Involvement of the accessory gene regulator (*agr*) in expression of type 5 capsular polysaccharide by *Staphylococcus aureus*

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The effect of an *agr* mutation on expression of type 5 capsular polysaccharide (CP) by *Staphylococcus aureus* Newman was investigated in different complex and synthetic media. CP expression by the *agr* mutant was strongly reduced in certain media but slightly in others, indicating that CP synthesis is positively controlled by *agr*. CP expression occurred in the post-exponential growth phase in both wild-type and mutant strains, suggesting that other regulatory systems could act in conjunction with *agr*.

Introduction

Capsular polysaccharides (CP) are produced by more than 90% of *Staphylococcus aureus* strains, and probably contribute to their virulence by increasing their resistance to phagocytosis (Karakawa *et al.*, 1985, 1988; Karakawa & Vann, 1982; Sompolinsky *et al.*, 1985). Among the eight capsular serotypes described, type 5 and type 8 account for about 70 to 80% of all isolates (Boutonnier *et al.*, 1989; Hochkeppel *et al.*, 1987; Lee *et al.*, 1990). Types 5 and 8 CP have been covalently coupled to *Pseudomonas aeruginosa* toxin A to form highly immunogenic conjugate vaccines and to test the hypothesis that antibodies specific for CP are protective against invasive diseases caused by *S. aureus* (Fattom *et al.*, 1990; Fournier, 1991).

During an investigation of the physiology of type 5 CP biosynthesis (Dassy *et al.*, 1991; Stringfellow *et al.*, 1991), it was shown that the specific yield of CP varied from one medium to another, and that the rate of CP production relative to the cell mass increased during the post-exponential phase of growth, suggesting that CP biosynthesis was regulated in a similar fashion to exoproteins involved in virulence (Foster, 1991). The

genes affecting capsule expression have recently been cloned from *S. aureus* strain M, a type 1 CP producer (Lee, 1992). Most of these genes appear to be clustered together on an 11 kb fragment. The capsular and exopolysaccharide genes of other bacteria are also clustered (Deretic *et al.*, 1991; Gray & Rolfe, 1990; Gottesman & Stout, 1991; Kroll, 1992). This clustering may facilitate coordinated expression and efficient regulation of CP biosynthetic genes.

The expression of extracellular and cell-bound proteins in *S. aureus* is controlled by a regulatory locus called accessory gene regulator (*agr*). Most of these exoproteins, many of which play a role in the pathogenesis of *S. aureus* infections, are positively regulated by *agr* and are mainly produced after the cessation of active exponential growth (Janzon & Arvidson, 1990; Peng *et al.*, 1988; Recsei *et al.*, 1986; Vandenesch *et al.*, 1992). The similarity between the temporal production of exoproteins and CP, and the fact that CP is also a potential virulence factor, prompted us to test the hypothesis that CP expression is controlled by *agr*.

In the present study, we compared the wild-type *S. aureus* strain Newman with its corresponding *agr* mutant for type 5 CP and exoprotein expression during growth in complex and synthetic media.

Methods

Bacterial strains and plasmids. *S. aureus* strain Newman (NCTC 8178) is a high producer of coagulase and clumping factor (Duthie & Lorenz, 1952; McDevitt *et al.*, 1992; Marston & Fahlberg, 1960). Strain DU

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Abbreviations: CP, capsular polysaccharide; MFM-YE, modified Frantz medium plus yeast extract diffusate; SEM, supplemented Eagle medium.

5869 is an *agr* mutant of strain Newman constructed by transducing the *agrA::Tn551* mutation from strain ISP546 (Recsei *et al.*, 1986) using bacteriophage 85. Strain ISP546 is a derivative of strain 8325-4 (Novick, 1967). Plasmid pRN6650 (pUC18 with the complete *agr* locus of strain 8325-4 cloned on a 6.1 kb insertion; Peng *et al.*, 1988) was kindly supplied by R. Novick (Public Health Research Institute, New York, USA).

DNA manipulation. Genomic DNA was isolated from *S. aureus* by the method of Pattee & Neveln (1975). DNA manipulation and hybridization were performed by standard techniques (Ausubel *et al.*, 1987).

Culture media. The modified Frantz medium (MFM-YE) is a semi-synthetic medium containing mineral salts, glutamic acid, cystine, lactose and yeast extract diffusate (Difco). Complex and semi-synthetic media were as described previously (Dassy *et al.*, 1991). Supplemented Eagle medium (SEM) and Base-21, containing mineral salts, nicotinamide, thiamine and amino acids, are synthetic media described by Stringfellow *et al.* (1991). Rabbit blood agar was from Sanofi Diagnostics Pasteur, France.

Growth conditions. Agar slants were incubated at 37 °C with loose caps permitting good air exchange. Flasks were inoculated with 2% (v/v) of their liquid volume (200 ml) from an overnight culture in the same medium, and placed in a rotary water-bath shaker (200 r.p.m.) at 37 °C.

Bacterial growth was followed by measuring the OD₆₂₀ of an appropriate dilution of the cell suspension.

Type 5 CP assay. The total type 5 CP content was measured by a two-step inhibition enzyme-linked immunosorbent assay using monoclonal antibodies as previously described (Boutonnier *et al.*, 1989; Dassy *et al.*, 1991).

Haemolysin. Haemolytic activity in the culture supernatant was determined according to Duncan & Cho (1971). One haemolytic unit (HU) is defined as that amount which liberated half of the haemoglobin in the test rabbit red cell suspension.

Acid phosphatase. Phosphatase activity in the culture supernatant was determined according to Barnes & Morris (1957), with some modifications. The substrate, *p*-nitrophenylphosphate (Sigma), was dissolved in 0.15 M-acetate buffer, pH 5.5, containing 0.01 M-MgSO₄ at a concentration of 1 mg ml⁻¹. A 0.1 ml sample was added to 1 ml of the substrate solution, and the reaction mixture was incubated for 30 min at 37 °C. The reaction was terminated by adding 2 ml 0.075 M-NaOH, and the yellow colour was measured as A₄₀₀. The spectrophotometer was adjusted to zero with a blank to which 0.1 ml non-inoculated medium was added instead of the sample. One unit of phosphatase activity was defined as the amount of enzyme which liberated 1 µmol *p*-nitrophenol h⁻¹.

Results

S. aureus strain Newman *agr::Tn551*

In order to test the role of the *agr* regulatory locus in expression of CP, the well-characterized *agrA::Tn551* mutation carried by the 8825-4 strain ISP546 was transduced into strain Newman, a strain which expresses type 5 CP. Erythromycin-resistant transductants were devoid of haemolytic activity for rabbit erythrocytes and produced elevated levels of protein A, characteristics of

agr mutants of strain 8325-4 (Recsei *et al.*, 1986). Genomic DNA from one transductant (DU5869) was analysed by Southern hybridization. A single *Hind*III fragment of greater than 16 kb hybridized to the *agr* probe pRN6650 in the parental strain, while two fragments of 13 kb and 10 kb from the mutant reacted with the probe DNA (data not shown). The 5.4 kb transposon *Tn551* carries two *Hind*III sites. The same results were obtained when DNA from strains 8325-4 and ISP546 was probed (data not shown). This shows that the *agr* locus was disrupted in strain DU5869 and that the *agr::Tn551* mutation was acquired by homologous recombination.

Production of exoproteins by S. aureus strains Newman and DU5869

In all the liquid media tested (Table 1), strain Newman produced haemolytic activity toward rabbit erythrocytes, while the haemolysin titres of the *Agr*⁻ strain DU5869 were lower. In contrast, phosphatase production appeared to be little affected (data not shown).

Expression of type 5 CP by strains Newman and DU5869

The pattern of production of type 5 CP by strain Newman in different liquid media (Table 1) was closely related to that observed previously with strain Reynolds, the prototype strain for type 5 CP (Dassy *et al.*, 1991; Stringfellow *et al.*, 1991). MFM-YE and medium 110 gave the highest yields. Similar results were obtained after overnight growth on different agar slants media (data not shown).

The *Agr*⁻ strain DU5869 produced no detectable type 5 CP in Brain Heart Infusion, Nutrient Broth and Mueller-Hinton media. However, very slow but significant amounts were detected in Columbia and 110 media, and substantial amounts were obtained in MFM-YE, synthetic SEM and Base-21 media. These levels were lower than that of the corresponding parental strain, and substantial variations were observed from one experiment to another resulting in relative high standard error values. Similar results were obtained after overnight growth on different media as agar slants (data not shown).

Production of haemolysin and type 5 CP during growth in batch culture

Haemolysin and type 5 CP production by strains Newmann and DU5869 were measured during growth in MFM-YE in shake flasks.

Strain Newman (Fig. 1) grew exponentially for 3 h,

Table 1. Type 5 CP and haemolysin production by *S. aureus* strains Newman and DU5869 (*Agr*⁻) growth for 24 h at 37 °C in shake flasks

Data shown are means $\pm$ SEM of 3 independent experiments.

Liquid medium	Strain	Growth (OD ₆₂₀)	pH	Type 5 CP (ng ml ⁻¹)	Haemolysin (HU ml ⁻¹)
Brain Heart Infusion	Newman	9.70 $\pm$ 0.14	8.23 $\pm$ 0.32	2200 $\pm$ 330	1100 $\pm$ 400
	DU5869	11.0 $\pm$ 0.50	8.23 $\pm$ 0.35	< 1	155 $\pm$ 100
Nutrient Broth	Newman	1.75 $\pm$ 0.35	8.19 $\pm$ 0.35	470 $\pm$ 340	260 $\pm$ 50
	DU5869	2.0 $\pm$ 0.46	8.23 $\pm$ 0.33	< 1	< 1
Mueller-Hinton	Newman	1.70 $\pm$ 0.17	7.80 $\pm$ 0.28	260 $\pm$ 170	50 $\pm$ 27
	DU5869	2.12 $\pm$ 0.12	7.86 $\pm$ 0.33	< 1	< 1
Columbia	Newman	10.30 $\pm$ 0.57	8.17 $\pm$ 0.28	5800 $\pm$ 2300	630 $\pm$ 400
	DU5869	11.70 $\pm$ 0.30	7.93 $\pm$ 0.02	50 $\pm$ 10	195 $\pm$ 25
Medium 110	Newman	6.71 $\pm$ 0.27	4.86 $\pm$ 0.16	7300 $\pm$ 1300	90 $\pm$ 70
	DU5869	5.33 $\pm$ 0.57	4.60 $\pm$ 0.11	75 $\pm$ 61	< 2
MFM-YE	Newman	3.04 $\pm$ 0.35	7.66 $\pm$ 0.26	4300 $\pm$ 2400	160 $\pm$ 60
	DU5869	2.90 $\pm$ 0.12	7.41 $\pm$ 0.03	1000 $\pm$ 1300	< 1
SEM	Newman	1.25 $\pm$ 0.30	6.43 $\pm$ 0.31	1900 $\pm$ 1300	ND
	DU5869	1.94 $\pm$ 0.11	5.70 $\pm$ 0.41	450 $\pm$ 100	ND
Base-21	Newman	7.16 $\pm$ 0.90	7.30 $\pm$ 0.14	2200 $\pm$ 1500	ND
	DU5869	7.65 $\pm$ 1.18	7.45 $\pm$ 0.42	260 $\pm$ 240	ND

ND, not determined.

Fig. 1. Growth (○), pH (■), type 5 CP production (□) and haemolysin production (▲) by *S. aureus* strain Newman in MFM-YE medium in a shake flask at 37 °C.

followed by a slow growth phase for a further 8 h. Haemolysin activity increased exponentially for 8 h, then reached a plateau at 8 to 11 h, before falling between 11 and 25 h. The level of type 5 CP first increased slowly for 8 h, and then, after a pronounced rise between 8 and 11 h, increased at a lower rate for 14 h. The pH decreased for 2 h and then increased between 2 and 26 h.

The plot of exoprotein and CP concentration vs extent

Fig. 2. Relationship between type 5 CP production (□) haemolysin production (▲) and growth of *S. aureus* strain Newman. The arrow indicates the end of the exponential growth phase. The data are from the experiment depicted in Fig. 1.

Fig. 3. Growth (○), pH (■) and type 5 CP production (□) by *S. aureus* strain DU5869 in MFM-YE medium in a shake flask at 37 °C.

of growth (Fig. 2) shows the differential rates of expression (Coleman *et al.*, 1978; Monod *et al.*, 1952). The differential rates for each factor increased at different

Fig. 4. Relationship between type 5 CP production and growth of *S. aureus* strain DU5869. The arrow indicates the end of the exponential growth phase. The data are from the experiment depicted in Fig. 3.

stages of growth; at the end of the exponential phase for haemolysin, and at the end of the slow growth phase for CP.

The *Agr*⁻ strain DU5869 (Fig. 3) showed similar growth and type 5 CP production to that observed with strain Newman. No haemolytic activity was detectable. The pH decreased for at least 11 h and then increased. The variations in the differential rate of CP formation (Fig. 4) were also similar to those observed with strain Newman.

Discussion

The *Agr*⁻ mutant of strain Newman exhibited a similar phenotype to *Agr*⁻ mutants of other strains of *S. aureus* with respect to the regulation of exoprotein synthesis (Björklind & Arvidson, 1980; Engels & Kamps, 1982). They showed a drastic reduction of haemolytic activity and a slight reduction in phosphatase production.

The kinetics of haemolysin production by strain Newman were closely related to those observed with other *S. aureus* strains (Duncan & Cho, 1971; Jassim *et al.*, 1989). It was assumed that the decrease in haemolytic activity during the stationary phase was the result of denaturation caused by continued shaking at 37 °C. The haemolysin produced by strain Newman is probably γ -toxin. The haemolytic zone around colonies growing on agar plates incorporating rabbit erythrocytes was enhanced substantially when the medium was made up with agarose which lacks the sulphonated polymers known to inhibit γ -toxin (Clyne *et al.*, 1992). Furthermore, antiserum to γ -toxin (obtained from M. Clyne, Trinity College, Dublin) inhibited haemolysis (data not shown). Strain Newman probably does not express significant amounts of α -toxin, because transduction of the *hla::Em^r* mutation (O'Reilly *et al.*, 1986) into this strain did not affect the size of haemolysis on agar plates incorporating rabbit erythrocytes.

Strain Newman has previously been grown in chemically defined media (Marston & Fahlberg, 1960), and CP was shown to be produced by *S. aureus* in such media (Stringfellow *et al.*, 1991). Significant differences for total growth and final pH between *Agr*⁺ and *Agr*⁻ strains was only observed with synthetic SEM medium, which contains limited amounts of essential amino acids (Stringfellow *et al.*, 1991). Moreover, differences were observed in the pH changes that occurred during growth of the *Agr*⁺ and *Agr*⁻ strains, suggesting a difference in metabolism. Recsei *et al.* (1986) showed that intracellular proteins as well as exoproteins were regulated by *agr*.

The kinetics of type 5 CP production by strains Newman and Reynolds were subtly but significantly different. The increase of the differential rate of CP formation occurred at the end of the exponential phase for strain Reynolds (Dassy *et al.*, 1991) and at the end of the slow growth phase for strain Newman. This indicates that regulation of type 5 CP expression may be more complex than that of exoproteins. In addition, CP expression appears to be positively controlled by *agr*. However, this *agr* control seems to be partially overruled in MFM-YE, SEM and Base-21 media, and the expression of CP by the *agr* mutant in MFM-YE also occurs in the post-exponential growth phase. This *agr* override could be due to the presence of a single medium component acting as a signal for the sensor protein of a two-component regulatory system (Gross *et al.*, 1989; Stock *et al.*, 1989) or to the effect that these media have on the metabolism of the bacteria. This suggests that another regulatory mechanism can interact with *agr*, and is consistent with the interaction of distinct sensor-regulator systems observed among other bacteria (Wanner, 1992), and with previous observations relating to the expression of exoproteins by *S. aureus* which suggested that other regulatory systems could act in conjunction with *agr* (Coleman *et al.*, 1989; Compagnone-Post *et al.*, 1991; Janzon *et al.*, 1986; Regassa *et al.*, 1991, 1992; Smeltzer *et al.*, 1992; Vandenesch *et al.*, 1992). A regulatory locus affecting exoprotein expression and distinct from *agr* was recently identified in *S. aureus* (Cheung *et al.*, 1992).

Recently, *agr* expression was shown to be affected by the pH of the culture medium (Regassa & Betley, 1992). Alkaline pH results in a decrease in *agr* expression and consequently in the expression of *agr* target genes. This is consistent with our previous results showing that post-exponential CP production was observed in the pH range 6–7, but not at pH 8 (Dassy *et al.*, 1991).

Some coagulase-negative staphylococci have been shown to produce extracellular proteins (Donham *et al.*, 1988) and slime (Bayston & Rodgers, 1990), mostly during the post-exponential growth phase. It is tempting to speculate that these species possess a common

regulatory mechanism exhibiting some homologies with *S. aureus agr*.

During bacterial infections, the pathogenic organism uses adaptative responses to sense environmental conditions. These responses require the coordinate expression of a number of bacterial genes, many of which play a role in virulence (Dorman, 1991; Mekalanos, 1992). The genetics of capsule and exopolysaccharide expression have been examined in several bacterial species (Deretic *et al.*, 1991; Frosch *et al.*, 1991; Gray & Rolfe, 1990; Gottesman & Stout, 1991; Kroll, 1992), but it has only recently been shown that elements of genetic regulation of exoprotein and exopolysaccharide were shared in *Xanthomonas campestris* (Tang *et al.*, 1990, 1991) and *Pseudomonas aeruginosa* (Cacalano *et al.*, 1992). Our results show that the same situation occurs in *S. aureus*. Further investigation of the molecular genetics of *S. aureus* capsule expression and its regulation is now warranted.

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