Epidemiology, variable genetic organization and regulation of the EDIN-B toxin in Staphylococcus aureus from bacteraemic patients

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INTRODUCTION

The impact of Staphylococcus aureus as a hospital- and community-acquired pathogen is due in particular to its ability to produce and secrete a great variety of toxins. Major secreted toxins (exotoxins) of S. aureus include haemolysins, the superantigenic enterotoxins and toxic shock syndrome toxin, Panton–Valentine leukocidin, and exfoliative toxins (Lowy, 1998). Recently, it has been reported that S. aureus also produces exotoxins of the C3-transferase family of ADP-ribosyltransferases (EDIN-A, -B and -C). The prototype of these staphylococcal toxins was termed epidermal cell differentiation inhibitor (EDIN-A) by the group of Sugai in 1990 (Czech et al., 2001; Sugai et al., 1990, 1992; Wilde et al., 2001; Yamaguchi et al., 2001, 2002a). It could be demonstrated that EDIN-B/C3Stau, like its homologues from clostridia, specifically ADP-ribosylates and inactivates the Rho-family GTP binding protein RhoA, but unlike the clostridial toxins it also modifies RhoE and Rnd3 (Sugai et al., 1992; Wilde et al., 2001).

Rho GTP binding proteins are universal regulators of eukaryotic cell function and have been identified as primary targets of virulence factors from diverse bacterial species (Aktories & Barbieri, 2005). The central function of Rho GTP binding proteins is considered to be regulation of the actin cytoskeleton, but they also control cell cycle progression, transcriptional activity, intracellular vesicle transport and cell transformation (Jaffe & Hall, 2005). By downregulating Rho GTP binding proteins, bacterial pathogens can block crucial immune cell functions such as chemotaxis, phagocytosis and antigen presentation (Aepfelbacher et al., 2007; Aktories & Barbieri, 2005; Bokoch, 2005). Furthermore, it is well accepted that glucosylation of Rho GTPases by Toxin-A and Toxin-B of Clostridium difficile is the causative pathomechanism of pseudomembranous colitis (Genth et al., 2008). However, at present it is not known whether and how the Rho ADP-ribosylating toxins of Clostridium spp. and S. aureus are involved in pathogenicity.

Recombinant and S. aureus-produced EDINs have been reported to enter cells by endocytosis and to inhibit...
endothelial cell wound repair, as well as to cause transient macroapertures and loss of barrier function in endothelium (Aepfelbacher et al., 1997; Boyer et al., 2006). These EDIN effects most likely are due to RhoA inhibition and suggest that EDIN contributes to the pathophysiology of S. aureus systemic infection. Yet, the first EDIN effects described were inhibition of terminal differentiation of keratinocytes and induction of hyperplasia in mouse skin (Sugai et al., 1992), which is consistent with the epidermis being the primary infection site of S. aureus. A role for EDIN in S. aureus skin invasion is also supported by molecular epidemiological findings. edin-B and exfoliative toxin D (etd) are organized in one operon on a chromosomal pathogenicity island, and similarly, edin-C and exfoliative toxin B (etb) are located in close vicinity on a recently described S. aureus virulence plasmid (Yamaguchi et al., 2001). Moreover, edin-A is often associated with eta (Yamaguchi et al., 2002b). The exfoliative toxins are serine proteases which cleave the cell–cell contact protein desmoglein-1, thereby causing skin exfoliation (Ladhani et al., 1999; Yamaguchi et al., 2002a). An association of the exfoliative toxins A and B with the staphylococcal scalded skin syndrome (SSSS), a group of blistering skin diseases ranging from localized forms like bullous impetigo to generalized exfoliation of the skin, is well documented (Ladhani et al., 1999).

Expression of virulence genes in S. aureus is controlled by a complex network of regulatory components, most of which act at the transcriptional level. This is thought to allow the tailored production of groups of virulence proteins during different types and stages of infection. It is assumed for instance that during initiation of infection, bacterial adhesins favour tissue colonization, whereas at later stages, exotoxins promote bacterial spread and blockage of immune cell responses. Details of the complex gene-regulatory principles in S. aureus have been described in recent reviews (Arvidson & Tegmark, 2001; Cheung et al., 2004, 2008; Göerke & Wolz, 2004; Novick, 2003). A few general principles of virulence factor regulation in staphylococci have been worked out particularly well and are briefly summarized below.

The accessory gene regulator (agr) quorum-sensing system induces transcription of RNA III during transition of the post-exponential to the stationary phase of S. aureus growth. It has been well established that RNA III in most cases upregulates transcription of secreted protein genes and downregulates genes encoding cell surface proteins (Arvidson & Tegmark, 2001; Morfeldt et al., 1988; Peng et al., 1988; Recsei et al., 1986).

The staphylococcal accessory regulator (sar) gene locus encodes a family of DNA binding proteins (SarA family; reviewed by Cheung et al. 2008). SarA can also control RNA III expression and therefore affects some of the same virulence genes as agr (Cheung et al., 1997). However, sarA mutations can have inhibitory or stimulatory effects on agr-regulated transcripts. This depends on many variables, including the genetic background of the S. aureus strains containing the sarA mutation. SarA can also regulate transcription of virulence genes in an agr-independent manner, to which belong hla, tst-1 and ftnA (Chan & Foster, 1998; Chien et al., 1999; Wolz et al., 2000).

The purpose of this study was to gain more information about the prevalence, organization and regulation of the EDIN-B gene and protein.

**METHODS**

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli was routinely grown on Luria–Bertani (LB) agar; S. aureus was grown on Columbia blood agar or on brain heart infusion agar, when necessary, containing the appropriate antibiotics: 100 μg ampicillin ml⁻¹, 10 μg chloramphenicol ml⁻¹, 5 μg tetracycline ml⁻¹, 100 μg kanamycin ml⁻¹. Liquid cultures were grown in trypticase soy broth (TSB) (Becton Dickinson) supplemented with antibiotics for selection and plasmid maintenance at 37 °C and 200 r.p.m. in a shaking incubator (Sartorius). For protein and RNA preparations, cells were diluted 1:100 from an overnight liquid culture in the same medium and grown at 37 °C and 200 r.p.m. in a shaking incubator (Sartorius).

Clinical isolates and patients. Clinical strains were collected from blood cultures of patients hospitalized in the University Medical Center Hamburg-Eppendorf between 2004 and 2006. Species identification was performed using the plasma coagulase tube test and the automated identification system VITEK 2 (bioMérieux) with the Gram Positive Identification Card (GPI). All patients suffered from bloodstream infections due to S. aureus with ≥ 2 positive blood culture sets. edin-B-positive strains (for PCR strategy see below) were found in five cases of device-related infections (central venous catheter, urinary catheter or cardiac pacemaker), one case of chronic osteomyelitis, one case with unknown focus, and two cases of post-operative wound infection.

Identification and sequence analysis of etd pathogenicity island-positive S. aureus blood culture strains. DNA manipulations were performed using standard methods (Sambrook et al., 1989). Chromosomal DNA from staphylococci was prepared with the ChargeSwitch gDNA bacteria kit (Invitrogen) according to the manufacturer’s instructions, with the modification that lysostaphin (15 U ml⁻¹, Sigma) was added at the cell lysis step. For PCR screening of the etd pathogenicity island sequence, primers were designed according to the published etd pathogenicity island sequence, GenBank accession number AB057421, and amplifications were performed with the DyNAzyme DNA Polymerase kit (Finnzyme) under conditions recommended by the manufacturer. The primers and PCR conditions are summarized in Table 2. S. aureus TY114 served as a positive control. Nucleotide sequencing was carried out using purified PCR amplicons at MWG Biotech. The sequences were analysed with the Vector NTI Advance 10 software package and compared with the publicly available etd pathogenicity island sequence (GenBank accession number AB057421).

spa typing of edin-B-positive S. aureus. The PCR and sequencing reaction of the S. aureus protein A (spa) were performed as described by Harmsen et al. (2003). The chromatograms were analysed with the Ridom StaphType software (Ridom) and with the Based Upon Repeat Patterns (BURP) algorithm (Ridom); the evolutionary distance between strains was examined as described by Mellmann et al. (2007).

Construction of plasmid pRBpi and cloning. Chromosomal DNA from S. aureus TY114 was used for amplification of a 2735 bp
fragment of the etd pathogenicity island with the Triple Master PCR kit (Eppendorf) and primers Pl_for and Pl_rev (Table 2), thereby introducing a Sau3A restriction site at the 5’ end and an EcoRI restriction site at the 3’. Primers were designed according to the published etd pathogenicity island sequence, GenBank accession number AB057421. Additionally, information on the presumed promoter region was obtained from bioinformatics analysis using the TSSpred software (Gordon et al., 2006), available at http://eresearch.fit.qut.edu.au/downloads. The software detects transcription start sites for the promoters recognized by σ70, the primary sigma factor of E. coli (Gordon et al., 2006). This search revealed seven putative transcription start sites within 250 bp upstream of the etd start codon. Therefore, the resulting PCR fragment encompasses ORF1 (etd), orf2, ORF3 (edin-B), and a 259 bp region upstream of ORF1, the anticipated natural promoter region (Fig. 3). The amplicon was cloned into the TA-cloning vector pCR4. Sequencing of the insert demonstrated that no point mutations had been artificially introduced during amplification. Using the restriction sites introduced by PCR, the insert was excised with EcoRI and SalI. The fragment was directionally cloned into pRB473, a promoterless shuttle vector (Bruckner, 1992). The resulting construct was termed pRBpi. Cloning procedures were performed using E. coli TOP10 as a host. pRBpi was then transformed into the restriction-deficient S. aureus RN4220 strain by electroporation (Augustin & Gotz, 1990). The plasmid was subsequently transduced into S. aureus RN6390 wild-type, agr and sarA strains, as well as into S. aureus Newman wild-type and its agr and sarA mutants, using phage 80a or phage 11, as previously described (Mack et al., 2001).

Protease activity of S. aureus Newman and RN6390 on skimmed milk agar. Protease activity was analysed on a peptone/yeast agar containing 0.8 % (w/v) skimmed milk powder (Merck), as described elsewhere (Karlsson & Arvidson, 2002). Liquid cultures of bacteria were grown in tryptic soy broth (TSB) to OD600 0.4. Filter paper disks (diameter = 9 mm, Schleicher & Schuell) were placed in the centre of the agar plates. A 10 μl sample of the culture was applied to the filter paper disks and plates were incubated at 30 °C for up to 72 h. The experiment was done three times for each strain. The diameter of the proteolytic area was measured after 24, 48 and 72 h. For each time point, the mean of three experiments was determined.

Expression and purification of recombinant EDIN-B. The pQE system (Qiagen) was used to express 6 × His-tagged EDIN-B in E. coli. IPTG (1 mM) was used to induce expression for 2.5 h. Bacterial cells were lysed as recommended by the manufacturer and proteins were purified under native conditions.

Protein preparation and Western immunoblotting. In order to prepare proteins from culture supernatants, bacteria were grown overnight under continuous shaking at 200 r.p.m. and 37 °C in TSB containing appropriate antibiotics. This culture was diluted 1 : 100 in 10 ml of the same fresh medium. For comparing the EDIN-B amount in different genetic backgrounds, at each time point the samples were normalized to an equal OD600 in a SmartSpec photometer (Bio-Rad) using 2.5 ml macrocultures with a path length of 12.5 mm. In order to estimate the influence of proteases on the amount of EDIN-B, protease inhibitor 22-macroglobulin (0.25 U ml⁻¹, Roche) was added to the growth medium. The supernatants were collected by centrifugation at 4000 m s⁻² for 15 min at 4 °C. Proteins were precipitated overnight on ice with 10 % (v/v) TCA. After centrifugation at 20000 m s⁻² and 4 °C for 15 min, the pellet was washed three times with 1.5 ml acetone, dried at room temperature, and resuspended in 100 μl PBS. SDS-PAGE was performed by standard methods (Laemmli, 1970). Proteins were separated on 11 % (w/v) polyacrylamide gels and electrophoretically transferred to PVDF Western blotting membranes in a semi-wet transfer unit (Invitrogen). The Precision Plus prestained protein standard (Bio-Rad) was used for molecular mass determination. Membranes were blocked with 0.5 % (w/v) dehydrated skimmed milk overnight at 4 °C. Specific rabbit polyclonal anti-EDIN-B antibodies (a gift of T. Yamaguchi, University of Hiroshima, Japan) diluted 1 : 5000 in PBS/0.1 % (v/v) Tween 20 (PBS-Tween 20) were used for a 1 h incubation at room temperature. Bound antibodies were detected by incubation with an anti-rabbit F(ab’)2 fragment conjugated with streptavidin–horseradish peroxidase (Amersham Bioscience) diluted 1 : 10 000 in PBS-Tween 20 for 1 h at room temperature. Membranes were developed with the ECL Western blotting detection reagent (Amersham Bioscience) and exposed to X-ray films (Kodak). All immunoblotting experiments were performed at least two times.
<table>
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<th>Function or reference</th>
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<td>Pi_rev</td>
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*Safl restriction site underlined.
†EcoRI restriction site underlined.

**Detection of anti-EDIN-B antibodies in human serum.** SDS-PAGE and electrophoretic transfer of recombinant EDIN-B to PVDF Western blotting membranes were performed as described above. The membranes were incubated with serial dilutions of human sera ranging from 1:1000 to 1:100 000 for 1 h. Bound antibodies were detected by incubating the membrane with a γ-chain-specific anti-human antibody conjugated with streptavidin–horseradish peroxidase from goat (Sigma) diluted 1:10 000 in PBS–Tween 20 for 1 h at room temperature.

**RNA preparation and real-time PCR.** For quantification of transcripts, total RNA was isolated from liquid cultures. Overnight cultures supplemented with the required antibiotics were diluted 1:100 into fresh TSB and cultivated up to OD₆₀₀ 0.8 and for 18 h. Samples were immediately collected on ice and centrifuged at 5000 × g for 5 min, resuspended in 2 ml ice-cold PBS, and mixed with RNase-free DNase (Promega), as described previously (Knobloch et al., 2004). The absence of DNA was confirmed by real-time PCR using primers gyr_F1 and gyr_R1, as described below. To evaluate the efficiency of the primers and the PCR, serial dilutions of chromosomal DNA (S. aureus TY114) were made and PCR was performed as described below. For reverse transcription, the DNase-treated RNA samples were diluted 1:10, and 5 μl was used for first-strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). The cDNA solution was diluted 1:4, and 1.5 μl was used as a template for real-time PCR. Reaction mixes were prepared with a qPCR Mastermix Plus for SYBR Green I kit (Eurogentec) and primers edincycl_for, edincycl_rev, gyr_F1 and gyr_R1. Real-time PCR was run in an IQ thermal cycler (pH 8) and the bacterial cell walls were digested with 150 U lysostaphin ml⁻¹ at 4 °C for 30 min. Then, 350 μl of the RLT buffer supplied supplemented with 3.5 μl β-mercaptoethanol (Roth) was added, and the suspension was transferred to a Lysing Matrix B tube (Q-biogene) and homogenized with a FastPrep centrifuge (Savant Instruments). After centrifugation at 14 000 m s⁻², the extraction was performed as described by the manufacturer. Contaminating DNA was degraded by digesting the RNA samples with RNase-free DNase (Promega), as described previously (Knobloch et al., 2004). The absence of DNA was confirmed by real-time PCR using primers gyr_F1 and gyr_R1, as described below. To evaluate the efficiency of the primers and the PCR, serial dilutions of chromosomal DNA (S. aureus TY114) were made and PCR was performed as described below. For reverse transcription, the DNase-treated RNA samples were diluted 1:10, and 5 μl was used for first-strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). The cDNA solution was diluted 1:4, and 1.5 μl was used as a template for real-time PCR. Reaction mixes were prepared with a qPCR Mastermix Plus for SYBR Green I kit (Eurogentec) and primers edincycl_for, edincycl_rev, gyr_F1 and gyr_R1. Real-time PCR was run in an IQ thermal cycler

**Table 2.** PCR primers used in the study

http://mic.sgmjournals.org
(Bio-Rad) under the following conditions: 95 °C for 3 min, 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. All real-time PCR experiments were performed in triplicate and all RNA preparations were performed three times. Relative transcriptional levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) with gyrB as internal control and Ct being the threshold cycle value.

RESULTS

The gene for EDIN-B is part of a diverse pathogenicity island present in specific S. aureus clonal lineages

To test the prevalence and genetic organization of edin-B in S. aureus blood culture isolates from septic patients, genomic DNA of 121 independent isolates was screened for edin-B by PCR (primers are listed in Table 2). Eight of the 121 S. aureus strains (7%) carried edin-B. All edin-B-positive strains were analysed by SmaI macrorestriction and PFGE, demonstrating that all but one isolate are closely related (Murchan et al., 2003; Tenover et al., 1995) (data not shown). A clonal relationship was further confirmed by spa typing, which assigned two strains each to t078 and t081 and one strain each to t216, t287, t349 and t401. spa types t078, t081, t349 and t401 are closely related as determined using the BURP algorithm (Ridom) (Harmsen et al., 2003). spa type t216 is unrelated to the other spa types because the cost distance is more than 4. Spa type t287 had to be excluded from further analysis because it only consists of three repeats (Mellmann et al., 2007). Cluster analysis using the spa typing data demonstrates that spa types t078, t081, t349 and t401 belong to clonal complex (CC) 25. Spa type t216 corresponds to multi locus sequence type (MLST) ST-59, which is unrelated to CC 25 (www.ridom.de/spa-server/). The edin-B-positive reference strain S. aureus TY114 was assigned to spa type t1481, which is also unrelated to CC 25. Additional analysis showed that all edin-B-positive blood culture isolates belong to agr type 1 (data not shown). In S. aureus TY114 in which the etd pathogenicity island was first described, edin-B and etd are part of a seven-ORF chromosomal genetic element flanked by 5 bp direct repeats (Yamaguchi et al., 2002a). The exact organization of the edin-B/etd pathogenicity island of TY114 is depicted in Fig. 1(a). To determine the chromosomal position and the molecular architecture of the etd pathogenicity island, all edin-B-positive sepsis
strains were subjected to direct nucleotide sequencing (primers are listed in Table 2). These data suggested that in our isolates the edin-B/etd pathogenicity island is located at the same chromosomal region as in TY114 but that it is variably composed.

Assembly of the DNA sequence fragments and comparison with TY114 (GenBank accession number AB057421) revealed three different genotypes characterized by the absence of IS256 (genotype 1), IS256/hsdS (genotype 2) or IS256/hsdS/hsdM/etd (genotype 3) (Fig. 1a). Thus, all edin-B-positive blood culture isolates harbour orf2, a predicted serine protease, and lack IS256, which suggests that the presence of this mobile genetic element represents an exception. One strain lacks etd, which loosens the hitherto tight epidemiological association of EDIN and exfoliative toxin genes. Western blotting experiments showed that all edin-B-positive strains expressed EDIN-B independently of the underlying genotype (Fig. 1b).

Detection of anti-EDIN-B antibodies in the serum of infected patients

If EDIN-B is produced during infection, anti-EDIN antibodies should be detectable in the serum of patients. Three sera from patients infected with edin-B-positive S. aureus strains were screened for the presence of anti-EDIN-B antibodies by Western blotting using recombinant EDIN-B as an antigen. In all three sera, anti-EDIN-B antibodies could be found. Antibody titres in sera of two patients were about 1:100,000, but in the serum of a patient suffering from chronic osteomyelitis an antibody titre of >1:100,000 was determined (Fig. 2). Six sera from patients infected with edin-B-negative S. aureus (three of which are shown in Fig. 2; numbers 4–6) and 10 sera from healthy blood donors (data not shown) contained no anti-EDIN-B antibodies, even after prolonged exposure of the blot membrane. These data indicate that EDIN-B is in fact produced during human infection and that continuous exposure to this virulence factor boosts antibody production.

The S. aureus regulator sarA controls transcription of edin-B

To get a first idea about the modalities of edin-B transcription, RNA was isolated from S. aureus TY114 in the exponential (4 h after inoculation) and stationary growth phases. Transcriptional analysis using real-time PCR according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) revealed that edin-B transcription is upregulated 15-fold in the exponential growth phase as compared with the stationary phase (data not shown).

With the intention of identifying regulators of edin-B, we employed S. aureus strains of two well-characterized genetic backgrounds, S. aureus RN6390 and Newman, and their isogenic agr and sarA mutants. These strains were transduced with plasmid pRBpi (see Fig. 3 for a schematic diagram of the insert), which was constructed by cloning etd (ORF1), orf2, edin-B (ORF3), and a 259 bp region upstream of the etd start codon comprising the anticipated natural promoter region of this operon into the promoterless shuttle vector pRB473 (Bruckner, 1992). Evidence for putative promoter structures upstream of etd was obtained from a bioinformatics analysis using the TSSpreded software (Gordon et al., 2006) (see Methods and Fig. 3 for further information). Additionally, RT-PCR using primers Etd_for and Edin_B rev with RNA prepared from S. aureus TY114 indicated that etd, orf2 and edin-B are expressed as one transcript spanning the three genes (data not shown).

For transcriptional analysis, RNA was prepared from equal amounts of bacteria derived from the exponential (OD$_{600}$ 0.8, 3–4 h after inoculation) or stationary phase of growth (18 h). Relative RNA levels were determined by real-time PCR according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). edin-B transcription in agr and sarA mutants of RN6390 and Newman was calculated compared with the respective wild-type strains. Interestingly, the sarA mutant of RN6390 showed a 500-fold downregulation of edin-B transcription in the exponential growth phase as compared with wild-type RN6390. No edin-B transcript could be detected in RN6390 sarA in the stationary growth phase (Fig. 4). Likewise, the sarA mutant of Newman displayed a significant three- and 13-fold reduction of edin-B transcription in the exponential and stationary growth phases, respectively (Fig. 4). Neither the agr mutant of RN6390 nor that of Newman exhibited a significant reduction of edin-B transcription (Fig. 4). These data show that on the level of transcription the edin-B/etd operon is influenced by the sarA, but not by the agr system.
Extracellular EDIN-B protein levels are sensitive to bacterial proteases

Besides control by transcriptional regulation, the level of exotoxins in staphylococcal growth supernatants can be controlled by post-transcriptional mechanisms. EDIN-B protein levels were determined in culture supernatants of TY114 at different growth phases. At time points ranging from 5 to 19 h, protein preparations from growth supernatants of identical numbers of bacteria (as determined by OD_{600}) were assayed (Fig. 5a). Together with the results from transcriptional analysis, these experiments demonstrate that EDIN-B is produced and secreted in a growth phase-dependent mode during transition from the exponential to the stationary phase of growth. Representative S. aureus strains belonging to the three genotypes identified by us (Fig. 1a) displayed growth phase-dependent EDIN-B production identical to that of TY114 (our unpublished data).

We next analysed EDIN-B protein amounts in growth supernatants (from 4 to 18 h after inoculation) of RN6390 and Newman wild-type and their respective sarA and agr mutants. Surprisingly, no EDIN-B protein was detected in the growth supernatant of RN6390 wild-type. However, a high level of EDIN-B was found in the supernatant of the RN6390 agr mutant, whereas again no EDIN-B could be detected in the supernatant of the RN6390 sarA mutant. At first sight, the failure to detect EDIN-B in supernatants of S. aureus RN6390 wild-type seems to be in conflict with the data showing an edin-B transcript in this strain. However, S. aureus RN6390 is known to be a functional sigmaB mutant, which leads to a constitutively elevated agr level (Giachino et al., 2001; Horsburgh et al., 2002). Since agr positively regulates the expression and secretion of staphylococcal proteases (Shaw et al., 2004), we speculated that EDIN-B in the growth supernatant of RN6390 wild-type might be removed through degradation by secreted proteases. Consequently, in the agr mutant these proteases should be produced to a lesser extent, which would explain why most of the secreted EDIN-B remains intact there. We tested this hypothesis with two experimental approaches. First, levels of EDIN-B were measured in the absence and presence of the broad-spectrum plasma protease inhibitor a2-macroglobulin at 8 and 18 h of growth (Fig. 5c). Supporting our hypothesis and verifying the transcriptional and translational regulation of edin-B, we observed a decrease in EDIN-B protein levels in the presence of the protease inhibitor.

Fig. 3. Schematic representation of the cloning strategy of pRBpi and localization of presumed transcription start sites. In order to analyse the regulation of edin-B, a 2735 bp PCR fragment of the etd pathogenicity island from S. aureus TY114 was integrated into the multiple cloning site of the promoterless shuttle vector pRB473 using Sall and EcoRI restriction sites. The insert consisted of etd, orf2, edin-B, and a 259 bp fragment upstream of etd, the putative promoter region. The positions of seven assumed transcription start sites identified by bioinformatics search (Gordon et al., 2006) are indicated (1–7). etd, exfoliative toxin D; edin-B, epidermal cell differentiation inhibitor-B; orf2, serine protease-like protein; hsdM, DNA methylase M. Representation not to scale.

Fig. 4. Regulation of edin-B transcription. Transcription of edin-B was analysed in RN6390 agr and sarA and Newman agr and sarA compared with their respective wild-type strains. Relative transcription was calculated using the $2^{-ΔΔC_{T}}$ method with gyrB as the reference gene (Livak & Schmittgen, 2001). Grey bars, RNA preparations from exponential growth phase; white bars, preparations from stationary growth phase. Means and standard deviations from three independent RNA preparations are given, and each transcription analysis was performed in triplicate. Asterisk: in the stationary growth phase, RN6390 sarA showed no edin-B transcript after 40 PCR cycles. The horizontal dotted line marks up- or downregulation (±2.5-fold). Values between these lines are defined as being not significantly different from 1.
tional data, a strong band of EDIN-B appeared in the supernatant of RN6390 wild-type in the presence of the protease inhibitor. Furthermore, addition of the protease inhibitor had little influence on EDIN-B amounts in supernatants of the agr mutant (Fig. 5c). Consistent with the lack of detectable edin-B transcription (Fig. 4), addition of the protease inhibitor did not reveal any EDIN-B in the RN6390 sarA mutant. Second, we directly assessed the proteolytic activity of the S. aureus strains investigated using a skimmed milk agar assay (Table 3, Supplementary Fig. S1). This assay indicated that S. aureus RN6390 wild-type in fact displays a significant proteolytic activity, whereas its agr mutant shows much less of this activity, best seen at the 24 and 48 h time points. Therefore, we propose
that EDIN-B is absent in RN6390 wild-type due to proteolytic degradation and absent in the RN6390 sarA mutant due to a lack of edin-B transcription. The results of EDIN-B protein regulation in RN6390 were compared with the respective results in Newman. EDIN-B was detected in Newman wild-type, which is consistent with its rather low proteolytic activity as compared with RN6390 wild-type (Fig. 5b, Table 3). EDIN-B was also detected in the Newman sarA mutant, which is readily explicable by less pronounced inhibition of edin-B transcription as compared with the RN6390 sarA mutant. Finally, an anticipated derepression of protease activity in the Newman sarA mutant (Lindsay & Foster, 1999) could be confirmed in the skimmed milk assay, and as a result, it was possible to increase the supernatant level of EDIN-B in this mutant by addition of the protease inhibitor (Fig. 5c, Table 3). Taken together, these data demonstrate that the level of EDIN-B in the extracellular medium is dependent on transcriptional and post-transcriptional mechanisms. For edin-B transcription, the sarA system plays a crucial role in strains RN6390 and Newman. Global regulatory systems are apparently responsible for the production of proteases which degrade the EDIN-B protein in the extracellular space. Here, the level of EDIN-B-degrading proteases is strongly dependent on the genetic background and the regulatory systems, which are altered in the S. aureus strains investigated.

**DISCUSSION**

We consider the EDIN proteins (A, B and C) to be relevant exotoxins of S. aureus not only because they belong to the Rho-GTPase-modifying bacterial virulence factors but also because the genes encoding EDIN are genetically associated with the exfoliative toxin genes and with certain invasive S. aureus lineages. Also, the effects of EDIN on keratinocyte differentiation and endothelial function support the idea that this toxin family contributes to the invasiveness of S. aureus.

The prevalence of EDIN genes (edin-A, -B or -C) in S. aureus has been determined in previous studies. EDIN genes were detected in 4% (three out of 81) of nasal isolates and in 8% (15 out of 196) of heterogeneous clinical isolates from Germany (Czech et al., 2001) as well as in 15% (13 of 88) of bullous impetigo isolates from Japan (Yamaguchi et al., 2002b). In the German S. aureus isolates, edin-B was predominant (about 90% of edin-positives), whereas in Japanese isolates, edin-C was the predominant subtype (about 80% of edin-positives) (Czech et al., 2001; Yamaguchi et al., 2002b). A study focusing on etd/edin-B prevalence in French S. aureus isolates revealed no difference between clinical and colonization isolates (10% versus 9% prevalence, respectively) (Yamasaki et al., 2006). We show here that the prevalence of edin-B in invasive blood culture isolates of S. aureus (7%) is not substantially different from that of the S. aureus populations investigated previously. Importantly, in a recent German study, the etd pathogenicity island was found in 10% of blood culture isolates (eight out of 88) but not (none out of 107) in nasal colonization isolates (Holtfreter et al., 2007). The study by Holtfreter and co-workers indicates a clear association of edin-B/etd with invasive strains of S. aureus. Consistent with those findings, our edin-B-positive strains belong to spa type t078 or a closely related spa type and to CC 25. CC 25 is one of the major meticillin-susceptible S. aureus clonal lineages in European countries (Deurenberg & Stobberingh, 2008). In community-acquired MRSA (CA-MRSA) populations, edin-B and/or etd were found in Pantone–Valentine-leukocidin-positive (lukS-PV lukF-PV) strains belonging almost exclusively to CC 80/spa type t044, the most frequent CA-MRSA clone in Europe and Tunisia (Ben Nejma et al., 2008; Holmes et al., 2005; Monecke et al., 2006; Vandenesch et al., 2003; Witte et al., 2007). Neither the edin-B-positive invasive strains of Holtfreter and co-workers nor those of our study carried mecA (our unpublished observations; Holtfreter et al., 2007). In addition, all of our strains were lukS/lukF-negative (our unpublished observations). Altogether, it appears that prevalence data cannot easily be employed to assess the contribution of EDIN to S. aureus pathogenicity. In this regard it has been proposed that the correlation of virulence factors with invasiveness of S. aureus may be obscured by the underlying clonal population structure of the staphylococci (Peacock et al., 2002).

Taken together, edin-B has been associated with two invasive S. aureus clonal lineages, CC 25 and relatives and CA-MRSA belonging to clonal complex 80/spa type t044 in Europe and Tunisia (Ben Nejma et al., 2008; Holmes et al., 2005; Holtfreter et al., 2007; Monecke et al., 2006; Vandenesch et al., 2003). In a Japanese study, edin (mostly

**Table 3. Diameter of proteolytic area on skimmed milk agar**

<table>
<thead>
<tr>
<th>Time</th>
<th>6390 wt</th>
<th>Newman wt</th>
<th>6390 agr</th>
<th>Newman agr</th>
<th>6390 sar</th>
<th>Newman sar</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>15.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>48 h</td>
<td>24.7</td>
<td>10</td>
<td>17</td>
<td>0</td>
<td>24.7</td>
<td>16.3</td>
</tr>
<tr>
<td>72 h</td>
<td>27</td>
<td>10.7</td>
<td>22.7</td>
<td>0</td>
<td>26</td>
<td>21</td>
</tr>
</tbody>
</table>

wt, Wild-type.
the plasmid-encoded *edin-C*) was detected in 15% of bullous impetigo isolates, whereas other clinical isolates or nasal isolates showed only a very low prevalence (estimated to be below 5%). The *edin*-positive isolates from Japan were to a large part *mecA*-positive, although they were phenotypically not MRSA (Yamaguchi *et al.*, 2002b). Hence, *edin* genes are apparently transferred on mobile genetic elements into fundamentally different genetic backgrounds in different regions of the world. Yet, it is intriguing that in almost all isolates, *edin-A*, *edin-B* and *edin-C* are genetically associated with *eta*, *etd* and *eth*, respectively (Yamaguchi *et al.*, 2001, 2002a, b). Taken together, *edin-B* and *etd* are associated with more invasive, partially *mecA*-positive strains of *S. aureus*, but the presence of these two virulence factors alone does not determine invasiveness.

In our invasive *S. aureus* strains, *edin-B* was detected within variants of the original *edin-B/etd* pathogenicity island (Yamaguchi *et al.*, 2002a). It is well accepted that *S. aureus* pathogenicity islands show diversification via rearrangements and deletions (Hacker & Kaper, 2000; Sung *et al.*, 2008). Evolutionary diversification has also been described for the staphylococcal cassette chromosome *mec* (SCC*mec*) gene complex (Chongtrakool *et al.*, 2006) and the staphylococcal superantigen-like proteins (*ssl*-cluster) (Fitzgerald *et al.*, 2003). According to one hypothesis, different alleles can evolve from an ancestral gene cluster by introduction into distinct genetic backgrounds, whereby individual components of the cluster are lost (Deurenberg *et al.*, 2007; Fitzgerald *et al.*, 2003). Because the *edin-B/etd* pathogenicity island variants of the German blood culture isolates are almost exclusively found in *spa* types related to t078, it is reasonable to assume that these variants developed by intra-lineage (vertical) genetic rearrangements (Lindsay & Holden, 2006). Yet, the presence of *edin-B/etd* in the distant *spa* type t044 (CC 80) also demonstrates that the *edin-B/etd* pathogenicity island has properties of a mobile genetic element that can be transferred horizontally.

In this study we were able to show for the first time, to our knowledge, that anti-EDIN-B antibodies are produced by patients infected with *edin-B*-positive *S. aureus*. Exceptionally high anti-EDIN-B titres were found in the serum of a patient suffering from chronic *S. aureus* osteomyelitis. This preliminary observation indicates that the toxin is produced *in vivo* and elicits a humoral immune response (Clarke *et al.*, 2006). The kinetics of antibody production in disease and reconvalescence and the potential of EDIN-B antibodies in modulating the infection process remain to be determined.

It is thought that *S. aureus* can adapt to different environments and infection phases by tight transcriptional and (post)translational regulation of its virulence factors (Chan & Foster, 1998; Goerke *et al.*, 2001). We report here that *edin-B* transcription is significantly diminished in *sarA* mutants of *S. aureus* Newman and RN6390. Regulation by *sarA* has likewise been described for the alpha toxin gene (*bla*) and the toxic shock syndrome toxin gene (*tst*) in *S. aureus* 8325-4 (Cheung *et al.*, 2004; Dunman *et al.*, 2001). By using *agr* mutants of Newman and RN6390 we also could demonstrate that the *agr* system has no significant effect on *edin-B* transcription. Virulence factors known to be regulated by the *agr* system include extracellular proteases such as the V8 serine protease and various exotoxins such as the Panton–Valentine leukocidin (Bronner *et al.*, 2004; Novick, 2003). Consistent with the drastically reduced levels of *edin-B* transcript in the *sarA* mutant of RN6390, no EDIN-B protein could be found in the growth supernatant of this mutant. However, no secreted EDIN-B could be detected in the RN6390 wild-type either, despite a significant level of *edin-B* transcript. In contrast, EDIN-B was readily detectable in the *agr* mutant of RN6390, although the level of *edin-B* transcript in this strain was similar to that of RN6390 wild-type. For interpreting these data it has to be considered that RN6390, which is a derivative of 8325, displays a functional sigmaB deficiency due to a mutation in the rsbW regulator rsbU (Bischoff *et al.*, 2004; Horsburgh *et al.*, 2002). Because sigmaB is a negative regulator of *agr*, this results in an artificially high activity of the *agr* regulatory system. As a consequence, the production and release of extracellular proteases are strongly enhanced in RN6390 (Oscarsson *et al.*, 2006b). It is known that bacterial proteases can cleave bacterial proteins, thereby readapting the staphylococci to changing environmental conditions (McAleese *et al.*, 2001; Shaw *et al.*, 2004). Furthermore, in rsbU-positive strains (clinical isolates and an rsbU-positive derivative of 8325), a transcription profile distinct from RN6390 can be seen for various virulence factors including extracellular proteases (Blevins *et al.*, 2002; Cassat *et al.*, 2006; Oscarsson *et al.*, 2006a). By using a protease inhibitor and direct measurement of protease activity we verified that the EDIN-B protein secreted by the RN6390 wild-type is certainly removed by proteolytic degradation. In the light of the above considerations it may be deduced that the *agr* mutant of RN6390 produces less proteases, and that this coincides with increased EDIN-B levels. In contrast, the Newman strain produced the most proteases when *sarA* was mutated (Blevins *et al.*, 2002), and this again was paralleled by a positive effect of the protease inhibitor on EDIN-B levels.

At present, it remains unclear whether SarA modulates *edin-B* expression directly or indirectly, for example by influencing the activity of additional regulators such as SarT, ArlRS and SaeRS. Preliminary data from our laboratory demonstrate that inactivation of *sae* has no influence on *edin-B* transcription (data not shown). Nevertheless, additional work using a combination of regulatory mutants is required before it is possible to specifically ascribe an exact mechanism of global regulation for *edin-B*. These studies will also have to address the question of whether sigmaB is involved in *edin-B* regulation. Taken together, the extracellular level of EDIN-B is dependent on both *sarA*-regulated transcription.
of edin-B and secreted proteases, the production of which is governed by different global regulatory systems. To decipher more definitely the role of EDIN toxins in *S. aureus* virulence, studies using purified toxins or specifically constructed *S. aureus* mutants in experimental models of infection are warranted.

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