Recombination at the coagulase locus in *Staphylococcus aureus*: plasmid integration and amplification

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The integrating plasmid pCOA18, comprising pUC18 linked to a mutated coagulase (coa) gene from *Staphylococcus aureus*, and constructed by substituting coa sequences with a tetracycline (Tc)-resistance marker (Δcoa::Tc'), was transformed into *Staphylococcus aureus* RN4220, where it underwent recombination with the chromosomal coa locus. Allele-replacement mutants were recovered at a low frequency directly after transformation. The majority of transformants carried pCOA18 integrated in the chromosome by a single Campbell-type recombination event. The majority of integrants contained tandem repeats of pCOA18 and expressed high levels of resistance to Tc (>30 μg ml⁻¹) compared to the single-copy integrants and allele-replacement mutants (15 μg ml⁻¹). Transduction of a single-copy integrant to a Coa+ recipient allowed the cointegrant to be resolved and allele-replacement recombinants to be selected. In addition, growth of a single-copy integrant on high concentrations of Tc (>30 μg ml⁻¹) selected for amplified derivatives at a frequency of It was estimated that up to 19 copies of pCOA18 could occur in a tandem array in the chromosome.

**Introduction**

The isolation of site-specific mutations in genes coding for putative virulence factors has provided new opportunities to analyse the virulence mechanisms of bacteria (Foster, 1992; Finlay, 1992). In *Staphylococcus aureus*, allele-replacement mutagenesis and transposon mutagenesis with Tn.551 (Pattee, 1981) and Tn916 (Jones et al., 1987) are commonly used methods for isolating site-specific mutations (Foster & McDevitt, 1992). These transposons insert into target DNA sequences essentially at random by illegitimate recombination whereas mutations in chromosomal genes can be specifically targeted by homologous recombination events such as directed plasmid insertion or allele-replacement with in-vitro-constructed mutant alleles.

Several procedures based on the instability of plasmid vectors have been employed to isolate allele-replacement mutants in *S. aureus*. A shuttle plasmid carrying the mutated allele was introduced into *S. aureus* along with a second incompatible plasmid (O'Reilly et al., 1986; Patel et al., 1987; Phonimdaeng et al., 1990). The incompatible plasmid was selected by growth in selective liquid media for 100–200 generations. In this way, the shuttle plasmid was eliminated from the majority of cells in the population and rare recombinants with replacement mutations in the chromosomal locus were detected.

Temperature-sensitive plasmids and integrating plasmid vectors have been used recently to deliver mutated alleles for allele-replacement (Sloane *et al.*, 1991; McDevitt *et al.*, 1992). Temperature-sensitive plasmids can be eliminated by growth at restrictive temperatures while integrating vectors lack a functional origin of replication and so will be lost during the growth that occurs after the plasmid is introduced by transformation. Integrating plasmids can be used for mutagenesis only if the frequency of plasmid transformation is high. Protoplast transformation in *S. aureus* is often not sufficiently reliable to use integrating plasmids. Recently, plasmid transformation frequencies of 10⁵ per μg of DNA have been achieved by electroporation (Oskouian & Stewart, 1990). This has facilitated the use of integrating plasmids in *S. aureus* (McDevitt *et al.*, 1992).

This paper reports experiments with an integrating vector carrying a deletion-substitution mutation in the coagulase gene (Δcoa::Tc') of *S. aureus*. The objective was to determine if insertions of the plasmid into the coa locus or allele-replacement mutants could be directly isolated following transformation by electroporation. The majority of plasmid integrants obtained after
Table 1. *Staphylococcus aureus* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Relevant phenotype</th>
<th>Properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newman</td>
<td>Coa⁺</td>
<td>High level of coagulase</td>
<td>Duthie &amp; Lorenz (1952)</td>
<td></td>
</tr>
<tr>
<td>RN4220</td>
<td>Coa⁺</td>
<td>Mutant of 8325-4 capable of stably maintaining shuttle plasmids</td>
<td>Kreiswirth et al. (1983)</td>
<td></td>
</tr>
<tr>
<td>DU5857</td>
<td>coa::pCOA18⁻</td>
<td>Coa⁺ Te⁻</td>
<td>Amplified pCOA18 cointegrate of RN4220</td>
<td>This study</td>
</tr>
<tr>
<td>DU5861</td>
<td>Δcoa::Te⁻</td>
<td>Coa⁻ Te⁻</td>
<td>Allele-replacement mutant of RN4220</td>
<td>McDevitt et al. (1992)</td>
</tr>
<tr>
<td>DU5862</td>
<td>coa::pCOA18⁻</td>
<td>Coa⁻ Te⁻</td>
<td>Amplified pCOA18 cointegrate of RN4220 with deletion in coa</td>
<td>This study</td>
</tr>
<tr>
<td>DU5863</td>
<td>coa::pCOA18</td>
<td>Coa⁺ Te⁺</td>
<td>Single-copy pCOA18 integrant of RN4220 on left-hand side of coa</td>
<td>This study</td>
</tr>
<tr>
<td>DU5864</td>
<td>coa::pCOA18</td>
<td>Coa⁺ Te⁺</td>
<td>Single-copy pCOA18 integrant of RN4220 on right-hand side of coa</td>
<td>McDevitt et al. (1992)</td>
</tr>
<tr>
<td>DU5865</td>
<td>coa::pCOA18⁻</td>
<td>Coa⁺ Te⁺</td>
<td>Amplified pCOA18 integrant derived from DU5864. Coagulate amplified</td>
<td>This study</td>
</tr>
<tr>
<td>DU5881</td>
<td>coa::pCOA18⁻</td>
<td>Coa⁺ Te⁺</td>
<td>Amplified pCOA18 integrant derived from DU5864</td>
<td>This study</td>
</tr>
<tr>
<td>DU5884</td>
<td>coa::pCOA18⁻</td>
<td>Coa⁺ Te⁺</td>
<td>Amplified pCOA18 integrant derived from DU5881</td>
<td>This study</td>
</tr>
<tr>
<td>DU5885</td>
<td>coa::pCOA18⁻</td>
<td>Coa⁺ Te⁺</td>
<td>Amplified pCOA18 integrant expressing highest level of resistance to Te. Derived from DU5881</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table 2. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Host</th>
<th>Markers</th>
<th>Relevant properties</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCW59</td>
<td><em>S. aureus</em></td>
<td>Cm⁺ Tc⁺</td>
<td>Contains a gene for Tc⁺ on a 2.35 kb HindIII fragment</td>
<td>Wilson et al. (1981)</td>
</tr>
<tr>
<td>pBluescript</td>
<td><em>E. coli</em></td>
<td>Ap⁺</td>
<td>Cloning vector</td>
<td>Short et al. (1988)</td>
</tr>
<tr>
<td>pCOA14</td>
<td><em>E. coli</em></td>
<td>Ap⁺</td>
<td>5.4 kb <em>Kpn</em>I coa fragment in <em>pUC19</em></td>
<td>Phonimdaeng et al. (1988)</td>
</tr>
<tr>
<td>pCOA18</td>
<td><em>E. coli</em></td>
<td>Ap⁺ Tc⁺</td>
<td>pBluescript with a 5.4 kb <em>Kpn</em>I fragment carrying the Δcoa::Te⁺ mutation</td>
<td>McDevitt et al. (1992)</td>
</tr>
</tbody>
</table>

transformation unexpectedly carried tandem multimers of the plasmid in the *coa* locus. Campbell integrants could be resolved by transduction to yield replacement mutants.

**Methods**

**Bacterial strains and plasmids.** The bacterial strains and plasmids are listed in Tables 1 and 2 respectively.

**Bacterial growth media, antibiotics and chemicals.** *Escherichia coli* strains harbouring plasmids were routinely grown in L-broth and L-agar (Miller, 1972). Ampicillin (100 µg ml⁻¹) was incorporated as appropriate. *S. aureus* strains were grown in Trypticase soy broth or agar incorporating the antibiotic tetracycline (Tc) at concentrations from 2-100 µg ml⁻¹ where appropriate. Laboratory chemicals used were of analytical grade obtained from Sigma or were the best grade available from BDH.

**Introduction of plasmid DNA into S. aureus by electrotransformation.** Cells were grown for 16 h in TSB at 37 °C and diluted in 100 ml of fresh
TSB at 37 °C until the OD₅₆₀ had reached 0.2. Cells were collected by centrifugation at 10000 g and washed twice with ice-cold 0.5 M sucrose. The cells were then resuspended in 1 ml of ice-cold 0.5 M sucrose and incubated on ice for 15 min. The cells were then centrifuged and resuspended in 1 ml of ice-cold 5 M sucrose. Cells (100 µl) and DNA at a concentration of 1 µg ml⁻¹ were mixed in a sterile microfuge tube and incubated on ice for 1 min. The mixture was transferred to a chilled cuvette of a Bio-Rad Gene Pulser and samples were electroporated at 2.5 kV, 25 µF capacitance with resistance varying from 200-600 Ω. The cells were then removed from the cuvette, diluted in sterile TSB and plated on TSA containing the appropriate selective drug. This procedure was adapted from Oskouian & Stewart (1990).

Manipulation of DNA. DNA-modifying enzymes were purchased from Boehringer or Promega and were used according to the manufacturers' instructions. DNA manipulations were performed using standard procedures (Sambrook et al., 1989; Ausubel et al., 1987). DNA hybridization was performed by the method of Southern (1975). S. aureus genomic DNA was purified by a modification of the method of Mekalanos (1983) as described by O’Reilly et al. (1986). Probe DNA was nick-translated (Rigby et al., 1977) by using [α-²³P]dATP (New England Nuclear).

Measurement of coagulase activity. Coagulase activity was determined by adding 0.5 ml of S. aureus culture supernatants diluted 1:2 and 0.5 ml of a 1:3 dilution in PBS of rabbit plasma. The expression of coagulase resulted in clot formation after 24 h incubation at 37 °C (Anderson et al., 1982).

Transduction. Transduction in S. aureus was performed by the method of Asheshov (1966) by using bacteriophage 85.

Amplification of the single-copy integrant strain DU5864. In order to isolate amplified derivatives of strain DU5864, cultures were grown in 1 µg Tc ml⁻¹ to induce the Tc resistance determinant and dilutions were plated on TSA incorporating Tc at concentrations from 50-200 µg ml⁻¹. Colonies were isolated from the plate containing 50 µg Tc ml⁻¹ and they were maintained on plates containing 30 µg Tc ml⁻¹. The amplified strain DU5881 was plated on Tc concentrations ranging from 100-400 µg ml⁻¹. Colonies grew on concentrations of Tc up to 120 µg ml⁻¹. Several of these were picked and maintained on agar containing 50 µg Tc ml⁻¹.

Estimation of copy number using densitometry. The relative intensities of the bands on autoradiograms were determined by scanning each lane with a CD-50 chromatogram-densitometer (Shimadzu). The densitometer was set to measure reflectance at 600 nm and the scanning window was set to cover an entire band. The different heights of the three peaks observed for the single-copy integrant reflected the homology that each fragment had with the pCOA14 probe. When calculating the copy number, this was taken into account by dividing the peak height of the 5.4 kb KpnI fragment by that of the 2.5 kb KpnI fragment for the single-copy integrant. A ratio of 1/9 was obtained. It was assumed that this ratio was maintained in all samples. In the amplified strains, the peak height of the amplified 2.5 kb KpnI fragment was multiplied by 1.9 and then divided by the corresponding peak height of the single-copy 5.4 kb KpnI fragment.

Measurement of tetracycline resistance. The efficiency of plating (e.o.p.) on Tc medium was determined with cultures that had been grown in 1 µg Tc ml⁻¹. Dilutions of the broth culture were spread on plates containing 30 µg Tc ml⁻¹. The e.o.p. was defined as the ratio of colonies growing at 30 µg ml⁻¹ compared to drug-free agar.

For IC₅₀ determinations, cultures were grown overnight in 1 µg Tc ml⁻¹ to induce expression of resistance and were then diluted to 10³ cells ml⁻¹ and 100 µl of each strain was plated onto agar containing Tc in the range 5-200 µg ml⁻¹. The plates were incubated at 37 °C for 18 h. The arbitrary end-point was defined as the concentration of drug at which there was reduction in colony size of about 50%.

Results
Transformation with the integrating plasmid pCOA18
Plasmid pCOA18 comprises the 2.95 kb E. coli vector plasmid pBluescript carrying the coa locus of S. aureus 8325-4 with a deletion-substitution mutation – a 2.35 kb insert carrying a Tc gene from the S. aureus plasmid pCW59 replaces the coa gene (McDevitt et al., 1992). Transduction in S. aureus by electroporation and to study the structure of the recombinants. It was anticipated that the majority of the Tc' transformants would contain the plasmid integrated in the coa locus by a single cross-over (Campbell-type integration) and that double recombination causing allele-replacement with Δcoa::Tc' might be detected at a lower frequency.

Plasmid pCOA18 DNA was transformed into S. aureus strain RN4220 by electroporation selecting for Tc resistance. The frequency of transformation was 1×10³ per µg DNA. A control experiment with pCW59 DNA gave 2×10³ transformants per µg DNA. Fifty Tc' transformants were purified and tested for their level of resistance to Tc and for their expression of coagulase. Representatives were analysed by transduction and by Southern hybridization.

Phenotype analysis of pCOA18 transformants
Fifty Tc' transformants of strain RN4220 were tested for the expression of coagulase by the tube test and only two were negative (Table 3). One of these (strain DU5861) expressed high-level resistance such as DU5862 (strain DU5862) expressed a higher level of resistance. However, when the low-level resistant strains were plated on 30 µg Tc ml⁻¹, colonies grew at a frequency of 1×10⁻³. In contrast, Tc' transformants which expressed high-level resistance such as DU5862 grew with 100% efficiency at 30 µg Tc ml⁻¹.

Transduction analysis
Bacteriophage 85 was propagated on representatives of each of the phenotypic groups described above. The ability of the phage to transduce Tc resistance to the coagulase-proficient strain Newman was evaluated.
were found among the 50 tested and 19/31 continued to isolated directly after transformation. The frequency of single-copy integrants, which express low level Tc'. In one case (donor strain DU5857), no Coa- transductants fold higher than for phage propagated on strains such as DU5861 (allele replacement) or DU5863 and DU5864. Transduction of Tc' into strain Newman was about 100-fold higher than for phage propagated on the Coa- strain DU5864, while strain DU5857 was that expressed high-level Tc'. One (strain DU5865) was a Coa- transformant of strain Newman that was selected from the low-level Tc' putative Campbell allele-replacement mutation (Sloane et al., 1991). Indeed, six of 31 Tc' transductants which were tested were Coa-*. This demonstrated that the Coa- mutation of this strain was not caused by the Tc' marker.

The low-level Tc' Coa+ transformants (DU5863 and DU5864) were probably derived from an integration of the pCOA18 plasmid in the coa locus of the chromosome by a Campbell-type recombination event. It has been reported that transduction of an integrated plasmid carrying an insertion mutation in a chromosomal gene and selection for the marker associated with the mutated gene can promote plasmid loss and the generation of an allele-replacement mutation (Sloane et al., 1991). Indeed, six of 31 Tc' transductants of strain Newman that were selected from lysates of bacteriophage 85 propagated on strain DU5864 were Coa-. Each of these expressed low-level Tc' and when plated on 30 μg Tc ml-1 no colonies grew.

Transducing phage was propagated on two strains that expressed high-level Tc'. One (strain DU5865) was selected from the low-level Tc' putative Campbell insertion strain DU5864, while strain DU5857 was isolated directly after transformation. The frequency of transduction of Tc' into strain Newman was about 100-fold higher than for phage propagated on strains such as DU5861 (allele replacement) or DU5863 and DU5864 (single-copy integrants), which express low level Tc'. In one case (donor strain DU5857), no Coa- transductants were found among the 50 tested and 19/31 continued to express high-level resistance (> 30 μg ml-1). However, with donor strain DU5865, 2/28 were Coa- and expressed low-level Tc' (15 μg ml-1) while the rest were Coa+ and expressed a higher level of Tc'.

Transformants were streaked on agar containing Tc and many showed a mixture of small and large colonies. The small colonies either gave a mixture of small and large colonies again upon restreaking or they could not be propagated. Large colonies formed a homogeneously sized population when restreaked. More than half the transductants selected and restreaked on 2 μg Tc ml-1 could grow on Tc concentrations above 30 μg ml-1.

### Southern hybridization analysis

Southern hybridization experiments were performed to investigate the structure of the coa loci in the transductants described above. Plasmids pCOA14 (which carries the wild-type strain 8325-4 coa gene on a 5.4 kb KpnI fragment in pUC19) and pCW59 (which contains the 2.35 kb HindIII Tc' fragment used to inactivate the coa gene in pCOA18) were nick-translation and used as probes.

Fig. 1 shows the results of hybridizing genomic DNA of the putative allele-replacement mutant of RN4220 strain DU5861. It also examines the properties of strain DU5857, a Coa+ transformant of RN4220 expressing high-level Tc' and when plated on 30 μg Tc ml-1 no colonies grew.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Resistance to Tc (IC₅₀, μg ml⁻¹)</th>
<th>No. of Coa⁺ transductants</th>
<th>Parental strain</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transformants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DU5861</td>
<td>15</td>
<td>1 x 10⁻⁸</td>
<td>RN4220</td>
</tr>
<tr>
<td>DU5862</td>
<td>50</td>
<td>1 x 10⁻⁶</td>
<td>RN4220</td>
</tr>
<tr>
<td>DU5863*</td>
<td>15</td>
<td>1 x 10⁻⁵</td>
<td>ND</td>
</tr>
<tr>
<td>DU5864*</td>
<td>15</td>
<td>1 x 10⁻⁵</td>
<td>ND</td>
</tr>
<tr>
<td>DU5857†</td>
<td>50</td>
<td>1 x 10⁻⁶</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Amplified derivatives</strong></td>
<td></td>
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</tr>
<tr>
<td>DU5865</td>
<td>50</td>
<td>10⁻⁷</td>
<td>DU5864</td>
</tr>
<tr>
<td>DU5881</td>
<td>50</td>
<td>10⁻⁷</td>
<td>DU5864</td>
</tr>
<tr>
<td>DU5884</td>
<td>80</td>
<td>10⁻⁷</td>
<td>DU5864</td>
</tr>
<tr>
<td>DU5885</td>
<td>125</td>
<td>10⁻⁷</td>
<td>DU5864</td>
</tr>
</tbody>
</table>

ND, Not done.

* Representative of the 6/50 Coa+ transformants expressing low-level Tc'.
† Representative of the 42/50 Coa+ transformants expressing high-level Tc'.

When Tc' transductants were selected with phage propagated on a low-level Tc' Coa⁻ transformant (strain DU5861), all of the five derivatives tested were Coa⁻ and expressed the same low-level Tc resistance as the parental strain. This suggested that strain DU5861 carries the Acoa : Tc' mutation rather than an integrated plasmid. In contrast, with phage propagated on the Coa- strain DU5862 none of the 8 Tc' transductants which were tested were Coa-. This demonstrated that the Coa⁻ mutation of this strain was not caused by the Tc' marker.
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3.05
2.95
2.5

Fig. 1. Southern hybridization analysis of strains RN4220, DU5857 (RN4220 amplified integrant) and DU5861 (RN4220 Δcoa::Tc'). Chromosomal DNA from strain RN4220 wild-type was cleaved with KpnI (lanes A and D) and with EcoRV (lane G). DNA from strain DU5857 was cleaved with KpnI (lanes B and E) and with EcoRV (lane H). Chromosomal DNA of strain DU5861 was cleaved with KpnI (lanes C and F) and with EcoRV (lane I). Samples in lanes A–C and lanes G–I were probed with labelled pCOA14 while samples in lanes D–F were probed with labelled pCW59. The DNA loadings in each lane were similar (about 10 µg).

which hybridized to the coa probe (Fig. 1, lane B), two of which also hybridized to the Tc' probe (Fig. 1, lane E). The fragments of 3.05 kb, 2.95 kb and 2.5 kb were more intense than fragments in strain DU5861, suggesting that they were present in multiple copies in strain DU5857.

The structures were investigated further by cleaving with EcoRV, an enzyme which cuts both within the Tc' insert and within sequences flanking the coa gene and the Δcoa::Tc' substitution in pCOA18 (Fig. 2). Fragments of > 15 kb and 3.65 kb hybridized when DNA of the parental strain RN4220 was probed with pCOA14 DNA (Fig. 1, lane G). Similar sized fragments were also visible in the strain DU5857 sample (Fig. 1, lane H). However, additional fragments of 6.75 kb and 1.73 kb were observed and these were more intensely labelled. The simplest explanation for these findings is that plasmid pCOA18 became integrated into the coa locus by crossing-over on the left of the coa gene, and the plasmid was subsequently amplified by tandem duplication (Figs 2 and 3), a process that probably occurs by unequal crossing-over during chromosome replication (Anderson & Roth, 1977). However, it is also possible that multimers of pCOA18 were transformed and became integrated in the chromosome. The amplified KpnI and EcoRV fragments are from within pCOA18 while the single copy 5.4 kb KpnI fragment and the 3.65 kb and > 15 kb EcoRV fragments were exclusively composed of coa DNA flanking the amplified region (Fig. 2). The
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**Fig. 2. Structure of the coa region in strains RN4220 Coa⁺, DU5861 (RN4220 Δcoa::Tc'), the single-copy integrant strains DU5864 (A) and DU5863 (B) and the amplified integrant strains DU5857 and DU5862.** The closed box represents the coagulase gene while the elevated line represents the inserted Tc' fragment that replaces the coa gene in Δcoa::Tc'. The double line represents vector plasmid sequences while the single line represents flanking coa sequences. The deletion present in strain DU5862 is indicated. In the single-copy integrant strain DU5864 (A), pCOA18 has integrated on the right-hand side of the coa gene while in strain DU5863 (B), pCOA18 has integrated on the left-hand side. Restriction endonuclease cleavage sites are abbreviated as follows: V, EcoRV; K, KpnI; amplified fragments are marked *. 

allele-replacement mutant strain DU5861 (RN4220 Δcoa::Tc') had three EcoRV fragments that hybridized to the pCOA14 probe, one of 3-65 kb, one of 1-73 kb and one of > 15 kb (Fig. 1, lane I). The 3-65 kb fragment was from sequence located 5' to the coa gene and was also present in the wild-type strain and in strain DU5857 (Fig. 2). The 1-73 kb fragment is composed of both coa and Tc' sequences (there is an EcoRV site located within the Tc' insert; Fig. 2). The large fragment was also a hybrid formed between the Tc' insert and 3' coa sequences (Fig. 2) but was too large for any differences in size from the wild-type > 15 kb fragment to be detected under the conditions used.

Two Coa⁺ transformants, strains DU5863 and DU5864, expressing low-level but amplifiable Tc' (putative single-copy Campbell integrants) were analysed by
Fig. 3. Integration of pCOA18 into the chromosome and amplification by unequal crossing-over. The top part of the diagram shows a single recombination event between homologous sequences located to the left of the chromosomal coa gene (open box) and the mutated coa gene (Δcoa::Tc'; elevated line) on pCOA18 that results in integration of the plasmid into the chromosome. The thick lines represent plasmid vector sequences and are not drawn to scale. The middle part of the diagram indicates one possible unequal crossing-over event between homologous sequences flanking the coa+ and Δcoa::Tc' alleles that could occur after chromosome replication. The result is one chromosome carrying two copies of Δcoa::Tc' and the plasmid vector (lower part of diagram). A second unequal crossing-over event can occur between duplicated copies of the integrated pCOA18 during subsequent chromosome replication to give three copies of pCOA18 in a tandem array. Further amplification can occur during subsequent rounds of replication.

Southern blotting analysis of amplified derivatives derived from the single-copy integrant strain DU5864

Amplified derivatives of the single-copy integrant strain DU5864 were selected by plating on 30 μg Tc ml⁻¹. One derivative, strain DU5865, was obtained in a single selection step but analysis of the fragment pattern of
DNA from this strain suggests that the amplified structure is more complex than that of strains DU5857 or DU5862. Cleavage with KpnI revealed fragments of 2.5 kb, 2.95 kb, 3.05 kb and 5.4 kb, all of which appeared to be amplified (Fig. 4, lane D). Comparing the intensity of the 5.4 kb fragment in strain DU5857 (Fig. 1, lane B) with that of strain DU5865 (Fig. 4, lane D) suggests that the wild-type coa gene had been amplified as well as pCOA18 (Fig. 5). The fragments above 5.4 kb in Fig. 4, lane D probably represent partial cleavage products. In addition, EcoRV fragments of both 8.35 kb and 6.75 kb were present in the same sample and both appeared to be amplified (Fig. 4, lane I), whereas in strain DU5863 only the 6.75 kb fragment was present (Fig. 4, lane J) and in strain DU5864 only the 8.35 kb fragment was present (Fig. 4, lane H). These data suggest that the coa gene had been amplified along with the Δcoa::Tc′ fragment. This could have occurred by two distinct unequal crossing-over events as shown in Fig. 5.

Strain DU5881 was derived from strain DU5864 by a single selection step on 100 μg Tc ml⁻¹. Southern blotting analysis of DNA from this strain showed KpnI and EcoRV fragment patterns (Fig. 6, lanes G and K) similar to those of strain DU5857 (Fig. 1, lanes B and H), which was isolated directly after electroporation. However, strain DU5881 had an additional single-copy EcoRV fragment of 8.35 kb, as the plasmid was previously shown to have integrated to the right of the coa locus in strain DU5864 (Fig. 2).

Strain DU5881 was then plated on higher concentrations of Tc to determine if further amplification events could be selected. A derivative was isolated (strain DU5884) which expressed resistance to 80 μg Tc ml⁻¹. None of the colonies was capable of growing on
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Fig. 5. Amplification of Δcoa::Tc' and coa in strain DU5865. The top part of the diagram indicates the structure that would be generated if pCOA18 integrated into the chromosome by a single cross-over event to the right of the Tc' insertion (see Fig. 3). During chromosome replication, unequal crossing-over could occur on the right of the coa alleles to generate the structure in the middle of the diagram. A second unequal cross-over event on the other side of the coa alleles would generate the structure shown at the bottom of the diagram where both coa' and Δcoa::Tc' are duplicated. This alternating structure could be further amplified during subsequent rounds of replication. V, EcoRV; K, KpnI; amplified fragments are marked *.

Fig. 6. Ethidium-bromide-stained gel and Southern blotting analysis of amplified strains. Chromosomal DNA from the single-copy integrant strain DU5864 was cleaved with KpnI (lanes B and F) and with EcoRV (lane J). DNA from the amplified strain DU5881 was cleaved with KpnI (lanes C and G) and with EcoRV (lane K) while DNA from the further amplified strain DU5884 was cut with KpnI (lanes D and H) and with EcoRV (lane L). DNA from the amplified strain DU5885 was cleaved with KpnI (lanes E and I) and with EcoRV (lane M). Uncut DNA from strain DU5885 (lane A) was run as a control to show the absence of extrachromosomal multimeric plasmid DNA. Lanes A–E were stained with ethidium bromide while lanes F–M were probed with labelled pCOA14. The DNA loading in each lane was similar (about 10 μg).
concentrations above 80 µg Tc ml⁻¹ when tested by the IC₉₀ method. Southern hybridization analysis revealed that this strain had a pattern of KpnI and EcoRV fragments (Fig. 6, lanes H and L) similar to strain DU5881, except that the amplified bands seemed to be even more intense, suggesting that additional amplification had occurred. Amplified KpnI fragments in stains DU5881 and DU5884 were clearly evident in ethidium-bromide-stained gels (Fig. 6, lanes C and D). Strain DU5885 was also isolated after two selection steps and expressed resistance to 125 µg Tc ml⁻¹. Amplified bands similar to strain DU5884 were observed in a stained gel (Fig. 6, lane E), and Southern blots with KpnI digests (Fig. 6, lane I) and EcoRV digests (Fig. 6, lane M). Extrachromosomal bands were not visible in a sample of uncut DNA from strain DU5885 (Fig. 6, lane A), indicating that the plasmid multimers are integrated in the chromosome. Also, there was no suggestion that excised monomers were present.

Autoradiograms of Southern blots with KpnI digests of the single-copy integrant strain DU5864 and the amplified-derivatives stains DU5881, DU5884 and DU5885 were scanned by a densitometer in order to estimate the number of tandem copies of pCOA18. Using the intensity of the single copy 5.4 kb KpnI coa fragment as a reference, the copy number of the amplified 2.5 kb KpnI fragment was estimated to be 9 (strain DU5881), 16 (strain DU5884) and 19 (strain DU5885). This correlated with resistance levels of 50 µg Tc ml⁻¹, 80 µg Tc ml⁻¹ and 125 µg Tc ml⁻¹ respectively.

**Discussion**

The underlying objective of this project was to investigate the possibility that allelic replacement mutants could be isolated in *S. aureus* with an integrating plasmid carrying an *in-vitro*-constructed mutation. Coagulase-deficient mutants were isolated and these have been extremely valuable in determining the relationship between coagulase and clumping factor and in analysing fibrinogen binding proteins of *S. aureus* (McDevitt *et al.*, 1992). The characterization of some of the transformants isolated when pCOA18 was introduced into *S. aureus* strain RN4220 has led to the identification of a number of interesting structures. The majority of these transformants occurred as a result of a single recombination event between the mutated and wild-type alleles where the entire plasmid had integrated into the chromosome by a Campbell-type recombination event. Recombination was shown to have occurred on either side of the wild-type gene. A double recombination event resulted in allele-replacement between the mutated and wild-type genes due to homologous sequences flanking the mutation. As predicted, this was a much rarer event. Unexpectedly, the majority of Campbell insertions appeared to have multiple copies of pCOA18 in tandem arrays.

The 50 transformants chosen for analysis could be divided into three classes on the basis of phenotype and transduction analysis and by Southern hybridization using a coa probe and a probe for the Tc' insertion.

1. Allele-replacement mutants were deficient in coagulase activity and expressed a low level of Tc' (15 µg ml⁻¹) from the single-copy Tc' determinant. Variants expressing a higher level of Tc' could not be selected. The Tc' and the Coa⁻ phenotypes were 100% co-transducible, proving that the coa mutation was caused by the inserted Tc fragment. Southern blotting data was consistent with allelic replacement; no plasmid vector DNA was detected and single copies of fragments consistent with the Δcoa::Tc' replacement were present in the chromosome.

2. Single-copy Campbell integrants which were Tc' and Coa⁺. Transformants were isolated where integration had occurred on either side of the chromosomal coa locus. Single-copy integrants expressed the same low level of Tc' as the replacement mutant, but the population of cells in a colony or broth culture contained derivatives which expressed higher levels of Tc'. These strains had amplified plasmid sequences as demonstrated by Southern hybridization. Transduction of a single-copy Campbell integrant into a wild-type strain generated Coa⁻ derivatives at a frequency of 19%. These transductants had lost the plasmid but retained the Δcoa::Tc' mutation and had the same properties as derivatives described in (1) above.

3. Campbell integrants which carried multiple copies of pCOA18 in a tandem array were Coa⁺ and were resistant to high levels of Tc' (up to 125 µg ml⁻¹). The frequency of transduction of Tc' to a Coa⁺ recipient was 100-fold higher than that of single-copy determinants. The majority of transductants were Coa⁻ and expressed high levels of Tc', and thus probably had retained amplified copies of the plasmid sequences. An amplified pCOA18 integrant was isolated which was Coa⁻ (strain DU5862) due to a deletion in the adjacent coa gene.

An explanation is lacking for the high incidence of multimerized pCOA18 among the Tc' transformants isolated in strain RN4220. The plasmid was predominantly monomeric when examined by gel electrophoresis (data not shown). However, dimers and multimers will always be present in any population of pUC- or pBR322-based plasmids (Summers & Sherratt, 1984). It is feasible that multimeric plasmids are preferentially transformed by electroporation into *S. aureus*. The possibility that monomeric plasmids that were transformed into the same cell underwent recombination prior to integration is less likely.
In *Bacillus subtilis*, the degree of amplification of a drug-resistance determinant that was forced to integrate into the chromosome is dependant on the concentration of the drug in the growth media (Young, 1984). However, in the case described here the transformants were selected on a low concentration of Tc (2 μg ml⁻¹), which is well below the MIC for a single copy of the Tc' determinant. Indeed the single-copy integrants and replacement mutants did not grow any more slowly than the amplified derivatives when compared on agar containing 2 μg Tc ml⁻¹. However, as transformants were not induced for Tc' prior to plating, colonies containing multiple copies may have been better adapted for growth when initially plated on agar containing 2 μg Tc ml⁻¹.

Tandem repeats of pCOA18 integrated in the chromosome could be selected by growing a single-copy integrant on high levels of Tc. This was presumably achieved by unequal recombination between two integrated plasmids as depicted in Fig. 3. This type of *in situ* gene amplification has been reported before in *S. aureus* (Matthew & Stewart, 1988) and in *Bacillus subtilis* (Young, 1983; 1984). As in this case, when a demand was made for increased expression of a drug-resistance gene, amplified drug-resistance determinants were selected (Matthew & Stewart, 1988; Young, 1984).

Densitometry was used to estimate the number of tandem copies of the amplified integrated plasmid in three strains. Strain DU5881, which was isolated after a single selection step, had about nine copies per chromosome. Strains DU5884 and DU5885, which had been isolated after two selection steps, had 16 and 19 copies per chromosome respectively. The tandemly amplified sequences appeared to be quite stable because after prolonged growth (100 generations) in drug-free broth, the single-cell resistance level had only dropped from 125 μg Tc ml⁻¹ to 75 μg Tc ml⁻¹. Reiterated sequences are also stable in the *Bacillus subtilis* chromosome (Young & Ehrlich, 1989).

Transducing phage propagated on strains that contained amplified copies of pCOA18 in the chromosome transferred the Tc' marker at a frequency about 100-fold higher than for single-copy determinants. Many of the transductants formed very small colonies which either failed to grow when replated or formed a mixture of small and large colonies. We suspect that abortive transduction is occurring with linear fragments or with circular molecules comprising tandem arrays of pCOA18 being introduced into the recipient by transduction and cells growing initially due to the presence of multiple copies of the Tc' determinant.

Integrating plasmids introduced by electroporation offer an alternative approach to temperature-sensitive and incompatible plasmids for genetic manipulation in *S. aureus*. If the transformation frequency is sufficiently high, allelic-replacement may be detected directly after plating transformants. Alternatively, transduction of a single-copy integrant allows the allele-replacement recombination event to be readily selected. The disadvantage of the integrating plasmid approach is that it is confined to the recipient strain RN4220. Mutagenesis can be performed in many other strains, even those in different phage groups, only if replicative plasmid-delivery systems are employed.

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References


