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Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*

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Summary. Insertion of the erythromycin-resistance transposon Tn*551* into the *Staphylococcus aureus* chromosome at a site which maps between the *purB* and *ilv* loci has a pleiotrophic effect on the production of a number of extracellular proteins. Production of alpha, beta and delta hemolysin, toxic shock syndrome toxin (TSST-1) and staphylokinase was depressed about fifty-fold while protein A production was elevated twenty-fold. Hybridization analysis showed that the defect in expression of TSST-1 and alpha hemolysin was at the transcriptional level. Inability of the mutant strain to express either a cloned TSST-1 gene or the chromosomal gene indicates that the transposon has inactivated a trans-active positive control element. This element has been designated *agr* for accessory gene regulator.

Key words: Gene regulation – Pathogenicity – Exoproteins – Pleiotropism – *S. aureus*

Staphylococcus aureus synthesizes a number of extracellular proteins which play a major role in the pathogenesis of staphylococcal disease (Smith 1979). Mutations which affect exoprotein production are often pleiotropic (Bjorklind and Arvidson 1980; Coleman 1981; Forsgreen et al. 1971; Yoshikawa et al. 1974) suggesting that exoprotein genes may be coordinately regulated in some cases. With the recent cloning of several exoprotein structural genes (Kehoe et al. 1983; Kreiswirth et al. 1983; Lofdahl et al. 1983; Sako et al. 1983; Shortle 1983) it has become possible to investigate the mechanisms of regulation of exoprotein gene expression. We demonstrate here the existence of a locus on the *S. aureus* chromosome that controls the synthesis of a number of exoproteins including toxic shock syndrome toxin (TSST-1) and α hemolysin. This locus, which has been named *agr* for accessory gene regulator, encodes an element that acts at the level of mRNA.

Mallonee et al. (Mallonee et al. (1982) previously reported that insertion of the erythromycin-resistance transposon Tn*551* into a chromosomal locus called *hla* virtually eliminated production of extracellular alpha hemolysin by *S. aureus* ISP546. The *hla* locus mapped between the *purB* and *ilv* loci and was linked to determinants that affect the synthesis of enterotoxin A and β-lactamase. DNA from *S. aureus* ISP546 was used to transform *S. aureus* RN4282 (previously designated 3–14) (Kreiswirth et al. 1983), a clinical isolate that produces α, β and δ hemolysin, TSST-1, staphylokinase, lipase, nuclease, protein A and a number of other extracellular proteins. The α hemolysin-negative phenotype of the Tn*551*-containing transformant RN4256 (Kreiswirth et al. 1983) indicated that it was derived by in vivo recombination between homologous regions on the ISP546 and RN4282 chromosomes. RN4282 and RN4256 grow at similar rates in liquid medium and exponential cultures have essentially identical cellular protein profiles as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1, lanes 1 and 2). However, analysis of stationary-phase cultures shows that the mutation results in a marked decrease in the production of numerous extracellular proteins (Fig. 1, lanes 5 and 6). As shown in Table 1, the expression of α, β and δ hemolysins, TSST-1 and staphylokinase are all depressed. In contrast, synthesis of extracellular protein A is elevated twenty-fold in mutant cells. The differences in expression of TSST-1 and protein A are clearly illustrated by an immunoblot analysis (Fig. 2) using affinity-purified anti-TSST-1 antibodies. Protein A gives a signal in this reaction as a consequence of binding to the Fc fragment of all immunoglobulins. It is also noteworthy that expontential cells produce extremely low levels of exoproteins (not shown) and that the whole-cell protein patterns of RN4256 and RN4282 in stationary phase are as different as are their exoprotein patterns, but in the opposite direction. This difference must also be related to the Tn551 insertion and is consistent with the overproduction of protein A by the mutant strain.

To test whether Tn*551* (which has a M_r 3.5×10^6 daltons) was inserted into either the α hemolysin or TSST-1 structural gene, chromosomal DNA from wild-type and mutant cells was analyzed by Southern blot hybridization. A 7.6 kbp *Hin*dIII fragment containing the α hemolysin structural gene and its flanking sequences (Kehoe et al. 1983) and a 10.6 kbp fragment containing the TSST-1 structural gene and its flanking sequences (Kreiswirth et al. 1983) were used as probes. The identity of the hybridization patterns obtained with DNA from mutant and wild-type cells using either probe (Fig. 3 and unpublished data) indicates that neither gene was the site of the insertion. The Wood 46 (strain from which the α-hemolysin gene was cloned) and RN4282 α hemolysin genes and their 5′ flanking sequences appear homologous since ClaI fragments of 700 bp (internal to the structural gene), 500 bp (containing the 5′ end of the gene) and 1.5 kbp (immediately upstream from the gene) are present in both digests. The Wood 46

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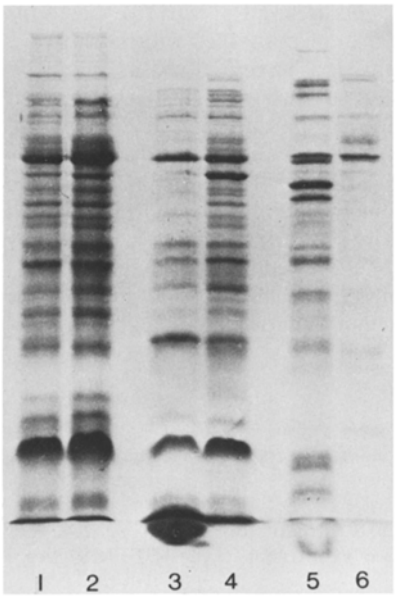

Fig. 1. Electrophoretic analysis of proteins synthesized by *S. aureus* RN4282 (TSST-1$^+$) and RN4256 (TSST-1$^-$). Cells grown to mid-logarithmic and stationary phase on CY medium (Novick and Brodsky 1972) were harvested by centrifugation, washed with cold water and acetone-dried (Bhaduri and Demchick 1983). The culture supernatant was concentrated twenty-fold by ultrafiltration using an Amicon YM-10 membrane. Samples were boiled for 5 min in sample buffer (Laemmli 1970), separated on a 12.5% SDS-polylacrylamide gel, and stained with Coomassie brilliant blue R. Lanes 1, 2, cell extracts of RN4282 and 4256 in exponential phase; lanes 3, 4, cell extracts of RN4282 and 4256 in stationary phase; lanes 5, 6, supernatants of RN4282 and 4256 in stationary phase

Table 1. Production of selected exoproteins by *S. aureus* RN4282 (TSST-1$^+$) and RN4256 (TSST-1$^-$)

Product	4282	4256
	Relative activity	
TSST-1	100	1
Staphylokinase	100	2
Alpha hemolysin	+ + +	−
Beta hemolysin	+ + +	−
Delta hemolysin	+ + +	−
Secreted protein A	5	100

TSST-1 and protein A in supernatants of cultures grown to stationary phase on beef-heart medium were determined by radial immunoassay (Vaerman 1981). Alpha and beta hemolysins were determined by measuring zones of hemolysis around colonies grown on infusion agar containing 5% rabbit or sheep blood respectively. Delta hemolysin was determined on sheep blood agar as described (Elek and Levy 1950). Staphylokinase was determined on human plasma agar (Devriese and Kerckhove 1980).

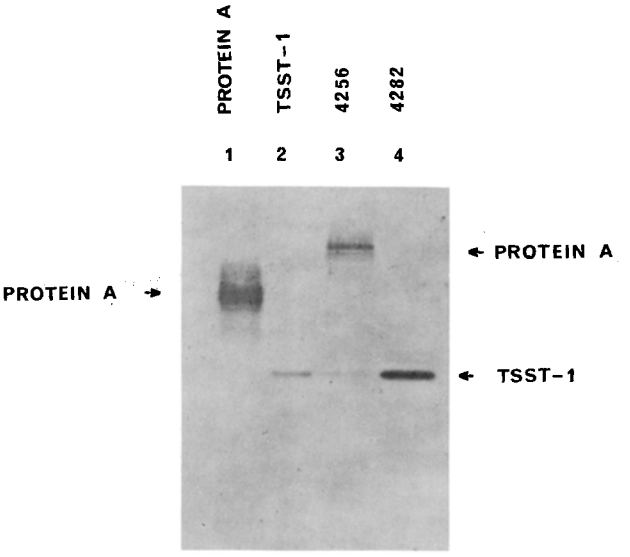

Fig. 2. Immunoblot analysis (Blake et al. 1984) of stationary phase culture supernatants of RN4256 and RN4282 grown in beef heart infusion broth. Samples were separated by SDS-polyacrylamide gel electrophoresis, transferred electrophoretically to nitrocellulose, and reacted with affinity-purified antibody to TSST-1. Lane 1, protein A purified from *S. aureus* treated with lysostaphin; 2, purified TSST-1; 3 and 4, stationary phase supernatants of RN4256 and RN4282 respectively. Protein A isolated from different subcellular fractions and strains may vary in composition, which would account for the observed heterogeneity in migration

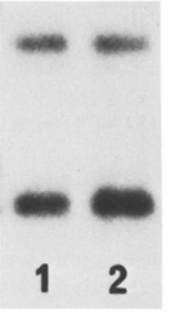

Fig. 3. Southern blot analysis of *Cla*I digested DNA from RN4282 (lane 1) and RN4256 (lane 2). DNA was electrophoresed in 1% agarose in 50 mM Tris, 50 mM boric acid, 1 mM EDTA pH 8.0 and transferred to nitrocellulose (Southern 1975). Filters were prehybridized at 65° C for 2 h in a solution containing 5 × SSC–0.5% SDS, denatured salmon sperm DNA (50 μg/ml) and 5 × Denhardt's solution (Denhardt 1966) and then hybridized at 65° C for 16 h with 2×10^6 cpm of ^{32}P-labeled pRN6100, which contains the TSST-1 gene (Kreiswirth et al. 1983). Filters were washed twice in 2 × SSC–0.5% SDS at 65° C and twice in 1 × SSC–0.1% SDS at room temperature and exposed to Fuji RX film for 24 h at −70° C. The upper and lower bands are approximately 8 and 4 kbp in size respectively

ClaI 1.6 kbp fragment containing the 3′ end of the gene and its flanking sequence is not present in the 4282 digest, however, suggesting that the gene may be located at variable positions on the chromosome.

Northern blot analysis of mRNA from late log cultures using radiolabeled TSST-1 DNA as probe showed the presence of the TSST-1 transcript in wild-type cells (Fig. 4, lanes 1 and 2). This transcript is about 1 kb in size and is therefore apparently monocistronic, since the secreted form of TSST-1 has a M_r 22,000 (Igarashi et al. 1984). The intracellular concentration of TSST-1 mRNA is reduced at least fiftyfold in mutant cells (Fig. 4, lanes 3 and 4). Additionally, the TSST-1 transcript is much more abundant in stationary phase than in exponential cells (Fig. 4, lanes 1 and 2), consistent with the observation that exoproteins are produced primarily in stationary phase. Although the expression de-

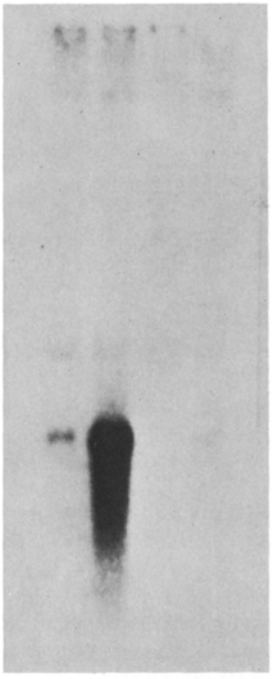

Fig. 4. Northern blot analysis of whole cell RNA from RN4282 and RN4256. RNA from cells grown on CY medium was extracted in the presence of guanidinium thiocyanate (Chirgwin et al. 1979) and electrophoresed on 1.5% agarose containing 20 mM morpholinopropane sulfonic acid, pH 7.0, 2.2 M formaldehyde and ethidium bromide (1 μg/ml). RNA was transferred to nitrocellulose, and filters were prehybridized and hybridized with ^{32}P-labeled pRN6107, which contains a 1 kbp *Bam*HI fragment derived from the TSST-1 gene, as described in Fig. 2. RNA is from: lanes 1 and 2, RNA4282 in exponential and stationary phase respectively; lanes 3 and 4, RN4256 in exponential and stationary phase respectively

fect in RN4256 is clearly at the mRNA level, expression of the TSST-1 mRNA in mutant and wild-type cells shows the same linkage to growth phase. We have observed a similar large decrease in the level of the α hemolysin transcript as a result of the insertion (data not shown).

We have tested for expression of the cloned TSST-1 gene by transferring the gene from pBR322 to the *S. aureus* plasmid pE194, generating the recombinant plasmid pRN6201 (Kreiswirth et al. 1984). Transformation of *S. aureus* RN4220, a non-TSST-1 producing wild-type strain, with pRN6201 resulted in the production of 25 μg of toxin per ml of culture supernatant (about twice the amount produced by the clinical isolate RN4282). However, transformants derived from RN4256 produced little or no toxin (0.05 μg per ml of supernatant), even though the recombinant plasmid was stably maintained at an equivalent copy number in the two strains. The results indicate that a functional *agr* locus is required for expression of the cloned TSST-1 gene and that this requirement is exerted in trans.

In summary, we have identified a trans-acting regulatory element required for expression of a number of staphylococcal exoprotein genes. The element acts at the level of mRNA and either enhances the rate of transcription of its target genes or stabilizes the transcripts. Two of the targets of *agr* regulation, *tst* and *hly*, are present in some but not all strains of *S. aureus*, map at variable positions on the chromosome and may be contained wihtin transposable genetic elements. *Agr* may have evolved as a mechanism to control expression of such elements. The marked increase in the production of extracellular protein A and of several intracellular proteins in mutant cells also raises the possibility that the *agr* product functions as a negative regulatory element in some cases. The fact that all or most of the affected proteins are produced only in stationary phase suggests that *agr* regulates genes that code for accessory proteins. Experiments are in progress to characterize the structure and mechanism of action of the *agr* product.

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