Journal of Immunological Methods 397 (2013) 18–27

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Rapid detection and semi-quantification of IgG-accessible *Staphylococcus aureus* surface-associated antigens using a multiplex competitive Luminex assay

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ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 23 July 2013
Accepted 29 July 2013
Available online 15 August 2013

Keywords:
Staphylococcus aureus
Competitive Luminex
Rapid surface characterization

ABSTRACT

The surface characterization of *Staphylococcus aureus* is currently labor intensive and time consuming. Therefore, we developed a novel method for the rapid yet comprehensive characterization of *S. aureus* cell-surface-associated proteins and carbohydrates, based on a competitive Luminex assay. In this assay, various *S. aureus* bacteria or culture supernatant dilutions are exposed to human pooled serum. *S. aureus* specific IgGs from the serum are captured to the IgG-accessible staphylococcal antigens present on the bacterial surface, or in supernatant, in a dose dependent manner. The remaining non-captured IgGs are then quantified by incubation with Luminex beads to which the individual recombinant *S. aureus* surface proteins and carbohydrates are covalently linked. The signals obtained are inversely proportional to the antigen concentration measured, and binding patterns were dependent on the particular *S. aureus* isolate used, growth conditions and growth phase. For example, the detection of cell wall teichoic acid (WTA) and peptidoglycan (PG) carbohydrate structures increased significantly when bacteria entered the stationary growth phase, whereas the amounts of clumping factors (ClfA and ClfB) decreased significantly. Using this technique, the appearance of several *S. aureus* gene knockout strains were studied. We demonstrated that in an *S. aureus srtA* gene knockout strain, srtA-dependent cell-surface-associated proteins were detectable yet not covalently linked to the PG layer. In conclusion, we show that this multiplex competitive Luminex assay is a suitable method for the rapid and simultaneous determination of *S. aureus* IgG-accessible cell-surface-associated proteins and carbohydrates in various growth phases and growth conditions in different *S. aureus* strains.

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1. Introduction

Staphylococcus aureus is a bacterial species naturally colonizing human skin, preferably the moist squamous epithelia of the anterior nares. Approximately 20% of the human population is permanently colonized (Peacock et al., 2001; Wertheim et al., 2005). In the majority of these cases, this colonization goes unnoticed. Under certain circumstances, colonization may lead to an invasive disease accompanied by the expression of a broad spectrum of secreted and cell-surface-associated bacterial virulence factors. The cell-surface-associated virulence factors include proteins, carbohydrates and teichoic

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http://dx.doi.org/10.1016/j.jim.2013.07.016

acids that are immobilized in the peptidoglycan (PG) scaffold (Foster and McDevitt, 1994; Lowy, 1998). The majority of the cell-surface-associated surface proteins contain a conserved pentapeptide LPXTG motif for recognition by the cell-membrane bound sortase A (srtA) (Marraffini et al., 2006). SrtA is responsible for the covalent anchoring of these cell-surface-associated proteins to the PG layer (Schneewind et al., 1995). The majority of cell-surface-associated surface proteins are involved in the binding to the host proteins, such as fibrinogen and fibronectin (Foster and Hook, 1998). This binding or adhesion can lead to subsequent colonization (Foster and Hook, 1998; Clarke and Foster, 2006). Emerging resistance of *S. aureus* towards different antibiotics, including methicillin (Smith et al., 1999; Chambers and Deleo, 2009), and *S. aureus* virulence, both contribute to the high morbidity and mortality associated with this bacterial species. It is generally acknowledged that an anti-staphylococcal vaccine would decrease the costs, morbidity and mortality related to *S. aureus* infection.

Currently, no suitable vaccine is available which protects humans from *S. aureus* infections (Verkaik et al., 2011; Jansen et al., 2013). To increase insights into the development of an anti-staphylococcal vaccine, knowledge regarding the composition of the bacterial surface is of importance. The exact determination of the composition of the bacterial surface is however still challenging, since the *S. aureus* surface is highly dynamic and cell-surface-associated antigen expression is growth phase-, growth condition- and strain dependent (Lowy, 1998; Fischetti, 2006; Resch et al., 2006; Hecker et al., 2010; McCarthy and Lindsay, 2010). Further, the presence of isoforms of different surface proteins makes the standardization of surface characterization even more complicated (Loughman et al., 2008; Burke et al., 2010). The most commonly used methods to determine cell-surface-associated proteins are: two dimensional electrophoresis (2DE) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF). Although these techniques are highly accurate and make detection of unknown antigens possible, they are time consuming and labor intensive (Nandakumar et al., 2005; Taverna et al., 2007; Hecker et al., 2010).

In this study, we aimed to develop an easy method to rapidly determine and quantify known IgG-accessible cell-surface-associated proteins and carbohydrates of *S. aureus* bacteria using multiplex competitive Luminex technology. We have previously used multiplex Luminex assay for semi-quantitative measurements of antibody responses in serum to *S. aureus* cell-surface-associated proteins and carbohydrates coupled to Luminex beads (Verkaik et al., 2010; den Reijer et al., 2013). In this case, antibodies directed against the majority of *S. aureus* cell-surface-associated proteins and carbohydrates were found in sera of both healthy and infected adults (Dryla et al., 2005; Colque-Navarro et al., 2010; Verkaik et al., 2010). Therefore, we hypothesized that the antibodies present in healthy human pooled serum (HPS) might be captured in a dose dependent fashion upon exposure to increasing numbers of bacteria. We succeeded in developing a rapid and simultaneous determination technique for cell-surface-associated proteins and carbohydrates of *S. aureus* bacteria using HPS. This technique facilitates the determination of cell-surface-associated proteins and carbohydrates in different growth phases and growth conditions in various *S. aureus* wild type (WT) and gene knockout (KO) strains.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Eleven blood culture derived *S. aureus* strains from individual patients were used in this study. All *S. aureus* strains were previously screened for the presence of genes encoding the staphylococcal proteins used in our study (Verkaik et al., 2010). As well as clinical strains, isolate 8325-4 and its isogenic *spa*, *clfB* and *srtA* KO variants were used. The *srtA* KO strain was grown on BHA (Brain Heart Infusion Agar) plates supplemented with erythromycin (3 μg/ml); the *spa* and *clfB* KO's were grown on tetracycline (3 μg/ml) BHA plates. Overnight cultures were used to inoculate BHI (Brain Heart Infusion) medium; bacteria were grown until mid exponential phase (0.5 OD_{600nm}) or early stationary phase (1.0 OD_{600nm}). Stationary grown bacteria were diluted to 0.5 OD_{600nm} in BHI, before use. To detect cell-surface-associated proteins and carbohydrates upon iron limited conditions, RPMI (Sigma Aldrich) medium supplemented with 1% casamino acids (BD) and 200 μM 2,2′ bipyridine pestanal (Sigma Aldrich) was used (Torres et al., 2006). Strain 8325-4 was used as an internal control for all competitive Luminex experiments.

2.2. Capture of specific S. aureus antibodies directed against IgG-accessible S. aureus cell-surface-associated proteins and carbohydrates

The exponential or stationary bacterial cultures (*from 2.1*) were centrifuged at 3700 ×*g* (2,100 ×*g* when bacterial supernatant was collected) at 4 °C during 10 min. The bacterial pellet was re-suspended in 1 ml assay buffer (PBS, 1% bovine serum albumin, 0.5% sodium azide, pH 7.4) and tenfold serial dilutions were made. Threefold serial dilutions in assay buffer were made from bacterial supernatant. To determine the covalent binding of srtA-dependent cell-surface-associated proteins, the bacterial cell pellet was treated with 1% SDS in PBS (Merck-Schuchardt) at 60 °C during 5 min. The SDS-treated pellet was washed twice with PBS before dilutions were made (only the SDS-treated bacterial pellet was studied, not the supernatant). 100 μl of the bacterial pellet dilutions or the bacterial supernatant dilutions were co-incubated with 100 μl 1/200 diluted HPS upon continuous shaking 800 rpm, at 4 °C during 35 min (thermomixer plate shaker, Eppendorf). After incubation with HPS, the bacterial supernatant samples were stored at 4 °C until use. The bacterial pellet samples were spun down at 2,400 ×*g* during 10 min after the incubation with HPS. 80 μl of the bacterial pellet-derived supernatant was collected, the pellet was discarded. These supernatants were once more centrifuged at 2,400 ×*g* during 15 min. 60 μl of supernatants were stored at 4 °C until use (the pellet was discarded). It was proposed, that *S. aureus* specific IgGs from the serum would be captured to the IgG-accessible staphylococcal antigens on bacterial surface or in supernatant in a dose dependent manner. The amount of remaining non-captured IgGs directed to the cell-surface-associated proteins and carbohydrates was determined using a Luminex platform (xMAP, Luminex Corporation, Austin, Texas, USA). The obtained signal (MFI strength) would be then inversely proportional to the IgG-accessible cell-surface-associated

proteins and carbohydrates concentration on the bacterial surface or in the supernatant. As alternative for HPS we used IgG that was purified from HPS using a HiTrap ProteinG HP, (GE Healthcare) according to the manufacturer's protocol. After titration, the IgG was diluted in assay buffer at the same concentration as previously mentioned for HPS.

2.3. Antigens and coupling procedure

The levels of the specific non-captured *S. aureus* cell-surface-associated proteins and carbohydrates antibodies were simultaneously quantified in the supernatants using Luminex bead based flow cytometry (Luminex Corporation). Antigens used in this assay were fibronectin-binding proteins (FnbpA and FnbpB), clumping factors (ClfA and ClfB), serine-aspartate repeat proteins (SdrE and SdrD), iron-regulated surface determinants (IsdH and IsdA), *S. aureus* surface protein G (SasG), wall teichoic acid (WTA), peptidoglycan (PG), *S. aureus* alpha-toxin (A-tox), *S. pneumoniae* choline-binding protein A (CbpA) and human Metapneumovirus (hMPV-99); they were all coupled to the SeroMAP carboxylated beads (Luminex Corporation) as described previously (Martins et al., 2006; Verkaik et al., 2008; den Reijer et al., 2013). The final concentration of the beads was adjusted to 3000 beads/μl and beads were stored until use at 4 °C in the dark. A-tox (not expressed during the exponential growth phase) and the non-*S. aureus* proteins CbpA and hMPV-99 were used as negative controls. The expression and the purification of the majority of recombinant proteins used have been described previously (Verkaik et al., 2009). The constructs were kindly provided by T. Foster (Trinity College, Dublin, Ireland). PG was kindly provided by K. van Kessel, University Medical Centre Utrecht, Utrecht, The Netherlands. WTA was kindly provided by A. Peschel, Interfaculty Institute of Microbiology and Infection Medicine, Cellular and Molecular Microbiology Division, University of Tübingen, Tübingen, Germany.

2.4. Luminex assay

To perform a Luminex assay 50 μl of supernatant was incubated with 50 μl of antigen coupled beads master mix to a final concentration of 3000 beads/μl in separate wells of a 96-well filter microtiter plate (Millipore Corporation) for 35 min at room temperature on a thermomixer plate shaker (Eppendorf). The following steps have already been described previously (Verkaik et al., 2009). In brief after incubation, the plate was washed twice in assay buffer (PBS, 1% bovine serum albumin, 0.5% sodium azide, pH 7.4). Subsequently, the beads were resuspended in 50 μl assay buffer and co-incubated with R-phycoerythrin (RPE)-conjugated goat anti-human IgG (Fcγ fragment specific, Jackson Immuno Research, 1/200 dilution) for 30 min at room temperature on a plate shaker. After this second incubation, the plate was washed with assay buffer and the beads resuspended in 100 μl of assay buffer. The plate was analyzed by Luminex technology (BMD) using Luminex IS software (version 2.2). All data are based on two independent experiments. The coefficient of variation (CV) was calculated for the duplicate experiments. The values were averaged if the CV value was lower than 25% and the SEM calculated. Based on the CV values, the detection limits were set on a noise: signal ratio > 2.0 and average MFI values > 1000.

2.5. Competitive Luminex data semi-quantification, shift calculation

Using the competitive Luminex assay, log dose-response curves of specific antigens were constructed for different conditions and bacterial strains. In each assay, exponentially grown WT 8325-4 *S. aureus* (10^8 bacteria) were included as an internal reference control. The relative signal for the antigens tested in the test samples was calculated by comparing the signals of the test samples with that of the reference control (normalized to 1 unit per bacterium (Fig. 3A).

2.6. Influence of Protein A on cell-surface-associated protein and carbohydrate detection at the S. aureus surface

The bacterial strains, 8325-4 WT and 8325-4 *spa* KO, were grown to exponential phase (*2.1*). The bacterial pellet was re-suspended in 1/100 dilution of fluorescently labeled (DyLight 633 NHS Esther, Thermo Scientific) human IgG Fc-protein (Fitzgerald), in a final volume of 10 μl and incubated for 1 h with continuous agitation at ambient temperature in the dark. IgG Fc-protein labeling was performed according to the manufacture's protocol. The bacteria were washed 3 times with PBS and their fluorescence was determined on a FacsCanto II™ flow cytometer and analyzed using FacsDiva™ software (both BD Biosciences).

2.7. Statistical analysis

Statistical analysis was performed using the Prism 5.0 package (GraphPad software, San Diego, CA, USA). The unpaired *t*-test was used for data analysis, with a value of $P < 0.05$ being considered statistically significant.

3. Results and discussion

3.1. Detection of S. aureus IgG-accessible cell-surface-associated proteins and carbohydrates with the competitive Luminex assay

To demonstrate that the Luminex technique can be used for the detection of IgG-accessible cell-surface-associated proteins and carbohydrates, we analyzed an exponentially grown strain of 8325-4 *S. aureus* (Fig. 1). The dose dependent

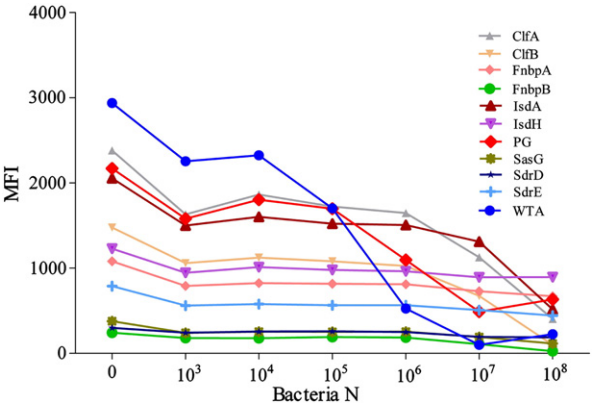

Fig. 1. *S. aureus* cell-surface-associated proteins and carbohydrates antibodies are inhibited in a dose dependent manner by the addition of increasing concentrations of *S. aureus* bacteria.

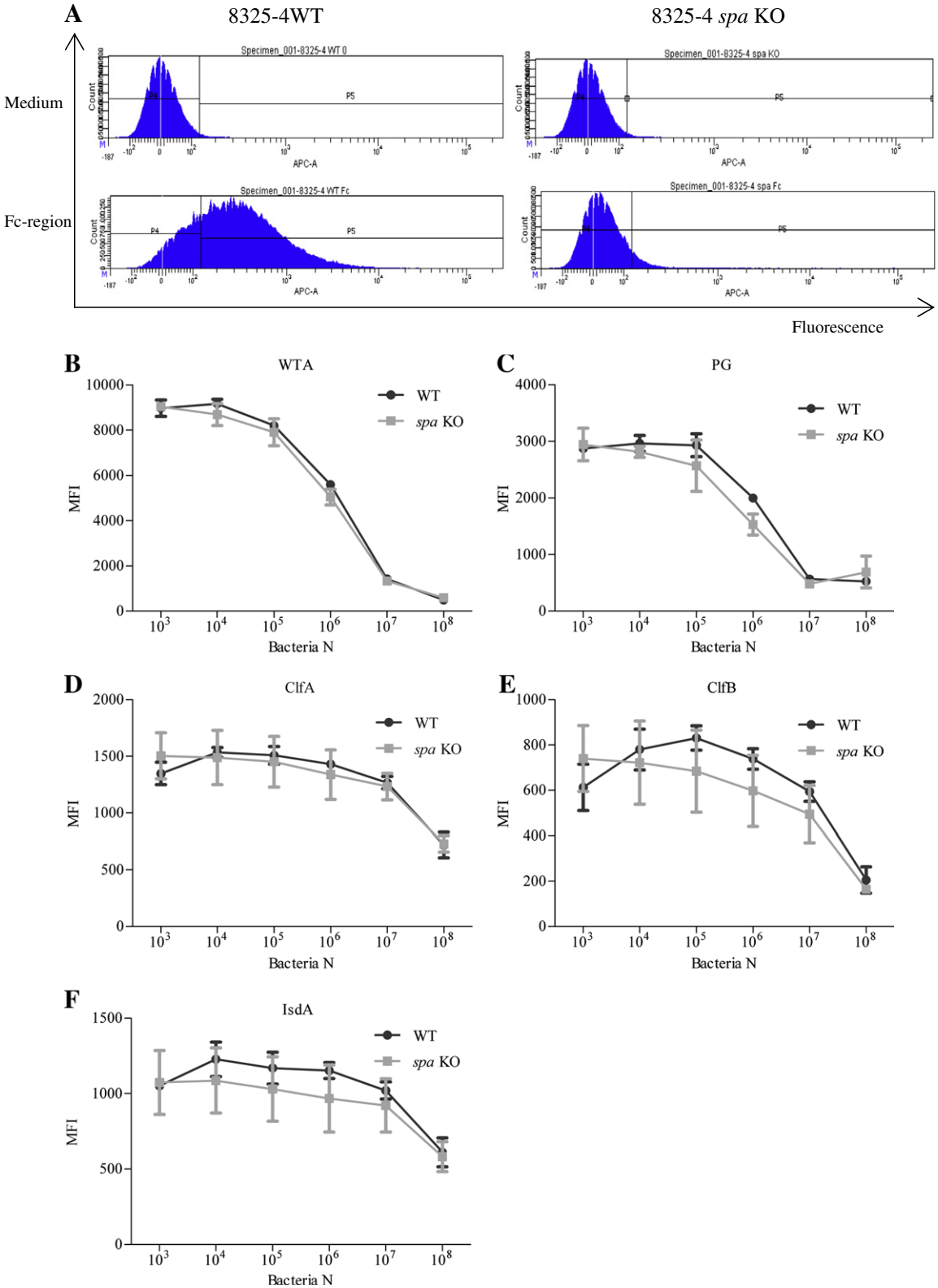

Fig. 2. The presence of Protein A on the *S. aureus* surface has no effect on overall specific *S. aureus* antibody binding to cell-surface-associated proteins and carbohydrates. (A) A representative FACS experiment (performed in duplicate). (B-F) Competitive Luminex data of 8325-4 WT strain and its isogenic *spa* KO *S. aureus* strain are shown.

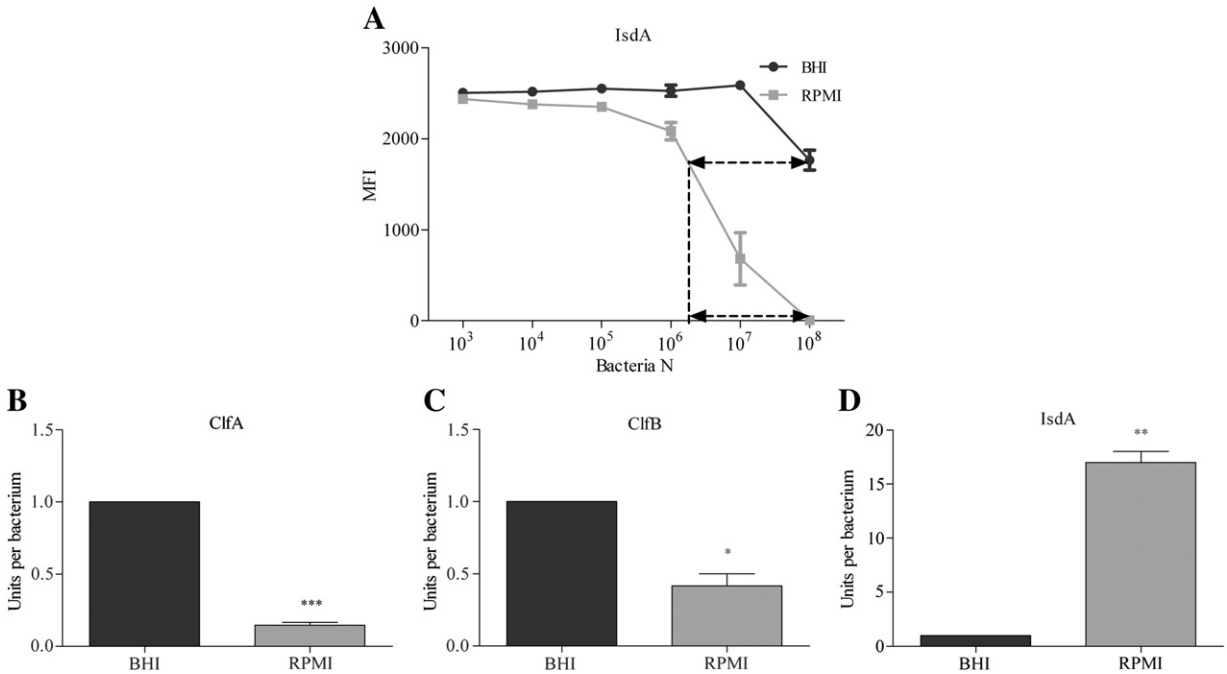

Fig. 3. The IsdA cell-surface-associated protein is detected in high levels in iron limiting conditions. The shift calculation is depicted. (A) Shift calculation of Luminex signal. (B–D) 8325-4 WT *S. aureus* cell-surface-associated proteins signals in BHI and RMPI are depicted.

absorption of several *S. aureus* cell-surface-associated protein and carbohydrate-specific antibodies was detected using HPS. The signal obtained (MFI strength) was inversely proportional to the IgG-accessible antigen concentrations present on the bacterial surface. Based on the signals obtained using the control antigens (CbpA, hMPV-99 and A-tox), we determined that the assay was most representative in the bacterial concentration range of 10^3 to 10^8 per assay, which were used as cut-off values. These cut-off values were used during the analysis of all subsequent data. SasG, SdrE, SdrD, FnbpA/B and IsdH signals were all under the 1000 MFI detection limit (Fig. 1) and were therefore not included for further analysis.

3.2. The competitive Luminex assay is surface Protein A independent

To evaluate the specificity of the competitive Luminex assay, the role of Protein A was analyzed in detail. Protein A is known to bind both the Fc- and the Vh3-region of immunoglobulins (Foster, 2005; Atkins et al., 2008). To verify Protein A expression on the surface of WT 8325-4 bacteria, but its absence on an isogenic *spa* KO *S. aureus* bacteria, both strains were stained with fluorescently labeled IgG Fc-region and analyzed with FACScan (Fig. 2A). The *spa* KO *S. aureus* strain failed to bind any fluorescently labeled Fc-fragments. In contrast, the WT strain observed to be stained brightly. No significant differences were found between the 8325-4 WT and its isogenic *spa* KO *S. aureus* strain cell-surface-associated proteins signals (Fig. 2B–F). This indicates that the decrease in signal of specific immunoglobulins was due to their capture by cognate antigens in the sample, rather than by non-specific binding to Protein A. This also suggests, that Protein A is blocked by non-*S. aureus* specific antibodies present in the HPS used in our assay, or that the affinity of antibodies towards their cognate antigens is stronger than their affinity towards Protein A on the bacterial surface.

Finally, to increase the sensitivity of our assay we analyzed *S. aureus* strain 8325-4 with purified IgG. No significant differences were obtained in comparison to the HPS data (data no shown).

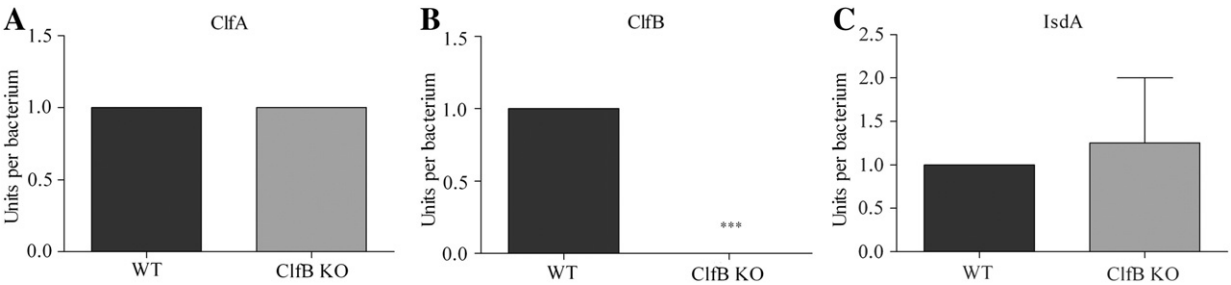

Fig. 4. The detection of cell-surface-associated proteins remains unchanged in a clfB KO *S. aureus* strain, except the ClfB signal.

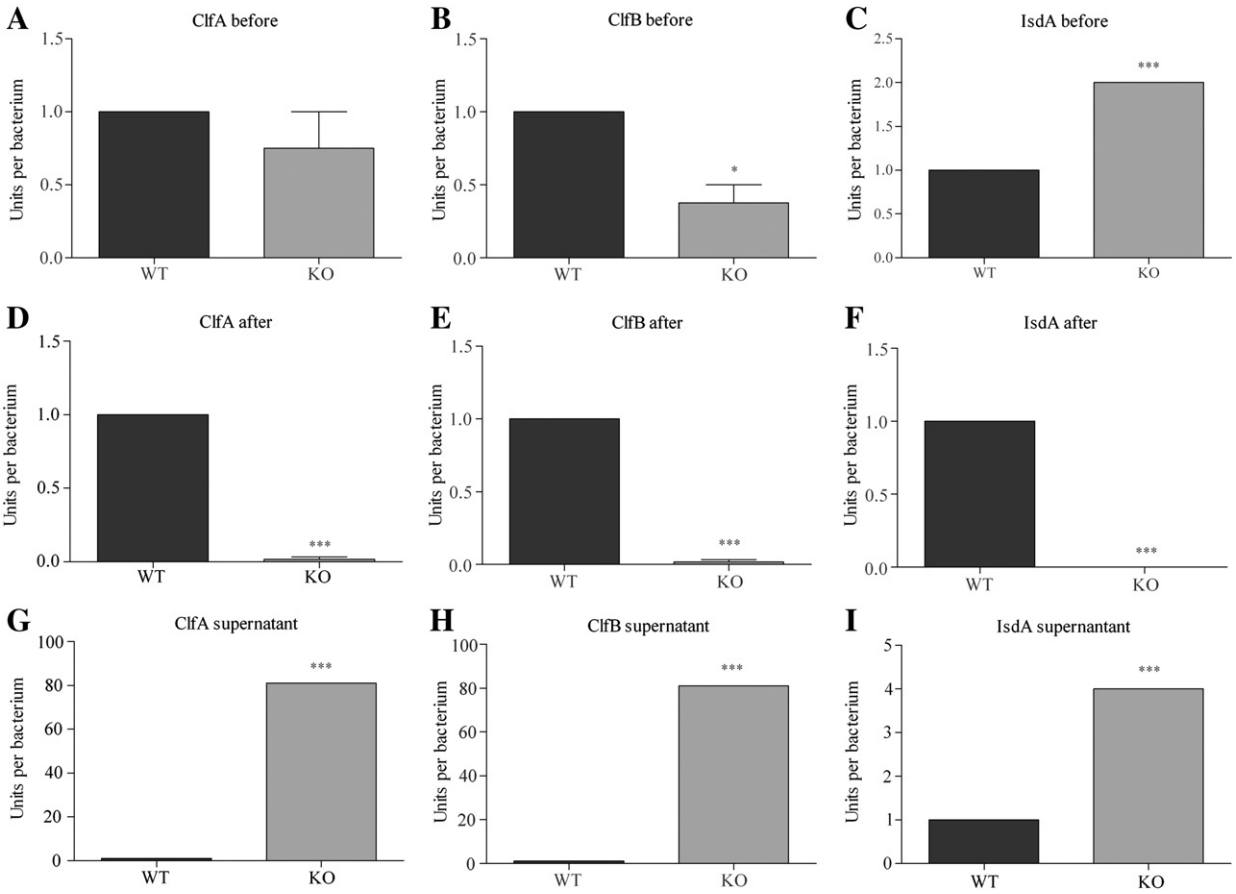

Fig. 5. The sortase-A-dependent cell-surface-associated proteins are non-covalently attached to the cell surface of srtA KO *S. aureus* and are also secreted in increased levels into the srtA KO *S. aureus* supernatant during the exponential phase. WT and *srt*A KO bacteria cell-surface-associated proteins detection was measured (A-C) before and (D–F) after SDS treatment. (G–I) The sortase-A-dependent cell-surface-associated proteins in the supernatant from WT and *srt*A KO bacteria are shown.

3.3. Competitive Luminex assay data semi-quantification, shift calculation

We analyzed the internal control *S. aureus* strain upon iron limiting conditions (Fig. 3A). To quantify the IsdA detection upon these conditions, we normalized the IsdA signal at 10^8 bacteria obtained in BHI to 1 unit per bacterium. By determining the shift in dose-response, the relative signal per bacterium was calculated in RPMI (Fig. 3A). Fig. 3B–D show the effect of iron limitation on the detection of IsdA, ClfA and ClfB. We detected an almost 20-fold increase of IsdA ($P = 0.0039$) under iron limiting conditions in comparison to iron rich conditions (Fig. 3D). On the other hand, the detection of ClfA and ClfB decreased ($P = 0.0006$, $P = 0.0199$, respectively) in comparison to iron rich conditions (Fig. 3B–C). The lower expression of ClfA and ClfB proteins upon iron limiting conditions has already been previously described by others (Ythier et al., 2012). This may for example, be caused by masking of ClfA and ClfB by IsdA protein, decreasing the binding between specific IgG to ClfA and ClfB proteins on the surface of WT *S. aureus* bacteria. Steric hindrance has been previously described for SasG (Corrigan et al., 2007). However, this phenomenon has not been described for other surface proteins. Therefore, the possible role of IsdA in steric hindrance still has to be elucidated.

3.4. S. aureus KO's characterization with the competitive Luminex technique

We wondered if the competitive Luminex assay is a suitable tool to check protein expression of *S. aureus* KO strains. Therefore, we screened *clfB* and *srtA* 8325-4 KO *S. aureus* strains. We found no ClfB signal in the *clfB S. aureus* KO strain ($P < 0.0001$) in comparison to the WT strain (Fig. 4B). The detection of other cell-surface-associated proteins remained unchanged in both strains (Fig. 4A and C).

Additionally, we tested the presence and the IgG-accessibility of several srtA-dependent cell-surface-associated proteins and their level of covalent binding to the PG layer at the bacterial surface during the exponential growth phase in WT and *srtA* KO 8325-4 stains. We found differences in IsdA detection, which was 2-fold higher ($P < 0.0001$) in the *srtA* KO strain (Fig. 5C). We detected a 2-fold decrease ($P = 0.0377$) in ClfB signal on the *srtA* KO bacterial surface compared to the WT bacteria (Fig. 5B). No differences in ClfA signal were detected (Fig. 5A). We conducted an SDS treatment to determine whether these cell-surface-

associated proteins are covalently bound to the PG layer of *srtA* KO *S. aureus* strain. This treatment resulted in significant differences of the detection of ClfA ($P = 0.0003$), ClfB ($P = 0.0003$) and IsdA ($P < 0.0001$) srtA-dependent cell-surface-associated proteins on the *srtA* KO 8325-4 strain in comparison to WT 8325-4 strain (Fig. 5D–F). To demonstrate the broader application of this technique, we looked for srtA-dependent surface proteins in the bacterial supernatant in both the *srtA* KO and the WT stain. Significantly higher signals ($P < 0.0001$) to all tested srtA-dependent surface proteins were detected in the *srtA* KO supernatant in comparison to the WT bacteria supernatant (Fig. 5G–I). Based on our Luminex data, we propose that the srtA-dependent cell-surface-associated proteins of the *S. aureus srtA* KO strain can be non-covalently associated to the surface and secreted into its environment. We suggest that the cell-surface-associated protein precursors of the *S. aureus srtA* KO are retained in the bacterial plasma membrane by means of the C-terminal positively charged domain and the membrane spanning hydrophobic domain. Schneewind et al. (1992) already showed that these two domains are essential for the endogenous

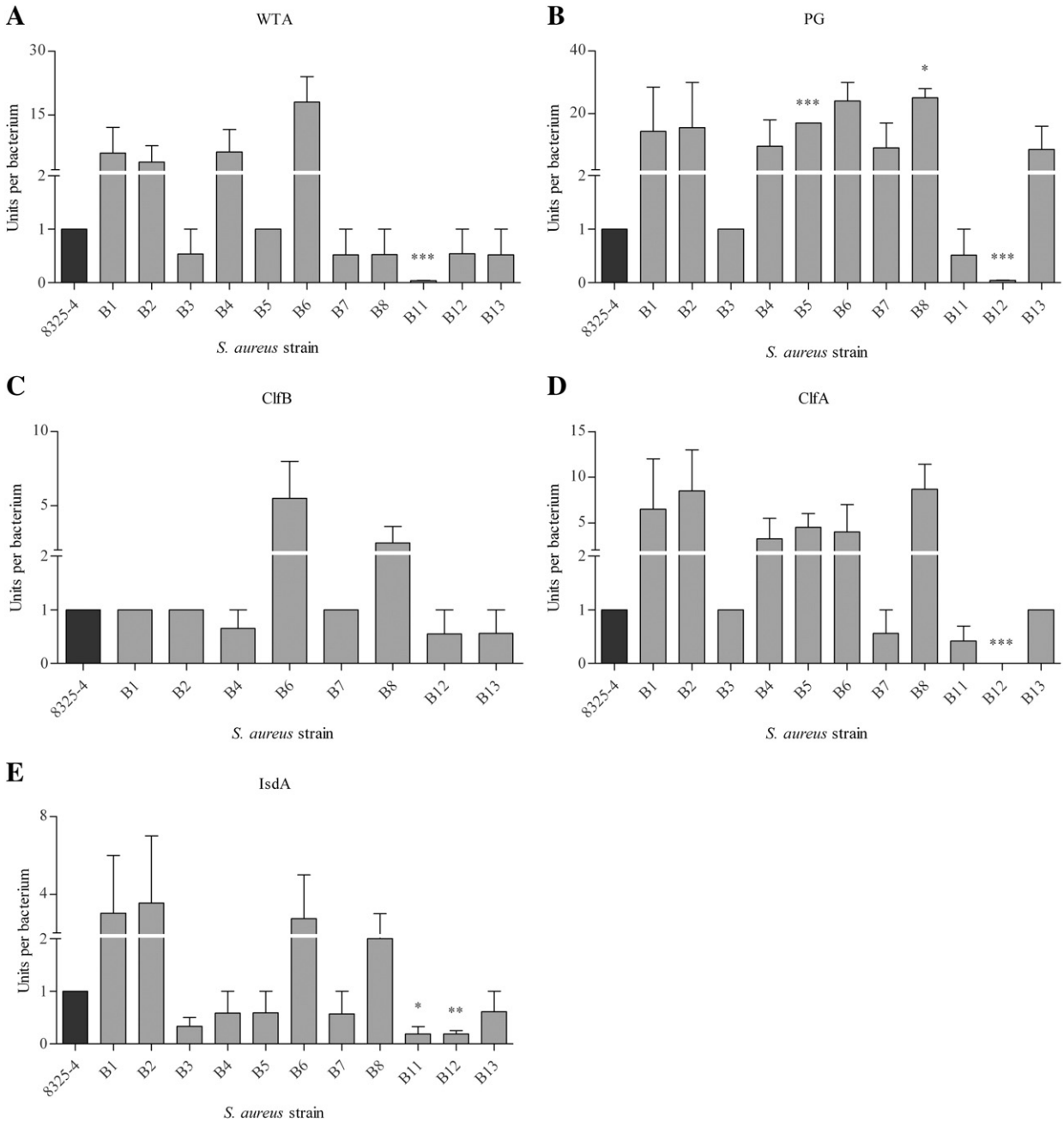

Fig. 6. Cell-surface-associated protein and carbohydrate detection for 11 *S. aureus* blood culture derived samples grown in vitro to exponential growth phase. The black bar represents the 8325-4 strain. Gray bars represent the shifts of the individual signals of 11 blood culture derived *S. aureus* strains towards signals obtained using the 8325-4 WT strain.

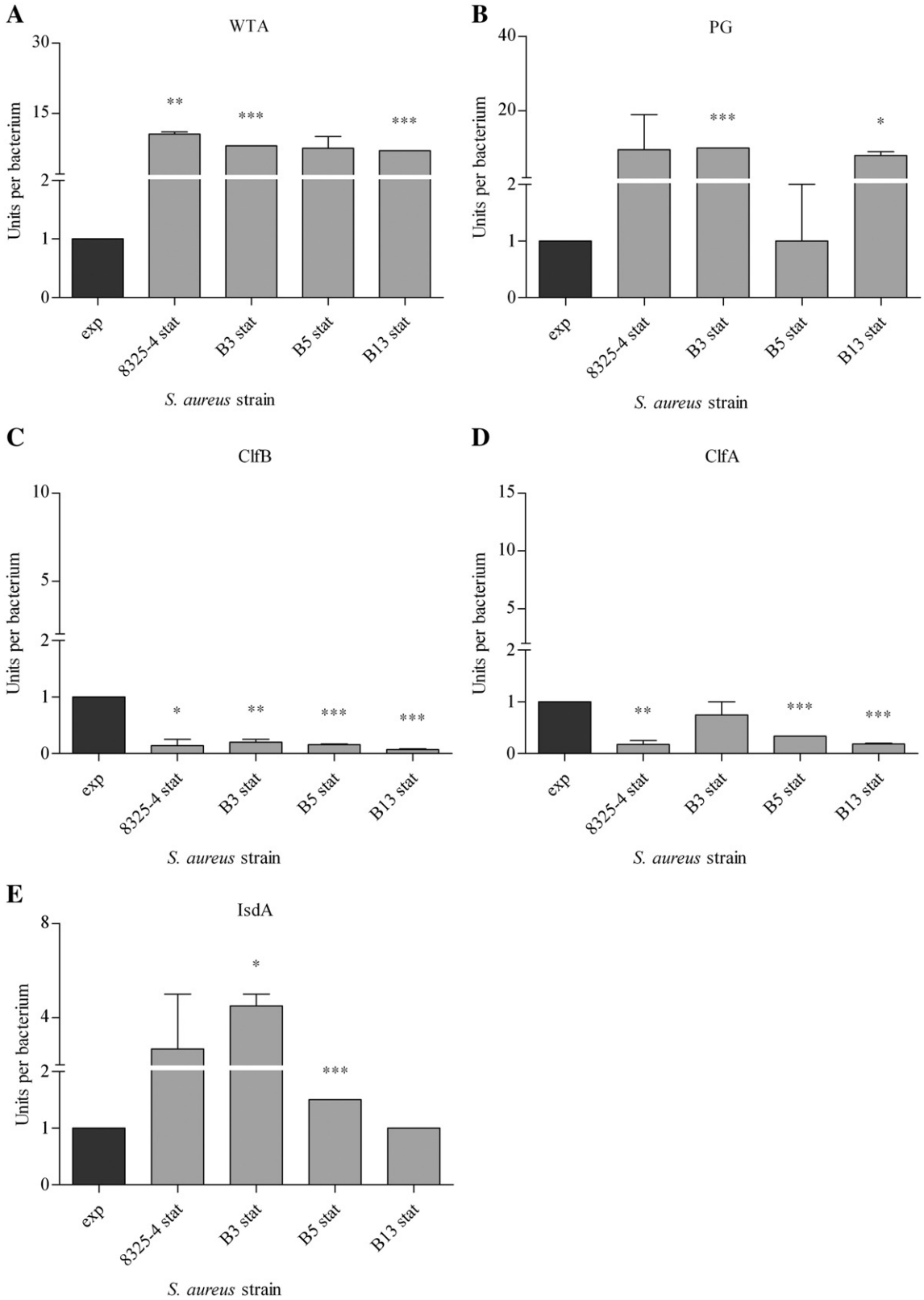

Fig. 7. Detection of cell-surface-associated proteins and carbohydrates in blood culture derived *S. aureus* strains grown in vitro to stationary phase. The black bar represents the exponential phase of tested strains and the gray bars the stationary phase of the corresponding *S. aureus* strain.

surface protein's retention in the cell-membrane, before being specifically processed by srtA transpeptidase (Schneewind et al., 1992).

3.5. Detection of IgG-accessible cell-surface-associated proteins and carbohydrates of blood culture derived S. aureus strains

To demonstrate that the competitive Luminex assay can be used for analysis of different *S. aureus* strains, we selected 11 blood culture derived *S. aureus* isolates. We observed heterogeneous shifts of cell-surface-associated proteins and carbohydrate signals of 11 exponentially grown blood culture derived *S. aureus* strains compared with the cell-surface-associated proteins and carbohydrates signals of 8325-4 strain. Based on WTA shifts, strains could be subdivided into two groups, one showing increased signals, and the other group showing decreased signals in comparison to the internal control strain (Fig. 6A). Signals for PG were higher (for two strains significantly higher; $P = 0.0153$; $P < 0.0001$) in the majority of the blood culture derived *S. aureus* isolates in comparison to the internal control strain (Fig. 6B). The ClfB signals were comparable to the internal control standard for most of the blood culture derived *S. aureus* strains tested; except for two strains in which we observed increased signals (Fig. 6C). Two ClfB measurements were not included in the graph because the signals obtained were not reproducible. The signals observed for ClfA and IsdA were rather diverse with lower, similar and higher signals being observed between the blood culture derived *S. aureus* strains (Fig. 6D–E). The heterogeneous results obtained from the analysis of the blood culture derived *S. aureus* strains might be explained by the genetic diversity of the *S. aureus* strains that cause bacteremia (Lindsay and Holden, 2004). Additionally, our heterogeneous data are in accordance with the heterogeneous immune responses measured against these strains as previously obtained by Verkaik et al. (2010).

To determine the growth phase dependent changes on the surface of *S. aureus*, we tested four strains cultured until the exponential and stationary growth phase (Fig. 7). We were able to detect for all strains almost 10 times more WTA in the stationary growth phase in comparison to the same bacterial strains from the exponential phase, this increase was significant ($P = 0.0028$; $P < 0.0001$; $P < 0.0001$) in three strains (Fig. 7A). The PG signals increased in three strains, in two significantly ($P < 0.0001$; $P = 0.0198$) when grown to stationary phase in comparison to the exponential phase (Fig. 7B). The signal for ClfA decreased on average 7-fold in the stationary growth phase in three strains tested ($P = 0.0082$; $P < 0.0001$; $P = 0.0004$) in comparison to bacteria from the exponential growth phase (Fig. 7C). We detected significantly less ClfB in all strains ($P = 0.0156$; $P = 0.0002$; $P = 0.0044$; $P = 0.0001$) in the stationary phase in comparison to bacteria from the exponential phase (Fig. 7D). Finally, signals for IsdA either did not change or significantly increased in two strains ($P = 0.0004$; $P = 0.0198$) in the stationary growth phase in comparison to bacteria from the exponential phase (Fig. 7E).

4. Conclusion

We developed a competitive Luminex assay for rapid and simultaneous screening of IgG-accessible cell-surface-associated proteins and carbohydrates of various *S. aureus* bacteria in different growth conditions and growth phases. Additionally, this method is suitable for *S. aureus* WT and KO strain comparisons. This multiple and rapid *S. aureus* surface determination method offers some advantages in comparison to existing protein screening techniques, as reviewed by Dreisbach et al. (2011). To our knowledge, this is the first technique which enables screening of carbohydrate structures. Our method is based on the interaction between IgG and IgG-accessible cell-surface-associated proteins and carbohydrates. However, this method cannot discriminate between the cell-surface-associated proteins and carbohydrates that are not present at the bacterial surface and those that are not IgG-accessible, i.e. those that are sterically hindered. Therefore, only IgG-accessible and previously identified antigens are detected, which might be a limitation of the technique if complete surveys of the surface are required. However, we are convinced that this rapid technique will be useful in initial mapping studies of the *S. aureus* surface for proteins and carbohydrates that might play a role during target assessment in *S. aureus* vaccine development.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

We kindly thank Dr. John Hays for correcting this manuscript, Department of Medical Microbiology & Infectious Diseases, Erasmus Medical Centre Rotterdam, Rotterdam, The Netherlands. This work was supported by ZonMW; grant title: Exploiting *Staphylococcus aureus* sortase for anti-infective purposes with the project number: 50-51700-98-055. The funding organization was involved in financial support for conducting the research only.

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