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Mass spectrometry as a rapid and powerful alternative to antibodies for detecting LPXTG wall-associated proteins of *Staphylococcus aureus*

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Abstract

Functional characterization of transformed or natively present bacterial virulence proteins can be achieved employing various model systems. A prerequisite is to verify the correct expression of the transformed protein or the presence of the native protein in the microbe. Traditionally, antibodies are raised against the protein or a peptide thereof, followed by Western blot analysis or by fluorescence-activated cell sorting. Alternatively, the protein-coding gene can be fused with a downstream reporter gene, the expression of which reports the simultaneous expression of the upstream recombinant protein. Although being powerful, these methods are time consuming, especially when multiple proteins must be assessed. Here we describe a novel way to validate the expression of Gram-positive surface proteins covalently attached to the peptidoglycan. Eighteen out of the 21 known LPXTG-motif carrying cell wall-associated proteins of *Staphylococcus aureus* were cloned in *Lactoccocus lactis* either alone, in combinations or as truncated forms, and their correct expression was assessed by liquid chromatography coupled to mass spectrometry (LC–MS). The method is rapid, sensitive and precise. It can identify multiple proteins in transformed constructs without the time and cost needed for raising and testing multiple sets of antibodies.

Keywords: Liquid chromatography; LPXTG motif; Mass spectrometry; Recombinant protein; *Staphylococcus aureus*

1. Introduction

Staphylococcus aureus expresses multiple surface proteins, several of which were shown to be implicated in disease processes [1–5]. At least 21 of these proteins are covalently attached to the peptidoglycan via the conserved LPXTG motif [6,7]. Some of them were demonstrated to be microbial surface molecule recognizing adherence matrix molecules (MSCRAMMs) [8,3], whereas the functions of others have remained elusive.

In addition to their multiplicity, these proteins have sometimes redundant functions, as exemplified by clumping factors A and B (ClfA and ClfB) [8,9], which bind fibrinogen, and fibronectin-binding proteins A and B (FnBPA and FnBPB), which bind fibronectin, fibrinogen and elastin [10–12]. Therefore, studying their individual function in gene-inactivated *S. aureus* mutants is difficult. To circumvent this limitation, systems were developed in which the *S. aureus* determinants are expressed individually or in tandem in surrogated bacteria lacking the rest of the *S. aureus* surface features [4,13,14]. Using this strategy, Que et al. [4] observed that binding to fibrinogen was necessary for colonization of damaged valves in experimental endocarditis. However, ClfA was not sufficient to provoke invasive and persistent infection, because lactococci producing only fibrinogen-binding proteins were progressively eradicated after valve colonization in vivo. Conversely, binding to fibronectin was necessary for invasion and persistence – by triggering internalization into endothelial cells surrounding the vegetations – but not sufficient to colonize the valves at first. Hence, both binding functions needed to cooperate to produce invasive and

Abbreviations: MSCRAMMs, microbial surface molecule recognizing adherence matrix molecules; ECM, extracellular matrix proteins; CFU, colony forming unit; GPF, gas phase fractionation

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persistent disease, and these two functions are provided by the set of *S. aureus* MSCRAMMs.

In a parallel study, Widmer et al. [15] cloned 18 of the 21 LPXTG wall-attached proteins of *S. aureus* in *Lactococcus lactis* and tested the recombinant bacteria for adherence to a variety of host extracellular matrix proteins (ECM), adherence to platelet–fibrin clots, internalization into cultured endothelial cells, and infectivity in rats with experimental endocarditis.

Before testing the lactococci for an acquired function of virulence/infectivity, it was important to assess their correct expression in the surrogate organisms. Detecting 18 individual proteins by Western immuno-blotting [16] or gene-reporter systems [17] would have required the generation of many individual antibodies or gene-reporters, not even considering that antibodies could cross-react and reporter fusion-proteins could affect the functionality of the polypeptide. To circumvent tedious and costly antibody production and protein purification for all 18 constructs, we developed a novel approach using the high separation of liquid chromatography coupled to the high peptide sequencing capability of mass spectrometry (LC–MS), in order to assess the correct expression and cell-surface localization of the recombinant proteins. Peptides derived from the enzymatic digestion of the total peptidoglycan-attached protein complement of each of the 18 recombinant lactococcal strains were separated, sequenced and identified in order to validate the correct expression of each construct. In addition, 10 constructs expressing truncated proteins and/or proteins expressed in tandem were tested to assess the appropriateness of the method to detect internal deletions and multiple protein expression.

2. Materials and methods

2.1. Bacerial cell wall preparation

Crude-bacterial cell wall aliquots were prepared as previously described [18]. Briefly, cells were grown at 30 °C in GM17 medium (M17 medium containing 0.5% glucose) without agitation until an OD_{600} of 0.4, cooled on ice and centrifuged 10 min at 6000 rpm at 4 °C. Supernatant was removed, cells were resuspended in 40 ml PBS, 40 ml 8% boiling SDS was immediately added and the suspension was further boiled for 15 min with continuous stirring. Boiled cells were cooled at room temperature and centrifuged for 10 min at 9400 × *g* at 25 °C, washed twice with 1 M NaCl, several times with Milli-Q water until no more foaming appeared upon vortexing and finally resuspended in 1 ml water. Cell lysis was further performed using FastPrep (Qbiogene, Morgan Irvine, CA, USA) with glass beads by three cycles of spinning for 45 s at 6.5 m/s with cooling on-ice between cycles. Glass beads were settled on ice and the supernatant recovered. One milliliter of Milli-Q water was added to the beads, a single FastPrep cycle applied and the supernanant recovered. Beads were resuspended in 1 ml Milli-Q water, transferred to a fresh Falcon tube, 20 ml Milli-Q water was added and beads vortexed before the supernatant was recovered again. The combined supernatants were centrifuged for 5 min at 1500 × *g* at 4 °C to sediment all non-broken cells. The clarified supernatant was transferred to a 30 ml Nunc ultracentrifugation tube and centrifuged for 20 min at 16,000 rpm at 4 °C. The pellet containing the cell walls was finally resuspended in 1.5 ml Milli-Q water.

2.2. Sample preparation for mass spectrometry

Crude-bacterial cell wall aliquots (5×10^8 cells) were dried using a SpeedVac and resuspended in 100 μl of 100 mM ammonium bicarbonate (NH_4HCO_3) at pH 7.8. Proteins were reduced for 30 min at 50 °C with10 μl 45 mM 1,4-dithio-DL-threitol (DTT) (Fluka, Buchs, Switzerland) and further alkylated for 60 min at room temperature in the dark with 10 μl of 100 mM iodoacetamide (Fluka). The resultant protein mixture was digested overnight at 37 °C with trypsin (Promega, Madison, USA) using an enzyme:protein ratio of 1:50 (w/w). The tryptic peptides were cleaned on SepPak tC18 cartridges (Waters, Milford, USA) and eluted with 2 × 1 ml 80% acetonitrile in two separate 1.5 ml centrifuge tubes. Solutions of purified peptides were partly concentrated under vacuum for 30 min and then pooled in one centrifugal tube for further vacuum concentration to reach a final volume of approximately 10 μl. Drying was interrupted every 30 min and the peptide mixture thoroughly vortexed to minimize sample loss. Peptides were diluted by adding 190 μl 0.1% formic acid and stored at −20 °C until MS processing.

2.3. LC–MS/MS

LC–MS/MS data were acquired using an HCTultra ion-trap mass spectrometer (Bruker, Bremen, Germany) coupled on-line to an Ultimate 3000 HPLC system (Dionex, Sunnyvale, USA) equipped with an analytical Magic C18 reversed-phase column (100 mm × 0.075 mm, 5 μm) (Spectronex, Basel, Switzerland). Ten microliters out of the 200 μl purified peptide solution was diluted with 40 μl 0.1% formic acid and peptides were analyzed using gas phase fractionation, performing a triplicate analysis in three different mass ranges, that are: (i) 400–700 Da, (ii) 700–1000 Da and (iii) 1000–1400 Da. For each replicate, 10 μl of the diluted mixture was loaded and washed for 10 min with 2% (v/v) acetonitrile/0.1% (v/v) formic acid on a C18 PepMap100 trapping column (5 mm × 0.3 mm, 5 μm) (Dionex, Sunnyvale, USA) at a flow rate of 20 μl/min prior to elution with a linear gradient of 2–50% (v/v) acetonitrile/0.1% (v/v) formic acid over 60 min at a flow rate of 0.3 μl/min. Peptides were analyzed using the "peptide scan" option of the HCTultra system, consisting of a full-scan MS spectrum acquisition in "standard-enhanced" mode (8100 *m/z*/s) for charge state assignment based on the ^{13}C isotope envelope followed by three MS/MS scans in "ultra scan" mode (26,000 *m/z*/s) on the three most abundant ions as well as exclusion of singly charged ions and preferred charge state set to doubly for MS/MS selection.

2.4. Database search

MS/MS data were extracted in MGF file format (Mascot Generic Format) using the Data Analysis software v3.3

(Bruker, Bremen, Germany) and submitted to Mascot (Matrix Science, London, UK) using the *L. lactis* protein database (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/Lactococcus_lactis) to which the 18 protein sequences of *S. aureus* were added. Searches were performed defining trypsin as the protease allowing one missed cleavage. Monoisotopic masses, peptide mass deviation tolerance of 100 ppm and MS/MS mass deviation tolerance of 0.3 Da were set as mass parameters. Methionine oxidation and cysteine carbamidomethylation were set as variable, respectively, fixed modifications. Search results were firstly filtered with a p-value of 0.01 and then, secondly, by setting the ion-score cut-off to the minimal individual ion score indicating identity or extensive homology for $p < 0.01$. Lastly, remaining filtered peptide identifications were manually confirmed with regard to their respective MS/MS spectra. Mascot reports a probability-based "ion score", which is defined as $-10 \times \log_{10}(p)$, where p is the probability that the observed match between experimental data and the database sequence is a random event.

3. Results

3.1. General methodology

Fig. 1 depicts (a) the detection of recombinant ClfA and FnBPA in lactococcal wall preparations by Western immunoblotting [15] and (b) the peptides detected by LC–MS, which led to the identification of ClfA and FnBPA. Immuno-blotting required a total protein mass from an equivalent of $\geq 10^7$ CFU (in absolute number) for reliable detection. By contrast, LC–MS-based protein expression validation could be performed with up to 10× less microorganisms, which makes the technique

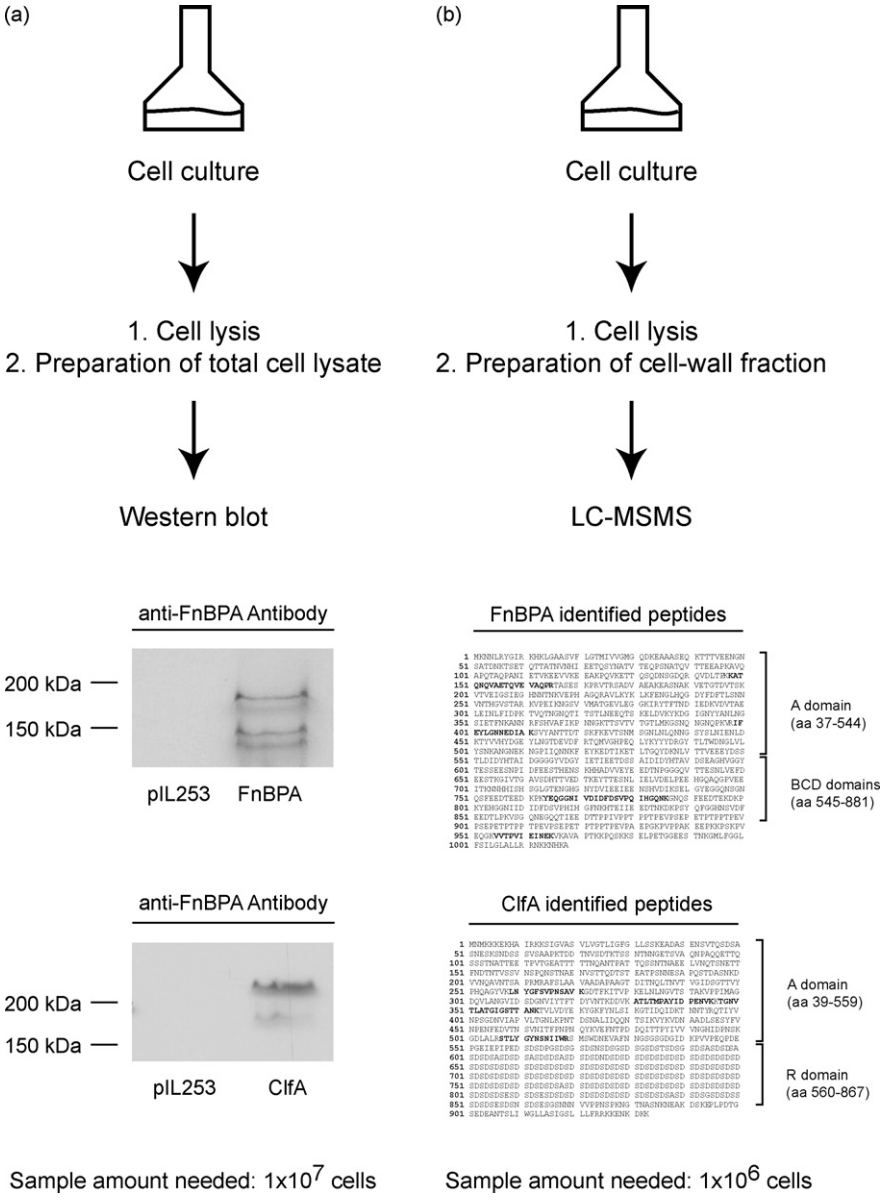

Fig. 1. Schematic comparison of our presented approach. (a) Western blot analysis using a specific antibody to detect the recombinant protein in a total cell lysate separated by SDS-PAGE. (b) The recombinant protein is detected and identified by liquid chromatography tandem MS analysis after processing the bacterial cell-wall preparation. Identified and validated peptide sequences are marked in bold in the full-length sequence of the proteins, FnBPA and ClfA, respectively.

appropriate for samples containing low bacterial numbers such as those derived from clinical samples. For example, infected urines contain up to 10^7 CFU/ml.

The cell wall-anchored proteins were digested with trypsin and the resulting peptides were purified by solid-phase extraction (C_{18} cartridges) prior to mass spectrometric analysis. The purified peptides were then subjected to C_{18} reversed-phase chromatography on-line coupled to ESI ion-trap tandem mass spectrometry. The peptides were analyzed by gas phase fractionation (GPF) [19], which consisted in a triplicate analysis covering three adjacent mass ranges compared to more standard triplicate analysis of all three ranges together. This approach permitted a more specific detection of low abundant peptides in the very complex bacterial cell-wall fractions. Only a narrow mass range is scanned at the time, which results in superior selection of minor peptides for peptide sequencing (MS/MS). Thus, the increased fragment spectra content of the mass spectrometric data improves protein identification with better scores and higher protein coverage.

The resulting MS/MS spectra were searched with Mascot [20] against a custom-built database containing all protein sequences of *L. lactis* plus the sequences of the 18 cell-surface protein of *S. aureus* to be tested.

3.2. Expression of S. aureus LPXTG wall-anchored proteins in lactococci

Fig. 2 depicts the positions and numbers of peptides identified for all 18 LPXTG-proteins of *S. aureus* cloned in lactococci. Table 1 provides information on the peptide sequences and identification scores obtained for each of the transformed proteins. Sixteen of the 18 cloned species were detected. In the case of SasD, exceptionally only one peptide was detected but the corresponding sequencing spectra (MS/MS spectra) resulted in a high Mascot database search score and hence a reliable protein identification. For three proteins, namely SdrD, SasH and FnBPB, no peptides were detected in the first bacterial preparations. Re-sequencing of the plasmids revealed missing nucleotides resulting in frame shifts in each of these constructs, a finding which explained the absence of any related peptide. A new corrected SdrD-containing construct was re-assessed and the SdrD protein

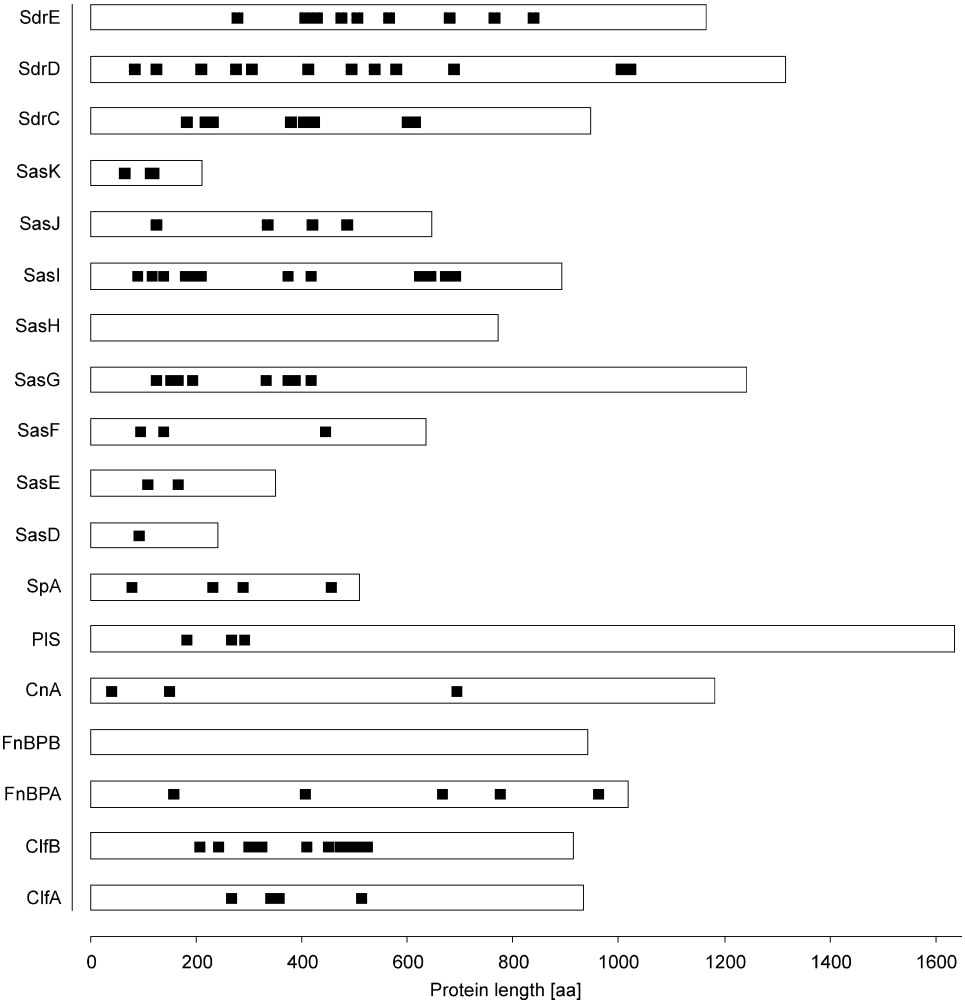

Fig. 2. Representation of peptides identified in the 18 individually-expressed proteins constructs expressed in *L. lactis*. Each recombinant protein is represented proportionally to its respective length, the N-terminal being situated at the left part of the figure and the C-terminal and the wall-anchoring domain at the right part of the figure. Black squares indicate the centered position of the identified peptides. Detailed information for each peptide is presented in Table 1.

Table 1
List of peptides identified for the 18 LPXTG wall-associated protein constructions of *Staphylococcus aureus*

Protein name (protein length)	Start	End	Length	Sequence	Mascot score	Sequence coverage (%)
ClfA (933)	259	271	13	K.LNYGFSVPNSAVK.G	40	6.22
	331	345	15	K.ATLTMPAYIDPENVK.K	35	
	347	363	17	K.TGNVTLATGIGSTTANK.T	96	
	507	519	13	R.STLYGYNSNIIWR.S	33	
ClfB (913)	197	211	15	R.SLAVAEPVVNAADAK.G	110	16.76
	229	250	22	K.TTFDPNQSGNTFMAANFTVTDK.V	95	
	296	303	8	K.ATYDILTK.T	50	
	304	316	13	K.TYTFVFTDYVNNK.E	74	
	317	331	15	K.ENINGQFSLPLFTDR.A	74	
	402	411	10	R.VLGNTWVYIK.G	51	
	442	456	15	K.LSDSYYADPNDSNLK.E	54	
	466	477	12	R.IYYEHPNVASIK.F	42	
	484	498	15	K.TYVVLVEGHYDNTGK.N	56	
	502	515	14	K.TQVIQENVDPVTNR.D	97	
	516	529	14	R.DYSIFGWNNENVVR.Y	76	
FnBPA (1018)	148	165	18	K.KATQNQVAETQVEVAQPR.T	98	8.06
	399	411	13	R.IFEYLGNNEDIAK.S	30	
	656	672	17	K.GIVTGAVSDHTTVEDTK.E	19	
	764	786	23	K.YEQGGNIVDIDFDSVPQIHGQNK.G	92	
	955	965	11	K.VVTPVIEINEK.V	36	
FnBPB (940)	N/A	N/A	N/A	N/A	N/A	N/A
can (1183)	32	47	16	R.DISSTNVTDLTVSPSK.I	105	3.72
	137	156	20	R.NLTQTNTSDDKVATITSGNK.S	74	
	688	695	8	K.YTVEELTK.V	28	
PlS (1637)	172	191	20	K.TEETDKATTEEAPAAEETSK.A	38	4.09
	256	275	20	K.AEETNKVETEEAPAAEETNK.A	34	
	276	302	27	K.AATEETPAVEDTNAKSNSNAQPSETER.T	59	
SpA (508)	70	84	15	K.DDPSQSANVLGEAQK.L	97	13.39
	219	238	20	K.EQQNAFYEILHLPNLNEEQR.N	29	
	277	296	20	K.EQQNAFYEILHLPNLTEEQR.N	85	
	447	459	13	K.NMIKPGQELVVDK.K	39	
SasD (241)	85	95	11	K.LYDATQNIADK.Y	84	4.56
SasE (350)	101	114	14	K.YYFQTVLNNASFWK.E	70	7.71
	157	169	13	K.VHIVVPQINYNHR.Y	27	
SasF (635)	89	98	10	K.TASQIDEIIK.R	37	5.67
	131	141	11	K.DLNEVSSNVDR.G	67	
	435	449	15	K.HFASTGDTSSDDILK.A	44	
SasG (1243)	118	128	11	K.EVADVAEVQPK.S	72	8.21
	145	155	11	R.SVDEGSFDITR.D	75	
	159	171	13	K.NVVESTPITIQGK.E	54	
	185	196	12	K.KPTDLGVSEVTR.F	53	
	322	338	17	K.TNFLNYADNSTNTSDGK.F	93	
	366	378	13	K.TWETSITDLGLSK.N	69	
	379	391	13	K.NQAYNFLITSSQR.W	74	
	410	421	12	K.GSEFTFTPEAPK.T	51	
SasH (772)	N/A	N/A	N/A	N/A	N/A	N/A
SasI (891)	82	95	14	K.NYPAADESLKDAIK.D	44	20.88
	110	119	10	R.EQVNFQLLDK.N	32	
	133	141	9	K.DPADVYYTK.K	40	
	172	186	15	R.LVSYSPVPEDHAYIR.F	90	
	187	197	11	R.FPVSDGTQELK.I	54	
	198	216	19	K.IVSSTQIDDGEETNYDYTK.L	107	
	364	381	18	R.QFYHYASTVEPATVIFTK.T	68	
	408	425	18	K.LPVELVSYDSDKDYAYIR.F	74	
	617	626	10	R.TLIFPYIPDK.A	51	
	627	634	8	K.AVYNAIVK.V	34	
	635	649	15	K.VVVANIGYEGQYHVR.I	93	

Table 1 (*Continued*)

Protein name (protein length)	Start	End	Length	Sequence	Mascot score	Sequence coverage (%)
	659	680	22	K.DDDTSQNNTSEPLNVQTGQEGK.V	83	
	681	697	17	K.VADTDVAENSSTATNPK.D	114	
SasJ (645)	117	130	14	K.ATNNTYPILNQELR.E	42	8.68
	327	341	15	K.SAITEFQNVQPTNEK.M	76	
	415	424	10	R.TIIFPYVEGK.T	31	
	475	491	17	K.EATPATPSKPTPSPVEK.E	59	
SasK (211)	60	68	9	K.LDNLIDGTK.D	26	17.54
	104	122	19	K.HPDNLIDGTKDPNVINQLK.N	36	
	114	122	9	K.DPNVINQLK.N	50	
SdrC (947)	171	188	18	R.MAVNTVAAPQQGTNVNDK.V	52	12.04
	210	221	12	K.TEFWATSSDVLK.L	67	
	224	233	10	K.ANYTIDDSVK.E	23	
	373	383	11	K.QTFVTNLTGYK.F	32	
	393	412	20	K.IYEVTDQNQFVDSFTPDTSK.L	44	
	413	429	17	K.LKDVTDQFDVIYSNDNK.T	83	
	592	604	13	K.DADNMTLDSGFYK.T	47	
	608	620	13	K.YSLGDYVWYDSNK.D	61	
SdrD (1315)	76	88	13	K.VDMQQLNQEDNTK.N	48	13.99
	114	130	17	K.ESVQSTTGNKVEVSTAK.S	38	
	197	221	25	K.SNDETLVDNNSNSNNENNADIILPK.S	66	
	268	278	11	K.IVPAQDYLSLK.S	44	
	297	312	16	K.YSDTVQVYGLNPEDIK.N	91	
	402	418	17	K.NSIGSAFTETVSHVGNK.E	84	
	486	497	12	K.ELTDVTNQYLQK.I	67	
	525	547	23	K.FQYTNSESPTLVQMATLSSTGNK.S	119	
	572	582	11	K.IGNYVWEDTNK.N	79	
	682	694	13	K.YNLGDYVWEDTNK.N	89	
	998	1010	13	K.DADNMTLDSGFYK.T	85	
	1014	1026	13	K.YSLGDYVWYDSNK.D	78	
SdrE (1166)	267	285	19	R.FAVAQPAAVASNNVNDLIK.V	85	12.35
	397	413	17	K.TVPNETSLNLTFATAGK.E	70	
	414	440	27	K.ETSQNVTVDYQDPMVHGDSNIQSIFTK.L	30	
	466	480	15	K.VDIAGSQVDDYGNIK.L	106	
	499	510	12	K.VNSDQQLPQSNR.I	63	
	554	571	18	K.YTPTSDGELDIAQGTSMR.T	110	
	673	683	11	K.FETPTGYLPTK.V	27	
	758	769	12	K.VIGTTTTDASGK.Y	39	
	831	843	13	K.YSLGDYVWYDSNK.D	80	

could be identified this time by MS/MS data of twelve tryptic peptides. These results clearly show the power of the LC–MS-based method not only to verify the correct expression of recombinant proteins, but also in case of failure, to indicate potential nucleotide sequence errors in the corresponding plasmids.

3.3. Validation of expression of truncated and tandem-expressed proteins

After having demonstrated the applicability of this method to full-length proteins by high protein sequence coverage (wide distribution of numerous peptides over the protein sequence in question), we extended the approach to characterize a series of truncated protein constructs, as well as proteins expressed in tandems. Fig. 3 summarises the LC–MS/MS-derived sequence coverage of full-length FnBPA as compared to three truncated forms. They lacked either the A domain (containing the fibrinogen-binding and elastin-binding regions of FnBPA) or the B–C–D domains (conferring binding to fibronectin) or all the functional domains except for the wall-anchoring portion [12,14]. In each case, the missing domains were confirmed by absence of any domain-related peptides in the LC–MS spectra. This result correlated accurately with the loss of the binding phenotypes in vitro and in vivo [21,4]. Fig. 4 shows the LC–MS protein sequence coverage for the constructs of tandem-expressed proteins. ClfA/FnBPA stands for a lactococcal recombinant expression whole-length ClfA and a truncated FnBPA with only the C–D domains. For this recombinant strain it was recently demonstrated that fibrinogen-binding and fibronectin-binding can cooperate to produce invasive infection in vivo [4]. The other tandem expressing recombinants have not been published previously, and all these strains demonstrated binding to their specific ligands in vitro; including dual binding to collagen and fibronectin for recombinant Cna/FnBPA-CD, and multiple binding to collagen, fibronectin, fibrinogen and

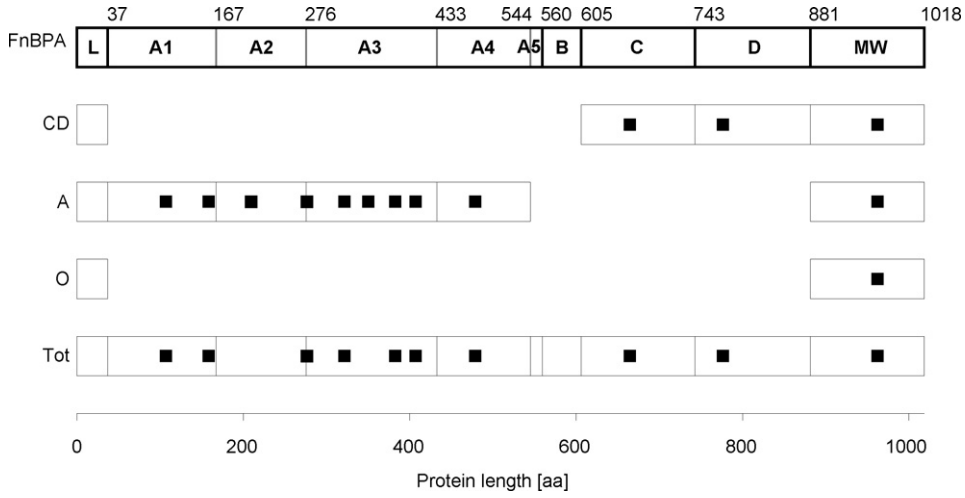

Fig. 3. Representation of peptides identified in three variants of the FnBPA protein. At the top of the figure, the full-length FnBPA is represented with an indication of the different domains present in this protein according to Massey et al. [12]. Below, the different constructions are shown including: (i) full-length FnBPA (Tot), (ii) FnBPA from which all the functional domains were deleted, except for the signal peptide (O), (iii) FnBPA from which the A and B domains were deleted (CD) and (iv) FnBPA from which the B–C–D domains were deleted (A). Missing rectangles mean that this part of the protein has been removed in the gene construction. Black rectangles indicated the identified peptides. Missing black squares indicate the absence of any peptide detection in the corresponding domain. Detailed information for each peptide is presented in Table 2.

elastin for recombinant Cna/FnBPA (data not shown). Binding to Fc-antibody fragments by Spa and binding of Pls to ECM was not tested. Thus, the new approach does not only yield information on the successful expression of a single intact protein, but also allows in-depth analysis of the primary structure and confirmation of domain structures in different variants. Table 2 summarizes the protein sequences coverage for each of the transformed proteins by highlighting the peptides identified.

4. Discussion

An integrated view of gene expression ideally assesses transcriptional, post-transcriptional, translational and post-

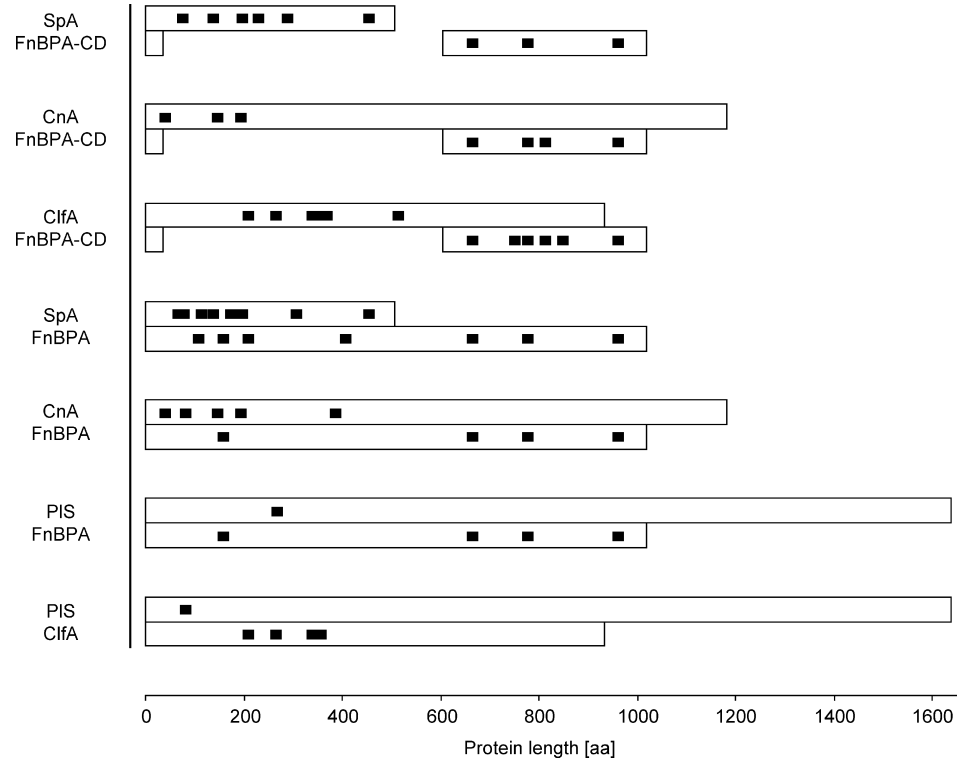

Fig. 4. Representation of peptides identified in the tandem-expressed constructs. Details are as in Fig. 1 and additional information for each peptide is presented in Table 3.

Table 2
List of peptides identified for each variant of FnBPA

Variant name (protein length)	Start	End	Length	Sequence	Mascot score	Sequence coverage (%)
Tot (1018)	98	114	17	K.AVQAPQTAQPANIETVK.E	48	15.6
	149	165	17	K.ATQNQVAETQVEVAQPR.T	103	
	267	282	16	K.NGSVVMATGEVLEGGK.I	67	
	311	331	21	K.TVQTNGNQTITSTLNEEQTSK.E	84	
	375	386	12	K.TTSVTVTGTLMK.G	52	
	399	411	13	R.IFEYLGNNEDIAK.S	30	
	472	483	12	R.TQMVGHPEQLYK.Y	61	
	656	672	17	K.GIVTGAVSDHTTVEDTK.E	19	
	764	786	23	K.YEQGGNIVDIDFDSVPQIHGQNK.G	92	
	955	965	11	K.VVTPVIEINEK.V	36	
O (174)	955	965	11	K.VVTPVIEINEK.V	64	6.3
A (681)	98	114	17	K.AVQAPQTAQPANIETVK.E	51	22.6
	149	165	17	K.ATQNQVAETQVEVAQPR.T	103	
	201	216	16	K.VTVEIGSIEGHNNTNK.V	42	
	267	282	16	K.NGSVVMATGEVLEGGK.I	76	
	311	331	21	K.TVQTNGNQTITSTLNEEQTSK.E	92	
	339	357	19	K.DGIGNYYANLNGSIETFNK.A	27	
	375	386	12	K.TTSVTVTGTLMK.G	46	
	399	411	13	R.IFEYLGNNEDIAK.S	89	
	472	483	12	R.TQMVGHPEQLYK.Y	57	
	955	965	11	K.VVTPVIEINEK.V	57	
CD (450)	656	672	17	K.GIVTGAVSDHTTVEDTK.E	108	11.3
	764	786	23	K.YEQGGNIVDIDFDSVPQIHGQNK.G	68	
	955	965	11	K.VVTPVIEINEK.V	56	

translational regulation. However, when the study is restricted to protein function, ensuring that the polypeptide of interest is present in the right cellular compartment comes first. This is usually achieved by raising antibodies against the protein of interest or constructing reporter systems. Although powerful, these methods become cumbersome where it comes to detecting more than a few polypeptides at a time. Typically, ensuring the presence of the 21 LPXTG wall-anchored proteins of *S. aureus* would necessitate as many different antibody sets, and testing their simultaneous expression would require multiple experiments.

The method described herein detected a total of 34 *S. aureus* LPXTG proteins expressed either individually or in tandem in *L. lactis*. The total analysis time including data interpretation required approximately 1 week. In three cases, the expected proteolytic peptides were not detected, but also no phenotype was observed (data not shown). In two of these cases (SdrD and FnBPB), re-sequencing of the plasmid montages revealed errors that could be corrected, thus further validating the system. In the third case (SasH), the protein could not be expressed for as yet unknown reasons.

The reliability of the method was further attested by the fact that in seven cases the detection of recombinant *S. aureus* MSCRAMMs in the wall preparations of lactococci conferred new phenotypes to the surrogate bacteria. This included known adherence properties of ClfA, ClfB, FnBPA, FnBPB and Cna, plus new binding function including binding to keratin for SdrE, and binding to fibrinogen for Pls [15]. Eventually, expression of truncated proteins from which specific binding domains had been deleted, as well as tandem-expressed proteins, could also be confirmed and correlated with the expected loss or gain of function in the recombinant lactococci. Thus, the new approach does not only yield information on the successful expression of a given protein, but also allows in-depth analysis of the primary structure and confirmation of domain structures in different variants. One particular peptide (aa267–aa282), spanning the boundaries of domain A2–A3 is an example of how this information can be exploited to confirm domain organization. This may serve as a control in the case of variants with either swapped or inverted domains, resulting in specific peptide sequences amenable to targeted MS analysis.

Beyond validating the presence of proteins, the method opens new perspectives for analyzing the dynamics of protein expression both in the test tube and during in vivo infections. In living cells, functional expression of proteins is not only regulated at the transcriptional level. For instance, certain surface proteins of *S. aureus* are processed by wall-associated proteolytic enzymes after having been exported to the cell surface [11,22]. Conversely, surface proteins could remain functionally active after prolonged periods of time after the transcription of their constitutive gene has been switched off. The LC–MS method described here would be appropriate to study such phenomena. Processing specific proteome sub-fractions, as performed herein, allows for greatly simplifying the whole proteome, which might be more difficult to analyze. In addition, the technique is also appropriate for protein quantification, for instance by spiking selected ("proteotypic") stable-isotope labelled peptides – representative of the proteins of interest – into the protein mixture, as recently described [23].

Table 3
List of peptides identified for each tandem expressed constructions

Protein name (protein length)	Start	End	Length	Sequence	Mascot score	Sequence coverage (%)
Clfa (933)	200	212	13	K.DVVNQAVNTSAPR.M	74	6.22
	259	271	13	K.LNYGFSVPNSAVK.G	28	
	331	345	15	K.ATLTMPAYIDPENVK.K	27	
	347	363	17	K.TGNVTLATGIGSTTANK.T	94	
Pls (1637)	68	93	26	K.GNVQTIEQSSANSNESDIPEQVDVTK.D	32	1.59
FnBPA (1018)	149	165	17	K.ATQNQVAETQVEVAQPR.T	113	6.68
	656	672	17	K.GIVTGAVSDHTTVEDTK.E	119	
	764	786	23	K.YEQGGNIVDIDFDSVPQIHGQNK.G	75	
	955	965	11	K.VVTPVIEINEK.V	42	
Pls (1637)	256	275	20	K.AEETNKVETEEAPAAEETNK.A	42	1.22
FnBPA (1018)	149	165	17	K.ATQNQVAETQVEVAQPR.T	109	6.68
	656	672	17	K.GIVTGAVSDHTTVEDTK.E	118	
	764	786	23	K.YEQGGNIVDIDFDSVPQIHGQNK.G	33	
	955	965	11	K.VVTPVIEINEK.V	31	
CnA (1183)	32	47	16	R.DISSTNVTDLTVSPSK.I	65	5.49
	75	84	10	K.VAWPTSGTVK.I	20	
	137	156	20	R.NLTQTNTSDDKVATITSGNK.S	31	
	190	198	9	R.WFLNINNEK.S	28	
	382	391	10	K.ALPSGDYILK.E	19	
FnBPA (1018)	98	114	17	K.AVQAPQTAQPANIETVK.E	52	11.20
	149	165	17	K.ATQNQVAETQVEVAQPR.T	120	
	201	216	16	K.VTVEIGSIEGHNNTNK.V	32	
	399	411	13	R.IFEYLGNNEDIAK.S	64	
	656	672	17	K.GIVTGAVSDHTTVEDTK.E	92	
	764	786	23	K.YEQGGNIVDIDFDSVPQIHGQNK.G	73	
	955	965	11	K.VVTPVIEINEK.V	53	
SpA (508)	62	69	8	R.NGFIQSLK.D	46	23.43
	70	84	15	K.DDPSQSANVLGEAQK.L	46	
	103	122	20	K.DQQSAFYEILNMPNLNEEQR.N	17	
	131	144	14	K.DDPSQSTNVLGEAK.K	63	
	161	180	20	K.EQQNAFYEILNMPNLNEEQR.N	100	
	189	202	14	K.DDPSQSANLLAEAK.K	77	
	297	311	15	R.NGFIQSLKDDPSVSK.E	30	
	447	459	13	K.NMIKPGQELVVDK.K	32	
FnBPA-CD (313)	656	672	17	K.GIVTGAVSDHTTVEDTK.E	108	38.98
	738	763	26	K.SELGYEGGQNSGNQSFEEDTEEDKPK.Y	40	
	764	786	23	K.YEQGGNIVDIDFDSVPQIHGQNK.G	68	
	802	824	23	K.YEHGGNIIDIDFDSVPHIHGFNK.H	43	
	836	857	22	K.DKPSYQFGGHNSVDFEEDTLPK.V	37	
	955	965	11	K.VVTPVIEINEK.V	56	
ClfA (933)	200	212	13	K.DVVNQAVNTSAPR.M	78	8.47
	259	271	13	K.LNYGFSVPNSAVK.G	42	
	331	345	15	K.ATLTMPAYIDPENVK.K	52	
	347	363	17	K.TGNVTLATGIGSTTANK.T	104	
	364	371	8	K.TVLVDYEK.Y	29	
	507	519	13	R.STLYGYNSNIIWR.S	59	
FnBPA-CD (313)	656	672	17	K.GIVTGAVSDHTTVEDTK.E	77	23.64
	764	786	23	K.YEQGGNIVDIDFDSVPQIHGQNK.G	70	
	802	824	23	K.YEHGGNIIDIDFDSVPHIHGFNK.H	42	
	955	965	11	K.VVTPVIEINEK.V	43	
can	32	47	16	R.DISSTNVTDLTVSPSK.I	65	3.80
(1183)	137	156	20	R.NLTQTNTSDDKVATITSGNK.S	33	
	190	198	9	R.WFLNINNEK.S	29	
FnBPA-CD (313)	656	672	17	K.GIVTGAVSDHTTVEDTK.E	98	16.29
	764	786	23	K.YEQGGNIVDIDFDSVPQIHGQNK.G	80	
	955	965	11	K.VVTPVIEINEK.V	44	

Table 3 (*Continued*)

Protein name (protein length)	Start	End	Length	Sequence	Mascot score	Sequence coverage (%)
SpA (508)	62	84	23	R.NGFIQSLKDDPSQSANVLGEAQK.L	90	20.67
	131	144	14	K.DDPSQSTNVLGEAK.K	63	
	189	202	14	K.DDPSQSANLLAEAK.K	44	
	219	238	20	K.EQQNAFYEILHLPNLNEEQR.N	61	
	277	296	20	K.EQQNAFYEILHLPNLTEEQR.N	87	
	447	460	14	K.NMIKPGQELVVDKK.Q	34	

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