Staphylococcus aureus newman requires eap and emp biology essay

Science, Biology

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Abstract

Staphylococcus aureus strain Newman, which is negative for cna (the gene for collagen adhesin) and secretes only truncated FnBPs, produces two broad-spectrum adhesins, Eap (extracellular adherence protein) and Emp (extracellular matrix binding protein). The Eap selectively recognizes extracellular matrix aggregates but Eap and also Emp binds promiscuously to monomeric matrix macromolecules including fibronectin, fibrinogen, collagen, and vitronectin. Previous studies of the Eap and Emp functions were mostly performed with monomeric proteins that did not allow dissection of its precise role because in host the suprastructural organisation presents binding epitopes for adhesions that are not found on the surfaces of individual matrix macromolecules. In present study, the interaction of Eap and Emp is determined with human skin and cartilage suprastructures using S. aureus Newman, isogenic single mutants (Δeap and Δemp) and a double mutant (Δeap/Δemp). We found that (i) intact S. aureus Newman adhered

equally well to collagen 1, human skin and cartilage suprastructures, in contrast mutants deficient either for Eap or Emp negative or both adhered to a significantly lesser extent. Adherence of the double mutant was restored upon complementation with the eap and emp genes. (ii) Anti-Eap or anti-Emp F(ab´)2 fragments blocked adherence but preimmune serum did not. (iii) The rEmp blocked adherence but as expected rEap promoted adherence presumably by acting as a ligand. (iv) The rEap or rEmp binds in a dose response to skin and cartilage extracts. (v) The wild type Newman adhered to higher extent (to cultured Ea. hy926 cells) and internalized by HUVEC and Ea. hy 926 cells than the isogenic single and double mutants for Eap and Emp proteins. Taken together these results suggest a potential role for Eap and Emp in S. aureus pathogenicity by promoting adhesion to complex eukaryotic substrates, especially for cna-negative S. aureus strains, in collagen-rich tissues. This may be important for the pathogenesis of osteomyelitis since collagen adhesin (Cna) is not found in ~50% of these isolates.

Introduction

S. aureus is an important human pathogen that can cause serious invasive diseases, as osteomyelitis or tissue infections. S. aureus expresses a variety of virulence factors, as different surface proteins and toxins, that enable the bacteria to establish an infection. Adhesins are a group of surface proteins that mediate adherence to host matrix proteins, plasma proteins and cells. The specific bacterial interaction with host proteins not only allows for adhesion and colonization of tissues but is also pivotal for S. aureus invasion

of non-professional phagocytes such as epithelial or endothelial cells by (50-52). Specific interaction of S. aureus has previously been described for various host structures, as fibronectin, fibrinogen, vitronectin, thrombospondin, bone sialoprotein, glycosaminoglycans, elastin, collagens (8, 20, 46). Several S. aureus genes coding for cell surface proteins are now well characterised. The staphylococcal adhesins with the sortase consensus motif LPXTG were termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (42). Another term, SERAMs (secretable expanded repertoire adhesive molecules) is used for an expanding spectrum of microbial proteins, which do not contain the LPXTG motif for covalent linking to peptidoglycan (4, 5, 27). The two anchorless, cell surface-associated adhesins Eap (extracellular adherence protein) and Emp (extracellular matrix binding protein) bind a broad spectrum of host protein (14, 21-23) and qualify as SERAMs. PCR analysis revealed that the eap and emp genes are present in almost all S. aureus isolates, whereas they were not found in any S. epidermidis isolates or other Gram-positive cocci (24) (22). Eap shows structural homology to the C-terminal domain of bacterial superantigens but lacks superantigen activity (9, 34). Eap was recently shown to curb acute inflammatory responses, to enhance internalization of the microorganism into eukaryotic cells, to inhibit wound healing, and to function as a potent angiostatic agent (1, 14, 17, 55). Hence, Eap is a critical factor in S. aureus infection. Emp is a S. aureus surface protein different from previously described secreted adhesive molecules such as coagulase, the Fg-binding protein Efb, Map/Eap, or a novel 30-kDa S. aureus phosphatase. The observation that emp is present in all S. aureus isolates, taken together with

the high-affinity binding of rEmp to Vn (?) and the decreased adhesion of an Emp-deficient mutant to fibrinogen and fibronectin, underlines its putative importance in staphylococcal pathogenesis. Furthermore, most recent work reports on regulation and role of Emp in biofilm formation in iron restricted conditions (16, 18, 22, 26). Matrix macromolecules in situ are aggregated into suprastructures such as fibrils, microfibrils, filaments, and networks. Matrix suprastructures are usually composed of several molecular species. The ultrastructure of skin collagen contains collagen I, the fibrillar collagen types III and V, the fibril-associated collagens with interrupted triple helical domains (FACITs), types XII, XIV, and XXII (37). The structure is further complexed by inclusion of non-collagenous macromolecules, such as the small leucin-rich proteoglycans decorin, fibromodulin, lumican, and biglycan (30). Cartilage fibrils contain fibrillar collagens II and XI, (2, 32). Depending on their precise tissue origin, cartilage fibrils also contain the FACIT collagen IX, or the leucin-rich proteoglycans decorin, or both (13). The binding of S. aureus to host tissues is a complex multifactorial process. S. aureus uses multiple adhesins for binding to host cells and matrix, which can often mutually compensate the loss-of-function of a given adhesin. Recently, we demonstrated that S. aureus strain Newman produces only truncated FnBPs, which are not cell wall anchored, but quantitatively secreted into the culture supernatants, due to a point mutation in fnbpA and fnbpB (12). This can be used as a tool for studying the role of Eap and Emp for adherence to fibrinogen and fibronectin, two functions which are mediated by FnBPs, in a Cna and FnBP-negative background. Strain Newman produces ClfA which also binds to fibrinogen, but Eap and Emp have been shown to substantially

contribute to this function (21, 22). A defined mutant deficient for more than one adhesin is required for studying their combined effect on adherence. Since both Eap and Emp display a largely overlapping spectrum of ligands, and that all S. aureus strains tested so far carry both genes (22, 24), their precise differential adhesive functions are difficult to address by single knock-out mutants. Thus, we constructed an isogenic double mutant (Δeap+Δemp) of S. aureus Newman and tested them for their adhesive and invasive properties. Furthermore,, in this study an attempt was made to explore binding characteristics of S. aureus Newman Eap and Emp knock-out mutants to authentic suprastructural fragments from skin and cartilage.

Materials and Methods

Bacterial strains and culture media. The bacterial strains and plasmids used in this study are listed in table 1. For cultivation of staphylococci, brain heart infusion (BHI) broth or agar (Merck, Darmstadt, Germany); tryptic soy broth (TSB) or agar (Difco, Detroit, USA); Muller Hinton broth or agar (Mast, Merseyside, UK) and Luria-Bertani (LB) broth or agar (Difco) were used. For cultivation of E. coli LB broth or agar was used. DNA manipulations and transformations. Manipulations were performed according to standard procedures (48). Genomic DNA was isolated using the QIAamp DNA Mini Blood Kit (Qiagen, Hilden, Germany) with the modification that recombinant lysostaphin (20U/ml) was used for lysis of staphylococci. Plasmid DNA was prepared using the Qiagen Plasmid Mini kit (Qiagen). DNA fragments were isolated from agarose gels using the QIAEX II Gel extraction kit (Qiagen). Selection for resistance to antibiotics in E. coli or S. aureus was performed

with ampicillin (100 µg/ml, Sigma), erythromycin (10 µg/ml, Serva, Heidelberg, Germany), gentamicin (5 µg/ml, Serva), tetracycline (10 µg/ml, Sigma, Deisenhofen, Germany) and chloramphenicol (10 µg/ml, Serva). Construction of the double mutant (i) insertion of gentamicin gene in emp. A 2. 0-kb PCR product of gentamicin gene amplified from plasmid DNA of pPQ132 (33) using primers Gm-F and Gm-R (Table 1) was ligated into the EcoRV linearized pQEmpN (22) containing emp as insert. The ligation mixture was transformed into freshly prepared competent E. coli DH5✔ cells (48), and the transformation mixture was plated onto LB plates containing ampicillin and gentamicin. One representative plasmid containing the emp:: GmR fragment was designated as pMH11. (ii) Construction of a emp:: GmR shuttle vector. The emp:: GmR fragment was isolated from pM11 as a 3. 0 kb fragment by restriction with Kpn I and BamH I and ligated into a shuttle vector pBT2 (3) with abiliy to replicate in E. coli and staphylococci The ligation mixture was transformed into E. coli TG1 cells and subsequently plated onto LB plates containing ampicillin and gentamicin. One representative plasmid conferring resistance to antibiotics was designated as pMH12. (iii) inactivation of emp. pMH12 was first propagated in restriction-deficient S. aureus SA113, and then electroporated (31) into an eap:: ermB deletion mutant of S. aureus Newman (23). For construction of an emp allelic replacement mutant, the method described by Palma et al (41) was used. S. aureus Newman ✍eap containing pMH12 was cultivated overnight in LB in the presence of erythromycin, gentamicin and chloramphenicol with shaking at 32oC. The overnight culture was reinoculated (1: 20) into LB medium with erythromycin and gentamicin and grown at the nonpermissive temperature

(43oC) overnight. This selects for clones with the plasmid integrated by single recombination into the chromosomal DNA. The culture was reinoculated (1: 20) and grown in LB medium at 43oC for 24 hours with only erythromycin selecting for stable chromosomal integration (emp:: GmR) and concomitant loss of chloramphenicol resistance. Various dilutions of this culture were plated on LB plates containing erythromycin and gentamicin, then incubated overnight at 43oC. Chloramphenicol-sensitive and gentamicin-resistant colonies were detected by replica plating onto plates containing chloramphenicol or erythromycin and gentamicin at 37oC overnight. Clone number 23, sensitive for chloramphenicol and resistant against gentamicin was termed DM23, and selected for further analysis. Correct insertion of the GmR fragment was confirmed by PCR method. For clarity DM23 was designated as ΔDM (Δeap/Δemp). Complementation of the double mutant. (i) Construction of pXREap+Emp. The PCR products of eap and emp were amplified with sets of primers PII + PIII and P4S + P5 (Table 1), repectively using S. aureus Newman genomic DNA as a template. The eap PCR product was restricted with BamHI and KpnI, and the emp PCR product with KpnI and SalI. Both PCR products were ligated to each other and then further ligated into BamHI and SalI linearized pXR100 (49). The ligation mixture was transformed into protoplasts of S. carnosus TM300 and plated onto TSA plates containing chloramphenicol. One representative clone expressing Eap and Emp on xylose induction was detected with an anti-Eap/anti-Emp antibody mix and designated as pXREap+Emp. (ii) The plasmid pXREap+Emp was transformed into Newman Δeap/Δemp by electroporation. Transformants were grown on TSA plates containing

chloramphenicol, erythromycin, and gentamicin. One representative clone expressing Eap and Emp upon xylose induction was identified by Western blot, using a mixture of anti-Eap and anti Emp antibodies, and was designated as compl. DM (Δeap/Δemp [pXREap+Emp]). PCR amplification for collagen adhesin cna. For PCR amplification of the cna gene in strains wild type Newman and its derived mutants, Cowan 1 and Philips, cna-specific primers (Table 1) were used. Expression and purification of recombinant Eap and Emp in E. coli. The PCR product of eap and emp (lacking the signal peptide) were amplified from genomic DNA of S. aureus Newman with sets of primers eapP3 + eapP2 (21) and empP3 + empP4 (22) respectively. The PCR product were ligated into theQIAexpress pQE30 vector (Qiagen). The ligation mixtures were then transformed into E. coli M-15 and resultants Eap and Emp expression vectors were designated as pQEapN and pQEmpN respectively. His-tagged Eap/Emp fusion proteins were expressed and purified according to the protocol provided by the manufacturer (Qiagen). Recombinant Eap and Emp co-migrated in SDS-PAGE with Eap and Emp homologues from S. aureus strain Newman extracted with 2% SDS and also showed binding to biotin-labeled fibronectin, fibrinogen, and vitronectin in ligand overlay assays (data not shown). Cell surface protein preparation. Staphylococci were grown in BHI broth at 37°C with 160 rpm for 18 h and bacteria were pelleted by centrifugation at 10, 000x g for 2 min. The bacterial pellet was resuspended in extraction buffer (125 mM Tris-HCl pH 7. 0 + 2% sodium dodecyl sulfate [SDS, Sigma]), heated at 95°C for 3 min and centrifuged at 10, 000x g for 3 min. The liquid supernatant was passed through a Nap-10 column (Amersham Pharmacia Biotech Europe GmbH,

Freiburg, Germany) containing Sephadex G-25 and the eluate was stored at –20oC. SDS-PAGE and ligand overlay analysis. To 20 µl of cell surface extract, 5 µl of 5x sample buffer (60 mM Tris-HCl, pH 6. 8, 25% glycerol, 2% SDS, 14. 4 mM 2-mercaptoethanol and 0. 1% bromophenol blue was added, heated at 95°C for 3 min, then separated in a SDS-PAGE minigel. For Western ligand blot analysis, proteins separated by SDS-PAGE were electrophoretically transferred (Trans-blot SD, Bio-Rad, Munich, Germany) onto a nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany), and then the membrane was blocked with 3% BSA (bovine serum albumin fraction V, Sigma). Fibronectin (Fn) (Chemicon, Temecula CA, USA), fibrinogen (Fg) (Calbiochem, San Diego CA, USA), collagen type I (Cn) (Sigma; Sigma product #7774) or vitronectin (Vn) purified by the method of Yatohgo et al. (60) were labeled with biotin (Roche, Mannheim, Germany). Blotted proteins on nitrocellulose membrane were exposed to biotinylated ligands and subsequently detected using an avidin alkaline phosphatase color reaction (Bio-Rad). Polyclonal antibodies. Polyclonal antibodies against the purified rEap and rEmp were raised separately in a set of two rabbits by standard procedures. After collection of preimmune sera, each rabbit was immunized subcutaneously with 50 µg of antigen in complete Freund’s adjuvant (Sigma). Second and third injections of antigen in incomplete Freund’s adjuvant were given subcutaneously 2 and 4 weeks later, respectively. Blood was obtained 2 weeks after the last antigen injection. Naturally occurring antistaphylococcal antibodies were complexed by mixing serum with 10 volumes of cell surface protein preparations from mutants, which does not produce the Eap or Emp, and immunocomplexes were partially removed by centrifugation (15, 000 x

g, 30 min, 4oC). Isolation of IgG fraction from crude antiserum was achieved using protein A column (Pierce, Rockford, IL 61105, USA). For isolation of F(ab´)2 fragment from IgG fraction, a kit " ImmunoPureR F(ab´)2 preparation kit " from Pierce was used. Preparation of human skin and cartilage extrat. Normal human skin was obtained with informed consent from patients undergoing plastic surgery. Isolation of authentic supramolecular fragments from the dermo-epidermal junction zone was performed as previously described (29). Briefly, the epidermal layer of skin was removed after treatment with neutral buffer containing 1 M NaCl for 4 h, and the dermal layer was frozen in liquid nitrogen. A layer of 200 µm was removed from the dermal surface by a dermatome and was repeatedly homogenized in 150 mM NaCl, 2 mM sodium phosphate buffer, pH 7. 4 containing a mixture of protease inhibitors. Between each homogenization step, tissue debris was removed by centrifugation. The supernatants containing the fibrillar fragments were used for analysis in electron microscopy. In addition, fragments from supramolecular aggregates specifically containing collagen IV or fibronectin were purified from crude skin extracts and separated by immuno-magnetic beads with antibodies to collagen IV or fibronectin, respectively (Villone, Bruckner and Hansen, unpublished). Fragments of suprastructural aggregates were obtained from human articular cartilage recovered after joint replacement surgery(29, 36). Briefly, slices of cartilage were twice homogenized in 15 volumes of 150 mM NaCl, 2 mM sodium phosphate-buffer, pH 7. 4 (PBS) containing a mixture of protease inhibitors and the homogenates were cleared by centrifugation. This procedure was repeated twice. Finally, partial purification and concentration of the

suprastructures contained in the combined supernatants was achieved by high speed centrifugation. The final pellet was resuspended in PBS and stored at 4oC. Adherence of staphylococci to Cn. The microtiter plate wells were separately coated with Cn (1 to 7 µg/well) in PBS (without Ca and Mg, Dulbeco´s, PAA Lab. GmbH, Austria) overnight at 4oC. Wells were washed with PBST (PBS+0. 05% Tween 20) and blocked with 3% BSA in PBS for 1 hour at 37C. Bacteria were grown in BHI over night at 37oC with shaking (160 rpm), washed with PBS and adjusted to an OD540 of 1. 0. A 100 µl bacterial suspension was added to each wells and plate was incubated at 37oC. After 1 h, wells were washed with PBST, adherent bacteria were stained with 0. 1% safranin dye for 1 min and washed to remove unbound dye and plate was read at 490 nm in microplate reader (Molecular Devices, CA94089, USA). Adherence to Fn- Fg- and Cn- coated coverslips. A radiometric assay described earlier (57) was used to determine the binding of bacteria to coated surfaces. Briefly, an inoculum (40 µl) containing 4 x 106 CFU of [3H]thymidine-labeled staphylococci was incubated with a polymethylmethacrylate (PMMA) coverslip preadsorbed with Fn or Fg in a tube containing 960 µl of PBS supplemented with 0. 5% human serum albumin and incubated at 37°C for 1 h in a shaking water bath. Thereafter, the PMMA coverslips were removed, washed with PBS three times, and the adherent counts per minute were determined. Adherence of staphylococci to human skin/ cartilage extrat. Polystyrene microtiter plates were coated with 5 µg/ml of human skin extract or cartilage extract in PBS overnight at 4°C. After washing with PBST, the wells were blocked by incubation with 1% (w/v) BSA in PBS for 1 h at 37oC. 100 µl bacteria adjusted to an OD540 of 1. 0

were added to each well and plate was incubated at 37oC for 1 h. Wells were washed with PBST and to each well 100 µl of 1: 1500 dilution of polyclonal antistaphylococcal antibody (Abcam, plc, Cambridge, UK)) was added and plate was again incubated at 37oC for 1 h. Wells were washed with PBST and to each well 100 µl of 1: 1500 dilution of AP-conjugated anti rabbit antibody raised in goats (Dako, Eching, Germany) was added and plate was placed for 1 h at 37oC. Wells were washed four times with PBST and with AP colour buffer. To each well 100 µl of phosphatase substrate (Sigma, S0942) was added and plate was read with in 20 min at 405 nm in Versamax microplate reader (Molecular Devices, CA94089, USA). Effect of rEap/ Emp and anti-Eap/anti-Emp on adherence. To observe the effect of rEap or rEmp or both together, recombinants proteins were added to wells coated with human skin or cartilage extracts. Plate was incubated for 1 h at 37oC and then unbound recombinants proteins were washed off with PBST. To look for effect of anti-Eap or anti-Emp or both in combinations, bacteria were first exposed to F(ab ´)2 in PBS+0. 1% BSA for 1 h at 37oC. Then bacteria were added to wells coated with human skin or cartilage extract. Rest of the method was same as described above. Adherence of staphylococci to Ea hy 926 cells. Briefly, endothelial cells were plated in a 96-well polystyrene microtiter plate and plate was incubated at 37oC in a CO2 incubator for upto 5 days until a confluent layer of cells was obtained. The wells were washed with PBS and fixed with ice cold methanol at -20oC for 10 min. The wells were then blocked with 1% BSA for 1 h at 37oC. To each well 100 µl of bacterial suspension in PBS adjusted to an OD540nm of 1. 0 was added and plate was incubated at 37oC for 1 h. After washing with PBST, the wells were again

blocked with 1% BSA in PBS for 1 h at 37oC. Wells were washed with PBST and to each well 100 µl of 1: 1500 dilution of antistaphylococcal antibody was added and plate was incubated at 37oC for 1 h. Wells were washed with PBST and to each well 100 µl of 1: 1500 dilution of AP-conjugated anti rabbit antibody raised in goats was added and plate was placed for 1 h at 37oC. Wells were washed four times with PBST and with AP colour buffer. To each well 100 µl of phosphatase substrate (sigma, S0942) was added and plate was read with in 20 min at 405 nm in Versamax microplate reader (Molecular Devices, CA94089, USA). Binding of Eap and Emp to human skin and cartilage extract. Polystyrene microtiter plates wells were coated with 5 µg /ml of human skin extract or cartilage extract in PBS overnight at 4°C. After washing with PBST, the wells were blocked by incubation with 1% BSA in PBS for 1 h at 37oC. To each well 100 µl of 6xHis-Eap or 6xHis-Emp suspension in PBS was added and plate was incubated at 37oC for 1 h. Wells were washed with PBST and to each well 100 µl of 1: 2000 dilution of anti-His HRP (horseradish peroxidase) antibody (Qiagen) was added and plate was incubated at 37oC for 1 h. Wells were washed four times with PBST and with substrate buffer. To each well 100 µl of colour substrate (ABTS, Sigma) was added and plate was read with in 20 min at 415 nm in Versamax microplate reader (Molecular Devices, CA94089, USA). Binding of rEap and rEmp to staphylococci. S. aureus Newman was grown with shaking (160 rpm) overnight at 37oC in BHI. Washed in PBS and fixed with 1% formaldehyde in PBS for 2 h. 100 µl fixed bacteria were added to each well of a microtitre plat and plate was placed at 37oC. After 2 h plate was washed with PBST and bacteria were fixed with ice cold methanol for 10 min at -20oC. Then plate

was washed with PBST and blocked with 1% BSA in PBS for 1 h at 37oC. After washing with PBS, 6xHis- Eap or 6xHis-Emp was added to each well and plate was incubated with shaking at 37oC for 1 h. Wells were washed with PBST and bound rEap or rEmp were determined as described above. Cell culture. Endothelial cells from human umbilical vein (HUVEC) were isolated by collagenase treatment as described previously (25) and grown on fibronectin-coated dishes in M199 supplemented with 20 mM Hepes (pH 7. 4), 2 mM glutamine, 10% (v/v) human serum, 10% (v/v) newborn calf serum, ECGS (150 mg ml-1), 5 IU ml-1 heparin, penicillin (100 IU/ml) and streptomycin (100 mg/ml) at 37oC in a 5% CO2 atmosphere. The human endothelial cell line Ea. hy926 (6), kindly provided by Volker Gerke (Münster, Germany), was maintained in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics (as HUVEC). The human epithelial cells (Adenovirus type 5 DNA-transformed primary human embryonic kidney cells, 293 cells) were obtained from the ATCC (#CRL-1573), were maintained in DMEM/Nut mix F- 12 (containing Glutamax I, a stable glutamine dipeptide), were supplemented with 10% fetal calf serum (FCS), 50 IU/ml penicillin, and 50 mg/ml streptomycin, and were split 1: 4 twice weekly, by trypsinization. Before the cells were used in the experiments, they had been passaged for a maximum of 35 times after freezing. Preparation of fluorescein-5-isothiocyanate (FITC)–labelled bacteria. For FITC-labeling, bacteria were prepared as described elsewhere (28, 51). In brief, bacterial cultures were grown overnight in 5 ml of BHI at 37oC with shaking at 160 rpm. Bacteria were washed with PBS, were fixed in 1% formaldehyde in PBS for 1 h, and then were washed again. The bacteria subsequently were treated with 3 ml

of 0. 5-mol/L NaHCO3 buffer (pH 9. 5) supplemented with 100 mg/mL FITC (isomer I [Molecular Probes]; solubilized in 150 mL of DMSO) for 1 h at 37oC. Finally, bacteria were resuspended in PBS that contained 1% HSA (W/V), and they were used within 24 h after preparation. Suspensions were normalized for an optical density of 1 at 540 nm, after gentle sonication in a water bath. Flow cytometric invasion assay. The flow cytometric invasion assay was performed as described previously with minor modifications (51). A fresh bacterial culture was used for each experiment. Briefly, endothelial cells were plated at 4x105 cells in 24-well plates the day before the assay. Cells were washed with DMEM, and then 0. 5 ml of 1% HSA, 10 mM Hepes (pH 7. 4) in DMEM and 50 ml of FITC-labelled bacteria suspension (OD540 1. 0) were added to cells. Culture dishes were pre-incubated for 1 h at 4oC to allow sedimentation of bacteria and were then shifted to 37oC for 3 h, to allow for invasion. Finally, cells were harvested, were treated with 20 mmol/L monensin (for 10 min at ambient temperature) to neutralize fluorescence quenching, and were analyzed by flow cytometry. Results were normalized according to the mean fluorescence intensity of the respective bacterial preparation, as determined by flow cytometry. The invasiveness of the laboratory strain Cowan I was set as 100%, and the results are shown as means ± SEM of three independent experiments performed in duplicates.

Results.

PCR amplification for the collagen adhesin gene cna. Using cna-specific primers (Table 1), the PCR products were obtained for S. aureus strains Cowan 1 and Phillips but not for strains Newman, Δeap, Δemp, ΔDM, and

compl. DM (data not shown). This is in accordance with previously published data (54) where strain Newman is reported to lack cna. Genotypic and phenotypic characterization of the Eap+Emp-deficient double mutant. We constructed a double mutant of strain Newman, deficient for Eap and Emp, by allelic replacement of emp in the established Δeap mutant (23). Genotypic characterization. The double cross-over emp:: GmR allelic replacement in the ΔDM mutant was confirmed by PCR amplification. The expected PCR products of 1 kb and 3. 0 kb from the parent strain Newman and the ΔDM mutant, respectively, were obtained using primers P2 and P4 (Table 1). The observed size increases for the PCR product generated from the ΔDM mutant with primers P2 and P4 as well as amplification of the GmR cassette of the ΔDM mutant but not of wild type strain Newman DNA using primers Gm-F and Gm-R (Table 1) confirmed the allelic replacement of the wild type emp gene by emp:: GmR (Fig. 1). Phenotypic characterization. (i) Biochemical analyses. Phenotypic comparison based on the biochemical profiles of the strains Newman, eap, emp and ΔDM by conventional microbiological tests (slide and tube coagulation test, Gram´s staining) and commercial identification test kits (API-Staph system, Pastorex latex slide agglutination for clumping factor, protein A and capsular polysaccharides) did not reveal any differences (data not shown). (ii) Analysis of surface proteins. For determination of cell surface protein expression patterns, proteins were extracted with 2% SDS and analyzed by SDS-PAGE and ligand overlay analysis. In Coomassie Blue-stained SDS-PAGE, the protein band pattern of an SDS extract from the mutants was similar to that of wild type Newman, with the exception that Eap, Emp and both Eap and Emp were

missing in Δeap, Δemp and ΔDM, respectively (Fig. 2, upper panel). Western immunoblots probed with a mixture of antibodies raised in rabbits against recombinants Eap and Emp, lacked recognition in cell surface protein extracts of Δemp, Δeap and ΔDM, bands at the position of Emp, Eap and both Emp and Eap, respectively. The ΔDM mutant was complemented with emp and eap and investigated for expression of functional Emp and Eap. Both adhesins were not detected in 2% SDS surface protein extracts of ΔDM, analyzed either by SDS-PAGE or by Western immunoblot assay, but detectable in extract of xylose-induced Compl. DM (Fig. 2, lower panel). Furthermore, functional Emp and Eap recognizing Fn, Vn and Fg were present in SDS extracts of the complemented mutant Compl. DM (data not shown). Adherence of staphylococci to immobilized ligands. We investigated defined mutants for a combined effect of Eap and Emp. Compared to wild type strain Newman, adherence of the Δemp mutant to immobilized Fn, Fg, and Cn was reduced by 60? %, 30? %, and 50 %, respectively. Adhernece of the Δeap mutant was only weakly affected (Discussion Binding to host structures and subsequent invasion of host cells essentially contribute to severe S. aureus infections, as osteomyelitis or wound and deep tissue infections, and are mediated by a wide variety of cell surface adhesions. A number of these adhesins covalently bind to peptidoglycan via the LPXTG consensus sequence, such as FnBPA, FnBPB, ClfA, ClfB, and the collagen binding protein (Cna). Another group of secreted S. aureus proteins includes the 19 kDa fibrinogen-binding protein Efb/Fib, staphylococcal coagulase, the Map (‘ major histocompatility complex type II analog protein’)/Eap homologues and Emp. Map/Eap homologues are prominent molecules in

S. aureus cell wall extracts of strain Newman (35). Eap and Emp possess extended-spectrum binding functions for eukaryotic ligands displaying binding activities for bone sialoprotein, fibronectin, fibrinogen, vitronectin, thrombospondin, and collagen-1 (22, 35), and osteopontin (27). A Δeap mutant of strain Newman shows that Eap does not contribute significantly to adherence to fibronectin and fibrinogen. Accordingly, reduced adherence of the Δemp mutant to fibronectin and fibrinogen suggests an important contribution of Emp to the overall adherence of strain Newman. S. aureus fibronectin-binding proteins (FnBPs) are important for binding to immobilized fibronectin, fibrinogen, and host cell invasion. Strain Newman, contains a centrally located point mutation in both fnbA and fnbB resulting in a stop codon. This leads to truncation of both FnBPs at the end of the C-domain, truncated FnBPs lack the cell wall anchor motif (LPETG) and are completely secreted into the culture supernatants (12). Therefore, strain Newman is only weakly adherent to immobilized fibronectin. This mutation may explain some of the earlier reported conflicting data with strain Newman (Lit?). Since strain Newman expresses functional ClfA and ClfB (38), the loss of FnBPs, and Eap/Emp may be partially compensated for by the Clfs with respect to adherence to fibrinogen. Binding of S. aureus to collagen by collagen-specific adhesins, such as collagen adhesin (Cna) is thought to be important in the pathogenesis of musculoskeletal infections like septic arthritis and osteomyelitis, as well as wound infections, since these infections take place in collagen-rich tissues. Vaccination with a recombinant fragment of collagen adhesin provides protection against S. aureus-mediated septic death (39). So far collagen adhesin (cna) is the only the reported adhesin that specifically

binds to collagen (44). Using gene knock out (disruption) studies in strain Philips and gene transfer to strain CYL316, Cna has been implicated as a virulence factor in ostemyelitis (43). A mutant of S. aureus strain PH100 with insertionally inactivated cna shows reduced virulence in animal models of endocarditis and septic arthritis (19, 23). A strong collagen binding characteristic has been attributed to S. aureus strains isolated from patients with arthritis and osteomyelitis, and has been implicated in the pathogenesis of septic arthritis (43). Recently, it has been shown by truncation studies that the virulence properties of Cna depend on its collagen-binding ability (59). However, a substantially higher prevalence in osteomyelitis isolates as compared to colonizing isolates has not been shown so far: The prevalence of the gene cna is only 40% (10 out of 25 isolates) (53), and only a moderate difference was seen in a larger study comparing 334 carrier and invasive disease isolates (32% and 52% in carrier and invasive isolates, respectively) (45). A further study with 216 isolates from bacteremic patients found a slightly higher total prevalence, no difference between the patient groups with or without bone or joint involvement (cna-positive: 57 % and 56 %, respectively) (47). In addition, there are conflicting data on the role of Cna: collagen binding is reported not to be a prerequisite for the development of endocarditis, osteomyelitis, or septic arthritis (47), and adherence of S. aureus to collagen is reported not to be a major virulence determinant for septic arthritis, osteomyelitis, or endocarditis (56). These studies suggest that in a substantial proportion of invasive isolates, other adhesins are important for collagen binding. This is also true for experimental models, since strain Newman is cna-negative. S. aureus isolates not harboring cna

may adhere to collagen I through enhanced expression of multiple adhesins like Eap or Emp. Our work reveals that …. Short Summary of results of the paper ….. native Emp and Eap from S. aureus, and recombinant Eap and Emp expressed either in E. coli or S. carnosus TM300 are binding equally well to collagen I in ligand overlay assays, rEmp binds to collagen I in a Biacore study and differential binding of Δeap, Δemp, Δeap/Δemp, and the parent strains in solid phase microtitre plate assay with immobilized collagen I (unpublished data). Taken together, our results suggest that Eap and Emp are potential candidates and alternative adhesins for collagen binding in S. aureus strains not harboring cna. As the rate of S. aureus strains with resistance to antibiotics (e. g. MRSA, VRSA) continuously increases, new antimicrobial therapies are needed. Identification of all bacterial products having binding affinity for collagen is necessary to develop therapeutic strategies to prevent bone and soft tissue infections.

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Legends.

Figure 1. Construction of a Δeap/Δemp double mutant in S. aureus strain Newman. The left panel is a schematic representation of the genetic map of emp in strain Newman and of the emp:: GmR insertion. Forward and reverse primers used for PCR amplification are shown. The size of the emp gene is 1.

0 kb, and the GmR cassette (not drawn to scale) is 2. 0 kb. The emp:: GmR construct on the pBT2 shuttle vector was electroporated into S. aureus Newman Δeap. The right panel is showing the PCR product amplified from genomic DNA of strain Newman (lanes 1 and 3) and ΔDM (lanes 2 and 4) with primers P2 and P4 (lanes 1 and 2) and GmR primers Gm-F and Gm-R (lanes 3 and 4). Figure 2. Analysis of cell surface-associated proteins. Lane 1, wild type strain Newman; lane 2, Δeap; lane 3, Δemp; lane 4, ΔDM; lane 5, ΔDMemp; and lane 6, Compl. DM. (Upper panel) Coomassie Blue-stained SDS-PAGE. (Lower panel) Western immunoblot analysis of blotted proteins probed with a mixture of antiserum directed separately against recombinant Emp and Eap in rabbits. Bound immunoglobulins were detected using AP-labeled anti-rabbit antibodies raised in sheep. Cell surface proteins were extracted as described in Materials and Methods. Molecular weight standards are in kDa. Figure 3. (A) Adherence to immobilized Fg, Fn and Cn. PMMA coverslips were coated with either human serum albumin, Fg, Fn or Cn I. A radiolabeled bacterial suspension of strains: 1, WT strain Newman; 2, Δeap; 3, Δemp: 4, ΔDM; and 5, Compl. DM was added to coverslips and incubated at 37°C with shaking. After 1 h the bacterial suspension was removed, the coverslips were washed with PBS, and the adherent radioactivity was measured. Figure 4. Adherence to immobilized Cn. Microtitre plate wells were coated separately with various concentrations of Cn (1 to 7 µg/ml) overnight and subsequently blocked with 3% BSA for 1 hour at 37°C. Bacteria were grown in BHI medium at 37ºC for 18 h, washed with PBS, adjusted to an OD540 of 1. 0 and added to microtitre wells. After 1 h at 37oC, wells were washed with PBST, adherent bacteria were stained with

safranin dye for 1 min and washed with PBST and the optical density at 490 nm was determined. The strains included are: WT strain Newman, Δeap, Δem, ΔDM and Compl. DM. Binding to BSA was used as a control. Experiments were performed three times in triplicates. Figure 5. Binding of soluble rEap and rEmp to S. aureus Newman. The indicated amounts of 6xHis-Eap or 6xHis-Emp were added to microtitre plate wells coated with cells of strain Newman. Bound 6xHis- Eap or 6xHis-Emp was determined using anti 6xHis-HRP antibody with ABTS colour reaction at OD 415 nm as described in methods and materials.. Figure 6. Binding of soluble rEap and rEmp to human skin and cartilage extract. The indicated amounts of 6xHis-Eap or 6xHis-Emp were added to microtitre plate wells coated with human skin or cartilage extract. Bound 6xHis- Eap or 6xHis-Emp was determined using anti 6xHis-HRP antibody with ABTS colour reaction at OD 415 nm as described in methods and materials. Figure 7 Adherence of S. aureus strain Newman, Δeap, Δ emp, ΔDM and compl. DM to human skin extract (A) and cartilage extract (B) and to various concentrations of human skin extract (C). Microtitre plate wells were coated with human skin extract (5 µg/ml) subsequently blocked with 1% BSA. Bacteria were grown in BHI broth overnight, washed with PBS and added to microtitre wells. After 1 h, wells were washed, to adherent bacteria antistaphylococcal antibody was added. AP conjugated anti-rabbit in goat antibody and AP colour substrate was used with optical density at 405 nm. Binding to BSA was used as a control. Experiments were performed three times in triplicates. Figure 8. Adherence of various staphylococcal strains to human skin extract (A) and cartilage extract (B). Microtitre plate wells were coated separately with human skin

extract or cartilage (5 µg/ml) subsequently blocked with 1% BSA. Bacteria were grown in BHI broth overnight, washed with PBS and added to microtitre wells. After 1 h, wells were washed, to adherent bacteria antistaphylococcal antibody was added. AP conjugated anti-rabbit in goat antibody and AP colour substrate was used with optical density at 405 nm. Binding to BSA was used as a control. Experiments were performed three times in triplicates. Figure 8. Effect of antibodies raised against Eap or Emp on adherence of strain Newman to skin or cartilage extract. Bacteria were first exposed to (Fab)2 fragments isolated from IgG fraction of anti-Eap or anti-Emp or both in combinations in PBS+0. 1% BSA for 1 h at 37oC. Then bacteria were added to wells coated with human skin or cartilage extract. Rest of the method was same as described for figure 8. Figure 9. Effect of rEap and rEmp on adherence of strain Newman to human skin and cartilage. The rEap or rEmp or both together were added to wells coated with human skin or cartilage extracts. Plate was incubated for 1 h at 37oC and then unbound recombinants proteins were washed off with PBST. Rest of the method was same as described for figure 8. Figure 10. Adherence to Ea. hy 926 cells of S. aureus strain Newman, Δeap, Δ emp, ΔDM and complemented ΔDM. Microtitre plate wells were coated with endothelial cells, subsequently blocked with 1% BSA. Bacteria were grown in BHI broth overnight, washed with PBS and added to microtitre wells. After 1 h, wells were washed, to adherent bacteria antistaphylococcal antibody was added. AP conjugated anti-rabbit in goat antibody and AP colour substrate was used with optical density at 405 nm. Figure 12. Invasion of Ea. hy 926 cells (A) HUVEC (B) and 293 cells by (C) S. aureus strain Newman, Δeap, Δ emp, ΔDM and

complemented ΔDM. Total internalized bacteria was measured by flow cytometry with fixed bacteria. Results are the means SEM of 3 independent experiments run in duplicates, expressed as relative invasiveness, compared with strain Cowan 1. S. carnosus was used as a negative control.

Table 2. Primer sequences used in this study

PrimerSequenceReferencePIIICTC GGA TCC AAG GAG TGA TAA TTT ATG AAATTT AAG TC(23)PIICTC GGT ACC TTA AAATTT AAT TTC AAT GTC TAC TTT TTT AAT GTC(23)P5CTC GGTACC AAG GAG AAA TAA CAGATG AAA AAG AAA TTA GTT TTA(22)P4SCTC GTC GAC TTA TAC TCG TGG TGC TGG TAA GC(22)P2CTC GGA TCC ATG AAA AAG AAA TTA TTA GTT TTA ACT(22)P4CTC GGT ACC TTA TAC TCG TGG TGC TGG TAA GC(22)Gm-FCTC GAT ATC AGA GGA GCC GTT CTT ATG GAC(33)Gm-RCTC GAT ATC AGA ACA GGA GTC TGG ACT TGA(33)cna-ForGCA CGA GAT ATT TCA TCA ACG ATC AAC GAA TG(54)cna-RevTTA TGA GTT AAA TCT TTT TCT TAA AAT TAA ATA CAG TCC C(54)eapP35′ CTC GGA TCC GCA GCT AAG CCA TTA GAT AAA TCA TCA AGT TCG TTA CAC C3′(23)eapP25′ CTC GG TACC TTA AAA TTT AAT TTC AAT GTC TAC TTT TTT AAT GTC 3(23)empP3CTC GGA TCC GCA TCA GTG ACA GAG AGT GTT GAC(22)empP4CTC GGT ACC TTA TAC TCG TGG TGC TGG TAA GC(22)Restriction enzyme sites are underlined.

Strain	Relevant Genotype or Plasmid	Properties	Reference or Source
S. aureus			
Cowan 1	Reference isolate	Isolated from septic arthritis	ATCC 12598
8325-4	Reference isolate, rsbU	Cured of prophages and plasmids	(11)
DU5883	fnbA:: TcR fnbB:: EmR	FnBPA --, FnBPB --	(11)
DU5883 (pFNBA4)	DU5883 (pFNBA4) CmR	FnBPA	8325-4 ++
+	(11)		
Newman	Reference isolate		
ClfA positive (high level)			
T. foster, Dublin, Ireland			
Philips	Reference isolate		
Clinical isolate, osteomyelitis case			
(43)			
SA4	Clinical isolate		
University Hospital, Muenster, Germany			
C47	Clinical isolate		
University Hospital, Muenster, Germany			
Wood 46	Reference isolate		

ATCC 10832

SA113Reference isolate

ATCC 35556

AH12Newman eap :: EmR

Eap - -

(23)

Emp50Newman emp :: EmR

Emp - -

(22)

DM23Newman eap :: EmR emp :: GmR

Eap - , Emp -

This study

Compl. DMDM23 (pCXREap+Emp) CmR

Eap +++, Emp +++

This study

S. carnosusTM300Reference isolate, WTNo expression of known adhesins(10)Escherichia coliDH5αCloning hostStratageneM15Expression vectorQiagenTG1Cloning hostStratagenepQEmpNpQE30+empEmp expression vector(22)pQEapNpQE30+eapEap expression vector(21)