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

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EDIN-B (epidermal cell differentiation inhibitor-B; also termed C3Stau) is an exotoxin of *Staphylococcus aureus* which ADP-ribosylates and inactivates Rho GTP binding proteins. The EDIN-B gene (*edin-B*) and the gene for exfoliative toxin D (*etd*) make up the central part of a recently described pathogenicity island. Here we evaluated the prevalence and genetic organization of the *edin-B/etd* pathogenicity island in invasive *S. aureus* isolates, and characterized *edin-B* transcription and EDIN-B production using artificial constructs transduced in *S. aureus* strains RN6390 and Newman. We found that eight out of 121 (7 %) *S. aureus* blood culture isolates harbour *edin-B*, which is organized in three novel variants of the original *edin-B/etd* pathogenicity island. In the serum of patients infected with *edin-B*-positive *S. aureus*, significant titres of anti-EDIN-B antibodies could be detected. Regulation of *edin-B* transcription depended on the *sarA* but not on the *agr* regulatory system. Furthermore, retrieval of EDIN-B protein secreted by *S. aureus* RN6390 required the presence of α 2-macroglobulin to inhibit the activity of extracellular proteases. These data suggest that the EDIN-B toxin is produced during human infection, is part of a highly variable pathogenicity island and can be controlled by the *sarA* gene regulon and secreted bacterial proteases.

Received 24 April 2009

Revised 21 October 2009

Accepted 23 October 2009

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

Abbreviation: CC, clonal complex.

A supplementary figure, showing protease activity on skimmed milk agar, is available with the online version of this paper.

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; Goerke & 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 *fnbA* (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, urine 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, 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

Table 1. Strains and plasmids used in the study

Strain or plasmid	Genotype and relevant properties	Source or reference
<i>E. coli</i> strain		
TOP10	Cloning host, genotype F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) F80 <i>lacZ</i> Δ M15 <i>ΔlacX74recA1 deoR araD139 Δ(ara-leu)7697 galU galK rpsL</i> (StrR) <i>endA1 nupG</i>	Invitrogen
<i>S. aureus</i> strains		
RN4220	8325-4 derivative, restriction-deficient cloning host	Novick (1991)
RN6390	Related to 8325-4, <i>rsbU</i> ⁻	Peng <i>et al.</i> (1988)
RN6390 <i>agr</i>	<i>agr::tetM</i>	Novick <i>et al.</i> (1993)
RN6390 <i>sarA</i>	<i>sar::kana</i>	C. Wolz
Newman	Wild-type strain	Duthie & Lorenz (1952)
Newman <i>agr</i>	<i>agr::tetM</i>	Wolz <i>et al.</i> (1996)
Newman <i>sarA</i>	<i>sar::kana</i>	This work
TY114	Isolate from wound infection, detected at the Clinical Laboratory Department in Hiroshima City Hospital, <i>etd</i> pathogenicity island-positive	Yamaguchi <i>et al.</i> (2002a)
Plasmids		
pCR4	<i>E. coli</i> vector for direct cloning of PCR fragments	Invitrogen
pRB473	Promoterless shuttle vector for expression in <i>E. coli</i> and staphylococci	Bruckner (1992)
pRBpi	pRB473 containing <i>etd</i> , <i>orf2</i> , <i>edin-B</i> and their anticipated natural promoter region	This work
pQEedin-B	Expression of 6 × His-tagged EDIN-B	M. Sugai

fragment of the *etd* pathogenicity island with the Triple Master PCR kit (Eppendorf) and primers Pi_for and Pi_rev (Table 2), thereby introducing a *SalI* restriction site at the 5' end and an *EcoRI* restriction site at the 3' end. 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 σ^{70} , 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 80 α 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 μ l 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 macrocuvettes with a path length of 12.5 mm. In order to estimate the influence of proteases on the amount of EDIN-B, protease inhibitor α 2-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 20 000 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 μ l 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 2. PCR primers used in the study

Primer name	Primer sequence (5'–3')	Function or reference
Pi_for	<u>GTCGACCAGTAATAGACATGTGATTCCCTC</u> *	Amplification of <i>etd</i> pathogenicity island fragment
Pi_rev	CACTTATATAAA <u>GAATTC</u> GGTGTAAGTAC†	Amplification of <i>etd</i> pathogenicity island fragment
EdinB_for	ATCTCAATAGCTGCCGAGACTA	PCR screening of clinical strains
EdinB_rev	TATCAGAAAGTTTAACTGCTCTACA	PCR screening of clinical strains
Orf2_for	ATGCTATTAGTTGCTAACTATCAAATAGT	PCR screening of clinical strains
Orf2_rev	CAGAACCAGATTGTCCAGAAGTA	PCR screening of clinical strains
Orf7_for	ATAGTGCATATTAAGTTCAGACTTTATG	PCR screening of clinical strains
Orf7_rev	GTTCTGTAGAGAATATAAGATGCCA	PCR screening of clinical strains
hsdS_for	TGACCAGATCTTACAGACATGACC	PCR screening of clinical strains
hsdS_rev	GATTACAGATTGCTATTAATGAACG	PCR screening of clinical strains
hsdM_for	ATATCTCGGTATATTTAGGTTGTAATC	PCR screening of clinical strains
hsdM_rev	TATGCAGATGCCTTGTCAGG	PCR screening of clinical strains
Etd_for	GGGGAGACTATAGCTTCTGGTGTATTA	PCR screening of clinical strains
Etd_rev	TCCAACATGAATACCACTAAGTCT	PCR screening of clinical strains
Edinicycl_for	GCAGCACTAGCAGGTAGGC	Real-time PCR of <i>edin-B</i>
Edinicycl_rev	AGCATATTCTGTCCCTCTAGGC	Real-time PCR of <i>edin-B</i>
gyrB_F1	GACTGATGCCGATGTGGA	Real-time PCR of <i>gyrB</i> ; Harraghy <i>et al.</i> (2005)
gyrB_R1	AACGGTGGCTGTGCAATA	Real-time PCR of <i>gyrB</i> ; Harraghy <i>et al.</i> (2005)
EdinPI_5for	TTTCAACTGTACCATTGCTG	Nucleotide sequencing of <i>etd</i> pathogenicity island
EdinPI_3rev	TGTCGAATAGTAGAGATGAAGCAT	Nucleotide sequencing of <i>etd</i> pathogenicity island
HsdS5_rev	GTCATGCTCTGAAGATCTGGTCA	Nucleotide sequencing of <i>etd</i> pathogenicity island
Orf7_3for	ACATTTGGCATCTTATATTCTCTAC	Nucleotide sequencing of <i>etd</i> pathogenicity island
HsdS_for3370	CTTTATTGCAATTACTGATTGA	Nucleotide sequencing of <i>etd</i> pathogenicity island
HsdM_rev4028	CTAGAAGCCGTGATTGGCT	Nucleotide sequencing of <i>etd</i> pathogenicity island
HsdM_for4741	ATATCGCTGAACAGACCGAT	Nucleotide sequencing of <i>etd</i> pathogenicity island
Etd_rev6199	GAATTATCTACTGAATACAATGAGC	Nucleotide sequencing of <i>etd</i> pathogenicity island
Etd_for6037	GTCAATATTTTGGGTATACTGAGTC	Nucleotide sequencing of <i>etd</i> pathogenicity island
EDINB_rev7308	TTTCCCCATTAGTAGCTTCA	Nucleotide sequencing of <i>etd</i> pathogenicity island
EDINB_for7676	CAACTAGTTAGTGGTGCAGCAC	Nucleotide sequencing of <i>etd</i> pathogenicity island
ORF7_rev8912	GTTTCAGGTAATAAAGGAGCAGT	Nucleotide sequencing of <i>etd</i> pathogenicity island
ORF7_for8758	GAAATATCCAAAAATTTCTCATATAG	Nucleotide sequencing of <i>etd</i> pathogenicity island
ORF8_rev10556	TGTTCACTTGGAAATAATGGC	Nucleotide sequencing of <i>etd</i> pathogenicity island
ORF9_rev11259	CAGTAATACCATCTACTGAGATCG	Nucleotide sequencing of <i>etd</i> pathogenicity island

**SalI* 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 m s⁻² and 4 °C for 5 min, resuspended in 2 ml ice-cold PBS, and mixed with RNeasy Protect Bacteria Reagent (Qiagen) to irreversibly inhibit the RNases. RNA was prepared with the RNeasy Bacteria kit (Qiagen) with the following modifications: after incubation with the RNase inhibitor, samples were resuspended in 200 μ l Tris-EDTA buffer

(pH 8) and the bacterial cell walls were digested with 150 U lysostaphin ml⁻¹ at 4 °C for 30 min. Then, 350 μ l of the RLT buffer supplied supplemented with 3.5 μ l 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 μ l 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 μ l 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 edinicycl for, edinicycl rev, gyr_F1 and gyr_R1. Real-time PCR was run in an IQ thermal cycler

(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 $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001) with *gyrB* as internal control and C_T 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 *Sma*I 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

Fig. 1. Identification of novel genotypes of the *etd* pathogenicity island and their distribution in *S. aureus* sepsis strains. (a) Sequencing and comparison with the reference strain *S. aureus* TY114 revealed three novel genotypes (1, 2 and 3) of the *etd* pathogenicity island in *edin-B*-positive *S. aureus* blood culture isolates. In all strains investigated the pathogenicity island (black arrows) is inserted in the same chromosomal region as that of *S. aureus* strain N315 (grey arrows), thereby replacing a 98 bp gene fragment. IS256, insertion sequence element 256; SA2004, putative uncharacterized protein of N315; SA2005, uncharacterized hydrolase of N315; *hsdS*, site-specific DNase; *hsdM*, DNA methylase M; *etd*, exfoliative toxin D; *orf2*, serine protease-like protein; *edin-B*, epidermal cell differentiation inhibitor-B. Representation not to scale. (b) Production of EDIN-B by the different genotypes. Exoprotein preparations from overnight liquid cultures of the *edin-B*-positive blood culture strains were separated by SDS-PAGE, blotted onto PVDF membranes and developed with a rabbit anti-EDIN-B antibody.

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 : 1000, 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

Fig. 2. Detection of specific anti-EDIN-B antibodies in human serum. Recombinant EDIN-B was run on SDS-PAGE gels and immunoblotted with different patient sera (patients 1–6; serum dilutions indicated). Patients 1–3, positive blood culture with *edin-B*-positive *S. aureus* isolate; patients 4–6, positive blood culture with *edin-B*-negative *S. aureus* isolate. Patient 1 was suffering from chronic osteomyelitis: an EDIN-B band was visible up to a serum dilution of 1 : 100 000. Upper panel, exposure time 5 min for patient sera 1–3 and 10 min for patient sera 4–6; lower panel, exposure time 5 s.

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 $2^{-\Delta\Delta C_T}$ 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 TSSpred 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 (OD₆₀₀ 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 $2^{-\Delta\Delta C_T}$ 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.

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 *SalI* 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.

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

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 $2^{-\Delta\Delta C_T}$ 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.

from 5 to 19 h, protein preparations from growth supernatants of identical numbers of bacteria (as determined by OD₆₀₀) 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 $\alpha 2$ -macroglobulin at 8 and 18 h of growth (Fig. 5c). Supporting our hypothesis and verifying the transcrip-

Fig. 5. Influence of global regulators on EDIN-B levels. (a) Growth phase-dependent expression of EDIN-B. In order to monitor EDIN-B formation during TY114 growth, samples of the bacterial growth suspension were taken at the indicated time points, normalized to the same OD₆₀₀. Supernatants were immuno-blotted using an anti-EDIN-B antibody. (b) The level of EDIN-B in growth supernatants of indicated strains at designated time points was determined as in (a). (c) Levels of EDIN-B in the growth supernatants of indicated strains in the absence or presence of the protease inhibitor α 2-macroglobulin were determined as in (a). WB, Western blot.

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

Table 3. Diameter of proteolytic area on skimmed milk agar

wt, Wild-type.

Time	Diameter of proteolytic area (mm) for strain:					
	6390 wt	Newman wt	6390 agr	Newman agr	6390 sar	Newman sar
24 h	15.7	0	0	0	0	12
48 h	24.7	10	17	0	24.7	16.3
72 h	27	10.7	22.7	0	26	21

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 methicillin-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 Panton-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

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 *etb*, 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

(*hla*) and the toxic shock syndrome toxin gene (*tsst*) 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.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SPP1130). Phage 11 and *S. aureus* RN6390 *sarA* were kindly provided by C. Wolz, University of Tübingen, Germany, and the rabbit anti-EDIN-B antibody by T. Yamaguchi, University of Hiroshima, Japan. pRB473 was a gift of R. Brückner, University of Kaiserslautern, Germany. We thank A. Peschel, University of Tübingen, Germany, and S. Foster, University of Sheffield, UK, for providing bacterial strains. We particularly appreciate the help of Claudia Trasak with EDIN purification, Western blotting and bacterial growth analysis. We are grateful to S. Scherpe, M. Christner and M. Wolters from our laboratory for *spa* typing and for help with the interpretation of data from transcription analysis.

REFERENCES

- Aepfelbacher, M., Essler, M., Huber, E., Sugai, M. & Weber, P. C. (1997). Bacterial toxins block endothelial wound repair. Evidence that Rho GTPases control cytoskeletal rearrangements in migrating endothelial cells. *Arterioscler Thromb Vasc Biol* 17, 1623–1629.
- Aepfelbacher, M., Trasak, C. & Ruckdeschel, K. (2007). Effector functions of pathogenic *Yersinia species*. *Thromb Haemost* 98, 521–529.
- Aktories, K. & Barbieri, J. T. (2005). Bacterial cytotoxins: targeting eukaryotic switches. *Nat Rev Microbiol* 3, 397–410.
- Arvidson, S. & Tegmark, K. (2001). Regulation of virulence determinants in *Staphylococcus aureus*. *Int J Med Microbiol* 291, 159–170.
- Augustin, J. & Gotz, F. (1990). Transformation of *Staphylococcus epidermidis* and other staphylococcal species with plasmid DNA by electroporation. *FEMS Microbiol Lett* 54, 203–207.
- Ben Nejma, M., Mastouri, M., Bel Hadj Jrad, B. & Nour, M. (2008). Characterization of ST80 Pantone–Valentine leukocidin-positive community-acquired methicillin-resistant *Staphylococcus aureus* clone in Tunisia. *Diagn Microbiol Infect Dis* (in press), doi:10.1016/j.diagmicrobio.2008.02.010.
- Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bachi, B. & Projan, S. (2004). Microarray-based analysis of the *Staphylococcus aureus* σ^B regulon. *J Bacteriol* 186, 4085–4099.
- Blevins, J. S., Beenken, K. E., Elasri, M. O., Hurlburt, B. K. & Smeltzer, M. S. (2002). Strain-dependent differences in the regulatory roles of *sarA* and *agr* in *Staphylococcus aureus*. *Infect Immun* 70, 470–480.
- Bokoch, G. M. (2005). Regulation of innate immunity by Rho GTPases. *Trends Cell Biol* 15, 163–171.
- Boyer, L., Doye, A., Rolando, M., Flatau, G., Munro, P., Gounon, P., Clément, R., Pulcini, C., Popoff, M. R. & other authors (2006). Induction of transient macroapertures in endothelial cells through RhoA inhibition by *Staphylococcus aureus* factors. *J Cell Biol* 173, 809–819.
- Bronner, S., Monteil, H. & Prevost, G. (2004). Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. *FEMS Microbiol Rev* 28, 183–200.
- Bruckner, R. (1992). A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. *Gene* 122, 187–192.
- Cassat, J., Dunman, P. M., Murphy, E., Projan, S. J., Beenken, K. E., Palm, K. J., Yang, S. J., Rice, K. C., Bayles, K. W. & Smeltzer, M. S. (2006). Transcriptional profiling of a *Staphylococcus aureus* clinical isolate and its isogenic *agr* and *sarA* mutants reveals global differences in comparison to the laboratory strain RN6390. *Microbiology* 152, 3075–3090.
- Chan, P. F. & Foster, S. J. (1998). Role of SarA in virulence determinant production and environmental signal transduction in *Staphylococcus aureus*. *J Bacteriol* 180, 6232–6241.
- Cheung, A. L., Eberhardt, K. & Heinrichs, J. H. (1997). Regulation of protein A synthesis by the *sar* and *agr* loci of *Staphylococcus aureus*. *Infect Immun* 65, 2243–2249.
- Cheung, A. L., Bayer, A. S., Zhang, G., Gresham, H. & Xiong, Y. Q. (2004). Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*. *FEMS Immunol Med Microbiol* 40, 1–9.
- Cheung, A. L., Nishina, K. A., Trottonda, M. P. & Tamber, S. (2008). The SarA protein family of *Staphylococcus aureus*. *Int J Biochem Cell Biol* 40, 355–361.
- Chien, Y., Manna, A. C., Projan, S. J. & Cheung, A. L. (1999). SarA, a global regulator of virulence determinants in *Staphylococcus aureus*, binds to a conserved motif essential for *sar*-dependent gene regulation. *J Biol Chem* 274, 37169–37176.
- Chongtrakool, P., Ito, T., Ma, X. X., Kondo, Y., Trakulsomboon, S., Tiensasitorn, C., Jamklang, M., Chavalit, T., Song, J. H. & Hiramatsu, K. (2006). Staphylococcal cassette chromosome *mec* (SCCmec) typing of methicillin-resistant *Staphylococcus aureus* strains isolated in 11 Asian countries: a proposal for a new nomenclature for SCCmec elements. *Antimicrob Agents Chemother* 50, 1001–1012.
- Clarke, S. R., Brummell, K. J., Horsburgh, M. J., McDowell, P. W., Mohamad, S. A., Stapleton, M. R., Acevedo, J., Read, R. C., Day, N. P. & other authors (2006). Identification of *in vivo*-expressed antigens of *Staphylococcus aureus* and their use in vaccinations for protection against nasal carriage. *J Infect Dis* 193, 1098–1108.
- Czech, A., Yamaguchi, T., Bader, L., Linder, S., Kaminski, K., Sugai, M. & Aepfelbacher, M. (2001). Prevalence of Rho-inactivating epidermal cell differentiation inhibitor toxins in clinical *Staphylococcus aureus* isolates. *J Infect Dis* 184, 785–788.
- Deurenberg, R. H. & Stobberingh, E. E. (2008). The evolution of *Staphylococcus aureus*. *Infect Genet Evol* 8, 747–763.
- Deurenberg, R. H., Vink, C., Kalenic, S., Friedrich, A. W., Bruggeman, C. A. & Stobberingh, E. E. (2007). The molecular evolution of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* 13, 222–235.
- Dunman, P. M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., Brown, E. L., Zagursky, R. J., Shlaes, D. & Projan, S. J. (2001). Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. *J Bacteriol* 183, 7341–7353.
- Duthie, E. S. & Lorenz, L. L. (1952). Staphylococcal coagulase; mode of action and antigenicity. *J Gen Microbiol* 6, 95–107.
- Fitzgerald, J. R., Reid, S. D., Ruotsalainen, E., Tripp, T. J., Liu, M., Cole, R., Kuusela, P., Schlievert, P. M., Järvinen, A. & Musser, J. M. (2003). Genome diversification in *Staphylococcus aureus*: molecular evolution of a highly variable chromosomal region encoding the staphylococcal exotoxin-like family of proteins. *Infect Immun* 71, 2827–2838.
- Genth, H., Dreger, S. C., Huelsenbeck, J. & Just, I. (2008). *Clostridium difficile* toxins: more than mere inhibitors of Rho proteins. *Int J Biochem Cell Biol* 40, 592–597.
- Giachino, P., Engelmann, S. & Bischoff, M. (2001). σ^B activity depends on RsbU in *Staphylococcus aureus*. *J Bacteriol* 183, 1843–1852.

- Goerke, C. & Wolz, C. (2004). Regulatory and genomic plasticity of *Staphylococcus aureus* during persistent colonization and infection. *Int J Med Microbiol* **294**, 195–202.
- Goerke, C., Fluckiger, U., Steinhuber, A., Zimmerli, W. & Wolz, C. (2001). Impact of the regulatory loci *agr*, *sarA* and *sae* of *Staphylococcus aureus* on the induction of α -toxin during device-related infection resolved by direct quantitative transcript analysis. *Mol Microbiol* **40**, 1439–1447.
- Gordon, J. J., Towsey, M. W., Hogan, J. M., Mathews, S. A. & Timms, P. (2006). Improved prediction of bacterial transcription start sites. *Bioinformatics* **22**, 142–148.
- Hacker, J. & Kaper, J. B. (2000). Pathogenicity islands and the evolution of microbes. *Annu Rev Microbiol* **54**, 641–679.
- Harmsen, D., Claus, H., Witte, W., Rothganger, J., Turnwald, D. & Vogel, U. (2003). Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. *J Clin Microbiol* **41**, 5442–5448.
- Harraghy, N., Kormanec, J., Wolz, C., Homerova, D., Goerke, C., Ohlsen, K., Qazi, S., Hill, P. & Herrmann, M. (2005). *sae* is essential for expression of the staphylococcal adhesins Eap and Emp. *Microbiology* **151**, 1789–1800.
- Holmes, A., Ganner, M., McGuane, S., Pitt, T. L., Cookson, B. D. & Kearns, A. M. (2005). *Staphylococcus aureus* isolates carrying Pantón–Valentine leukocidin genes in England and Wales: frequency, characterization, and association with clinical disease. *J Clin Microbiol* **43**, 2384–2390.
- Holtfreter, S., Grumann, D., Schmutde, M., Nguyen, H. T., Eichler, P., Strommenger, B., Kopron, K., Kolata, J., Giedrys-Kalemba, S. & other authors (2007). Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. *J Clin Microbiol* **45**, 2669–2680.
- Horsburgh, M. J., Aish, J. L., White, I. J., Shaw, L., Lithgow, J. K. & Foster, S. J. (2002). σ^B modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol* **184**, 5457–5467.
- Jaffe, A. B. & Hall, A. (2005). Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol* **21**, 247–269.
- Karlsson, A. & Arvidson, S. (2002). Variation in extracellular protease production among clinical isolates of *Staphylococcus aureus* due to different levels of expression of the protease repressor *sarA*. *Infect Immun* **70**, 4239–4246.
- Knobloch, J. K., Jager, S., Horstkotte, M. A., Rohde, H. & Mack, D. (2004). RsbU-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative sigma factor σ^B by repression of the negative regulator gene *icaR*. *Infect Immun* **72**, 3838–3848.
- Ladhani, S., Joannou, C. L., Lochrie, D. P., Evans, R. W. & Poston, S. M. (1999). Clinical, microbial, and biochemical aspects of the exfoliative toxins causing staphylococcal scalded-skin syndrome. *Clin Microbiol Rev* **12**, 224–242.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lindsay, J. A. & Foster, S. J. (1999). Interactive regulatory pathways control virulence determinant production and stability in response to environmental conditions in *Staphylococcus aureus*. *Mol Gen Genet* **262**, 323–331.
- Lindsay, J. A. & Holden, M. T. (2006). Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. *Funct Integr Genomics* **6**, 186–201.
- Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25**, 402–408.
- Lowy, F. D. (1998). *Staphylococcus aureus* infections. *N Engl J Med* **339**, 520–532.
- Mack, D., Bartscht, K., Fischer, C., Rohde, H., de Grahl, C., Dobinsky, S., Horstkotte, M. A., Kiel, K. & Knobloch, J. K. (2001). Genetic and biochemical analysis of *Staphylococcus epidermidis* biofilm accumulation. *Methods Enzymol* **336**, 215–239.
- McAleese, F. M., Walsh, E. J., Sieprawska, M., Potempa, J. & Foster, T. J. (2001). Loss of clumping factor B fibrinogen binding activity by *Staphylococcus aureus* involves cessation of transcription, shedding and cleavage by metalloprotease. *J Biol Chem* **276**, 29969–29978.
- Mellmann, A., Weniger, T., Berssenbrugge, C., Rothganger, J., Sammeth, M., Stoye, J. & Harmsen, D. (2007). Based Upon Repeat Pattern (BURP): an algorithm to characterize the long-term evolution of *Staphylococcus aureus* populations based on *spa* polymorphisms. *BMC Microbiol* **7**, 98.
- Monecke, S., Slickers, P., Hotzel, H., Richter-Huhn, G., Pohle, M., Weber, S., Witte, W. & Ehrlich, R. (2006). Microarray-based characterisation of a Pantón–Valentine leukocidin-positive community-acquired strain of methicillin-resistant *Staphylococcus aureus*. *Clin Microbiol Infect* **12**, 718–728.
- Morfeldt, E., Janzon, L., Arvidson, S. & Lofdahl, S. (1988). Cloning of a chromosomal locus (*exp*) which regulates the expression of several exoprotein genes in *Staphylococcus aureus*. *Mol Gen Genet* **211**, 435–440.
- Murchan, S., Kaufmann, M. E., Deplano, A., de Ryck, R., Struelens, M., Zinn, C. E., Fussing, V., Salmenlinna, S., Vuopio-Varkila, J. & other authors (2003). Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus*: a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. *J Clin Microbiol* **41**, 1574–1585.
- Novick, R. P. (1991). Genetic systems in staphylococci. *Methods Enzymol* **204**, 587–636.
- Novick, R. P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol Microbiol* **48**, 1429–1449.
- Novick, R. P., Ross, H. F., Projan, S. J., Kornblum, J., Kreiswirth, B. & Moghazeh, S. (1993). Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J* **12**, 3967–3975.
- Oscarsson, J., Kanth, A., Tegmark-Wisell, K. & Arvidson, S. (2006a). *SarA* is a repressor of *hla* (α -hemolysin) transcription in *Staphylococcus aureus*: its apparent role as an activator of *hla* in the prototype strain NCTC 8325 depends on reduced expression of *sarS*. *J Bacteriol* **188**, 8526–8533.
- Oscarsson, J., Tegmark-Wisell, K. & Arvidson, S. (2006b). Coordinated and differential control of aureolysin (*aur*) and serine protease (*sspA*) transcription in *Staphylococcus aureus* by *sarA*, *rot* and *agr* (RNAIII). *Int J Med Microbiol* **296**, 365–380.
- Peacock, S. J., Moore, C. E., Justice, A., Kantzanou, M., Story, L., Mackie, K., O'Neill, G. & Day, N. P. (2002). Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. *Infect Immun* **70**, 4987–4996.
- Peng, H. L., Novick, R. P., Kreiswirth, B., Kornblum, J. & Schlievert, P. (1988). Cloning, characterization, and sequencing of an accessory gene regulator (*agr*) in *Staphylococcus aureus*. *J Bacteriol* **170**, 4365–4372.
- Recsei, P., Kreiswirth, B., O'Reilly, M., Schlievert, P., Gruss, A. & Novick, R. P. (1986). Regulation of exoprotein gene expression in *Staphylococcus aureus* by *agr*. *Mol Gen Genet* **202**, 58–61.

- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shaw, L., Golonka, E., Potempa, J. & Foster, S. J. (2004). The role and regulation of the extracellular proteases of *Staphylococcus aureus*. *Microbiology* **150**, 217–228.
- Sugai, M., Enomoto, T., Hashimoto, K., Matsumoto, K., Matsuo, Y., Ohgai, H., Hong, Y. M., Inoue, S., Yoshikawa, K. & Suganaka, H. (1990). A novel epidermal cell differentiation inhibitor (EDIN): purification and characterization from *Staphylococcus aureus*. *Biochem Biophys Res Commun* **173**, 92–98.
- Sugai, M., Hashimoto, K., Kikuchi, A., Inoue, S., Okumura, H., Matsumoto, K., Goto, Y., Ohgai, H., Moriishi, K. & other authors (1992). Epidermal cell differentiation inhibitor ADP-ribosylates small GTP-binding proteins and induces hyperplasia of epidermis. *J Biol Chem* **267**, 2600–2604.
- Sung, J. M., Lloyd, D. H. & Lindsay, J. A. (2008). *Staphylococcus aureus* host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. *Microbiology* **154**, 1949–1959.
- Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**, 2233–2239.
- Vandenesch, F., Naimi, T., Enright, M. C., Lina, G., Nimmo, G. R., Heffernan, H., Liassine, N., Bes, M., Greenland, T. & other authors (2003). Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton–Valentine leukocidin genes: worldwide emergence. *Emerg Infect Dis* **9**, 978–984.
- Wilde, C., Chhatwal, G. S., Schmalzing, G., Aktories, K. & Just, I. (2001). A novel C3-like ADP-ribosyltransferase from *Staphylococcus aureus* modifying RhoE and Rnd3. *J Biol Chem* **276**, 9537–9542.
- Witte, W., Strommenger, B., Cuny, C., Heuck, D. & Nuebel, U. (2007). Methicillin-resistant *Staphylococcus aureus* containing the Panton–Valentine leukocidin gene in Germany in 2005 and 2006. *J Antimicrob Chemother* **60**, 1258–1263.
- Wolz, C., McDevitt, D., Foster, T. J. & Cheung, A. L. (1996). Influence of *agr* on fibrinogen binding in *Staphylococcus aureus* Newman. *Infect Immun* **64**, 3142–3147.
- Wolz, C., Pohlmann-Dietze, P., Steinhuber, A., Chien, Y. T., Manna, A., van Wamel, W. & Cheung, A. (2000). *agr*-independent regulation of fibronectin-binding protein(s) by the regulatory locus *sar* in *Staphylococcus aureus*. *Mol Microbiol* **36**, 230–243.
- Yamaguchi, T., Hayashi, T., Takami, H., Ohnishi, M., Murata, T., Nakayama, K., Asakawa, K., Ohara, M., Komatsuzawa, H. & Sugai, M. (2001). Complete nucleotide sequence of a *Staphylococcus aureus* exfoliative toxin B plasmid and identification of a novel ADP-ribosyltransferase, EDIN-C. *Infect Immun* **69**, 7760–7771.
- Yamaguchi, T., Nishifuji, K., Sasaki, M., Fudaba, Y., Aepfelbacher, M., Takata, T., Ohara, M., Komatsuzawa, H., Amagai, M. & Sugai, M. (2002a). Identification of the *Staphylococcus aureus* *etd* pathogenicity island which encodes a novel exfoliative toxin, ETD, and EDIN-B. *Infect Immun* **70**, 5835–5845.
- Yamaguchi, T., Yokota, Y., Terajima, J., Hayashi, T., Aepfelbacher, M., Ohara, M., Komatsuzawa, H., Watanabe, H. & Sugai, M. (2002b). Clonal association of *Staphylococcus aureus* causing bullous impetigo and the emergence of new methicillin-resistant clonal groups in Kansai district in Japan. *J Infect Dis* **185**, 1511–1516.
- Yamasaki, O., Tristan, A., Yamaguchi, T., Sugai, M., Lina, G., Bes, M., Vandenesch, F. & Etienne, J. (2006). Distribution of the exfoliative toxin D gene in clinical *Staphylococcus aureus* isolates in France. *Clin Microbiol Infect* **12**, 585–588.

Edited by: J. A. Lindsay