Label-free differential 2D-LC-MS/MS

The LC-MS/MS system consisted of an LTQ XL IT mass spectrometer (ThermoScientific, San José, CA) equipped with a microflow ESI source and interfaced to an LCPackings Ultimate Plus Dual gradient pump, Switchos column switching device, and Famos Autosampler (Dionex, Amsterdam, Netherlands). Two RP peptide traps C18 Pepmap 100 Dionex (0.30 mm x 5 mm) were used in parallel with two analytical BioBasic-C18 columns from ThermoScientific (0.18 mm x 150 mm). Samples (6.5 µl) were injected and desalted on the peptide trap equilibrated with solvent C at a flow rate of 30 µl/min. After valve switching, peptides were eluted in backflush mode from the trap onto the analytical column equilibrated in solvent D (5% ACN v/v, 0.05% v/v formic acid in water) and separated using a 100-min gradient from 0 to 70% solvent E (80% ACN v/v, 0.05% formic acid in water) at a flow rate of 1.5 µl/min.

The mass spectrometer in positive mode was set up to acquire one full MS scan in the mass range of 300–2000 m/z, followed by five MS/MS spectra of the five most intense peaks in the mass range 300–1600 m/z. The dynamic exclusion feature was enabled to obtain MS/MS spectra on coeluting peptides, and the exclusion time was set at 1.5 min.

Protein identification and quantification

Raw data collection of approximately 222,000 MS/MS spectra per 2D-LC-MS/MS experiment was followed by protein identification using SEQUEST. In details, peak lists were generated using extract-msn (ThermoScientific) within Proteome Discoverer 1.3. From raw files, MS/MS spectra were exported with the following settings: peptide mass range: 350–3500 Da, minimal total ion intensity 500. The resulting peak lists were searched against a target-decoy *Pseudomonas aeruginosa* protein database (release 25.10.2011, 11128 entries comprising forward and reversed sequences obtained from Uniprot) using Sequest by

comparison with the theoretical spectra of all possible peptides fragments from the target-decoy database. The following parameters were used: trypsin was selected with proteolytic cleavage only after arginine and lysine, number of internal cleavage sites was set to 1, mass tolerance for precursor and fragment ions was 1.0 Da, considered modifications were +15.99 Da for oxidized methionine, +125.12 for N-ethylmaleimide on cysteines and +57.05 Da for carboxyamidomethylcysteine. Peptide matches were filtered using the q-value and Posterior Error Probability calculated by the Percolator algorithm ensuring an estimated false positive rate below 5% (1). The filtered SEQUEST output files for each peptide were grouped according to the protein from which they were derived using the multiconsensus results tool within Proteome Discoverer. The analysis was repeated on biological triplicates for each strain. Sampling statistics such as unique peptides, spectral counts and sequence coverage were exported in Microsoft Excel spreadsheets. The spectral counts data were normalized by dividing the protein spectral count in a particular experiment by the average spectral count across all the proteins in that experiment. Relative quantification of protein abundance was estimated by calculating the ratio of normalized spectral counts, statistical significance was tested with the unpaired Student's t-test, and significance was defined as a p 0.05 (two-tail two-sample equal variance test). Statistical analysis was performed using the Microsoft Excel spreadsheet software.

Reference

1. Kall L, Canterbury JD, Weston J, Noble WS and MacCoss MJ, Nat Methods 4:923-925, 2007