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agr function in clinical *Staphylococcus aureus* isolates

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The accessory gene regulator (*agr*) of *Staphylococcus aureus* is a global regulator of the staphylococcal virulon, which includes secreted virulence factors and surface proteins. The *agr* locus is important for virulence in a variety of animal models of infection, and has been assumed by inference to have a major role in human infection. Although most human clinical *S. aureus* isolates are *agr*$^+$, there have been several reports of *agr*-defective mutants isolated from infected patients. Since it is well known that the *agr* locus is genetically labile *in vitro*, we have addressed the question of whether the reported *agr*-defective mutants were involved in the infection or could have arisen during post-isolation handling. We obtained a series of new staphylococcal isolates from local clinical infections and handled these with special care to avoid post-isolation mutations. Among these isolates, we found a number of strains with non-haemolytic phenotypes owing to mutations in the *agr* locus, and others with mutations elsewhere. We have also obtained isolates in which the population was continuously heterogeneous with respect to *agr* functionality, with *agr*$^+$ and *agr*$^-$ variants having otherwise indistinguishable chromosomal backgrounds. This finding suggested that the *agr*$^-$ variants arose by mutation during the course of the infection. Our results indicate that while most clinical isolates are haemolytic and *agr*$^+$, non-haemolytic and *agr*$^-$ strains are found in *S. aureus* infections, and that *agr*$^+$ and *agr*$^-$ variants may have a cooperative interaction in certain types of infections.

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INTRODUCTION

agr is a well-studied central transcriptional regulator that controls the expression of virulence-associated protein genes in *Staphylococcus aureus*. The *agr* locus (Fig. 1) consists of two divergent transcription units driven by promoters P2 and P3. The P2 operon encodes a two-component signalling module, of which AgrC is the receptor and AgrA is the response regulator. It also encodes two proteins, AgrB and D, which combine to produce and secrete an autoinducing peptide (AIP) that is the ligand for AgrC. AgrA functions to activate transcription from its own promoter and from the *agr*P3 promoter, which drives the synthesis of RNAIII, the effector of target gene regulation (Novick *et al.*, 1993). RNAIII also encodes δ-haemolysin (Janzon *et al.*, 1989), the expression of which serves as a surrogate for *agr* functionality.

Although its importance for pathogenesis in animal models is well established (Abdelnour *et al.*, 1993; Arvidson & Tegmark, 2001; Gillaspy *et al.*, 1995; Novick, 2003), and most clinical isolates are *agr*$^+$, the isolation from clinical material of *S. aureus* strains expressing *agr* poorly, or not at all (Fowler *et al.*, 2004; Sakoulas *et al.*, 2002), raises the question of the precise role of *agr* in human *S. aureus* disease (Li *et al.*, 1997; Nozohoor *et al.*, 1998). This question is compounded by the fact that *agr*-defective mutants arise frequently in laboratory cultures (Bjorklind & Arvidson, 1980; McNamara & Iandolo, 1998; Somerville *et al.*, 2002). Consequently, *agr*-defective genotypes found among clinical isolates could be the result of mutations occurring during subculture after isolation. Accordingly, we studied *agr* functionality in a series of clinical *S. aureus*

Abbreviations: AIP, autoinducing peptide; MRSA, meticillin-resistant *Staphylococcus aureus*.

A supplementary figure and supplementary tables with details of isolates and primers are available with the online version of this paper.

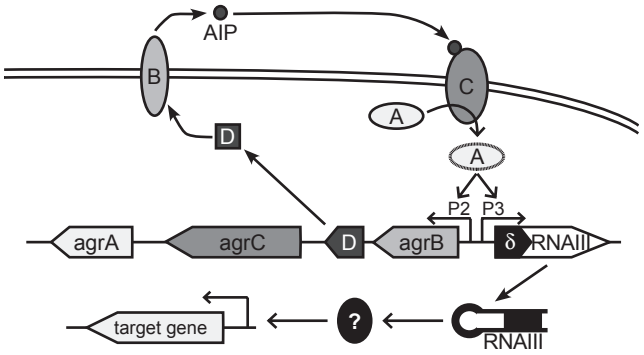

Fig. 1. The *agr* locus (see text for details).

isolates that were handled with a minimum of manipulation to avoid post-isolation mutations. We report that *agr*-defective mutants occur in clinical material, that these arise and persist during infections, and that they are not the result of post-isolation handling; thus, they represent an important subset of clinical *S. aureus*.

METHODS

Isolation and maintenance of *S. aureus* clinical isolates. *S. aureus*-positive culture specimens were kindly donated by the NYU Tisch Hospital microbiology laboratory. The specimens were catalogued to indicate the age and sex of the patient, days to positive culture, diagnosis, type of specimen, antibiotics used to treat the patient, and antibiotic sensitivities of the isolates (see Supplementary Table S1, available with the online version of this paper). All specimens were obtained from primary cultures: blood specimens were from culture bottles; urine samples were from urine receptacles, etc. Swabs (from wounds, etc.) were placed in 1.0 ml CYGP broth (Novick, 1991) and snap-frozen with dry-ice/ethanol. Before freezing, GL (Novick, 1991) plates were inoculated with the specimen (blood, urine, wound, sputum). Cultures from these plates were tested for *agr* group (described below), cross-streaked against RN4220 to assess haemolysin activity (described below), and then resuspended in CY broth and frozen in dry-ice/ethanol to create a secondary stock, which was stored at –80 °C. All subsequent studies were performed with bacteria grown from the original specimen. The secondary stock was used to confirm results.

Laboratory bacterial strains and culture conditions. Laboratory bacterial strains and plasmids are listed in Table 1. Strains RN9688, RN9689, RN9690 and RN9691 were used for *agr* typing as described by Wright *et al.* (2005b). RN4220 was used to score haemolysin production. CYGP broth cultures, inoculated with bacteria grown overnight on GL agar at a cell density of 15 Klett units ($\sim 2 \times 10^8$ cells ml^{-1}), unless otherwise specified, were incubated at 37 °C with shaking at 250 r.p.m.

***agr* typing.** *agr* type was determined by a luciferase plate assay as previously described (Wright *et al.*, 2005b).

Assessment of haemolysin activity on SBA plates. Haemolytic activities were determined by cross-streaking perpendicularly to RN4220, which produces only β-haemolysin (Traber & Novick, 2006), on a sheep blood agar (SBA) plate. This test can usually identify the three staphylococcal haemolysins active on SBA – α, β and δ (see Fig. 2) – because of the interactions between them: β-haemolysin enhances lysis by δ-haemolysin, but inhibits lysis by α-haemolysin (Elek & Levy, 1950). To determine δ-haemolysin production by single colonies, we prepared SBA plates by spreading 400 µl of a sterile twofold-concentrated RN4220 supernatant before plating a suitable dilution of the strain to be tested. Note that the β-haemolysin produced by RN4220 enables detection of δ-haemolysin (see Fig. 2).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference
Strains		
RN4220	Non-lysogenic derivative of NCTC 8325 that accepts *E. coli* DNA; has *agrA*-8A mutation	Peng *et al.* (1988)
RN6734	Prototypical *agr*-I (derived from NCTC 8325)	Ji *et al.* (1997)
RN7206	*agr*-null derivative of RN6734	Ji *et al.* (1997)
RN6607	Prototypical *agr*-II	Ji *et al.* (1997)
RN3984	Prototypical *agr*-III	Ji *et al.* (1997)
RN4850	Prototypical *agr*-IV	Jarraud *et al.* (2000)
RN9688	RN7206 with pRN7141 + pRN7130 *agr*-I tester	Wright *et al.* (2005b)
RN9689	RN7206 with pRN7141 + pRN7129 *agr*-II tester	Wright *et al.* (2005b)
RN9690	RN7206 with pRN7141 + pRN7131 *agr*-III tester	Wright *et al.* (2005b)
RN9691	RN7206 with pRN7141 + pRN7128 *agr*-IV tester	Wright *et al.* (2005b)
Plasmids		
pRN7141	pMK4*lux* with *agr*P3 driving *lux*	Wright *et al.* (2005a)
pRN6959	pRN5548 with P*bla* driving *agrBD*-III	Ji *et al.* (1997)
pRN6961	pRN5548 with P*bla* driving *agrB*-III	Ji *et al.* (1997)
pRN6918	pRN5548 with P*bla* driving *agrC*-II	Ji *et al.* (1997)
pRN7130	*agr*P2 driving *agrAC*-I	Wright *et al.* (2004)
pRN7129	*agr*P2 driving *agrAC*-II	Wright *et al.* (2004)
pRN7131	*agr*P2 driving *agrAC*-III	Wright *et al.* (2004)
pRN7128	*agr*P2 driving *agrAC*-IV	Wright *et al.* (2004)

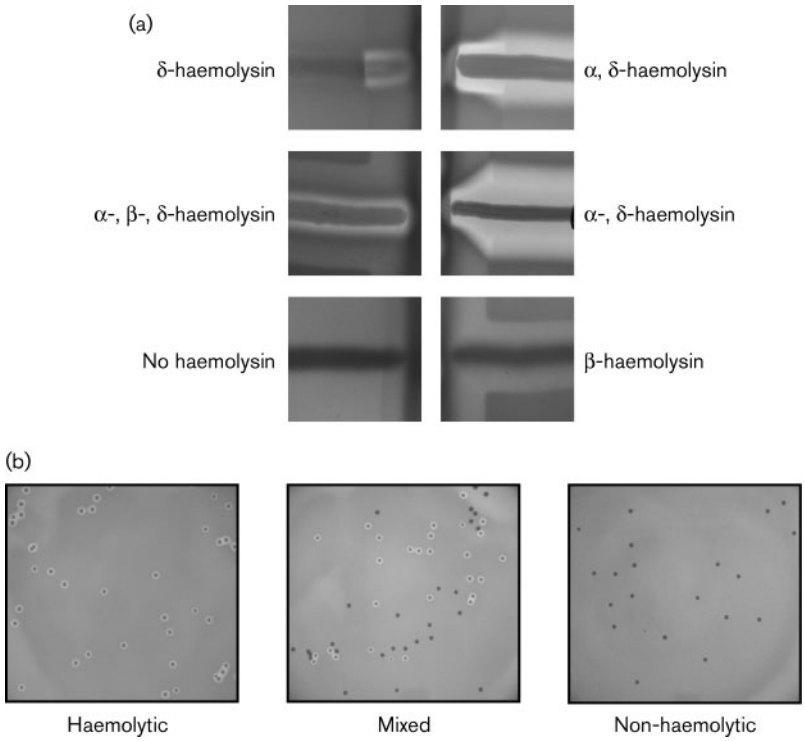

Fig. 2. Haemolytic activities. (a) Strains were tested against RN4220; (b) analysis of single colonies for δ-haemolysin. See Methods for details.

DNA sequencing. Chromosomal DNA isolation as well as sequencing of the *agr* locus and the SSR region of *spa* were performed as described by Traber & Novick (2006). Primers are listed in Supplementary Table S2.

Northern blot analysis. Total cellular RNA was prepared according to a standard protocol (Traber & Novick, 2006), and Northern blot analysis was performed as described by Kornblum *et al.* (1988). PCR primers used to prepare DNA probes are listed in Supplementary Table S2. In all blots, 16s rRNA was used as a loading control.

Exoprotein profiles. Exoprotein profiles were generated as described by Traber & Novick (2006). Culture supernatants, prepared by two cycles of centrifugation, were precipitated with a 10 % volume of 50 % trichloroacetic acid, and the pellet analysed by SDS-PAGE (Laemmli, 1970).

RESULTS

Isolation, maintenance and initial typing of *S. aureus* clinical isolates

A total of 146 *S. aureus* infection isolates, obtained between 1 August 2001 and 1 June 2002 from 102 patients with documented staphylococcal disease, were kindly provided by the Tisch Hospital bacteriology laboratory. Two or more cultures were obtained from 34 of these patients.

agr typing

S. aureus strains have been divided into four *agr* specificity groups (Jarraud *et al.*, 2000; Ji *et al.*, 1997). Of the 102 patients, 51 had an *agr*-I strain, 34 an *agr*-II, 14 an *agr*-III and 1 an *agr*-IV strain. One patient (no. 75) was infected by strains of two different *agr* groups: an *agr*-I strain in two blood cultures, and an *agr*-II strain in a wound culture. Five strains could not be typed by the luciferase plate test and were typed by direct *agrD* sequencing (Ji *et al.*, 1997). One strain could not be typed and has not been studied further. The intensity of the bioluminescence produced in the typing assay (Wright *et al.*, 2005b) varied considerably from strain to strain, representing variation in AIP levels. Low levels of bioluminescence likely represented the basal activity of the *agr* P2 promoter, seen when the *agr* autoinducing circuit is not activated owing to genotypic defects. Therefore, the production of detectable AIP cannot be taken as an automatic indication of a functioning *agr* autoinduction circuit. Strains that produced no reaction in the plate test may have had mutations in *agrB* or *agrD*.

agr typing by other investigators has generated widely varying distributions of the *agr* types, presumably representing variations in the local prevalence of strains of the different *agr* groups (Gilot & van Leeuwen, 2004; Jarraud *et al.*, 2002; Shopsin *et al.*, 2003). The distribution of *agr* types among our isolates does not represent a significant departure from the overall results of other series, nor have we encountered any significant correlation between clinical features and *agr* type in this series. The *agr* typing results and haemolytic activities of the entire set of isolates are summarized in Supplementary Table S1.

Characterization of non-haemolytic strains

Of the 146 cultures collected, 33, from 26 patients, were non-haemolytic on SBA. In 10 different cases, we obtained,

from a single patient, separate cultures that had different haemolysin patterns. Many of these contained mixtures of haemolytic and non-haemolytic staphylococci, some of which are described in detail below. Eleven of the pure non-haemolytic strains were analysed to establish the basis for their lack of haemolytic activity. Nine strains had mutations in the *agr* locus that could have inactivated *agr* function, Of these nine, three could be complemented and three could not; these are presumed to have other genotypic causes for their non-haemolytic phenotype and are to be studied further. Three were resistant to the antibiotics used to select for the complementing plasmids and were not tested for complementation (see Table 2). Two of the non-haemolytic strains had the wild-type *agr* sequence corresponding to their *agr* group. These results are shown in Table 2.

Sixteen of the 33 non-haemolytic and 73 of the 146 haemolytic isolates (Supplementary Table S1) were meticillin-resistant *S. aureus* (MRSA). Therefore, the SCCmec element was not implicated in the development of *agr* defects. Loss of *agr* function has been associated with the development of vancomycin resistance (Sakoulas *et al.*, 2002). However, in this series, there was no correlation between vancomycin treatment and the development of *agr* defects (Supplementary Table S1); nonetheless, all of the patients were treated with some type of antibiotic, raising the question of whether antibiotics in general could drive selection for *agr* negatives.

Transcription patterns and P3 activity in non-haemolytic clinical isolates

For several non-haemolytic strains, the level of bioluminescence seen in the *agr* typing test seemed too high to represent the basal level of P2 promoter activity. In these strains, the defect in haemolysin production may be a result of mutations inactivating α- or δ-haemolysin, or impairing RNAIII transcription, timing, function or stability. To differentiate these possibilities we tested the 11 above-mentioned strains for their ability to activate the P3 promoter, transcribe RNAIII and transcribe the RNAIII-regulated exoprotein genes *hla* and *spa*, with results shown in Fig. 3(a).

Two strains, RN9771 and RN9774, demonstrated a transcription pattern that closely resembled that of the *agr*$^+$ strain. RNAIII was produced at wild-type levels, plasmid-carried *agr*P3-*lux* was strongly activated, *hla* was transcribed moderately strongly, and little or no detectable *spa* transcript was produced. This transcription pattern, coupled with lack of haemolytic activity, is typical of mutants with delayed *agr* activation, as described elsewhere (Traber & Novick, 2006) and discussed further below. Strain RN9771 had a complementable mutation in *agrC* while RN9774 had the wild-type *agr* sequence.

The second pattern was shown by strain RN9764, and is very similar to the pattern seen in the *agr* null control RN7206. It produced only a trace of RNAIII, activated P3 *lux* weakly and transcribed *hla* extremely weakly but had relatively strong *spa* transcription. RN9764 has a complementable mutation in *agrB*, indicating that it is *agr*-defective. It also has a probably unimportant mutation in *agrC*.

In the third pattern, strains RN9765, RN9772, RN9900 and RN9901, there was little or no detectable RNAIII; there were relatively strong *spa* bands, as expected, but also high levels of *hla* transcription. These strains had various *agr* mutations, some complementable, others not, but more importantly, are likely to have mutations that can bypass the requirement of RNAIII for *hla* transcription (McNamara *et al.*, 2000). Testing for such mutations is currently in progress.

Finally, the fourth pattern, as seen in strains RN9902, RN9903, RN9904 and RN9906, shows no RNAIII, and a

Table 2. *agr* mutations and complementation data for non-haemolytic isolates

Strain	*agr* group	*agr* mutations*	Complementation‡
RN9764	III	*agrB* V119L (aa), *agrC* E220D (aa)	*agrBD*-yes; *agrB*-yes
RN9765	I	*agrC* S116C (aa)	*agrCA*-no
RN9771	II	Intergenic G218A† *agrC* L245S (aa)	*agrC*-yes
RN9772	III	*agrC*, many mutations	ND
RN9774	III	Wild-type	
RN9900	I	*agrC* aa151 stop	*agrCA*-yes; *agrC*-yes
RN9901	III	*agrB* V119L (aa); *agrC* aa127 (nt378) FS; aa145 stop	*agrA*-no; *agrB*-ND
RN9902	Ia	Wild-type	
RN9903	I	RNAIII T228C, *agrC* IS*256* insertion	ND
RN9904	Ia	*agrA*, aa63 (nt487) FS; aa175 stop	*agrA*-no
RN9906	Ia	*agrC*, del. nt302–305 aa100	ND

*Numbers refer to nucleotide (nt or unspecified) or amino acid (aa) coordinates in the GenBank sequences for the respective genes. FS, frameshift.
†Silent polymorphism.
‡ND, No data.

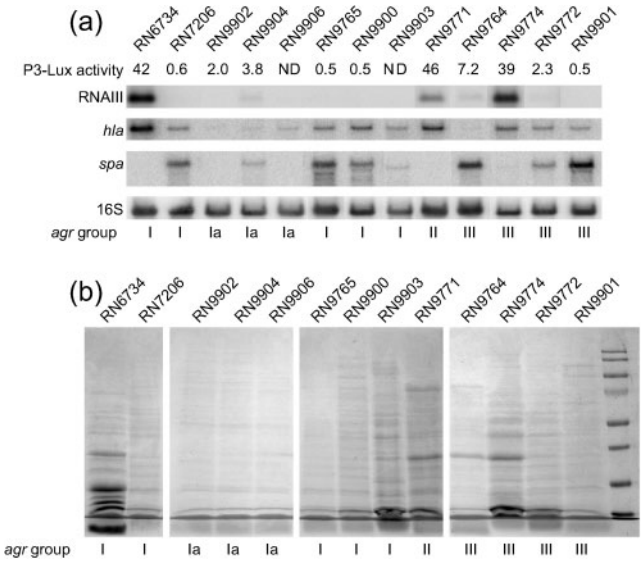

Fig. 3. Transcription and translation in non-haemolytic, *agr*-typable clinical isolates. (a) Northern blot analysis of post-exponential-phase RNA (t=4 h), with strains RN6734 and RN7206 as agr^+ and agr^- standards. Relative luciferase activities of an agrP3-*lux* fusion (Wright *et al.*, 2005a) in each strain are listed above. (b) SDS-PAGE analysis of exoprotein profiles at t=6 h.

very weak *hla* transcript, but low to no *spa*. RN9902 is especially interesting as it had a wild-type *agr* sequence, suggesting a mutation in an upstream gene required for *agr* expression. Strains RN9903, RN9904 and RN9906 all have mutations in the *agr* locus that probably account for their low RNAIII levels, but would not account for low *spa* transcript levels. RN9903 and RN9906 were not tested for complementation owing to their resistance phenotypes. RN9904, with a clearly inactivating frameshift in *agrA*, was not complemented by an *agrA* clone and presumably has a mutation elsewhere that prevents *agr* expression.

As a further characterization of the phenotypes of these strains, we determined exoprotein profiles (Fig. 3b). Strains of the same *agr* type group generally had similar exoprotein profiles; however, whenever RNAIII was not detected, there were few or very weak exoprotein bands. These strains did not significantly activate the *agr*P3-*lux* fusion (Fig. 3a) and are probably *agr*-defective. However, the two non-haemolytic strains that transcribed RNAIII and *hla* strongly activated the agrP3-*lux* fusion, had robust exoprotein profiles, and were clearly agr^+. Thus, using haemolysin as a measure of *agr* functionality will sometimes result in false negatives.

Late RNAIII transcription

As relatively late transcription of RNAIII is associated with failure to translate δ-haemolysin and α-haemolysin (Traber & Novick, 2006), we hypothesized that late RNAIII expression could be responsible for the failure of the agr^+ strain, RN9774, to produce α- or δ-haemolysin. As shown in Fig. 4(a), transcription of RNAIII in strain RN9774 was indeed delayed by 1–2 h as compared to RN6734. Transcription of *hla* was also delayed, as well as somewhat lower in intensity than in RN6734. Finally, *spa* was transcribed in RN9774 and was downregulated concomitantly with RNAIII activation, between 2 and 3 h, as opposed to RN6734, in which *spa* transcription was inhibited at all time points. Note that in the assay of plasmid-carried *agr*P3-*lux* activity, luciferase activity was determined with overnight plate cultures, which are in early stationary phase, and any delay in *agr* activation would not have been apparent.

We next hypothesized that the late *agr* activation in RN9774 could be the result of low AIP activity. To test this, we added a 1/10 volume of 6 h culture supernatant from strain RN9774, or from another *agr*-III strain, RN3984, or from the *agr* null strain RN7206 or sterile CYGP broth, to early-exponential-phase cultures of RN9774. We grew the cultures for 6 h after the addition of supernatant and analysed exoprotein production. As shown in Fig. 4(b), post-exponential *agr*-III supernatant from RN3984 or from

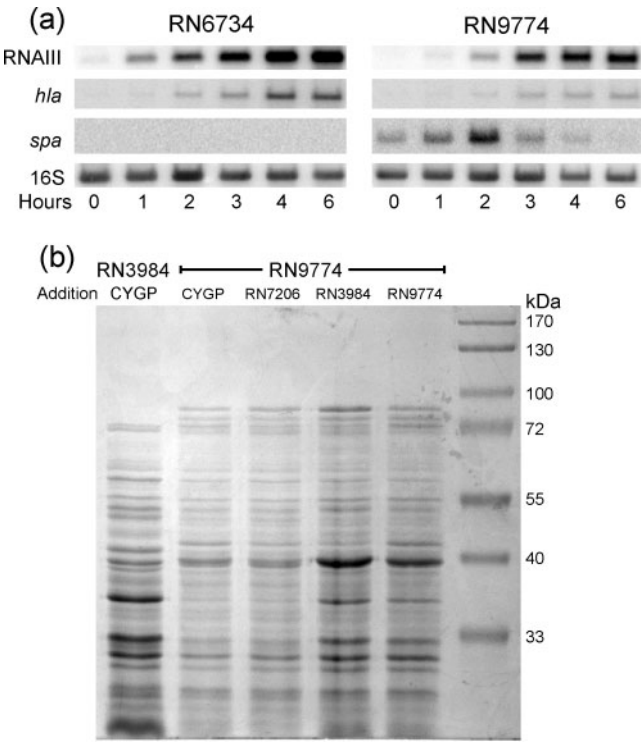

Fig. 4. Transcription and exoprotein production in non-haemolytic strain RN9774. (a) Northern blot of temporal transcription of RNAIII, *hla* and *spa* in RN9774 compared to RN6734. (b) SDS-PAGE analysis of the effect of conditioned medium on exoprotein production. Lane 1, strain RN3984 treated with cygp; lanes 2–5, RN9774 treated with CYGP or with conditioned medium from RN7206 (agr^-), RN3984 or RN9774.

RN9774 stimulated the production of many exoproteins, generating an exoprotein profile similar to that of the wild-type *agr* strain RN3984. The results with strain RN9774 indicate that the timing of *agr* activation may be an important factor in the regulation of virulence factor production *in vitro*.

Clinical isolates with mixtures of *agr*$^+$ and *agr*$^-$ staphylococci

As mentioned above, several of the non-haemolytic isolates were obtained from patients from whom haemolytic isolates were also obtained. Among these, five haemolytic and two non-haemolytic blood cultures were isolated from patient 60 and a haemolytic and a non-haemolytic isolate were obtained from patient 28. The cultures from each patient were otherwise identical (see below), suggesting that the primary cultures contained congenic haemolytic and non-haemolytic substrains. As this would not have been detected by cross streaking (see Fig. 2a), we diluted and plated these isolates for single colonies on SBA prespread with RN4220 supernatant (See Methods and Fig. 2).

For these two patients, many of the frozen primary cultures were mixed (Table 3). From patient 28, both wound cultures were mixed. From patient 60 three cultures were mixed, (one each from days 8, 9 and 10), and four were >99 % pure (one each from days 5 and 9, both positive; two from day 11, one positive, one negative). The mixed cultures from patient 60 were highly variable in composition, containing 85 % δ-haemolysin-positive (1140 of 1342 colonies scored), 3 % positive (22 of 777) and 24 % positive (330 of 1389) respectively. Each culture was independently plated three times and the relative proportion of haemolytic and non-haemolytic colonies was always similar. The variability of the proportions of haemolytic and non-haemolytic colonies in the cultures from patient 60 probably represents sampling variability owing to the very small number of bacteria that are ordinarily present in any blood sample.

Table 3. Overview of mixed clinical isolates

Clinical culture			Haemolysin	
Number*	Source	Day isolated	Whole culture	Single colony
28-1	Wound	1	δ^+	Mix $\delta^{+/-}$
28-5	Wound	5	δ^-	Mix $\delta^{+/-}$
60-5	Blood	5	δ^+	Mix $\delta^{+/-}$
60-8	Blood	8	δ^+	>99 % δ^+
60-9a	Blood	9	δ^+	>99 % δ^+
60-9b	Blood	9	δ^+	Mix $\delta^{+/-}$
60-10	Blood	10	δ^-	Mix $\delta^{+/-}$
60-11a	Blood	11	δ^-	>99 % δ^-
60-11b	Blood	11	δ^+	>99 % δ^+

*Patient number and day of isolation: e.g. 60-9b=patient 60, day 9 second isolate.

Genotyping of mixed cultures

These mixed cultures could have arisen by mutation or could represent co-infection by two distinct lineages. To distinguish between these alternatives, we isolated single colonies from each of the cultures from patients 28 and 60. Since these mixtures had probably resulted from *in vivo* mutations, we analysed these strains in rather more detail than the non-haemolytic isolates described above. For patient 60, from each of the mixed cultures we examined three haemolytic and three non-haemolytic colonies, and from the homogeneous cultures we also examined three colonies. From patient 28, we examined a haemolytic and a non-haemolytic colony from each culture (Table 4). For each isolate we determined *Cla*I restriction patterns (Supplementary Fig. S1), and *spa* types (not shown), which indicated that all of the isolates from patients 28 and 60 were identical, and sharply different from the patient 9 strains, suggesting that a common nosocomial multi-resistant MRSA strain had infected patients 28 and 60.

agr sequencing

We sequenced the *agr* locus in representative non-haemolytic colonies from patients 28 and 60. Of the patient 60 isolates, we sequenced three from each culture containing non-haemolytic organisms, and in the patient 28 isolates, one non-haemolytic isolate from each culture. The non-haemolytic isolates from patient 60 contained four different mutations, three in *agrA*, and one in *agrC* (T399P) (Fig. 5, Table 4). Two of the *agrA* mutations had

Table 4. Mutations in cultures from patients 28 and 60

Isolate number*	*agr* mutation	Alternative name
60-5-1	*agrA* 8A frameshift	*agrA*-8A
60-5-2	*agrA* 6A frameshift	*agrA*-6A
60-5-3	*agrA* deletion nt473–482	*agrA*-del
60-9b-1	*agrA* 8A frameshift	*agrA*-8A
60-9b-2	*agrA* 8A frameshift	*agrA*-8A
60-9b-3	*agrA* 8A frameshift	*agrA*-8A
60-10-1	*agrC*-T399P	
60-10-2	*agrC*-T399P	
60-10-3	*agrC*-T399P	
60-10-4	wt, *agr*$^+$	
60-11a-1	*agrA* 8A frameshift	*agrA*-8A
60-11a-2	*agrA* 8A frameshift	*agrA*-8A
60-11a-3	*agrA* 8A frameshift	*agrA*-8A
28-1-4	wt, *agr*$^-$	
28-5-4	*agrC* deletion nt1105–1115	*agrC*-del

*E.g. 60-11a-2=patient 60, day 11, first culture, second colony.

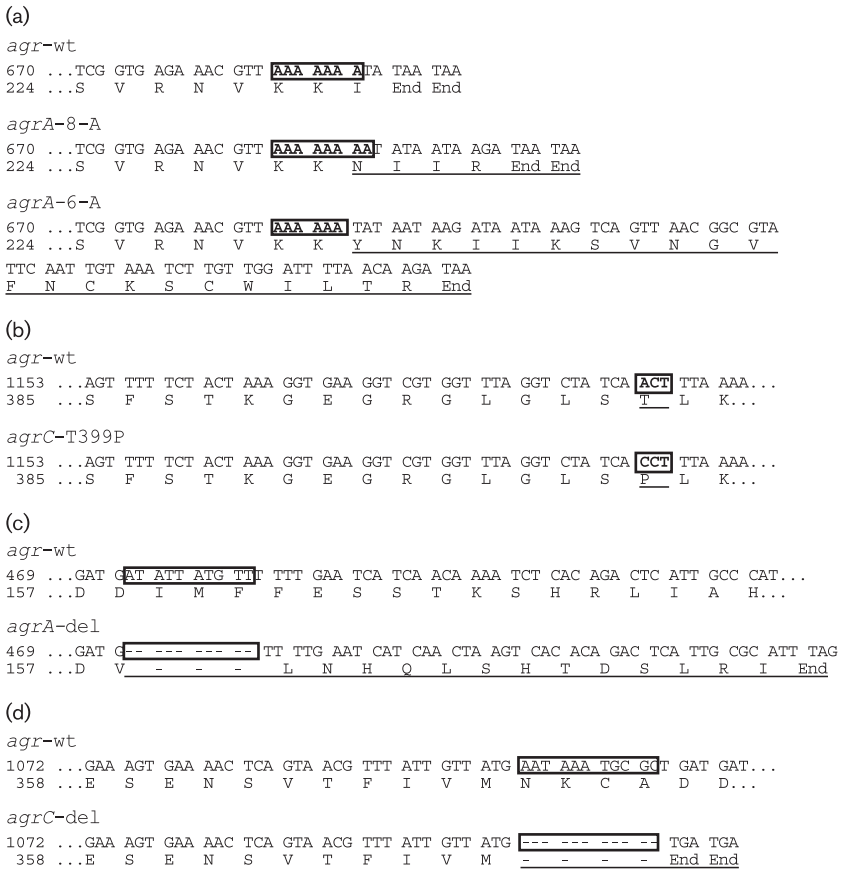

Fig. 5. Sequences of mutations shown in Table 4. (a) Frameshift mutations in *agrA*. (b) Nucleotide change in *agrC*. (c) Deletion in *agrA*. (d) Deletion in *agrC*.

an insertion or deletion of an adenine in a string of seven adenines near the C terminus. The six-adenine mutation (6A) is predicted to add 21 amino acids and the eight-adenine mutation (8A), three. A second TAATAA stop sequence was brought into frame by the *agrA*-8A mutation. The run of seven adenines and the additional stop sequence was conserved in all four *agr* groups (GenBank accession numbers: Group I, CP000046; Group II, BA000018; Group III, BX571857; and Group IV, DQ229853, sequenced for this paper). The third mutation in *agrA* was a 10 bp central deletion of nucleotides 473–482 that is predicted to truncate the protein at residue 172. Two non-haemolytic variants were present in the patient 28 series – one with a complementable 11 nucleotide deletion in *agrC* (1105–1115), causing a frameshift in the histidine kinase domain, and one with the wild-type *agr* sequence.

The various *agr* mutations from patient 60's cultures were isolated at different times, as shown in Fig. 6. The *agrA*-8A mutation was present in at least one colony on days 5, 9, and 11; the other mutants were each present in one culture only. The culture from day 5 contained three different mutants, *agrA*-8A, *agrA*-6A and *agrA*-del, those from days 9 and 11 had only the *agrA*-8A mutant, while the strain from day 10 had the *agrC*-T399P mutation and the *agrA*-8A mutation. Wild-type strains and *agrA*-8A mutants were present throughout. As noted, these differences in the composition of blood cultures are probably a result of sampling variation. Alternatively, the singly occurring mutants may have been released from the endocarditic vegetations only briefly.

The RNAIII transcription patterns and exoprotein patterns for the *agrA*-8A and *agrA*-6A strains have been presented elsewhere (Traber & Novick, 2006). Briefly, transcription of RNAIII in the *agrA*-8A strain was delayed when compared with the *agr* wild-type strain. In the *agrA*-6A strain, RNAIII is not detectably transcribed, suggesting that *agrA* is inactive. Since the C-terminal end of proteins homologous to AgrA is critical for DNA binding (Koenig *et al.*, 2004) we

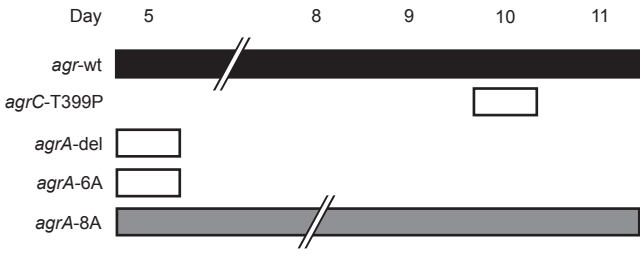

Fig. 6. Timing of the appearance of *agr* mutations in isolates from patient 60.

suggest that the added amino acids may have compromised DNA-binding activity; alternatively, the mutant proteins may be unstable. The *agrA*-8A mutation had an exoprotein profile that more closely resembled the completely defective *agrC*-T399P mutant than the *agr*-wt isolate, perhaps owing to its delayed *agr* activation, as seen with RN9774.

Genotypic stability

Having the wild-type parent of naturally occurring agr^- mutants enabled a test of the frequency of agr^- mutations in a known genetic background and thus a test of the frequency of such mutations occurring during post-isolation handling. This test, using one of the agr^+ isolates from patient 60, involved repeated subculturing of 48 h broth cultures, in which the bacteria were in deep stationary phase. Cultures were plated for single colonies on SBA + RN4220 supernatant, and then diluted 100-fold and incubated again for 48 h. Approximately 500 colonies were scored for each subculture. Non-haemolytic colonies began to appear after three subcultures, representing about 20 generations of growth and three stationary-phase challenges. The non-haemolytic portion increased to 20 % after four subcultures, and to >99 % after five. While this result is consistent with the occurrence and increased fitness *in vitro* of agr^- mutants, it effectively rules out the possibility that the agr^- mutants present in the mixed cultures from the patients represent post-isolation mutations, because the number of generations required for the appearance of *agr* mutations is far greater than could possibly have been experienced by these isolates during handling. Since there is no reason to suspect that the *agr* locus in the agr^+ isolate from patient 60 is different in stability from that in the other isolates, this result supports the conclusion that the mutations identified occurred before or during the infections rather than afterwards during handling. For the four isolates with complementable *agr* mutations, we tested for the stability of the mutations. For one of the strains, it was possible to obtain a PCR product for the mutated region of the *agr* locus directly from the frozen clinical specimen. For two of the other three, we were unable to obtain a PCR product and resequenced the *agr* region using a new primary subculture. For all three strains, the same mutation was identified as that reported above (not shown). For the fourth strain, the primary culture was lost.

DISCUSSION

In this report, we have shown that non-haemolytic *S. aureus* variants arise and survive in clinical material and are, in at least one case, almost certainly not the result of post-isolation mutations. We conclude that agr^- strains are involved in clinical disease.

In the series reported here, some 15 % of isolates were non-haemolytic. These occurred in *agr* groups I, II and III. A few of the non-haemolytic isolates had complementable mutations in *agrA*, *B* and *C*. Some non-haemolytic strains with *agr* mutations were not complementable by *agr* clones, suggesting that genotypic factors outside the *agr* locus were responsible, and that the observed *agr* mutations were secondary and adventitious. There were also a few non-haemolytic isolates with a wild-type *agr* sequence, suggesting the existence of genes upstream of *agr* in the exoprotein pathway, and required for *agr* expression. The identity of any such genes remains to be determined, and experiments addressing this are in progress.

Among the non-haemolytic strains, RN9774 had an apparently normally functioning *agr* locus, as revealed by its ability to activate an *agr*P3-*lux* fusion as well as to transcribe RNAIII. Although this strain could have mutations in *hla*, accounting for its inability to produce α-haemolysin, as has been described previously (O'Reilly *et al.*, 1990), its failure to produce δ-haemolysin is more difficult to explain, since it has the wild-type RNAIII/*hld* sequence. One possibility is that it expresses *agr* late in the overall growth curve, which has previously been shown to result in a lack of δ-haemolysin translation (Traber & Novick, 2006). We conclude that haemolysin production, which has been commonly used as an indicator of *agr* functionality, is usually but not always accurate when examining clinical isolates.

We believe that the mixed cultures we obtained from these blood cultures were representative of a mixture in the patient rather than a post-isolation mutation for the following reasons. First, we obtained the wound isolates directly from the infected material retrieved from the patient, leaving little opportunity for mutations to occur. Indeed, when we monitored the appearance of agr^- mutants in an *agr*-wt strain, although such mutants appeared it took three 48 h passages before they became detectable.

Additional support came from further characterization of isolates from patients for whom we had at least two separate specimens. For patient 28, both cultures were mixed; for patient 60, three were mixed, three were positive and one was negative. The evidence that a mixture was present in patient 60, rather than representing post-isolation mutations, is especially compelling, because in addition to mixed cultures, we obtained a positive and a negative culture on the same day. Finally, we performed single-colony analysis of these specimens multiple times on multiple days. Each specimen contained a reproducible ratio of haemolytic to non-haemolytic organisms. Taken in sum, these data strongly suggest that although there is a low level of instability of the *agr* locus in these strains, as evidenced by the appearance of agr^- mutants during long-term growth in broth, the clinical specimens described here clearly represent the occurrence and persistence of mixed infections.

The results described above confirm recent reports that agr^- and other exoprotein-defective variants occur and

persist in clinical material (Fowler *et al.*, 2004; Sakoulas *et al.*, 2002), and extend these observations to include skin and soft tissue infections, pneumonia and osteomyelitis. However, since *agr*-negative variants arise commonly *in vivo* as well as *in vitro*, the occurrence of such organisms in clinical material does not mean that they actually initiated the infection. In fact, *agr*-negative strains have not been shown to initiate infections, and it is suggested that they may be genotypic dead-ends in the ecology of the organism. In this connection, it is noted that *agr* expression seems to be important during the early stages of an infection (Wright *et al.*, 2005b), although it is expressed poorly, if at all, during later stages, both in experimental models and in material from chronic human infections such as the cystic fibrosis lung, owing either to metabolic depression or to mutations (Goerke *et al.*, 2000). These ideas have important implications for the natural history of staphylococcal infection and are currently under study.

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