Proteomic and Transcriptomic Profiling of Staphylococcus aureus Surface LPXTG-proteins: Correlation with agr Genotypes and Adherence Phenotypes*  

Mathilde Ythier‡, Grégory Resch‡, Patrice Wardel§, Alexandre Panchaud‡, Aurélie Gfeller‡, Paul Majcherczyk‡, Manfredo Quadroni§, and Philippe Moreillon‡¶

Staphylococcus aureus infections involve numerous adhesins and toxins, which expression depends on complex regulatory networks. Adhesins include a family of surface proteins covalently attached to the peptidoglycan via a conserved LPXTG motif. Here we determined the protein and mRNA expression of LPXTG-proteins of S. aureus Newman in time-course experiments, and their relation to fibrinogen adherence in vitro. Experiments were performed with mutants in the global accessory-gene regulator (agr), surface protein A (Spa), and fibrinogen-binding protein A (ClfA), as well as during growth in iron-rich or iron-poor media. Surface proteins were recovered by trypsin-shaving of live bacteria. Released peptides were analyzed by liquid chromatography coupled to tandem mass-spectrometry. To unambiguously identify peptides unique to LPXTG-proteins, the analytical conditions were refined using a reference library of S. aureus LPXTG-proteins heterogeneously expressed in surrogate Lactococcus lactis. Transcriptomes were determined by microarrays. Sixteen of the 18 LPXTG-proteins present in S. aureus Newman were detected by proteomics. Nine LPXTG-proteins showed a bell-shape agr-like expression that was abrogated in agr-negative mutants including Spa, fibronectin-binding protein A (FnBPA), ClfA, iron-binding IsdA, and IsdB, immunomodulator SasH, functionally uncharacterized SasD, biofilm-related SasG and melittin resistance-related FmtB. However, only Spa and SasH modified their proteomic and mRNA profiles in parallel in the parent and its agr-mutant, whereas all other LPXTG-proteins modified their proteomic profiles independently of their mRNA. Moreover, ClfA became highly transcribed and active in fibrinogen-adherence tests during late growth (24 h), whereas it remained poorly detected by proteomics. On the other hand, iron-regulated IsdA-B-C increased their protein expression by >10-times in iron-poor conditions. Thus, proteomic, transcriptomic, and adherence-phenotype demonstrated differential profiles in S. aureus. Moreover, trypsin peptide signatures suggested differential protein domain exposures in various environments, which might be relevant for anti-adhesin vaccines. A comprehensive understanding of the S. aureus physiology should integrate all three approaches. Molecular & Cellular Proteomics 11: 10.1074/mcp.111.014191, 1123–1139, 2012.

Staphylococcus aureus is a highly successful opportunistic pathogen that can produce a wide variety of diseases (1). Moreover, it has a unique ability to develop antibiotic resistance, which reflects its extraordinary capacity to adapt and survive in a great variety of environments. Over the last decades molecular and genetic dissections of S. aureus have revealed a great number of surface adhesins, secreted enzymes, and toxins that might be implicated in pathogenesis (2–5). In particular, cell-wall-associated surface adhesins—referred to as microbial surface components recognizing adherence matrix molecules or MSCRAMMs1—mediate binding to extracellular matrix and plasma components, enabling staphylococci to colonize and invade host tissues and cells, as well as to escape immune defenses (6–8). Surface proteins are also implicated in biofilm formation (9), which promotes chronic infections and helps bacteria to escape antibiotic-induced killing.

1 The abbreviations used are: MSCRAMMs, Microbial Surface Components Recognizing Adherence Matrix Molecules; Agr, Accessory gene regulator; ClfA, Clumping factor A; ClfB, Clumping factor B; Cna, Collagen adhesion; -Ts, Elongation factor TS; FmtB, Factor affecting methicillin resistance in the presence of Triton X-100; FnbpA, Fibronectin-binding protein A; GM17, Glucose M17 medium; Isd, Iron-regulated surface determinants; L. lactis, Lactococcus lactis; LC-MS/MS, Liquid-Chromatography coupled to tandem Mass-Spectrometry; OD, Optical Density; PBS, Phosphate Buffer Saline; Pls, Plasmin-sensitive protein; RPMI, Roswell Park Memorial Institute; Sas, Staphylococcus aureus surface protein; Sdr, Serine-aspartate repeat protein; SP, Signal Peptide; Spa, Protein A; S. aureus, Staphylococcus aureus; TSB, Tryptic Soy Broth; TSST-1, Toxic Shock Syndrome Toxin 1.
Proteomic and Transcriptomic Profiling of S. aureus LPXTG-proteins

MSCRAMMs encompass several surface components including proteins, teichoic acids, lipoteichoic acids, and maybe polysaccharidic capsules, which have been implicated in tissue colonization and disease to various extents (5, 10, 11). Important surface proteins include polypeptides covalently attached to the peptidoglycan via a conserved anchoring mechanism. After membrane translocation, a transpeptidase called “sortase” cleaves the exported protein at a specific LPXTG motif present at its C terminus, and attaches its penultimate threonine to a side-chain of the peptidoglycan stem peptides, i.e. a pentaglycine in the case of S. aureus (12). Twenty-one genes encoding LPXTG-proteins have been identified by in silico analysis of S. aureus genomes (13). Experimental deletion or heterologous expression of these proteins helped identify their physiological functions and infer their roles in diseases (14, 15). However, although highlighting the multiple facets of S. aureus pathogenesis, none of these experiments provided a comprehensive view of the infection process. For instance, none of the gene inactivation experiments could entirely abrogate the S. aureus disease capacity, suggesting that infection is a multifactorial process. Moreover, experimental results may be difficult to interpret, because of the complex regulatory gene network operating in S. aureus (e.g. agr, sae, srpAB, sarS, sarA, sarR, sarS, sarT, sarV, sarU, sarY, and rot) (3, 16–22). As an example, the TSST-1 toxin is positively regulated by the “global accessory gene regulator” agr when bacteria are grown in vitro. However, the agr-regulation pathway may become overruled by other regulators in vivo, as TSST-1 can be expressed in animals even in the absence of agr (23).

Several approaches have been used to understand the pathogenic behavior of S. aureus. These include genomics, transcriptomics, and more recently proteomics (15, 24–29). In particular, proteomics might provide the most realistic picture of the infective process, because it detects the very end-products of gene biosynthetic pathways, which may eventually determine a biological phenotype. Moreover, post-translational protein regulation (or modification) may affect the stability and function of proteins independently of their upstream transcriptional or translational regulation, e.g. proteins may persist longer than their encoding mRNAs. Therefore, in addition to transcriptional and translational regulation, understanding the behavior of an organism requires both qualitative and quantitative assessment of its protein equipment over time.

Here we describe a semiquantitative proteomic approach based on trypsin digestion (i.e. trypsin shaving) of surface-exposed proteins and spectral counting of resulting peptides. This technique was applied to time course analysis in order to determine the level of LPXTG-proteins expressed in a variety of conditions known to affect the expression of their corresponding mRNAs. Transcriptomic controls were performed in similar conditions using microarrays. Experimental conditions included mutants inactivated in the global regulator agr, which promotes expression of adhesin mRNAs in post-exponential growth phase, and shuts it off in the stationary phase (16, 30, 31). In addition, growth alteration in iron-poor or iron-rich media, promoting or repressing the expression of mRNAs of LPXTG siderophore proteins, respectively (32, 33). Eventually, the adherence phenotype to host matrix proteins was determined. The results show that the time course profiles of LPXTG-proteins detected on the bacterial surface do not systematically follow the time course profile of their encoding mRNAs (16, 30, 31), and that some of these proteins can be functionally very active, e.g. in case of adherence to fibrogen, despite the fact that they are poorly detected in vitro. The results also suggest that surface proteins may adopt different conformations and expose different portions on the surface of different bacteria. Indeed, trypsin digestion released different sets of peptides when LPXTG-proteins were expressed in parent S. aureus or surrogate L. lactis, as exemplified by protein A (Spa), clumping factor A (ClfA), clumping factor B (ClfB) and fibronectin-binding protein A (FnBPA). Our work extends previous proteomic studies in S. aureus (27, 34, 35) and adds a level of subtlety in the continuum of transcriptional to post-translational protein regulation. Notably, the differences in domain exposure in various bacterial backgrounds might have unforeseen implications in vaccine development.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Bacterial strains used in this study are listed in Table I. Staphyloccocal strains included the well-described S. aureus Newman, one isogenic mutant (S. aureus ALC355) deleted in the global regulator agr (36), one isogenic mutant (S. aureus DU5873) deleted in the protein A gene (spa) (37), and one isogenic mutant (S. aureus DU5852) deleted in the clumping factor A gene (clfA) (38). Lactococcal recombinants were used for method validation and included the18 previously described L. lactis constructs, each expressing a different staphylococcal LPXTG-protein (39–41) (see below). Staphylococci were grown at 37 °C either in tryptic soy broth (TSB, Becton Dickinson, NJ) or agar, or in Roswell Park Memorial Institute culture medium 1640 (RPMI, Life Technology, Carlsbad, CA), without agitation. Lactococci were grown at 30 °C in GM17 broth (M17 medium containing 0.5% glucose, Becton Dickinson, NJ) supplemented with 5 µg/ml of erythromycin (Sigma-Aldrich) without agitation, or on GM17 agar. Growth was followed by colony counting on plates and OD600 nm measurements of the different cultures using a spectrophotometer (Ultraspex 500 pro, GE Healthcare). Bacterial stocks were kept frozen at −80 °C in 20% (v/v) glycerol.

Bacterial Cell Preparation for Proteolysis of Surface Proteins—Three different protocols were tested including (1) bacterial cell wall purification (35) prior to proteolysis, (2) bacterial cell wall purification followed by teichoic acid removal with hydrofluoric acid for 48 h (42) prior to proteolysis, and (3) trypsin surface shaving of live bacteria according to a slightly modified described methods (34). Protocols (1) and (2) (described in supplemental experimental procedures) appeared too stringent and resulted in the loss of up to two-thirds of the released peptides. Therefore, trypsin-shaving was used and is described here. In brief, bacteria were grown in 300 ml liquid cultures in the different media described above. At various times of the logarithmic or stationary growth phases, samples (between 10 and 100 ml depending on the cell density) were removed, immediately chilled at 4 °C, and harvested by centrifugation. Pellets were washed three
times with ice-cold phosphate-buffered saline (PBS) and finally re-suspended in 1 ml of the same buffer. To allow semiquantitative comparisons between the proteomes of different samples, cell concentration were adjusted to 1 × 10⁶ bacteria/ml in all samples. Cell counts were validated by optical microscopy (Neubauer cell) and viable colony counts on nutrient agar. There were <0.5 log₃ differences between the Neubauer cell and viable counts, indicating that the large majority of cells were alive. Samples were then shaved for 1 h with 1 µg/ml (final concentration) of trypsin (Promega, Madison, WI) at 37 °C, after which they were chilled at 4 °C and bacterial cells removed by centrifugation for 10 min at 4000 rpm and 4 °C. Supernatants containing trypsin-shaved peptides were filtered (0.22 µm) and freeze-dried until further use.

**Peptide Preparation for LC-MS/MS Analysis**—The freeze-dried shaved peptides were rediluted in 100 µl of 100 mM ammonium bicarbonate, reduced with 10 µl of 45 mM dithiothreitol (Sigma-Aldrich) for 30 min at 60 °C, and alkylated with 10 µl of 100 mM iodoaceticamide (Sigma-Aldrich) for 30 min at room temperature in the dark. The resultant mixtures were digested a second time at 37 °C with 1 µg of trypsin (Promega) for 4h. The digested peptides were desalted through Sep-Pak TC18 cartridges (Waters, Milford, MA) following the manufacturer’s recommendations and eluted with 1 ml of 60% (v/v) and 1 ml of 30% (v/v) acetonitrile (Merck). Solutions of purified peptides were pooled, dried under vacuum, and kept at −20 °C.

**Liquid Chromatography-MS/MS Analysis and Protein Identification**—Samples were analyzed on a hybrid linear trap LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) interfaced via a TrVersa Nanomate (Advion Biosciences, Norwich, UK) to an Agilent 1100 nano HPLC system (Agilent Technologies, Waldbronn, Germany). Solvents used for the mobile phase were 95:5 H₂O/acetonitrile (v/v) with 0.1% formic acid (solvent A) and 5:95 H₂O/acetonitrile (v/v) with 0.1% formic acid (solvent B).

Solutions of purified peptides were loaded onto a trapping micro-column ZORBAX 300SB C18 (5 mm × 300 µm ID, 5 µm, Agilent) in H₂O/acetonitrile 97:3 (v/v) + 0.1% formic acid at a flow rate of 10 µl/min. After 5 min, they were back-flush eluted and separated on a reversed-phase nanocolumn ZORBAX 300SB C18 column (75 µm ID × 15 cm, 3.5 µm, Agilent) at a flow rate of 300 nl/min with a 7-step gradient from 5 to 85% acetonitrile in 0.1% formic acid as following: (1) 5 min at 0% of solvent B, (2) from 0 to 25% of B in 35 min, (3) from 25 to 50% B in 15 min, (4) from 50 to 90% in 5 min, (5) 90% B during 10 min, (6) from 90 to 5% in 5 min, and finally (7) 15 min at 0% (total time: 90 min).

For spraying, a 400 nozzle ESI Chip (Advion Biosciences) was used with a voltage of 1.65 kV, and the mass spectrometer capillary temperature was set at 200 °C. In data-dependent acquisition controlled by Xcalibur 2.0 software (Thermo Scientific), the four most intense precursor ions detected in the full MS survey performed in the Orbitrap (range 350–1500 m/z, resolution 60000 at m/z 400) were selected and fragmented. MS/MS was triggered by a minimum signal threshold of 10,000 counts, carried out at relative collision energy of 35%, and with isolation width of 4.0 amu. Only precursors with a charge >1 were selected for CID fragmentation and fragment ions were analyzed in the LTQ linear trap. The m/z of fragmented precursors was then dynamically excluded, with a tolerance of 0.01 amu from any selection during 120 s. From raw files, MS/MS spectra were exported as mgf files (Mascot Generic File, text format) using the extract.msn.exe script from Thermo Scientific.

MS/MS spectra were analyzed using Mascot 2.2 (Matrix Science, London, UK). Mascot was set up to search the UniProteomics Database (SWISSPROT + TrEMBL, www.expasy.org) restricted to other Firmicutes taxonomy (database release used was 13.2 of April 8th 2008, 527,426 sequences after taxonomy filter). For time course experiments, a subset database of UniProt was used (2,594 sequences), which contained only proteins of *S. aureus* strain Newman, as well as sequences of *S. aureus* LPXTG-proteins expressed in *L. lactis* clones. Trypsin (cleavage at K, R, not before P) was used as the enzyme definition. Mascot searches were done with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10 ppm. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine and glutamine, and oxidation of methionine were specified as variable modifications.

**Scaffold** (version Scaffold_2.06_02, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications, and to perform data set alignment. Peptide identifications were accepted if they could be established at a probability >90.0% as specified by the PepPeptide Prophet algorithm (43). Protein identifications were accepted if they could be established at a probability >95.0% and contained at least one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (44). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Relative quantification of proteins between samples was based on spectral counting (45). Spectral counts were normalized by Scaffold (semiquantitative values) to take into account variations of protein amounts between samples.

**Microarrays**—Total RNAs from two independent triplicates of 100 ml bacterial cultures of *S. aureus* Newman and its isogenic agr-mutant were harvested at OD₆₀₀ nm of 0.2, 0.6, 1.8, and 2.2 by centrifugation at 4000 rpm at 4 °C for 10 min and processed as follows. Resuspended bacterial cells were first lysed in 100 µl TE containing 800 µg/ml lysostaphin (Sigma-Aldrich, Saint Louis, USA) for 1 h at room temperature. Total RNA were further purified and stabilized using the RNeasy Protect Bacteria mini kit (Qiagen) following the manufacturer’s recommendations. All RNA quantities were assessed by NanoDrop®ND-1000 spectrophotometer and the RNA quality was assessed using RNA 6000 NanoChips with the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, USA). Triplicates were equitably pooled to obtain at least 10 µg of RNA. For each sample, 10 µg of total RNA were reverse transcribed using dUTP for enzymatic fragmentation; 2 µg of the resulting sense cDNA was fragmented by UDG (uracil DNA glycosylase) and APE 1 (apurinic/apyrimidinic endonuclease 1) and biotin-labeled with TdT (terminal deoxynucleotidyl transferase) using the GeneChip® WT Terminal labeling kit (Affymetrix Cat. no. 900671, Santa Clara, CA). Affymetrix GeneChip *S. aureus* Genome Array (Affymetrix, Cat. no. 900514) were hybridized with 1.8 µg of biotinylated target, at 45 °C for 16 h washed and stained according to the protocol described in Affymetrix GeneChip® Expression Analysis Manual (Fluidics protocol FS450_0007).

The arrays were scanned using the GeneChip® Scanner 3000 7G (Affymetrix) and raw data was extracted from the scanned images and analyzed with the Affymetrix Power Tools software package (Affymetrix).

All statistical analyses were performed using the free high-level interpreted statistical language R and various Bioconductor packages (http://www.Bioconductor.org). Hybridization quality was assessed using the Expression Console software (Affymetrix). Normalized expression signals were calculated from Affymetrix CEL files using RMA normalization methods. Differential hybridized features were identified using Bioconductor package "limma" that implements linear models for microarray data (46). The p values were adjusted for multiple testing with Benjamini and Hochberg’s method to control the false discovery rate (FDR). Probe sets showing a FDR < 0.05 were considered significant.

**Bacterial Adherence to Solid-phase Extracellular Matrix Compounds**—We used a previously described in *vitro* adherence assay to measure the ability of *S. aureus* to adhere to increasing concentra-
shaving of
were grown to the early stationary growth phase, harvested by cen-
trifugation, and bacteria were shaven with 1 μg/ml of trypsin for various periods of time (1h, 4h, and 8h). Released peptides were processed as described under “Experimental Procedures” and identified by LC-MS. Studied LPXTG-proteins are indicated at the bottom of the graph. Columns indicate the numbers of unique peptides (nonredundant) recovered. Because a maximum of unique peptides were detected after 1 h, this incubation time was further used in all experiments of trypsin-shaving.

tions of surface-adsorbed fibrinogen, fibronectin, and collagen. Briefly, 96-well plates (Nunc-Immuno plates; MaxiSorp surface; Thermo Fisher Scientific) were filled with 100 μl of twofold serial dilutions of fibrinogen (1 mg/ml initial concentration; Sigma-Aldrich), fibronectin (250 μg/ml initial concentration; Sigma-Aldrich) and colla-
gen I and VI (20 μg/ml initial concentrations; Sigma-Aldrich). The last well served as a negative control and was filled with 100 μl of PBS without ligand. After washing, bovine serum albumin (Sigma-Aldrich) was added to each well to block nonspecific binding sites. Bacterial cultures were harvested at different times during growth by centrifugation (4000 × g at 4 °C for 20 min). Cells were re-suspended in PBS and bacterial cell concentrations were adjusted to 5.10^9 CFU/ml. Fifty microliters (i.e., 2.5.10^8 cells) were added to each well. Plates were incubated for 1.5 h at 37 °C, after which wells were washed with PBS and fixed at 55 °C. Adherent bacteria were detected by staining with crystal violet, and the OD570 nm was determined with an enzyme-

RESULTS

Trypsin-shaving of Live Cells—As mentioned under “Experimental Procedures,” initial attempts to recover LPXTG-proteins by trypsin digestion of purified staphylococcal cell walls resulted in too much contamination with nonwall proteins, and/or poor recovery of LPXTG-proteins (described in supplemental Experimental procedures). In contrast, trypsin-shaving decreased contamination with nonwall proteins by ≥5 times and reproducibly released similar sets of peptides from individual LPXTG-proteins. Fig. 1 indicates that the recovery of peptides during trypsin treatment of live staphylo-
cocci was time-dependent, and that 1 h of treatment appeared optimal. This duration was experimentally amenable for serial extractions during time course experiments and thus was used in all subsequent experiments. The decrease in peptide recovery after longer incubation periods is not ex-
plained, but could be because of concomitant proteolysis by intrinsic S. aureus proteases (see Discussion section).

Construction of a Reference Peptide Library of S. aureus LPXTG-proteins Expressed in Lactococci—An important prerequisite to this study was to unambiguously identify the trypsin peptide signatures of S. aureus LPXTG-proteins using the LC-MS/MS system described herein. This was achieved thank to a preliminary analysis of each of these proteins expressed in L. lactis, which does not carry S. aureus proteins. This permitted alleviation of certain ambiguities regarding to different adhesin denominations in UniProt, because of redundancies or isoforms. It also allowed verifying if an observed set of peptides could be attributed to a unique parent protein, or whether peptides were found in different proteins showing sequences similarities. To address these questions, we reinvestigated the 18 lactococcal clones successfully expressing unique S. aureus MSCRAMMs (Table I) (35) using the shaving technique, and assigned the obtained peptides to the corresponding proteins. Table II shows that the shaving procedure generated sets of peptides (between 3 and 59 pep-
tides) for 16 out of the 18 LPXTG-proteins studied. The great majority of the detected peptides was specifically assignable to a unique parent LPXTG-protein (Table II). Only few peptides were redundant between more than one protein species, for instance between IsdB and IsdH, Spa and Pls, and between SasD, SasE, and SasC. No peptides were detected for IsdB and SasH when expressed in lactococci (Table II). Possible explanations could be either poor expression in this particular organism, or poor detectability of these peptides by LC-MS/MS.

This allowed constructing a concordance table between UniProt protein nomenclatures (shown in Table III) as well as a dedicated sequence database specific for S. aureus Newman (supplemental Table S1). Specifically, Table III also presents the number of unique peptides and the percentage of peptide coverage of each of the LPXTG-proteins detected in recombinant L. lactis. Coverage varied from 6% to 60% (median 31%).

Profiling of LPXTG-proteins in S. aureus Newman and Its agr- Mutant in Various Growth Conditions—Time course pro-
filing of the surface proteome of S. aureus was performed during growth from early logarithmic (OD_600 nm = 0.2) to late stationary (OD_600 nm = 2.2) phases (Fig. 2). At each time point, the proteomic analysis assessed the relative quantity of pro-
teins in 1 × 10^9 bacterial cells. This semiquantification was based on spectral counting (45) normalized to take into account the variations of protein amounts between samples (n = 3 to 4). Spectral counting measures the number of times that a peptide is selected for fragmentation during a LC-MS/MS analysis, and is correlated to abundance of specific peptides and proteins (45). As dynamic exclusion has a direct impact on spectral counting, a value of 120 s was chosen as a compromise between redundant peptide fragmentations required for better quantitation accuracy and the need of
selecting low abundant peptides for a higher proteome coverage.

**Overall Protein Profiling**—Fig. 3 depicts the results obtained for the wild-type *S. aureus* Newman and its agr- mutant grown either in iron-rich TSB (Fig. 3A and 3C, respectively) or iron-poor RPMI (Fig. 3B and 3D, respectively). Overall, 16 of the 21 putative LPXTG-proteins described in *S. aureus* (13) were successfully identified in our tests. Three (*i.e.* Cna, Pls and SasK) of the 5 undetected species had no gene counterparts in the genome of *S. aureus* Newman (GenBank Accession Number AP009351) (48) and thus were not expected to be found, and two (Srap and SasC) remained undetected, maybe because of poor detectability of their corresponding peptides by LC-MS/MS. As a negative control, no Spa was detected at the surface of the spa-negative mutant DU5873, whereas the profile of the other LPXTG-proteins remained unaffected in this mutant (data not shown).

**Effect of agr**—Fig. 3 indicates that the amounts of several LPXTG-proteins depended on agr integrity and growth conditions. When wild-type *S. aureus* Newman was grown in TSB, nine of the 16 LPXTG-proteins (*i.e.* Spa, FnBPA, ClfA IsdA, IsdB, SasD, SasG, SasH, and FmtB) showed a time-dependent agr-like bell-shape expression, with an increase in abundance during late logarithmic growth followed by a decrease up to the late stationary phase (Fig. 3A). Although there were some variations between individual adhesins, as well as few relatively unexpected findings (*e.g.* poor detection of ClfA, see below), such a time-dependent expression pattern is in general agreement with proteins regulated by agr (16, 30, 31). On the other hand, ClfB was present quite early during growth, and was rather stable until it disappeared in late stationary phase, and SdrD and SdrE reproducibly presented a biphasic expression pattern. Iron-regulated IsdH was not detected in TSB grown staphylococci, most probably because this rich medium provides ample iron for growth (see below).

Strikingly, this agr-like pattern was abrogated when the isogenic agr-negative mutant of *S. aureus* Newman was tested in similar conditions (Fig. 3C). In this case, the bell-shape pattern was replaced by a continuing increase of protein quantities into the late stationary phase for all nine proteins mentioned above. Moreover, several proteins became either detectable or became more expressed in the agr mutant, including FnBPB, ClfA, SdrC, SdrE, SasF, and SasG. This is compatible with the loss of agr-mediated down-regulation of surface protein synthesis during stationary growth phase (16). Besides, some protein decreased (*e.g.* FmtB), whereas the atypical patterns of ClfB, SdrD, and SdrE persisted.

**Effect of Iron**—The experiments were repeated in the iron-poor medium RPMI. Of note, the growth rate of strains was substantially slower than in TSB (Fig. 2). Nevertheless, when wild type *S. aureus* Newman was tested in this condition (Fig. 3B), the global expression profile was very similar to that in
TSB, except for the sharp increase in iron-regulated surface determinants IsdA, IsdB, and IsdH (32, 33). The IsdC determinant of the iron-capturing system was not analyzed herein. IsdC is processed by Sortase B and associated to the peptidoglycan via a NPQTN module, and its expression should increase as well (32, 33). Aside from these major changes, minor differences were also observed, notably increase in the presence of iron of SasD and SasH, and decrease of SdrE (Figs. 3A and 3B).

When the isogenic agr-negative mutant was examined in RPMI (Fig. 3D), the loss of the agr bell-shape pattern was much less striking than in TSB. Nevertheless, some obvious modifications occurred such as a significant (p < 0.05) increase of the detection of SasF and a decrease for SasH.

**Transcriptome Analysis**—To assess the relationships between the profiles of time course expression of LPXTG-proteins and their mRNAs, we determined the parallel time course transcriptomes of the parent S. aureus Newman and its agr-mutant grown in TSB. Transcriptomic results indicated that the two organisms segregated very well at the level of their global transcriptomes. In addition, all duplicated microarray experiments clustered together, using all 7668 Probe sets, indicating high reproducibility and consistency of the data (supplemental Fig. S1). Fig. 4 presents the dynamics of the relative changes in mRNA amounts for specific transcripts in the parent S. aureus Newman and its agr-mutant. Note that these are relative changes—not absolute mRNA quantities—with regard to a basal value arbitrarily fixed at 1 for the first time point of the growth curve, i.e. at OD₆₀₀ nm/₀.₂. Therefore, the relative dynamics of proteomic and transcriptomic profiles (Fig. 3 and 4, respectively) can be compared.

Considering agr-related genes, the agr+ parent demonstrated a linear increase (by 1.6-fold) of the RNAIII transcript over the whole growth duration. Conversely, the agr-mutant did not show any hybridization to the structural genes of the agr locus (i.e. argA, agrB, argC, and argD), as well as a >300-fold decrease in hybridization to RNAIII and hemolysin δ as compared with the parent strain (supplemental Table S2). Thus, the transcription of agr was genuinely silenced in the mutant (supplemental Table S2). As an additional control, the mRNA of the gene of protein A (spa), which is typically regulated by agr, followed an agr-like bell-shape profile in the parent strains whereas this profile was flattened in the mutant (Fig. 4), as previously described (30). One additional LPXTG-protein gene sasH adopted an agr-like bell-shape pattern in the parent, which was modified in the agr-mutant. Moreover, the transcription of sasD and fmtb showed statistically significant modifications (p < 0.001) in the late growth phase, i.e. at OD₆₀₀ nm of 2.2, in the agr-mutant. Of note, the transcript of clfA showed a sharp increase in the late growth phase, i.e. at OD₆₀₀ nm of 1.8 (p < 0.001) and 2.2 (p < 0.05), in both the parent and the agr-mutant (Fig. 4), an observation that comes in support to recent observations of clfA regulation using the RNAseq technology (49). Thus, at least four of the LPXTG-

| Lactococci recombinant expressing staphylococcal LPXTG-proteins | Spa | CNA | CIB | FnbpA | FnbpB | IsdA | IsdB | IsdH | SdrC | SdrD | SdrE | SasD | SasF | SasG | SasH | SasK | Cna | Pls | Pil |
|---------------------------------------------------------------|-----|-----|-----|-------|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Number of unique peptides released from single LPXTG proteins | Spa | 17  | 9   | 20   | 10    | 5   | 28  | 0   | 28  | 4   | 0   | 4   | 3   | 28  | 5   | 13  | 20  | 0   | 5   | 3   |
|                                                                 | CNA | 5   | 10  | 9    | 0     | 4   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | CIB | 5   | 10  | 10   | 5     | 28  | 4   | 0   | 28  | 4   | 0   | 4   | 3   | 28  | 5   | 13  | 20  | 0   | 5   | 3   |
|                                                                 | FnbpA| 20  | 4   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | FnbpB| 10  | 20  | 4    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | IsdA | 4   | 4   | 4    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | IsdB | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | IsdH | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | SdrC | 4   | 4   | 4    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | SdrD | 3   | 4   | 4    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | SdrE | 28  | 4   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | SasD | 5   | 13  | 20   | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | SasF | 28  | 4   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | SasG | 5   | 13  | 20   | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | SasH | 4   | 4   | 4    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | SasK | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | Cna  | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | Pls  | 20  | 4   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
|                                                                 | Pil  | 0   | 0   | 0    | 0     | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |
TABLE III
UniProt nomenclature, numbers of unique peptides, and sequence coverage of LPXTG wall-associated protein obtained in lactococcal recombinants shaving experiments

| Protein name | Accession number | UniProt protein name                                      | Number of unique peptides | Sequence coverage (%) |
|--------------|------------------|-----------------------------------------------------------|---------------------------|-----------------------|
| ClfA         | A3F6G7_STAAU     | Clumping factor A                                         | 9                         | 31                    |
|              | A3F6G8_STAAU     | Clumping factor A                                         |                          |                       |
| ClfB         | CLFB_STAAB       | Clumping factor B precursor-Newman                         | 20                        | 41                    |
|              | A5IW57_STAA9     | LPXTG-motif cell wall anchor domain precursor-JH9          |                          |                       |
|              | A6US14_STAA2     | LPXTG-motif cell wall anchor domain precursor-JH1          |                          |                       |
|              | A7X714_STAA1     | Clumping factor B-Mu3/ATCC 700698                          |                          |                       |
|              | CLFB_STAAB       | Clumping factor B precursor-NCTC 8325                     |                          |                       |
|              | CLFB_STAAC       | Clumping factor B precursor–COL                           |                          |                       |
|              | CLFB_STAAM       | Clumping factor B precursor-Mu50/ATCC 700699               |                          |                       |
|              | CLFB_STAAN       | Clumping factor B precursor-N315                           |                          |                       |
| FnBPA        | FNBA_STAAB       | Fibronectin-binding protein A precursor-NCTC 8325          | 10                        | 19                    |
| FnBPB        | ABYYQ2_STAAT     | Fibronectin-binding protein B-USA300/TCH1516               | 5                         | 15                    |
|              | Q2FE04_STAAB     | Fibronectin binding protein B-USA300                       |                          |                       |
|              | Q2G1T5_STAAB     | Fibronectin binding protein B, putative-NCTC 8325          |                          |                       |
|              | Q53682_STAAU     | Fibronectin binding protein B                              |                          |                       |
|              | Q5HD53_STAAC     | Fibronectin binding protein B-COL                          |                          |                       |
| Cna          | A2P2A0_STAAU     | Collagen adhesion                                         | 3                         | 6                     |
|              | Q6GDB3_STAAR     | Collagen adhesion                                         |                          |                       |
|              | PLS_STAAC        | Putative surface protein SACOL0050 precursor–COL          | 8                         | 8                     |
|              | PLS_STAUU        | Surface protein precursor                                 |                          |                       |
|              | Q9LC00_STAUA     | Putative uncharacterized protein                          |                          |                       |
| SasD         | A6QDB8_STAAB     | Putative uncharacterized protein-Newman                   | 5                         | 39                    |
|              | A8Y260_STAAT     | Cell wall surface anchor protein-USA300                    |                          |                       |
|              | Q2FKC5_STAAB     | Cell wall surface anchor protein-USA300                    |                          |                       |
|              | Q2G260_STAAB     | Putative uncharacterized protein-NCTC 8325                 |                          |                       |
|              | Q2YLUU8_STAAB     | Surface protein-bovine RF122                              |                          |                       |
|              | Q5HJN4_STAAC     | Cell wall surface anchor family protein-COL                |                          |                       |
| IsdA         | ISDA_STAAB       | Iron-regulated surface determinant protein A precursor–Newman | 4                         | 12                    |
|              | A6IS16_STAA9     | LPXTG-motif cell wall anchor domain precursor-JH9          |                          |                       |
|              | A6V007_STAAB     | LPXTG-motif cell wall anchor domain precursor-JH1          |                          |                       |
|              | A7X148_STAAB     | Cell surface protein-Mu3/ATCC 700698                      |                          |                       |
|              | A8Z1RO_STAAT     | Iron-USA300/TCH1516                                       |                          |                       |
|              | ISDA_STAAB       | Iron-regulated surface determinant protein A precursor–Newman | 13                        | 26                    |
|              | ISDA_STAAC       | Iron-regulated surface determinant protein A precursor–COL | 20                        | 16                    |
|              | ISDA_STAAM       | Iron-regulated surface determinant protein A precursor-Mu50/ATCC 700699 | 28                        | 47                    |
|              | ISDA_STAAN       | Iron-regulated surface determinant protein A precursor-N315 |                          |                       |
|              | ISDA_STAAS       | Iron-regulated surface determinant protein A precursor-MSSA476 |                          |                       |
|              | ISDA_STAASW      | Iron-regulated surface determinant protein A precursor-MW2 |                          |                       |
| SasF         | A6OKDS_STAAB     | Putative uncharacterized protein-Newman                   |                          |                       |
|              | Q6G620_STAAS     | Putative surface anchored protein-MSSA476                  |                          |                       |
|              | Q8NUK1_STAAB     | Putative uncharacterized protein MW2567-MW2                |                          |                       |
|              | Q6T1N1_STAAB     | Surface protein SasF                                      |                          |                       |
| SasG         | A6OJ2Y_STAAB     | Putative uncharacterized protein-strain Newman            |                          |                       |
|              | Q5HD57_STAAC     | Cell wall surface anchor family protein-strain COL         |                          |                       |
|              | SASG_STAAB       | Surface protein G precursor-strain NCTC 8325               |                          |                       |
| IsdH         | A6QHRA_STAAB     | Haptoglobin-binding surface anchored protein–Newman       |                          |                       |
|              | A8Z2P9_STAAT     | Cell wall surface anchored protein-USA300/TCH1516           |                          |                       |
### TABLE III—continued

| Protein name | Accession number | UniProt protein name | Number of unique peptides | Sequence coverage (%) |
|--------------|------------------|----------------------|---------------------------|-----------------------|
| ISDH_STA3    |                  | Iron-regulated surface determinant protein H precursor-USA300 |                          |                       |
| ISDH_STAAC   |                  | Iron-regulated surface determinant protein H precursor-COL    |                          |                       |
| IsdB         | ISDB_STAEE       | Iron-regulated surface determinant protein-Newman             | 0                         | 0                     |
| SasK         | A7X6X3_STA1A     | Putative uncharacterized protein-Mu3/ATCC 700698              | 5                         | 31                    |
| Q7A3B0_STAAN |                  | Putative uncharacterized protein SA2381-N315                  |                          |                       |
| Q99R43_STAAM |                  | Putative uncharacterized protein-Mu50/ATCC 700699             |                          |                       |
| SdrC         | SDRC_STAEE       | Serine-aspartate repeat-containing protein C precursor-Newman | 20                        | 31                    |
| SdrD         | SDRD_STAEE       | Serine-aspartate repeat-containing protein D precursor-strain Newman | 49                        | 53                    |
| SdrE         | A8YZR1_STAAT     | Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SDRE-USA300/TCH1516 | 59                        | 60                    |
| Spa          | A6QD95_STAEE     | Immunoglobulin G binding protein A-Newman                     | 17                        | 42                    |
|             | A1KDX0_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | A1KYD8_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | A1KE04_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | A1KE29_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | A1KE57_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | A1KE58_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | A1KE61_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | A1KE85_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | A1KEA2_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | A4L7T5_STAOU     | Immunoglobulin G binding protein A precursor                  |                          |                       |
|             | A5NY2_STA9       | LPXTG-motif cell wall anchor domain precursor-JH9             |                          |                       |
|             | A6TXP6_STA2A     | LPXTG-motif cell wall anchor domain precursor-JH1             |                          |                       |
|             | A7XWBO_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | A8YZ26_STAAT     | Immunoglobulin G binding protein A-USA300/TCH1516             |                          |                       |
|             | Q2FKE8_STA3      | Immunoglobulin G binding protein A-USA300                      |                          |                       |
|             | Q2UW16_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | Q2UW21_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | Q2UW30_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | Q2UW31_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | Q2UW33_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | Q2UW54_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | Q2UW59_STAOU     | Immunoglobulin G binding protein A                            |                          |                       |
|             | Q5HJ08_STAAC     | Immunoglobulin G binding protein A-COL                        |                          |                       |
|             | Q6GD14_STAAS     | Immunoglobulin G binding protein A-MSSA476                    |                          |                       |
|             | Q8NYT0_STA2W     | IMMUNOGLOBULIN G BINDING PROTEIN A-MW2                        |                          |                       |
|             | SPA1_STA2W       | Immunoglobulin G binding protein A precursor-NCTC 8325        |                          |                       |
protein mRNAs (spa, sasD, sasH and fmtB) showed clear modifications between the agr/H11001 and agr- strains, and two of them (spa and sasH) had a clear agr-like profile. On the other hand, most of the other LPXTG-protein genes adopted various mRNA profiles that were essentially not affected by inactivation of agr.

When comparing mRNA and proteomic profiles, the expression of Spa clearly followed an agr-like profile at both the transcriptional and translational levels, which was abrogated in the agr- mutant. SasH followed a relatively similar pattern. On the other hand, FnBPA and to a lesser extend FnBPB, ClfA, ClfB, IdsB, SdrC SdrE, and SasF modified their protein expression patterns between the two mutants, but not their mRNA profiles. Only SasG did not modify its proteins and mRNA profiles in both parent strains. Therefore, although the transcriptome profile was remarkably predictive of the LPXTG-protein profile in some cases, it appears that additional factors were interfering with the physical presence—or access to trypsin—of several adhesins at the bacterial surface. Of note, Srap was detected at the transcriptional but not at the protein level.

**Correlation Between Proteomic Expression Profiles and In Vitro Adherence Phenotypes**—Fig. 3A shows that 9/16 LPXTG proteins detected in *S. aureus* Newman (i.e. Spa, FnBPA, Cifa, IsdA, IsdB, SasD, SasG, SasH, andFmtB) followed an agr-like expression pattern. Therefore, we tested whether the in vitro adherence profile of this organism followed a similar pattern when grown in the same conditions. Fig. 5 indicates that adherence to fibrinogen adopted differential profiles depending on both the growth medium and the presence or not of an intact agr.

In TSB (Fig. 5A), binding of the parent *S. aureus* Newman to fibrinogen was more pronounced during exponential growth and decreased in the early stationary phase, thus obeying an agr-like pattern. As a control, the clfA mutant was virtually unable to bind to immobilized fibrinogen, indicating that ClfA was largely responsible of the phenotype. The early stationary phase drop was even more pronounced in the agr- mutant (16, 30, 31). Finally, adherence increased again after 24 h in both strains, a phenotype that did not correlate with the proteomic detection of ClfA (Fig. 3), but correlated well with the clfA gene transcription profile (Fig. 4). In consequence, ClfA-mediated binding to fibrinogen did not strictly follow an agr pattern in these experimental conditions, and senescent
Bacteria were still able to bind fibrinogen to a substantial extent. Conversely, binding to fibrinogen adopted an agr-like bell-curve in RPMI (Fig. 5B) and this bell-curve was abrogated in the agr-inactivated mutant. Besides, binding to fibronectin and collagens was quasi null (data not presented), which is coherent with the fact that in S. aureus Newman, the genes encoding for fibronectin binding and collagen binding are either truncated (for fnA and fnB) or absent (for cna).

Trypsin Releases Different Sets of Peptides from LPXTG-proteins Expressed on the Surface of S. aureus or L. lactis—S. aureus had a much lower adherence score to fibrinogen than ClfA-positive L. lactis—in vitro adherence tests (Fig. 5). This difference could result from a lower expression of ClfA on the surface of S. aureus than on the surface of L. lactis, or from differences in the accessibility to ClfA-binding domains when the protein is exposed on the surface of S. aureus versus L. lactis, or from both reasons.

Fig. 6 compares the ClfA, ClfB, Spa, and FnBPA-specific sets of peptides released by trypsin shaving of the surface of recombinant lactococci or S. aureus Newman. In case of ClfA, nine peptides were released from lactococci expressing ClfA and eight from the surface of S. aureus Newman. Thus, the peptides numbers were quite similar. However, among those, three peptides were specific for lactococci and two were specific of S. aureus. Therefore, although the majority of the released peptides were similar (i.e. 6/9 in lactococci and 6/8 in S. aureus), some were specific of the host bacteria, suggesting that different portions of the protein were accessible to trypsin digestion on the surface of the two microorganisms. Details on these peptides are presented in supplemental Table S1. This small difference in peptide numbers was also true for ClfB and Spa (Fig. 6). Regarding to ClfB, among 20 and 21 released peptides 3 and 4 were specific for L. lactis and S. aureus, respectively. For Spa, among 23 and 29 released peptides, 0 and 5 were specific for L. lactis and S. aureus,
Fig. 4. Expression profiles of mRNA from LPXTG-proteins in time course experiments. The parent *S. aureus* Newman and its agr-mutant were grown in TSB, and harvested at four different time-points (i.e. at OD₆₀₀ nm of 0.2, 0.6, 1.8, and 2.2) before being processed for RNA extraction. The transcriptomes were analyzed by microarray as described under “Experimental Procedures.” The amounts of mRNA at the different time points are represented as fold changes compared with a value arbitrarily fixed at 1 for the first time point (i.e. at OD₆₀₀ nm = 0.2). Thus, all measures are reported as relative values. The results represent the mean of ≥two determinations on two separate chips, with relative variations between individual values of ≤15%. Asterisks above the columns indicate that the values are statistically significantly different (*, p < 0.05; **, p < 0.01 and ***, p < 0.001) from the previous time point. p values were adjusted for multiple testing with Benjamini and Hochberg's method to control the false discovery rate (FDR).
respectively. Moreover, in these cases, some peptides, which were recovered in the same LC-MS runs, displayed redundancies between partial and complete hydrolysis (see overlapping black boxes in Fig. 6). Partial hydrolysis could result from a too short duration of trypsin digestion. However, extending the length of digestion to more than 1h did not yield more peptides (Fig. 1). Therefore, partial trypsin hydrolysis of LPXTG-proteins might depend on other factors, possibly including protein conformation and trypsin accessibility.

Finally, an unexpected observation was that twice as many peptides were released from FnBPA expressed in *S. aureus* than from FnBPA-positive *L. lactis* (18 versus nine peptides, respectively). This observation is interesting because the fnbPA gene of *S. aureus* Newman carries a stop codon, which leads to a premature arrest of the transcription and the translation of a protein devoid of the C-terminal LPXTG anchoring domain. Hence, this truncated protein could be free-floating in the cell envelope of *S. aureus* Newman and thus more accessible to trypsin digestion. Such a possibility would support the differential trypsin accessibility of other surface proteins expressed in either of the two tested bacteria, as suggested above.

**DISCUSSION**

*S. aureus* produces a plethora of virulence determinants (50), which are regulated by a complex network of two-component regulatory systems, DNA-binding proteins, and small RNAs (3, 16, 21, 30, 51, 52). This explains why there is no simple approach to assess the presence or absence of each individual pathogenic feature along the successive steps of infection. Previous experiments in which specific genes were inactivated were sometimes difficult to interpret, particularly when bacteria were equipped with multiple genes encoding redundant or complementary functions (13, 40, 53). Moreover,
gene regulation may vary between in vitro and in vivo conditions (23). To integrate this multilevel information, experimental systems should allow appraising quantitative snapshots of global macromolecule expression in both in vitro and in vivo conditions. Although this is possible at the level of mRNA (11, 29, 31), its equivalent at the protein level is as yet less developed (27–29). Individual proteins can be quantitatively evaluated by Western blotting or in situ hybridization. However, these methods are not amenable to evaluate multiple proteins simultaneously, because of the need of numerous different antibodies and the limited number of dyes that can be used together in a single experiment. Here, we attempted to bypass this limitation by using a proteomic approach. Because we previously contributed to the understanding of the role of S. aureus surface adhesins using heterologous gene expression (13, 39–41, 53, 54), we intentionally concentrated our efforts on the analysis of the time course detection of the 21 known S. aureus LPXTG surface proteins.

Initial analyses indicated that the whole proteome of purified cell walls was much more complex than expected, revealing numerous proteins that were not anticipated to be found in the peptidoglycan and its appendages. The unexpected presence of these species was considered as contamination, at least in the setting of crude bacterial walls purified after mechanical cell breakage. However, this kind of contamination persisted both after harsher purification (e.g., removal of teichoic acids), and in the trypsin-shaving protocol, which was performed on /H11022/99,9% integral cells as assessed by microscopy and colony counts. Therefore, the question as to whether some cytoplasmic proteins might be constitutive parts of the normal wall environment, as also suggested by others (27, 34), remains open.

**FIG. 6.** Comparison of the sets of peptides released by trypsin digestion of several LPXTG-proteins expressed on the surface of recombinant L. lactis or S. aureus Newman. Sets of unique peptides of ClfA, ClfB, Spa, or FnBPA released by trypsin shaving of recombinant L. lactis or S. aureus Newman are shown. Data were extracted from the experiments presented in Table II and Fig. 3. Major protein domains and gross amino acid numbering are indicated. Precise amino acid numbering of the peptides is presented in supplemental Table S1.

Trypsin-released peptides are represented by the inserted black boxes. Note that some peptides displayed both completely and partially digested species simultaneously (indicated by overlapping boxes). LPXTG motifs are indicated by thin yellow bars and the peptide removed by sortase in gray. SP stands for signal peptides, which are indicated as green boxes. The ligand binding domains are highlighted in blue and S.D. repeat regions in purple.
Proteomic and Transcriptomic Profiling of *S. aureus* LPXTG-proteins

We previously showed that LPXTG-proteins from *S. aureus* could be heterogeneously expressed in *L. lactis* and individually detected by LC-MS/MS in the recombinant lactococci (35, 41). On this basis we constructed a peptide library specific to each of these LPXTG-proteins. This library was indispensable to further quantify LPXTG-proteins in the more complex *S. aureus* environment. With some exceptions, the amounts of LPXTG-proteins in *S. aureus* increased up to the early stationary growth phase, and decreased thereafter. This bell-shape behavior is reminiscent of agr-regulated surface proteins such as protein A, which is expressed during logarithmic growth and repressed in stationary phase (3, 30). In the present experiments, comparisons between proteomic and transcriptomic profiles confirmed this parallelism for protein A, which is in accordance with previous studies (29, 31, 55). Moreover, we identified at least one additional LPXTG-protein, SasH, that demonstrated similar profile modifications between protein and mRNA detection in the parent strain and its agr−mutant, suggesting that it was also under tight control by agr. Conversely, however, several LPXTG-proteins modified their proteomic profiles between the parent and the mutant despite the fact that transcriptomic profiles remained unchanged. This suggests that, in addition to mRNA, protein expression was further affected by additional factors at the post-transcriptional level, e.g., via interference with mRNA, or post-translational level via protein modification (56) or protease degradation (29, 31, 55).

This was particularly relevant when comparing proteomic profiles with adherence phenotypes. Taking fibrinogen binding as a model, the present results show that adherence was indeed affected by both agr integrity and growth conditions, but did not follow an absolute agr paradigm. For instance, in TSB, adherence decreased in the early stationary phase of growth and re-increased later on (at 24 h) without a clear correlate with measured amounts of surface ClfA, but with a clear correlate with increasing clfA mRNA. Likewise, in RPMI adherence tended to decrease over time, without a good proteomic correlate either (mRNA was not measured in this condition). This seeming incoherence most likely reflects our lack of understanding of the subtlety of the wall environment, which implicates additional factors that may affect the phenotype. Indeed, the Gram-positive envelope is not a static envelope is constantly traversed by secreted molecules including ≥10 different proteases (57), among which some were shown to regulate LPXTG-proteins by protein degradation (e.g., ClfB) (29, 31, 55). These could be responsible for the progressive decreases in recovery of unique peptides over time, as observed in Fig. 1.

Alternatively, mutual interactions between various wall polymers may influence the exposure of protein binding domains to the extracellular milieu. This was recently exemplified with recombinant ClfA, where artificial lengthening or shortening of the spacer region (R-repeats) between the proximal wall anchor and the outermost binding domains increased or decreased adherence to fibrinogen, respectively, because they modified the exposure of distal binding domains to their ligand fibrinogen (58). These authors reported similar variations in the presence of absence of an expo-polysaccharide capsule. Therefore, the bacterial surrounding may influence the access of exogenous ligands or proteases to LPXTG-protein domains, a phenomenon that observed with trypsin herein.

Apart from these differences, some other proteins also demonstrated differential regulation between TSB and RPMI, including genes of the iron-capturing *isd* locus, as well as sizable increases in SasD and SasH in iron starvation. The increase in *isd* genes is expected in low iron medium (32, 33). On the other hand, the reason for the increase in SasD and SasH is more difficult to interpret. Although the physiological role of SasD is as yet unclear, SasH (recently renamed AdsA (59)) is a cell wall associated adenosine synthase that converts adenosine-monophosphate into adenosine, a strong immunomodulator helping staphylococci to escape phagocyte-induced killing. Hence, SasH (or AdsA) could well be coregulated with the siderophore locus *isd*, which expression is induced in experimental *S. aureus* nasal colonization (11). In this setting, expression of *isd* could be required for survival in the low-iron mucosal environment, while SasH could be required to dampen host defenses and promote bacterial persistence. Eventually, the mRNA of Srap was detected but its encoded protein was not, suggesting the possible lack of access to trypsin shaving.

The present study yielded other interesting observations. First, FnBPA was detected in both purified walls and trypsin-shaving experiments, despite the fact that it lacks the LPXTG-anchoring module and the entire cell wall proximal α-W regions in *S. aureus* Newman, because of the presence of a stop codon (60). This is also true for its FnBPB counterpart, which in contrast was barely detected at all our experiments. One possibility for this difference is that the 741 (out of 1018) amino acids of truncated FnBPA is enough for nonspecific wall attachment, whereas the 678 (out of 940) residues of truncated FnBPB is too short. Alternatively, the two proteins could have been differentially expressed in the present experimental conditions, a possibility which has yet to be demonstrated. Another noteworthy observation was the fact that trypsin released different sets of peptides from LPXTG-proteins expressed in *S. aureus* or in recombinant *L. lactis*. Thus, some kind of differential domain hindrance or exposure must have taken place in the two bacterial backgrounds, as suggested by others (27, 34). In the same line, a few LPXTG-proteins were not detected at all (i.e., Srap and SasC) in *S. aureus* Newman, although they were detected by LC-MS/MS in recombinant lactococci (35, 41). This raises the question of
their conditional expression, as observed in the present experiments and by others for *isd* genes (32, 33), or of differential access of trypsin digestion.

Taken together, two sets of conclusions can be drawn from these results. First, from the biological point of view, they indicate that some LPXTG-proteins followed an *agr*-like regulation, which was abrogated in *agr*-negative mutants and correlated with the mRNA transcription profile in the parent strain and its *agr*- mutant. On the other and, several LPXTG-proteins varied their expression without a good mRNA correlate, and remained functionally active for prolonged periods of time, such as, for instance, for fibrinogen-binding. This study revises somewhat the dogma that surface adhesins are essentially active during the exponential growth phase, to colonize new sites, and shut off after colonization, to facilitate bacterial detachment and colonization of other sites. Moreover, it also reveals that the bacterial wall environments are different in *S. aureus* and *L. lactis*, potentially resulting in different exposure of LPXTG-proteins at the bacterial surface, which in turns might lead to a differential accessibility for trypsin digestion. Whether this altered access has functional consequences for bacterial adherence remains to be determined. Likewise, whether this could influence the protective efficacy of blocking antibodies might be relevant for vaccine development.

Second, from the technical point of view, the results open the way to semiquantitative and time course proteome analysis of multiple *S. aureus* pathogenic polypeptides simultaneously. Hence, they add to other recently published proteomic analyses (27, 29, 34). One theoretical limitation of trypsin-shaving is that it peptide-release is limited to the trypsin-accessible proteins. Thus, it may underestimate proteins buried deeper in the multipolymeric wall. However, preliminary purification of cell walls or removal of teichoic acids further decreased the recovery of LPXTG-protein peptides, suggesting that trypsin-shaving was a good compromise in this complex surrounding. Another limitation is strain-dependence, which may require recharacterization of each singular organism. However, the same remark is valid for any physiologic or phylogenic characterization of any isolates. Finally, the method could help determining the real-time behavior of numerous bacterial adhesins not only *in vitro*, but possibly also *in vivo*. For the latter case, targeted mass spectrometry techniques based on selected reaction monitoring (61, 62) could be easily developed to specifically detect and quantify bacterial surface molecules in complex matrices such as those obtained from animal models or clinical samples.

Acknowledgments — We thank Alexandra Paillusson, Mélanie Dupasquier, Sylvain Pradrervand and Keith Harshman from the Center of Integrative Genomics (University of Lausanne, Switzerland) for stimulating discussions and outstanding technical support.

* This work was supported by grant 32003B-113854 to P.M. from the Swiss National Science Foundation.

[5] This article contains supplemental Fig. S1 and Tables S1 and S2.

To whom correspondence should be addressed: Department of Fundamental Microbiology, University of Lausanne, 1015 Lausanne, Switzerland. Tel.: +41216925601; Fax: +41216925605; E-mail: philippe.moreillon@unil.ch.

**REFERENCES**

1. Gordon, R. J., and Lowy, F. D. (2008) Pathogenesis of methicillin-resistant Staphylococcus aureus infection. *Clin. Infect. Dis.* **46**, 5350–5359
2. Cheung, A. L., Projan, S. J., and Gresham, H. (2002) The Genomic Aspect of Virulence, Sepsis, and Resistance to Killing Mechanisms in *Staphylococcus aureus*. *Curr. Infect. Dis. Rep.* **4**, 400–410
3. Novick, R. P. (2003) Autoinduction and signal transduction in the regulation of *staphylococcal virulence*. *Mol. Microbiol.* **48**, 1429–1449
4. Pragman, A. A., and Schlievert, P. M. (2004) Virulence regulation in *Staphylococcus aureus*: the need for in vivo analysis of virulence factor regulation. *FEMS Immunol. Med. Microbiol.* **42**, 147–154
5. Patti, J. M., Allen, B. L., McGavin, M. J., and Höök, M. (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. *Annu. Rev. Microbiol.* **48**, 585–617
6. Foster, T. J., and Höök, M. (1998) Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol.* **6**, 484–488
7. Nilsen, I. M., Patti, J. M., Bremell, T., Höök, M., and Tarkowski, A. (1998) Vaccination with a recombinant fragment of collagen adhesin provides protection against *Staphylococcus aureus*-mediated septic death. *J. Clin. Invest.* **101**, 2640–2649
8. Peacock, S. J., Foster, T. J., Cameron, B. J., and Berendt, A. R. (1999) Bacterial fibronectin-binding proteins and endothelial cell surface fibronec-tin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells. *Microbiology* **145**, 3477–3486
9. Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., and Penadés, J. R. (2001) Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J. Bacteriol.* **183**, 2888–2896
10. Weidenmaier, C., Kokai-Kun, J. F., Kristian, S. A., Chanturiya, T., Kalbacher, H., Gross, M., Nicholson, G., Neumeister, B., Mond, J. J., and Peschel, A. (2004) Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nat. Med.* **10**, 243–245
11. Burian, M., Rautenberg, M., Kohler, T., Fritz, M., Krämer, B., Unger, C., Hoffman, W. H., Peschel, A., Wolz, C., and Goerke, C. (2010) Temporal expression of adhesion factors and activity of global regulators during establishment of *Staphylococcus aureus* nasal colonization. *J. Infect. Dis.* **201**, 1414–1421
12. Mazmanian, S. K., Liu, G., Ton-That, H., and Schneewind, O. (1999) *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. *Science* **285**, 760–763
13. Roche, F. M., Massey, R., Peacock, S. J., Day, N. P., Visai, L., Speziale, P., Lam, A., Pallen, M., and Foster, T. J. (2003) Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. *Microbiology* **149**, 643–654
14. Clarke, S. R., and Foster, S. J. (2006) Surface adhesins of *Staphylococcus aureus*. *Adv. Microb. Physiol.* **51**, 187–224
15. Lindsay, J. A., Moore, C. E., Day, N. P., Peacock, S. J., Witney, A. A., Stabler, R. A., Hussain, S. E., Butcher, P. D., and Hinds, J. (2006) Microarrays reveal that each of the ten dominant lineages of *Staphylococcus aureus* has a unique combination of surface-associated and regulatory genes. *J. Bacteriol.* **188**, 666–676
16. Novick, R. P., Ross, H. F., Projan, S. J., Kornblum, J., Kreiswirth, B., and Moghazeh, S. (1993) Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.* **12**, 3967–3975
17. Giraud, A. T., Cheung, A. L., and Nagel, R. (1997) The sae locus of *Staphylococcus aureus* controls exoprotein synthesis at the transcriptional level. *Arch. Microbiol.* **168**, 52–59
18. Vanwood, J. M., McCormick, J. K., and Schlievert, P. M. (2001) Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. *J. Bacteriol.* **183**, 1113–1123
19. Fournier, B., and Hooper, D. C. (2000) A new two-component regulatory system involved in adhesion, autolysis, and extracellular proteolytic ac-
tivity of Staphylococcus aureus. J. Bacteriol. 182, 3955–3964
20. Cheung, A. L., Bayer, A. S., Zhang, G., Gresham, H., and Xiong, Y. Q. (2004) Regulation of virulence determinants in vitro and in vivo in Staphylococcus aureus. FEMS Immunol. Med. Microbiol. 40, 1–9
21. Cheung, A. L., Koomey, J. M., Butler, C. A., Projan, S. J., and Fischetti, V. A. (1992) Regulation of exoprotein expression in Staphylococcus aureus by a locus (sar) distinct from agr. Proc. Natl. Acad. Sci. U.S.A. 89, 6462–6466
22. Said-Salim, B., Dunnan, P. M., McAleece, F. M., Macapagal, D., Murphy, E., McNamara, P. J., Arvidson, S., Foster, T. J., Projan, S. J., and Kreiswirth, B. N. (2003) Global regulation of Staphylococcus aureus genes by Rot. J. Bacteriol. 185, 610–619
23. Yarwood, J. M., McCormick, J. K., Paustian, M. L., Kapur, V., and Schlievert, P. M. (2002) Repression of the Staphylococcus aureus accessory nuclease, NucA, in culture and in vivo. J. Bacteriol. 184, 1095–1101
24. Fell, E. J., Cooper, J. E., Grundmann, H., Robinson, D. A., Enright, M. C., Berendt, T., Peacock S. J., Smith, J. M., Murphy, M., Spratt, B. G., Moore, C. E., and Day, N. P. J. (2003) How clonal is Staphylococcus aureus? J. Bacteriol. 185, 3307–3316
25. Kuroda, M., Onta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., Cui L., Oguchi, A., Aoki, K., Nagai, Y., Lian, J., Ito, T., Kanamori, M., Matsumoto, H., Yama, A., Murakami, H., Hosoyama, A., Mizutani-U., Y., Takahashi, N. K., Sawai, T., Inoue, R., Kaito, C., Sekimizu, K., Hirakawa, H., Kuhara, S., Goto, S., Yabuzaki, J., Kanehisa, M., Yamaishita, A., Oshima, K., Furuya, K., Yoshino, C., Shiba, T., Hattori, M., Ogasawara, N., Hayashi, H., and Hiramatsu, K. (2001) Whole genome sequencing of meticillin-resistant Staphylococcus aureus. Lancet 357, 1225–1240
26. Mueller, D. C., Gorkin, R. F., Boelens, H. A., Snijders, S. V., Peeters, J. K., Moorhouse, M. J., van der Spek, P. J., van Leeuwen, B. W., Simons, G., Verbrugh, H. A., and van Belkum, A. (2004) Natural population dynamics and expansion of pathogenic clones of Staphylococcus aureus. J. Clin. Invest. 114, 1732–1740
27. Dreisbach, A., Hempel, K., Buist, G., Hecker, M., Becker, D., and van Dijl, J. M. (2010) Profiling the surfacome of Staphylococcus aureus. Proteomics 10, 3082–3096
28. Francois, P., Schena, A., Hochstrasser, D., and Schrenzel, J. (2010) Proteomic approaches to study Staphylococcus aureus pathogenesis. J. Proteomics 73, 701–708
29. Jones, R. C., Deck, J., Edmondson, R. D., and Hart, M. E. (2008) Relative quantitative comparisons of the extracellular protein profiles of Staphylococcus aureus UAMS-1 and its sarA, agr, and sarA agr regulatory mutants using one-dimensional polyacrylamide gel electrophoresis and nano-chromatography liquid chromatography coupled with tandem mass spectrometry. J. Bacteriol. 190, 5265–5278
30. Huntinger, E., Boisset, S., Saveau, C., Benito, Y., Geissmann, T., Nama, A., Lina, G., Etienne, J., Ehresmann, B., Ehresmann, C., Jacquier, A., Vandenesch, F., and Romby, P. (2005) Staphylococcus aureus RNAIII and the endoribonuclease III coordinately regulate spa gene expression. EMBO J. 24, 821–835
31. Dussap, C. G., Ungethüm, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., Brown E., Laguzinsky, R. J., Shaia, D., and 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
32. Mazmanian, S. K., Skaar, E. P., Gaspar, A. H., Humayun, M., Gornicki, P., Jelenska, J., Joachmiak, A., Missiakas, D. M., and Schneewind, O. (2003) Passage of heme-iron across the envelope of Staphylococcus aureus. J. Exp. Med. 201, 1627–1635
33. Widmer, E., Que, Y. A., Entenza, J. M., and Moreillon, P. (2006) New concepts in the pathophysiology of infective endocarditis. Curr. Infect. Dis. Rep. 8, 271–279
34. Majcherczyk, P. A., Rubli, E., Heumann, D., Glauser, M. P., and Moreillon, P. (2004) Identification of Teichoic acids are not required for Streptococcus pneumoniae and Staphylococcus aureus cell walls to trigger the release of tumor necrosis factor by peripheral blood monocytes. Infect. Immun. 71, 3707–3713
35. Keller, A., Nesvizhskii, A. I., Kolker, E., and Aebersold, R. (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal. Chem. 74, 5383–5392
36. Nesvizhskii, A. I., Keller, A., Kolker, E., and Aebersold, R. (2003) A statistical model for identifying proteins by tandem mass spectrometry. Anal. Chem. 75, 4646–4658
37. Liu, H., Sadygov, R. G., and Yates, J. R. 3rd (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal. Chem. 76, 4193–4201
38. Smyth, G. K. (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, 102209/1544–6150, 2004
39. Ythier, M., Entenza, J. M., Bille, J., Vandenesch, F., Bes, M., Moreillon, P., and Sakwinska, O. (2010) Natural variability of in vitro adherence to fibrinogen and fibronectin does not correlate with in vivo infectivity of Staphylococcus aureus. Infect. Immun. 78, 1711–1716
40. Baba, T., Bae, T., Schneewind, O., Takeuchi, F., and Hirama, K. (2008) Genome sequence of Staphylococcus aureus strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. J. Bacteriol. 190, 300–310
41. Beaume, M., Hernandez, D., Farinelli, L., Deluen, C., Linder, P., Gaspin, C., Romby, P., Schrenzel, J., and Francois, P. (2010) Cartography of methicillin-resistant S. aureus transcripts: detection, orientation and temporal expression during growth phase and stress conditions. PloS One 5, e10725
42. McCarthy, A. J., and Lindsay, J. A. (2010) Genetic variation in Staphylococcus aureus surface and immune evasion genes is lineage associated: implications for vaccine design and host-pathogen interactions. BMC Microbiol. 10, 173
43. Pichon, C., and Felden, B. (2005) Small RNA genes expressed from Staphylococcus aureus genomic and pathogenicity islands with specific expression among pathogenic strains. Proc. Natl. Acad. Sci. U.S.A. 102, 14249–14254
44. Boisset, S., Geissmann, T., Huntingter, E., Fechter, P., Bendriri, N., Possedko, M., Chevalier, C., Heffer, A. C., Benito, Y., Jacquier, A., Gaspin, C., Vandenesch, F., and Romby, P. (2007) Staphylococcus aureus RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. Genes Dev. 21, 1353–1366
45. Que, Y. A., Francois, P., Haefiger, J. A., Entenza, J. M., Vaudaux, P., and Moreillon, P. (2001) Reassessing the role of Staphylococcus aureus clumping factor A in Lactococcus lactis subsp. cremoris using a new shuttle vector. Infect. Immun. 69, 6296–6302
46. Piroth, L., Que, Y. A., Widmer, E., Panchaud, A., Piu, S., Entenza, J. M., and Moreillon, P. (2008) The fibronogen- and fibronectin-binding domains of Staphylococcus aureus fibronectin-binding protein A synergistically pro-
mote endothelial invasion and experimental endocarditis. *Infect. Immun.* 76, 3824–3831

55. McAleese, F. M., Walsh, E. J., Sieprawska, M., Potempa, J., and 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

56. Waridel, P., Ythier, M., Gfeller, A., Moreillon, P., and Quadroni, M. (2012) Evidence for a new post-translational modification in *Staphylococcus aureus*: hydroxymethylation of asparagine and glutamine. *J. Proteomics* 75, 1742–1751

57. Zdzalik, M., Karim, A. Y., Wolski, K., Buda, P., Wojcik, K., Brueggemann, S., Wojciechowski, P., Eick, S., Calander, A. M., Jonsson, I. M., Kubica, M., Polakowska, K., Miedzobrodzki, J., Władyka, B., Potempa, J., and Dubin, G. (2012) Prevalence of genes encoding extracellular proteases in *Staphylococcus aureus* - important targets triggering immune response in vivo. *FEMS Immunol. Med. Microbiol.* in press

58. Risley, A. L., Loughman, A., Cywes-Bentley, C., Foster, T. J., and Lee, J. C. (2007) Capsular polysaccharide masks clumping factor A-mediated adherence of *Staphylococcus aureus* to fibrinogen and platelets. *J. Infect. Dis.* 196, 919–927

59. Thammavongsa, V., Kern, J. W., Missiakas, D. M., and Schneewind, O. (2009) *Staphylococcus aureus* synthesizes adenosine to escape host immune responses. *J. Exp. Med.* 206, 2417–2427

60. Grundmeier, M., Hussain, M., Becker, P., Heilmann, C., Peters, G., and Sinha, B. (2004) Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion because of loss of the cell wall anchor function. *Infect. Immun.* 72, 7155–7163

61. Lange, V., Malmström, J. A., Didion, J., King, N. L., Johansson, B. P., Schäfer, J., Rameseder, J., Wong, C. H., Deutsch, E. W., Brusniak, M. Y., Bühlmann, P., Björck, L., Domon, B., and Aebersold, R. (2008) Targeted quantitative analysis of *Streptococcus pyogenes* virulence factors by multiple reaction monitoring. *Mol. Cell. Proteomics* 7, 1489–1500

62. Lange, V., Picotti, P., Domon, B., and Aebersold R. (2008) Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol. Syst. Biol.* 4, 222

63. Simon, D., and Chopin, A. (1988) Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. *Biochimie* 70, 559–566

64. Duthie, E. S., and Lorenz, L. L. (1952) *Staphylococcal* coagulase; mode of action and antigenicity. *J. Gen. Microbiol.* 6, 95–107