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Cloning, Characterization, and Sequencing of an Accessory Gene Regulator (*agr*) in *Staphylococcus aureus*

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We have previously identified a gene in *Staphylococcus aureus*, *agr*, whose activity is required for high-level post-exponential-phase expression of a series of secreted proteins. In this paper, we describe the cloning of this gene in *Escherichia coli* by using an inserted transposon (Tn*551*) as a cloning probe. The cloned gene, consisting of a 241-codon open reading frame containing the site of the transposon insertion, was recloned to an *S. aureus* vector, pSK265, and shown to be functional in *S. aureus*. Activity was evaluated by determinations of α-hemolysin, β-hemolysin, and toxic shock syndrome toxin-1 production in early-stationary-phase cultures. The cloned gene showed considerable variation with respect to different exoproteins and different host strains compared with the chromosomal *agr* determinant; this variation could not be attributed to the higher copy number of the cloned gene and probably reflects inapparent subtleties of the regulatory system.

In *Staphylococcus aureus*, as in most other bacteria, there is a class of accessory proteins, many of which are secreted, that are produced in laboratory cultures at the end of exponential growth and during the stationary phase (5, 6). At the same time, the production of many proteins essential for growth and cell division is shut off. The regulation system that governs this changeover can be regarded as a metabolic toggle switch that is set at the end of exponential phase for accessory protein synthesis. When a new growth cycle is initiated (e.g., by dilution into fresh medium), the switch is reset for the synthesis of exponential-phase proteins. Neither the nature of the switch nor the identity of the metabolic factors involved is known. Pleiotropic mutations affecting the production of accessory proteins in *S. aureus* have been described by several groups (1, 10, 34), and it is likely that their analysis may be informative about this regulation system. Commonly, these mutations block post-exponential-phase synthesis of the following proteins: serine protease, nuclease, lipase, fibrinolysin, α-hemolysin, β-hemolysin, δ-hemolysin, enterotoxin B, and toxic shock syndrome toxin-1 (TSST-1), whereas production of certain other exoproteins, including protein A and coagulase, is increased (1, 25). One such mutation is a transposon Tn*551* insertion isolated by Mallonee et al. (12) and referred to as *hla* on the basis of its α-hemolysin-negative phenotype. Studies of the mutant strain revealed that its α-hemolysin structural gene is intact (22) and that its pleiotropic regulatory phenotype is correlated with a lack of mRNA corresponding to the genes whose expression is blocked (25; B. Kreiswirth and R. P. Novick, unpublished data). Consequently, with the concurrence of Pattee (P. A. Pattee, personal communication), we redesignated the gene *agr* (accessory gene regulator) (25). We report here the cloning, initial characterization, and sequencing of the *agr* gene and show that the cloned gene restores the secretory protein phenotype of *agr* mutants and also of spontaneous exoprotein-deficient (Exp^-) mutants.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 contains the pedigrees of the staphylococcal strains used; Table 2 lists the plasmids. We have listed the pedigrees because we noticed retrospectively that certain of the 8325 derivatives in our stock collection that were used in these experiments are deficient in exoprotein production (Exp^-), whereas others are proficient (Exp^+). The genetic basis of the apparent lability of this trait is unknown. Note that RN450, derived from RN25 by UV-induced curing of φ13, expresses β-hemolysin, in keeping with the observations of others that φ13 lysogeny inactivates the β-hemolysin gene (T. Foster, personal communication). The β-hemolysin activity of RN450 is as low as it is in *agr* mutants such as ISP546. pSK265 (kindly provided by S. Khan) is a derivative of pC194 (7) with the polylinker region of pUC18 inserted at its unique *Hin*dIII site. RN4220 is a nitrosoguanidine-induced mutant of RN450 that is efficiently transformed with DNA from *Escherichia coli* (11).

Media and growth conditions. CY broth (17) was used for liquid cultures, shaken at 37°C, and monitored turbidimetrically with a Klett-Summerson photoelectric colorimeter read at 540 nm. GL agar (17) was supplemented with antibiotics as indicated. Tetracycline (Tc), chloramphenicol (Cm), and erythromycin (Em) were used at 5 μg/ml.

Protoplast transformation was performed by the method of Chang and Cohen (2) as modified for *S. aureus* (20). Transduction was with phage 80α as described (16). Plasmid copy numbers were determined by fluorimetric densitometry of ethidium bromide-stained agarose gels (23). Plasmid stability was assessed by scoring the cultures used for exoprotein measurements for retention of the plasmid Cm^r marker.

Analysis of exoproteins. β-Lactamase was assayed colorimetrically with nitrocefin as the substrate (21); α-hemolysin was assayed by serial dilution of supernatants taken from early-stationary-phase cultures with 0.5% whole defibrinated rabbit blood as the substrate. Samples were incubated for 90 min at 37°C and then held at 4°C for 30 min. Activity was determined by reading the samples turbidimetrically (Klett-Summerson colorimeter, red filter) and interpolating to cal-

TABLE 1. Strains used

Strain[a]	Exoprotein expression	Production			Lysotype
		α-Hemolysin	β-Hemolysin	TSST-1	
NTCC 8325	+	+	−	−	φ11, φ12, φ13
RN25	+	+	−	−	φ13
RN450	−	Low	Low	−	
RN4220[b]	−	Low	Low	−	
RN1478	+	+	+	−	
ISP479	+	+	+	−	
ISP546[b]	−	−	Low	−	
RN6390[b]	+	+	+	−	
RN4282[b]	+	−	−	+	Unknown
RN4256[b]	−	−	−	−	Unknown

[a] The pedigree, beginning with NTCC 8325, is as follows: from 8325 after UV treatment, RN25; from RN25 after UV treatment, RN450; from RN450 after nitrosoguanidine mutagenesis, RN4220; from RN450 after transduction with pRN3032 (Tn*551* donor), RN1478; from RN1478 after transduction with Cd^r revertant of pRN3032, ISP479; from ISP479 cured of pRN3032, RN6390; from ISP479 after Tn*551* insertion, ISP546. Also, from RN4282 transformed with Tn*551* from ISP546, RN4256.

culate the 50% lysis point. Activities (hemolytic units [HU]) are expressed as the reciprocal of the dilution giving 50% lysis; β-hemolysin was assayed in the same manner but with sheep instead of rabbit blood. Since α- and β-hemolysins are not absolutely specific for rabbit and sheep erythrocytes, respectively, strains Wood 46 (which produces only α-hemolysin) and TC82 (which produces only β) were used to determine the relative activities of the two hemolysins on each of the two blood cell types, and these relative activities (10% for β-hemolysin on rabbit blood versus sheep blood and 3% for α-hemolysin on sheep versus rabbit blood) were used to correct the titers of strains that produced both. Although β-hemolysin inhibits the activity of α-hemolysin on sheep blood agar plates, no such inhibition was observed in the Tris-saline buffer used for the α-hemolysin titrations. TSST-1 was assayed immunologically as described by Schlievert et al. (29).

Restriction mapping and cloning. Restriction endonucleases were purchased from Boehringer Mannheim Biochemicals and used as described by the manufacturer. Restriction mapping and fragment isolations were performed with ethidium bromide-Cs-Cl-purified plasmid DNA samples (4, 19). For molecular cloning, specific fragments were eluted from polyacrylamide gels, phenol extracted, and ethanol precipitated. For ligation, samples were combined in approximately equimolar ratios and incubated for 16 to 24 h at 14°C at a DNA concentration of at least 10 μg/ml and a ligase concentration of 40 U/ml. Cloning with pBR322, pUC18, and m13 in *E. coli* was performed as described by Maniatis et al. (13). *E. coli* clones were verified by restriction analysis and by blot hybridization (30) as required. DNA sequencing was done by the dideoxynucleotide methods of Sanger and co-workers (27, 28) with m13mp10 and m13mp11 clones and a universal m13 sequencing primer purchased from Pharmacia.

Blot hybridization. Southern blot hybridization was performed by standard methods (30) with nick-translated (26) samples of gel-purified DNA as probes. Northern blot hybridization was performed by the procedure of Thomas (32) with whole-cell RNA purified by extraction with guanidinium isothiocyanate followed by centrifugation through a CsCl step gradient. Nick-translated samples of gel-purified DNA fragments were used as probes.

RESULTS

Cloning of *agr*. Strain RN4256 (11) was constructed in connection with an earlier study of TSST (11) by transforming a naturally occurring toxic shock strain, RN4282, for erythromycin resistance (Em^r) with DNA from ISP546, the original Tn*551*-induced *agr* mutant isolated by Mallonee et al. (12). A *Bgl*II fragment of plasmid pRN3174 containing one end of Tn*551* (19) was used to probe a *Bgl*II digest of chromosomal DNA of RN4256 (25) for Tn*551* sequences. A single 4.7-kilobase (kb) fragment hybridized to the probe. DNA from the region of the gel containing this fragment was eluted and cloned to pBR322 in *E. coli*, and positive clones were identified by hybridization with the same probe. One such clone contained about 4 kb of chromosomal sequences flanking the inserted transposon. A *Cla*I fragment derived from this clone, containing the end of the transposon plus about 2.8 kb of the flanking DNA, was then used to probe a lambda library prepared from a partial *Sau*IIIa digest of RN4282 chromosomal DNA. Positive plaques were purified by subculture and used to prepare lambda DNA for restriction analysis and for subcloning in pUC18. Restriction analysis of three clones (not shown) suggested that overlapping regions with different ends had been cloned. Initial subcloning of one of the λ derivatives yielded a 4.0-kb *Eco*RI-*Pst*I fragment that was used for further study. This fragment (Fig. 1) was found to contain sequences homologous to the *Cla*I probe plus about 2.3 kb of DNA distal to the original Tn*551* insertion site. To test the cloned fragment for *agr* activity, we ligated it to an appropriately digested sample

TABLE 2. Plasmids used

Plasmid	Description	Source or reference
pSK265	pC194::pUC19 PL[a]	9
pWN2018	pC194::*ori* ColE1::*bla*::pUC18 PL	33
pWN2019	pC194::*ori* ColE1::*bla*::pUC19 PL	33
pRN3032	pI258 *blaZ401 cad-52 seq-36*	18
pRN3174	pI258Δ94 (*mer*→*asi*)	19
pRN6583	pSK265::*agr*-A2	This work
pRN6584	pSK265::*agr*-A4	This work
pRN6585	pSK265::*agr*-A6	This work
pRN6632	pSK265::*agr*-A10	This work
pRN6661	pSK265::*agr*-A12	This work
pRN6662	pSK265::*agr*-A14	This work
pRN6663	pSK265::*agr*-A16	This work
pRN6664	pSK265::*agr*-A17	This work
pRN6599	pWN2019::*agr*-A8	This work
pRN6598	pWN2018::*agr*-A8	This work

[a] PL, Polylinker.

TABLE 3. Expression of *agr*-regulated exoproteins

agr fragment[b]	Activity[a] in host strain:									
	RN6390 (*agr*⁺)		ISP546 (*agr*)		RN4220 (*agr*)		RN4282 (*agr*⁺)			RN4256 (*agr*)
	α	β	α	β	α	β	α	β	TSST-1	TSST-1
None	1,000	320	<10	96	91	260	25	22	3.2	<0.02
A2	360	290	120	250	1,600	3,100	21	20	3.2	0.8
A4										0.2
A6										0.2
A10			<10	78	36	440				<0.02
A14			100	260	1,200	2,900				0.1
A16			76	290	910	2,600				0.1
A17			57	250	1,000	2,800				0.1

[a] α and β, α-hemolysin and β-hemolysin, respectively. Hemolysin activities are expressed as hemolytic units per milligram (dry weight) of cells; TSST-1 activity is expressed as micrograms of protein per milliliter.
[b] pSK265 derivative containing the indicated fragment (see Fig. 1).

of pSK265 DNA and used the ligation mixture to transform *S. aureus* RN4220, with selection for chloramphenicol resistance. Plasmid DNA from one such transformant was used to transform two pairs of isogenic *agr*⁺/*agr* mutant strains, RN4282/RN4256 and RN6390/ISP546. As shown in Table 3, transformants of RN4256 (e.g., RN6122) produced TSST-1 and transformants of ISP546 (e.g., RN6114) produced α-hemolysin. However, the TSST-1 and α-hemolysin activities

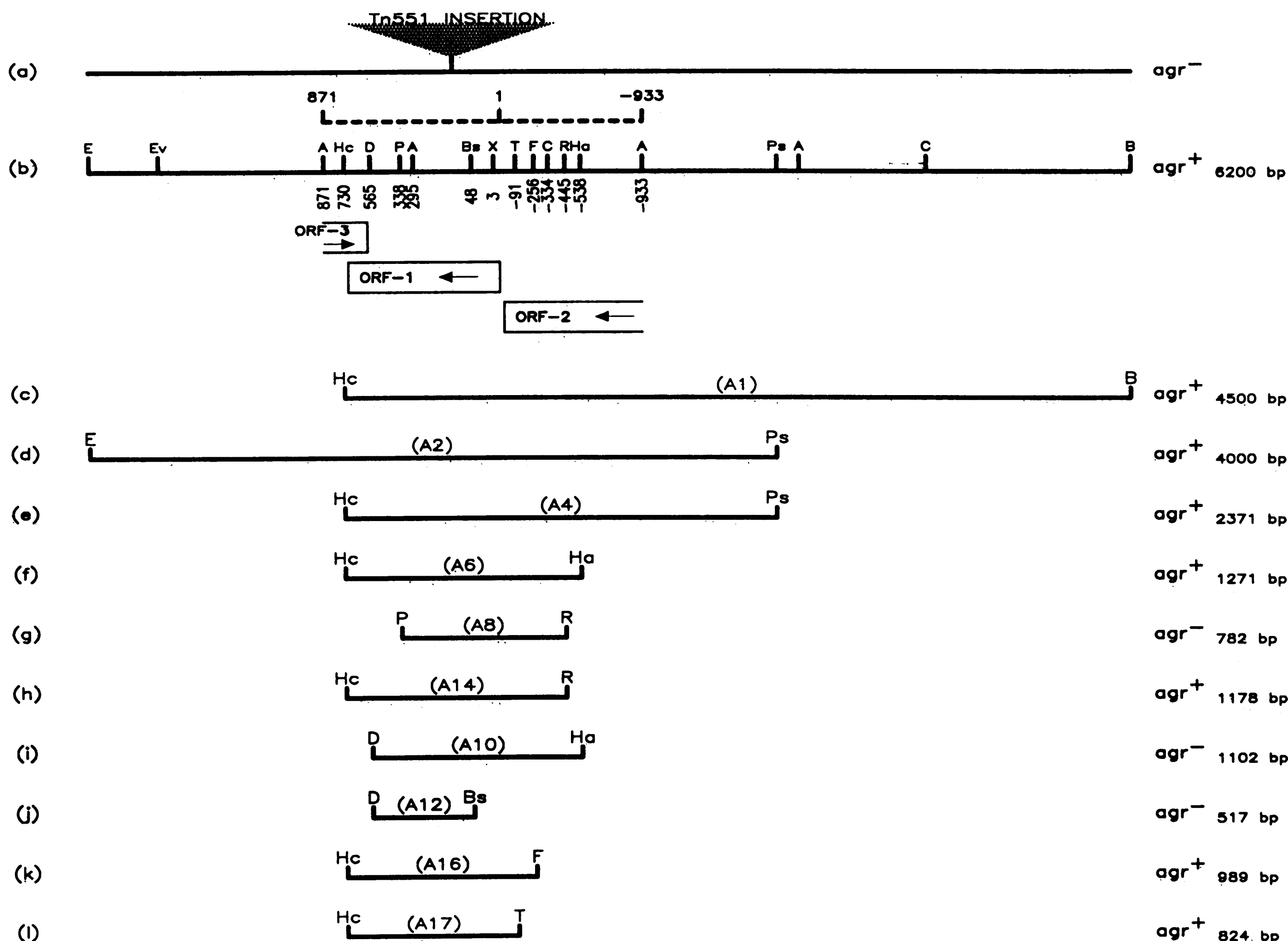

FIG. 1. Cloning and mapping the *agr* gene. Line a represents chromosomal DNA with Tn*551* inserted in the *agr* region. Line b is a restriction map of the lambda clone containing *agr*. Abbreviations: A, *Acc*II; B, *Bgl*II; Bs, *Bst*EII; C, *Cla*I; D, *Dde*I; E, *Eco*RI; Ev, *Eco*RV; F, *Fnu*DII; Ha, *Hae*III; Hc, *Hinc*II; P, *Pvu*II; Ps, *Pst*I; R, *Rsa*I; T, *Tth*III-I; X, *Xmn*I. The extent of the sequenced region is indicated by a dashed line, and the three major ORFs identified are located and oriented as shown by the boxes below. Lines c to l represent various restriction fragments that were subcloned into pSK265 and scored for *agr* activity in RN4220.

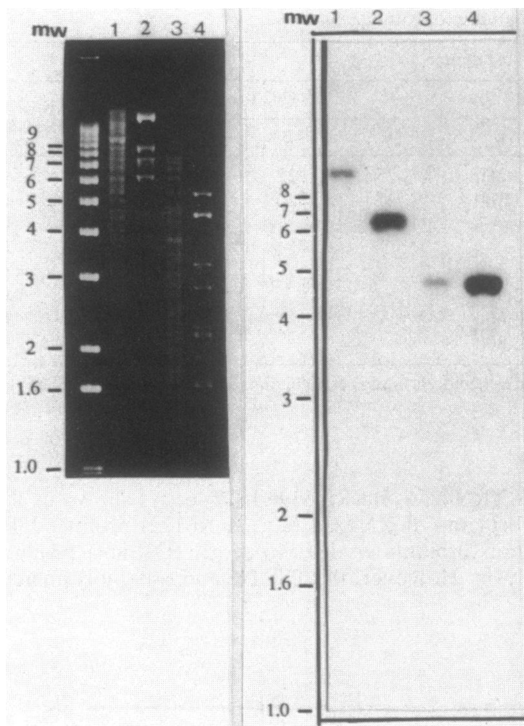

FIG. 2. Blot hybridization analysis of λ *agr* clone. RN4282 chromosomal DNA and DNA prepared from the *agr*-positive λ clone diagrammed in Fig. 1 were digested with restriction enzymes, separated by agarose gel electrophoresis, stained with ethidium bromide, and UV-photographed (left panel). The gel was then blotted to nitrocellulose and hybridized with a nick-translated sample of the A6 fragment (see Fig. 1). Tracks: 1 and 3, chromosomal DNA digests, 2 and 4, λ DNA digests. Samples were digested with *Bgl*II and *Eco*RI (tracks 1 and 2) or with *Bgl*II and *Hin*cII (tracks 3 and 4). mw, Molecular weight markers (in thousands).

of these transformants were not as high as those of the isogenic agr^+ strains RN4282 and RN6390, respectively. Whereas TSST-1 production by RN4282 was unaffected by the A2 clone, α-hemolysin production by RN6390 was reduced by about 70%. A number of possibilities were considered that might account for the variability of the response to the cloned *agr* determinant. These possibilities are addressed below.

Southern blot hybridization analysis. To test for the possibility that a rearrangement had occurred during cloning or that the λ clones included noncontiguous DNA fragments, we compared chromosomal and λ clone restriction patterns by blot hybridization with the *agr*-specific probe A6 (Fig. 1). As shown in Fig. 2, the largest agr^+ fragment, A1, was present in both chromosomal and λ clone digests (lanes 3 and 4), suggesting that contiguous DNA has been cloned and that there was no gross rearrangement during cloning. The difference in size between the *Bgl*II-*Eco*RI fragments shown in lanes 1 and 2 is due to the presence of an *Eco*RI site at the λ insert junction. We therefore consider it unlikely that DNA rearrangements could account for the difference in *agr* activity between the native and cloned genes.

Subcloning. The *agr* determinant was mapped by subcloning. Various subfragments derived from the A2 fragment were inserted into the polylinker site of pSK265 and used to transform *S. aureus* RN4220. Additionally, to test for the possibility that the A2 clone did not contain the entire *agr* determinant, we cloned from the *agr*-containing lambda fragment a segment containing about 2.2 kb to the right of A2 but lacking about 1.6 kb to the left, as shown in Fig. 1 (clone A1). Although this fragment was successfully cloned to pUC18, we were unable to clone it in *S. aureus*, using the high-copy vector pSK265. For each subclone, the plasmid insert was verified by restriction analysis, and the derivative clone was transferred to various agr^+ and *agr* host strains. The resulting derivative strains were scored for production of α- and β-hemolysins and TSST-1. The results of this subcloning are shown schematically in Fig. 1, and the exoprotein values are listed in Table 3. A number of subclones failed to activate the production of α-hemolysin. One of these, A10, in conjunction with the smallest agr^+ clone, A17, defined the shortest segment, about 0.8 kb in length and containing the site of the Tn*551* insertion in RN4256, that had detectable *agr* activity. The positive clones were remarkable in that they produced different *agr* responses with respect to one another, with respect to the three different exoproteins analyzed, and with respect to the various *agr* mutant host strains. RN4282 expressed only a trace of either α-hemolysin or β-hemolysin, and so the RN4282/RN4256 pair was not studied for the effects of *agr* clones on the expression of these products. TSST-1 was expressed well in RN4282 and was not detectable in the *agr* derivative RN4256. None of the agr^+ subclones restored full TSST-1 production in RN4256; all showed lower activities that seemed to represent at least two distinct levels of expression. In ISP546, none of the clones restored full α-hemolysin activity, and as with TSST-1 in RN4256, there appeared to be several levels of activity correlated with the size of the *agr*-bearing fragment. β-Hemolysin activity in the ISP546 derivatives was approximately the same for all of the agr^+ clones tested and was only slightly, if at all, lower than that observed with the agr^+ parental strain, RN6390. RN4220 is derived from RN450, a spontaneous exoprotein-deficient mutant similar to the Exp^- mutants described previously by Bjorklind and Arvidson (1). In RN4220, all of the agr^+ clones strongly stimulated the expression of the two hemolysins—α to nearly the level shown by RN6390, and β to considerably higher levels—suggesting that the Exp^- phenotype of RN4220 is due to a defect in *agr* expression. The *agr* defect in RN4220, however, is clearly different from that in ISP546. Thus, on the one hand, there was considerable residual expression of both hemolysins in RN4220 and both were stimulated about 10-fold by the cloned *agr* determinant in this strain. On the other hand, α-hemolysin was not detectably expressed in ISP546 and was stimulated at least 30-fold by *agr*, whereas β-hemolysin showed considerable activity in this *agr* strain but was stimulated only about 3-fold by the *agr* clones. Genotypic variability among ISP546, RN6390, and RN4220 is unlikely to be a major factor, as the three are coancestral; however, RN4220 has been through a nitrosoguanidine mutagenesis, which could have had significant genotypic consequences. It is also unlikely that variations in plasmid copy number or stability could be responsible, as the *agr*-bearing clones were stable in all three strains and had copy numbers that were indistinguishable from one another (although higher than that of the parental plasmid, pC194), about 90 copies per cell (data not shown).

Sequence analysis. We have determined the sequence of a 1.8-kb *Acc*I fragment containing the site of the Tn*551* insertion inactivating *agr*, as shown in Fig. 1. Subfragments derived from this segment were cloned to m13mp10 or m13mp11 and sequenced by the dideoxynucleotide method

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-90  ACAATGTCTTATTAGATACAATTATCGAAAATGGTTTCTTTTATTCAAAAAGTTGAAATT

-30  ATTAACAACTAGCCATAAGGATGTGAATGTATGAAAATTTTCATTTGCGAAGACGATCCA
                       SD          MetLysIlePheIleCysGluAspAspPro

 31  AAACAAAGAGAAAACATGGTTACCATTATTAAAAATTATATAATGATAGAAGAAAAGCCT
     LysGlnArgGluAsnMetValThrIleIleLysAsnTyrIleMetIleGluGluLysPro

 91  ATGGAAATTGCCCTCGCAACTGATAATCCTTATGAGGTGCTTGAGCAAGCTAAAAATATG
     MetGluIleAlaLeuAlaThrAspAsnProTyrGluValLeuGluGlnAlaLysAsnMet

151  AATGACATAGGCTGTTACTTTTTAGATATTCAACTTTCAACTGATATTAATGGTATCAAA
     AsnAspIleGlyCysTyrPheLeuAspIleGlnLeuSerThrAspIleAsnGlyIleLys

211  TTAGGCAGTGAAATTCGTAAGCATGACCCAGTTGGTAACATTATTTTCGTTACGAGTCAC
     LeuGlySerGluIleArgLysHisAspProValGlyAsnIleIlePheValThrSerHis

271  AGTGAACTTACCTATTTAACATTTGTCTACAAAGTTGCAGCGATGGATTTTATTTTTAAA
     SerGluLeuThrTyrLeuThrPheValTyrLysValAlaAlaMetAspPheIlePheLys

331  GATGATCCAGCTGAATTAAGAACTCGAATTATAGACTGTTTAGAAACTGCACATACACGC
     AspAspProAlaGluLeuArgThrArgIleIleAspCysLeuGluThrAlaHisThrArg

391  TTACAATTGTTGTCTAAAGATAATAGCGTTGAAACGATTGAATTAAAACGTGGCAGTAAT
     LeuGlnLeuLeuSerLysAspAsnSerValGluThrIleGluLeuLysArgGlySerAsn

451  TCAGTGTATGTTCAATATGATGATATTATGTTTTTTGAATCATCAACAAAATCTCACAGA
     SerValTyrValGlnTyrAspAspIleMetPhePheGluSerSerThrLysSerHisArg

511  CTCATTGCCCATTTAGATAACCGTCAAATTGAATTTTATGGTAATTTAAAAGAACTGAGT
     LeuIleAlaHisLeuAspAsnArgGlnIleGluPheTyrGlyAsnLeuLysGluLeuSer

571  CAATTAGATGATCGTTTCTTTAGATGTCATAATAGCTTTGTCGTCAATCGCCATAATATT
     GlnLeuAspAspArgPhePheArgCysHisAsnSerPheValValAsnArgHisAsnIle

631  GAATCTATAGATTCGAAAGAGCGAATTGTCTATTTTAAAAATAAAGAACACTGCTATGCA
     GluSerIleAspSerLysGluArgIleValTyrPheLysAsnLysGluHisCysTyrAla

691  TCGGTGAGAAACGTTAAAAAAATATAATAAGATAATAAAGTCAGTTAACGACGTATTCAA
     SerValArgAsnValLysLysIle----

751  TTGTAAATCTTGTTGGATTTTAACAAGATAACTAGCAAATGCACTGTATAGCTGGCTTTT

811  TAATTTTATTAACAAAATTAAATATGACGCGTGAATTAAAAAATGATGTAACGTCTCTTT

871  GTAT
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FIG. 3. Nucleotide sequence of the *agr* reading frame. The predicted amino acid sequence and putative ribosome-binding site (Shine-Dalgarno sequence, SD) are also shown.

(28). The sequenced region, indicated in Fig. 1, revealed two major open reading frames (ORFs) in the right-to-left direction, ORF1 and ORF2, and a third, ORF3, in the other direction. ORF1, extending from positions 1 to 714, included the Tn*551* insertion site and was fully contained within fragment A17, the smallest agr^+ fragment; it is therefore likely to represent *agr*. ORF2 began to the right of the sequenced region and ended at nucleotide position −38; ORF3 began to the left of the known sequence and ended at position 577, overlapping ORF1. The subcloning data are also consistent with the assignment of *agr* to ORF1: all subclones that contained this ORF were agr^+, whereas all that interrupted it were *agr* defective. In Fig. 3 is shown the sequence of the ORF1 region, indicating the predicted amino acid sequence of the *agr* protein.

Gene fusion analysis. In a preliminary attempt to localize the *agr* promoter, we used a pair of promoter-probe vectors, pWN2018 and pWN2019, that we developed recently for gene fusion analysis (33). These vectors contain the pUC18 polylinker in either orientation followed by a promoterless derivative of the *S. aureus* plasmid pI258 β-lactamase (*bla*) gene. In the absence of a cloned promoter, neither of these plasmids directs the synthesis of detectable β-lactamase (33). The A8 fragment was chosen for this experiment because it includes 445 nucleotides (nt) 5′ to the start of ORF-2 and terminates within the reading frame. The fragment was excised from pSK265 by double digestion with *Pst*I and *Sst*I and ligated to similarly digested preparations of each of the two vectors, and the resulting ligation mixtures were used to transform RN4220 protoplasts, with selection for the Cm^r marker of the vector. Note that this strategy should produce derivatives of pWN2019 with the *agr* insert oriented in the same direction as the *bla* determinant of the vector and derivatives of pWN2018 with the insert in the

opposite orientation. Restriction analysis of plasmids recovered from the transformants confirmed the expected orientations, and one of each type was transferred to RN6390, RN4220, and ISP546 and analyzed for β-lactamase production in exponential- and post-exponential-phase cultures. A pWN2018 derivative (insert oriented opposite to the *bla* gene) produced no detectable β-lactamase, whereas a pWN2019 derivative (insert oriented in the same direction as *bla*) produced about 1 U of enzyme per mg (dry weight) of cells in all three strains. These activities were rather low, about 0.05% of that seen with a constitutive mutation of the native β-lactamase expression system (20), were unaffected by the presence of an active *agr* determinant, and showed no change during the transition from exponential to post-exponential phase.

***agr* transcription.** In Fig. 4 is shown the autoradiogram of a Northern (RNA) blot hybridization analysis of whole-cell RNA probed with the *agr*-specific fragment A6. RNA was prepared from both exponential- and post-exponential-phase cells and from the agr^+ and *agr*-defective strains RN6390 and ISP546, respectively. As shown in the autoradiogram, the Tn*551* insertion in *agr* blocked detectable transcription of the *agr* region. It is assumed that the promoter identified by the *bla* fusions is too weak to have been picked up in this blot. Additionally, this rather preliminary experiment suggests that *agr* is transcribed more actively in post-exponential- than in exponential-phase cells; there is, moreover, the hint of several *agr*-specific bands, at least one of which is present in the post-exponential-phase preparation but absent from the exponential-phase cells. From the positions of other transcripts in parallel gels (not shown), the largest species is estimated to be about 3 to 4 kb in length.

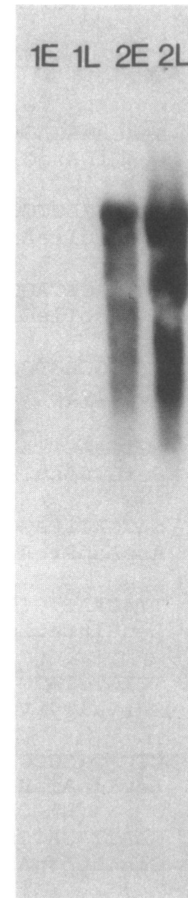

FIG. 4. Northern blot analysis of whole-cell RNA from early- and late-phase cell cultures of ISP546 and RN6390. RNA was extracted from cells grown on CY medium in the presence of guanidinium thiocyanate and purified by the method of Chirgwin et al. (3). A 10-μg portion of each sample was heat-denatured and electrophoresed on 1.5% agarose containing 20 mM morpholinopropanesulfonic acid, pH 7.0, 2.2 M formaldehyde, and ethidium bromide (1 μg/ml). RNA was transferred to a Nytran filter (Schleicher & Schuell) by using the Janssen semidry electroblotter, and the filter was prehybridized and hybridized at 65°C and then probed with a 582-bp *Dde*I-*Bst*EII fragment that is contained within *agr* subclone pRN6585 (*Streptococcus epidermidis*, Fig. 1). Lanes 1E and 1L and 2E and 2L correspond to early- and late-phase cell cultures, respectively, from ISP546 (*agr* mutant) and RN6390 (agr^+), respectively. Probes were labeled with ^{32}P by nick translation (12). Early (E) refers to mid-exponential-phase RN6390 cells that are not expressing α-hemolysin; late (L) refers to early-stationary-phase cells that are expressing α-hemolysin maximally.

DISCUSSION

The data presented in this report demonstrate that *agr* is a *trans*-activator of a series of exoprotein genes that are expressed during the post-exponential phase of growth in *S. aureus*. The cloned *agr* determinant encodes a potential polypeptide of 241 amino acid residues, and the results of subcloning suggest that the *agr* product is, in fact, this polypeptide. This conclusion is strengthened by the demonstration of a polypeptide of the predicted size following cloning of fragment A16 to a Gemini vector under control of the T7 promoter and overexpression in *E. coli* following the induction of T7 polymerase (31; unpublished data). Earlier studies (25) have suggested that *agr* acts at the level of transcription. *agr* itself, however, does not appear to be involved directly in the temporal aspect of the regulation, as the residual activity of *agr*-sensitive genes is still temporally regulated in *agr* mutants (8, 25). An important question is that of the regulation of *agr* itself. On the one hand, we have identified, by means of gene fusion analysis, an *agr* promoter that is probably located less than 100 nt 5′ to the *agr* start. This promoter is rather weak and has about the same activity in agr^+ and *agr*-defective host strains and the same activity in post-exponential-phase as in exponentially growing cells. On the other hand, Northern blotting with an *agr* probe revealed strong transcriptional activity that was absent in *agr*-defective strains, showed considerable growth phase dependency, and was initiated about 2 kb upstream. Morfeldt and co-workers, who have independently cloned *agr* (14), have identified a strong leftward promoter, just to the right of the *Pst*I site (Fig. 1), that directs the synthesis of a 3.5-kb *agr* transcript. This transcript was similarly absent from *agr* mutants and is likely to correspond to the major transcript shown in Fig. 4. Together, these results suggest that there are at least two *agr* promoters, a strong one to the right of the *Pst*I site and a weak one on the A8 fragment, close to the start of the gene. The absence of the large transcript in *agr* mutant strains suggests the possibility that a functional *agr* product is required for the activation of the upstream promoter. This would mean that *agr* is part of an operon that has both growth-phase-dependent and -independent components and that includes ORF2 and possibly other products as well.

Measurements of three *agr*-regulated exoproteins with various agr^+ subclones, in various host strains, revealed a complex pattern of expression that will probably be inter-

pretable only when the entire regulatory system has been worked out. Incomplete restoration of expression by the *agr* clone may be due in part to the copy number of the vector; Morfeldt et al. (14) demonstrated normal expression when their cloned gene was reinserted into the chromosome at the *agr* locus. Copy number effects cannot, however, explain either the interstrain variability that we have observed or the difference in expression levels seen with different *agr* clones. For example, there seem to be three distinct levels of expression for α-hemolysin in the three strains ISP546 (*agr*), RN4220 (*agr* mutant), and RN6390 (agr^+), but only two levels for β-hemolysin. These results suggest that there may be two distinct *agr* activities; at unit dosage, β-hemolysin would require only one of these for expression at the wild-type level, whereas α-hemolysin would require both. These activities could be correlated with the two different promoters thus far identified—the weak one just 5′ to ORF1 and the much stronger one upstream—and it seems probable that ORF2 may also be involved. Results with the cloned fragments also suggest a difference between α-hemolysin and β-hemolysin regulation in that β-hemolysin expression was stimulated to the same extent by all of the agr^+ clones, whereas α-hemolysin, and also TSST-1, showed three more or less distinct levels correlated with removal of sequences 5′ to *agr*. Our present view is that the variability in expression is likely to be a combination of gene dosage effects plus subtle consequences of genome organization that will be revealed only by further study. With respect to other regulatory systems, *agr* could be a member of the class of recently characterized cytoplasmic transcriptional activators (24). However, computer searches have thus far failed to reveal any significant amino acid sequence similarity between the predicted *agr* polypeptide and these activators, *Bacillus subtilis* sigma factors, or other proteins in the Dayhoff data base. More extensive computer analyses are currently in progress.

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