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The Effects of Osmotic Stress on *Salmonella enterica* serovar Typhimurium: a quantitative proteomic study of a cell membrane fraction

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OVERVIEW

- Cell membrane fractions from *Salmonella enterica* were enriched to study the effect of osmotic stress on membrane protein expression profiles.
- Relative quantification, at the protein level, was performed using a label-free, LC-MS based technique
- The effect of the ompR regulator gene on osmotic stress was studied through the comparative analysis of a wild type with a mutant strain, deficient in ompR.
- A significant number of membrane related proteins were identified as up or down regulated under conditions of osmotic stress. A number of these had not previously been reported as stress related gene products.

INTRODUCTION

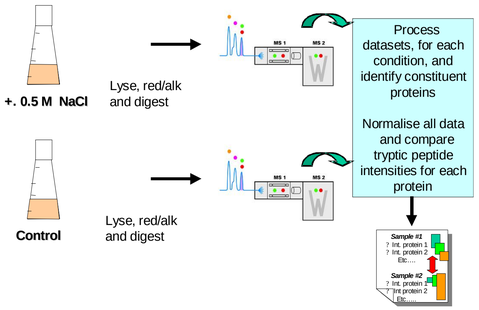
- *Salmonella enterica* serovar Typhimurium is the causal agent of acute gastroenteritis , causes 3.6M deaths per year (WHO)
- *S. enterica* invades and survives within macrophages and typhoid fever results from a systemic infection, characterised by the presence of bacteria in the liver, spleen and bone marrow. Specific immune responses are mounted against *Salmonella* outer membrane proteins (OMPs).
- Studies on the molecular features and regulation of *Salmonella* OMPs and porins should aid in further understanding their role during bacterium-host interactions.
- In this study we have investigated the response of *Salmonella* to osmotic stress. Specifically, we have looked at the change in protein expression profiles of the cell membrane when *Salmonella* are exposed to high concentrations of NaCl.
- In addition we have looked at a mutant strain deficient in ompR. Cell membrane extracts were obtained from the wild type and ompR mutant *Salmonella* after exposure to NaCl and also from a control grown under normal conditions. These extracts were digested and analysed by replicate LC-MS experiments on a Q-Tof mass spectrometer.

METHODS

Salmonella production and cell membrane extraction

- 2 X 50 ml culture of *Salmonella typhymurium*, SL1344 (ompR- or wild type), in Luria Broth were grown overnight at 37°C with shaking.
- When the A_{600} = 0.5, 50 ml of pre-warmed NaCl/LB solution was added to the salt stressed flask and 50 ml of pre-warmed LB added to the control.
- The cultures were incubated for a further 60min at 37°C. The cultures were chilled and harvested by centrifugation at 6,000 rpm for 10 min at 4°C, washed with 250 ml of HEPES buffer and re-centrifuged.
- Each pellet was re-suspended in 1.6 ml of HEPES buffer and the cells lysed by sonication. Following lysis of cells by sonication and removal of unbroken cells via a low-speed spin, samples were centrifuged at 40,000 x *g* to prepare an unfractionated membrane fraction for further analysis.
- Cell membrane fractions were solubilized in 1% RapiGest™ *SF* (Waters, Milford, MA), warmed to 80°C, sonicated for 15 mins, then heated at 95°C for 5 mins followed by a further 15 mins sonication.
- Solubilized proteins were reduced with 5mM dithiothreitol and alkylated with 10mM iodoacetamide. The proteins were then digested with 1% (w/w) sequence grade trypsin (Promega, UK) overnight (16hrs) at 37°C. RapiGest *SF* was removed by the addition of HCl followed by centrifugation. Samples were diluted with 0.1% formic acid to a final concentration of 1ug/ul prior to analysis.

Method overview



LC-MS

- Liquid chromatography was carried out using a nanoACQUITY™ UPLC™ system (Waters, Milford, MA) which provides direct flow down to a few nanolitres per minute. The system was configured with a 75µm analytical column packed with Atlantis® dC18 3.0µM stationary phase (Waters, Milford, MA).
- Samples were loaded onto a trapping column in 2% acetonitrile +0.1% formic acid and eluted using a two hour gradient; 0-40% acetroniltrile containing 0.1% formic acid.
- The Q-Tof™ Premier mass spectrometer (Waters Micromass, Manchester UK) was programmed to acquire data as described previously (1) where the energy applied to the collision cell is alternated between a low (8 eV) and elevated (23—33 eV) energy.
- An integration time of 1.5 seconds was used for each function with the reference channel was sampled every 30 seconds. Glu-Fibrinopeptide B was infused as a reference in the NanoLockSpray™ source.
- The TOF resolution, used in all experiments, was greater than 17,500 FWHM, whilst mass measurement in both modes of acquisition was typically 5ppm or better.
- Protein identification was achieved by searching a *Salmonella* species-specific database using Protein Expression System Informatics, (Waters, Manchester, UK).

RESULTS

Changes in Protein Expression

- The most notable changes in protein expression, between the control and salt stressed samples, and additionally the wild type and ompR-ve mutant are summarised in ***Figures 1 & 2***.
- In general, proteins down regulated in response to osmotic stress, exhibit a more pronounced down regulation in the ompR-ve mutant
- In particular, the ompR-ve strain shows a more exaggerated down-regulation for D-galactose binding periplasmic protein, elongation factor G and malate dehydrogenase.
- The WT strain shows a more pronounced down regulation of Glycerol kinase and a currently unnamed protein.

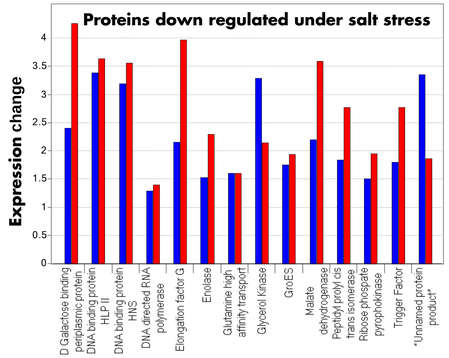


Figure 1. A summary of the key proteins down regulated under osmotic stress
(WT – blue, ompR-ve – red)

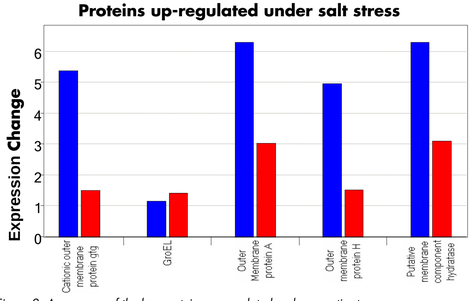


Figure 2. A summary of the key proteins up regulated under osmotic stress

(WT- blue, ompR-ve – red)

- As seen in ***figure 2***, the expression of some membrane related proteins is greater in the wild type than in the ompR-ve mutant.

Example data- up regulation of ompA

- Data acquired from the oa-TOF consisted of low energy data containing intact peptide multiply charged ions and in the elevated energy data, the corresponding fragment ion data.
- The bioinformatics were used to process the data, and produce the results summarised in figures 1&2.
- However, to provide added credence to the results we decided to manually verify the up-regulation of the integral membrane protein ompA
- Figure 3 shows exact mass chromatograms for a tryptic peptide, m/z 689.89 originating from ompA.
- Software integration of the peak areas shows a six-fold up regulation of this peptide between the control and salt stressed.

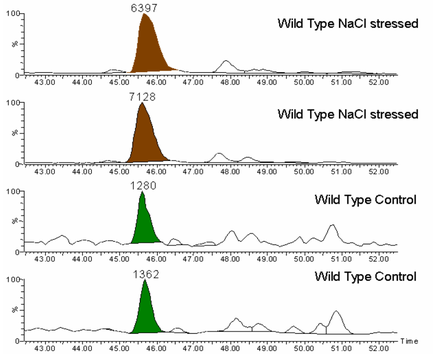


Figure 3. Integrated mass chromatograms in duplicate are shown for the tryptic peptide from residues 281-292 of the ompA protein expressed in the wild type under control and stressed conditions. A comparison of the integrated areas is used to determine the fold change of the protein between conditions.

- The peptide at m/z 689.89 elutes at a retention time of 45.5 mins, with the mass spectrum shown below in figure 4. The low energy spectrum provides the exact mass of the peptide, as well as all the co-eluting species.

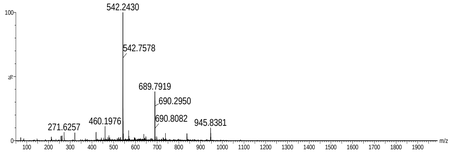


Figure 4. Low energy mass spectrum at 45.5 mins. The doubly charged ion at m/z 689.89 is a tryptic peptide from ompA, used to calculate the peak areas shown in Fig 3. .

- The corresponding elevated energy spectrum contains fragment ions from all of the peptides present in the collision cell at that point in time.

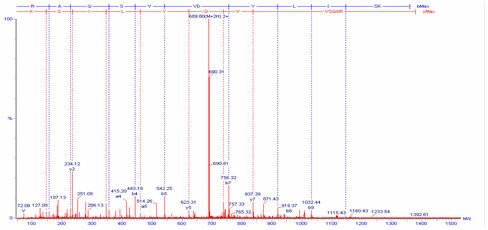


Figure 5. Combined and subtracted elevated energy spectrum from around 45.5 mins showing fragment ions for the peptide RAQSVVDYLISK from ompA.

- The elevated energy spectrum for m/z 689.89 is shown in Figure 5; annotated with the sequence RAQSVVDYLISK from ompA. Good coverage of both the y'' and b-ions is observed, with the b-ions prominent due to the presence of an N-terminal arginine residue

DISCUSSION AND CONCLUSION

- This method allows label free quantitative comparisons to be made between conditions to give a ratio for change in protein expression. The ability to rapidly and conveniently measure a range of components without recourse to labelling or tagging, significantly simplifies experimental analyses and may also substantially improve proteome coverage.
- The outer membrane porins, ompA and ompH are up regulated in both the WT and ompR-ve strain when exposed to osmotic shock.
- Other key proteins (e.g. Tol import, lipoprotein E) have been identified and are up-regulated under osmotic stress conditions in the ompR-ve strain.
- The results show a general down regulation in metabolic enzymes associated with the Krebs cycle. Previous results have indicated that the cells may be switching to anaerobic metabolism, and are generally reducing their metabolism to conserve energy.

REFERENCES

1. 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)

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