amounts of *sec*$^+$ mRNA or extracellular SEC were performed at least twice; representative blots are shown in Fig. 1, 2, 4, 5, 7, and 8.

Labeled probes. Protocols used for isolation of *E. coli* plasmid DNA, DNA endonuclease restriction digestions, isolation of DNA fragments from agarose gels, and labeling of double-stranded DNA with ^{32}P have been described elsewhere (3).

The probe used for detection of *sec*$^+$ mRNA in Northern blot analysis and *sec*$^+$-containing restriction endonuclease fragments in Southern blot analysis was derived from pMJB124 (7). It was a 562-bp *Ssp*I fragment that consisted solely of *sec*$^+$ sequence (designated probe C-562) (7). The probe used for detection of *agr*-containing restriction endonuclease fragments in Southern blot analysis was a 1.2-kbp insert fragment of pRN6656 (33).

Western blot (immunoblot) analysis. Culture samples were centrifuged to remove cellular debris, and the supernatant fluids were used for Western blot analysis. To compare relative concentrations of accumulated extracellular SEC from two different cultures, twofold serial dilutions of supernatants were prepared in uninoculated medium. Samples were electrophoresed through a 12.5% acrylamide gel and electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell). Filters were treated with rabbit antiserum prepared against SEC (kindly provided by Merlin S. Bergdoll, University of Wisconsin-Madison, Madison). Signals were visualized with the ProtoBlot System AP (Promega Corp., Madison, Wis.). Intensity of the signals was quantified by densitometry (GS 300 densitometer; Hoefer Scientific Instruments, San Francisco, Calif.). Supernatant samples prepared from RN450 cultures were used as negative controls; signals were observed only in the region corresponding to proteins of >30,000 molecular weight. Presumably, these signals were due to protein A. The calculated molecular weight of SEC is 27,438 (7). No proteins corresponding to 30,000 molecular weight or smaller were observed by Western blot analysis of RN450 culture supernatants. Unless otherwise stated, the same amounts of culture supernatants were analyzed for samples that are indicated as having the same dilution factor.

RESULTS

Steady-state levels of *sec*$^+$ mRNA for different growth phases. Supernatant samples prepared from FRI1230 cultures grown in the shake flask system when the culture had OD_{540} values of 1, 2, 3, 4, 5, and 6 were analyzed by Western blot (data not shown). This analysis revealed that the majority of extracellular SEC accumulates postexponentially. In a separate experiment, samples for Western and Northern analyses were taken from an FRI1230 batch culture both during and after exponential growth (Fig. 1). The highest concentration of steady-state *sec*$^+$ mRNA was observed for samples obtained following exponential growth, correlating with the large increase in extracellular, accumulated SEC. Based on these experiments, samples for Northern blot analysis of steady-state *sec*$^+$ mRNA were prepared from cultures during postexponential growth, but prior to the time when the OD_{540} of the culture remained constant.

Effect of *agr* on *sec*$^+$ expression. Two *sec*$^+$-containing strains were constructed that had inactive *agr* alleles. First, the tetracycline resistance (Tmn^r) chromosomal marker of ISP484 was transduced into FRI1230, forming MJB466; Tmn^r served as an outside marker for FRI1230 in the next cross. The Tn*551* insertionally inactivated *agr* of ISP546 was transferred into *sec*$^+$ strains MJB466 and FRI400, resulting in the erythromycin resistance (Erm^r) transductants MJB407 and MJB392, respectively. Acquisition of Erm^r by MJB466 and FRI400 correlated with loss of hemolytic activity on Trypticase soy agar that contained 5% (vol/vol) sheep erythrocytes; this was consistent with the Erm^r derivatives being Agr$^-$ (20, 34), unlike their respective parent strains. Physical evidence that MJB407 and MJB392 had Tn*551* inserted within *agr* like ISP546 came from Southern blot analysis of genomic DNA of the parent, donor, and recipient strains (data not shown). Genomic DNA digested with *Bgl*I or *Eco*RI was analyzed by Southern blots hybridized to an *agr*-containing probe by a previously described method (3). Examination of autoradiograms revealed that the banding patterns of the transductants were identical to that of ISP546 and different from that of their parent strains (data not shown). These patterns were consistent with both MJB407 and MJB392 having only one Tn*551* insertion and this insertion being within *agr*. In addition, Southern blot analysis with the ^{32}P-labeled C-562 probe revealed that each of the parent and recipient strains had a 6-kbp *Cla*I fragment that contained *sec*$^+$; thus, acquisition of Tn*551* had not disrupted *sec*$^+$ (data not shown).

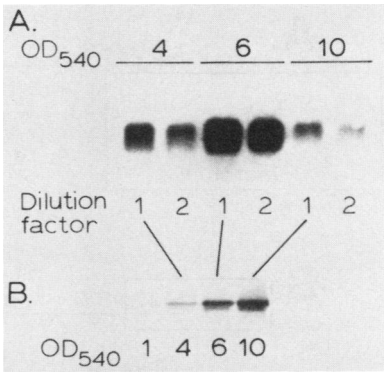

FIG. 1. Northern (A) and Western (B) blot analyses of FRI1230 samples prepared by using shake flasks with medium consisting of 3% N-Z amine type A and 1% yeast extract. Cultures with an OD_{540} of 1 were in the exponential phase. Cultures with values of 4, 6, and 10 were in the postexponential phase. (A) Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a Nytran membrane, and reacted with a ^{32}P-labeled *sec*$^+$-specific probe, C-562. Samples with the same dilution factor contain the same amount of total cellular RNA. (B) Samples of culture supernatant (40 μl) 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 Protoblot Western Blot AP System.

Both pairs of parent and recipient strains had the same growth patterns as judged by monitoring the OD_{540} of cultures grown in the shake flask system. Appropriate samples for Western analysis of accumulated extracellular SEC and Northern analysis of steady-state *sec*$^+$ mRNA were collected when the cultures had attained an OD_{540} of 5.0 or 6.0. As determined by densiometric scans of Western blots, the MJB392 sample contained 16- to 32-fold less SEC than the FRI400 sample and the MJB407 sample had at least 4-fold less SEC than the MJB466 sample (Fig. 2A). Northern blot analysis showed that samples from both MJB392 and MJB407 contained two- to threefold less *sec*$^+$ mRNA compared with samples obtained from their respective parent

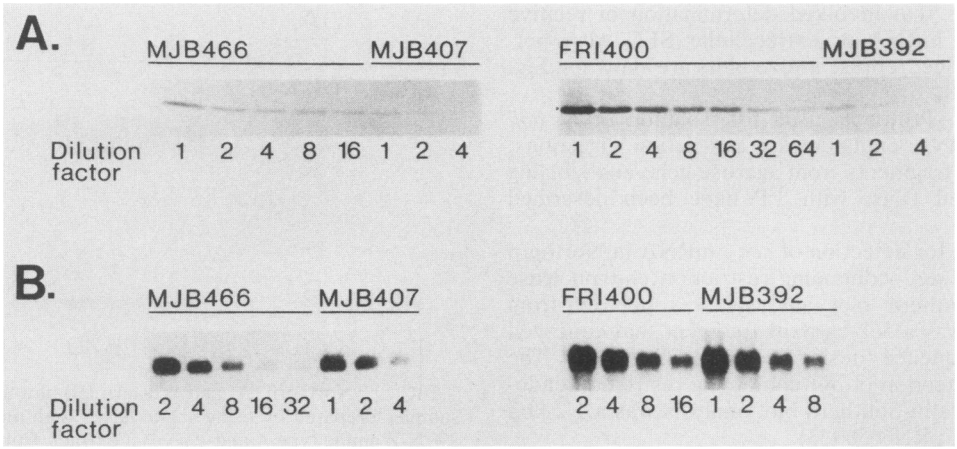

FIG. 2. Western (A) and Northern (B) blot analyses of samples prepared from MJB466 and FRI400 cultures, Agr^+ parent strains, and their respective Agr^- derivatives, MJB407 and MJB392. Each strain was grown in an Erlenmeyer flask that contained 3% N-Z amine type A and 1% yeast extract. Samples were collected when the OD_{540} of the cultures was 5.0 (MJB466 and MJB407) or 6.0 (FRI400 and MJB392). (A) 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 by using the Protoblot Western Blot AP System. Samples prepared from different strains with the same dilution factor contain the same amount of culture supernatant. (B) Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a Nytran membrane, and reacted with a ^{32}P-labeled sec^+-specific probe, C-562. Different samples with the same dilution factor contain the same amount of total cellular RNA.

strains (Fig. 2B). These results were observed in three separate experiments (Fig. 2 contains representative data).

Effect of glucose on sec^+ expression. The glucose effect on extracellular SEC production by FRI1230 was examined first by using the shake flask system. Samples were prepared when the cultures had OD_{540} values of 2.5, 6.0, and 9.0 and glucose concentrations of 16, 12, and 3 mg/ml, respectively. Based on OD_{540} readings, growth of the test and control cultures was very similar (Fig. 3). Supernatants from exponential-phase cultures (OD_{540}, 2.5) grown in medium containing or lacking glucose had similar SEC concentrations (Fig. 4A). Differences in the concentration of extracellular SEC and sec^+ mRNA between the control and test cultures were observed following exponential growth (Fig. 4A). Maximal steady-state sec^+ mRNA was detected when the cultures reached an OD_{540} of 6.0, which corresponded to postexponential growth (Fig. 4B). Analysis of samples obtained when both the control and test cultures had an OD_{540} of 6.0 revealed that the control culture contained at least 32-fold more accumulated SEC and steady-state sec^+ mRNA by visual inspection (Fig. 4; all dilutions for the Northern blot are not shown).

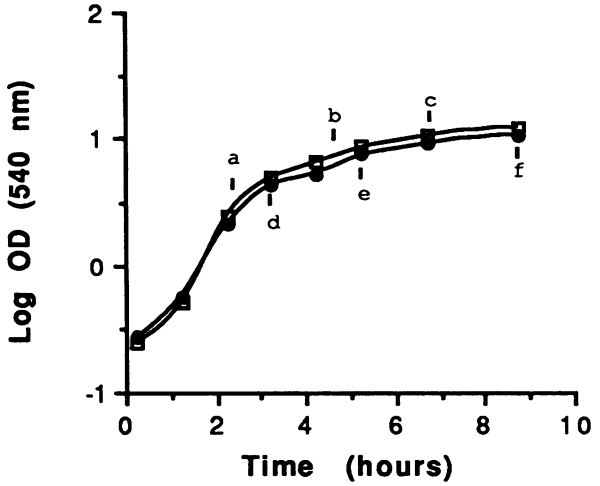

FIG. 3. Growth curves for FRI1230 grown in shake flasks with medium consisting of 3% N-Z amine type A and 1% yeast extract that either contained (□) or lacked (●) glucose. The logarithm of the OD_{540} for the cultures was plotted against the time that had elapsed since inoculation. The times at which samples were prepared for Western and Northern blot analyses are indicated by the lowercase letters. OD_{540} values were 2.5 for the test (a) and control (d) cultures; 6.0 for the test (b) and control (e) cultures; and 9.0 for the test (c) and control (f) cultures.

FRI1230 grown in the presence of glucose and the control culture which lacked added glucose showed very comparable growth (Fig. 3); however, the pHs for the two cultures diverged. During the course of the experiment, the pH of the control culture increased from 6.5 to 8.0. In contrast, the test culture exhibited a drop in pH from 6.5 to 5.0. A difference between the accumulated SEC in the test and control cultures was not apparent until after the pHs of the cultures diverged (data not shown). Other workers have demonstrated that staphylococcal enterotoxin production is sensitive to the pH of the medium (15, 16, 36). Therefore, with the shake flask system, it was not possible to differentiate between glucose affecting sec^+ expression due to pH variations as opposed to another mechanism independent of pH.

To study the effect of glucose on accumulated SEC under conditions of controlled pH and maintained glucose concentration, experiments were carried out in bench top fermentors. In the initial experiments utilizing the fermentor system, glucose concentrations were maintained, but the pH of the cultures was not controlled. Under these conditions, the pH of the control culture increased from 6.5 to 7.2 and the pH of the test culture decreased from 6.5 to 5.0. Samples for Western analysis were prepared both 1 and 2 h after the exponential growth phase, corresponding to 4 and 5 h of incubation, respectively. Comparison of the culture supernatants by Western blot analysis showed that samples from

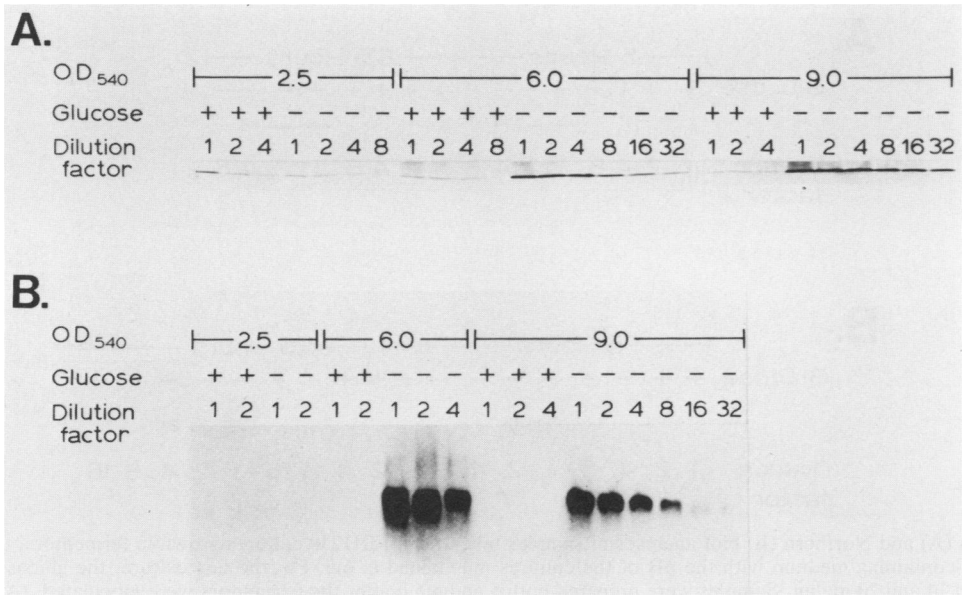

FIG. 4. Western (A) and Northern (B) blot analyses of FRI1230 samples taken from cultures grown in shake flasks with medium consisting of 3% N-Z amine type A and 1% yeast extract that either contained or lacked glucose. OD_{540} refers to the ODs of the culture when the samples were prepared. (A) Samples 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 by using the Protoblot Western Blot AP System. Samples with the same dilution factor contain the same amount of culture supernatant. (B) Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a Nytran membrane, and reacted with a ^{32}P-labeled *sec*$^+$-specific probe, C-562. Different samples with the same dilution factor contain the same amount of total cellular RNA.

the control culture had at least eightfold more SEC than samples from the glucose-containing test cultures (Fig. 5).

The glucose effect on *sec*$^+$ expression was examined using the fermentor system with the pH of the cultures maintained at 6.5. As judged by OD_{540} values, the growth of the control culture was slightly slower than that of the test culture (Fig. 6). Samples were prepared 5 and 6.5 h after inoculation, corresponding to 1.5 and 3 h after the end of the exponential growth phase. In three separate experiments, supernatants from the control culture had about fourfold more SEC than supernatants from the glucose-containing culture (Fig. 7A). Analysis of Northern blots probed with ^{32}P-labeled C-562 in separate experiments revealed that samples prepared from the control culture had approximately fourfold more steady-state *sec*$^+$ mRNA than samples from the cultures grown in glucose-containing medium (Fig. 7B).

In an effort to determine whether the glucose effect on *sec*$^+$ expression required an intact *agr*, an Agr$^-$ strain (MJB407) was examined under conditions of maintained

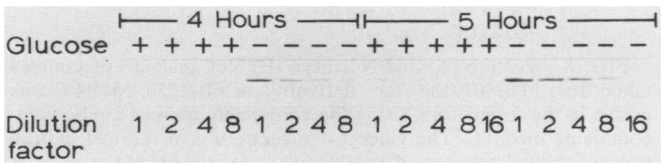

FIG. 5. Western blot analysis of samples prepared from FRI1230 cultures grown in fermentors, using the completely defined amino acid-containing medium. The glucose concentration of the test culture was maintained between 14 and 20 mg/ml. pHs of the cultures were not maintained. After 4 h of incubation, both cultures had an OD_{540} equal to 4.5. When the samples were prepared after 5 h of incubation, the test culture had an OD_{540} of 5.0, but the control culture had reached an OD_{540} of 6.0. Samples with the same dilution factor contain the same amount of culture supernatant. 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 by using the Protoblot Western Blot AP System.

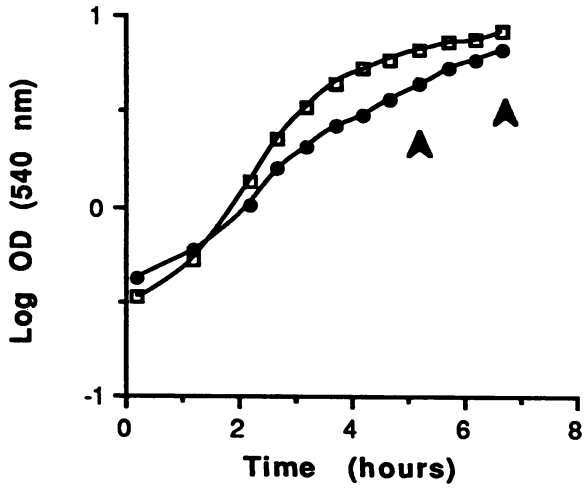

FIG. 6. Growth curves for FRI1230 grown in fermentors, using the completely defined amino acid-containing medium that either contained (□) or lacked (●) glucose. The pHs of the cultures were maintained at 6.5. The logarithm of the OD_{540} for the cultures was plotted against the time that had elapsed since inoculation. Samples were prepared for Western and Northern blots at both 5 and 6.5 h after inoculation as indicated by the arrows. The respective OD_{540} values for the control and test cultures were 3.8 and 5.8 after 5 h of incubation and 5.8 and 7.2 after 6.5 h of incubation.

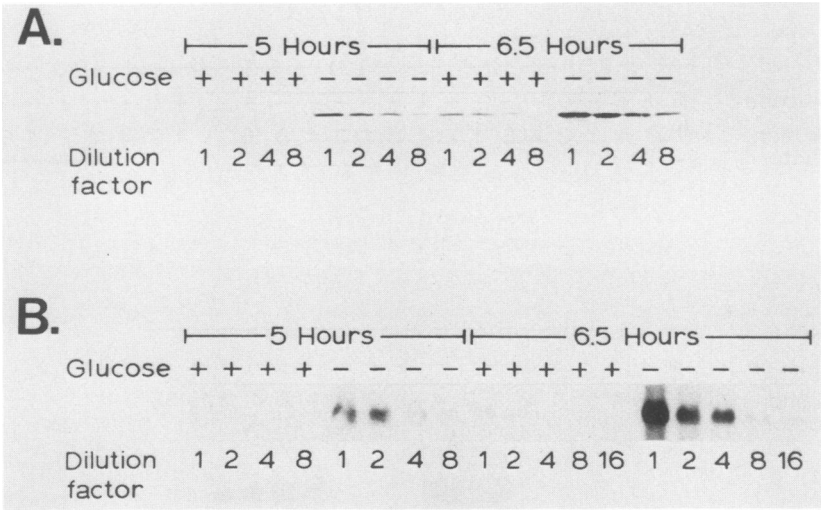

FIG. 7. Western (A) and Northern (B) blot analyses of samples taken from FRI1230 cultures grown in fermentors, using the completely defined amino acid-containing medium with the pH of the cultures maintained at 6.5. For the test culture, the glucose concentration was maintained between 14 and 20 mg/ml. Samples were prepared both 5 and 6.5 h after the fermentors were inoculated. (A) 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 by using the Protoblot Western Blot AP System. Differential amounts of culture supernatant were analyzed to adjust for differences in OD_{540} values between the control and test cultures. The respective OD_{540} values for the control and test cultures were 3.8 and 5.8 after 5 h of incubation and 5.8 and 7.2 after 6.5 h of incubation. The undiluted samples from the control culture both contain 40 μl of supernatant, but the 5-h sample from the test culture contains 26 μl and the 6.5-h sample contains 32 μl. (B) Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a Nytran membrane, and reacted with a ^{32}P-labeled *sec*$^+$-specific probe, C-562. Different samples with the same dilution factor contain the same amount of total cellular RNA.

glucose concentration and pH, utilizing the fermentors. To achieve similar growth patterns for the control and test cultures of MJB407, it was necessary to increase the air flow to 0.8 liter/min. Western blot analysis of culture supernatants was consistent with the control culture having at least eightfold more SEC than the test culture (Fig. 8A). Northern blot analysis revealed that the control culture contained 16-fold more *sec*$^+$ mRNA than the test culture (Fig. 8B).

DISCUSSION

Western blot analysis of culture supernatants of FRI1230 prepared when the culture had OD_{540} values of 1 through 6 revealed that there was a dramatic increase in the amount of extracellular SEC during postexponential growth as compared with the exponential phase. This increase in the amount of extracellular SEC occurring after the exponential phase is similar to that observed for SEB production by *S. aureus* S6. SEC and SEB production are unlike SEA production by *S. aureus* FRI100: SEA production paralleled growth and apparently stopped during the transition into stationary phase (10). Northern blot analysis of FRI1230 samples taken postexponentially had a greater concentration of steady-state *sec*$^+$ mRNA than samples taken from exponential-phase cultures (Fig. 1 and 4). The correlation between the increase in extracellular SEC and steady-state *sec*$^+$ mRNA following the exponential phase was consistent with an increase in *sec*$^+$ transcription or *sec*$^+$ mRNA stability.

agr affects postexponential phase expression of several proteins (20, 34). For *seb*$^+$, *hly*, *spa*, and *tst*, the *agr* product is thought to act at the level of transcription (14, 20, 34). We demonstrated that an intact *agr* was required for maximal *sec*$^+$ expression by strains MJB466 and FRI400 (Fig. 2A). Each of the Agr$^-$ *sec*$^+$ strains had two- to threefold less *sec*$^+$

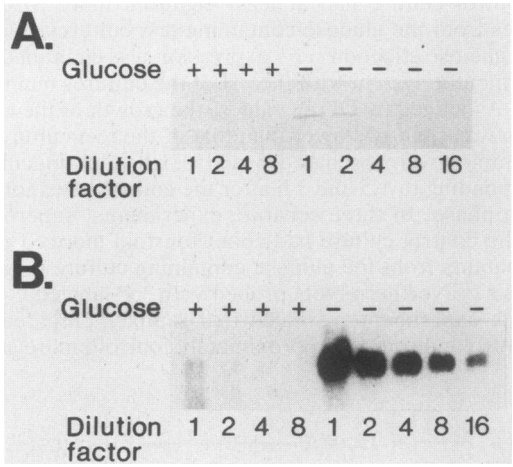

FIG. 8. Western (A) and Northern (B) blot analyses of samples taken from MJB407, the Agr$^-$ derivative of FRI1230. MJB407 was grown in the fermentors, using the completely defined amino acid-containing medium. The glucose concentration of the test culture was maintained between 14 and 20 mg/ml, and the pH of the cultures was held constant at 6.5. Samples were prepared after 6.5 h of incubation. The OD_{540} values for the test and control cultures were 8.2 and 5.2, respectively. (A) 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 by using the Protoblot Western Blot AP System. The undiluted supernatant samples for the test and control cultures contain 25 and 40 μl, respectively, to adjust for differences in OD_{540} values between the two cultures. (B) Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a Nytran membrane, and reacted with a ^{32}P-labeled *sec*$^+$-specific probe, C-562. Different samples with the same dilution factor contain the same amount of total cellular RNA.

mRNA compared with their respective parents (Fig. 2B). These observations were consistent with the *agr* effect on *sec*$^+$ expression being at the level of transcription or *sec*$^+$ mRNA stability. It is curious that there was such a large difference in SEC concentration between FRI400 and MJB392 (at least 16-fold) and only a two- to threefold difference in steady-state *sec*$^+$ mRNA; this result suggests that in FRI400 *agr* may affect *sec*$^+$ expression via some posttranscriptional process such as at the level of translation or secretion. The apparent discrepancy between steady-state *sec*$^+$ mRNA and SEC for the FRI400-MJB392 pair was not seen for the MJB466-MJB407 pair, allowing for the possibility of a strain-specific mechanism affecting *sec*$^+$ expression in FRI400, but not in MJB466.

Enterotoxin production is affected by the pH of the growth medium (15, 16, 36). The decrease in pH for cultures grown in glucose-containing media is probably due to acidic end products of glucose fermentation. Part of the apparent glucose effect on SEC production in cultures in which pH was not maintained may have been due to changes in the pH of the medium during growth. Attempts have been made to utilize nonfermentable glucose analogs to demonstrate that the glucose effect on SEA and SEB production is not solely due to a drop in pH of the medium (18, 40). It is difficult to interpret these experiments because these analogs inhibit growth of enterotoxin-producing *S. aureus* strains (18, 40). Our attempts to prevent pH fluctuation of the medium in the shake flask system, using K_2HPO_4 as a buffer, were unsuccessful; 1% K_2HPO_4 was not able to buffer sufficiently and 2% K_2HPO_4 inhibited FRI1230 growth. Because of these problems in the shake flask system, we switched to a fermentor system that allowed the pH of the culture to be maintained. The medium containing 3% N-Z amine type A and 1% yeast extract resulted in poor growth and low SEC production in the fermentor system. Therefore, we chose a completely defined amino acid-containing medium that had been used by Jarvis and co-workers in fermentors (22). Using the fermentor system, the glucose effect on *sec*$^+$ expression was compared under similar conditions of maintained and nonmaintained pH; based on densiometric analysis of Western blots from separate experiments, there was a slightly greater glucose effect under conditions of nonmaintained pH (Fig. 5 and 7A).

In both the shake flask and fermentor systems, FRI1230 grown in medium containing glucose produced less SEC and contained less steady-state *sec*$^+$ mRNA compared with samples prepared from cultures grown in medium that lacked glucose (Fig. 4 and 7). These observations were consistent with the glucose effect on *sec*$^+$ expression being at the level of transcription, although we have not ruled out an effect on *sec*$^+$ mRNA stability. Recently, Oskouian and Stewart reported that the glucose effect on the staphylococcal *lac* operon is also at the level of steady-state mRNA, based on the comparison of the amount of *lac* mRNA present when cultures were grown in either the presence or the absence of added glucose (30).

agr affects postexponential-phase expression of several staphylococcal proteins including SEB, α-hemolysin, β-hemolysin, and lipase which are also affected by glucose (4, 11, 14, 22, 34). We observed that maximal *sec*$^+$ expression was dependent on *agr*, and results from the shake flask system were consistent with the glucose effect on *sec*$^+$ expression occurring after the exponential phase (Figs. 2 and 4A). Therefore, it was conceivable that the glucose effect on *sec*$^+$ expression was mediated through *agr*. To address this question, MJB407, an Agr$^-$ derivative of FRI1230, was examined. This strain exhibited decreased levels of extracellular SEC and *sec*$^+$ mRNA when grown in the presence of glucose, suggesting that an intact *agr* is not required for the glucose effect on *sec*$^+$ expression (Fig. 8).

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