The Multifunctional *Staphylococcus aureus* Autolysin Aaa Mediates Adherence to Immobilized Fibrinogen and Fibronectin

Christine Heilmann,* Jörg Hartleib, Muzaffar S. Hussain, and Georg Peters

Institute of Medical Microbiology, University of Münster, Domagkstr. 10, D-48149 Münster, Germany

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Staphylococci can cause a wide spectrum of infections, including endocarditis, osteomyelitis, and sepsis, which is reflected by the numerous virulence factors they produce, among them a recently identified new class of adhesins, namely, the multifunctional autolysins/adhesins. Here we report the identification and molecular characterization of Aaa, a novel autolysin/adhesin from *Staphylococcus aureus*. The gene encoding Aaa was cloned from the clinical isolate *Staphylococcus aureus* 4074. DNA sequence analysis revealed that *aaa* encodes a deduced protein of 334 amino acids with a predicted molecular mass of 35.8 kDa. Aaa contains three N-terminal repetitive sequences that comprise features of a peptidoglycan-binding domain, the LysM domain. The expression of *aaa* by *Escherichia coli* and its subsequent characterization revealed that Aaa possesses bacteriolytic activity as well as adhesive properties, such as binding to extracellular matrix proteins. Real-time biomolecular interaction analysis demonstrated that the interaction of Aaa with fibrinogen, fibronectin, and vitronectin is dose dependent and saturable and occurs with a high affinity. Furthermore, we demonstrate that Aaa binds to the α and β chains of fragment D of fibrinogen. Immunofluorescence microscopy revealed that Aaa is located at the cell surface. Finally, an *aaa* knockout mutant showed reduced adherence to surface-adsorbed fibrinogen and fibronectin, strongly suggesting a role for Aaa in the colonization of host factor-coated polymer surfaces and/or host tissue.

Staphylococcus aureus is a major human pathogen responsible for a variety of community-acquired and nosocomial infections, ranging from mild skin diseases, abscesses, and soft tissue diseases to life-threatening infections such as osteomyelitis, endocarditis, pneumonia, and sepsis (37). The adherence of bacteria to host tissue or to implanted medical devices plays a crucial role in the development of disease. The adherence of bacteria to biological or artificial surfaces can occur either directly or via bridging molecules, such as the extracellular matrix and plasma proteins fibrinogen (Fg), fibronectin (Fn), and vitronectin (Vn), and may be followed by proliferation of the bacteria into multilayered cell clusters, leading to the formation of a three-dimensional structure of microorganisms known as a biofilm. Various genes for host factor-binding proteins from *S. aureus* that typically belong to the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family (49) have been cloned and sequenced (8, 11, 13, 24, 25, 28, 29, 41, 48, 50). However, the mechanisms leading to colonization are not yet completely understood.

Recently, a new class of staphylococcal adhesins, the autolysins/adhesins, was proposed. Members include AtlE from *Staphylococcus epidermidis* (20), the homologous Aas protein from *Staphylococcus saprophyticus* (22), and AtlC from *Staphylococcus caprae* (1). These surface-associated proteins have both enzymatic (amidase and glucosaminidase) and adhesive functions (1, 17, 20, 22). AtlE is involved in initial attachment of the cells to a polymer surface and in biofilm formation. It also binds to Vn, suggesting a role not only in colonizing

polymer surfaces but also in colonizing host factor-coated material and host tissue. Recent results demonstrated the importance of AtlE in *S. epidermidis* pathogenicity: an *atlE* mutant strain was significantly less virulent than the wild type in an intravascular catheter-associated infection model in rats (55). Aas and AtlC bind to Fn, and Aas also agglutinates sheep erythrocytes (1, 22). Recently, we identified a novel autolysin with adhesive functions, Aae, from *S. epidermidis* (21). Here we describe the molecular characterization of Aaa, a homologous autolysin/adhesin from *S. aureus*. Furthermore, our results demonstrate that Aaa is located at the cell surface, has bacteriolytic activity, and is involved in the adherence of *S. aureus* to surface-adsorbed Fg and Fn.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The clinical strain *S. aureus* 4074 (19) was used to clone the *aaa* gene and to generate the *aaa*-deficient mutant. The protein A-deficient strain *S. aureus* Wood 46 (44) was used for immunofluorescence microscopy; the presence of the *aaa* gene in that strain was confirmed by PCR (see below). Recombinant plasmids prepared in *Escherichia coli* were passaged in the restriction-negative mutant *S. aureus* SA113 (27). Cloning hosts were *E. coli* INVαF' (Invitrogen, Groningen, The Netherlands) and *E. coli* TG1.

For cloning of *aaa*, the vector pCR2.1 (Invitrogen) was used, and for the expression of a six-His-tagged fusion protein, the Qiaexpress vector pQE30 (QIAGEN, Hilden, Germany) was used. The *aaa*-deficient mutant was constructed by using the plasmids pEC4 and pBT9 (9).

Staphylococci were routinely cultivated in brain heart infusion (BHI) broth or Trypticase soy broth (TSB). *E. coli* strains were grown in Luria-Bertani (LB) medium. Trypticase soy agar (TSA), BHI agar, and LB agar contained 1.4% agar. Selection for resistance to antibiotics in *E. coli* was performed with 100 µg/ml ampicillin and 200 µg/ml erythromycin, and selection for that in *S. aureus* was performed with 10 µg/ml chloramphenicol and 5 µg/ml erythromycin.

DNA manipulations, transformation, PCR, DNA sequencing, and websites. DNA manipulations and the transformation of *E. coli* were performed according to standard procedures (56). *S. aureus* was transformed with plasmid DNA by electroporation (36). Plasmid DNA was isolated using a QIAGEN plasmid kit according to the instructions of the manufacturer (QIAGEN). Chromosomal

* Corresponding author. Mailing address: Institute of Medical Microbiology, University of Münster, Domagkstr. 10, D-48149 Münster, Germany. Phone: 49-251-8355357. Fax: 49-251-8355350. E-mail: heilmann@uni-muenster.de.

DNA from *S. aureus* was isolated according to the procedure of Marmur (40). PCR was carried out with *Taq* DNA polymerase (Boehringer Mannheim) or with PCR High Fidelity Supermix (Invitrogen) in accordance with the protocol of the supplier. The primers were synthesized by MWG-Biotech (Ebersberg, Germany).

The following primers were used (restriction sites are underlined). For cloning and sequencing of the *aaa* gene, the primers were primer CH55 (5'-CAG GGA TCC GGA AAG TTA AGC AAG AGG AGG-3' [BamHI site]) and primer CH57 (5'-GCA CTG CAG CAT TAT ATA TTT ATA TAC GTA AGA C-3' [PstI site]). For the construction of the six-His-Aaa fusion protein, the primers were primer CH94 (5'-CAG GGA TCC GCT ACA ACT CAC ACA GTA AAA CCG GG-3' [BamHI site]) and CH57 (see above). For the construction of the *aaa*-deficient mutant, the primers were AaaKn1 (5'-CAA ATT CAG ATG CAG TAA AGC C-3' [including nucleotides 517 to 538 upstream of the *aaa* start codon]), AaaKn2 (5'-GTT TAA GCA CTG TAG GAA CCG-3' [including nucleotides 1315 to 1335 downstream of the *aaa* start codon]), AaaKn3 (5'-CAG GTC GAC CCG CAT TTG CTT GAG TTG CCG C-3' [including nucleotides 55 to 76 of the *aaa* gene and a Sall site]), and AaaKn4 (5'-GCT AAT AAC TGG GAT AAC GCA GCG G-3' [including nucleotides 769 to 793 downstream of the *aaa* start codon]).

To determine the prevalence of *aaa*, the *aaa* gene was amplified from genomic DNAs of various *S. aureus* clinical isolates and *S. aureus* strains Wood 46 and Mu50 by using the primers CH56 (5'-CAG GGA TCC GTG CAA AAA AAA GTA ATT GCA GC-3' [BamHI site]) and CH57 (see above).

The PCR-amplified *aaa* gene was cloned into the vector pCR2.1 by use of a TA cloning kit (Invitrogen) according to the manufacturer's instructions. The DNA sequences of both strands of *aaa* were determined by the dideoxy chain termination method using a cycle sequencing protocol with a GeneAmp PCR system 2400 (Perkin-Elmer) on an ABI Prism 310 genetic analyzer (Perkin-Elmer) or by MWG-Biotech using a LI-COR DNA sequencer.

The DNA and deduced protein sequences were analyzed using the program PC/GENE (IntelliGenetics). The protein sequences were compared with those of known proteins using the programs BLASTP (2) and FASTA (51). Alignments were done using the program ClustalW from the European Bioinformatics Institute (Cambridge, United Kingdom). Homologous *aaa* or its promoter sequences were analyzed from *S. aureus* strains COL (The Institute for Genomic Research [http://www.tigr.org/]), N315 (34), Mu50 (34), MW2 (3), NCTC8325 (26), MSSA476, and MRSA252 (both from The Wellcome Trust Sanger Institute [http://www.sanger.ac.uk/Projects/S_aureus/]). The Pfam accession number of the LysM motif is Pf01476 and is available at http://pfam.wustl.edu and at http://www.sanger.ac.uk/Software/Pfam (4, 31, 54). The signal peptide of Aaa was predicted by using the program SignalP at http://www.cbs.dtu.dk/services/SignalP/.

Construction and purification of six-His-Aaa fusion protein and production of anti-Aaa antiserum. For the construction of the His-tagged fusion protein, the primers CH94 and CH57 were used to amplify the *aaa* gene without the nucleotide sequences encoding the signal peptide from the genomic DNA of *S. aureus* 4074, introducing a BamHI site at the 5' end and a PstI site at the 3' end. The PCR-amplified fragment was cloned into the vector pQE30 so that the *aaa* gene was in frame with the His codons. A representative clone that expressed the *aaa* gene was designated *E. coli*(pQAaa). The respective His-tagged fusion protein was purified under denaturing conditions via its His tag using Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Ni-NTA spin kit; QIAGEN) according to the protocol of the supplier. The yield of the fusion protein was in the range of 150 µg per 10 ml culture volume, as determined by the Pierce BCA protein assay (Pierce Europe B.V., BA oud-Beijerland, The Netherlands). For use in Biacore experiments, ligand affinity blot analysis, Western blot analysis, and immunization of rabbits, the Aaa protein solution was dialyzed against 0.1 M phosphate buffer (pH 7.0) to remove urea. The purified Aaa protein was used to immunize rabbits, which was performed by Eurogentec (Belgium) according to their standard immunization program.

Protein isolation, SDS-PAGE, detection of bacteriolytic activity, and Western blot and ligand affinity blot analysis. Surface-associated proteins of *S. aureus* 4074 and the *aaa* mutant were prepared from cultures that were grown overnight in BHI broth at 37°C. The cells were harvested by centrifugation, and the cell pellet was resuspended in 1 volume of Laemmli (SDS) sample buffer and then heated for 5 min at 95°C. Crude cell lysates of *E. coli*(pQAaa) were prepared from noninduced and induced (by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside [IPTG] and continued growth for 5 h) cultures by harvesting the cells, resuspending the cell pellet in sample buffer, and heating the suspension for 5 min at 95°C. Additionally, as a negative control, a crude cell lysate was prepared from an induced culture of *E. coli*(pQE30). After centrifugation, 10 µl of supernatant containing SDS-soluble, surface-associated staphylococcal pro-

teins, 10 µl of cell lysate from *E. coli*, or 4 µl of the purified protein (containing 1.5 µg) was subjected to SDS-PAGE (10% or 12.5% separation gel and 4.5% stacking gel). Proteins were stained with Coomassie brilliant blue R250 (0.1%).

Zymographic analysis of the bacteriolytic activities of protein preparations was performed as described earlier (20). Briefly, surface-associated proteins or crude cell lysates were isolated as described above and separated by SDS-PAGE on a polyacrylamide gel containing heat-inactivated staphylococcal cells (0.05%) as a substrate in the separation gel (12.5%). After electrophoresis, the gels were washed and then incubated in phosphate buffer at 37°C. Bands with bacteriolytic activity were observed as clear zones in the opaque gel. After photography against a dark background, the clear zones appeared as dark bands.

For Western blot analysis, surface-associated proteins were prepared and separated by SDS-PAGE as described above and then transferred to a nitrocellulose membrane (Schleicher & Schuell). The membranes were then blocked in Tris-buffered saline (TBS)-5% bovine serum albumin (BSA) (overnight) and washed three times with TBS-0.05% Tween 20 (TBST). Afterwards, the nitrocellulose membranes were incubated for 2.5 h with the anti-Aaa antiserum diluted 1:2,000 in TBST-1% normal goat serum (NGS) (Dako Cytomation GmbH, Hamburg, Germany). As a negative control, incubation was performed in TBST-1% NGS with (1:2,000) or without preimmune serum. The reaction of proteins with specific antibodies was detected by incubation (1.5 h) with an anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase (AP) conjugate (Dako) diluted 1:2,000 in TBST-1% NGS and by the subsequent color reaction.

To determine the binding domains of Fg for Aaa, we performed ligand affinity blot analysis. For this, the disulfide bonds between the Fg chains (Aα, Bβ, and γ chains) were reduced by heating Fg (Calbiochem, Darmstadt, Germany) in sample buffer (Bio-Rad). Additionally, fragments D (85-kDa protein band under nonreducing conditions; Calbiochem) and E (50-kDa protein band under nonreducing conditions; Calbiochem) of human Fg were tested. The Fg chains (5 µg per lane) or Fg fragments D and E under nonreducing conditions (5 µg per lane) were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane. Afterwards, the membranes were blocked in TBST-5% BSA and then incubated with 2 µg/ml purified Aaa in TBST-1% BSA. As a negative control, incubation was performed in TBST-1% BSA without a ligand or with 2 µg/ml six-His-dihydrofolate reductase (purified from a culture of *E. coli* harboring the control plasmid pQE40 [QIAGEN]) in TBST-1% BSA. Bound proteins were detected by incubation with a Ni-NTA-AP conjugate (QIAGEN) diluted 1:3,500 in TBST-1% BSA and by the subsequent color reaction (Bio-Rad).

Real-time BIA for quantification of molecular interactions. Experiments were performed on a BIAcore 2000 instrument according to the general procedures recommended by the manufacturer. Aaa was immobilized on the sensor chip C1 to analyze its interaction with the extracellular matrix proteins Fg, Fn, and Vn. For immobilization, Aaa was covalently coupled to the sensor chip surface C1 via primary amine groups. To prepare the surface of the sensor chip, a freshly prepared 0.1 M glycine-NaOH (pH 12), 0.3% Triton X-100 solution was injected at a flow rate of 20 µl/min (twice, with 30 µl each time). Afterwards, to activate the sensor chip, a mixture of 50 µl NHS-EDC [0.1 M N-hydroxysuccinimide (NHS) and N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (EDC)] (amine coupling kit; Biacore AB) was injected at a flow rate of 5 µl/min. The autolysin Aaa (approximately 8 µg/ml) in 10 mM sodium acetate buffer (pH 5.0) was immobilized on the surface. Remaining NHS-ester groups on the sensor chip surface were then blocked by the injection of 55 µl of 1 M ethanolamine (pH 8.5) (amine coupling kit; Biacore AB). Nonspecific binding of the extracellular matrix proteins to the sensor surface was avoided by coating the surface with 0.2 mg/ml BSA in HBS buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant, pH 7.4) at a flow rate of 5 µl/min. HBS buffer was also used as the running buffer. After Aaa was immobilized on the C1 sensor chip surface (211 pg Aaa/0.26 mm²), extracellular matrix proteins in increasing concentrations were injected over the sensor surface for the kinetic studies at a flow rate of 30 µl/min. The binding of extracellular matrix proteins was monitored and presented in an overlay plot of the sensorgrams (a plot of resonance units versus time). Kinetic data were determined at 25°C. For some experiments, Fg was preincubated with different concentrations of peptides (HHLGGAKQAGDV, GRGDSP [both from Bachem AG, Weil am Rhein, Germany] and FYQVLNMPNLNA [synthesized by Eurogentec]) before injection over the sensor surface. For regeneration of the sensor chip surface after each injection, 100 mM glycine (pH 10.5) was used, resulting in a return to baseline. Kinetic data were analyzed using biomolecular interaction analysis (BIA) evaluation software (version 3.1) from Biacore AB as described previously (32).

Immunofluorescence microscopy. Immunofluorescence microscopy was performed essentially as described before (20). Briefly, an aliquot (500 µl) of a culture of *S. aureus* strain Wood 46, which is deficient in the IgG-binding protein A, was washed in phosphate-buffered saline (PBS; 500 µl) and then incubated

with 500 μ l of rabbit anti-Aaa antibodies or preimmune serum diluted 1:500 in PBS for 2 h at 37°C. Afterwards, the cells were washed three times with PBS, and bound rabbit antibodies were detected by incubation with 500 μ l of fluorescein-conjugated anti-rabbit IgG (Sigma) diluted 1:500 in PBS for 1 h at 37°C. Thereafter, cells were washed three times with PBS and resuspended in 200 μ l of distilled water. Aliquots (15 μ l) were applied to slides, air dried, and viewed with a fluorescence microscope (Zeiss, Oberkochen, Germany).

Construction of an *aaa*-deficient mutant in *S. aureus* by gene replacement (*aaa::Ery^r*). (i) **Cloning of the *aaa* gene.** The *aaa* gene was amplified from the chromosomal DNA of *S. aureus* 4074 by PCR with primers AaaKn1 and AaaKn2, yielding a 1,873-bp DNA fragment. The amplified DNA fragment was cloned into the vector pCR2.1, creating plasmid pA2.

(ii) **Insertion of the *ermB* gene.** Plasmid pA2 was amplified by inverse PCR using primers AaaKn3 and AaaKn4, which deleted an internal 693-bp fragment of the *aaa* gene, yielding a 5.1-kb DNA fragment with a *Sal*I restriction site at the 5' end and a blunt end at the 3' end. The deleted fragment was then replaced by the 1.4-kb erythromycin cassette *ermB* taken from plasmid pEC4, creating the 6.5-kb plasmid pA210. The *aaa-ermB-aaa* fragment was isolated from plasmid pA210 as a 2.6-kb *Sst*I and *Xba*I fragment that was then ligated to the temperature-sensitive shuttle vector pBT9. *E. coli* was transformed with the ligation mixture, and the resulting plasmid was designated pBA3.

(iii) **Insertional inactivation of *aaa* in *S. aureus* 4074.** Plasmid pBA3 was propagated in strain SA113 and then transferred to *S. aureus* 4074 by electroporation. For gene disruption experiments, one transformant was cultivated in TSB with chloramphenicol (10 μ g/ml) at 30°C overnight. The culture was diluted 1:1,000 in fresh medium containing erythromycin (5 μ g/ml) and grown at 44°C. After an identical second culture, a 1:1,000 dilution was plated onto TSA containing erythromycin (5 μ g/ml) and incubated at 44°C. Resulting colonies were checked for resistance to chloramphenicol. Successful recombination in erythromycin-resistant and chloramphenicol-sensitive colonies was verified by PCR.

Adherence of *S. aureus* 4074 and its *aaa* mutant to Fg- and Fn-coated coverslips. To quantify the capacity of strains to adhere to surface-adsorbed Fg or Fn, we performed a radiometric adherence assay essentially as described before (15, 62). Briefly, 50- μ l quantities of a solution of Fn or Fg (200 μ g/ml) were adsorbed onto polymethylmethacrylate coverslips (60 min at 37°C). As a negative control, binding to BSA (5 mg/ml) was estimated. Coverslips were then washed three times with PBS and transferred into tubes. For radiolabeling, bacteria were grown for 4 h in Mueller-Hinton broth supplemented with [³H]thymidine as described previously (30) and then washed three times. Thereafter, coverslips were incubated with 1 ml of Ca²⁺-Mg²⁺-PBS containing labeled staphylococci (4 $\times$ 10⁷ CFU) and 0.5% BSA in a stirred water bath (60 min at 37°C). Afterwards, unbound bacteria were removed from coverslips by washing three times with PBS, and adherent radioactivity was determined.

Statistical analysis. Data are given as means $\pm$ standard errors of the means. Statistical analysis was performed using the Wilcoxon matched-pair signed rank test for nonparametric data. *P* values of <0.05 were considered to indicate statistically significant differences.

Nucleotide sequence accession number. The EMBL/GenBank/DBJ accession number of the *aaa* DNA sequence is AJ250906.

RESULTS

Identification of the autolysin/adhesin Aaa in *S. aureus* and cloning of the *aaa* gene. Recently, we identified a novel autolysin with adhesive properties in *S. epidermidis* (21). We found that *S. aureus* produces a homologous protein, which we termed Aaa (autolysin/adhesin from *S. aureus*), and we sought to characterize its function in more detail. We amplified a DNA fragment containing the *aaa* gene by PCR from *S. aureus* 4074 genomic DNA, yielding a 1,060-bp DNA fragment. The DNA fragment was cloned into the vector pCR2.1 in *E. coli*, creating plasmid pCAaa.

Nucleotide sequence of *aaa* and amino acid sequence analysis of the deduced protein. The nucleotide sequence of the cloned *aaa* gene was determined for both strands. *aaa* consists of 1,002 nucleotides and encodes a deduced protein of 334 amino acids (aa) with a predicted molecular mass of 35.8 kDa. It starts with the start codon GTG and is preceded by a putative ribosome-binding site at a distance of 8 bp. Putative -10

(CATAAT; nucleotides -79 to -84) and -35 (ATGTCA; nucleotides -110 to -104) promoter sequences were deduced from homologous DNA sequences from strain *S. aureus* Col. Aaa contains a putative signal peptide in the first 25 aa. The predicted *aaa* gene product is composed of 32.4% hydrophobic, 7.5% basic, and 3.3% acidic aa. The theoretical pI value of Aaa is 9.67. The aa sequence of Aaa is 100% identical to those of the putative homologous proteins from *S. aureus* strains COL, N315 (34), MW2 (3), NCTC8325 (26), and MSSA476 and shares 97.9% identical aa with Aaa from strain MRSA252. The *aaa* gene of strain Mu50 (34) encodes a putative protein with only 267 aa. Its predicted aa sequence is 100% identical to the aa sequence of Aaa from strain 4074 except for a 67-aa deletion from aa 90 to aa 157.

A sequence comparison of Aaa with known proteins in databases revealed two domains. The N-terminal domain of Aaa contains three direct repeated sequences. The repeats Aaa-2 (66 aa, starting at Y-93) and Aaa-3 (65 aa, starting at Y-160) are 65% identical, and the repeat Aaa-1 (56 aa, starting at H-29) shares only 32% identical aa with Aaa-3. The Aaa repeats are highly homologous to a consensus sequence that has been termed the LysM domain (for lysin motif) (Fig. 1A). The LysM domain is a part of various cell wall lytic enzymes (4, 31, 54). The N-terminal Aaa repeats are homologous to those of other autolysins that harbor the LysM domain, including the 35-kDa autolysin/adhesin Aae from *S. epidermidis* (three repeats, 71% identity) (21), the 51-kDa cell wall-associated endopeptidase LytF from *Bacillus subtilis* (five repeats, 42% identity) (38), the putative 35-kDa endopeptidase LytE from *B. subtilis* (three repeats, 42% identity) (39), the 70-kDa *N*-acetylmuramoyl-L-alanine amidase from *Enterococcus faecalis* (five repeats, 37% identity) (6), and the 50-kDa invasion-associated protein p60 of *Listeria* species (three repeats, 35% identity) (10) (Fig. 1A). The C-terminal domain of Aaa (starting at S-225) shows the highest similarity to the 35-kDa autolysin/adhesin Aae from *S. epidermidis* (87% identical aa) (21), the secretory, highly antigenic, in vivo-expressed protein SsaA from *S. epidermidis* (56% identical aa) (35), ORF1 from *S. aureus* (55% identical aa) (K. Wieland and F. Götz, unpublished), and the extracellular protein ScaB from *Staphylococcus carnosus* (53.6% identical aa) (B. Krismer, J. Montenbruck, and F. Götz, unpublished) (Fig. 1B). The functions of the last three proteins remain unknown.

Expression and purification of six-His-Aaa in *E. coli*. For expression of the *aaa* gene in *E. coli*, the PCR-amplified fragment was cloned into the expression vector pQE30. A representative clone expressing the *aaa* gene contained the plasmid pQAaa. Subsequently, Aaa was purified from *E. coli*(pQAaa) via its His tag using Ni-NTA affinity chromatography. SDS-PAGE of the affinity-purified fusion protein revealed an approximately 36-kDa protein. A protein with the same size was present in a cell lysate of an induced culture of *E. coli*(pQAaa) and was absent from cell lysates of an induced culture of *E. coli*(pQE30) (not shown) and a noninduced culture of *E. coli*(pQAaa) (Fig. 2).

Bacteriolytic activity of Aaa. To analyze the bacteriolytic activity of Aaa, the His-tagged Aaa protein as well as cell surface-associated *S. aureus* proteins and cell lysates of *E. coli*(pQAaa) and *E. coli*(pQE30) was investigated using zymographic analysis. SDS-PAGE was performed with a separation

FIG. 1. (A) Alignment of the deduced amino acid sequences of the repetitive sequences of the autolysin/adhesin Aaa (334 aa) from *S. aureus* 4074 (Aaa-1 to Aaa-3), Aae (324 aa) from *S. epidermidis* (Aae-1 to Aae-3), LytE (334 aa) from *B. subtilis* (LytE-1 to LytE-3), LytF (488 aa) from *B. subtilis* (LytF-1 to LytF-5), p60 (524 aa) from *Listeria ivanovii* (P60-1 to P60-3), and *N*-acetylmuramoyl-L-alanine amidase (641 aa) from *E. faecalis* (Am-1 to Am-5) (only the first 44 amino acids of the repeats are shown). The consensus sequence indicates the LysM domain that is proposed to have a general peptidoglycan-binding function. Bold letters indicate amino acids that match the consensus sequence. The letters in the second and third line in the consensus sequence show alternative amino acids. (B) Alignment of the deduced C-terminal amino acid sequences of the autolysin/adhesin Aaa from *S. aureus* 4074, Aae from *S. epidermidis* (324 aa), the secretory antigen SsaA (257 aa) from *S. epidermidis*, ORF1 (255 aa) from *S. aureus* (EMBL database accession number X97985), and the extracellular protein SceB (263 aa) from *S. carnosus* (EMBL database accession number U96107). Asterisks indicate identical amino acids, colons indicate very similar amino acids, and dots indicate somewhat similar amino acids. Gaps (dashes) were filled in to maximize homologies.

gel containing heat-inactivated *S. carnosus*, *S. aureus*, or *Micrococcus luteus* cells as a substrate for lytic enzymes. Aaa extracted from the *S. aureus* surface as well as His-tagged Aaa produced by *E. coli* exhibited bacteriolytic activity when *S. carnosus* cells (Fig. 3) and *S. aureus* cells (not shown) were used as the substrate. Smaller proteins than the 35.8-kDa Aaa protein that showed bacteriolytic activity presumably represented degradation products of Aaa (Fig. 3). No bacteriolytic activity was detected with cell lysates from noninduced cultures of *E. coli*(pQAaa) (Fig. 3) or induced cultures of *E. coli*(pQE30) (not shown). No Aaa-associated bacteriolytic activity was detected when *M. luteus* cells were used as the substrate in the separation gel (not shown).

FIG. 2. Expression and purification of six-His-Aaa. The image shows an SDS-PAGE gel (12.5% separation gel) with crude cell lysates (10 μ l) of noninduced *E. coli*(pQAaa) (lane 1), IPTG-induced *E. coli*(pQAaa) (lane 2), and six-His-Aaa (1.5 μ g) purified from *E. coli*(pQAaa) (lane 3). The arrow indicates the 35.8-kDa His-tagged Aaa protein purified from *E. coli*(pQAaa). The same protein band was absent from crude extracts of *E. coli*(pQAaa) before induction. Marker proteins are shown on the left.

Quantification of Fg-, Fn-, and Vn-binding activity of Aaa by real-time BIA. Biomolecular interaction analysis (BIA), based on the optical detection of surface plasmon resonance, was performed to study the adhesive properties of the autolysin Aaa. In BIA, interactions are analyzed between a ligand immobilized on a sensor chip and an analyte, which is in solution and flows over the surface of the chip. The use of this system allowed the study of the interaction of the autolysin Aaa (ligand) with the extracellular matrix proteins Fg, Fn, and Vn (analytes) without the need to label any of the proteins. After Aaa was immobilized on the C1 chip, various concentrations of

FIG. 3. Zymographic analysis of bacteriolytic activity of Aaa. The image shows an SDS-PAGE gel of crude cell lysates (10 μ l) of noninduced *E. coli*(pQAaa) (lane 1), IPTG-induced *E. coli*(pQAaa) (lane 2), six-His-Aaa (1.5 μ g) purified from *E. coli*(pQAaa) (lane 3), and cell surface-associated proteins (10 μ l) of *S. aureus* 4074 (lane 4). The separation gel (12.5%) contained heat-inactivated *S. carnosus* cells (0.05%) as a substrate for bacteriolytic enzymes. Bacteriolytic activity is visible as a clear zone after incubation in phosphate buffer. The arrows indicate Aaa-associated bacteriolytic activity.

Fg (concentrations ranging from 100 nM to 1,200 nM), Fn (concentrations ranging from 200 nM to 1,600 nM), or Vn (concentrations ranging from 500 nM to 1,300 nM) were sequentially injected over the immobilized protein for kinetic studies. The sensorgrams of the binding of the matrix proteins to Aaa were monitored as shown in Fig. 4. The interactions of Aaa with Fg, Fn, and Vn were dose dependent and saturable (Fig. 4A to C). In control experiments, Aaa did not bind to BSA (Fig. 4D). From the quantitative data on the kinetics of these interactions, the dissociation constants (K_D) were determined to be 12.3 nM for the interaction of Aaa with Fg, 22.1 nM for the interaction of Aaa with Vn, and 29.9 nM for the interaction of Aaa with Fn. To further characterize the binding regions, competition experiments were performed. For these experiments, the peptides HHLGGAKQAGDV and GRGDSP, which represent different binding sites in Fg for the platelet receptor GPIIb/IIIa (16, 33), and FYQVLNMPNLNA, which represents the ligand-binding site in protein A (unpublished results), were used. In a previous study, we found that protein A binds to von Willebrand factor (15) and also to Fg (unpublished results). We found that the peptide FYQVLNMPNLNA dose-dependently inhibited the interaction of Fg with Aaa (Fig. 4G). In contrast, the peptides HHLGGAKQAGDV (Fig. 4E) and GRGDSP (Fig. 4F) had no effect.

Surface location of Aaa. To detect the surface location of Aaa, an antiserum against Aaa that was raised in rabbits was used for immunofluorescence microscopy. The anti-Aaa antiserum strongly reacted with cells of the protein A-deficient strain *S. aureus* Wood 46, indicating the cell surface location of Aaa (Fig. 5A). Much less immunofluorescence was detected with the preimmune serum (Fig. 5B). However, there was some immunofluorescence detectable with the preimmune serum, which may be due to the IgG-binding and surface-associated protein Eap (14), which also seemed to react with the anti-Aaa antiserum and the preimmune serum in Western blot analysis (Fig. 6).

Characterization of *aaa*-deficient mutant. To analyze the function of Aaa in the context of the bacterial cell, we constructed an *aaa*-deficient mutant from *S. aureus* 4074 by gene replacement and compared it to its wild type.

(i) Confirmation of the *aaa* mutant. To verify that the 35.8-kDa autolysin Aaa was missing from protein preparations of the *aaa* mutant, surface-associated proteins were analyzed by zymographic analysis and Western blot analysis (Fig. 6). Zymographic analysis confirmed that a 35.8-kDa protein with bacteriolytic activity was present in the *S. aureus* wild type but missing from the *aaa* mutant (Fig. 6B). The larger protein bands with bacteriolytic activity presumably represent the products of proteolytic cleavage from the Atl precursor molecule. In Western blot analysis, a 35.8-kDa protein that reacted with the anti-Aaa antiserum was detected in protein preparations of the wild-type strain but was missing from protein preparations of the *aaa* mutant (Fig. 6C). In control experiments, no specific binding of Aaa to the preimmune serum or to the anti-digoxigenin-AP conjugate was observed (not shown). A larger and a smaller protein that reacted with the anti-Aaa antiserum and also with the preimmune serum (not shown) may represent Eap (also referred to as Map or p70) and its degradation products, respectively, which were reported to have an IgG-binding function (14). Like Aaa, Eap

does not seem to be covalently linked to the cell surface and thus can be extracted from the cell surface by SDS (25).

(ii) Colony morphology. Previously, we found that the colony morphology of the autolysin mutant *S. epidermidis atlE* differed from that of its wild type (18): the *atlE* mutant formed rough and dull colonies instead of shiny colonies like the wild type. In contrast, there was no difference in colony morphology phenotypes on TSA between the *aaa* mutant and its wild type, strain 4074 (not shown).

(iii) Growth in liquid culture and cell aggregation. Autolysins are involved in cell division and cell separation. Therefore, we compared the growth of the *aaa* mutant in liquid culture with that of its wild type. The *aaa* mutant revealed the same growth curve in TSB as the wild type, and both strains reached the same end OD₅₇₈ (data not shown). Cell aggregation is indicative of a loss of an enzyme involved in cell separation (20, 61, 64). To clarify whether Aaa is involved in cell separation, we observed liquid cultures of the *aaa* mutant and its wild type in TSB by using phase-contrast microscopy and could not detect any difference in cell cluster formation. With both strains, there were mainly single cells, pairs, and tetrads visible (not shown).

(iv) Adherence to surface-adsorbed Fg and Fn. To examine whether Aaa is involved in the adherence of *S. aureus* cells to Fg- or Fn-coated surfaces, we performed a radiometric adherence assay. Compared to that of the *S. aureus* wild type, the adherence of the *aaa* mutant to Fg and Fn was reduced approximately 50%, which was statistically significant (Fig. 7). With both strains, binding to Fg was more pronounced than binding to Fn. The adherence of the strains to BSA was comparable and very low.

Identification of Aaa-binding domains of Fg and Fn by ligand affinity blot analysis. Each Fg molecule consists of three pairs of nonidentical polypeptide chains ($\alpha\beta\gamma$) that are arranged into three globular domains, one central domain (E), and two distal domains (D), which are created after digestion with plasmin. To identify the binding domains for Aaa on Fg, the α , β , and γ chains were analyzed in a ligand affinity blot using six-His-Aaa as a probe followed by a Ni-NTA-AP conjugate. Aaa bound to the α chains and, to a lesser extent, to the β chains (Fig. 8B). Furthermore, fragments D and E of human Fg (nonreduced) were analyzed in a ligand affinity blot. Aaa bound to the D fragment but not to the E fragment (Fig. 8B). In the negative controls, no significant binding of the Ni-NTA-AP conjugate was detected (Fig. 8C and D). To identify the binding domain of Fn for Aaa, human Fn was digested with the protease thermolysin, which produces a 29-kDa N-terminal Fn fragment and three larger polypeptide fragments (46). In ligand affinity blot analysis, Aaa was detected binding to the 29-kDa Fn fragment (not shown).

Prevalence of *aaa*. To determine the prevalence of the *aaa* gene among clinical *S. aureus* isolates, we performed a PCR analysis using primers CH56 and CH57. An approximately 1-kb fragment representing the *aaa* gene was found to be present in all 30 clinical *S. aureus* strains tested as well as in strain Wood 46 (not shown). This indicates a very high prevalence of the *aaa* gene among clinical *S. aureus* isolates. PCR with the chromosomal DNA of strain Mu50 revealed a 0.8-kb fragment, which reflects the deletion that we identified within the *aaa* gene in the genome of Mu50 (see above; not shown).

FIG. 4. Determination of binding of Fg (A, E, F, and G), Fn (B), Vn (C), and BSA (D) to the immobilized autolysin/adhesin Aaa using the BIAcore system. After Aaa was immobilized on the C1 chip surface, Fg, Fn, Vn, and BSA were injected over the chip surface at a flow rate of 30 μ l/min. The binding of Fg, Fn, Vn, and BSA was monitored and presented in overlay plots of the sensorgrams (plots of resonance units [RU] versus time). (A) Concentrations of Fg (from bottom to top): 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, 700 nM, 800 nM, and 1,200 nM; (B) concentrations of Fn (from bottom to top): 200 nM, 300 nM, 400 nM, 700 nM, 1,200 nM, and 1,600 nM; (C) concentrations of Vn (from bottom to top): 500 nM, 600 nM, 700 nM, 800 nM, 900 nM, 1,000 nM, 1,100 nM, 1,200 nM, and 1,300 nM; (D) concentrations of BSA (from bottom to top): 0 nM, 100 nM, 500 nM, 1,000 nM, 2,000 nM, 5 μ M, and 10 μ M; (E) concentrations of Fg and peptide HHLGGAKQAGDV (from bottom to top): 0 nM Fg and 1,000 nM peptide, 100 nM Fg and 1,000 nM peptide, and 100 nM Fg and 0 nM peptide; (F) concentrations of Fg and peptide GRGDSP (from bottom to top): 0 nM Fg and 900 nM peptide, 100 nM Fg and 900 nM peptide, 100 nM Fg and 750 nM peptide, 100 nM Fg and 500 nM peptide, 100 nM Fg and 250 nM peptide, 100 nM Fg and 100 nM peptide, and 100 nM Fg and 0 nM peptide; (G) concentrations of Fg and peptide FYQVLNMPNLNA (from bottom to top): 0 nM Fg and 900 nM peptide, 100 nM Fg and 900 nM peptide, 100 nM Fg and 500 nM peptide, 100 nM Fg and 250 nM peptide, 100 nM Fg and 100 nM peptide, 100 nM Fg and 50 nM peptide, and 100 nM Fg and 0 nM peptide.

FIG. 5. Detection of Aaa at the cell surface by immunofluorescence microscopy. Cells of the protein A-deficient strain *S. aureus* Wood 46 grown overnight in BHI broth were incubated with the anti-Aaa antiserum raised in rabbits (A) or with preimmune serum (B). Bound antibodies were detected with fluorescein-conjugated anti-rabbit IgG antibodies. Cells were viewed with a fluorescence microscope. Wood 46 cells reacted with the anti-Aaa antiserum, indicating the cell surface location of Aaa. In comparison, the reaction of the cells with the preimmune serum was much weaker. Magnification, $\times 1,000$.

DISCUSSION

Recently, we reported the identification and cloning of an autolysin with adhesive properties, Aae, from *S. epidermidis* (21). Here we describe the molecular characterization of Aaa, a multifunctional autolysin/adhesin from *S. aureus* that is 76% identical to Aae. Furthermore, this study deals with the construction and characterization of an *aaa*-negative mutant. Generally, bacterial cell wall lytic enzymes or autolysins are peptidoglycan hydrolases with multiple functions. They are not only involved in cellular processes, such as cell division, cell separation, and cell wall turnover (for a review on *Bacillus subtilis* autolysins, see reference 59), but are also thought to be involved in pathogenesis. In vivo studies have shown that some autolysin-defective mutants are less virulent than their wild-type strains, including an *atlE* mutant of *S. epidermidis* (55), an *iap*-negative mutant of *Listeria monocytogenes* (53), an *ami* mutant of *L. monocytogenes* (42), and a *lytA* mutant of *Strep-*

tococcus pneumoniae (7). The 60-kDa invasion-associated protein p60 (Iap) from *L. monocytogenes* seems to be involved in adherence to and invasion of eukaryotic cells, and an *iap* mutant is strongly impaired in the ability to spread between host cells (47, 53). Another autolysin from *L. monocytogenes*, Ami, also seems to function in adherence to eukaryotic cells (43). The eukaryotic cell-binding activity of Ami has been located to the cell wall-anchoring domain (42). This domain consists of GW-containing repeats. Such GW repeats are also present within the repeat regions of the homologous autolysins Atl from *S. aureus*, AtlE from *S. epidermidis*, and Aas from *S. saprophyticus* (23, 42). Like these major staphylococcal autolysins, Aaa contains three repetitive sequences, but they are not homologous to the GW repeats. Instead, they are highly homologous to a consensus sequence that is part of several peptidoglycan hydrolases, namely, the LysM (lysin motif) domain (Fig. 1). Recently, the LysM domain of the major autolysin AcmA of *Lactococcus lactis* has been demonstrated to bind to peptidoglycan (60). The peptidoglycan-binding func-

FIG. 6. Surface-associated proteins (10 μ l) from *S. aureus* 4074 (lanes 1) and the *aaa* mutant (lanes 2) were separated by SDS-PAGE (10% separation gel), stained with Coomassie brilliant blue (A), and subjected to zymographic analysis (B) or Western blot analysis (C). A 35.8-kDa protein with bacteriolytic activity (B) that reacted with the anti-Aaa antiserum (C) was missing from the *aaa* mutant (indicated by an arrow). The sizes of the marker proteins are indicated on the left (A; Bio-Rad prestained marker [M]) and the right (C; MBI Fermentas prestained marker [M]).

FIG. 7. Adherence of *S. aureus* 4074 and its *aaa* mutant to immobilized Fg and Fn adsorbed to polymethylmethacrylate coverslips. [3 H]thymidine-labeled *S. aureus* 4074 or *S. aureus* *aaa* (4×10^7 CFU/ml) was incubated with coverslips that were preadsorbed with Fg, Fn, or BSA. Afterwards, the bacterial suspension was removed, the coverslips were washed, the adherent radioactivity was determined, and the CFU were calculated. Experiments were performed three times in quintuplicate. Results are means $\pm$ standard errors of the means. Statistical analysis was performed using the Wilcoxon matched-pair signed rank test for nonparametric data. *P* values of <0.05 were considered to indicate statistically significant differences. *, $P < 0.05$ for comparison with wild-type strain *S. aureus* 4074. Black bars, *S. aureus* 4074; gray bars, *aaa* mutant.

FIG. 8. Determination of binding domains of Fg for Aaa by ligand affinity blot analysis. Fragments D (lanes 2) and E (lanes 3) of Fg (nonreduced) and the Fg chains (α , β , and γ chains [reduced]) (lanes 1) were separated by SDS-PAGE (10% separation gel) (A) and transferred to nitrocellulose filters that were probed with six-His-Aaa (B) or six-His-dihydrofolate reductase (C) or without a ligand (D), followed by a Ni-NTA-alkaline phosphatase conjugate. The sizes of the marker proteins (MBI Fermentas prestained marker [M]) and the positions of the α , β , and γ chains of Fg are indicated.

tion of the LysM motif in Aaa might also be responsible for its surface-associated location, because Aaa does not have a C-terminal anchor region with the LPXTG motif that is typical for surface proteins of gram-positive bacteria (57). The LysM domain has also been found in proteins that are not involved in cell wall metabolism but that have been associated with adherence and pathogenesis, such as the elastin-binding protein EbpS of *S. aureus* (one copy of the LysM domain located in the C-terminal portion) (12), the immunoglobulin-binding protein A of *S. aureus* (one copy of the LysM domain located near the C terminus) (58), and intimin, which is an outer membrane protein required for the attachment of enteropathogenic *E. coli* to mammalian cells (one copy of the LysM domain located near the N terminus) (5). The function of the LysM domain in protein A and EbpS is not known. However, a function in anchoring these proteins to the cell surface seems unlikely, because protein A has a C-terminal anchor region with the LPXTG motif (57) and EbpS is an integral membrane protein (12).

Aaa was found to bind to the D fragment of Fg and, more precisely, to the α and β chains of the protein. These chains are also recognized by other staphylococcal Fg-binding proteins, such as Aae from *S. epidermidis* (21), Efb from *S. aureus*, which binds to the α chains (45), and Fbe from *S. epidermidis*, which binds to the β chains (52). Using BIA, the interactions between Aaa and extracellular matrix proteins were found to be dose dependent and saturable. The resulting K_D values were determined to be between 12.3 nM for the interaction of Aaa with Fg and 29.9 nM for the interaction of Aaa with Fn. Thus, our results indicate a very high affinity of the autolysin/adhesin Aaa for extracellular matrix proteins. Competition experiments revealed that the interaction of Fg with Aaa was inhibited by the peptide FYQVLNMPNLNA in a dose-dependent manner, confirming the specificity of the interaction between Fg and Aaa. This peptide represents a portion of protein A that we identified as a ligand-binding site (unpublished results). Therefore, we concluded that protein A and Aaa bind to the same site within Fg, which seems to be independent of the binding sites (HHLGGAKQAGDV and RGD) in Fg for the platelet receptor GPIIb/IIIa.

Aaa exhibits lytic activity against *S. carnosus* and *S. aureus*

(not shown) cells but not against *M. luteus* cells (not shown). Thus, it is likely that Aaa does not possess amidase and glucosaminidase activities, both of which cleave the peptidoglycans of staphylococcal as well as *M. luteus* cells. Aaa might have an endopeptidase activity that cleaves the amide bonds in the peptide cross-links. Further analyses are necessary to identify the enzymatic activity of Aaa.

To gain a better understanding of the function of Aaa, we constructed an *aaa*-deficient mutant. The *aaa* mutant did not differ from the wild type in its colony morphology, growth rate, and cell cluster formation, suggesting that Aaa does not play a role in cell separation or, more probably, that the function of Aaa in cell separation may have been taken over by other autolysins, among them the major autolysin Atl, which seemed to be more strongly expressed in the *aaa* mutant than in the wild type (Fig. 6B). Analogous to the recent finding that an *agr* mutation in *S. epidermidis* led to increased biofilm formation due to an increased expression of *atlE* (63), the increased expression of *atl* might also be responsible for the observed, slightly more pronounced capacity (approximately 1.7-fold) of the *aaa* mutant for biofilm formation than that of the *S. aureus* wild type (data not shown).

Immunofluorescence microscopy indicated that Aaa is located at the cell surface, which is the prerequisite for acting as an adhesin. Compared with the *S. aureus* wild type, the *aaa* mutant revealed an approximately 50% reduced adherence to surface-adsorbed Fg and Fn. This suggests that among several other *S. aureus* factors, which typically belong to the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) adhesin family (49), Aaa might be involved in the colonization of host factor-coated material or host tissue by *S. aureus*. The surface location of Aaa and the high prevalence of the *aaa* gene among clinical *S. aureus* isolates indicate its possible suitability as a vaccine candidate. Analyses of the *aaa*-negative mutant in animal models are warranted to determine the role that Aaa plays in staphylococcal pathogenicity.

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