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PROTEOMIC PROFILING OF METHICILLIN SENSITIVE (MSSA) AND METHICILLIN RESISTANT (MRSA) *STAPHYLOCOCCUS AUREUS* STRAINS BY LC-MS AND LC-MS/MS

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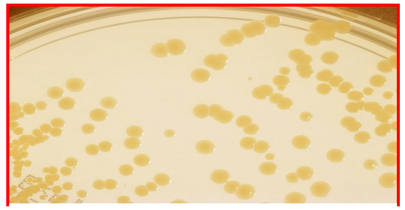
OVERVIEW

- We employed a **'label-free' mass spectrometry-based approach** to analyze the proteomic profiles from a total tryptic digest of *Staphylococcus aureus* (MRSA) and (MSSA) for the detection of potential markers for strain differentiation.
- The overlap of proteins identified between the strains was investigated.
- A number of putative biomarkers were identified and quantified. The confidence limits of these potential markers for clinical diagnostics will be established in larger studies.



INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become increasingly prevalent worldwide with particularly high incidences of its recovery from the 1960's in nursing homes and the community. Severe staphylococcal infections are associated with high rates of mortality and morbidity and have increased significantly during the 1990's in England and Wales causing a huge burden for the public health, with estimated costs of £5-10 million per annum. This increase coincides with the emergence of multi-drug resistance among strains and the difficulty in detecting this resistance. The vast increase in the use of antibiotics, namely mupirocin and intravenous vancomycin, may lead to the emergence and similar spread of vancomycin-resistant MRSA in hospitals since these infections are virtually untreatable with current antibiotics. There is a need for rapid detection of these organisms to facilitate control measures and, various phenotypic and genotypic methods have been applied. However, the correlation between drug resistance and measurement of antibiotic sensitivities among clinical isolates is not always clear due to heterogeneous expression. Consequently, there is a need for new markers to identify MRSA from sensitive (MSSA) strains. In the present study, comparative proteomic analysis of resistant and sensitive strains were undertaken with the aim of searching for subtle differences between strains that may identify new biomarkers for subsequent detection.

In this study we have used a previously described (1, 2) label free mass spectrometry-based approach to identify and quantify the protein profiles from four strains of *S. aureus* including two MRSA types [J95 (152415), J96 (270702)] and two MSSA isolates H591 and D547.

METHODS

Sample preparation

- *Staphylococcus aureus* MRSA types J95 (152415) and J96 (270702) and *Staphylococcus aureus* MSSA isolates H591 and D547 were cultured for 24 hours at 37°C on Columbia Blood Agar plates.
- Cells were harvested and the proteins extracted by a combination of freeze-thawing and mechanical disruption as described previously for *Streptococcus pneumoniae* (3).
- Proteins concentrations of all extracts were adjusted to give a final concentration of 4.5 mg ml^{-1}.
- 30µL aliquots of each sample were denatured with RapiGest™ SF surfactant (0.1%) (Waters Corp.), reduced (10 mM DTT) and alkylated (10 mM IAA).
- Samples were digested with sequencing grade Trypsin (Promega),1:50 (w/w) enzyme:protein ratio.
- The resulting mixture was diluted (x60) and spiked with internal standard (yeast Enolase) prior to analysis.

LC CONDITIONS

Waters nanoACQUITY™ UPLC System

Column: Trap cartridge, Symmetry® C18 (180µm x 20mm, 5µm particle size)
Analytical column BEH C18 (75µm x 100mm, 1.7 µm particle size)

Gradient: H_20/MeCN/formic acid at 300nL/min

MS CONDITIONS

Waters Q Tof Premier™ (oa-Tof) MS

Mode: ESI +ve at 17,000 resolution (FWHM) using "Expression" mode

Collision Energy: Function 1- 4eV; Function 2 - 15 to 40eV

Lock reference: Glu fibrinopeptide B

Calibration: NaI + CsI mix

DATA PROCESSING

Datasets were normalised and processed using Waters Protein Expression System Informatics and searched against a combined Saphylococcus database which contained ~5600 protein entries to identify the proteins present. The intensities of all matching peptides were then compared to ascertain the relative expression levels of proteins between the sample groups (MSSA and MRSA).

RESULTS

As expected the isolates showed a high degree of similarity in protein profiles since they belong to he same species and share 99-100% DNA sequence homology. Figure 1 shows low energy (precursor ion) BPI chromatograms from an LCMS analysis of an MSSA and an MRSA strain. Figure 2 below highlights the complexity of the data as a low energy and a time aligned elevated energy spectrum is displayed from an MRSA replicate at 24.3 minutes. Several precursor ions are present in the low energy spectrum and the elevated energy spectrum is complex.

Figure 1 illustrates low energy BPI chromatograms for 2 of the *Staphylococcus aureus* strains. Simultaneously, low and elevated energy chromatograms and spectra were collected.

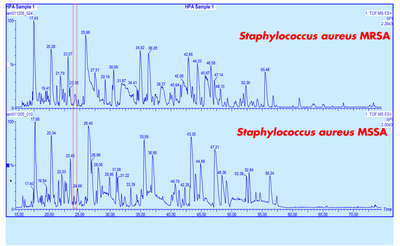
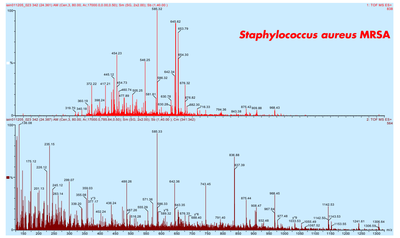


Figure 2 Typical low and elevated energy spectra are shown below. The low energy data are used for the quantification



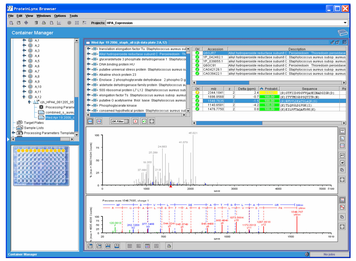
Qualitative Proteomic Analysis

The low energy function contains all detectable peptide pseudo molecular ions. In a complementary fashion, the resulting elevated-energy data provides extensive multiplexed fragmentation information for every precursor ion detected in the low energy mode. The elevated energy fragment ions are aligned to their related precursor ions in chromatographic space by time and profile. Proteins were identified from a databank search using PLGS 2.2.5, for each sample replicate and filtered at the 100% confidence limit. Between 92 and 176 proteins were identified from each strain and the results of one such search is presented in Figure 3.

The data summarised in Figure 4 demonstrates the reproducibility of the technique. The pie charts show the overlap of proteins detected initially between the MRSA J96 and MSSA H591strains for each of three replicate injections. The percentage overlap between the strains is consistent for each of the three injections. Figure 5 highlights differences within the strains selected.

Figure 6 shows the overlap between all four strains from all three replicate injections to be 50%.

Figure 3. Browser showing protein identification summary.



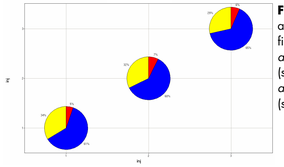


Figure 4 Percentage overlap of identified proteins from *S. aureus* MSSA H591 (sample 1) and *S. aureus* MRSA J96 (sample 4).

Figure 5 Percentage overlap of identified proteins between *S. aureus* MRSA J95 and J96 strains and *S. aureus* MSSA H591 and D547 strains.

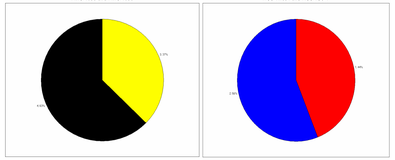


Figure 6 Percentage overlap of identified proteins from *S. aureus* MSSA H591 (sample 1) and *S. aureus* MRSA J96 (sample 4)

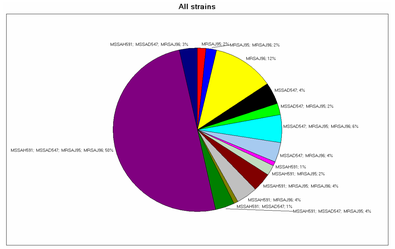


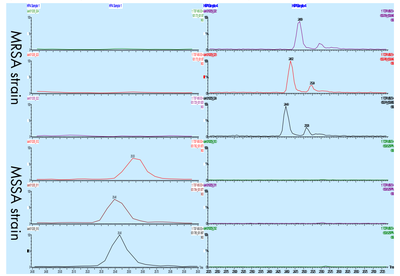
Table 1 shows an overview of the proteins detected from their characteristic peptides. Most represent key functions of the cell and include such proteins as e.g. glyceraldehyde phosphate dehydrogenase, protein synthesis e.g. DNA binding protein HU, Protein folding and cell signaling e.g Phosphoglycerate kinase.

Identified proteins

elongation factor Tu Staphylococcus aureus subsp aureus MRSA252
Alkyl hydroperoxide reductase subunit C Peroxiredoxin Thioredoxin peroxidase
alkaline shock protein 23 A5P23 Staphylococcus aureus
DNA binding protein HU Staphylococcus aureus subsp aureus MRSA252
Glyceraldehyde 3 phosphate dehydrogenase 1 GAPDH 1
hypothetical protein SAR0874 Staphylococcus aureus subsp aureus MRSA252
putative universal stress protein Staphylococcus aureus subsp aureus MRSA252
Enolase 2 phosphoglycerate dehydratase 2 phospho D glycerate hydro lyase Laminin binding protei
aldehyde dehydrogenase family protein Staphylococcus aureus subsp aureus MRSA252
isocitrate dehydrogenase Staphylococcus aureus subsp aureus MRSA252
elongation factor Ts Staphylococcus aureus subsp aureus MRSA252
Chaperone protein dnaK Heat shock protein 70 Heat shock 70 kDa protein HSP70
putative 30S ribosomal protein S1 Staphylococcus aureus subsp aureus MRSA252
Dihydrolipoyl dehydrogenase E3 component of pyruvate complex Dihydrolipoamide dehydrogenase Mem
Phosphoglycerate kinase
Cysteine synthase O acetylserine sulfhydrylase O acetylserine Thiol lyase CSase
putative aldehyde dehydrogenase Staphylococcus aureus subsp aureus MRSA252
succinyl CoA synthetase subunit beta Staphylococcus aureus subsp aureus MRSA252
Immunoglobulin G binding protein A precursor IgG binding protein A Staphylococcal protein A
L lactate dehydrogenase 2 Staphylococcus aureus subsp aureus MRSA252
Elongation factor G EF G 85 kDa vitronectin binding protein
Pyruvate dehydrogenase E1 component beta subunit
putative 50S ribosomal protein L25 Staphylococcus aureus subsp aureus MRSA252
succinyl CoA synthetase alpha subunit Staphylococcus aureus subsp aureus MRSA252
Triosephosphate isomerase TIM Triose phosphate isomerase
50S ribosomal protein L7 L12 Staphylococcus aureus subsp aureus MRSA252
Fructose bisphosphate aldolase Staphylococcus aureus subsp aureus MRSA252
Nucleoside diphosphate kinase NDK NDP kinase Nucleoside 2 P kinase
citrate synthase Staphylococcus aureus subsp aureus MRSA252
HmB Staphylococcus aureus
50S ribosomal protein L29 Staphylococcus aureus subsp aureus MRSA252
50S ribosomal protein L30
60 kDa chaperonin Protein Cpn60 groEL protein Heat shock protein 60
hypothetical protein SAR1936 Staphylococcus aureus subsp aureus MRSA252
DNA directed RNA polymerase alpha subunit Staphylococcus aureus subsp aureus MRSA252
PTS system glucose specific IIA component Staphylococcus aureus subsp aureus MRSA252
hypothetical protein SAR0985 Staphylococcus aureus subsp aureus MRSA252
Cell division protein RsZ
ornithine oxo acid transaminase Staphylococcus aureus subsp aureus MRSA252
FolD bifunctional protein includes methylenetetrahydrofolate dehydrogenase methenyltetrahydrofolat
Glutamine synthetase Glutamate ammonia ligase GS
putative pyruvate dehydrogenase E1 component alpha subunit Staphylococcus aureus subsp aureus MR5
glycerol kinase Staphylococcus aureus subsp aureus MRSA252
alanine poly phosphoribitol ligase subunit 2 D alanyl carrier protein DCP
50S ribosomal protein L6 Staphylococcus aureus subsp aureus MRSA252
50S ribosomal protein L18 Staphylococcus aureus subsp aureus MRSA252
putative lipoprotein Staphylococcus aureus subsp aureus MRSA252
30S ribosomal protein S7
Glu Leu Phe Val dehydrogenase C terminal Glu Leu Phe Val dehydrogenase dimerisation region Staphy
Virulence factor esxA
hypothetical protein SAR1945 Staphylococcus aureus subsp aureus MRSA252
transaldolase Staphylococcus aureus subsp aureus MRSA252
1 hypothetical protein SAR2264 Staphylococcus aureus subsp aureus MRSA252
hypothetical protein SAR1837 Staphylococcus aureus subsp aureus MRSA252
30S ribosomal protein S10 Staphylococcus aureus subsp aureus MRSA252
hypothetical protein SAR2021 Staphylococcus aureus subsp aureus MRSA252
30S ribosomal protein S16 Staphylococcus aureus subsp aureus MRSA252
glycine cleavage system protein H Staphylococcus aureus subsp aureus MRSA252
50S ribosomal protein L10 Staphylococcus aureus subsp aureus MRSA252
putative thiamine pyrophosphate enzyme Staphylococcus aureus subsp aureus MRSA252
50S ribosomal protein L15
hypothetical protein SAR1041 Staphylococcus aureus subsp aureus MRSA252

Many of the proteins identified had no known function and could only be described as hypothetical proteins. Analysis of the data showed that peptides and hence proteins unique to either the methicillin sensitive (~6%) or the methicillin resistant strain (~30%) were identified. A significant difference between resistant and sensitive strains was the presence of a peptide within a hypothetical lipoprotein. In particular a putative lipoprotein was identified in both samples although each strain had a unique peptide identification shown in the exact mass chromatograms in Figure 7, where the response from two peptides is shown from 3 replicates in the bottom panel from an MSSA strain and an MRSA strain in the top panel.

Figure 7 Mass chromatograms from the doubly charged ions (653.8 and 631.8) for 2 peptides, from 3 replicates for and MSSA strain (bottom panel) and an MRSA strain (top panel).



The peptides, SGEESEVLVADK and SGEESEVLVDDK identified are highlighted in the putative lipoprotein sequences below.

>gi|49485251|ref|YP_042472.1| putative lipoprotein [Staphylococcus aureus subsp. aureus MSSA476]
MKLKSLAVLSMSAVVLTACGNDTPKDETKSTESNTNQDTNTTKDVIALKDVKTS-PEDAVKKAEETYKGQKLKGISFENSNGEWAYKVTQQKSGEESEVLVADKNKKVI NKKTKEKDTVNENDNFKYSDAIDYKKAIKEGQKEFDGDIKEWSLEKDDGKLVYNI DLKKGNKKQEVTVDAKNGKVLKSEQDH

>gi|49482621|ref|YP_039845.1| putative lipoprotein [Staphylococcus aureus subsp. aureus MRSA252]
MKLKSLAVLSMSAVVLTACGNDTPKDETKSTESNTNQDTNTTKDVIALKDVKTS-PEDAVKKAEETYKGQKLKGISFENSNGEWAYKVTQQKSGEESEVLVDDKNKKVI NKKTKEKDTVNENDNFKYSDAIDYKKAIKEGQKEFDGDIKEWSLEKDDGKLVYNI DLKKGNKKQEVTVDAKNGKVLKSEQDQ

Quantitative Proteomic Analysis

Protein expression changes between the two groups (MSSA v MRSA) were determined by comparison of the data at the protein level with all the identified peptides contributing to the fold change of a particular protein (3). Some 16 proteins exhibited changes which are statistically significant between MRSA J96 and MSSA H591. Four proteins were up regulated between 1.16 and 1.84 fold in the MRSA J96 strain and four were down regulated between 0.39 and 0.82 fold in the MRSA J96 strain compared to the MSSA H591 strain.

CONCLUSIONS

- A label free, exact mass, mass spectrometry-based approach was used to analyze proteomic profiles from MSSA and MRSA and could be used to identify potential protein markers for strain differentiation.
- The detection of a lipoprotein biomarker for MRSA and MSSA strains from a total tryptic digest, to our knowledge represents the first such finding and provides an novel approach for further studies.
- No prior preparative steps such as 2-D gel electrophoresis nor fractionation methods were undertaken.
- Future studies, involving a larger number of strains will establish the confidence limits of this biomarker among drug-resistant strains of *S. aureus* and its potential use for the clinical diagnostic laboratory.

References

1. Bateman R et al, J Am Soc Mass Spectrom. 2002 Jul;13(7):792-803
2. McKenna et al. A Novel Approach to Protein Identification: A Direct Comparison to Traditional Mass Spectrometric Techniques. 52nd meeting of the ASMS. Poster presentation (2004).
3. Encheva et al., 2006, Proteomics in press

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