Variation in the size of the repeat region of the fibrinogen receptor (clumping factor) of *Staphylococcus aureus* strains

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The ability of *Staphylococcus aureus* to bind to fibrinogen and fibrin is believed to be an important factor in the initiation of foreign body and wound infections. Recently, the gene encoding the fibrinogen receptor (clumping factor, ClfA) of *S. aureus* strain Newman was cloned and sequenced. The ClfA protein possesses a highly unusual 308 residue dipeptide repeat region composed predominantly of Asp and Ser. Polymerase chain reaction analysis of seven different strains showed that the size of the *clfA* repeat coding region varies from 580 bp to 1320 bp. In contrast, the *clfA* region A is the same size in each strain. The size of the *clfA* repeat region did not correlate with the ability of these strains to form clumps in a solution of fibrinogen. Indeed, the strain with the smallest repeat size of 580 bp clumped almost as well as strain Newman. Each strain of *S. aureus* examined contained several high molecular mass proteins that reacted with anti-ClfA region A antibody. In some cases the molecular mass of the major protein varied in accordance with the length of the coding sequence for the repeat region R.

Keywords: *Staphylococcus aureus*, fibrinogen receptor, clumping factor

INTRODUCTION

Staphylococcus aureus is an important pathogen that is responsible for a variety of infections of both man and animals. It is a major cause of hospital acquired infections (Shanson, 1990) and can cause wound infections and infections associated with indwelling medical devices including catheters and surgical prostheses (Maki, 1982; Kristinsson, 1980). These infections can lead to life threatening sequels such as septicaemia and endocarditis. Colonization has been identified as a key step in the pathogenic process. When medical devices are introduced into the body they become coated with host plasma proteins including fibrinogen and fibronectin (Cottonaro *et al.*, 1981; Cheung & Fischetti, 1989; Vaudaux *et al.*, 1989). In particular, intact fibrinogen is the predominant protein deposited on biomaterials during short-term contact with blood (Vaudaux *et al.*, 1991). Prolonged contact results in degradation of fibrinogen and loss of its binding site for *S. aureus* and the emergence of fibronectin as the pre-eminent determinant of adherence (Vaudaux *et al.*, 1993). The ability of *S. aureus* to bind to these

deposited proteins is believed to be an important factor in the pathogenesis of foreign body infections.

The structure of the receptor for fibrinogen on the surface of *S. aureus* strain Newman (sometimes called the clumping factor, ClfA) has been deduced from the DNA sequence (McDevitt *et al.*, 1994). The deduced amino acid sequence of the ClfA protein revealed features characteristic of proteins located at the surface of Gram-positive bacteria, namely the sequence LPDTG which corresponds to the motif LPXTG, followed by a hydrophobic transmembrane domain and then several positively charged residues (Fig. 1). ClfA lacks an extensive proline-

Fig. 1. Schematic diagram showing the domain organization of the ClfA protein of *S. aureus* strain Newman. S, signal peptide; A, non-repeat region; R, repeat region; W, wall region; M, membrane spanning region; +, positively charged residues. F1 and F2, forward primers; R1 and R3, reverse primers. The location of LPDTG is indicated.

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and glycine-rich region characteristic of protein A and the fibronectin-binding proteins (Guss *et al.*, 1984; Signas *et al.*, 1989; Jonsson *et al.*, 1992). A 39 residue sequence that has all the features of a secretory signal sequence is located at the N-terminus of ClfA. This is followed by a 520 residue non-repeated region A (Fig. 1) which contains the fibrinogen-binding domain (D. McDevitt & T. J. Foster, unpublished data). A novel 308 residue repeat region (region R) is found between region A and the C-terminal wall- and membrane-associated regions (Fig. 1). It is composed of 154 dipeptide repeats predominantly of Asp and Ser and ends 28 residues upstream of LPDTG.

The dipeptides are coded for predominantly by an 18 bp DNA repeat, with a consensus of GAY TCN GAY TCN GAY AGY where N is any base and Y is T or C (McDevitt *et al.*, 1994). There are 52 DNA repeat units, two of which (numbers 45 and 51) consist of 12 bp. Of the 50 18 bp repeats, 31 encode Ser and Asp exclusively. The sequence of the *clfA* repeat region diverges at either end, which is consistent with the hypothesis that it evolved by intragenic recombination. This type of divergence is also found in the repeats of protein A (Uhlen *et al.*, 1984) and coagulase (Phonimdaeng *et al.*, 1990). The function of the ClfA repeat region is not known. It may act as a 'stalk' to project the biologically active fibrinogen-binding region from the cell surface or it may serve as a mechanism for attaching the protein to the cell wall similar to the proline and glycine repeats of protein A.

This study investigated the sizes of the *clfA* repeat regions in different clumping factor producing *S. aureus* strains using PCR. The repeat region was found to vary from 580 bp to 1320 bp. The repeat length did not correlate with the fibrinogen clumping titre. The variation in size of the clumping factor protein was investigated by Western immunoblotting.

METHODS

Bacterial strains. The strains are listed in Table 1.

PCR analysis. PCR was used to amplify both the region A and the repeat region R from the chromosomal *clfA* gene of the *S. aureus* strains in Table 1. To amplify the region A of *clfA* the forward primer 5' TATGCCATGGTAGGTACGTTAATCGG 3' (F1) (starting at position 67) and reverse primer 5' TACAAGCTTGCTCATCAGGTTGTTTCAGG 3' (R1) (ending at position 1651) were used. To amplify the *clfA* repeat region R the forward primer 5' ATTACCATGGAACAACCTGATGAGCC 3' (F2) (starting at position 1636) and reverse primer 5' GTCAAGCTTAGGTGAATTAGGCGG 3' (R3) (ending at position 2633) were used. The bp coordinates refer to the ATG codon of the *clfA* gene of strain Newman (accession number Z18852 SAU CF). It should be noted that these primers contain 5' extensions (underlined) which do not hybridize to *clfA* sequences. Thus the PCR products will be 18 bp and 16 bp larger, respectively, than predicted from the 5' and 3' coordinates. PCR was performed with a Cetus/Perkin Elmer DNA Thermocycler. Genomic DNA was isolated from *S. aureus* strains by the method of Bollet *et al.* (1991). Reaction mixtures (100 µl) contained 100 ng target DNA, 200 pM of forward and reverse primers, 1.5 mM MgCl₂, 2 µM of each dNTP, 10 mM Tris/HCl pH 9, 50 mM KCl, 0.1% Triton X-100 and 2.5 U *Taq*

DNA polymerase (Promega). The reaction mixtures were overlaid with 100 µl mineral oil and amplified for 30 cycles consisting of a 1 min denaturation period at 94 °C, a 1 min anneal period at 45 °C and a 1 min extension period at 72 °C. After amplification, 10 µl PCR product was analysed by agarose gel electrophoresis (1%, w/v, agarose). Standards were the 100 bp ladder purchased from Gibco-BRL.

Manipulation of DNA. DNA-modifying enzymes were purchased from Boehringer or from 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). Probe DNA was labelled by random priming using [α -³²P]dATP (Amersham) and the Prime-A-Gene Kit (Promega). PCR amplification of plasmid pCF14 (carrying the cloned *clfA* gene) using F1 and R1 primers resulted in a 1600 bp product which was gel-purified and used as the *clfA* region A probe. PCR amplification of plasmid pCF14 using F2 and R3 primers resulted in a 1020 bp product which was gel-purified and used as the *clfA* repeat region R probe.

Measurement of clumping titre. The clumping titres of *S. aureus* strains were measured as previously described (McDevitt *et al.*, 1992). Briefly, serial twofold dilutions of a 2% (w/v) solution of fibrinogen (Sigma) was made in phosphate-buffered saline (PBS; 0.145 M NaCl, 0.15 M sodium phosphate). Bacteria were grown for 15 h in 20 ml trypticase soy broth in a 250 ml flask shaken at 200 r.p.m. A suspension of 2×10^9 washed cells (20 µl) was added to 50 µl of the fibrinogen dilutions in microtitre trays. The reciprocal of the highest dilution of fibrinogen showing clumping after 3 min was recorded as the titre.

SDS-PAGE and Western immunoblotting. Proteins were extracted from *S. aureus* cells as follows. Bacteria were grown for 15 h in trypticase soy broth (20 ml) in a 250 ml flask shaken at 200 r.p.m. Cells were harvested by centrifugation at 12000 g and resuspended in 2 ml PBS containing 40 µg DNase I ml⁻¹, 40 µg RNase I ml⁻¹, 2 mM of the protease inhibitors PMSF and benzamidine, and recombinant lysostaphin (Applied Microbiology) at 50 µg ml⁻¹. Cells were incubated at 37 °C for 2 h followed by centrifugation at 30000 g for 30 min. The supernatants were stored in aliquots at -20 °C.

Escherichia coli strains harbouring plasmids were grown for 15 h with shaking at 200 r.p.m. in 50 ml L broth with ampicillin at 100 µg ml⁻¹. Cells were harvested by centrifugation at 12000 g and resuspended in 5 ml PBS containing 40 µg DNase I ml⁻¹, 40 µg RNase I ml⁻¹ and 2 mM each of PMSF, benzamidine and EDTA. Cells were lysed by passage through a French press followed by centrifugation at 30000 g for 30 min. The supernatants were stored in aliquots at -20 °C. The protein concentration was determined by the Bio-Rad protein assay method using human fibrinogen as a standard.

Proteins were separated by SDS-PAGE which was performed by standard procedures (Laemmli, 1970) with 7.5% (w/v) acrylamide gels. Proteins were transferred from the gel to nitrocellulose membranes (0.45 µm, Schleicher & Schuell) using a semidry blotter (Bio-Rad) and 48 mM Tris/HCl, 39 mM glycine (pH 9.2) and 20% (v/v) methanol. Filters were blocked with a 10% (w/v) solution of skim milk in PBS. ClfA proteins were detected with rabbit anti-ClfA antibodies prepared by immunization with purified region A of clumping factor that had been amplified by recombinant techniques (D. McDevitt & T. J. Foster, unpublished). The antibody was adsorbed with an *E. coli* TB1 lysate and used at a dilution of 1 in 1000 followed by

Table 1. Strains

Strain	Description	Source/reference
<i>Staphylococcus aureus</i>		
Newman	Archetypal clumping factor producer NCTC 8178	Duthie & Lorenz (1952)
DU5876	Clumping factor defective Tn917 mutant of strain Newman	McDevitt <i>et al.</i> (1994)
C304/11 Cowan	Bovine mastitis isolate NCTC 8530	Jones & Wiencke (1986)
V37	Septicaemia isolate from Dublin	Humphreys <i>et al.</i> (1989) O'Reilly <i>et al.</i> (1990)
V41	Septicaemia isolate from Dublin	Humphreys <i>et al.</i> (1989) O'Reilly <i>et al.</i> (1990)
V48	Septicaemia isolate from Dublin	Humphreys <i>et al.</i> (1989) O'Reilly <i>et al.</i> (1990)
V82	Septicaemia isolate from Dublin	Humphreys <i>et al.</i> (1989) O'Reilly <i>et al.</i> (1990)
<i>Escherichia coli</i>		
TB1	Host for chimeric plasmids	Yanisch-Perron <i>et al.</i> (1985)
TB1(pCF3)	pCF3 carries the <i>clfA</i> gene on a 7 kb <i>Hind</i> III fragment cloned in pUC18	McDevitt <i>et al.</i> (1994)
TB1(pCF14)	pCF14 carries the <i>clfA</i> gene on a 3.5 kb <i>Hind</i> III- <i>Kpn</i> I fragment cloned in pUC18	McDevitt <i>et al.</i> (1994)

incubation with protein A conjugated with peroxidase (1/500, Sigma).

RESULTS

Variation in the size of the *clfA* repeat region

Chromosomal DNA was isolated from seven different *S. aureus* strains from a variety of human and bovine sources (Table 1) and analysed by PCR. For amplification of the region A of *clfA*, primers F1 and R1 (designed to anneal to sequences flanking the region A of strain Newman) were used while region R was amplified with primers F2 and R3 (designed to anneal to sequences flanking the *clfA* repeat region R of strain Newman) (Fig. 1).

Amplification of the repeat region R of strain Newman generated a product of 1020 bp (Fig. 2a, lane 1). This correlated well with the fragment size predicted from the DNA sequence (1013 bp, including primers). Fig. 2(a) shows the variability in the size of the *clfA* repeat regions of six other strains compared to strain Newman. The sizes varied from 580 bp for strain V37 (lane 2) to 1320 bp for strain V48 (lane 7). Most strains had a significantly larger *clfA* repeat region compared to that of strain Newman. When analysis was expanded to a panel of 37 strains from various sources, 92% of strains (34/37) had a repeat region equal to or greater than that of strain Newman (data not shown).

Southern hybridization analysis using a specific probe for the *clfA* repeat region of strain Newman was performed

to ensure that the PCR products were amplified from the *clfA* repeat region. The major PCR product in each sample hybridized with the probe (Fig. 2b). A second fainter product was observed for some strains (Fig. 2a, lanes 4, 6 and 7). In each case these failed to hybridize to the probe showing that they represent non-specific products.

Amplification of region A of strain Newman generated a product of 1600 bp (Fig. 3a, lane 1). This correlated well with the fragment size predicted from the DNA sequence (1602 bp, including primers). In contrast to the *clfA* repeat region, the size of the region A was the same in all the strains tested (Fig. 3a). Southern hybridization analysis, using a specific probe for region A of strain Newman, was performed on the PCR products. Each of the PCR products hybridized with the region A specific probe (Fig. 3b).

Clumping factor activity of *S. aureus* strains

Strain Newman is the archetypal clumping factor producing strain of *S. aureus* (Duthie & Lorenz, 1952; Hawiger *et al.*, 1982). It consistently gave a high titre (2048) in the quantitative clumping assay with soluble fibrinogen (Table 2) whereas the clumping factor deficient mutant of strain Newman (DU5876) failed to clump even at the highest concentration. No correlation was found between the clumping titre and the size of the *clfA* repeat region. Strain V37 which had the smallest *clfA* repeat

Fig. 2. (a) PCR analysis of the *clfA* repeat region. Chromosomal DNA from the various strains was amplified by PCR using the F2 and R3 primers. (b) Southern hybridization analysis of the *clfA* repeat region PCR products. The PCR products isolated using primers F2 and R3 were probed with the *clfA* repeat region probe. Lanes: 1, strain Newman; 2, strain V37; 3, strain C304/11; 4, strain V82; 5, strain Cowan; 6, strain V41; 7, strain V48. The 100 bp ladder was used as size standards.

Fig. 3. (a) PCR analysis of the *clfA* region A. Chromosomal DNA from the various strains was amplified by PCR using the F1 and R1 primers. (b) Southern hybridization analysis of the *clfA* region A PCR products. The PCR products isolated using primers F1 and R1 were probed with the *clfA* region A probe. Lanes: 1, strain Newman; 2, strain V37; 3, strain C304/11; 4, strain V82; 5, strain Cowan; 6, strain V41; 7, strain V48.

clumped almost as strongly as strain Newman while strains V41 and V48, which had the largest *clfA* repeat regions, had clumping titres of 256 (Table 2). None of the strains tested had a higher clumping factor titre than strain Newman.

Analysis of clumping factor proteins

Proteins released from the cell by lysostaphin digestion of the clumping factor producing *S. aureus* strains and a lysate of *E. coli* expressing the cloned *clfA* gene were

Table 2. Clumping titres and sizes of the *clfA* repeat regions of the various strains

Strain	Clumping titre*	PCR fragment size (bp)†
Newman	2048	1020
DU5876	< 2	ND
V37	1024	580
C304/11	2048	990
V82	1024	1280
Cowan	2048	1290
V41	256	1320
V48	256	1320

ND, Not determined.

* Average of three experiments.

† Approximate size.

analysed by Western immunoblotting with anti-ClfA antibodies. The lysate of *E. coli* TB1(pCF3) (carrying the cloned strain Newman *clfA* gene) contained several immunoreactive proteins (Fig. 4, lane 2) which were

absent in a lysate of *E. coli* TB1 without the plasmid (Fig. 4, lane 1). The largest of these was 190 kDa. *S. aureus* strain Newman expressed a 190 kDa immunoreactive protein (Fig. 4, lane 3). A smaller immunoreactive protein of 130 kDa was also detected. Despite the presence of protease inhibitors and testing samples from cells harvested from mid-exponential to late-stationary phase, two proteins were always present. Both proteins were absent in the clumping factor mutant strain DU5876 (Fig. 4, lane 4) indicating that they are products of the *clfA* gene.

Two or more immunoreactive proteins were found in each of the other strains tested. In some strains a tentative correlation could be made between the largest immunoreactive protein and the size of the *clfA* repeat region detected by PCR but there were several exceptions. The largest immunoreactive protein of V37 (580 bp repeat region) was 170 kDa (Fig. 4, lane 5) while strains V41 and V82 which had the largest repeats (1320 bp) expressed immunoreactive proteins of > 200 kDa (Fig. 4, lanes 10 and 11). In strain Cowan (lane 8) an even higher molecular mass band is evident. This could represent an undenatured aggregate of ClfA or cross-reaction with another protein. The faint protein of 56 kDa in some of the samples is most probably protein A. This protein was absent in a sample of proteins released from a protein-A-deficient mutant of

Fig. 4. Western immunoblotting analysis of ClfA proteins. Samples from *E. coli* strains TB1 (lane 1) and TB1(pCF3) (lane 2) and from *S. aureus* strains Newman (lane 3), DU5876 (lane 4), V37 (lane 5), strain C304/11 (lane 6), strain V82 (lane 7), strain Cowan (lane 8), strain V41 (lane 9) and strain V48 (lane 10) were transferred to a nitrocellulose membrane. The membrane was incubated with anti-ClfA antibodies followed by protein A peroxidase. One hundred micrograms of protein extract was loaded for *E. coli* TB1, *E. coli* TB1(pCF3) and for strains Newman and DU5876 while 500 µg protein was loaded for strains V37, C304/11, V82, Cowan, V41 and V48.

strain Newman (data not shown). Using protein-A-peroxidase to detect IgG does not entirely eliminate detection of IgG bound by the non-immune mechanism to protein A.

DISCUSSION

The primary objective of this study was to determine if the *clfA* repeat region varied in size among *S. aureus* strains from different sources. This was achieved by PCR using primers designed to anneal to sequences directly flanking the strain Newman *clfA* repeat region. A PCR product was obtained with each of the seven strains tested suggesting that the primer binding regions are conserved. In addition, the fact that the 1020 bp PCR product from strain Newman was very close to the size (1013 bp) predicted from the DNA sequence suggests that the *clfA* repeat sizes from other strains are accurate estimates. Southern hybridization analysis confirmed that the PCR products are derived from the *clfA* repeat. A significant size variation in the *clfA* repeat regions was observed. The smallest *clfA* repeat region was 580 bp while the largest was 1320 bp. It is known that tandemly repeated DNA sequences can act as hotspots for recombination or slipped strand mispairing. This can result in the formation of deletions or duplications (Hollingshead *et al.*, 1987; Fischetti *et al.*, 1986; Stern *et al.*, 1986). Size variation has been reported in type M6 proteins of Group A streptococci and this was shown to be generated by intragenic recombination between repeats (Hollingshead *et al.*, 1987). Evidence for intragenic recombination being involved in the amplification of repeats is found in the gradient of homology seen between repeats of protein A of *S. aureus* (Uhlen *et al.*, 1984) and protein G of streptococci (Olsson *et al.*, 1987). The outermost repeats are most different from the consensus. This was observed in region R of the Newman *clfA* gene where mutations in the first or second bases of triplet codons in the outermost repeats have led to amino acid divergence from the Ser-Asp dipeptide. Region R consists of 50 × 18 bp (and 2 × 12 bp) defined by the codon usage for serine. Every third serine codon is AGY while the first and second are TCN. Thus region R must have evolved by unequal crossing over or slippage between 18 bp units (or multiples of 18 bp).

In principle such highly repetitive DNA should be unstable. For example the tandem repeats of CTCTT in the *Neisseria gonorrhoeae opa* genes are thought to change at a frequency of 1 in 100 (Stern *et al.*, 1986). PCR analysis of region R showed that the number of repeats varies from one strain to another. However, no evidence was found for variation within a strain occurring at a high frequency. We considered that the additional faint bands in strains V82, V41 and V48 could have been due to amplification of variant repeat regions with the population. However these bands did not react with the region R probe and are thus probably due to non-specific amplification events. Also, a line of strain Newman kept in this laboratory for 20 years had the same repeat length as the derivative obtained from the NCTC. The DNA sequence of the

region R 18 bp repeats in strain Newman vary considerably from one to another. Neighbouring 18 bp repeats differ by as many as six positions while only two pairs of adjacent 18 bp repeats are identical. Analysis of the 31 Ser-Asp encoding repeats shows that they are saturated for mutations in the third position with the mean number of differences being three (D. Shields, D. McDevitt & T. J. Foster, unpublished data). The high level of mismatching may reduce the potential for unequal crossing over and replication slippage and could have evolved to preserve the integrity of region R.

Many of the strains tested had a larger repeat region than strain Newman while strain V37 had a smaller repeat of 580 bp. The latter was able to clump almost as well as strain Newman. In addition, strains V41 and V48 which had repeats of 1320 bp had a low titre of 256. Thus, there is no correlation between the length of the repeat and the ability of the strains to clump in soluble fibrinogen.

The largest immunoreactive protein expressed by *E. coli* TB1(pCF3) (carrying the cloned *clfA* gene) was 190 kDa. The smaller immunoreactive proteins are probably degradation products which occurred despite the presence of protease inhibitors in the sample. In samples that were prepared from late-stationary phase cultures or which were repeatedly frozen and thawed, the 190 kDa band was missing. Alternatively, the smaller proteins may be due to premature translational termination. An immunoreactive protein of 190 kDa was also expressed by *S. aureus* strain Newman as well as a 130 kDa protein which may also be a degradation product. Both of these proteins were absent in the clumping factor deficient mutant indicating that they are products of the *clfA* gene. Previously we reported the size of the ClfA protein to be 130 kDa in an affinity blotting assay with fibrinogen and peroxidase labelled anti-fibrinogen antibodies (McDevitt *et al.*, 1994). The immunoblotting assay is much more sensitive and precautions were not taken in the early work to avoid proteolysis. The 130 kDa protein detected in the affinity blotting assay is most probably a truncated derivative of ClfA. The apparent molecular mass of the native ClfA protein of strain Newman thus appears to be 190 kDa.

Assuming that the largest immunoreactive protein is the native molecule, the ClfA proteins detected from some strains (e.g. strain V82) were smaller than expected. Nevertheless, in others there appeared to be a correlation between the size of the largest ClfA immunoreactive protein and the length of the *clfA* repeat region. Also, there did not appear to be an obvious correlation between the clumping titre and the amount of ClfA protein expressed, as judged qualitatively by examining the intensity of immunoreactive bands in Western blots (it should be noted that cells from the same culture were divided for the clumping assay, for the release of cell-wall-associated proteins and for the isolation of chromosomal DNA). However, strain Newman, which had the highest clumping titre, also expressed a high level of the ClfA protein, but strains C304/11 and Cowan which had the same clumping titre as Newman expressed significantly less protein. Perhaps variants have different affinity for

fibrinogen or for the antibody or there may be strain to strain variation in release of ClfA or in its stability.

Much remains to be determined about the unique dipeptide repeat region of ClfA. It is still unknown whether this region of the protein is surface-exposed or is exclusively associated with the peptidoglycan found in the cell wall. Antibodies raised against the repeat region will be used to address this issue.

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