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SHORT COMMUNICATION

Characterization of transcription within *sdr* region of *Staphylococcus aureus*

Izabela Sitkiewicz · Ireneusz Babiak · Waleria Hryniewicz

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Abstract *Staphylococcus aureus* is an opportunistic pathogen responsible for various infections in humans and animals. It causes localized and systemic infections, such as abscesses, impetigo, cellulitis, sepsis, endocarditis, bone infections, and meningitis. *S. aureus* virulence factors responsible for the initial contact with host cells (MSCRAMMs—microbial surface components recognizing adhesive matrix molecules) include three Sdr proteins. The presence of particular *sdr* genes is correlated with putative tissue specificity. The transcriptional organization of the *sdr* region remains unclear. We tested expression of the *sdrC*, *sdrD,* or *sdrE* genes in various in vitro conditions, as well as after contact with human blood. In this work, we present data suggesting a separation of the *sdr* region into three transcriptional units, based on their differential reactions to the environment. Differential reaction of the *sdrD* transcript to environmental conditions and blood suggests dissimilar functions of the *sdr* genes. SdrE has been previously proposed to play role in bone infections, whilst our results can indicate that *sdrD* plays a role in the interactions between the pathogen and human immune system, serum or specifically reacts to nutrients/other factors present in human blood.

Keywords *sdr* · *Staphylococcus aureus* · MSCRAMMs

I. Sitkiewicz · W. Hryniewicz (✉)
Department of Epidemiology and Clinical Microbiology, National Medicines Institute, Chełmska 30/34, Warszawa, Poland
e-mail: waleria@cls.edu.pl

I. Sitkiewicz
e-mail: isitkiewicz@cls.edu.pl

I. Babiak
Department of Orthopedics and Traumatology of Locomotory System, Medical University of Warsaw, Warsaw, Poland

Introduction

Staphylococcus aureus is an opportunistic pathogen responsible for various infections in humans and animals. *S. aureus* causes both localized and systemic infections, such as abscesses, impetigo, cellulitis, sepsis, endocarditis, bone infections, and meningitis. *S. aureus* is also responsible for diseases caused by secreted toxins such as enterotoxins, exfoliatins (scalded skin syndrome), or toxic shock syndrome toxin (Lowy 1998). At present, *S. aureus* is one of the most important causes of nosocomial infections, especially infections of surgical sites, catheters, and implants. *S. aureus* causes a large number of dangerous community acquired infections that have a significant impact on public health (Bartlett 2008). In addition to its high pathogenic potential, *S. aureus*

is a reservoir of multiple antibiotic resistance genes (Jensen and Lyon 2009).

The pathogenesis process is multifactorial, and it is very difficult to assess the role of particular virulence factors in the process. A group of *S. aureus* virulence factors responsible for the initial contact with host cells mediates adhesion of staphylococcal cells to the extracellular matrix of host cells and are called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). MSCRAMMs are cell surface proteins that recognize fibronectin-, fibrinogen-, collagen-, and heparin-related polysaccharides (Patti et al. 1994).

Sdr proteins (from SD Repeat), together with MSCRAMM proteins ClfA (clumping factor A) and ClfB (clumping factor B), are members of a structurally related family of cell wall anchored proteins (Josefsson et al. 1998). The characteristic feature of the family is the presence of R region containing multiple serine-aspartate repeats (Foster and Hook 1998; Josefsson et al. 1998; Ní Eidhin et al. 1998). The *sdr* locus encodes three proteins, SdrC, SdrD, and SdrE; however, not all three genes are present in all *S. aureus* strains (Sabat et al. 2006). Also, the transcriptional organization of the region remains unclear. Based on previous analyses (Peacock et al. 2002; Sabat et al. 2006), it was noticed that the *sdrC* gene is always present in the locus, while *sdrD* and *sdrE* are not. There also seems to be a correlation between carriage/invasive strains and the presence of the *sdrE* gene (Peacock et al. 2002). Strains carrying only the *sdrC* gene have a diminished potential to cause bone infections, which may be connected with the fact that one of the allelic variants of SdrE was previously identified as a bone sialoprotein-binding protein (Tung et al. 2003). SdrC binds β-neurexin 1 exodomain and expression of the protein increases adherence to cultured mammalian cells expressing β-neurexin on their surface (Barbu et al. 2010). Other Sdr proteins are involved in adherence to epithelial cells (Corrigan et al. 2009) and SdrD is crucial in abscess formation (Cheng et al. 2009).

In this work, we present data confirming a separation of the *sdr* region into three transcriptional units, based on their differential reaction to the environment. In addition, we present data that the *sdrD* gene might be involved in pathogenesis and invasiveness, as it is activated upon contact with human blood.

Materials and methods

Bacterial strains and growth conditions

Staphylococcus aureus strain 838/05 from the National Medicines Institute collection was grown in liquid trypticase soy broth (TSB) medium (Bio Merieux) with gentle aeration or on Columbia agar plates containing 5% sheep blood (Bio Merieux). The strain contains all the *sdr* genes i.e. *sdrC*, *sdrD*, and *sdrE*.

Media containing 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 5mM $FeCl_2$ were prepared by mixing sterile TSB medium with sterile stock solutions of the appropriate salt. To prepare TSB medium with 1 M NaCl, powdered medium was mixed with appropriate amount of NaCl, mixed with water and sterilized.

Growth curves for the 838/05 strain were determined for all experimental conditions in at least three independent replicates. Growth was determined by measuring OD_{595} of the culture versus media. To properly determine optical density, cultures were diluted 10–25 times in growth medium prior the measurement.

Sequence analysis

The presence of putative promoters and transcriptional organization of the *sdr* region was detected using the BPROM and FGENESB algorithms (www.softberry.com) based on region 611262 bp–623152 bp (GeneBank number CP000730.1) of the *S. aureus* subsp. *aureus* USA300_TCH1516 complete genome sequence (Highlander et al. 2007).

Sample collection for RNA isolation

Five milliliter samples of bacterial cultures in early (EL), mid- (ML), and 2 ml samples from late logarithmic (LL) and stationary phases were mixed with 2 volumes of RNA protect reagent (Qiagen), centrifuged to collect cells and frozen at −80°C until processing.

RNA isolation and cDNA synthesis

RNA was isolated from frozen bacterial samples using acid phenol:chloroform extraction. The bacterial cells were mechanically disrupted with glass

beads in the presence of acid phenol (Sambrook et al. 1989) and precipitated with ethanol. Residual chromosomal DNA was removed by treatment with DNAse I (Roche). 10 μg of total RNA was transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen) according to SuperScriptIII manufacturer.

Taqman analysis

Non labeled primers were purchased from Genomed and fluorescently labeled probes with MGB, and VIC or FAM tags were purchased from Applied Biosystems. Multiplex analysis with primers-probe sets for *gyrA* (calibrator) and one of the *sdrC*, *sdrD*, or *sdrE* sets was performed according to the manufacturer of the 7500 Real Time PCR System and TaqMan® Gene Expression Master Mix (Applied Biosystems). Primers used in the study are presented in Table 1.

The relative amount of *sdrC*, *sdrD*, or *sdrE* transcripts during growth was calibrated to the amount of *gyrA* transcript in the sample; next, the increase (or decrease) in the amount of *sdrC*, *sdrD*, or *sdrE* during growth was normalized to the amount of transcript in the early exponential growth phase (EL) using the $\Delta\Delta C_T$ method (Applied Biosystems 2001 User Bulletin #2). Briefly, C_T value representing amount of studied transcript is compared to the C_T value of the reference gene *gyrA*. The difference between C_T values is termed ΔC_T. The ΔC_T values are calculated for two experimental conditions and the difference between ΔC_T values is termed $\Delta\Delta C_T$. For the final calculation, $\Delta\Delta C_T$ value is used in the equation $2^{-\Delta\Delta C_T}$, and the result describes fold change value between two samples.

Table 1 Primers used in the study

Primer	Sequence (5′ → 3′)
gyrAFtaq	ACCAGTGAAATGCGTGAATCAT
gyrARtaq	CTGGCAATGCACGAGCAA
gyrAP	TTTAGATTATGCGATGAGTGTTA
sdrCFtaq	TGAAGCTAAAGCGGCAGAACA
sdrCRtaq	GGGCTGTCGTTTCATTTTTTG
sdrCP	ACGAATGGAGAATTAAATC
sdrDFtaq	AAGCGTTACAACCTGATTTGCA
sdrDrtaq	GCATCTACCTTTTTGTTTTCCTCATT
sdrDP	AATCAGTGGTAAATGTTCA
sdrEFtaq	TGCATCGATTTTAGTAGGTACGACAT
sdrERtaq	TTCTGTACTAGTGTTTTCAGCAGCTTT
sdrEP	ATTTTTGGTCTAGGGAACC

Interaction of *S. aureus* with human blood

50 ml of bacteria grown at 37°C in TSB medium to ML growth phase were collected by centrifugation and washed twice to remove the medium with sterile, pre-warmed to 37°C, PBS and re-suspended in 50 ml of pre-warmed sterile PBS. The time to prepare washed *S. aureus* cells was coordinated with blood collection from healthy volunteers, so the prepared cells could be immediately mixed with blood. 10 ml of cells were mixed with ~100 ml of mixed fresh human blood. Immediately after mixing blood with bacteria, 30 ml of the sample (Time 0) were mixed with two volumes of RNA Protect reagent (Qiagen), centrifuged to sediment cells, and frozen at −80°C. *S. aureus* cells were incubated with blood at 37°C with gentle mixing to avoid cell sedimentation. Additional samples were collected after 30 and 90 min of incubation of bacteria with blood. Samples collected after 30 and 90 min were treated the same as samples collected at the beginning of the experiment. RNA was isolated, and cDNA was generated, as described above. The expression level of the *sdr* genes was calibrated to the *gyrA* level and then normalized to the expression level at time 0 using the $\Delta\Delta C_T$ method.

Results and discussion

The transcriptional organization of the *sdr* locus has not been investigated so far. We performed basic sequence analysis with tools that allow identification of putative transcriptional units, promoters and terminators (BPROM and FGENESB; www.softberry.com). We detected three putative −10/−35 promoter sequences in front of each *sdr* gene (Fig. 1). In addition, we detected the presence of a sequence responsible for the formation of a loop forming a *rho* independent terminator between the *sdrC* and *sdrD* genes. Such organization suggests independent transcription of all three genes, or the presence of transcripts encompassing various parts of the region for example *sdrC* and *sdrD* + *sdrE*, or combinations of both.

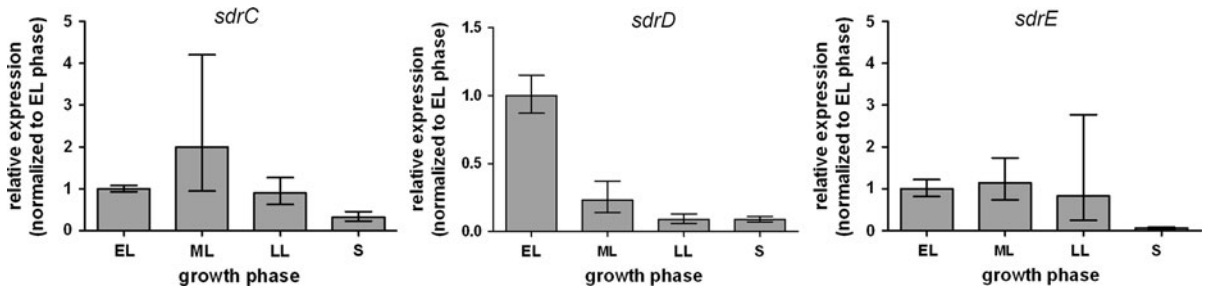

Fig. 1 Analysis of sdr locus organization. Fragment of genomic sequence of *S. aureus* strain USA300 with marked putative promoter −35 and −10 sequences of *sdrC*, *sdrD*, and *sdrE* (boxed) and a *rho* independent terminator (*bold*) in the intergenic region between *sdrC* and *sdrD*. Because of the length of the putative reading frames, for protein coding sequences of *sdrC*, *sdrD*, and *sdrE* genes only the start and stop codon are shown with *dots* marked inbetween (*bold italic, boxed*)

Fig. 2 Expression of *sdrC*, *sdrD*, and *sdrE* genes during growth at 37°C. Expression of studied genes was calibrated to *gyrA* level and normalized to expression in early log (EL) growth phase. *EL* early logarithmic, *ML* mid logarithmic, *LL* late logarithmic, and *S* stationary growth phase. *Error bars* 1 standard deviation

To test if all *sdr* genes are encoded by the same transcriptional unit, we determined their individual behavior during growth in rich laboratory medium at a standard 37°C. EL, ML, LL, and stationary (S) growth phases were determined based on growth properties of the strain. Growth curves were based on OD_{595} and determined for all experimental conditions in at least three independent replicates (data not shown). Transcript levels of *sdrC* and *sdrD* were comparable in EL phase, while transcript level of *sdrE* was about sixfold lower (data not shown). To compare transcript dynamics during growth, changes of the *sdrC*, *sdrD*, and *sdrE* transcript levels during bacterial growth were normalized to the basal level of their expression in the EL phase and set as 1. Consequently, we observed dissimilar expression patterns of the *sdrC*, *sdrD*, and *sdrE* genes in ML, LL, and S phases (Fig. 2). Expression of *sdrC* stays relatively steady and the transcript level declines in the S phase, with a similar pattern observed for *sdrE*; however, differences between EL and S phases are much greater for the *sdrE* gene. *sdrD* exhibits a different pattern; its expression is highest in the EL phase, significantly decreases in the ML phase, and stays low until the S phase. The differences in expression of individual genes of the *sdr* region are in concordance with in silico analysis of transcriptional organization of the region which suggests separation of the *sdr* region into three independent transcriptional units (Fig. 1).

To further characterize properties of the *sdr* region, we tested the influence on their transcription

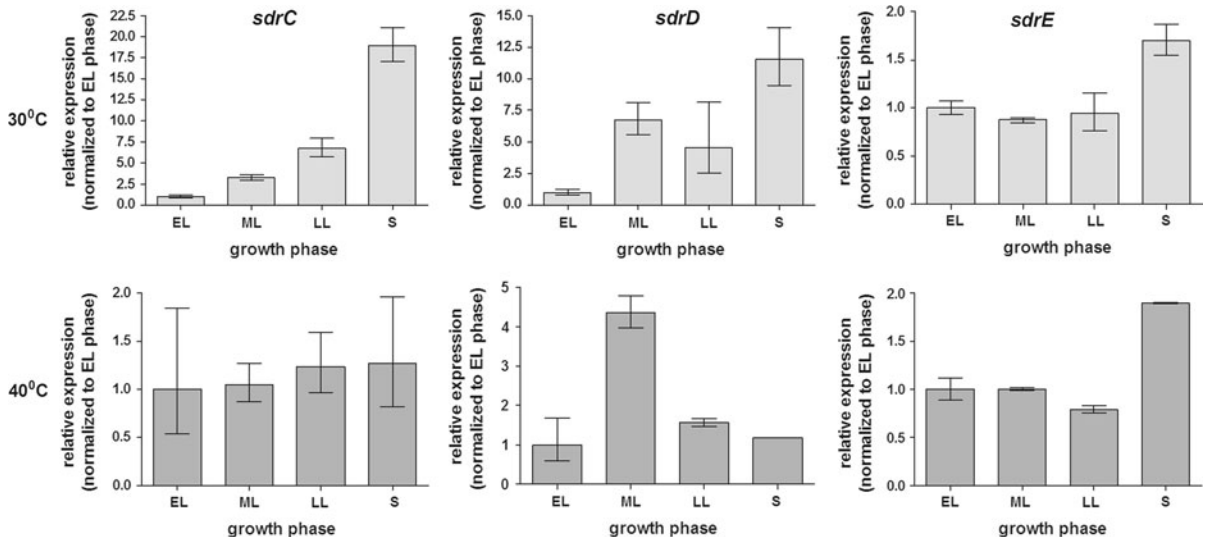

Fig. 3 Influence of low and high growth temperature on expression of *sdrC*, *sdrD*, and *sdrE* genes. Expression of studied genes was calibrated to *gyrA* level and normalized to expression in early log (EL) growth phase. *EL* early logarithmic, *ML* mid logarithmic, *LL* late logarithmic, and *S* stationary growth phase. *Error bars* 1 standard deviation

of multiple environmental stress factors such as temperature (30, 40°C) and osmotic shock (1 M NaCl), as well as divalent cations (Fe^{2+}, Ca^{2+}, Mg^{2+}). Stress factors influence expression of the *sdrC* and *sdrD* genes, but have no or minimal influence on the expression of *sdrE* (Figs. 3, 4). Low temperature caused over a 20-fold increase in the activation of *sdrC* transcription in the S phase when compared to the EL phase and over a tenfold increase of the *sdrD* transcript amount. Conversely to the dramatic increase caused by low temperature, high temperature does not influence the expression of *sdrC*. During growth at 40°C, the amount of *sdrD* peaks in the ML phase (about a fivefold increase), but later returns to a level comparable with the EL phase. Both low and high temperatures cause a minimal increase of the *sdrE* transcript, about 1.5-fold in the S phase when compared with the EL phase. Osmotic shock did not significantly influence expression of the *sdrD* and *sdrE* genes. Expression of the *sdrC* gene increased about sixfold in the LL phase (Fig. 4).

Next, we tested the influence of divalent cations of important biological functions, such as calcium, magnesium, and iron. Iron ions do not cause significant changes in the expression of *sdrC* and slightly decrease the expression of *sdrD* and *E* over time (Fig. 4). Magnesium and calcium ions have a profound effect on the expression of all genes, especially *sdrC* (Fig. 4). Magnesium ions cause over a 30-fold increase in the *sdrC* transcript amount in the LL phase, over a sixfold increase of the *sdrD* transcript amount in the ML–LL phases, and about a threefold increase in the *sdrE* transcript amount in the ML–S phases. A similar pattern of expression, though, to a lesser extent, was caused by the addition of calcium ions. Transcription of *sdrC* increased about 12-fold in the LL phase and *sdrD* about sixfold, while transcription of *sdrE* was only slightly increased (Fig. 4).

Taking into consideration the reaction of the three transcripts to environmental conditions we can conclude that *sdrC* is probably more related to the transition from exponential to stationary phase, while *sdrD* is more related to the logarithmic phase. Sequential differences in expression of cell wall anchored proteins may translate into temporal changes of cell envelope composition and can have a profound effect on bacterial virulence and their biological role. The differences can be connected to various steps of bacterial invasion, such as establishing the infection and attachment to specific tissues (Schwarz-Linek et al. 2004). The differences in expression can also be connected to the role of the proteins in the colonization of various environments of the organisms; for example, genes reacting to low temperatures might be involved in skin infections,

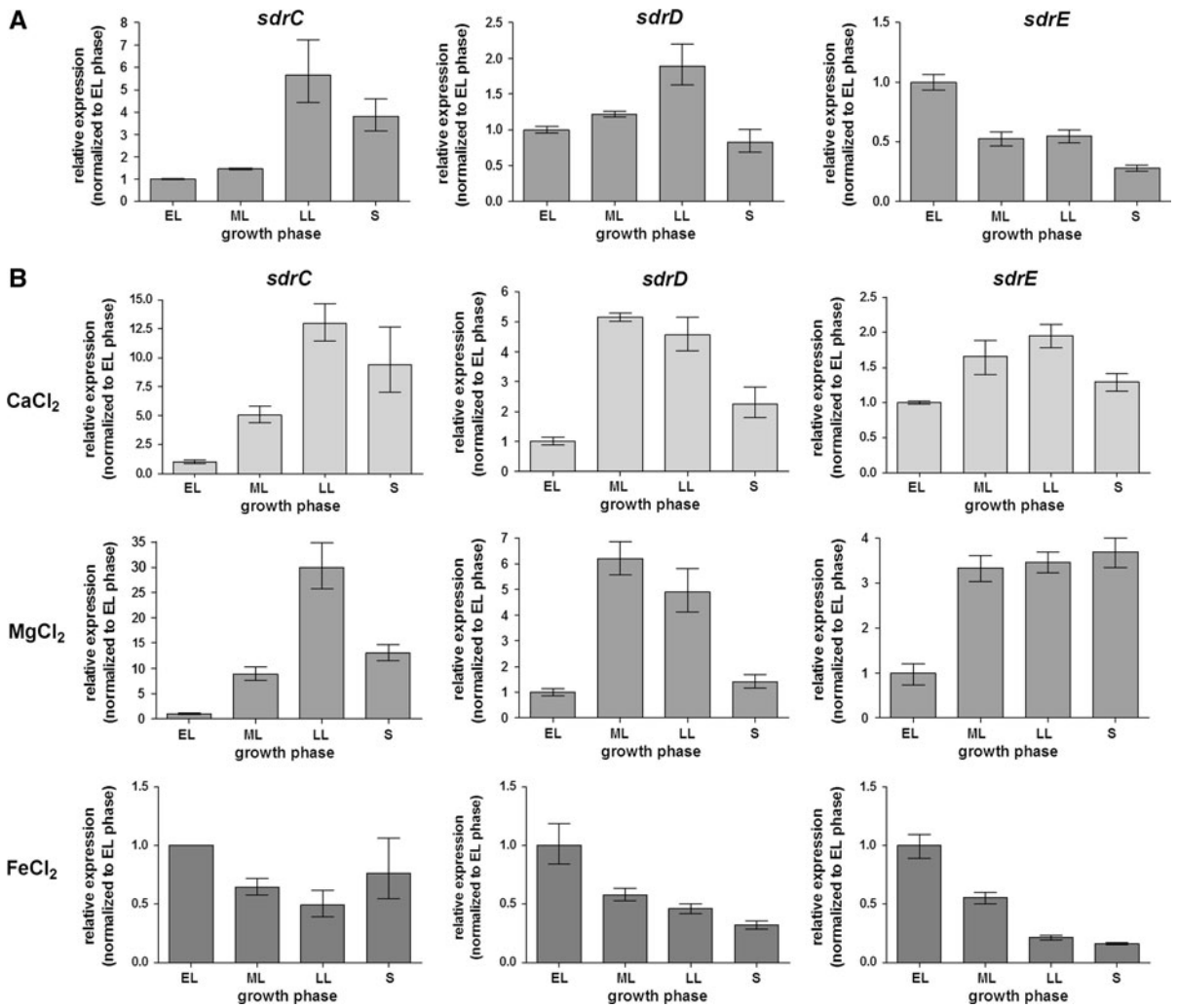

Fig. 4 Effect of in vitro growth conditions on the expression of *sdr* genes. **a** Influence of osmotic stress on the expression of *sdrC*, *sdrD*, and *sdrE* genes. **b** Influence of divalent ions on expression of *sdrC*, *sdrD,* and *sdrE* genes. Expression of studied genes was calibrated to *gyrA* level and normalized to expression in early log (EL) growth phase. *Error bars* 1 standard deviation

while genes activated by high temperatures might play a role in establishing massive, invasive infections such as bacteremia.

Many regulatory systems are responsible for the reaction of the pathogen to the environment, for example, the *covRS* system in *Streptococcus pyogenes* reacts to magnesium concentration and triggers the regulatory cascade in a response. The CovRS cascade is responsible for the regulation of multiple virulence factors and cell wall anchored proteins (Gryllos et al. 2003, 2007). On the other hand, calcium ions are often co-factors of enzymes and regulatory proteins. The strong reaction of magnesium and calcium ions may be a result of the influence of major regulatory pathways on *sdr* genes expression.

To further characterize the transcriptional response to the environment and its putative role in host-pathogen interactions, we used an ex vivo approach. The technique allows the study of the influence of well defined biological elements, like body fluids or cell cultures. This type of approach simplifies the data analysis by minimizing environmental factors and can be a base for further, more complex, in vivo analysis. Well defined experimental conditions with a known number of introduced components are the advantages of ex vivo experiments. For example, in

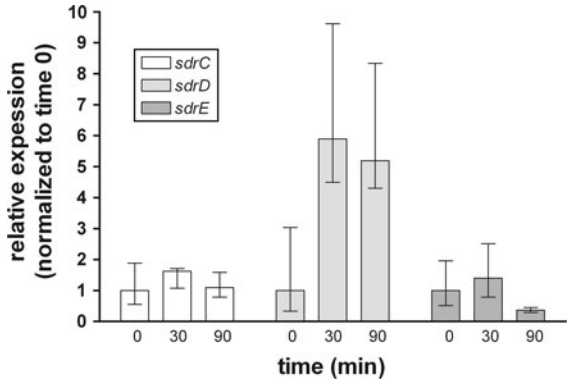

Fig. 5 Influence of human blood on expression of *sdrC*, *sdrD*, and *sdrE* genes. Expression of studied genes was calibrated to *gyrA* level and normalized to expression at time 0. *Error bars* 1 standard deviation

the experimental design of the influence of blood on the expression of bacterial genes, we can study well defined host tissue with a limited variety of known types of cells, in contrast to infected tissues. Previously, an ex vivo approach was successfully used to study gene expression in various streptococci (Graham et al. 2005; Mereghetti et al. 2008; Shelburne et al. 2005; Sitkiewicz et al. 2009).

In our experiments we studied the influence of complete human blood on the expression of the *sdr* genes. As a result, we observed a significant increase in *sdrD* expression after 30 and 90 min from the initial contact (Fig. 5). The changes in transcription of only the *sdrD* gene confirm separation of the region into three independent transcriptional units. This conclusion is further supported by the behavior of the *sdr* genes in multiple microarray experiments (*S. aureus* micro-array meta-database; http://www.bioinformatics.org/sammd/) in which the *sdrD* gene reacts in a different manner to the *sdrC* and *sdrE* genes. *sdrC* and/or *sdrE* are down-regulated by oxacillin, d-Cycloserine, and bacitracin, chlorination, SOS response, nitrite stress, cefoxitin, hemB and *sarA*; and up-regulated by *arlR*, in strains resistant to vancomycin, by *murF*, peracetic acid, *graRS*, in mild acid and when grown as biofilm. Conversely, *sdrD* exhibits the reverse behavior and is up-regulated by *sarA*, *rot* and *arlR*; and down-regulated by *traP*, in vancomycin resistant mutant and during growth in biofilm conditions.

Differential reaction of *sdrD* transcript levels to environmental conditions and blood suggests dissimilar functions of the *sdr* gene products. The Sdr proteins have been previously proposed to play role in bone infections (Tung et al. 2000) and in adherence to epithelial cells (Barbu et al. 2010; Cheng et al. 2009; Corrigan et al. 2009). Our results indicate that *sdrD* could also play a role in the interactions between the pathogen and human immune system, as it reacts to human blood. On the other hand, the differences in *sdrD* expression could be caused by the other factors such as serum proteins or nutritional differences between TSB medium and blood.

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