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RESEARCH ARTICLE

# Proteomic analysis of exoproteins expressed by enterotoxigenic *Staphylococcus aureus* strains

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Pathogenic bacteria excrete a variety of virulence factors into extracellular medium and to the cell surface which have essential roles in the colonization and insurrection of the host cells, and thus reflect the degree of bacterial pathogenicity. For the exploration of virulence factors expressed in the secreted proteome fraction, different *Staphylococcus aureus* strains were analyzed using gel-based bottom-up proteomic approach. A total of 119 distinct proteins were identified for the enterotoxin gene cluster (*egc*) negative and *seb* gene positive *S. aureus* American Type Culture Collection (ATCC) 14458 strain by the use of one- and 2-DE based proteomics. Detailed analysis of enterotoxin region of the 2-D map confirmed, beside the highly expressed staphylococcal enterotoxin B (SEB), the presence of enterotoxin-like proteins SElK and SElQ previously predicted by genotyping (Sergeev *et al.*, *J. Clin. Microbiol.* 2004, *42*, 2134–2143). Exoprotein patterns at the late-exponential (7 h) and stationary (24 h) phases of cellular growth show a high-level similarity in this region. Comparative analysis of enterotoxin region of five *S. aureus* strains including two clinical isolates (RIMD 31092 and A900322), a food derived strain (AB-8802) with highly prevalent *egc* positive operon and a nonenterotoxigenic reference strain (ROS) revealed the presence of different known enterotoxins and other virulence factors along with a number of core exoproteins. In addition, production of SElL (RIMD 31092) and SElP (A900322) was demonstrated for the first time at the protein level. Under the experimental conditions applied none of the enterotoxins encoded by the genes of *egc* operon was identified.

**Keywords:**
Extracellular proteins / Pyrogenic toxin superantigens / Staphylococcal enterotoxins / *Staphylococcus aureus* / Virulence factors

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**Abbreviations: egc,** enterotoxin gene cluster; **IAA,** iodoacetamide; **IDA,** information dependent analysis; **MW,** molecular weight; **SAgs,** superantigens; ***Sar*A,** staphylococcal accessory regulator; **SEB,** staphylococcal enterotoxin B; **SEI,** staphylococcal enterotoxin-like; **SEs,** staphylococcal enterotoxins; **TSS,** toxic shock syndrome; **TSST-1,** toxic shock syndrome toxin-1

## 1 Introduction

Proteome subset secreted into the extracellular environment of a cell is defined as the exported proteome or "secretome" [1, 2]. Prokaryotes have differently located secretion systems and pathways dedicated to the process of protein secretion [3, 4]. Recent studies suggested four distinct pathways for protein export of Gram-positive bacteria. In addition to the

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major "Sec" system, three additional pathways, the twin-arginine translocation or "Tat" pathway, the ATP-binding cassette (ABC) transport system, and the type IV prepilin-like export operate as "special-purpose" pathways [3]. Moreover, proteins without signal peptides could also be secreted by using nonspecific and/or currently unknown mechanisms [5]. Bacterial exoproteins are involved in many diverse and essential cell functions such as growth, development, defense responses, cell–cell interaction, and intercellular communication [6, 7]. In addition to core exoproteins, pathogenic bacteria secrete others that aid virulence and survival. These, often called virulence factors, are implicated in the colonization and subversion of host cells and thus in cytotoxicity.

*Staphylococcus aureus* is an important human bacterial pathogen that causes community- and hospital-acquired infections [8], food poisoning [9], and toxic shock syndrome (TSS) [10, 11]. The pathogenesis of the majority of *S. aureus*-caused diseases is a multifactorial process involving the coordinated synthesis of cell-wall associated adhesins and a variety of extracellular proteins in order to promote invasion/tissue penetration and cytotoxicity. Nearly all strains of *S. aureus* secrete a group of core exoproteins to facilitate host tissues degrade into nutrients required for bacterial growth. Some strains also produce pyrogenic toxin superantigens (SAgs) whose *in vivo* function is supposed to be the inhibition of the host immune responses [10, 12–14]. The term superantigenicity refers to the ability of such exotoxins to stimulate proliferation of T-lymphocytes regardless the antigen specificity of these cells [15, 16]. In addition to their functional similarities, pyrogenic toxin SAgs also share genetic and biochemical characteristics: they are single-chain, nonglycosylated, proteins with molecular weights (MW) ranging from 20 000 to 35 000 Da characterized by high sequence homology (22–80% identity) [11, 17]. They are moderately stable to chemical inactivation, proteolysis, and denaturation by boiling. Classical staphylococcal SAgs include (i) the five antigenic variants of staphylococcal enterotoxins (SEs) from A to E (*i.e.*, SEA, SEB, SEC, SED, and SEE), (ii) toxic shock syndrome toxin-1 (TSST-1), and (iii) exfoliatins A and B. To date, there are nine known SEs (SEA–H) (Table 1) whose existence have been proved experimentally at the protein level (such as partial to complete Edman sequencing, identification by MS, X-ray, or NMR structure, detection by antibodies, *etc.*). Recent data resulting from genome sequencing of several different *S. aureus* strains have revealed numerous novel SAg genes [18]. Their corresponding putative protein products called "staphylococcal enterotoxin-like" (SEl) SAgs have been proposed [14] (for recent nomenclature see Ref. [12]), and some of them have been produced recombinantly. In addition to the 9 characterized SEs, 11 additional SEl proteins (SElI–SElU) have been predicted by genotyping, but to date no valid data are available concerning the evidence of their expression at protein or transcript level (based on UniProtKB/Swiss-Prot proteindatabase data) (Table 1). SAgs genes are located on heterologous elements like plasmids and bacteriophages, usually referred to as pathogenicity islands, whose horizontal transfer between staphylococcal strains is supposed to play an important role in the evolution of *S. aureus* as pathogen [19]. They usually carry at least two SAgs and are responsible for most SAg-related human diseases. SAg genes, *seg, sei, selm, seln*, and *selo*, for example, are linked together on a pathogenicity island-like DNA element called enterotoxin gene cluster (*egc*, sometimes also referred as *egc1*), and are occasionally associated with TSS [18, 20]. Occurrence of SAg genes encoded by the *egc* cluster and staphylococcal pathogenic islands has been shown to be predominant in numerious *S. aureus* strains isolated from animal hosts [21]. While multiplex enterotoxin genotyping of *S. aureus* strains including novel and putative SEs genes today is feasible [21, 22], identification of their corresponding native protein products using classical immunoassays is hampered by the unavailability of the corresponding antibody. Hence, at present little is known about the expression and significance of secreted enterotoxin and superantigene-like proteins. Because of the possible significance of these enterotoxins for public health and food safety, greater knowledge of their occurrence and an efficient means of screening at the protein level is needed. It is under these circumstances where proteomics can be of value as it is capable of identifying both the expressed enterotoxins and the enterotoxin-like proteins.

In order to define the secretome of pathogenic bacteria and to monitor the expression of virulence factors upon external and/or internal stimuli, proteins isolated from cell culture media can be analyzed and identified by the use of proteomic approaches. Proteomics has been employed to define the secretome of *Bacillus subtilis* [2, 23], *Bacillus anthracis* [6], *Helicobacter pylori* [24], *Aeromonas hydrophylia* [25], and *S. aureus* [26–30] as well. Regarding the secretome of *S. aureus* it is expected that between 5 and 15% of the whole staphylococcal proteome is exported by specific and nonspecific pathways during bacterial lifetime in a growth-phase dependent manner [5, 31]. In a recent review, a comparative approach was applied to define the core and variant *S. aureus* secretomes by combing the results of genomic and proteomic studies [26]. Using genomic data and multiple BLAST searching of six *S. aureus* subspecies, 58 exported core proteins were predicted out of which 33 had been previously identified [30]. The predicted variant secretomes of the six strains includes more than 60 proteins containing most of the staphylococcal exotoxins and immunomodulating factors. In total, 70 and 86 proteins were identified either with known and without any signal peptides, respectively, in the six *S. aureus* strains studied [26].

In this work, we performed an analysis of the secretome of enterotoxigenic *S. aureus* strains never analyzed before with a proteomic approach. The proteins secreted into the culture medium at postexponential and stationary growth were analyzed by gel electrophoresis combined with bottom-up proteomics. 1-DE separation of proteins was followed by nano-HPLC-ESI-MS/MS in information dependent analysis

**Table 1.** Molecular mass, p*I*, and evidence on protein existence of known SEs, predicted enterotoxin-like proteins, and TSS toxin according to UniProtKB

| Abbr. name | Swiss-Prot accession number and entry name | Organism | Chain range | MW (Av.)[a] | p*I*[b] | Evidence[c] |
|---|---|---|---|---|---|---|
| SEA | **P0A0L2** ETXA_STAAW | MW2 | 25–257 | 27 093 | 6.59 | Protein |
| SEB | **P01552** ETXB_STAAU | Rosenbach 1884 | 28–266 | 28 368 | 8.25 | Protein |
| SEC1 | **P01553** ENTC1_STAAU | Rosenbach 1884 | 28–266 | 27 527 | 7.87 | Protein |
| SEC2 | **P34071** ENTC2_STAAU | Rosenbach 1884 | 28–266 | 27 585 | 6.35 | Protein |
| SEC3 | **P0A0L5** ENTC3_STAAU | Rosenbach 1884 | 28–266 | 27 584 | 6.68 | Protein |
| SED | **P20723** ETXD_STAAU | Rosenbach 1884 | 26–258 | 26 942 | 7.17 | Protein |
| SEE | **P12993** ETXE_STAAU | Rosenbach 1884 | 28–257 | 26 425 | 6.59 | Protein |
| SEG | **P0A0L8** ETXG_STAAU | Rosenbach 1884 | 26–258 | 27 041 | 6.02 | Protein |
| SEH | **P0A0M0** ETXH_STAAU | Rosenbach 1884 | 25–241 | 25 143 | 5.18 | Protein |
| SEI | **O85383** ETXH_STAAU | Rosenbach 1884 | 25–242 | 24 925 | 8.37 | Homology |
| SEIJ | **O85217** O85217_STAAU | Rosenbach 1884 | 1–268 | 31 230 | 8.86 | Predicted |
| SEIK | **O54476** O54476_STAAU | Rosenbach 1884 | 1–242 | 27 720 | 7.70 | Predicted |
| SEIL | **Q7A4K9** Q7A4K9_STAAN | N315 | 1–240 | 27 496 | 9.00 | Predicted |
| SEIM | **Q7A4W7** Q7A4W7_STAAN | N315 | 1–239 | 27 371 | 6.45 | Predicted |
| SEIN | **Q7A4X1** Q7A4X1_STAAN | N315 | 1–258 | 29 676 | 8.14 | Predicted |
| SEIO | **Q7A4W6** Q7A4W6_STAAN | N315 | 1–260 | 29 836 | 6.14 | Predicted |
| SEIP | **Q931M4** Q931M4_STAAM | Mu50 | 1–260 | 30 016 | 8.25 | Predicted |
| SEIQ | **Q8NVM3** Q8NVM3_STAAW | MW2 | 1–242 | 28 139 | 8.94 | Predicted |
| SEIR | **Q76LS8** Q76LS8_STAAU | Rosenbach 1884 | 1–259 | 30 018 | 8.89 | Predicted |
| SEIU | **Q6XXM4** Q6XXM4_STAAU | Rosenbach 1884 | 1–261 | 30 564 | 6.66 | Predicted |
| TSST-1 | **P06886** TSST-STAAU | Rosenbach 1884 | 41–234 | 21 945 | 6.95 | Protein |

a) MW (Av.), molecular weights (average).

b) p*I*s have been calculated using Expasy server http://www.expasy.ch/ MW/p*I* calculator. Sequences of mature and unprocessed proteins for SEs and SEIs, respectively, were used for the calculations as it is indicated in the column "Chain range."

c) Evidence: gives indication on the evidence of protein existence based on UniProt Knowledgebase. "Protein" indicates evidence at protein level for the existence of a protein. "Homology" is used to indicate that the existence of a protein is probable because clear orthologs exist in closely related species. The status "Predicted" is used for entries without evidence at protein, transcript, or homology levels.

(IDA), while 2-DE spots were analyzed by means of two different MS-based techniques: MALDI-TOF MS peptide mass fingerprint (PMF) and SELDI-MS/MS peptide sequencing. Enterotoxin production of five *S. aureus* strains including three *egc* positive isolates which potentially express SEG, SEI, SElM, SElN, and SElO was compared with the aim to identify strain-specific protein expression and exotoxins implicated in bacterial virulence.

## 2 Materials and methods

### 2.1 Materials

Tryptone, yeast extract and NaCl used to prepare Luria-Bertani (LB) broth were obtained from Oxoid (Hampshire, UK). Urea, CHAPS, DTT, iodoacetamide (IAA), ammonium bicarbonate ($NH_4HCO_3$) protease inhibitor cocktail, CHCA, angiotensin I (MW 1296.68), renin substrate (MW 1758.93), and adrenocorticotropic hormone fragment 18–39 (MW 2465.19) were purchased from Sigma–Aldrich Chemie (Stenheim, Germany). Immobiline DryStrip (IPGstrip), IPG buffer, DryStrip cover fluid, protein molecular weight markers for SDS-PAGE analysis, and agarose for IEF were obtained from Amersham Biosciences (Uppsala, Sweden). 30% Acrylamide/bis solution (37.5:1, 2.6%C), and CBB G-250 were purchased from BioRad (Hercules, CA, USA). Formic acid, TFA, Tris, SDS, glycine, bromophenol blue, ammonium persulfate, and TEMED were obtained from ICN (Aurora, Ohio). Glycerol, ACN, acetone, and TCA were purchased from Carlo Erba (Milano, Italy). MS sequence grade trypsin was from Promega (Madison, WI, USA). All other solvents were HPLC-grade and purchased from Merck (Whitehouse Station, NJ, USA).

### 2.2 Strains, culture conditions, and exoprotein extraction

Origin, source, and SEs genotype of *S. aureus* strains used in this study are reported in Table 2. Bacteria were cultured under gentle shaking (150 rpm) at 37°C in 50 mL LB media. Growth was monitored every hour by measuring OD (at 600 nm wavelength) and by counting colonies on plates. Exoprotein fractions were isolated and purified in the late-

**Table 2.** Source, origin, and genotype of *S. aureus* strains used for proteomic analysis. Protein concentrations measured by Bradford assay on the protein samples precipitated from the culture media in the stationary growth phase (for ATCC 14458 strain protein concentration at 7 h also reported)

| Sample ID | Source[a)] | Origin[b)] | Genotype[c)] | Method[d)] | References | $C_{prot}$ (μg/mL)[e)] |
|---|---|---|---|---|---|---|
| ATCC 14458 | ATCC | Feces of child with acute diarrhea | *seb*$^+$, *seg*$^+$, *selk*$^+$ | Oligo. microarray | [22] | 68 (24 h)<br>16 (7 h) |
| A900322 | DSAN | Clinical strain from a patient with TSS | (*seg*$^+$, *sei*$^+$, *selm*$^+$, *seln*$^+$, *selo*$^+$)‡, *selp*$^+$ | RT-PCR, oligo. microarray, PCR | [18, 22, 41] | 58 |
| RIMD 31092 | DSAN | Clinical MRSA strain | *seb*$^+$, *sec*$^+$, (*seg*$^+$, *sei*$^+$, *selm*$^+$, *seln*$^+$, *selo*$^+$)‡, *tsst-1*$^+$ | RT-PCR, PCR | [18, 41] | 14 |
| AB-8802 | DSAN | Raw poultry meat | (*seg*$^-$, *sei*$^+$, *selm*$^+$, *seln*$^+$, *selo*$^+$) *seg*$_v^+$, *sei*$_v^+$ | PCR | [41] | 61 |
| ROS | ISA | From a patient | Nonenterotoxigenic control strain | | – | 10 |

a) ATCC, American Type Culture Collection, Rockville, MD; DSAN, Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli Federico II, Portici, Italy; ISA, Istituto di Scienza dell'Alimentazione, CNR, Avellino, Italy.
b) MRSA, methicillin-resistant *S. aureus*, TSS, toxic shock syndrome.
c) ‡, complete *egc* cluster; *tsst-1*, toxic shock syndrome toxin-1 gene; *v*, variant.
d) Method applied for gene detection; oligo. microarray, oligonucleotide microarray.
e) $C_{prot}$, exoprotein concentration in culture supernatant measured at 24 h unless otherwise is stated.

exponential (7 h, OD: $3 \times 10^8$ cfu/mL) and the stationary phases of growth (24 h, OD: $5.6 \times 10^9$ cfu/mL). Cells were removed by centrifugation (12 500 × *g*, 15 min, 4°C) and supernatant was filtrated by passing through a 0.45-μm pore size filter (MILLEX-GS). Proteins were precipitated by adding three volume of acetone at −20°C for 2 h followed by centrifugation (10 000 × *g*, 5 min, 4°C). A second precipitation step was performed by using 10% w/v TCA on ice for 2 h. Suspension was centrifuged (5000 × *g*, 15 min, 4°C) and pellet was washed by ice-cold acetone. After centrifugation (5000 × *g*, 15 min, 4°C) pellet was resuspended in 1 mL of 20 mM Tris-HCl (pH 8) and protein concentration was measured using the Bradford protein assay.

### 2.3 1-DE

Sample (30 μg) was solved in 20 μL buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.2 M DTT, 0.02% bromophenol blue), boiled at 100°C for 6 min and loaded on 12–22% polyacrylamide gradient gel (16 cm × 20 cm) according to the method of Laemmli [32]. Electrophoresis was carried out using the Tris–glycine–SDS buffer system (25 mM Tris, 198 mM glycine, and 0.1% SDS) on a Protean II xi cell apparatus (BioRad) at 20 mA/gel until the dye front reached the bottom edge of the gel. Low molecular weight standards were used in the gel. Gel was fixed and stained with CBB G-250. Selected gel bands were cut manually and in-gel digestion was performed according to Shevchenko *et al.* [33] using 30 μL of 10 mM DTT in 100 mM $NH_4HCO_3$ for reduction, 30 μL of 55 mM IAA in 100 mM $NH_4HCO_3$ for alkylation, and 30 μL trypsin at 6 ng/ μL concentration in 25 mM $NH_4HCO_3$ for enzymatic digestion. Samples (1 μL) were analyzed by nanoflow-HPLC-ESI-MS/MS.

### 2.4 2-DE

2-DE was performed as described by O'Farrell [34]. The equipment was purchased from Amersham Biosciences. First-dimensional IEF was performed using the Ettan IPGphor, while the second dimensional SDS-PAGE was carried out using the Ettan DALT twelve System. Gels were poured using the Gel Caster system. Sample (350 μg) was solved in 350 μL rehydration solution (8 M urea, 2% CHAPS, 18 mM DTT, 0.5% v/v. IPG buffer pH 3–10 and 0.002% bromophenol blue) and applied by in-gel rehydration (according to the manufacturer's instructions) in IPGstrip 18 cm, pH 3–10 for 12 h; then proteins were focused up to 60 000 V·h at a maximum voltage of 8000 V. After IEF, proteins were reduced and alkylated by soaking the IPGStrips in the equilibration solution (6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl, pH 8.8) containing 130 mM DTT for 10 min at room temperature and then in equilibration solution containing 135 mM IAA for 5 min. The equilibrated IPGStrips were sealed on top of the SDS-PAGE gel (21 cm × 25 cm, 12% polyacrylamide), using 0.5% agarose. The second dimensional SDS-PAGE was carried out using the Tris–glycine–SDS buffer system (25 mM Tris, 198 mM glycine, and 0.1% SDS) at 5 W/gel for 1 h and then at 15 W/gel until the dye front reached the bottom edge of the gel. Protein spots were visualized by staining with CBB G-250. Three analytical replicates were analyzed *per* each sample.

### 2.5 Image analysis and spot processing

The 2-D-PAGE protein patterns were recorded as digitalized images using a high-resolution scanner (GS-710 Calibrated Imaging Densitometer, BioRad). Spot detection, quantiza-

tion, and analysis were performed using the PDQuest™ 2-D Analysis Software, Version 6.2 by BioRad. To correct for differences in sample loading or staining intensity among gels "total quantity in valid spot" normalization method was used. The triplicate gels of the same sample were grouped together with "replicate groups" function that allows determining the average quantities of their protein spots.

Spots of interest were processed using the Proteome Workstation (Waters, UK). Spots were excised and placed in a 96-well plate using a Spot Cutter (BioRad). MassPREP (Waters) liquid handling system was used for enzymatic digestion, peptide extraction and sample loading. Briefly, the method involves the following main steps: reduction with 10 mM DTT in 100 mM $NH_4HCO_3$; alkylation with 55 mM IAA in 100 mM $NH_4HCO_3$; overnight enzymatic digestion using trypsin at 6 ng/μL concentration; peptide extraction with 1% formic acid in 2% ACN. One microliter of the tryptic digests mixed with 1 μL solution of CHCA (2 mg/mL in 50% ACN, 0.1% v/v TFA) was loaded onto the MALDI target plate.

### 2.6 Protein identification

#### 2.6.1 Nanoflow-HPLC-ESI-MS/MS analysis

Peptide mixtures from 1-D gel bands tryptic digestion were analyzed by using nanoflow-HPLC-ESI-MS/MS. Separation was performed using an Integral 100Q HPLC system coupled on-line to a hybrid, quadrupole-TOF (QqTOF) mass spectrometer, QStar Pulsar (Applied Biosystems, Foster City, CA). Flow rate was split from 400 μL/min to 200 nL/min using a flow splitter, Accurate (LC Packings, Sunnyvale, CA). Sample was loaded, purified, and concentrated on a precolumn, PepMap C18, 5 mm length, 300 Å (LC Packings) using an external HPLC pump, Pheonix (Fisons-Instruments, Manchester, UK) at 20 μL/min flow rate. Peptides were separated using PepMap C18 capillary column, 15 cm length, 75 μm ID, 300 Å (LC Packings). Eluents used were: (A) 5% ACN in 0.08% HCOOH and 0.01% TFA and (B) 95% ACN in 0.08% HCOOH and 0.01% TFA. Gradient: 5–50% B in 90 min, 50–100% B in 5 min. Nanoflow-ESI-MS/MS experiments were performed in IDA mode. Precursor ions were selected using the following MS to MS/MS switch criteria: ions greater than *m/z* 300.0, charge state 2–4, intensity exceeds 15 counts, ion tolerance 50.0 mmu, former target ions were excluded for 60 s. CID was performed using nitrogen as collision gas. Tandem mass spectra were extracted; charge state deconvoluted and deisotoped using ABI Analyst version 1.1 software. All MS/MS samples were analyzed using X! Tandem (www.thegpm.org; version 2007.04.01.1). X! Tandem was set up to search the NC_002952.faa.pro database (23 865 entries) assuming the digestion enzyme trypsin. X! Tandem was searched with a fragment ion mass tolerance of 0.15 Da. Iodoacetamide derivative of cysteine was specified in X! Tandem as a fixed modification. Deamidation of asparagine and glutamine, oxidation of methionine and tryptophan, sulfone of methionine, tryptophan oxidation to formylkynurenin of tryptophan and acetylation of lysine and the N-terminus were specified in X! Tandem as variable modifications.

Criteria for protein identification: Scaffold (version Scaffold-01_06_18, Proteome Software, Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm [35]. Protein identifications were accepted if they could be established at greater than 90.0% probability and contained at least one identified peptides. In case only a single peptide identified for a protein MS/MS spectrum was manually validated and were accepted only in the case if they showed at least three consecutive *b*- or *y*-type fragment ions. PSORTb version 2.0.4 was used to predict subcellular protein localization http://www.psort.org/psortb [31]. Not signal peptide triggered protein secretion was predicted by the use of SecretomeP 2.0 (http://www.cbs.dtu.dk/services/SecretomeP-2.0/) [5].

#### 2.6.2 MALDI-TOF MS analysis

M@LDI reflectron TOFMS (Waters, USA) was used for analysis. External calibration was performed in the range of *m/z* 800–3500 using a mixture of three peptide standards (Angiotensin I, Renin Substrate, and ACTH) of known molecular masses. Internal calibration was performed using ACTH. MassLynx 4.0 software (Waters) was used for data processing and for peak list generation. Peak list containing the 40 most intense peaks of the spectrum was sent to MASCOT program (www.matrixscience.com) for PMF search against database NCBInr 20070623 (5 124 231 sequences; 1 772 943 954 residues). The following parameters were used for database search: type of search: PMF; enzyme: trypsin; fixed modifications: carbamidomethyl (C); variable modifications: oxidation (M); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ±150 ppm; and peptide charge state: 1+.

#### 2.6.3 SELDI-MS and MS/MS analysis

One microliter of sample was spotted on normal phase ProteinChip, NP20 (Ciphergen, Freemont, CA), dried and washed with ice-cold TFA 0.1% v/v. One microliter matrix solution, CHCA, 2 mg/mL in 50% ACN, 0.1% v/v TFA was added, dried and analyzed by QStar-Pulsar mass spectrometer (Applied Biosystems) equipped with a SELDI (Ciphergen) ion source and controlled by PS1000 interface. Nitrogen laser was used for ionization (VSL-337 ND-S, Laser Science, Franklin, MA, USA). Spectra were acquired in positive ion mode, in the range *m/z* 300–2500. The instrument was calibrated using known fragment ions of ACTH. CID was performed using argon as collision gas. Peak lists were generated by the Analyst QS program (version 1.1) which was used for database search in the MASCOT program both for PMF and MS/MS ion search using the same parameters as above.

## 3 Results and discussion

### 3.1 1- and 2-DE analysis of extracellular protein fraction of ATCC 14458

Extracellular protein fraction obtained from *S. aureus* ATCC 14458, a prototype strain for SEB [36], at 24 h of stationary growth phase ($OD_{600}$: $5.6 \times 10^9$ cfu/mL) was separated by 1-D SDS-PAGE followed by nanoflow-HPLC-ESI-MS/MS analysis of the in-gel tryptic digests. In the 20 gel bands (Fig. 1) selected for this analysis, 74 unique proteins were identified (Table 3 and Supporting Information Table). SEB was mainly found in the most heavily stained band (band 8 in Fig. 1 and Table 3) indicating its high extracellular concentration (note: SEB was also identified in a number of other bands due to its comigration with other proteins). To predict extracellular location and nonclassical (*i.e.*, not signal peptide triggered) protein secretion of the identified proteins we used Psortb2 and SecretomeP software, respectively. Based on Psortb2 analysis, 35 proteins (47%) have a cytoplasmic origin, 13 are extracellular, three originate in the cell wall, one in cytoplasmic membrane, and 22 have unknown localization (Table 3). Sixteen of the unknown and four cytoplasmic proteins led to a SecretomeP score higher than 0.5 indicating that these may well follow signal peptide independent secretion pathway(s). Beside SEB (band 8), other proteins with high PSortb2 extracellular scores were also identified (Table 3, Supporting Information Table) in this strain: triacylglycerol lipase (band 1), alpha-hemolysin (band 6), gamma-hemolysin component B (band 5), and component C (band 6), chain II (band 7), three serine proteases (bands 5, 9, and 15), one cysteine protease (staphopain) (band 11), autolysin (band 2), thermonuclease (band 12), and *N*-acetylmuramoyl-L-alanine amidase (band 1).

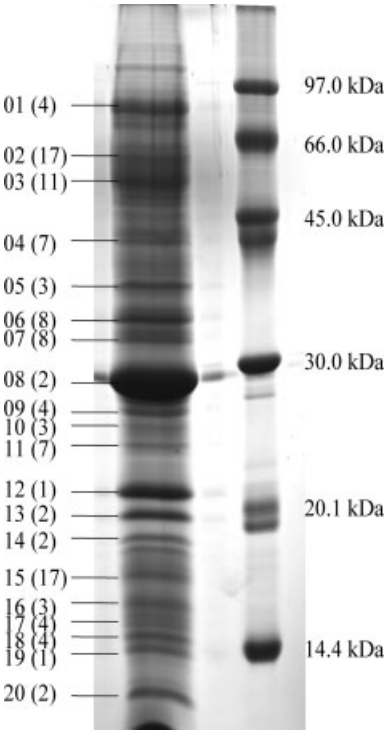



**Figure 1.** SDS gel electrophoresis of extracellular protein fraction of *S. aureus* ATCC 14458. Low molecular weight markers are indicated on the right. Bands 1–20 were analyzed by nanoflow-HPLC-ESI-MS/MS in IDA mode. Number of proteins identified in each gel bands is reported in brackets. Identified proteins are reported in Table 3 and in Supporting Information Table.

Analysis of the ATCC 14458 strain by 2-DE and MS (Fig. 2a) confirmed 19 proteins already identified by 1-DE proteomics, and resulted in 45 newly identified ones (Table 4). By this approach 22 exoproteins with high Psortb2 and SecretomeP scores including 13 exoproteins previously not identified in the 1-DE experiment could be identified. Triacylglycerol lipase (spots 11 and 12) and SEB (spots 64–67) were found highly expressed in characteristic train spots (Fig. 2a). Analysis of spots 62 and 63 (Figs. 2a,b and 3a,b) yielded the identification of two enterotoxin-like proteins, SElK and SElQ, respectively. To the best of our knowledge this is the first experimental evidence on the expression of native SElK and SElQ in a *S. aureus* strain. Genes *selk*, *sell*, and *selq* are often present on the same pathogenicity islands, transposons, and bacteriophages, sometimes in association with the gene for TSST-1. Superantigenic activity of SElK has already been demonstrated by the use of recombinant protein [37], while SElL and SElQ are enterotoxin-like proteins predicted by genotyping. In a recent study by Sergeev *et al.* [22], using a microarray-based genotyping assay capable of detecting 16 enterotoxin genes simultaneously, ATCC 14458 *seb* reference strain was found positive also for *selk* and *selq* genes. Our results thus confirm the production of the above enterotoxins at the protein level.

Combining the results obtained by the two bottom-up proteomics methods, here we identified 119 unique proteins in ATCC 14458 strain from which 46 (38%) have been predicted to be extracellular. Expression of *S. aureus* virulence factors is known to be regulated by a number of regulatory systems, amongst which accessory gene regulator (*agr*) locus, staphylococcal accessory regulator (*Sar*A), *S. aureus* exoprotein expression (*sae*), *Cod*Y a global virulence and stationary phase gene expression regulator, and SigmaB are the most studied. Recently a novel global regulator, *Mgr*A which regulates virulence factors in a fashion analogous to that of the *agr* (*i.e.*, exoproteins are up-regulated and surface proteins are down-regulated) has also been described at the transcriptional level [38]. *Mgr*A has been shown to affect autolysis and antibiotic resistance, and also the production of several virulence factors including capsules, protein A, and alpha-toxin. In our study we identified protein products of three global virulence regulators, *i.e.*, SarA, (band 16–17), MgrA (band 15), and CodY (band 8 in Fig. 1 and spot 61 in Fig. 2a) in ATCC 14458. To the best of our knowledge this is the first time that MgrA has been identified at protein level in a *S. aureus* strain.

**Table 3.** Proteins identified from the extracellular protein fraction of ATTC 14458 strain separated by 1-D gel electrophoresis (Fig. 1)

| Protein name[a)] | Acc. No.[b)] | MW[c)] | Extracell. score[d)] | SecP score[e)] | Gel band[f)] |
|---|---|---|---|---|---|
| SEB (COL) | gi\|57651597 | 31 435.9 | 10 | 0.937 | 2–4, 7, **8**, 9–11, 13, 15–18, 20 |
| Triacylglycerol lipase precursor (MW2) | gi\|21284319 | 76 806.7 | 9.73 | 0.943 | **1**–4, 7 |
| Gamma-hemolysin component B (N315) | gi\|15927999 | 36 711.1 | 9.73 | 0.900 | 5 |
| Thermonuclease precursor (COL) | gi\|57650135 | 25 119.9 | 10 | 0.970 | **12**, 15 |
| Immunodominant antigen B (Mu50) | gi\|15925628 | 19 370.2 | 3.33 | 0.977 | **15** |
| Enolase (Mu50) | gi\|15923766 | 47 116.9 | 0.01 | 0.088 | 2, **3**, 6–7 |
| Surface protein, putative (COL) | gi\|57650793 | 15 447.5 | 3.33 | 0.803 | 16, 17, **18** |
| Alpha-hemolysin precursor (Mu50) | gi\|15924153 | 35 975.3 | 10 | 0.899 | **6**, 13 |
| Catalase (Mu50) | gi\|15924324 | 58 612.2 | 0.01 | 0.555 | 2 |
| G-binding protein precursor (MW2) | gi\|21281813 | 53 774.3 | 0.01 | 0.939 | **2**, 3 |
| Phosphoenolpyruvate carboxykinase (Mu50) | gi\|15924781 | 59 377.4 | 2.5 | 0.725 | 2 |
| Autolysin (MW2) | gi\|21282665 | 137 407.2 | 9.98 | 0.908 | **2**, 4–5, 7, 9 |
| Gamma-hemolysin component C (Mu50) | gi\|15925410 | 35 583.8 | 9.73 | 0.904 | **6**, 15 |
| Inositol-monophosphate (Mu50) | gi\|15923380 | 52 850.6 | 0.73 | 0.067 | 3 |
| Ornithine-oxo-acid aminotransferase (Mu50) | gi\|15923947 | 43 417.5 | 0.73 | 0.105 | 4 |
| Glyceraldehyde-3-phosphate dehydrogenase 1 (Mu50) | gi\|15923762 | 36 280.9 | 0 | 0.101 | 4 |
| Glutamine-ammonia ligase (Mu50) | gi\|15924300 | 50 854.7 | 0 | 0.159 | 3 |
| Hypothetical protein SAV1854 (Mu50) | gi\|15924844 | 22 343.5 | 0.73 | 0.092 | 11 |
| Bifunctional GMP synthase/glutamine amidotransferase protein (Mu50) | gi\|15923381 | 58 202.4 | 0.73 | 0.069 | 2 |
| Formyltetrahydrofolate synthetase (Mu50) | gi\|15924722 | 59 871.6 | 0.73 | 0.069 | 2 |
| Succinyl-CoA synthetase subunit beta (Mu50) | gi\|15924235 | 42 056.3 | 0.73 | 0.044 | 4 |
| DNA-directed RNA polymerase alpha subunit (Mu50) | gi\|15925214 | 35 011.7 | 0.01 | 0.067 | 4 |
| Glycerophosphoryl diester phosphodiesterase (Mu50) | gi\|15923949 | 35 310.8 | 4.86 | 0.891 | 6, **7** |
| Similar to outer membrane protein precursor (Mu50) | gi\|15923297 | 33 351.9 | 3.33 | 0.876 | 7 |
| Hypothetical protein SAV0708 (Mu50) | gi\|15923698 | 16 045.2 | 3.33 | 0.883 | 15 |
| Aerobic glycerol-3-phosphate dehydrogenase (Mu50) | gi\|15924292 | 64 463.4 | 0.73 | 0.481 | 2 |
| Serine protease (Mu50) | gi\|15924802 | 26 140.5 | 9.73 | 0.856 | **15**, 18 |
| Elongation factor Tu (Mu50) | gi\|15923538 | 43 103.6 | 0.01 | 0.071 | 3, **15** |
| Hypothetical protein SAV0372 (Mu50) | gi\|15923362 | 21 273.7 | 3.33 | 0.880 | **10**, 11 |
| Map protein, programmed frameshift (COL) | gi\|57650691 | 77 059.5 | 3.33 | 0.821 | 2 |
| Formate acetyltransferase (Mu50) | gi\|15923216 | 84 861.9 | 0.01 | 0.144 | 1 |
| Xaa-His dipeptidase homolog (Mu50) | gi\|15924741 | 52 824.2 | 0.73 | 0.216 | 3 |
| Hypothetical protein SAV0613 (Mu50) | gi\|15923603 | 18 594.2 | 3.33 | 0.857 | 14 |
| Probable transmembrane sulfatase (RF122) | gi\|82750421 | 74 399.2 | 0.32 | 0.957 | 3 |
| Alkyl hydroperoxide reductase subunit C (Mu50) | gi\|15923371 | 20 976.6 | 0.01 | 0.943 | 11 |
| Serine protease (Mu50) | gi\|15924038 | 36 977.3 | 9.98 | 0.943 | 5 |
| Serine protease (Mu50) | gi\|15924799 | 25 641.1 | 9.73 | 0.973 | 9 |
| Transcriptional regulator (Mu50) | gi\|15923676 | 17 089.5 | 2.5 | 0.742 | 15 |
| ATP-dependent Clp protease proteolytic subunit homolog (Mu50) | gi\|15923758 | 21 513.6 | 0.17 | 0.059 | 11 |
| D-Alanine aminotransferase (Mu50) | gi\|54040804 | 31 907.9 | 0.73 | 0.206 | 6 |
| Lysyl-tRNA synthetase (Mu50) | gi\|15923507 | 56 719.2 | 0 | 0.102 | 2 |
| Staphylococcal accessory regulator A (Mu50) | gi\|15923606 | 14 718.0 | 2.5 | 0.211 | **16**, 17 |
| Hypothetical protein ebhA (Mu50) | gi\|15924424 | 72 2317.8 | 0.62 | n.a. | **9**, 10 |
| Glycyl-tRNA synthetase (Mu50) | gi\|15924555 | 53 620.3 | 0 | 0.330 | 3 |
| Similar to universal stress protein family (Mu50) | gi\|15924700 | 18 474.9 | 0.73 | 0.110 | 11 |
| Glucose-6-phosphate 1-dehydrogenase (Mu50) | gi\|15924495 | 56 965.2 | 0.73 | 0.396 | 2 |
| Glycerol kinase (Mu50) | gi\|15924291 | 55 612.6 | 2.5 | 0.173 | 3 |
| Fructose-bisphosphate aldolase (Mu50) | gi\|14247898 | 30 836.1 | 0.73 | 0.097 | 6 |
| Hit-like protein involved in cell-cycle regulation (Mu50) | gi\|15924828 | 15 944.9 | 0.73 | 0.203 | 15 |
| 50S ribosomal protein L17 (Mu50) | gi\|15925213 | 13 747.7 | 0.73 | 0.050 | 15 |
| Staphopain (Mu50) | gi\|15924899 | 44 204.2 | 9.73 | 0.887 | 11 |
| Glycine dehydrogenase subunit 2 (Mu50) | gi\|15924525 | 54 783.1 | 0.73 | 0.283 | 2 |
| 50S ribosomal protein L22 (Mu50) | gi\|15925235 | 12 834.9 | 0.73 | 0.044 | 15 |

**Table 3.** Continued

| Protein name[a)] | Acc. No.[b)] | MW[c)] | Extracell. score[d)] | SecP score[e)] | Gel band[f)] |
|---|---|---|---|---|---|
| Transcriptional repressor CodY (Mu50) | gi\|15924245 | 28 755.1 | 0.73 | 0.037 | 8 |
| Chaperone protein HchA (Mu50) | gi\|15923541 | 32 176.6 | 2.5 | 0.698 | 6 |
| Hypothetical protein SAV1338 (Mu50) | gi\|15924328 | 39 523.7 | 2.5 | 0.931 | 6 |
| Putative indole-3-pyruvate decarboxylase (Mu50) | gi\|15923178 | 60 527.4 | 2.5 | 0.235 | 2 |
| Asparaginyl-tRNA synthetase (Mu50) | gi\|15924444 | 49 127.5 | 0 | 0.168 | 2 |
| 1-Pyrroline-5-carboxylate dehydrogenase (Mu50) | gi\|15925544 | 56 867.5 | 0.01 | 0.106 | 2 |
| Hypothetical protein SA0022 (N315) | gi\|15925728 | 83 498.4 | 0 | 0.903 | 2 |
| Hypothetical protein SAV0387 (Mu50) | gi\|15923377 | 15 135.9 | 0.73 | 0.758 | 15 |
| Hypothetical protein SAV1626 (Mu50) | gi\|15924616 | 15 643.0 | 0.73 | 0.059 | 15 |
| ATP synthase B chain (Mu50) | gi\|15925097 | 19 539.3 | 0.73 | 0.517 | 15 |
| 50S ribosomal protein L13 (Mu50) | gi\|15925208 | 16 333.4 | 2.5 | 0.723 | 15 |
| Hypothetical protein SA1755 (N315) | gi\|15927517 | 17 072.6 | 3.33 | 0.856 | 15 |
| Hypothetical protein SAV1176 (Mu50) | gi\|15924166 | 17 271.7 | 2.5 | 0.107 | 15 |
| Dihydrolipoamide S-acetyltransferase comp. of pyruvate dehyd. Complex E2 (Mu50) | gi\|15924085 | 46 368.3 | 0.01 | 0.090 | 1 |
| *N*-Acetylmuramoyl-L-alanine amidase (Mu50) | gi\|15925634 | 69 227.3 | 9.73 | 0.938 | 1 |
| Naphthoate synthase (Mu50) | gi\|15924035 | 30 411.6 | 0.73 | 0.092 | 7 |
| Gamma-hemolysin chain II precursor (Mu50) | gi\|15925409 | 34 955.7 | 9.73 | 0.876 | 7 |
| DNA-binding protein II (Mu50) | gi\|15924463 | 9626.0 | 2.5 | 0.080 | 20 |
| Putative exported protein S (MSSA476) | gi\|49244200 | 15 838.2 | 2.5 | 0.793 | 19 |
| Aconitate hydratase (Mu50) | gi\|15924340 | 98 969.6 | 0.01 | 0.499 | 14 |
| Hypothetical protein SAV0981 (Mu50) | gi\|15923971 | 15 898.3 | 0.33 | 0.796 | **17**, 19 |

Protein names according to NCBI database containing the nine sequenced *S. aureus* strains.

a) *S. aureus* strains with known genome used for protein identification is indicated after the name of the protein in brackets.

b) Accession number of the identified protein in NCBInr sequence database used for protein identification.

c) Calculated protein average molecular mass including signaling peptide (Da).

d) Extracellular score calculated by software PSORTb2 (http://www.psort.org/psortb).

e) SecP score was used to predict nonclassical protein secretion (http://www.cbs.dtu.dk/services/SecretomeP-2.0/). Proteins predicted to be nonclassically secreted has a score exceeding the normal threshold of 0.5. n.a. is for not applied.

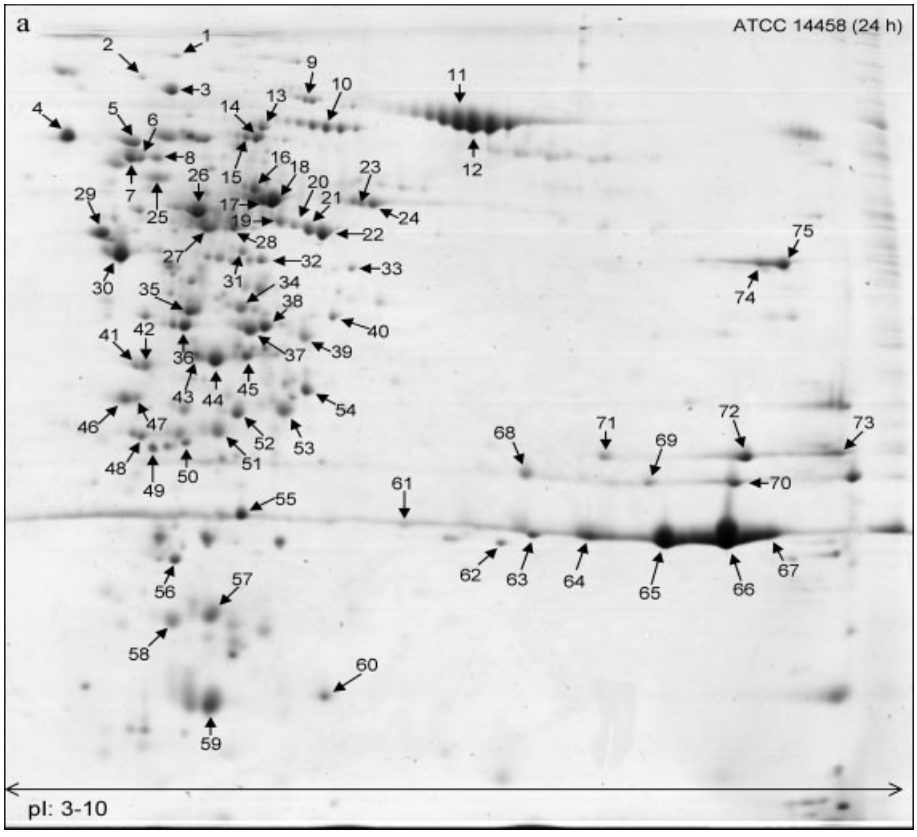
f) Gel band number as it is indicated in Fig. 1. If a protein was identified in more than one gel band, gel band number corresponding to the highest confidence identification is in bold underlined.

The conventional 2-DE approach was used to compare exoprotein expression of ATCC 14458 strain at two different time points of growth, *i.e.*, at 7 h in the late-exponential ($3 \times 10^8$ cfu/mL) and at 24 h in the stationary ($5.6 \times 10^9$ cfu/mL) growth phase. Total protein concentration increases five-fold during this time interval in the culture broth (Table 2), and expression pattern becomes significantly more complex (Figs. 2a and b). This could be explained by the accumulation of exoproteins over time and by the increased cellular autolysis leading to the release of cytosolic proteins into the extracellular medium. Accordingly, approximately 170 and 530 spots could be visualized in the 2-DE map at 7 h and at 24 h, respectively. Proteins (Table 4) expressed in both samples are indicated in Fig. 2b. Importantly, the presence of the three strain-specific enterotoxins, *i.e.*, SEB, SElK, and SElQ, could already be confirmed at 7 h together with a number of other proteins (prevalently virulence factors) in the zone of p*I* 5–9.5 and MW 20–50 kDa (Figs. 3a and b). In fact, this region seems to be qualitatively similar at the two time points studied and, therefore it was used to compare the different *S. aureus* strains.

### 3.2 Comparative 2-DE analysis of the enterotoxin region of different *S. aureus* strains

The *egc* locus of *S. aureus* harbors two enterotoxin genes (*seg* and *sei*) and three enterotoxin-like genes (*selm*, *seln*, and *selo*) [39]. Between the *sei* and *seln* genes there are ψ*ent1*/ψ*ent2* pseudogenes. Recently, a new enterotoxin-like genes, *selu* or *selu*$_v$, between *sei* and *seln* genes of the *egc* operon (*egc2*), was also identified to result from sequence divergence in the region of the pseudogenes ψ*ent1*/ψ*ent2*. *egc* encodes the expression of SEs SEG and SEI, and SEl proteins SElK, SElL, and SElM (*egc2* also for SElU and SElU$_v$) [18, 20, 39]. In our work, we compared the 2-DE exoprotein maps of three *egc* positive *S. aureus* isolates (*i.e.*, RIMD 31092,



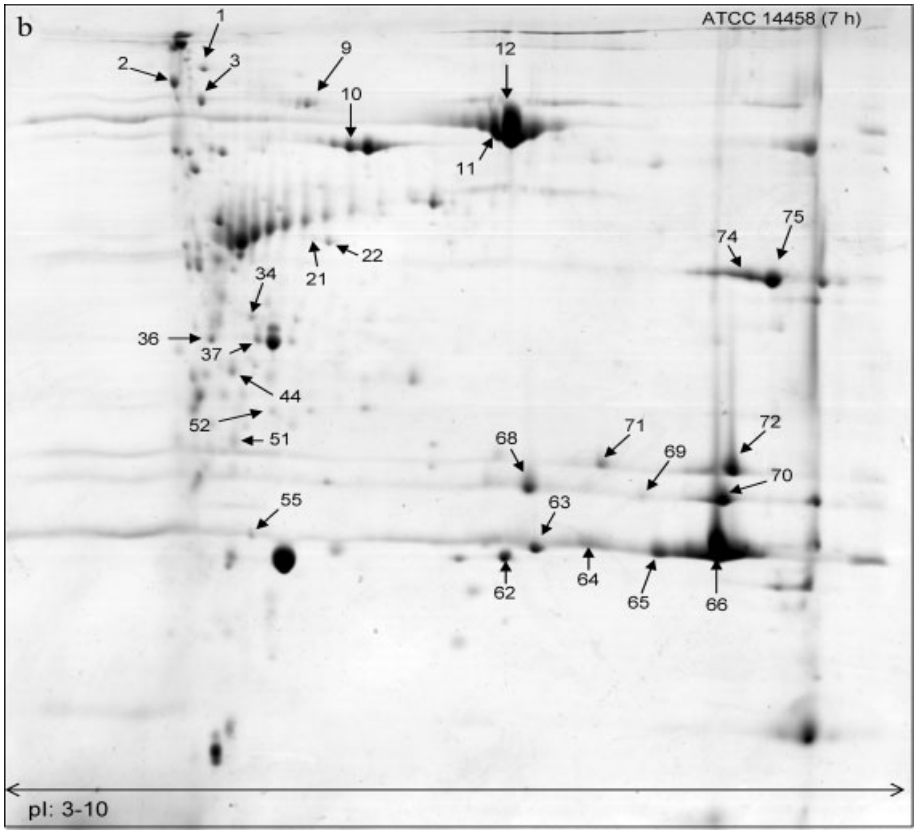




**Figure 2.** 2-DE images of the extracellular protein fractions of *S. aureus* ATCC 14458 strain (a) at 24 h in stationary growth, and (b) at 7 h in the late-exponential phase. Numbers refer to proteins identified according to Table 4.

**Table 4.** Proteins separated by 2-DE and identified by different MS-based methods (nanoflow-HPLC-ESI-MS/MS, MALDI-TOF MS, and SELDI-MS/MS) from the extracellular protein fraction from strains ATTC 14458 (7 and 24 h), ROS1, RIMD 31092, A900322, and AB-8802

| Spot | Acc. no. | Protein name | IP | MW | Score | Tech-nique | Seq. Cov./ Pep. Seq. | Extracell. score | SecP score |
|---|---|---|---|---|---|---|---|---|---|
| **ATCC 14458** | | | | | | | | | |
| 1 | gi\|15924340 | Aconitate hydratase (Mu50) | 4.83 | 98 969 | 129 | PMF | 24% | 0.01 | 0.499 |
| 2 | gi\|81775026 | Serine-aspartate repeat-containing protein E precursor (Mu50) | 4.24 | 124 038 | 138 | PMF | 26% | 0.00 | 0.950 |
| 3 | gi\|15923537 | Translational elongation factor G (Mu50) | 4.80 | 76 612 | 82 | PMF | 35% | 0.01 | 0.090 |
| 4, 78[a)] | gi\|15924665 | Trigger factor (Mu50) | 4.34 | 48 609 | 125 | PMF | 30% | 0.73 | 0.130 |
| 5 | gi\|1169381 | Chaperone protein dnaK (Heat shock protein 70) (STAAU) | 4.63 | 66 347 | 115 | PMF | 30% | 0.01 | 0.688 |
| 6,7 | gi\|15924074 | Phosphoenolpyruvate-protein phosphatase (Mu50) | 4.62 | 63 206 | 53 | PMF | 19% | 0.01 | 0.054 |
| 8 | gi\|15923765 | Phosphoglyceromutase (Mu50) | 4.74 | 56 454 | 42 | PMF | 17% | 0.01 | 0.156 |
| 9,10 | gi\|15923515 | Endopeptidase (Mu50) | 5.51 | 91 037 | 72 | PMF | 21% | 0.01 | 0.066 |
| 11,12 | gi\|15925661 | Triacylglycerol lipasi precursor (Mu50) | 6.58 | 76 662 | 138 | PMF | 29% | 9.73 | 0.943 |
| 13 | gi\|21283355 | Threonyl-tRNA synthetase1 (MW2) | 5.23 | 74 460 | 84 | PMF | 23% | 0.00 | 0.173 |
| 14 | gi\|21284285 | Arginine deiminase (MW2) | 5.09 | 46 914 | 46 | PMF | 32% | 0.73 | 0.103 |
| 15 | gi\|15924687 | Pyruvate kinase (Mu50) | 5.23 | 63 102 | 81 | PMF | 27% | 0.73 | 0.048 |
| 16 | gi\|15925321 | Urocanate hydratase (Mu50) | 5.23 | 60 663 | 48 | PMF | 15% | 0.73 | 0.079 |
| 17–18 | gi\|15924324 | Catalase (Mu50) | 5.27 | 58 612 | 80 | PMF | 29% | 0.01 | 0.563 |
| 19 | gi\|15923518 | Glutamyl-tRNA Synthetase (Mu50) | 5.21 | 56 288 | 53 | PMF | 24% | 0.17 | 0.226 |
| 20–22 | gi\|15923380 | Inositol monophosphate dehydrogenase (Mu50) | 5.61 | 52 850 | 160 | PMF | 43% | 0.73 | 0.067 |
| 23 | gi\|49242418 | Putative RNA binding protein (MRSA252) | 6.16 | 80 904 | 119 | PMF | 29% | 0.73 | 0.217 |
| 24 | gi\|15924722 | Formytetrahydrofolate synthetase (Mu50) | 5.69 | 59 872 | 60 | PMF | 34% | 0.01 | 0.072 |
| 25 | gi\|14247183 | Dihydrolipoamide succinyltransferase (Mu50) | 4.86 | 46 683 | 43 | PMF | 19% | 0.17 | 0.597 |
| 26 | gi\|15924086 | Dihydrolipoamide dehydrogenase (Mu50) | 4.95 | 49 451 | 81 | PMF | 27% | 0.17 | 0.055 |
| 27 | gi\|15924555 | Glycyl-tRNA synthetase (Mu50) | 4.99 | 53 620 | 69 | PMF | 26% | 0.00 | 0.330 |
| 28 | gi\|21282921 | Glutamine-ammonia ligase (MW2) | 5.08 | 50 841 | 129 | PMF | 25% | 0.01 | 0.159 |
| 29 | gi\|21283094 | 30S ribosomial protein S1 (MW2) | 4.51 | 43 287 | 108 | PMF | 28% | 0.01 | 0.127 |
| 30 | gi\|15923766 | Enolase (Mu50) | 4.55 | 47 117 | 163 | PMF | 30% | 0.01 | 0.089 |
| 31 | gi\|21283521 | Fumarate hydratase class-II (MW2) | 5.16 | 51 146 | 83 | PMF | 35% | 0.01 | 0.180 |
| 32 | gi\|15923960 | Coenzyme A disulfide reductase (Mu50) | 5.28 | 49 291 | 141 | PMF | 49% | 0.17 | 0.120 |
| 33 | gi\|15925103 | Serine hydroxymethyl transferase (Mu50) | 5.75 | 45 172 | 91 | PMF | 31% | 0.01 | 0.093 |
| 34 | gi\|15923763 | Phosphoglycerate kinase (Mu50) | 5.17 | 42 602 | 123 | PMF | 47% | 0.05 | 0.070 |
| 35 | gi\|15924236 | Succinyl-CoA synthetase alpha subunit (MW2) | 5.47 | 31 542 | 126 | PMF | 40% | 0.73 | 0.067 |
| 36 | gi\|49244956 | Glyceraldehyde-3-phosphate dehydrogenase 2 (MSSA476) | 5.95 | 36 979 | 30 | PMF | 21% | 0.01 | 0.142 |
| 37 | gi\|15923947 | Ornithine-oxo-acid transaminase (Mu50) | 5.21 | 43 418 | 45 | PMF | 19% | 0.73 | 0.105 |
| 38 | gi\|15924037 | Cysteine protease precursor (Mu50) | 5.68 | 44 595 | 49 | PMF | 19% | 9.55 | 0.929 |
| 39 | gi\|15924685 | Citrate synthase (Mu50) | 5.41 | 42 593 | 90 | PMF | 34% | 0.01 | 0.085 |
| 40 | gi\|21283381 | Alanine dehydrogenase (MW2) | 5.58 | 40 105 | 98 | PMF | 37% | 0.01 | 0.086 |
| 41 | gi\|15924084 | Pyruvate dehydrogenase E1 component beta subunit (Mu50) | 4.65 | 35 246 | 91 | PMF | 35% | 0.73 | 0.044 |
| 42 | gi\|21282661 | Serine protease; V8 protease; glutamyl endopeptidase (MW2) | 5.22 | 35 319 | 41 | PMF | 30% | 9.98 | 0.935 |
| 43 | gi\|15923544 | Branched-chain amino acid aminotrans-ferase homologue (Mu50) | 4.92 | 40 086 | 66 | PMF | 32% | 0.73 | 0.326 |
| 44 | gi\|15924247 | Elongation factor Ts (Mu50) | 5.15 | 32 493 | 135 | PMF | 59% | 0.01 | 0.045 |
| 45 | gi\|21282297 | Alcohol dehydrogenase (MW2) | 5.34 | 36 048 | 47 | PMF | 35% | 0.01 | 0.044 |
| 46–47 | gi\|14248355 | Similar to hydrolase (Mu50) | 4.72 | 31 005 | 69 | MS/MS | $^{44}$DHFTVVAVDR$^{53}$ | 0.73 | 0.611 |
| 48, 79[a)] | gi\|15923538 | Elongation factor Tu (Mu50) | 4.74 | 43 104 | 117 | PMF | 42% | 0.01 | 0.071 |
| 49 | gi\|15924518 | Elongation factor P (Mu50) | 4.75 | 20 554 | 64 | PMF | 44% | 0.01 | 0.424 |
| 50 | gi\|54040804 | D-Alanine aminotransferase (Mu50) | 4.95 | 31 908 | 89 | PMF | 44% | 0.73 | 0.206 |

**Table 4.** Continued

| Spot | Acc. no. | Protein name | IP | MW | Score | Technique | Seq. Cov./ Pep. Seq. | Extracell. score | SecP score |
|---|---|---|---|---|---|---|---|---|---|
| 51 | gi\|14247898 | Fructose-bisphosphate aldolase (Mu50) | 5.01 | 30 836 | 56 | MS/MS | $^{230}$INVNTENQIASAK$^{242}$ | 2.5 | 0.097 |
| 52 | gi\|15923754 | Thioredoxine reductase (Mu50) | 5.21 | 33 616 | 91 | PMF | 49% | 0.17 | 0.087 |
| 53 | gi\|15923503 | Cysteine synthase homolog (Mu50) | 5.38 | 32 976 | 83 | PMF | 53% | 0.73 | 0.154 |
| 54 | gi\|15924236 | Succynil-CoA synthetase alpha subunit (Mu50) | 5.47 | 31 542 | 81 | MS/MS | $^{119}$LVGPNCPGVITA DECKI$^{135}$ | 0.73 | 0.067 |
| 55 | gi\|15924700 | Similar to universal stress protein family (Mu50) | 5.60 | 18 475 | 29 | PMF | 34% | 0.73 | 0.110 |
| 56 | gi\|49484362 | Purine nucleoside phosphorylase (MRSA252) | 4.85 | 25 908 | 48 | MS/MS | $^{31}$HHNTYVTK$^{38}$ | 2.5 | 0.163 |
| 57 | gi\|15924543 | Superoxide dismutase (Mu50) | 5.08 | 22 711 | 94 | PMF | 44% | 9.55 | 0.549 |
| 58 | gi\|15923371 | Alkyl hydroperoxide reductase subunit C (Mu50) | 4.88 | 20 977 | 55 | MS/MS | $^{83}$AWHDHSDAISK$^{93}$ | 0.01 | 0.702 |
| 59 | gi\|15925172 | Alkaline shock protein 23 (Mu50) | 5.13 | 19 191 | 76 | MS/MS | $^{103}$VILEYGESAPK$^{113}$ | 2.5 | 0.948 |
| 60 | gi\|15923742 | Similar to ribosomal subunit interface protein (Mu50) | 5.15 | 22 213 | 84 | PMF | 53% | 0.73 | 0.097 |
| 61 | gi\|15924245 | Transcriptional repressor CodY (Mu50) | 5.87 | 28 755 | 37 | MS/MS | $^{70}$HIPSEYTER$^{78}$ | 0.73 | 0.037 |
| 62 | gi\|15625529 | Staphylococcal enterotoxin K (COL) | 7.70 | 27 727 | 92 | PMF | 44% | 10.00 | 0.908 |
| 63 | gi\|15625528 | Staphylococcal enterotxin Q (COL) | 8.33 | 28 185 | 93 | PMF | 52% | 10.00 | 0.949 |
| 64–67 | gi\|57651597 | SEB (COL) | 8.65 | 31 436 | 117 | PMF | 45% | 10.00 | 0.937 |
| 68 | gi\|14245862 | 1-Phosphatidylinositol phosphodiesterase precursor (Mu50) | 7.71 | 37 088 | 78 | PMF | 39% | 9.97 | 0.924 |
| 69–70 | gi\|15923949 | Glycerophosphoryl diester phosphodiesterase (Mu50) | 8.67 | 35 310 | 59 | PMF | 29% | 4.86 | 0.891 |
| 71–72 | gi\|15924153 | Alpha-hemolysin precursor (Mu50) | 7.94 | 35 975 | 52 | PMF | 32% | 10.00 | 0.915 |
| 73 | gi\|15925410 | Gamma-hemolysin component C (Mu50) | 9.29 | 35 584 | 15 | PMF | 4% | 9.73 | 0.904 |
| 74–75 | gi\|15923709 | Anion-binding protein (Mu50) | 9.04 | 74 400 | 163 | PMF | 38% | 0.01 | 0.957 |
| 76[a)] | gi\|15925634 | *N*-Acetylmuramoyl-L-alanine amidase (Mu50) | 5.96 | 69 243 | 78 | PMF | 21% | 9.73 | 0.938 |
| 77[a)] | gi\|15924570 | dnaK protein (Mu50) | 4.65 | 66 361 | 71 | PMF | 22% | 0.01 | 0.681 |
| **ROS** | | | | | | | | | |
| 78–79 | gi\|57285741 | Sulfatase family protein (COL) | 9.04 | 74 400 | 105 | MS/MS | $^{431}$SNTGDATVD GYIQTAR$^{446}$ | 0.01 | 0.957 |
| 80 | gi\|57650091 | Ribosomal subunit interface protein (COL) | 5.15 | 22 225 | 69 | MS/MS | $^{4}$FEIHGDNLTITDAIR$^{18}$ | 0.73 | 0.090 |
| | gi\|15928148 | Immunodominant antigen A (N315) | 6.11 | 24 204 | 58 | MS/MS | $^{148}$LSNGNTA-AAQIMAQ$^{168}$ | 9.98 | 0.902 |
| 81–82 | gi\|15923949 | Glycerolphosphoryl diester phosphodiesterase (Mu50) | 8.67 | 35 310 | 90 | MS/MS | $^{95}$DGHLVAMHDETVNR$^{108}$ | 4.86 | 0.891 |
| 83 | gi\|57284716 | Rod shape-determining protein MreC (COL) | 9.04 | 31 010 | 75 | MS/MS | $^{159}$FSSQVDLISTNTR$^{170}$ | 3.33 | 0.953 |
| **RIMD 31092** | | | | | | | | | |
| 84 | gi\|15923709 | Anion-binding protein (Mu50) | 9.04 | 74 400 | 159 | PMF | 39% | 0.01 | 0.957 |
| 85 | gi\|15924037 | Cysteine protease precursor (Mu50) | 5.68 | 44 595 | 74 | PMF | 29% | 9.55 | 0.929 |
| 86–87–88 | gi\|15923949 | Glycerolphosphoryl diester phosphodiesterase (Mu50) | 8.67 | 35 310 | 166 | PMF | 48% | 4.86 | 0.891 |
| 89–90 | gi\|15924153 | Alpha-hemolysin precursor (Mu50) | 8.70 | 35 975 | 94 | PMF | 43% | 10.00 | 0.915 |
| 91 | gi\|295141 | Enterotoxin (STAAU) | 6.69 | 27 709 | 139 | PMF | 59% | 10.00 | 0.894 |
| 92 | gi\|15925001 | TSST-1 (Mu50) | 8.80 | 26 447 | 106 | PMF | 57% | 10.00 | 0.954 |
| 93–94 | gi\|15924999 | Enterotoxin C3 precursor (Mu50) | 8.22 | 30 671 | 61 | MS/MS | $^{168}$NTISFEVQTDK$^{178}$ | 10.00 | 0.940 |
| 95–96 | gi\|57651597 | SEB (COL) | 8.25 | 28 368 | 126 | PMF | 50% | 10.00 | 0.937 |
| 97 | gi\|15923971 | Hypothetical protein SAV0981 (Mu50) | 9.28 | 15 898 | 40 | PMF | 43% | 3.33 | 0.796 |
| 98–99 | gi\|38259777 | Surface protein SasE (STAAU) | 9.70 | 16 537 | 46 | PMF | 50% | 0.07 | 0.947 |
| 100 | gi\|15925518 | Sortase (Mu50) | 8.37 | 23 598 | 60 | PMF | 45% | 3.33 | 0.940 |
| 101 | gi\|15924998 | Extracellular enterotoxin L (Mu50) | 9.00 | 27 496 | 80 | PMF | 37% | 10.00 | 0.940 |

**Table 4.** Continued

| Spot | Acc. no. | Protein name | IP | MW | Score | Technique | Seq. Cov./ Pep. Seq. | Extracell. score | SecP score |
|---|---|---|---|---|---|---|---|---|---|
| **A900322** | | | | | | | | | |
| 102 | gi\|15923297 | Similar to outer membrane protein precursor (Mu50) | 9.49 | 33 352 | 43 | MS/MS | $^{155}$GVDIYYISDR$^{164}$ | 3.33 | 0.876 |
| 103–104 | gi\|15924153 | Alpha-hemolysin precursor (Mu50) | 8.70 | 35 975 | 138 | PMF | 52% | 10.00 | 0.915 |
| 105–106 | gi\|15923949 | Glycerolphosphoril diester phosphodiesterase (Mu50) | 8.67 | 35 310 | 155 | PMF | 49% | 4.86 | 0.891 |
| 107 | gi\|15923085 | 1-Phosphatidylinositol phosphodiesterase precursor (Mu50) | 7.71 | 37 087 | 61 | PMF | 40% | 9.97 | 0.924 |
| 108 | gi\|15924938 | Enterotoxin P (Mu50) | 8.25 | 30 016 | 88 | PMF | 51% | 10.00 | 0.886 |
| 109–110 | gi\|15923310 | Glycerol ester hydrolase (Mu50) | 8.99 | 76 542 | 60 | MS/MS | $^{83}$EELQDFWHHLAD DLVK$^{98}$ | 9.55 | 0.930 |
| 111–112 | gi\|15925661 | Triacylglycerol lipase precursor (Mu50) | 6.58 | 76 662 | 133 | PMF | 30% | 9.73 | 0.944 |
| 113 | gi\|15923742 | Similar to ribosomial subunit interface protein (Mu50) | 5.15 | 22 213 | 68 | MS/MS | $^{4}$FEIHGDNLTITDAIR$^{18}$ | 0.73 | 0.097 |
| 114 | gi\|15925411 | Gamma-hemolysin gamma 2 component, HlgB (Mu50) | 9.25 | 36 658 | 98 | PMF | 43% | 9.55 | 0.899 |
| 115 | gi\|475839 | Leucocidin S component (STAAU) | 9.23 | 35 557 | 33 | MS/MS | $^{250}$GSSDTSEFEITYGR$^{263}$ | 9.73 | 0.904 |
| **AB-8802** | | | | | | | | | |
| 116–117–118 | gi\|21283669 | Beta-hemolysin (MW2) | 7.68 | 31 255 | 118 | PMF | 51% | 9.97 | 0.927 |
| 119–120–121 | gi\|15924153 | Alpha-hemolysin precursor (Mu50) | 8.7 | 35 975 | 118 | PMF | 48% | 10.00 | 0.915 |
| 122–123 | gi\|15923949 | Glycerophosphoryl diester phosphodiesterase (Mu50) | 8.67 | 35 310 | 93 | PMF | 43% | 4.86 | 0.891 |
| 124 | gi\|15923085 | 1-Phosphatidylinositol phosphodiesterase precursor (MW2) | 7.71 | 37 087 | 52 | PMF | 37% | 9.97 | 0.924 |
| 125–126 | gi\|15925661 | Triacylglycerol lipase precursor (Mu50) | 6.58 | 76 662 | 124 | PMF | 30% | 9.73 | 0.944 |

Protein names according to NCBI database containing nine sequenced *S. aureus* subspecies.
a) ID was obtained in the 2-D PAGE at 7 h.

A900322, and AB-8802) to that of the *egc* negative ATCC 14458 and to the nonenterotoxigenic ROS reference strains (Table 2). Enterotoxins comprising regions of 2-DE maps (Fig. 3) show very different protein patterns for the different strains. In the nonenterotoxigenic reference strain (ROS) there are only few proteins expressed in this region and no enterotoxins were detected. In contrast, protein patterns of enterotoxigenic strains are more complex in this region. RIMD 31092 strain expresses two isoforms of enterotoxin C3 in spots 92 and 94, and another SEC variant in spot 91 (Fig. 3d). Apart from an array of core exoproteins (Table 4c) this clinical isolate also produces TSST-1 (Fig. 3d, spot 92) one of the best characterized pyrogenic toxin SAgs known to be the major responsible agent of TSS, an acute, multisystem disease. In addition, we have found evidence of the expression of enterotoxin-like protein L (SElL, Fig. 3d, spot 101). This is the first time that the production of SElL has been confirmed for a *S. aureus* strain. The gene *sell* was cloned from *S. aureus* TSS isolate and the produced recombinant SElL has been shown to be pyrogenic and superantigenic in rabbit model [40]. By sequence similarities of recombinant SEL has been shown to belong to a new subfamily of SEs, along with the recently described SElI, SEK, and SElQ.

A900322 strain isolated from a patient with TSS is used as *seg*+ and *sei*+ reference strain [18, 20]. Previously, it was shown to have genotype *sea-*, *seb-*, *sec-*, *sed-*, *see-*, *seg*+, *seh-*, *sei*+, *seln*+, *selo*+, and *selm*+. It was recently found to be positive for *selp* gene as well [22]. Under the experimental conditions applied in this study, we did not detect SEG, SEI, SElN, SElO, and SElM in the culture broth of this strain. However, for the first time, we could demonstrate the presence of *selp* gene product, enterotoxin like P (SElP) in spot 108 (Fig. 3e). Other expressed virulence factors are from the leukocidin/hemolysin toxin family, *i.e.*, alpha- (Fig. 3e, spots 103 and 104) and gamma-hemolysin (spot 114) and leucocidin S (spot 115). Leucocidin/hemolysin toxins affect target cells by forming large exogenous pores in the plasma membrane. They are two-component and hetero-oligomeric cytotoxins composed of nonassociated soluble proteins, F (*e.g.*, LukF-PV, LukF-R, LukF-I, LukM, HlgB) and S (*e.g.*, LukS-PV, LukS-R, LukS-I, HlgA, HlgC).

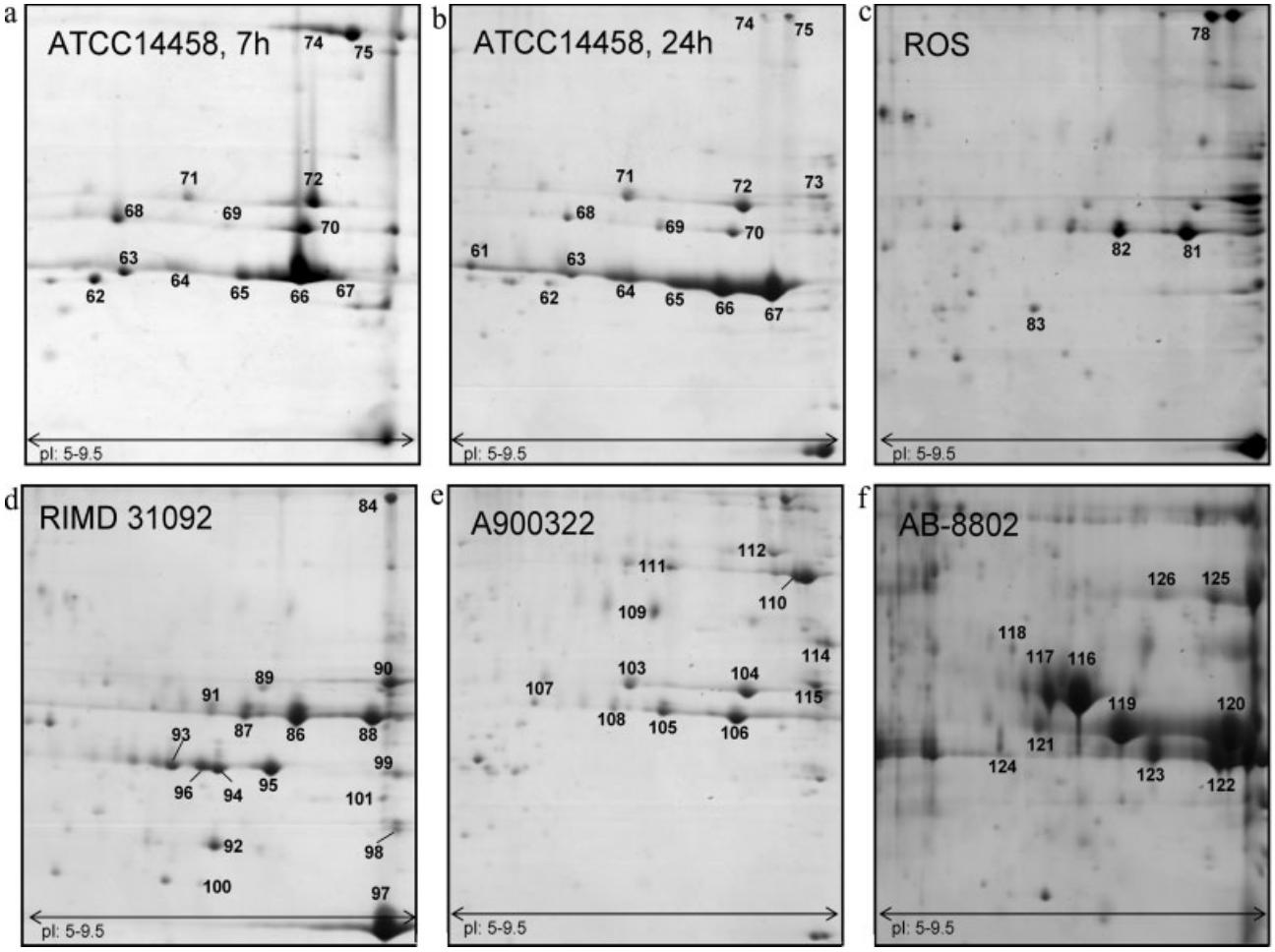



**Figure 3.** 2-DE images of exoprotein patterns of (a) ATCC 14458 at 7 h, (b) ATCC 14458 at 24 h, (c) ROS, (d) RIMD 31092, (e) A900322, and (f) AB-8802 *S. aureus* strains (Table 3) at 24 h in the p*I* 5–9.5 and MW 20–50 kDa ranges. Identified proteins are reported in Table 4.

AB-8802 food derived strain was found to express a high quantity of alpha- (spots 119–121, Fig. 3d) and beta-hemolysin (spots 116–118) together with some core exoproteins, such as glycerylphosphoryl diester phosphodiesterase (spots 122 and 123), 1-phosphatidylinositol phosphodiesterase (spot 124), and triacylglycerol lipase (spots 125 and 126). AB-8802 has been characterized by the presence of two variant enterotoxin gene nucleotide sequences, *i.e.*, $seg_v^+$ and $sei_v^+$ (Table 3) of *egc* operon [20, 41]. However, in our work none of the corresponding enterotoxins could be detected.

## 4 Concluding remarks

*S. aureus* releases a large number of exoproteins such as adhesins, toxins, and enzymes into the extracellular medium. Different strains have very different secreted protein expressions. Many of these proteins are believed to contribute to pathogenic activity of the bacteria or to enhance virulence. The pathogenicity of *S. aureus* is a complex process involving the coordinated production of a number of virulence factors which often have overlapping roles and can act either alone or in concert. Staphylococcal SAgs, in particular, are one of the leading causes of infections and food intoxications by *Staphylococcus*, and some of them are considered as potential bio-weapons as well. Their sensitive and reliable detection and characterization is therefore an important issue. Recent works employing genetic typing allowed the detection of specific staphylococcal SAgs, but they lack information on the expression of their corresponding gene products. This is the case of many known SEs discovered recently at the genome level (*sei* to *selu*). Detection of SEs at the protein level can be performed by immunoassay [42, 43], or alternatively by proteomics. Immunoassay of newly discovered SAgs is however limited by the unavailability of antibodies. Analyzing the secretome by proteomic tools can give a comprehensive picture of the expression both of core exoproteins and virulence factors under a given condition. In particular, studying the enterotoxin region of the 2-DE maps of different enterotoxigenic strains lets us iden-

tify various strain specific SAgs (SEB, TSST-1, and various SEC isoforms). More importantly, for the first time, expressions of four enterotoxin-like proteins, SElK and SElQ for ATCC 14458, SElL for RIMD 31092, and SElP for A900322) were proved. Genes of three of these proteins, *i.e.*, selk, selq, and selp had been previously predicted by oligonucleotide microarray assay [22], thus, here we confirm their production at the protein level. In addition, predicted protein products of *egc* positive strains (*i.e.*, SEG, SEI, SElM, SElN, and SElO) could not be confirmed under the experimental conditions used in this work.

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