Waters

A Quantitative Proteomic Study on the Effects of Osmotic Stress on *Salmonella*

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Introduction

Salmonella enterica serovar *typhi* is the causal agent of typhoid fever in humans, affecting a wide sector of the world population and causing an estimated 600,000 deaths per year. A further three million fatalities per year are caused due to acute gastroenteritis and diarrhoea. *S. enterica* serovar *typhi* invades and survives within macrophages, with typhoid fever a systemic infection, characterized by the presence of bacteria in the liver, spleen, and bone marrow. Specific immune responses are mounted against *Salmonella* outer membrane proteins (OMPs). In addition, *S. enterica* serovar *typhimurium* double *ompC* and *ompF* porin mutants have shown attenuated virulence, and *S. enterica* serovar *typhimurium* porins have been observed to trigger signal transduction in host cells. Hence, studies on the molecular features and regulation of *Salmonella* OMPs and porins should aid in further understanding their role during bacterium-host interactions. Regulation of certain OMP porins is controlled by the ompR gene.

In this study we have investigated the response of *Salmonella* to osmotic stress. Specifically, we have looked at the change in protein expression profiles when *Salmonella* is exposed to high concentrations of NaCl. In addition we have looked at a mutant strain deficient in ompR. Protein 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 protein extracts were digested with sequencing grade trypsin and analysed by triplicate LC-MS experiments on a Q-Tof mass spectrometer.

Methods

Salmonella production

2 X 50mL culture of *Salmonella typhymurium*, SL1344 (ompR- or wild type), in Luria Broth were grown overnight at 37°C with shaking. 2 x 390mL of fresh pre-warmed medium were inoculated with 10mL each of the overnight culture and incubated at 37 °C with shaking.

When the A_{600} = 0.5, 50mL of pre-warmed NaCl/LB solution was added to the salt stressed flask and 50mL of pre-warmed LB added to the control. The cultures were incubated for a further 60 min at 37 °C. The cultures were chilled and harvested by centrifugation at 6,000 rpm for 10 min at 4 °C, washed with 250mL of HEPES buffer and re-centrifuged.

Each pellet was re-suspended in 1.6mL of HEPES buffer and the cells lysed by sonication. The lysed samples were then centrifuged at 13,000 g for 15 min and the supernatant snap frozen.

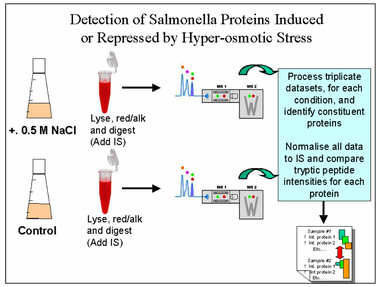


Figure 1. Analytical Strategy

Sample preparation

150µg (total protein) of the *Salmonella* protein extracts were diluted, and solubilised, by incubation at 80°C for 15 mins in 0.1% RapiGest™ SF before reduction with 5 mM dithiothreitol and alkylation with 10 mM iodoacetamide. The proteins were then digested with 1% (w/w) sequencing grade trypsin overnight (16hrs). RapiGest SF was cleaved by addition of HCl, followed by removal via centrifugation. Samples were diluted with 0.1% formic acid to a final concentration of 1µg/µL prior to analysis.

Mass Spectrometry and data processing

Analysis using the Waters CapLC™ Q-Tof LC-MS system was carried out using a 2hr reverse phase gradient (linear gradient from 5% to 40% Acetonitrile over 120 minutes). Samples were run in triplicate with the Q-Tof programmed to step between normal (10eV) and elevated collision energies (23-33eV) on the gas cell to provide both intact molecular ions, and the associated fragmentation spectrum for each eluting peptide. A scan time of 1.5s per function over the m/z range of 50-1990 *was used in* all experiments. Protein identification and quantitative information were extracted by the use of specialised algorithms, and searching against a *Salmonella* species-specific database.

Results and Discussion

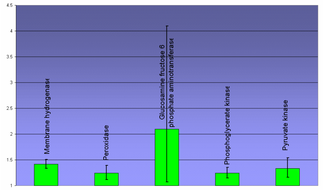


Figure 2- Metabolic enzymes up regulated in salt shock: There is a subtle up regulation of metabolic enzymes during salt shock. For example, there is an elevation of several enzymes involved in glycolysis (phosphoglycerate kinase, pyruvate kinase) suggesting perhaps that the energy requirement for the cell is increasing. A slight rise in peroxidase suggests the cell is undergoing some oxidative stress. There is a significant uncertainty on the expression ratio measurement for Glucosamine fructose-6-phosphate aminotransferase, with the extent of up regulation difficult to determine from the dataset. Despite this the software is confident that this protein is up regulated (95% confidence).

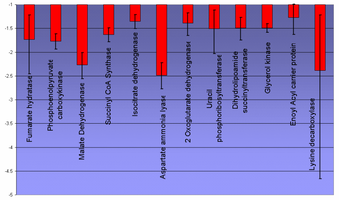
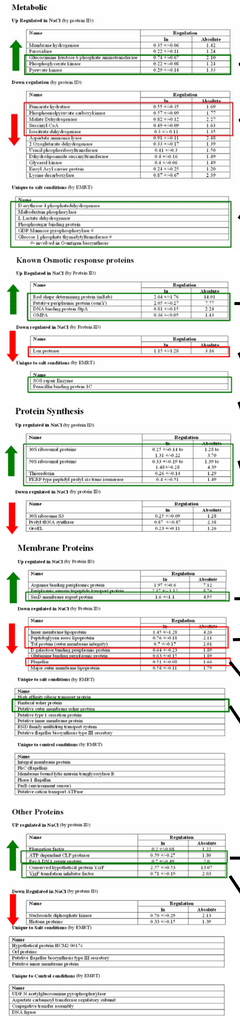


Figure 3- Metabolic enzymes down regulated in salt shock: The down regulation of metabolic enzymes is significant during conditions of salt shock. The first five enzymes in the histogram (fumarate hydratase to isocitrate dehydrogenase) are involved in the Krebs cycle and are down regulated by 1.5- 2 fold, suggesting a decrease in energy derived from aerobic respiration. This may be due to products from the glycolysis pathway being diverted to the trehalose pathway to cope with the low water conditions. Other pathways affected by metabolic down regulation appear to include fatty acid metabolism, amino acid synthesis and nucleic acid synthesis.



Under conditions of salt shock it appears that several glycolysis enzymes are up regulated, whilst many of the enzymes involved in the Krebs cycle are down regulated. This may be indicative that the cells are switching to a more anaerobic based respiration. An alternative suggestion is that sugars in the glycolysis pathway are being drawn off to the trehalose pathway, which is a known response in hyper osmotic stress (1). As a response to the decreasing availability of Acetyl CoA from glycolysis the enzymes associated with the Krebs cycle may be down regulated.

Also there are a number of sugar enzymes unique to the salt stress conditions, indicating that perhaps alternative carbohydrate sources are being utilised. O-antigen biosynthesis (a virulence related response) appears to have started.

Rod shape determining protein has been shown to elongate the cells to reduce their volume to surface area ratio (2), and is highly up regulated. OsmY, the hyperosmotically inducible periplasmic protein has been shown to be up regulated by around 8 times in *E.coli* under salt stress (3), and has clearly been up regulated in this case. The function of this protein is still largely unkown. StpA is a transcription factor, which stimulates a number of genes including virulence genes (4). Outer membrane protein A, or OmpA, is a β-barreled membrane protein thought to span the membrane and have the ability to work as a gated pore (5). It is this family of proteins that can stimulate the immune response.

The Lon protease is a repressor of cellular invasion and pathogenicity (7), down regulation of this indicates increased virulence.

Both the SOS repair enzyme and the *fts* gene products (of which the Penicillin binding protein is one) have been implicated in filament formation in Salmonella (6), which is a typical intercellular response to low water potential.

There are a variety of ribosomal proteins up regulated by differing amounts, indicating a general increase in protein production, supported by the up regulation of Thioredoxin.

SecD is a cytoplamic chaperone, responsible for translocation to the cell membrane

Parts of the Tol-Pal membrane integrity system are perhaps being down regulated to allow membrane growth. Also the down regulation may be a product of the required energy for maintenance being diverted towards other pathways.

Down regulation of Flagellin may imply that the cells no longer require motility as they are preparing for cellular invasion. Alternatively the cells may be conserving ATP as a survival mechanism.

Fimbrial usher proteins are used to bind to host cell glycoproteins prior to cellular invasion

ATP dependant ClpP is involved in the degredation of mis-folded proteins, generated by stress . Its up regulation under salt stress is therefore unsurprising. Also DNA repair factor is up regulated, perhaps alongside the SOS DNA repair system, suggesting that DNA damage occurs during salt stress.

The function of these highly conserved hypothetical proteins is largely unknown but is thought to be vital for life. The clear changes in their regulation here may be of great importance.

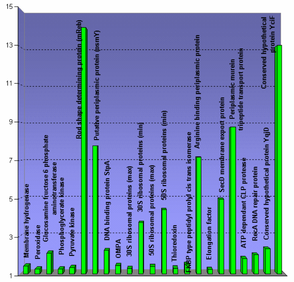


Figure 4- Overview of proteins up regulated in salt shock: Proteins related to the cell membrane appear to have been up regulated by the greatest amount, which is to be expected if we assume that the cell membrane is being strengthened to cope with the osmotic change. Rod shape determining protein (mReb) has been shown to elongate the cells to reduce their volume to surface area ratio and this is highly up regulated, presumably to strengthen the cell and reduce the effect of osmotic stress. A number of other processes look to be altered, including the regulation of highly conserved hypothetical gene products whose functions are unknown (YqiD, YciF). Protein synthesis mechanisms (ribosomal proteins & thioredoxin) are apparently up regulated, possibly to increase production of other promoted proteins, as well as the necessary machinery to export these products to the membrane (SecD). DNA repair appears to be initiated (RecA) and mis-folded salt damaged proteins are being degraded (CLP protease).

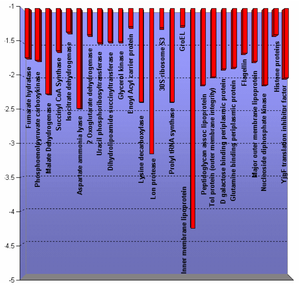


Figure 5- Overview of down regulated proteins in salt shock: The most notable down regulated group of proteins are those involved in the Krebs cycle, potentially indicating a switch to anaerobic respiration, although it should be noted that this group do not exhibit the largest expression changes. A rise in salt concentration often triggers more than just osmotic defence mechanisms, the change in osmolarity is also interpreted by the cell as transfer from fresh water to a gut environment. The large down regulation in the Lon protease (which represses virulence) is indicative of the cell beginning to switch to a cell invasion state. In correlation with this Flagellin is being down regulated, as the cell will no longer require motility once in the gut. Alternatively Flagellin is down regulated to conserve ATP for survival purposes (8)

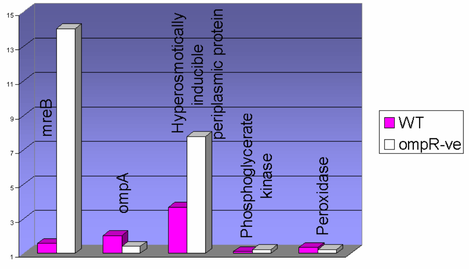


Figure 6- Comparison of salt stress protein regulation in ompR-ve mutant and Wild Type: Both the wild type and the ompR-ve mutant deal with salt stress in similar ways, PGK'ase and peroxidase are up regulated in equivalent amounts. The mutant appears to produce slightly less ompA, but there are far more profound effect for other proteins. The up regulation of the hyperosmotically inducible protein osmY is doubled in expression over that of the wild type. Expression of the rod shape determining protein mreB is highly elevated with respect to the wild type. Presumably these large increases in expression are to counteract the effects in under production of certain porins in the mutant.

Summary

- In the work presented here we have studied the effect of osmotic stress on *Samonella enterica* and compared the proteomic profiles obtained from a wild type and OMPR –ve mutant
- This study was performed using an LC-MS based approach on a Q-Tof mass spectrometer without using isotope labelling
- A significant number of up and down regulated proteins across a broad range of functions were identified and quantified
- In particular this approach allowed us to quantitatively study changes in membrane related proteins
- The important shape determining protein, mreB, was highly up-regulated in the wild type under conditions of salt stress and this was further accentuated in the OMPR –ve mutant

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