# A novel von Willebrand factor binding protein expressed by Staphylococcus aureus

Bjerketorp, Joakim; Nilsson, Martin; Ljungh, Åsa; Flock, Jan-Ingmar; Jacobsson, Karin; Frykberg, Lars

*Published in:*
Microbiology

2002

*Microbiology* (2002), **148**, 2037–2044 Printed in Great Britain

# A novel von Willebrand factor binding protein expressed by *Staphylococcus aureus*

Joakim Bjerketorp,[1] Martin Nilsson,[1] Åsa Ljungh,[2] Jan-Ingmar Flock,[3] Karin Jacobsson[1] and Lars Frykberg[1]

**Author for correspondence:** Lars Frykberg. Tel: +46 18673299. Fax: +46 18673392. e-mail: Lars.Frykberg@mikrob.slu.se

[1] Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, SE-75007 Uppsala, Sweden

[2] Department of Infectious Diseases and Medical Microbiology, Lund University, Sölvegatan 23, SE-22362 Lund, Sweden

[3] Department of Microbiology, Pathology and Immunology, Huddinge University Hospital, Karolinska Institutet, SE-14186 Huddinge, Sweden

**When a shotgun phage-display library of *Staphylococcus aureus* Newman was affinity selected (panned) against recombinant von Willebrand factor (vWf), a novel von Willebrand factor binding protein (vWbp) was found. Experimental data indicate that the interaction between vWbp and vWf is very specific and mediated by a region of 26 aa residues in the C-terminal part of vWbp. vWbp has an N-terminal secretory signal sequence but no cell wall anchoring motif, suggesting a soluble extracellular location. Mature vWbp could be purified from the culture supernatant and the identity of the protein was confirmed by N-terminal sequencing. vWbp migrates with an apparent molecular mass of 66 kDa and the deduced protein consists of 482 aa. The gene encoding vWbp, named *vwb*, was present in all *S. aureus* strains investigated.**

**Keywords:** receptin, virulence factor, phage display

## INTRODUCTION

*Staphylococcus aureus* is a pathogen responsible for a wide variety of diseases in humans and animals, including endocarditis, osteomyelitis, wound infections and mastitis. To interact successfully with the host, the bacterium has a large repertoire of components such as extracellular enzymes and toxins, as well as cell-wall-bound and secreted proteins that bind to proteins in host serum and extracellular matrix (Lowy, 1998). Proteins of microbial origin, cell-bound or soluble, which bind to mammalian proteins are generally considered as putative virulence factors and several such proteins from *S. aureus* have been molecularly characterized (Smeltzer, 2000). It should be noted that the outcome of virulence studies seems to be dependent on the animal model employed (Coulter *et al*., 1998; Darouiche *et al*., 1997). Nevertheless, studies of virulence in various animal models, with isogenic *S. aureus* mutants lacking a specific gene, have in several cases established the importance of a certain bacterial protein binding to a host protein, for some types of *S. aureus* infections. For example, the fibrinogen-binding proteins Efb (Palma *et al*., 1996) and ClfA (Moreillon *et al*., 1995), the collagen-binding protein Cna (Hienz *et al*., 1996; Patti *et al*., 1994; Rhem *et al*., 2000) and the IgG-binding protein A (Gemmell *et al*., 1997; Patel *et al*., 1987) are commonly considered as virulence factors. Further, a recent study showed that protein A also binds von Willebrand factor (Hartleib *et al*., 2000). Interestingly, in a vascular disease (Kawasaki disease) with symptoms and epidemiology overlapping with staphylococcal toxic shock syndrome, high protein A production was observed in the *S. aureus* strains isolated. This feature of the bacteria could possibly contribute to the pathological picture of this disease, with disturbed host haemostasis and an increased risk of vascular thrombosis (Wann *et al*., 1999).

The mature form of von Willebrand factor (vWf) is a large multifunctional glycoprotein, consisting of 2050 aa arranged in four different types of repeats (A through D). vWf exists as homodimers about 540 kDa in size, or multimers of different sizes up to 20000 kDa. Analysis by SDS-PAGE of plasma-derived vWf with reduced disulfide bonds reveals a predominant band with mobility corresponding to an apparent molecular mass of 225 kDa. The molecular mass of the vWf subunit, based

**Abbreviations:** HSA, human serum albumin; HRP, horseradish peroxidase; RT, room temperature; vWf, von Willebrand factor; vWbp, von Willebrand factor binding protein.

The GenBank accession number for the sequence reported in this paper is AY032850.

on its chemical composition, is approximately 270 kDa. vWf is synthesized exclusively by endothelial cells and megakaryocytes (Ruggeri, 1999). The endothelial cells generate a plasma pool of vWf with a concentration of $\sim 10$ μg ml$^{-1}$ as well as an intracellularly stored supply of vWf in Weibel–Palade bodies. Megakaryocytes are responsible for vWf stored within the α-granule of platelets. By supporting platelet adhesion and aggregation to exposed subendothelium in damaged blood vessels, vWf is an essential component in the maintenance of haemostasis, especially under conditions of rapid blood flow. vWf mediates platelet adhesion through two distinct platelet receptors: the glycoprotein (GP) Ib in the GP Ib–V–IX complex and the GP IIb–IIIa (also called integrin $\alpha_{IIb}\beta_3$). Furthermore, vWf transports and stabilizes the coagulation factor VIII. vWf contains an Arg-Gly-Asp (RGD) motif also recognized by the endothelial integrin $\alpha_V\beta_3$ and vWf can in addition bind to various subendothelial components, such as collagens (type I, III and VI) and heparin-like glucosaminoglycans (Ruggeri, 1999; Ruggeri & Ware, 1993). Malfunctional vWf, or a reduced amount of this glycoprotein, leads to one of several types and subtypes of von Willebrand disease, which is the most common inherited bleeding disorder (Mohlke *et al*., 1999).

In this investigation a shotgun phage-display library was made from chromosomal DNA of *S. aureus* strain Newman. The library was affinity selected (panned) against recombinant vWf, which resulted in the finding of a novel von Willebrand factor binding protein (vWbp).

## METHODS

**Proteins and reagents.** Human fibronectin, chicken IgG, human IgG, human serum albumin (HSA) and casein were purchased from Sigma, and human fibrinogen from IMCO. Human vitronectin was purified as described previously (Yatohgo *et al*., 1988). Recombinant vWf was a kind gift from Professor F. Dorner, Vienna, Austria. Recombinant vWf has been shown to mediate platelet aggregation and to promote binding of collagen and coagulation factor VIII with activity comparable to human-plasma-derived vWf (Fischer *et al*., 1997). Goat anti-vWf antibodies were purchased from Kordia; antibodies against recombinant vWbp (see below) developed in chicken were obtained from Immunsystem AB; mouse anti-E-tag antibodies and horseradish peroxidase (HRP)-labelled anti-mouse antibodies were from Amersham Biosciences. HRP-conjugated anti-chicken antibodies and HRP–anti-goat antibodies were from Sigma. Restriction enzymes and DNA manipulation enzymes were from MBI Fermentas or Amersham Biosciences. Oligonucleotides were from Invitrogen.

**Bacterial strains, growth conditions and helper phage.** *Escherichia coli* TG1 (Sambrook *et al*., 1989) was used for construction of the phage library and production of phage stocks. *E. coli* BL21(DE3) (Novagen) was used for expression of recombinant vWbp. *E. coli* was grown in Luria–Bertani (LB) broth or on LA plates (LB with 1·5 % agar) supplemented with 50 μg ampicillin ml$^{-1}$ (LA-amp) when appropriate. Phage R408 (Promega) was used as helper phage for production of phage stocks. The *S. aureus* strains used were Newman, 8325-4, Wood 46 and five different human clinical isolates kindly provided by Dr B. Christensson, University of Lund, Sweden. Clinical isolates of *Staphylococcus epidermidis* were used as negative controls. *S. aureus* Newman ΔEap, an isogenic mutant strain of *S. aureus* Newman in which the gene for staphylococcal extracellular adherence protein (Eap) has been deleted (J.-I. Flock, unpublished), was used for purification of vWbp. Staphylococci were grown in Tryptic Soya Broth (TSB, Oxoid).

**Construction of an *S. aureus* shotgun phage-display library.** A shotgun phage-display library was constructed from *S. aureus* strain Newman DNA as described previously (Jacobsson & Frykberg, 1995, 1996). In short, DNA fragments of approximately 0·5–5 kb, obtained by sonication, were ligated into the pG8SAET phagemid vector (Jacobsson & Frykberg, 1999). After transformation into *E. coli* TG1 and infection with helper phage R408, the final library consisted of $10^7$ individual clones and had a titre of $1{\cdot}5 \times 10^9$ c.f.u. ml$^{-1}$.

**Panning of the shotgun phage-display library.** Microwells (Maxisorp, Nunc) were coated with 50 μg vWf in 200 μl coating buffer (0·05 M $NaHCO_3$, pH 9·5) and incubated at room temperature (RT) with shaking for 1 h. The wells were then washed three times with phosphate-buffered saline containing 0·05 % Tween 20 (PBS-T). Two hundred microlitres of the phagemid library were then added to the vWf-coated wells, together with casein at a final concentration of 100 μg ml$^{-1}$. Panning was carried out at RT with shaking for 4 h. After washing extensively with PBS-T, bound phages were eluted with 200 μl elution buffer (0·05 M sodium citrate, 0·15 M NaCl, pH 2·0) at RT for 2 min. After neutralization in a tube with 25 μl 2 M Tris/HCl, pH 8·7, 0·001–50 μl volumes of the eluate were added to 25 μl stationary-phase *E. coli* TG1, together with LB broth to a final volume of 200 μl. The infection was allowed to proceed for 20–30 min at RT before the suspension was spread on LA-amp plates for determination of the number of c.f.u. in the eluate. The plates were incubated overnight at 37 °C, then 150 colonies were transferred to two identical plates for screening and sequencing. The rest of the colonies were collected and infected with 10 μl helper phage R408 ($10^{11}$ p.f.u. ml$^{-1}$) for production of enriched phage stocks. The infected bacteria were mixed with 5 ml 0·5 % soft agar, poured on an LA-amp plate and incubated at 37 °C overnight. The phages produced were recovered and the resulting phage stocks used for subsequent repannings, which were carried out as for the panning described above, except that repannings were accomplished in 2 h.

**Screening and sequencing of phagemid clones.** After each round of panning, 150 colonies were picked in an identical pattern to two LA-amp plates, transferred to nitrocellulose filters (Schleicher & Schuell) and subsequently screened for expression of the phagemid expression tag (E-tag) with the anti-E-tag antibody. Phagemid DNA from positive clones was prepared with a Qiagen Miniprep kit (Qiagen) according to the manufacturer's instructions. The inserted DNA fragments were sequenced using the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Biosiences) and the samples analysed using the ABI 377 DNA Sequencer (Perkin Elmer) according to the manufacturer's instructions. NTI Vector software (Informax) was used for handling the sequences obtained.

**Cloning of the *vwb* gene.** The *vwb* gene was PCR-cloned from *S. aureus* strain Newman using Pwo DNA polymerase (Roche Diagnostics) and the primers 5′-GAATTCTCATATGATT-CATGAAGAAGCC-3′ (upstream) and 5′-GAATTCGCC-ATGCATTAATTATTTGCC-3′(downstream). The primers were designed based on *S. aureus* sequence data available at

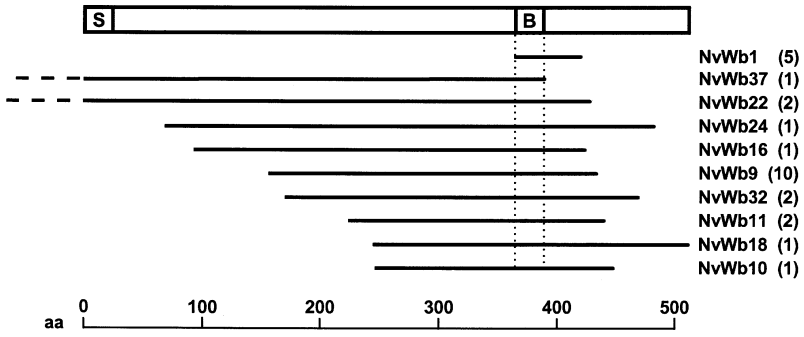


**Fig. 1.** Schematic representation of the vWbp protein aligned with the polypeptides encoded by inserts isolated from different phagemid clones obtained after panning an *S. aureus* phage-display library against recombinant vWf. S, signal sequence; B, vWf-binding region. Numbers in parentheses indicate how many times an individual clone was found. The broken line in two of the clones indicates that the insert contained DNA preceding the coding sequence.

TIGR (http://www.tigr.org/). The resulting PCR product was cloned into pUC18 for subsequent sequencing.

**Binding specificity of phagemid particles of NvWb32.** A phage stock of clone NvWb32 (Fig. 1) was prepared by infecting 500 µl of *E. coli* TG1 cells harbouring the phagemid with 10 µl helper phage R408 as above. After propagation in soft agar on an LA-amp plate, the phagemid particles were recovered as described above. The phage stock generated ($2 \times 10^{10}$ c.f.u. ml$^{-1}$) was used in an experiment to analyse the binding specificity of the phagemid particles, and also in an inhibition experiment. In the binding specificity experiment $10^9$ c.f.u. of the phage stock was panned against uncoated microwells and microwells coated with 2 µg of fibrinogen, fibronectin, vitronectin, vWf, IgG, HSA or casein. After 2 h of panning at RT the wells were washed, and the c.f.u. ml$^{-1}$ of the eluate determined as described above. In the inhibition experiment $5 \times 10^7$ c.f.u. ml$^{-1}$ of the phage stock was mixed with chicken antibodies, either unspecific or specific against vWbp, at a final concentration of 0·08–50 µg ml$^{-1}$. After 1 h pre-incubation at RT the mixtures were transferred to vWf-coated microtitre wells (1 µg vWf per well) and incubated for 2 h, followed by washing and determination of the c.f.u. ml$^{-1}$ of the eluate as above.

**Recombinant vWbp (rvWbp-part, rvWbp-mat) and purification of antibodies against rvWbp-part.** A part of the *vwb* gene, encoding amino acids 124–392 of the mature vWbp, was amplified using *Taq* polymerase and the PCR primers 5′-TTAATACCATGGCTAACCCTGAATTGAAAGACTT-3′ (upstream) and 5′-ATTATTATGCGTGTGATTTGAA-3′ (downstream). The PCR product was cleaved with *Nco*I, treated with T4 polynucleotide kinase, ligated into a *Nco*I- and *Sma*I-cleaved pTYB4 vector and subsequently electrotransformed into *E. coli* BL21(DE3) for expression and purification of recombinant protein using the IMPACT T7 expression system (New England Biolabs) with a self-cleavable affinity tag. This was done according to the manufacturer's instructions, yielding essentially pure protein (rvWbp-part) which was used for generation of antibodies in chicken by Immunsystem AB. The antibodies generated were affinity-purified on rvWbp-part immobilized on a HiTrap column (Amersham Biosciences). Later the part of the *vwb* gene encoding the whole mature vWbp (amino acids 1–482) was amplified using Pwo DNA polymerase and the PCR primers 5′-AATATACCATGGTGGTTTCTGGGGAGAAGAAT-3′ (upstream) and 5′-TTTGCCATTATATACTTTATTGAT-3′ (downstream). The same scheme as above was followed and the expressed recombinant whole mature vWbp was designated rvWbp-mat.

**Inibition of the binding between vWf and rvWbp-mat using specific antibodies.** Microwells were coated for 1 h with 100 µl of a solution containing 1 µg rvWbp-mat ml$^{-1}$ and coating buffer (0·05 M $NaHCO_3$, pH 9·5) at RT with shaking. The wells were then washed three times with PBS-T, after which PBS with chicken antibodies, either unspecific or specific against vWbp, were added and incubated for 30 min, whereupon $^{125}$I-labelled vWf (IODO-BEADS Iodination Reagent Kit, Pierce) was added to the wells and the mixture was further incubated for 1 h. The final concentrations of the antibodies were 0·016–50 µg ml$^{-1}$. The wells were washed three times with PBS-T and bound $^{125}$I-vWf was detected with a gamma-radiation counter (Searle).

**Purification and N-terminal sequencing of vWbp from *S. aureus*.** vWbp was purified from an *S. aureus* strain Newman ΔEap mutant (J.-I. Flock, unpublished). One hundred millilitres of an exponential-phase culture ($OD_{600}$ 3·0) was pelleted; the supernatant was sterile-filtered and subsequently passed through a HiTrap column with immobilized anti-vWbp antibodies. The eluate was collected in 1 ml fractions; the fractions were TCA-precipitated, the precipitates were dried and each fraction was resuspended in 10 µl water. The N-terminal sequence of the purified vWbp was determined by Edman sequencing by Bo Ek, Department of Plant Biology, Swedish University of Agricultural Sciences (SLU).

**SDS-PAGE and Western blot analysis.** Protein samples were prepared for gel electrophoresis by mixing equal volumes of protein solution and 2× sample buffer [1× sample buffer is 62·5 mM Tris/HCl pH 6·8, 10 % (v/v) glycerol, 2 % SDS, 5 % β-mercaptoethanol and 0·01 % bromophenol blue]. After boiling, the samples were analysed by SDS-PAGE using the PhastSystem (Amersham Biosciences) with PhastGel Gradient 4–15 % or 8–25 % gels and PhastGel SDS Buffer Strips. Proteins were blotted onto ECL nitrocellulose filters (Amersham Biosience). The presence of vWbp was detected either with chicken anti-vWbp antibodies and HRP–anti-chicken antibodies or with vWf, goat anti-vWf antibodies and HRP–anti-goat antibodies. Bound antibodies were detected with 4-chloro-1-naphthol (Sigma). $^{125}$I-labelled vWf was also used to detect vWbp, and bound $^{125}$I-vWf was visualized with Kodak BioMax MS film.

**Purification of vWf from human serum.** Human serum (15 ml) was passed over a HiTrap column with immobilized rvWbp-part. The eluate was collected in 500 µl portions, which were TCA-precipitated and resuspended in 5 µl distilled water. vWf was detected with goat anti-vWf antibodies and HRP-labelled anti-goat antibodies in a Western blot experiment as described above.

**Peptide sequencing by mass spectrometry.** Protein bands were excised from a Coomassie-blue-stained SDS-PAGE gel and cleaved with trypsin by in-gel digestion. Peptide analysis

was performed by electrospray ionization mass spectrometry according to Wilm *et al*. (1996) on a Q-Tof mass spectrometer using the Masslynx software (Micromass, Manchester, UK). This was done by Håkan Larsson, Department of Plant Biology, Swedish University of Agricultural Sciences (SLU).

**Detection of *vwb* in *S. aureus*.** Chromosomal DNA from eight different *S. aureus* strains and five *S. epidermidis* strains was prepared by using the DNeasy Tissue kit from Qiagen, supplemented with lysostaphin at a final concentration of 250 μg $ml^{-1}$ in the cell lysis step. DNA was cleaved with *Eco*RI, separated on a 0·7 % agarose gel and blotted to a nylon filter using the VaccuGene blotting system (Amersham Biosciences). After UV-fixation the filter was probed overnight at 65 °C with a $^{32}$P-labelled probe spanning the whole *vwb* gene. After washing, the filter was exposed to a Kodak BioMax MR film for 24 h at −70 °C.

## RESULTS AND DISCUSSION

### Panning against vWf reveals a novel vWf-binding protein

When the *S. aureus* library was panned repeatedly against vWf, a strong enrichment of binding phagemid particles occurred. This was manifested both as a large increase in the number of eluted phagemid particles and as an increase in the percentage of clones positive for E-tag expression in consecutive pannings. In the first panning $2 \times 10^4$ phagemid particles were recovered from the well and in the second and the third panning the number of phagemid particles was $5 \times 10^7$ and $2 \times 10^8$, respectively. Accordingly, the frequency of E-tag-positive clones increased from 8 %, to 70 % and to 94 % in the consecutive pannings. Expression of the E-tag reveals that a designed frameshift in the phagemid vector is corrected by the inserted DNA (Jacobsson & Frykberg, 1999). Thus, an enrichment of E-tag-positive clones suggests that an interaction between phagemid particles and ligand has occurred. This, together with the increased number of bound phagemid particles, strongly indicates a specific interaction between the ligand and the displayed polypeptides. After the second and third panning, 32 E-tag-positive clones were isolated and the inserts of the phagemids sequenced. Six of these clones did not harbour any gene of obvious interest and were considered background clones. However, the remaining 26 clones all contained overlapping inserts derived from the same gene (Fig. 1), suggesting that a gene encoding a vWf-binding protein had been identified. The gene was named *vwb* (*von Willebrand factor binding*). When compared to the sequence data from TIGR, the sequence obtained from the phage-display clones turned out to cover almost the entire *vwb*, except the last 44 nucleotides of the gene. The complete *vwb* gene was subsequently cloned from *S. aureus* strain Newman by PCR and the sequence was verified. From the alignment of the different phagemid clones, the vWf-binding region in the corresponding protein was mapped to 26 aa: TSPTTY-TETTTQVPMPTVERQTQQQI, which are equivalent to amino acids 333–358 in the deduced mature protein. The presence of the gene in different staphylococcal strains was tested by Southern blot hybridization with the whole *vwb* gene as a probe. The gene was present in all tested strains of *S. aureus* (Newman, 8325-4, Wood 46, and five different clinical isolates) but not in five *S. epidermidis* strains tested (data not shown).

### Sequence analysis of *vwb*

The *vwb* gene is located between the *clfA* and *emp* genes. *clfA* encodes clumping factor A, the main fibrinogen-binding adhesin in *S. aureus* (McDevitt *et al*., 1994; Ni Eidhin *et al*., 1998) and *emp* encodes an extracellular-matrix-protein-binding protein (Emp), a cell-surface protein with multiple binding activities (Hussein *et al*., 2001). The *clfA*, *vwb* and *emp* genes are all in the same orientation according to the newly published complete genome sequence of two *S. aureus* strains, N315 and Mu50 (Kuroda *et al*., 2001). The same gene organization is also seen in the different *S. aureus* sequencing projects in progress at TIGR (http://www.tigr.org/), University of Oklahoma (http://www.genome.ou.edu/) and the Sanger Centre (http://www.sanger.ac.uk/). The gene *vwb* encodes a previously uncharacterized protein now named von Willebrand factor binding protein (vWbp). Kuroda *et al*. (2001) denote the gene product as a possible staphylocoagulase (function unknown). The amino acid sequences of the staphylocoagulase and vWbp from strain N315 shared 25 % amino acid identity when a pairwise BLAST was performed at NCBI (http://www.ncbi.nlm.nih.gov/). Including the signal sequence, the deduced protein vWbp from strain Newman is composed of 508 aa starting with a leucine. The putative start codon TTG is preceded by a plausible ribosome-binding site (AGGAGA). Sequence analysis shows that the deduced mRNA transcript, after the translation stop codon, has a putative transcription terminator sequence with two stem–loop structures followed by a stretch of six U residues. It is not likely that *vwb* is co-transcribed with *clfA*, since the deduced *clfA* mRNA sequence also contains a putative transcription termination sequence. The sequence of *vwb* obtained from *S. aureus* strain Newman was found to be identical to the sequence available from TIGR (*S. aureus* strain COL) and one of the sequences from the Sanger Centre (a hyper-virulent community-acquired methicillin-sensitive *S. aureus* strain), was also found to be completely identical in all but one nucleotide, leading to the substitution of an isoleucine by a leucine. The *vwb* sequences from the published, complete genomes of *S. aureus* strains N315 (methicillin-resistant) and Mu50 (methicillin-resistant and vancomycin-resistant) are identical to each other but only 80 % identical to the *vwb* sequence from strain Newman. The second sequence from the Sanger Centre (the epidemic methicillin-resistant *S. aureus* strain EMRSA-16), shares about 80 % sequence identity at the nucleotide level compared to strain Newman and strain N315. The *vwb* sequence from the University of Oklahoma (strain 8325) is identical to *vwb* from strain Newman, except for one missing nucleotide. Also in one *vwb* sequence from the Sanger Centre (strain EMRSA-16) one nucleotide is missing but at a different position.

If correct, these frameshifts would lead to early disruptions of the *vwb* ORF but it seems more likely that the missing nucleotide in *vwb* in these two unfinished genomes is due to sequencing errors. Most of the differences between the *vwb* genes from the different strains are found in the first half of the gene. Very few differences are found in the C-terminal half of the deduced protein sequences (data not shown), which includes the vWf-binding domain.

The observed clustering of several genes encoding putative virulence factors is another interesting thing to consider, since many chromosomal virulence determinants are often associated in so-called virulence blocks (Hacker *et al*., 1997). The *clfA* gene is expressed in the exponential growth phase, but the expression is highest during postexponential growth (Ni Eidhin *et al*., 1998; Wolz *et al*., 1996). The conditions for expression of *vwb* and *emp* are not yet clarified at the mRNA level. At the protein level, Emp is detectable from mid-exponential growth and the concentration gradually increases up to stationary phase (Hussein *et al*., 2001). Preliminary data indicate that vWbp is expressed already early in the exponential phase, becomes more abundant during the exponential growth and decreases in the stationary growth phase (data not shown).

### Characterization of vWbp and its interaction with vWf

vWbp has a functional secretory signal sequence, with positively charged lysine residues in the N-terminal region, a central hydrophobic region and a more polar C-terminal region containing a signal peptidase cleavage sequence with alanines at the −3 and −1 positions. However, vWbp has no cell-wall-anchoring sequence typical of surface-bound proteins in Gram-positive bacteria. Thus, the protein should be found in a soluble form outside the bacteria. To further investigate vWbp, the part of the *vwb* gene encoding amino acids 124–392 of the mature vWbp was expressed in *E. coli* and the recombinant protein was purified (rvWbp-part). Chickens were subsequently immunized with rvWbp-part and the resulting anti-vWbp antibodies were used to detect vWbp in the concentrated culture supernatant of *S. aureus* strain Newman. In a Western blot, the anti-vWbp antibodies specifically recognized a protein of ~66 kDa. A protein of the same size was also detected by using vWf and anti-vWf-antibodies or by $^{125}$I-labelled vWf directly (Fig. 2).

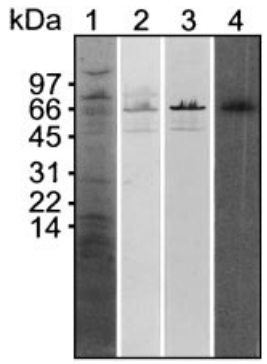


**Fig. 2.** SDS-PAGE and Western blot of culture medium from an exponential-phase culture of *S. aureus* strain Newman, concentrated 100-fold. Lanes: 1, Coomassie-blue-stained 8–25 % SDS-PAGE gel; 2, Western blot treated with chicken antibodies against vWbp and HRP-labelled anti-chicken antibodies; 3, Western blot (of an identical gel) treated with vWf, goat anti-vWf antibodies and HRP-labelled anti-goat antibodies to detect the interaction; 4, autoradiograph of a Western blot treated with $^{125}$I-labelled vWf.

To prove that the protein detected in the culture supernatant was indeed vWbp, attempts were made to purify the protein on immobilized anti-vWbp antibodies. Surprisingly, this resulted in isolation of the staphylococcal protein Map (Jönsson *et al*., 1995; McGavin *et al*., 1993)/Eap (Palma *et al*., 1999) as confirmed by N-terminal sequencing of the purified material. The N-terminals of mature Map and Eap are very similar, but not identical. Another *S. aureus* protein, p70 (Fujigaki *et al*., 1998), is also similar to Eap and Map (Smeltzer, 2000). Since these proteins with similar characteristics were isolated from different strains of *S. aureus* – Eap from strain Newman, Map from strain FDA 574, p70 from strain Wood 46 – they might be encoded by allelic variants of the same gene. Eap is known to bind to itself in addition to various host proteins. However, Eap does not bind chicken antibodies in an non-immunogenic manner, nor does it cross-react with the anti-vWbp antibodies (data not shown). From these results it is reasonable to assume that the isolation of Eap is not due to binding to the anti-vWbp antibodies, but instead is a result of a direct interaction between Eap and vWbp. Indeed, vWbp could be purified from the culture medium of an isogenic Eap-deletion mutant of *S. aureus* strain Newman. The N-terminal sequence of secreted vWbp was determined by Edman N-terminal sequencing. The resulting sequence was VVSGEKNPYV, which fully corresponds to the N-terminal sequence of the anticipated mature vWbp consisting of 482 aa. The predicted molecular mass of the mature vWbp from the amino acid sequence (56·5 kDa) is smaller than the apparent molecular mass deduced from polyacrylamide gels (~66 kDa). The same result was obtained under both reducing and non-reducing conditions. It is not uncommon that proteins have altered migratory properties in polyacrylamide gels, particularly the ones with unusual amino acid compositions. In vWbp, the percentages of acidic (DE) and basic (KR) amino acids are surprisingly high, but well balanced (DE 18 %, KR 19 %), giving the protein an estimated isoelectric point very close to 7. This means that the net charge under physiological (host) conditions should be close to zero. Phagemid particles displaying amino acids 139–428 of the mature vWbp (clone NvWb32) were assayed for binding to vWf and to a number of other mammalian proteins. The results showed that NvW32 bound at least 1000 times more efficiently to vWf than to any of the other proteins tested

**Table 1.** Binding study with phagemid particles displaying the vWf-binding region (clone NvWb32)

The phagemid particles were panned against different mammalian proteins and the number of bound phagemid particles was determined as c.f.u. $ml^{-1}$. The results from one typical experiment are shown.

| Ligand | Bound phagemid (c.f.u. $ml^{-1}$) |
|---|---|
| vWf | $3 \times 10^7$ |
| Fibronectin | $4 \times 10^4$ |
| Fibrinogen | $5 \times 10^3$ |
| Vitronectin | $1 \times 10^3$ |
| IgG | $1 \times 10^3$ |
| HSA | $1 \times 10^3$ |
| Casein | $2 \times 10^3$ |
| None (uncoated well) | $3 \times 10^3$ |

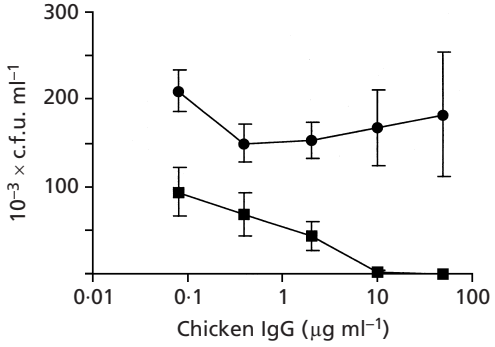


**Fig. 3.** Inhibition study with phagemid particles displaying the vWf-binding domain. The phagemid particles were panned against vWf in the presence of antibodies against vWbp (■) or unspecific antibodies (●) at different concentrations. The number of bound phagemid particles was determined as c.f.u. $ml^{-1}$. Values are mean ± SD from two experiments.

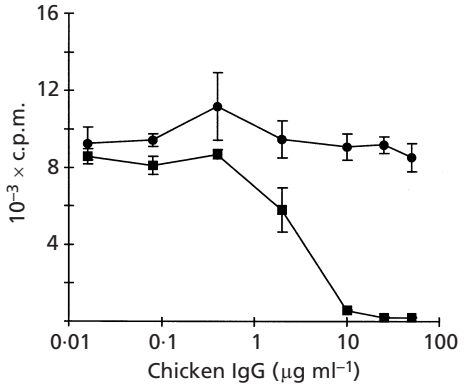


**Fig. 4.** Inhibition of binding of $^{125}$I-labelled vWf to immobilized rvWbp-mat by chicken antibodies, either specific against vWbp (■) or unspecific (●). Values are mean ± SD from two experiments.

(Table 1). This binding could be inhibited by specific antibodies against vWbp, while unspecific antibodies did not affect the interaction (Fig. 3). To perform a more direct inhibition experiment, the mature vWbp (482 aa) was expressed as recominant protein (rvWbp-mat) in *E. coli* and purified. The binding of $^{125}$I-vWf to rvWbp-mat immobilized in microwells was inhibited in a concentration-dependent manner by specific antibodies against vWbp, but not by unspecific antibodies (Fig. 4). These different experiments show that vWbp can bind vWf in both immobilized and soluble form. Further, we could affinity-purify vWf from human serum by passing it over a column with immobilized rvWbp-part (Fig. 5). The successful purification of vWf from a complex solution like human serum demonstrates the specificity of the interaction between vWbp and vWf. The protein band recognized by vWf-specific antibodies was further verified as vWf by peptide sequencing by electrospray ionization mass spectrometry (data not shown). The other two major bands were also sequenced by mass spectrometry and found to be properdin P (~53 kDa) and HSA (~66 kDa). Properdin P is mainly composed of six trombospondin type I repeats (Nolan *et al*., 1992; Lawler & Hynes, 1986), and plays an important role in the alternative pathway of the complement system by stabilizing the C3bBb complex (Fearon & Austin, 1975). Although a direct interaction between properdin P and vWf has to our knowledge not been demonstrated, trombospondin-1 (TSP-1) has been reported to interact with the vWf A3 domain via such repeats, also known as TSP-1 type I properdin domains (Xie *et al*., 2001). Thus, we find it more likley that the co-purification of properdin P is due to to an interaction between properdin P and vWF rather than binding to rvWbp-part. HSA, on the other hand, is most likely a contaminant due to the huge amount present in human serum.

### Possible consequences of the interaction between *S. aureus* and vWf

A recent publication demonstrated that protein A, besides its IgG-binding ability, also has affinity to vWf, and consequently protein A promotes adhesion of *S. aureus* to a vWf-coated surface (Hartleib *et al*., 2000). This means that *S. aureus* has at least two proteins recognizing vWf, one cell-surface-attached and one secreted as reported in this paper. To further complicate the picture, it has been reported that protein A in some strains exists in a soluble form, not bound to the surface of *S. aureus* (Lindmark *et al*., 1977). The function and importance of vWpb during *S. aureus* infections is not yet known. The frequency of *S. aureus* strains carrying the *vwb* gene is high (15/15 discussed in this paper), which indicates an important role for the encoded protein. Since the extracellular fibrinogen-binding protein, Efb, has been shown to contribute to virulence and to delay wound healing (Palma *et al*., 1996), it is tempting to speculate that vWbp also could have similar

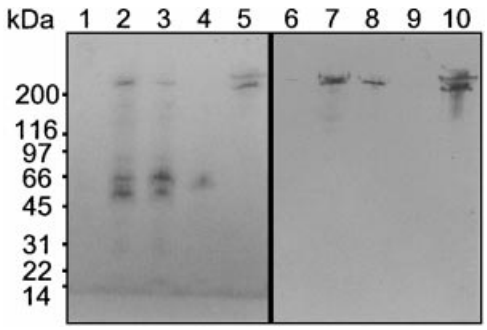


**Fig. 5.** Purification of vWf from human serum on a HiTrap column with immobilized rvWbp-part. The figure shows eluted fractions run on a Coomassie-blue-stained SDS-PAGE gel (left panel) and corresponding Western blot (right panel) treated with goat antibodies against vWf and secondary HRP-labelled anti-goat antibodies. Lanes: 1–4 and 6–9, eluate from the rvWbp-part column concentrated 100-fold; 5 and 10, recombinant vWf.

functions, since both fibrinogen and vWf are crucial components in the events leading to the formation of blood clots. Actually, in experimental settings mimicking a vascular wound during rapid blood flow as in the normal arteriole, platelet adhesion and aggregation require vWf but not fibrinogen (Savage *et al*., 1996; Ikeda *et al*., 1991; Weiss *et al*., 1989). Therefore, the bacteria may benefit from also disturbing the platelet–vWf interaction in the wound healing process. Further, it is interesting to consider the putative interaction between Eap, a sticky protein secreted by *S. aureus*, and vWbp. Eap can bind back to the bacteria, as well as to various components of the extracellular matrix, thus serving as an adhesive bridge between host cells and *S. aureus* (Palma *et al*., 1999). If vWbp binds vWf at sites of vascular damage and Eap binds to vWbp, these interactions may function as a homing device and subsequently direct the bacteria to the wound where colonization can occur.

## ACKNOWLEDGEMENTS

We thank Professor F. Dorner, Vienna, Austria, for the kind gift of recombinant vWf and Dr B. Christensson, University of Lund, Sweden, for kindly providing clinical isolates of *S. aureus*. We also thank Dr B. Ek and Dr H. Larsson, SLU, Sweden, for the protein sequencing work and Professor M. Lindberg, SLU, Sweden, for comments on this manuscript. This work was supported by the Swedish Foundation for Strategic Research, Infection & Vaccinology Research Program, grant 24/98, Biostapro AB and the Swedish Medical Research Council, grants K2000-16X-13058-02B and 06x-11229.

## REFERENCES

**Coulter, S. N., Schwan, W. R., Ng, E. Y. & 7 other authors (1998).** *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. *Mol Microbiol* **30**, 393–404.

**Darouiche, R. O., Landon, G. C., Patti, J. M., Nguyen, L. L., Fernau, R. C., McDevitt, D., Greene, C., Foster, T. & Klima, M. (1997).** Role of *Staphylococcus aureus* surface adhesins in orthopaedic device infections: are results model-dependent? *J Med Microbiol* **46**, 75–79.

**Fearon, D. T. & Austin, K. F. (1975).** Properdin: binding to C3b and stabilisation of the C3b-dependent C3 convertase. *J Exp Med* **142**, 856–863.

**Fischer, B. E., Schlokat, U., Reiter, M., Mundt, W. & Dorner, F. (1997).** Biochemical and functional characterization of recombinant von Willebrand factor produced on a large scale. *Cell Mol Life Sci* **53**, 943–950.

**Fujigaki, Y., Yousif, Y., Morioka, T., Batsford, S., Vogt, A., Hishida, A. & Miyasaka, M. (1998).** Glomerular injury induced by cationic 70-kD staphylococcal protein; specific immune response is not involved in early phase in rats. *J Pathol* **184**, 436–445.

**Gemmell, C. G., Goutcher, S. C., Reid, R. & Sturrock, R. D. (1997).** Role of certain virulence factors in a murine model of *Staphylococcus aureus* arthritis. *J Med Microbiol* **46**, 208–213.

**Hacker, J., Blum-Oehler, G., Muhldorfer, I. & Tschape, H. (1997).** Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol Microbiol* **23**, 1089–1097.

**Hartleib, J., Kohler, N., Dickinson, R. B. & 7 other authors (2000).** Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. *Blood* **96**, 2149–2156.

**Hienz, S. A., Schennings, T., Heimdahl, A. & Flock, J. I. (1996).** Collagen binding of *Staphylococcus aureus* is a virulence factor in experimental endocarditis. *J Infect Dis* **174**, 83–88.

**Hussein, M., Becker, K., von Eiff, C., Schrenzel, J., Peters, G. & Herrmann, M. (2001).** Identification and characterization of a novel 38·5-kilodalton cell surface protein of *Staphylococcus aureus* with extended-spectrum binding activity for extracellular matrix and plasma protein. *J Bacteriol* **183**, 6778–6786.

**Ikeda, Y., Handa, M., Kawano, K. & 9 other authors (1991).** The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *J Clin Invest* **87**, 1234–1240.

**Jacobsson, K. & Frykberg, L. (1995).** Cloning of ligand-binding domains of bacterial receptors by phage display. *Biotechniques* **18**, 878–885.

**Jacobsson, K. & Frykberg, L. (1996).** Phage display shot-gun cloning of ligand-binding domains of prokaryotic receptors approaches 100% correct clones. *Biotechniques* **20**, 1070–1076.

**Jacobsson, K. & Frykberg, F. (1999).** Gene VIII-based, phage display vectors for selection against complex mixtures of ligands. In *Expression Genetics: Accelerated and High-throughput Methods*, pp. 225–238. Edited by M. McClelland & A. B. Pardee. Natick, MA: Eaton Publishing.

**Jönsson, K., McDevitt, D., McGavin, M. H., Patti, J. M. & Hook, M. (1995).** *Staphylococcus aureus* expresses a major histocompatibility complex class II analog. *J Biol Chem* **270**, 21457–21460.

**Kuroda, M., Ohta, T., Uchiyama, I. & 34 other authors (2001).** Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* **357**, 1225–1240.

**Lawler, J. & Hynes, R. O. (1986).** The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. *J Cell Biol* **103**, 1635–1648.

**Lindmark, R., Movitz, J. & Sjoquist, J. (1977).** Extracellular protein A from a methicillin-resistant strain of *Staphylococcus aureus*. *Eur J Biochem* **74**, 623–628.

**Lowy, F. D. (1998).** *Staphylococcus aureus* infections. *N Engl J Med* **339**, 520–532.

**McDevitt, D., François, P., Vaudaux, P. & Foster, T. J. (1994).** Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. *Mol Microbiol* **11**, 237–248.

**McGavin, M. H., Krajewska-Pietrasik, D., Ryden, C. & Hook, M. (1993).** Identification of a *Staphylococcus aureus* extracellular matrix-binding protein with broad specificity. *Infect Immun* **61**, 2479–2485.

**Mohlke, K. L., Nichols, W. C. & Ginsburg, D. (1999).** The molecular basis of von Willebrand disease. *Int J Clin Lab Res* **29**, 1–7.

**Moreillon, P., Entenza, J. M., Francioli, P., McDevitt, D., Foster, T. J., François, P. & Vaudaux, P. (1995).** Role of *Staphylococcus aureus* coagulase and clumping factor in pathogenesis of experimental endocarditis. *Infect Immun* **63**, 4738–4743.

**Ni Eidhin, D., Perkins, S., François, P., Vaudaux, P., Hook, M. & Foster, T. J. (1998).** Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. *Mol Microbiol* **30**, 245–257.

**Nolan, K. F., Kalutz, S., Higgins, J. M., Goundis, D. & Reid, K. B. (1992).** Characterization of the human properdin gene. *Biochem J* **287**, 291–297.

**Palma, M., Nozohoor, S., Schennings, T., Heimdahl, A. & Flock, J. I. (1996).** Lack of the extracellular 19-kilodalton fibrinogen-binding protein from *Staphylococcus aureus* decreases virulence in experimental wound infection. *Infect Immun* **64**, 5284–5289.

**Palma, M., Haggar, A. & Flock, J. I. (1999).** Adherence of *Staphylococcus aureus* is enhanced by an endogenous secreted protein with broad binding activity. *J Bacteriol* **181**, 2840–2845.

**Patel, A. H., Nowlan, P., Weavers, E. D. & Foster, T. (1987).** Virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. *Infect Immun* **55**, 3103–3110.

**Patti, J. M., Bremell, T., Krajewska-Pietrasik, D., Abdelnour, A., Tarkowski, A., Ryden, C. & Hook, M. (1994).** The *Staphylococcus aureus* collagen adhesin is a virulence determinant in experimental septic arthritis. *Infect Immun* **62**, 152–161.

**Rhem, M. N., Lech, E. M., Patti, J. M., McDevitt, D., Hook, M., Jones, D. B. & Wilhelmus, K. R. (2000).** The collagen-binding adhesin is a virulence factor in *Staphylococcus aureus* keratitis. *Infect Immun* **68**, 3776–3779.

**Ruggeri, Z. M. (1999).** Structure and function of von Willebrand factor. *Thromb Haemostasis* **82**, 576–584.

**Ruggeri, Z. M. & Ware, J. (1993).** von Willebrand factor. *FASEB J* **7**, 308–316.

**Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

**Savage, B., Saldivar, E. & Ruggeri, Z. M. (1996).** Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell* **84**, 289–297.

**Smeltzer, M. S. (2000).** Characterization of staphylococcal adhesins for adherence to host tissues. In *Handbook of Bacterial Adhesion. Principles, Methods, and Applications*, pp. 411–444. Edited by Y. H. An & R. J. Friedman. Totowa, NJ: Humana Press.

**Wann, E. R., Fehringer, A. P., Ezepchuk, Y. V., Schlievert, P. M., Bina, P., Reiser, R. F., Hook, M. M. & Leung, D. Y. (1999).** *Staphylococcus aureus* isolates from patients with Kawasaki disease express high levels of protein A. *Infect Immun* **67**, 4737–4743.

**Weiss, H. J., Hawiger, J., Ruggeri, Z. M., Turitto, V. T., Thiagarajan, P. & Hoffmann, T. (1989).** Fibrinogen-independent platelet adhesion and thrombus formation on subendothelium mediated by glycoprotein IIb–IIIa complex at high shear rate. *J Clin Invest* **83**, 288–297.

**Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T. & Mann, M. (1996).** Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **378**, 466–469.

**Wolz, C., McDevitt, D., Foster, T. J. & Cheung, A. L. (1996).** Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* Newman. *Infect Immun* **64**, 3142–3147.

**Xie, L., Chesterman, C. N. & Hogg, P. J. (2001).** Control of von Willebrand factor multimer size by thrombospondin-1. *J Exp Med* **193**, 1341–1349.

**Yatohgo, T., Izumi, M., Kashiwagi, H. & Hayashi, M. (1988).** Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct Funct* **13**, 281–292.

Received 18 February 2002; accepted 5 March 2002.