Complementary Analysis of the Vegetative Membrane Proteome of the Human Pathogen Staphylococcus aureus*

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The Gram-positive bacterium Staphylococcus aureus is a serious human pathogen causing a wide variety of diseases, and its increasing resistance toward all available antibiotics makes its further investigation absolutely essential. We examined the membrane proteome of exponentially growing cells of S. aureus COL because this subproteome plays a major role in the virulence of the bacterium in its host. In general, an analysis of membrane proteins is impeded by their hydrophobic nature as well as by a high abundance of many cytosolic proteins. The implementation of three different technologies, one-dimensional gel-LC, two-dimensional LC, and a membrane shaving approach combined with MS/MS analyses, enabled an identification of 271 integral and 86 peripheral membrane proteins from exponentially growing cells. In particular, the latter approach that combined membrane shaving with a subsequent chymotrypsin digest of integral membrane domains of proteins greatly facilitated the detection of hydrophobic peptides derived from membrane-spanning segments (713 peptides, 60% of all peptides) and therefore yielded almost exclusively highly hydrophobic integral membrane proteins (96.7%). A comparison of the various methods disclosed the one-dimensional gel-LC and the shaving approach to be highly complementary techniques. A combination of them will reveal a most comprehensive view on membrane proteomes. Molecular & Cellular Proteomics 7: 1460–1468, 2008.

The cytoplasmic membrane of a cell represents the interface with its direct surroundings and is therefore the subcellular location at which information and substance interchange is taking place. The perception of and the response to specific environmental conditions such as physical stress or starvation are often mediated by proteins within the membrane. Pathogenic bacteria, for instance, depend on the interaction with their respective hosts to exert virulence and survive in the host.

The human pathogenic bacterium Staphylococcus aureus causes a wide variety of diseases ranging from minor skin infections (1, 2) to critical endocarditis (3, 4) and sepsis (5). The spreading of multiresistant S. aureus strains and the increasing number of lethal infections worldwide is most alarming. It is of utmost importance to characterize especially membrane, surface-associated, and secretory proteins of this organism because those subproteomes substantially determine its virulence. The proteome of S. aureus consists of ~2600 proteins (6, 7) of which more than one-quarter possess one or more transmembrane domains (TMDs) according to TMHMM 2.0 (8). Although first insights into the membrane proteome of S. aureus could be delivered (9, 10), our knowledge is still limited, and in particular the detection of proteins with multiple TMDs lags far behind. There are several special features of membrane proteins that render their analysis so difficult (11, 12). Their hydrophobic character conveys poor solubility in aqueous buffer and makes a separation via 2D PAGE very complicated (13). Furthermore membrane proteins are disguised by the high abundance of many cytosolic proteins requiring an efficient isolation procedure. Mass spectrometric analyses are generally preceded by protein digestion. Because membrane proteins are shielded by lipids they have to be solubilized by detergents or organic solvents to allow the enzymes to attack. Inauspiciously trypsin, the standard digestion enzyme used in preparation for mass spectrometry, exhibits very few cleavage sites in membrane proteins. Thus, alternative enzymes or cleavage procedures have to be considered to assure an efficient protein digestion (14). Previous experience with LC-MS/MS analyses of membrane proteins has revealed complications in the detection of hydrophobic peptides derived from TMDs (12). Proteins containing multiple membrane-spanning domains are especially hard to identify and are identified with only low sequence coverage if at all. Improved sequence coverage of membrane proteins is not only important for a more confident identification but also necessary for reliable protein quantitation based on several peptides and the detection of post-translational modifications. Some approaches have been introduced to improve sequence coverage (15–20), but the detection of TM peptides remains a challenge.

The abbreviations used are: TMD, transmembrane domain; IMP, integral membrane protein; TM, transmembrane; 1D, one-dimensional; 2D, two-dimensional; TEAB, triethylammonium bicarbonate buffer; SCX, strong cation exchange.
The aim of this work was to develop a protocol that facilitates the identification of integral membrane proteins (IMPs) by depleting contaminations from cytosolic proteins on the one hand and detecting peptides derived from TMDs on the other. Three different strategies were pursued to investigate the membrane proteome of exponentially growing cells of *S. aureus*. A comparison of the three approaches including their advantages and drawbacks will be raised.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions and Preparation of Protein Extracts—** *S. aureus* COL (21) was grown in Luria broth (Invitrogen) under vigorous agitation at 37 °C until the culture reached an *A*₅₆₀ of 1.5 (final *A*₅₆₀ in stationary phase, 7–8). The exponentially growing cells were harvested by centrifugation (8000 × *g*) for 10 min at 4 °C. Cell pellets were washed twice with ice-cold TBS buffer (50 mM Tris, 150 mM NaCl, pH 8.0) and resuspended in lysis buffer (50 mM Tris, 1 mM PMSF, 1 × Nuclease Mix from Amersham Biosciences). Cell disruption by homogenization with glass beads occurred in the RiboLyser (Thermo Fisher Scientific) in three cycles of 30 s at 6.5 m/s with intermittent cooling. Cell lysates were kept at room temperature for 30 min to allow nucleolytic digestion before removal of cell debris by centrifugation (8000 × *g* at 4 °C for 2 min). Supernatants were centrifuged another 10 min, and protein extracts were stored at −20 °C. Protein concentration was determined using Roti-Nanoquant (Roth, Karlsruhe, Germany).

**Proteinase K-Chymotrypsin Digestion of Membrane Proteins (Shaving Approach)—** An aliquot of 50 mg of protein extract was pelleted by ultracentrifugation at 100,000 × *g* for 1 h. The crude membrane fraction was homogenized in 500 μl of carbonate buffer (200 mM Na₂CO₃, pH 11.0) and further treated and digested with proteinase K (Promega) using a modified protocol described briefly by Speers et al. (20). The protein concentration of the homogenized pellet was adjusted to 1 mg/ml (total protein amount was 3.5 mg). The sample was incubated for 1 h on an overhead shaker (4 °C) and every 15 min homogenized using an insulin syringe. After the sample was brought to room temperature, solid urea was added to a concentration of 8 M. Protein reduction was performed at 50 °C in 5 mM tris(2-carboxyethyl)-phosphine hydrochloride for 30 min followed by an alkylation step in 10 mM iodoacetamide in the dark (15 min at room temperature). Proteinase K was added in an enzyme:protein ratio of 1:50 followed by incubation for 15 h in a thermomixer (37 °C at 900 rpm). One volume of 10% acetonitrile in water was added before the solution was cooled on ice for 15 min. Following an ultracentrifugation step of 1 h (100,000 × *g* at 4 °C) the supernatant was discarded. To remove residual urea from the pellet, it was rinsed with 50 mM triethylammonium bicarbonate buffer (TEAB), pH 7.8, and again ultracentrifuged (100,000 × *g* at 4 °C for 1 h).

For a chymotrypsin digest the pellet was resuspended in 200 μl of digestion buffer (50 mM TEAB, pH 7.8, 10 mM CaCl₂, and 0.5% RapiGest™ (Waters)) before 4 μg of the enzyme (emp Biotech, Berlin, Germany) were added. The digest was carried out while shaking (900 rpm) at 30 °C for 6 h. To remove RapiGest concentrated HCl was added to give a 250 mM HCl solution (pH < 2) followed by incubation at 37 °C for 45 min. The sample was centrifuged three times for 15 min at 4 °C (20,000 × *g*), each time keeping the supernatant that contained the peptides of the chymotryptic digest. An injection of 0.2 μl of the final peptide solution gave an adequate amount for LC-MS/MS analyses. The peptide concentration was therefore estimated to be 2 μg/μl.

**1D SDS-PAGE and Tryptic Digestion of Membrane Proteins (1D Gel Approach)—** Membrane proteins were purified from 100 mg of a crude protein extract (see “Growth Conditions and Preparation of Protein Extracts”) as described before (22), leaving out an extraction of proteins by n-dodecyl-β-D-maltoside treatment. The purified membrane pellet was homogenized in 500 μl of 50 mM TEAB, pH 7.8, resulting in a protein concentration of 1.7 μg/μl. An amount of 100 μg of purified membrane proteins was reduced and alkylated according to “Proteinase K-Chymotrypsin Digestion of Membrane Proteins (Shaving Approach)” and delipidated (18). Fifteen micrograms of such treated proteins were separated by 1D SDS-PAGE to give reasonable amounts for later LC-MS/MS analyses. A gel lane was cut into 12 equal pieces. Gel electrophoresis and in-gel trypsinization were performed as recorded previously (22).

**Chymotryptic-Tryptic Digestion of Membrane Proteins and Off-line Strong Cation Exchange (SCX) Chromatography (2D LC Approach)—** Fifty micrograms of purified membrane proteins (see “1D SDS-PAGE and Tryptic Digestion of Membrane Proteins (1D Gel Approach)”) were solubilized in 50 μl of digestion buffer, reduced, and alkylated as specified above (see “Proteinase K-Chymotrypsin Digestion of Membrane Proteins (Shaving Approach)”). Chymotrypsin was added in a ratio of 1:20, and the sample was shaken at 900 rpm at 30 °C for 3 h. Tryptsin was added (ratio, 1:50), and digestion was prolonged another 3 h, keeping the same conditions. The detergent RapiGest was removed by acidic hydrolysis as mentioned above (see “Proteinase K-Chymotrypsin Digestion of Membrane Proteins (Shaving Approach)”).

An aliquot of 6 μg of peptides of the combined chymotryptic-trypsin digestion was separated into 12 fractions by SCX chromatography as described previously (23). The salt plugs used to elute peptides were concentrated as follows: 3, 6, 10, 13, 16, 20, 25, 37.5, 75, 200, and 500 mM NH₄Cl. The flow-through was collected as well. Just as in the 1D SDS-PAGE approach (see “1D SDS-PAGE and Tryptic Digestion of Membrane Proteins (1D Gel Approach)”) 12 fractions were obtained to be finally measured by LC-MS/MS, but an equivalent of 6 μg of purified membrane proteins less starting material was needed probably because of a smaller recovery of peptides after an in-gel digest compared with an in-solution digest.

**Nano-HPLC—** For nano-HPLC analyses a nanoACQUITY™ UPLC™ System (Waters) was used. Peptides originating from the proteinase K-chymotrypsin digestion were loaded directly on an analytical column (nanoACQUITY UPLC column, BEH130 C₁₈, 1.7 μm, 100 μm × 100 mm; Waters) and washed for 30 min with 99% buffer A (0.1% acetic acid). Peptides were eluted from the column in a 5-h linear gradient going up to 60% buffer B (90% acetonitrile, 0.1% acetic acid). The flow rate during column loading and elution was set to 1 μl/min, and the analytical column was tempered at 60 °C (22).

Because of impurities and higher salt concentrations, peptides of the in-gel trypsin digestion and the fractionated sample of the chymotryptic-trypsin digestion were loaded onto a trapping column (nanoACQUITY UPLC column, Symmetry® C₁₈, 5 μm, 180 μm × 20 mm; Waters) and washed for 3 min with 99% buffer A at a flow rate of 10 μl/min. Peptides were then eluted and separated on the same analytical column as mentioned above going from 99% buffer A to 60% buffer B in 80 min.

**MS/MS Analysis—** The nanoACQUITY UPLC System was coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). After a full survey scan in the Orbitrap (m/z 300–2000) with a resolution of 30,000, MS/MS experiments of the three most abundant precursor ions were acquired in the LTQ via CID. The accurate masses of the precursor ions were determined by selected ion monitoring scans in the Orbitrap, applying a resolution of 60,000. Precursors were dynamically excluded for 30 s, and unassigned charge states as well as singly charged ions were rejected.

**Data Analysis—***.dta files were generated from *.raw files using BioworksBrowser 3.2 EF2 (Thermo Fisher Scientific). Charge state deconvolution and deisotoping were not performed. All MS/MS sam-
To pursue strategy A comprises a separation via 1D SDS-PAGE of the proteins before the gel is cut into 12 slices, and the proteins are digested in-gel using trypsin. Method B consists of a crude membrane extract as starting material and fractionates the complex peptide mixture by SCX chromatography. The shaving protocol (C) starts out with a proteinase K digest of membranes. Only the pellet of the following ultracentrifugation step is kept and further digested with chymotrypsin. Each of the methods ends with a peptide analysis implementing reversed phase chromatography (RPC) coupled to MS/MS.

Samples were analyzed using SEQUEST version 27.12 (Thermo Fisher Scientific), applying the following search parameters: peptide tolerance, 10 ppm; tolerance for fragment ions, 1 amu; b- and y-ion series; an oxidation of methionine (15.99 Da) and a carboxamidomethylation (57.02 Da) of cysteine were considered as variable modifications (maximal three modifications per peptide). For the three different digestion strategies varying settings for the enzyme had to be used. Samples of the in-gel tryptic digestion were searched with trypsin cutting fully enzymatically and allowing one missed cleavage site. Proteins subjected to a combined chymotryptic-tryptic digestion were searched with an enzyme cutting fully enzymatically at the amino acids KRWYFL. Four missed cleavage sites were allowed because often the enzyme does not cut at all leucine residues in leucine clusters, which are intrinsic to TMDs. The samples originating from a proteinase K-chymotrypsin digestion were searched three times: first defining no enzyme specificity because proteinase K cuts unspecifically, second with chymotrypsin cutting fully enzymatically at WYFL (four missed cleavage sites allowed), and third with chymotrypsin cutting partially enzymatically (four missed cleavage sites) to identify peptides that were cut by proteinase K at one end and by chymotrypsin at the other. All samples were searched against a target-decoy database (5236 entries) that was composed of all protein sequences of S. aureus COL extracted from the National Center for Biotechnology Information (NCBI) bacteria genomes and an appended set of the reversed sequences created by BioworksBrowser 3.2 EF2 (24). The resulting *.dta and *.out files were uploaded in Scaffold™ version 01_06_19 (Proteome Software Inc., Portland, OR) (25). Protein hits were stringently filtered with the requirement that two different peptides (peptides in a modified form do not count as different) must pass the following SEQUEST filter: XCorr for doubly charged peptides must be 2.2 and for triply or higher charged peptides must be 3.75. The ΔCn score was set to 0.26. For the determination of peptides overlapping with TMHMM 2.0-predicted TMDs a user-written Excel spreadsheet was used to calculate the number of amino acids located within the membrane.

RESULTS

Protein Identifications—Three different strategies were used to study the vegetative membrane proteome of S. aureus (Fig. 1). It had to be assured that differences in the results of the single approaches are not caused by differences in the protein content of the S. aureus cells. Therefore, all three approaches started out with the same biological material and were accomplished in technical replicates. The first strategy comprised a 1D gel-LC-MS/MS analysis that was carried out twice and led to an identification of 493 and 526 proteins, respectively (1D gel approach). In the second approach 2D LC-MS/MS analyses of chymotryptic-trypsin-digested membrane proteins were performed, resulting in 289 protein identifications in the first and 262 protein identifications in the second replicate (2D LC approach). Peptides originating from the proteinase K-chymotrypsin method (shaving approach) were separated only in one dimension but over a long LC gradient that was run in six replicates (number of protein identifications in single replicates: 121, 118, 112, 122, 132, and 135) approaching a saturation in the number of protein identifications. Detailed information on the protein identification for each of the three techniques introduced is provided in supplemental Tables S4–S6. Furthermore the corresponding Scaffold files and results of single replicates including MS/MS spectra for all peptide identifications are available upon request.

Because a comparison of the three strategies is the focus of this work, the data were filtered in a stringent manner to produce only the most confident results (database search against the target-decoy database gave no false positive hits), thereby risking loss of some information. There were 572 proteins identified from the 1D gel after combining the results of replicates using Scaffold. The 2D LC method gave rise to 321 proteins in total, and 182 proteins were detected after membrane shaving. Thus, 1D gel-LC-MS/MS yielded 3 times as many proteins compared with membrane shaving. When

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2 Schäfer, J., Baumann, C., and Schwarz, J. (2004) Poster presented at the 52nd American Society for Mass Spectrometry (ASMS) Conference, Nashville, Tennessee (May 23–27, 2004).
considering the number of proteins predicted to possess TMDs, the 1D gel and the shaving strategy perform comparably well. This is because of the low proportion of IMPs identified by the 1D gel method (179 proteins, 31.3%), whereas 96.7% of the proteins identified by the shaving strategy contain TMDs (176 proteins). By 2D LC-MS/MS 133 IMPs (41.3%) were detected. A comparison of IMPs with multiple membrane-spanning domains revealed an even more dramatic difference between the methods. Eighty-six percent of the proteins identified by the shaving protocol have three or more TMDs (158 proteins). In the 1D gel and 2D LC methods only 15.2% (87 proteins) and 26.1% (84 proteins) represent these highly hydrophobic IMPs, respectively. The distribution of the number of membrane-spanning domains over the proteins identified by each of the strategies is shown in Fig. 2.

Most of the IMPs are very hydrophobic due to their location inside the membrane. The GRAVY index of a protein is a measure of its hydrophobicity (27): the larger the GRAVY the more hydrophobic the protein is and the higher its chance of a TM location. Most of the proteins detected by 1D gel-MS/MS and 2D LC-MS/MS possess negative GRAVY values, reflecting a high degree of contamination by cytosolic proteins (Fig. 3). By contrast, the major fraction of the proteins identified after membrane shaving show highly positive GRAVY indices of larger than 0.5. Also their average GRAVY of 0.58, which is larger than the average GRAVY of all theoretical IMPs (0.41), indicates this method to be specific for membrane proteins with multiple TMDs.

Peptide Identifications—A detailed evaluation concerning the transmembrane location of identified peptides was carried out using a user-written Excel spreadsheet. The difference in protein identifications between the three technologies is in good accordance with observations made on the peptide level. With a number of 3877 most peptides were identified in the 1D gel approach (compared with 1824 peptides by 2D LC and 1188 peptides by the shaving method), but only a negligible number of 23 peptides originate from TMDs of membrane proteins (0.6%). More peptides located inside the membrane could be identified carrying out the 2D LC technique (149 peptides, 8.2%), and with 713 peptides (60.0%) by far the most of such peptides were found after a proteinase K treatment of the membrane with subsequent chymotryptic digestion. Fig. 4 illustrates the distribution of TM peptides according to their number of amino acids integral to the membrane. It is obvious that the detection of TM peptides, particularly of those with many residues within the membrane, is a significant strength of the membrane shaving procedure. The 1D gel approach favors identification of peptides originating from loops of membrane proteins. This is plausible because arginine and lysine, the cleavage sites of trypsin, are rarely found within TMDs. In consideration of this fact, one has to assume that the 1D gel approach and the shaving strategy are highly complementary techniques covering different regions of membrane proteins.

Three Approaches: Overlap—A total of 271 IMPs was identified, but only 68 of them were accessible by all three methods (Fig. 5). The 2D LC approach is interchangeable with the other two technologies as only two proteins were exclusively
found by this method. Fifty-five membrane proteins uniquely identified from the 1D gel (78% contain only one or two TMDs) and 65 membrane proteins only detected after membrane shaving (94% have three or more TMDs) clearly show these techniques to be complementary. A list of all IMPs identified in this study is in supplemental Table S2.

**Biases in Protein Identification**—According to the TMHMM 2.0 algorithm (8) there is clear bias toward IMPs with an even number of membrane-spanning domains in the theoretical membrane proteome of *S. aureus*. Considering the percentage of the proteins identified according to their number of TMDs, two independent trends are noticeable. First with an increasing number of TMDs the identification rate rises from 20% of IMPs with three TMDs to 67% of proteins containing 13 TMDs. This is most likely because of an increase in molecular weight with the number of TMDs a protein contains, and therefore the chance to detect peptides of this protein increases (19). Identification rates with regard to the number of TMDs and average molecular mass are given in Table I. Even more interesting is the second trend that reflects higher identification rates for proteins with an odd number of TMDs (t test significance, >99%) (Fig. 6). This is directly opposed to the fact that membrane proteins with an even number of TMDs are more frequent. The observation was supported by the implementation of a different prediction algorithm for TMDs. Applying the SOSUI (28) algorithm to our data resulted in the same trend of a preferential identification of proteins having an odd number of TMDs with >96% t test significance (data not shown). It is difficult to give a rational explanation for this observation. We made the case that proteins with an odd number of TMDs have higher abundance than proteins with an even number of TMDs. A scan of the codon adaptation indices (29) of all IMPs provided information on the translation efficiency of these proteins, but no difference in the codon adaptation indices of proteins with odd and even numbers of TMDs could be demonstrated (data not shown). Further investigation is therefore required to identify the origin of the finding to be of biological or bioinformatic nature.

**Physiological Classification of Proteins**—Most of the detected IMPs can be assigned to specific functional classes. More than one-quarter of them are components of transporters (e.g. ATP-binding cassette transporters), and 33 proteins are involved in the synthesis and turnover of the cytoplasmic membrane, cell wall, or cell division. A further major category comprises proteins facilitating resistance to drugs and antibiotics or playing another important role in the virulence of *S. aureus*. This latter category of proteins is expected to increase in stationary phase because many virulence factors are known to be induced after cells become stationary (30, 31). Twenty-eight percent of the 271 IMPs identified at present have no known function. An allocation of the IMPs to diverse categories is given in Fig. 7 and in supplemental Table S2.

For a complete examination of the membrane proteome of *S. aureus* one cannot omit the fraction of peripheral proteins, which is more difficult to identify. A fraction of these peripheral proteins is known to be involved in the interaction with the host cell or with other cells and is called the cell wall associated or the outer membrane protein. The structure of these proteins is rather hydrophilic and therefore hard to detect by gel electrophoresis. But even more problematic is the fact that these proteins are not able to pass through the membrane into the wash buffer. Thus, we were not able to detect any of these proteins by gel electrophoresis. A list of all peripheral proteins is given in supplemental Table S3.

![Fig. 4. Distribution of total identified peptides regarding their number of amino acids that are integral to the membrane according to TMHMM 2.0 (8).](image1)

Because only 23 of 3877 total peptides identified via 1D gel-LC-MS/MS showed an overlap with TMDs, no graph was created for this method. About 8% of the peptides identified by 2D LC-MS/MS were partially located within the membrane (149 peptides). The most powerful approach to address this crucial kind of peptide is the membrane shaving technique yielding 713 integral membrane peptides (60%).

**Fig. 5. Number of identified IMPs for different approaches and overlap of the three protein populations.** In total 271 IMPs could be identified, but only 68 of them were covered by all three methods. Particularly the 1D gel-LC and the shaving protocol display a complementary character with 55 and 65 unique protein identifications, respectively. Most notably, proteins exclusively identified by 1D gel-LC-MS/MS contain primarily one or two TMDs, whereas the proteins only detected after membrane shaving comprise multiple TMDs.
membrane proteins. In our assay, primarily 1D gel-LC and 2D LC experiments were well suited to reveal this subgroup of membrane proteins. In total 86 peripheral membrane proteins could be detected, and although no TMDs are predicted, their location at the membrane can be anticipated by their function. A list of peripheral membrane proteins and their assignment to different functional classes, e.g. substrate-binding and ATP-binding proteins of ATP-binding cassette transporters or enzymes involved in the synthesis and turnover of the cellular membrane and wall, are given in supplemental Table S3.

### DISCUSSION

No method of choice exists yet in the field of membrane proteomics. There is rather a wide variety of methods used to analyze this special kind of protein. According to the literature very promising strategies are based on a separation of membrane proteins via SDS-PAGE (32), shotgun approaches using multidimensional separation on the peptide level (17), and methods involving membrane shaving (20). We tied in with these valuable results of other groups and strongly focused on sample preparation, most of all on protein digestion, because this is thought to be one of the most crucial points in the analysis of integral membrane proteins and TMDs (12). From our optimization procedures three different protocols have emerged to be the most successful concerning an identification of membrane proteins. This study provides a comparison of those approaches applied to the vegetative membrane proteome of *S. aureus*. Although analytical techniques were the focus of this work, we also report the largest validated set of membrane proteins of exponentially growing populations of this serious human pathogen to date (9, 10). As demonstrated before, microorganisms do not express their entire set of proteins under certain growth conditions. The Gram-positive bacterium *Bacillus subtilis*, which is closely related to *S. aureus*, expresses only 60% of all open reading frames during exponential phase in a chemically defined minimal medium (22). Assuming a similar expression rate for *S. aureus*, at least 60–70% of the theoretically expressed IMPs have been detected in this study. Considering that *S. aureus* will express additional proteins in response to changing physiological and environmental conditions, such as stationary phase induced by stress or starvation (31, 33–35), one can expect an identification of even more IMPs, covering a high proportion of the total membrane proteome of this pathogenic bacterium.

It is generally known that detection of peptides resulting from protein regions that are located within the membrane is

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**Fig. 6.** Normalized identification (norm. ID) rates against the number of TMDs. Identification rates depending on the number of TMDs were normalized by the average molecular mass of the corresponding protein population to eliminate the bias in identification rates caused by protein size (Table I). IMPs with one and two TMDs were omitted because of more frequent false TMD predictions for these proteins. Also IMPs having 15 and 21 TMDs were not considered because there are only two proteins for each of the populations, which do not provide meaningful values. A preferential identification of IMPs containing odd numbers of TMDs is clearly displayed in the graph (t test significance, >99%).

**Fig. 7.** Physiological classification of identified IMPs. For more than one-quarter of all identified IMPs the function still has to be elucidated. The three largest functional categories, transport and binding proteins, proteins involved in the synthesis and turnover of the cellular membrane/wall and cell division, and proteins important for drug resistance and virulence, account for almost one-half of the IMPs (134 proteins).

**Table I**

| No. of TMDs | No. of proteins | ID rate % | Ø theoretical molecular mass kDa | ID rate normalized by Ø molecular mass |
|-------------|----------------|-----------|-----------------------------------|----------------------------------------|
| 1           | 184           | 35.3      | 34.6                              | 1.02                                   |
| 2           | 65            | 49.2      | 35.9                              | 1.37                                   |
| 3           | 45            | 20.0      | 17.5                              | 1.14                                   |
| 4           | 62            | 30.6      | 25.3                              | 1.21                                   |
| 5           | 55            | 40.0      | 32.1                              | 1.25                                   |
| 6           | 61            | 27.9      | 34.8                              | 0.80                                   |
| 7           | 24            | 41.7      | 38.8                              | 1.07                                   |
| 8           | 27            | 33.3      | 43.8                              | 0.76                                   |
| 9           | 26            | 61.5      | 42.8                              | 1.44                                   |
| 10          | 44            | 45.5      | 47.4                              | 0.96                                   |
| 11          | 20            | 60.0      | 49.6                              | 1.21                                   |
| 12          | 41            | 48.8      | 54.6                              | 0.89                                   |
| 13          | 12            | 66.7      | 55.6                              | 1.20                                   |
| 14          | 17            | 47.1      | 55.8                              | 0.84                                   |
| 15          | 2             | 100.0     | 73.1                              | 1.37                                   |
| 21          | 2             | 100.0     | 89.5                              | 1.12                                   |
| Total       | 687           | 39.4      | 37.0                              | 1.06                                   |
quite difficult (15, 19, 20). According to the current opinion (12), one reason for this obstacle is the length and hydrophobicity of peptides after a trypsinization of IMPs, which hampers their extraction from the gel in the case of an in-gel digestion and which leads to a poor recovery from reversed phase material during chromatographic separation. Furthermore, the low abundance of membrane-embedded peptides in a complex mixture of highly concentrated hydrophilic peptides most likely impedes their detection. In our study the complexity of peptide mixtures, finally analyzed by reversed phase LC-MS/MS, differs for each of the applied methods. We lowered the complexity in two of the three approaches by prefractionation, whether on the protein level by SDS-PAGE (1D gel approach) or on the peptide level by SCX (2D LC approach). Because we could not take advantage from prefractionation in the shaving approach (SCX was not well suited for a fractionation of hydrophobic peptides), we lengthened the LC gradient to compensate for this drawback. Despite a two-dimensional separation in the 1D gel as well as in the 2D LC approach, integral membrane peptides were still found to be strongly underrepresented, an observation also made by other groups regardless of prefractionating the sample (36) or lowering sample complexity in vacuo via gas phase fractionation (37). Also an increase in the number of precursor ions selected for fragmentation in MS/MS experiments will not increase the fraction of low abundance hydrophobic peptides as we observed in studies in which the five most abundant ions were selected\(^3\) and as presented by others (20, 38). Statements on poor ionization efficiencies and poor fragmentation qualities of hydrophobic peptides could be refuted as well (26, 39). Those arguments led us to the conclusion that the concentration of high abundance hydrophilic peptides especially from soluble proteins is still very high in the mixture of the 1D gel and the 2D LC strategy, hence preventing an extensive mass spectrometric detection of low abundance hydrophobic peptides from IMPs.

According to the accepted literature (12), at the current technological state only an efficient removal of hydrophilic proteins and hydrophilic protein segments will enable the detection of TMD peptides. To our knowledge there are only two groups who have been particularly successful in a detection of peptides from TMDs (19, 20). Both of them started with a membrane shaving procedure to enrich integral membrane segments. Speers et al. (20) combined membrane shaving by proteinase K with a cyanogen bromide (CNBr) treatment of the remaining membranes and analyzed the peptides by \(\mu\)LC-MS/MS at elevated temperature, enabling a detection of many transmembrane peptides. The application of CNBr to membrane-spanning domains is more meaningful than a conventional trypptic digestion because methionine is more frequent within TMDs than lysine and arginine, resulting in more and shorter peptides available for LC-MS/MS analysis. Still methionine is a rare amino acid, and therefore residues more common in TMDs should be considered as cleavage sites. One could make use of the characteristically high content of leucine and aromatic amino acids in hydrophobic domains of membrane proteins. As shown by Fischer and Poetsch (14) and Fischer et al. (19) digestion with chymotrypsin that cuts C-terminal of the mentioned amino acids is perfectly suited for these critical regions of IMPs and provides even more theoretical peptides from membrane-spanning segments. Therefore, we combined the enrichment of TMDs by membrane shaving and a chymotryptic digestion of the remaining membrane pellet solubilized in the detergent RapiGest.

Because the nonspecific digestion of the membrane by proteinase K is very efficient, one can start the protocol with a rather crude membrane extract. There is no need for long membrane purification procedures as required in the 1D gel or 2D LC approach. Still almost all of the proteins identified by the shaving protocol are integral to the membrane. Only for six proteins was no TMD predicted; four of them represent lipoproteins, and two are very alkaline ribosomal proteins.

According to Speers et al. (20) LC-MS/MS analyses of peptides in the shaving protocol were performed at 60 °C to increase the number of TM peptides. Whereas this group observed an increase in peptide identification of up to 500%, elevated temperature conveyed only a small effect in our experiments. This may be due to a shorter length of chymotryptic peptides compared with peptides obtained by CNBr treatment and the resultant difference in elution behavior from reversed phase material. Additional reasons may include the different column dimensions and column material as well as the higher back pressure and flow rate used in our study.

The shaving-chymotrypsin protocol described here led to an identification of 713 TM peptides. Comparing this number with recently published data of Fischer et al. (19), who detected 135 TM peptides from *Corynebacterium glutamicum* by their SIMPLE (Specific Integral Membrane Peptide Level Enrichment) method, and with Speers et al. (20), who identified 246 TM peptides from HeLa cells by proteinase K shaving and CNBr treatment, great progress was made concerning the detection of the nontrivial population of peptides located within the membrane, although a comparably small proteome was investigated.

The shaving protocol theoretically only allows an analysis of integral domains of membrane proteins, so this technique strongly discriminates against peripheral membrane proteins as well as proteins with only a small number of TMDs. These proteins can be covered by technologies not including a membrane shaving such as the 1D gel and the 2D LC approach. Both protocols involve a two-dimensional separation of the sample. However, in a 1D gel-LC experiment complete proteins are separated in the first dimension, and peptides are separated in the second step, whereas the 2D LC technique implements two peptide separation levels. Possibly the lower

\(^3\) H. Hahne, S. Wolff, M. Hecker, and D. Becher, unpublished data.
degree of complexity in the first separation dimension is the reason why the 1D gel approach yielded more protein identities than the 2D LC protocol in this survey.

In summary, it became apparent that a membrane shaving procedure giving access to very hydrophobic domains of membrane proteins and 1D gel-LC-MS/MS best suited for the detection of soluble regions are highly complementary technologies. A combination of these two approaches will result in the most comprehensive analysis currently possible.

This study aimed at the preparation of new analytical tools in the most relevant field of membrane proteomics. The presented techniques should be applicable to a wide variety of species and can therefore be generally used for an extensive identification, a reliable quantitation, and an elucidation of post-translational modifications of membrane proteins. Our study also revealed a large fraction of the theoretical membrane proteome of exponentially growing S. aureus cells and provides the basis for further investigations, for instance on staphylococcal virulence, that will be of great interest for the medical and immunological community.

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