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
5869 is an agr mutant of strain Newman constructed by transducing the 
agr::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 OD_{600} 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-MgSO_{4} 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 A_{405}. 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 *agr::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 *HindIII* 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 *HindIII* 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±SEM of 3 independent experiments.

<table>
<thead>
<tr>
<th>Liquid medium</th>
<th>Strain</th>
<th>Growth (OD₆₂₀)</th>
<th>pH</th>
<th>Type 5 CP (ng ml⁻¹)</th>
<th>Haemolysin (HU ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain Heart Infusion</td>
<td>Newman</td>
<td>9.70±0.14</td>
<td>8.23±0.32</td>
<td>2200±330</td>
<td>1100±400</td>
</tr>
<tr>
<td></td>
<td>DU5869</td>
<td>11.0±0.50</td>
<td>8.23±0.35</td>
<td>&lt;1</td>
<td>155±100</td>
</tr>
<tr>
<td>Nutrient Broth</td>
<td>Newman</td>
<td>1.75±0.35</td>
<td>7.89±0.35</td>
<td>470±340</td>
<td>260±50</td>
</tr>
<tr>
<td></td>
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<td>2.0±0.46</td>
<td>8.23±0.33</td>
<td>&lt;1</td>
<td>&lt;1</td>
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<tr>
<td>Mueller–Hinton</td>
<td>Newman</td>
<td>1.70±0.17</td>
<td>7.86±0.28</td>
<td>260±170</td>
<td>50±27</td>
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<td>&lt;1</td>
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<tr>
<td>Columbia</td>
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<td>10.30±0.57</td>
<td>8.17±0.28</td>
<td>5800±2200</td>
<td>630±400</td>
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<td></td>
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<td>7.93±0.02</td>
<td>50±10</td>
<td>195±25</td>
</tr>
<tr>
<td>Medium 110</td>
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<td>6.71±0.27</td>
<td>4.86±0.16</td>
<td>7300±1300</td>
<td>90±70</td>
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<td>4.60±0.11</td>
<td>75±61</td>
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<tr>
<td>MFM-YE</td>
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<td>3.04±0.35</td>
<td>7.66±0.26</td>
<td>4300±2400</td>
<td>160±60</td>
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<td>7.41±0.03</td>
<td>1000±1300</td>
<td>&lt;1</td>
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<td>SEM</td>
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<td>6.43±0.31</td>
<td>1900±1300</td>
<td>ND</td>
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<tr>
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<td>Base-21</td>
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<tr>
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<td>7.45±0.42</td>
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<td>ND</td>
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</tbody>
</table>

ND, not determined.

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 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
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 (Björklin & 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' 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; Vandenbosch 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 \textit{S. aureus agr}.

During bacterial infections, the pathogenic organism uses adaptive 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; Frosh 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 \textit{Xanthomonas campestris} (Tang et al., 1990, 1991) and \textit{Pseudomonas aeruginosa} (Cacalano et al., 1992). Our results show that the same situation occurs in \textit{S. aureus}. Further investigation of the molecular genetics of \textit{S. aureus} capsule expression and its regulation is now warranted.

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\section*{References}


