Coagulase deficiency in clinical isolates of *Staphylococcus aureus* involves both transcriptional and post-transcriptional defects

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Summary. The molecular basis of the non-expression of coagulase was investigated for 14 coagulase-negative isolates of *Staphylococcus aureus* obtained from different clinical samples. These isolates had typical *S. aureus* characteristics such as production of clumping factor, DNAase and protein A, but, with one exception, failed to produce detectable amounts of α-haemolysin. All 14 strains had DNA homologous to the coagulase gene (coa), but a coa-specific transcript was found in only seven of them. α-Haemolysin mRNA was detected in only eight strains without direct correlation to coa-mRNA expression. Thus, coagulase and α-haemolysin deficiencies in *S. aureus* may involve either transcriptional or post-transcriptional alterations although additional regulatory factors may influence the expression of both genes.

Introduction

*Staphylococcus aureus*, an important human pathogen, is usually identified by the presence of either staphylocoagulase or fibrinogen affinity factor (clumping factor). Although most strains of *S. aureus* produce both these proteins, some pathogenic isolates may lack one or the other. Coagulase-negative staphylococci are still often considered to be incidental contaminants, and many laboratories do not identify such strains further; therefore, atypical coagulase-negative *S. aureus* strains in clinical specimens may be misidentified, leading to inappropriate therapeutic decisions.

Staphylocoagulase is an extracellular protein produced by most strains of *S. aureus*. It reacts specifically with prothrombin in a stoichiometric process resulting in the formation of staphylothrombin which can convert fibrinogen into fibrin in a way similar to the physiological effect of thrombin and is detected by its ability to induce coagulation in rabbit plasma.

Failure to detect coagulase activity may result from technical insufficiencies. An inoculum of at least 10⁶ cfu/ml is required for a reliable positive result in the standard tube test for coagulase and, since *S. aureus* does not grow in rabbit plasma, longer incubation times do not compensate for smaller inocula. Temperature, humidity and CO₂ concentration have been shown to influence coagulase expression, and different batches of rabbit plasma can differ in sensitivity. On the other hand, false positive reactions due to prothrombin activation without staphylothrombin formation can occur. They are caused by proteases or pseudo-coagulase and are generally inhibited by EDTA. Once these technical difficulties have been resolved, there remains a number of isolates with a typical phenotypic and pathological profile of *S. aureus*, except that they produce no detectable staphylocoagulase.

The coagulase gene (coa) is expressed preferentially during exponential growth and can be repressed by a regulatory element designated agr. This regulatory gene down-regulates coagulase and protein A, and up-regulates most other exoproteins including α-haemolysin, enterotoxins B, C and D, toxic shock syndrome toxin (TSST)-1, and others. Another regulatory locus (sar) affects exoprotein expression in *S. aureus*, and sar mutants produce low amounts of coagulase but over-produce α-haemolysin. Mutation at either of these loci could, theoretically, give a coagulase-negative phenotype. A third regulatory locus, xpr, has been described but it does not influence coa expression. None of these regulatory loci would
be expected to produce the coagulase-negative, α-haemolysin-negative, protein A-positive phenotype typical of the isolates described in this study.

Materials and methods

Bacterial strains and characterisation

Fourteen coagulase-negative isolates of *S. aureus* were collected from various clinical sources including blood cultures, pus, bronchoalveolar lavage fluid, sputum and cerebrospinal fluid by eight different laboratories in France and one in England. They were identified by a gallery of biochemical tests (API ID32 Staph, bioMérieux, Marcy-l'Étoile, France). They were tested for fibrinogen affinity factor (clumping factor) by a haemagglutination test (Staphylidise, bioMérieux), thermostable DNAase by a DNA-toluidine blue agar (Diagnostic Pasteur, Marnes-la-Coquette, France), protein A by a haemagglutination test (AureA kit, bioMérieux) and the presence of specific *S. aureus* tRNA by DNA-RNA hybridisation (Accuprobe System, Gen-Probe, Biotechnie, Rungis, France). Phage typing was done with the international basic set of 23 *S. aureus* bacteriophages. Enterotoxins A, B, C and D and TSST-1 production were detected by ELISA procedures. Culture supernates from post-exponential phase culture (6 h after inoculation) were assayed for α-haemolysin production on rabbit erythrocytes as described previously. Titres were expressed as haemolytic units (HU)/mg (dry weight) of cells, and compared to that of *S. aureus* Wood 46 as positive control. Susceptibility to a range of anti-staphylococcal antibiotics, including oxacillin, was determined for all strains by the standard Kirby-Bauer disk diffusion method.

*S. aureus* 8325-4 from which the coagulase gene has been cloned and sequenced (table I), and four coagulase-positive isolates of *S. aureus* from clinical specimens served as positive controls for coagulase tests. Six coagulase-negative staphylococci (two *S. epidermidis*, two *S. haemolyticus*, one *S. lugdunensis* and one *S. schleiferi*) provided the negative controls. Other bacterial strains and plasmids used are listed in table I.

Measurement of coagulase activity

The tube coagulase test was performed in duplicate with different batches of rabbit coagulase plasma (bioMérieux and Difco) with either citrate or EDTA as anticoagulant. The test was performed by mixing 0.1 ml of an overnight culture in brain heart infusion broth with 0.5 ml of reconstituted plasma, incubating the mixture at 37°C for 4 h and observing the tube for clot formation. Coagulase titration was performed as described by McDevitt et al., i.e., staphylococci were grown overnight in brain heart infusion broth and the culture was centrifuged at 5000 rpm for 15 min.

Coagulase activity was assayed by adding 0.5 ml of serial two-fold dilutions of culture supernate in PBS to 0.5 ml of rabbit plasma (bioMérieux) diluted 1 in 3 in distilled water. The titre was the reciprocal of the highest dilution of the supernate that showed evidence of clotting after incubation for 18 h at 37°C.

Total DNA extraction and Southern blotting

Whole-cell DNA was isolated from staphylococci according to a standard procedure. HindIII restriction endonuclease digests were prepared according to the manufacturer’s instructions (Boehringer Mannheim, Meylan, France). DNA fragments were separated by agarose gel electrophoresis, vacuum-transferred to positively charged nylon membranes (Boehringer Mannheim) and cross-linked by UV light.

Total RNA extraction and Northern blotting

Whole-cell extracts from cultures grown to exponential phase in brain heart infusion broth were prepared as described previously and used as the source of RNA. Samples containing total RNA from 7.5 x 10⁷ cells were separated by electrophoresis on agarose 1% gels containing 2.2 M formaldehyde. The nucleic acids were vacuum-transferred to nylon membranes (Boehringer Mannheim) and cross-linked by UV light. Before hybridisation, the positions of the staphylococcal 16 and 23 S rRNA were marked on the filter for use as size markers.

DNA and RNA probes

An 871-bp EcoRI-HindIII fragment, internal to the coa-gene, was isolated from pCOA54 (table I) and labelled with digoxigenin-11-dUTP by random priming according to the manufacturer’s instruction (Boehringer Mannheim). For Northern blot hybridisation, this fragment was subcloned in pBluescript II KS +/−. This plasmid was designated pLUG12 (table I). A coa-specific RNA probe was synthesised by in-vitro T3 polymerase transcription of pLUG12 with digoxigenin-labelled UTP (Boehringer Mannheim) as substrate. An hla probe was a 0.7-kb ClaI fragment internal to the hla structural gene, isolated from pDU1150, a pBR derivative containing the entire hla gene (kindly provided by T. Foster). This fragment was radiolabelled with [α-32P]dATP by random priming.

Hybridisation

Hybridisations were performed under stringent conditions at 68°C in 5 x SSC (Southern blots) or at 42°C in 5 x SSC plus formamide 50% v/v (Northern blots). Detection was performed with the DIG kit (Boehringer Mannheim) according to the manufacturer’s
Table I. Reference strains and plasmids used in this study

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<th>Strain</th>
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<th>Relevant genotype or phenotype</th>
<th>Source or origin</th>
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<td>8325-4</td>
<td>coa+ agr+, NCTC 8325 cured of</td>
<td>R. Novick&lt;sup&gt;22&lt;/sup&gt;</td>
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Table II. Phenotypic and genotypic characters of 14 coagulase-negative isolates of S. aureus

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<th>Fibrinogen affinity factor</th>
<th>Protein A</th>
<th>Thermostable DNAase</th>
<th>Toxins</th>
<th>Haemolysin HU/mg†</th>
<th>HindIII digest coa-hybridising band (in kb)</th>
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*Coagulase titre was 17749 for the control strain S. aureus 8325-4.
†Haemolytic titre of strain Wood 46 was 207 HU/mg; w, weak hybridisation signal.

instructions. The chemoluminescence was generated by hydrolysis of Lumigen PPD substrate—4-methoxy-4-(3-phosphatheyphyl)spiro(1,2-dioxetane-3,2'-adamantane)—by alkaline phosphatase conjugated to anti-digoxigenin antibodies (Boehringer Mannheim). Alternatively, Northern blot hybridisation with the radiolabelled hla probe was performed as described previously.<sup>10</sup> The staphylococcal rRNAs were used as size markers and internal controls in all Northern blots.

Results

The 14 coagulase-negative isolates were identified as S. aureus by the biochemical gallery with a percentage of identification > 99%. This was confirmed by S. aureus rRNA-specific probing, which was positive for all strains. Eight strains were typable with a conventional set of S. aureus phages, yielding seven different patterns. Only three isolates were resistant to oxacillin and to several other antibiotics. The tube coagulase test was negative for all strains with different batches of both the bioMérieux and Diço rabbit plasma. Coagulase titres were < 2 for 13 strains and 4 for one strain compared with 17749 for the control strain 8325-4 (table II). The results of additional tests are summarised in table II, showing that most of the strains produced fibrinogen affinity factor, protein A and thermostable DNAase. Several strains also produced one of the following toxins: enterotoxin A, B or TSST-1 (table II). Haemolytic titration revealed that 13 strains gave haemolytic titres ≤ 30 HU/mg (table II). One strain had a titre of 128 HU/mg versus 207 HU/mg for the control strain Wood 46.

Southern blot hybridisation with the coa probe

All S. aureus strains tested, including those deficient in coagulase production, gave a positive hybridisation signal with the coa-probe (fig. 1). A single HindIII restriction fragment of 7 kb for S. aureus 8325-4, of 1.3, 7 or 9.2 kb for two, three and nine coagulase-negative S. aureus isolates, respectively, (table II) and of 7 or 9.2 kb for the control coagulase-positive strains was recognised. No hybridisation was detected with the control coagulase-negative strains (S. epidermidis, S. haemolyticus, S. lugdunensis and S. schleiferi).
Fig. 1. Southern blot hybridisation of total DNA from representative isolates of coagulase-negative S. aureus and from the control strain S. aureus 8325-4. Total DNA was cleaved with HindIII. Samples were hybridised with the 871-bp digoxigenin-11-dUTP-labelled EcoRI/HindIII fragment of the coa gene.

Fig. 2. Northern blot analysis of whole-cell RNA from representative isolates of coagulase-negative S. aureus and from the control strain S. aureus 8325-4. Samples containing total RNA from 7.5 x 10^7 cells in early exponential phase of growth were analysed by Northern blot hybridisation with the in-vitro synthesised coa probe (see Materials and methods).

Northern blot hybridisation with the coa and hla probes

Samples collected from early exponential phase cultures were Northern blotted with an in-vitro-synthesised digoxigenin-labelled RNA probe and showed the presence of a specific transcript in coagulase-positive S. aureus (fig. 2, S. aureus 8325-4) and in seven of the 14 coagulase-negative isolates of S. aureus tested (table II); two strains with positive transcripts and four with negative results are shown in fig. 2. In some cases, the transcript appeared shortened
as compared with controls (fig. 2, strain A900201). As most of our coagulase-negative isolates of *S. aureus* were also low α-haemolysin producers, and α-haemolysin is expressed post-exponentially, samples from post-exponential phase cultures of the same strains were probed with the *hla* probe (table II). The *hla*-specific transcript was detected in five of the seven *coa*-mRNA producers and in three of the seven *coa*-mRNA-negative strains (table II). It should be noted that strain A900361 had no detectable *hla* transcript but showed weak haemolytic activity (18 HU/mg, table II), that could be caused by a haemolysin other than the α-haemolysin.

**Discussion**

Despite their lack of coagulase and a low or undetectable level of α-haemolysin production, the 14 isolates studied here, appear to be authenticated as *S. aureus* by various biochemical and genetic tests and by the clinical context of their isolation. No accumulation of associated mutations was observed. They all carried at least a large part of the *coa* gene, as Southern blot hybridisation with a probe derived from the highly conserved region of the three coagulase genes sequenced was positive in all cases. A *Hind*III length polymorphism occurs in the *coa* region, and the fragment sizes seen in the majority of our samples were the same as those of the control strains (7 and 9-2 kb), although two isolates had a shorter 1.3-kb fragment (fig. 1, table II).

Defects at different levels of *coa* expression are presumably present in the different strains since Northern blotting showed the presence of a *coa*-specific transcript in only seven of 14 coagulase-negative isolates. Among these seven strains, the weak coagulase activity detected in strain A880130 (table II) may be due to mutation in the active domain of the protein or to the effect of pseudo-coagulase or proteases as has been described by Wegynowicz et al.

For the other six strains that showed a *coa*-mRNA transcript and no coagulase activity, a translational defect is the most probable explanation, and preliminary experiments by Western blotting of culture supernates favour this hypothesis, as none of these strains showed a coagulase-specific band with antisera against two of the eight coagulase serotypes (unpublished results). No detectable *coa* mRNA was present in the other cases, suggesting faulty transcription.

The possibility that coagulase and α-haemolysin deficiencies were linked by a common mechanism was investigated by Northern blotting with the *hla* probe. The *hla*-specific transcript was detected in eight strains only, without direct correlation between the presence of this transcript and that of the *coa* transcript (table II), suggesting that coagulase and α-haemolysin deficiencies were independent phenomena. Nevertheless, α-haemolysin deficiency is unusual, as 85-95% of *S. aureus* strains from clinical specimens produce this haemolysin. As the 14 coagulase-negative isolates of *S. aureus* were selected solely on the basis of coagulase deficiency, the associated defect in α-haemolysin production must have arisen from unknown selective pressures leading to this double deficiency. Furthermore, the absence of *hla* transcript in two strains producing TSST-1 (strains A7144 and A900293) (table II) should be noted since an *hla*-translational defect has been described regularly in such strains.

Three regulatory elements are known to affect transcription of exoprotein genes in *S. aureus*—*xpr*, *sar* and *agr*. Coagulase expression is not reduced in *xpr* mutants. None of our strains showed the over-expression of α-haemolysin typical of *sar* mutants, indeed they all had haemolysin titres below that of the control strain (Wood 46) and showed transcription deficiencies in six of the 14 strains. Furthermore, the undetectable level of coagulase activity of our isolates would be unusual in *sar* mutants that produce low but detectable amount of coagulase. The expression of *agr* RNAIII results in down-regulation of coagulase and protein A, so over-expression of *agr* could result in a coagulase-negative phenotype. As protein A is expressed normally in most of our strains (12 of 14) and α-haemolysin is not over-produced, the possibility of repression of *coa* by over-expression of *agr* is unlikely.

The phenomenon described here indicates that *S. aureus* strains may have multiple independent mutational defects in individual exoprotein genes such as in *coa* and *hla*, rather than pleiotropic regulatory mutations. In addition to the dissociation between coagulase and α-haemolysin transcription, the differences between strains in phenotypic characters such as phage type, enterotoxin and TSST-1 production, make it improbable that they could have a recent common origin. Such phenotypes may be expected to re-occur in other circumstances.

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**References**


