Multi-omic profiling to assess the effect of iron starvation in *Streptococcus pneumoniae* TIGR4

Irene Jiménez-Munguía[1], Mónica Calderón-Santiago[2], Antonio Rodríguez-Franco[1], Feliciano Priego-Capote[2], Manuel J. Rodríguez-Ortega[1] *.

[1]Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba; Campus de Excelencia Internacional CeiA3, Córdoba (Spain).

[2]Departamento de Química Analítica, Universidad de Córdoba; Campus de Excelencia Internacional CeiA3, Córdoba (Spain).

*Corresponding author:

Manuel J. Rodríguez-Ortega

Address: Departamento de Bioquímica y Biología Molecular, Edificio "Severo Ochoa" planta baja, Campus de Rabanales, Universidad de Córdoba. 14071 Córdoba, Spain.

Tel: +34 957 218519

Fax: +34 957 218856

e-mail: mjrodriguez@uco.es

Abstract

We applied multi-omics approaches (transcriptomics, proteomics and metabolomics) to study the effect of iron starvation on the Gram-positive human pathogen *Streptococcus pneumoniae* to elucidate global changes in the bacterium in a condition similar to what can be found in the host during an infectious episode. We treated the reference strain TIGR4 with the iron chelator deferoxamine mesylate. DNA microarrays revealed changes in the expression of operons involved in multiple biological processes, with a prevalence of genes coding for ion binding proteins. We also studied the changes in protein abundance by 2-DE followed by MALDI-TOF/TOF analysis of total cell extracts and secretome fractions. The main proteomic changes were found in proteins related to the primary and amino sugars metabolism, especially in enzymes with divalent cations as cofactors. Finally, the metabolomic analysis of intracellular metabolites showed altered levels of amino sugars involved in the cell wall peptidoglycan metabolism. This work shows the utility of multi-perspective studies that can provide complementary results for the comprehension of how a given condition can influence on global physiological changes in microorganisms.

Introduction

Bacteria need a plethora of factors for optimal growth, being iron an essential micronutrient. Within the human body, the free concentration of this element is approximately 10^{-18} M (Yang et al. 2014), which is too low to hold a growth capable of supporting bacterial infections. The low concentrations of free iron within the host are due to the scavenging of this element by different high-affinity proteins (e.g. transferrin, lactoferrin, haemoglobin, etc.) (Froehlich et al. 2009). Therefore, in order to survive in the host, pathogens need to develop especial strategies to uptake the minimum amount that they require of such a nutrient (Nanduri et al. 2008), as the direct extraction of this metal cation from host iron-containing proteins or by capturing ferric-binding siderophores from host environments via ABC transporters (Ge & Sun 2014). Moreover, the capacity of bacterial pathogens for iron acquisition represents itself an important virulence determinant (Kanaujia et al. 2015). In addition, pathogens can also modify their energetic metabolism to adapt it to the environmental situation within the host.

Streptococcus pneumoniae, also known as the pneumococcus, is a Gram-positive microorganism that lives as a commensal in the human respiratory tract and that, under appropriate circumstances, becomes pathogenic, being able to cause high morbidity and mortality (Blasi et al. 2012; Olaya-Abril et al. 2014b). This bacterium is a major cause of mucosal diseases such as otitis media and sinusitis, and is a prevalent pathogen in different invasive diseases including pneumonia, bacteremia, meningitis, and sepsis (O'Brien et al. 2009). Pneumococcal pneumonia, which is the major clinical manifestation of pneumococcal infections, affects mainly young children and the elderly although all age groups may undergo it. It has been estimated that almost one million children die every year because of pneumococcal diseases, with >90% of these deaths occurring in developing countries (Johnson et al. 2010). Pneumococcal infections represent also a high burden of disease in adults of developed countries. Actually, around 25,000 deaths are registered every year in the

United States in adults > 50 years of age, and in European countries the pneumococcus also causes significant mortality and morbidity (Olaya-Abril et al. 2013; Weycker et al. 2010). Therefore, understanding the basics of host-pathogen interactions is critical to effectively fight against infections.

In the context of systems biology, the use of massive analysis platforms is highly valuable to understand biological processes (Fondi & Lio 2015; Kohlstedt et al. 2014). However, single –omic datasets, although powerful, offer a partial view of a biological system (Grady et al. 2017). Studying the responses of any biological system to a given condition using transcriptomics, proteomics and/or metabolomics can greatly help to elucidate the global adaptation to such a condition, i.e. stress, pathological status, nutrient availability/limitation, etc (Dall'Agnol et al. 2014; Feng et al. 2011; Fu et al. 2013; Yang et al. 2012). To this regard, we have approached from the *in vitro* bacterial culture to what it should actually occur *in vivo*, mimicking the iron restriction in order to describe the global changes that the pneumococcus undergoes and, therefore, to understand mechanisms of adaptation when it infects the host. To this end, we have studied the responses of the reference pneumococcal strain TIGR4 in an iron-deprived medium, at three different –omic levels: transcriptomics, proteomics and metabolomics. We have identified sets of genes, proteins and metabolites that are differentially expressed/synthesized under the studied nutrient restriction.

Materials and methods

Bacterial strains and culture conditions

S. pneumoniae TIGR4 was grown without agitation at 37 ºC in air with 5% CO_2 in Todd Hewitt Broth (THB) until mid-exponential phase (OD_{600} = 0.3), and kept at -80 ºC with 20% glycerol. Three different biological replicates were made for proteomics and metabolomics experiments, and 4 replicates for transcriptomics (20 mL, 100 mL, and 200 mL cultures to perform transcriptomics, metabolomics, and proteomics experiments, respectively). Each set of replicates was standardised by inoculum, using starter cultures from glycerol-kept vials, in order to prepare the standard inoculum that was further added to the different biological replicates for each "ome" extraction. Iron-depleted cultures were prepared by adding deferoxamine (DFO) mesylate salt (Sigma) dissolved in water, at 100 μM, according to the dose used for this chelator in other works for the pneumococcus (Trappetti et al. 2011).

Protein extracts

Total cellular proteins and secreted proteins were obtained as described (Mitsuwan et al. 2017). Briefly, to obtain cellular proteins, pellets were washed three times in sterile phosphate buffered saline (PBS) pH 7.4. Bacterial cell wall was digested at 37 ºC with top-down agitation by adding 100 U mutanolysin (Sigma–Aldrich). Protoplasts were resuspended in 4 mL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 0.005% bromophenol blue, 0.5% Bio-lyte 3-10 ampholytes (Bio Rad)) and disrupted by sonication (6 cycles; 20-s pulses, 90% amplitude). Proteins were recovered by centrifugation (5,000 × *g,* 7 min), dialyzed and concentrated using a centrifugal filter device (Amicon Ultra15, 10 kDa; Millipore). To obtain secretome fractions, proteins were precipitated from the supernatants with 10% trichloroacetic acid, after removing cell debris through filter devices (0.22 μm,

Millipore). Protein pellets were washed twice with 1 mL of ice-cold absolute ethanol. Finally, proteins were air-dried and resuspended in 500 μL of rehydration buffer.

Two-dimensional polyacrylamide gel electrophoresis and image analysis

Protein samples were cleaned using the 2-D clean up kit (Life Sciences) according to manufacturer's instructions. Proteins were resuspended in 200 μL of rehydration buffer and quantified by the Bradford method (Bradford 1976). Five hundred μg of protein were subjected to isoelectric focusing (IEF) on 18 cm Immobiline DryStrips immobilized pH gradient (IPG) gel strips (4 – 7 pH linear gradient (Life Sciences)). The strips were loaded onto a BioRad Protean IEF Cell system, and IEF was performed at 20 ºC using the following conditions: 2 h of passive rehydration, 50 V for 10 h followed by a voltage-ramp (250 V for 15 min; 500 V for 30 min; 1,000 V for 1 h; 2,000 V for 1 h; 5,000 V for 1 h; 8,000 V for 2 h); finally, proteins were focused on 70,000 Vh. Before the second dimension, the IPG strips were first soaked for 15 min in equilibration solution (50 mM Tris- HCl buffer, pH 8.8, 6 M urea, 30% v/v glycerol, 2% SDS, and bromophenol blue traces) containing 2.5 mg/mL DTT, and subsequently soaked for 15 min in equilibration solution containing 45 mg/mL iodoacetamide. The second dimension was performed on 12% polyacrylamide gels, using the Protean plus Dodeca Cell system (Bio Rad). Gels were run at 90 V until the dye reached the bottom. Then, gels were stained with brilliant blue G-colloidal solution (Sigma-Aldrich) according to manufacturer's instructions. Gels were scanned with a GS-800 densitometer (Bio-Rad). Digitized images were analyzed with PD-Quest v8.1.0 (Bio Rad). Two analytical gels were made per sample (i.e. biological replicate and protein extraction). Consistent spots were considered as those whose presence remained constant at the three biological replicates. Spots showing a consistent change in the intensity value of at least 2-fold change were included in the quantitative analysis.

Protein identification by MALDI-TOF/TOF MS/MS

Spots excision, protein digestion, peptide desalting and mass spectrometry analysis was performed as already described by our group (Mitsuwan et al. 2017) with slight modifications: after mass spectra acquisition using a MALDI-TOF/TOF (4800 Proteomics Analyzer, Applied Biosystems) mass spectrometer in the m/z range 800 to 4,000, Mascot 2.0 search engine (Matrix Science Ltd., London) was used for protein identification running on GPS Explorer^{TM} software v3.5 (Applied Biosystems) over the National Center for Biotechnology Information (NCBI) protein database (updated monthly). Search setting allowed one missed cleavage with the selected trypsin enzyme, *Streptococcus pneumoniae* for taxonomy restrictions, cysteine carbamidomethylation as a fixed modification, methionine oxidation as a variable modification, a MS/MS fragment tolerance of 0.2 Da, and a precursor mass tolerance of 10 ppm. Identifications with a Mascot score >70 (p-value < 0.05) were considered as significant.

Inductively coupled plasma-mass spectrometry (ICP-MS) analysis

Iron concentration in THB medium was determined by ICP-MS. Five ml aliquots of samples were chemically digested with 2 mL concentrated nitric acid on a hot plate with a heating ramp of 20 ºC until reaching 130 ºC, maintaining this temperature for two hours. High purity deionized water was added to the digested samples to a final volume of 25 mL. Rh-solution was added as internal standard (final concentration of 10 µg/L). The isotope ^{56}Fe and the internal standard were analyzed with a NexION 350X instrument (PerkinElmer), equipped with a PFA concentric microFlow nebulizer and a cyclonic PFA spray chamber, and operated at 1600W in He collision mode. Results were expressed as µg of Fe per liter of culture.

RNA isolation

147 Cells resuspended in 1 mL of Tri-Reagent (Sigma–Aldrich) were disrupted by vortexing (20
148 min) with 0.5 g of glass beads (Sigma–Aldrich). After recovering the supernatant by
149 centrifugation (1 min, 12,000 × *g*, 4 ºC), 200 μL of chloroform were added. Samples were
150 centrifuged again (12,000 × *g* for 15 min; 4 ºC) and 500 μL of ice-cold isopropanol were
151 added. After 15 min incubation (4º C), samples were centrifuged (30 min, 12,000 × *g*, 4 ºC)
152 and washed with 500 μL of 70% ice-cold ethanol. RNA was air-dried and resuspended in 40
153 μL of distilled water previously treated with 1% of diethylpyrocarbonate (DEPC). Samples
154 were treated with DNAse (Ambion) according to manufacturer's instructions.

155 **RNA amplification, labeling and hybridization to DNA microarrays**

156 RNA quality was assessed using a TapeStation (Agilent Technologies). The RNA integrity
157 number (RIN) ranged between 7.0 and 9.2. Samples with RIN > 7 were considered for
158 analysis. RNA concentration and dye incorporation was measured using a UV-VIS
159 spectrophotometer (Nanodrop 1000, Agilent Technologies). Hybridization to custom 8 × 15K
160 Gene Expression Microarrays (ID 044371, Agilent Technologies) containing the whole
161 genome of *S. pneumoniae* TIGR4 was conducted following manufacturer's protocol using a
162 two-color (Cy3 and Cy5) Microarray-Based Gene Expression Analysis (v. 6.5, Agilent
163 Technologies). Microarrays were then washed and scanned using a DNA Microarray Scanner
164 (Model G2505C).

165 **Gene expression analysis**

166 Microarray hybridization data were obtained with the Feature Extraction Software v. 10.7
167 (Agilent Technologies), using the default variables. Data analysis was performed using the R
168 Limma *Bioconductor* package (Ritchie et al. 2015), according to a direct two color design. A
169 total of 8 microarrays were done, corresponding to 4 biological replicates for each condition
170 using swapped and random mixtures to cope with batch effects. Functional annotation of the

differentially expressed genes was done using embedded BlastX included into the *Blast2GO* program (Conesa et al. 2005) using the public NCBI nr database. Raw feature intensities were corrected using the *Normexp* background correction algorithm. An initial within-array normalization was done using spatial and intensity-dependent *Loess* method, followed by a between-array *Aquantile* normalization. Normalized data are shown in Figs. S1 and S2. Differential expression was ordered according their adjusted *p*-values, and the expression of each gene is reported as the $\log_2$ ratio of the value obtained of each condition compared to control condition. A gene was considered differentially expressed if it displayed an adjusted *p*-value less than 0.05 by the Student *t*-test. Finally, over- and under- expressed genes were analyzed in terms of gene ontology by using a hypergeometric analysis (GOStats package). Prediction of operons was obtained from the DOOR database (Dam et al. 2007; Mao et al. 2009).

Preparation of intracellular metabolite samples

Cell pellets were washed twice with PBS and resuspended in lysis buffer (PBS and 30% sucrose) containing 100 U mutanolysin. Samples were incubated at 37 ºC overnight. Metabolic quenching was achieved by adding ice-cold 50% methanol. Cells were disrupted by sonication (6 cycles: 20 s, 90% amplitude). After centrifugation (5,000 × *g*, 7 min), cell debris was separated through 0.22 µm membrane filters (Millipore). Then, supernatants were collected and ultracentrifuged (100,000 × *g*; 1.5 h, 4 ºC). Finally, metabolite samples were kept at -80 ºC before analysis.

Analysis of intracellular metabolites

Intracellular metabolite samples were analyzed by LC–QTOF MS/MS using an Agilent 1200 Series LC system coupled to an Agilent 6540 UHD Accurate-Mass QTOF hybrid mass spectrometer equipped with dual electrospray (ESI) source as described (Mitsuwan et al.

2017) without modifications. Briefly, chromatography was performed using a C18 reverse-phase analytical column (Mediterranean, 50 mm × 0.46 mm *i.d.,* 3 µm particle size; Teknokroma, Barcelona, Spain), thermostated at 25 °C. The mobile phases were 5% ACN (phase A) and 95% ACN (phase B) both with 0.1% formic acid as ionization agent. The LC pump was programmed with a flow rate of 0.8 mL/min with the following elution gradient: 3% phase B was kept as initial mobile phase constant from min 0 to 1; from 3 to 100% of phase B from min 1 to 13. A post-time of 5 minutes was set to equilibrate the initial conditions for the next analysis. The injection volume was 3 µL and the injector needle was washed for 10 times between injections with 80% methanol. The parameters of the electrospray ionization source, operating in negative and positive ionization mode, were as follows: the capillary and fragmentor voltage were set at ±3.5 kV and 175 V, respectively; N_2 in the nebulizer was flowed at 40 psi; the flow rate and temperature of the N_2 as drying gas were 8 L/min and 350 °C, respectively. MS and MS/MS data were collected in both polarities using the centroid mode at a rate of 2.6 spectra per second in the extended dynamic range mode (2GHz). Accurate mass spectra in auto MS/MS mode were acquired in bot MS and MS/MS *m/z* ranges of 60-1,100 Da. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses by using the signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H,1H,3H-tetrafluoropropoxy) phosphazine or HP-921] in positive ion mode; while in negative ion mode ions with m/z 119.0362 (proton abstracted purine) and m/z 966.0007 (formate adduct of HP-921) were used. The auto MS/MS mode was configured with 2 maximum precursors per cycle and an exclusion window of 0.25 min after 2 consecutive selections of the same precursor. The collision energy selected was 20 V.

Metabolomics data processing and identification

MassHunter Workstation software (version 5.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used to process all data obtained by LC–QTOF in auto MS/MS mode. Treatment of raw data files was initiated by extraction of potential molecular features (MFs) with the suited algorithm included in the software. For this purpose, the extraction algorithm considered all ions exceeding 1,000 counts with a single charge state. Additionally, the isotopic distribution to consider a molecular feature as valid should be defined by two or more ions (with a peak spacing tolerance of 0.0025 *m/*z, plus 10.0 ppm in mass accuracy). Adducts formation in the positive (+Na) and negative (+HCOO) modes, as well as neutral loss by dehydration were included to identify features corresponding to the same potential metabolite. Then, MFs, characterized by their retention time, intensity in the apex of chromatographic peak and accurate mass, were exported in compound exchange format files (.cef files) and imported into Mass Profiler Professional (MPP) software package (version 12.1, Agilent Technologies, Santa Clara, CA, USA) for alignment and further processing. MPP allowed statistical analysis by Volcano plot (a combination of analysis of variance and fold change analysis). The MS/MS METLIN Personal Compound and Database Library (PCDL) was used to identify significant compounds using both MS and MS/MS information to assure metabolite identification. To identify compounds with no MS/MS information in the METLIN Database, MetaCyc database, and the MassBank database were used.

Statistical analysis

All the quantitative analyses (transcriptomics, proteomics, and metabolomics) were performed from three or four independent biological replicates, and the results are expressed as the mean ± standard deviation. Paired data were analyzed by univariate analysis using the Student's *t*-test. Principal component analysis (PCA) was done with the web-based software NIA array analysis tool (http://lgsun.grc.nia.nih.gov/anova/index.html) (Sharov et al. 2005). *p*-Values lower than 0.05 were considered statistically significant.

Results

Effect of iron starvation on pneumococcal growth *in vitro*

S. pneumoniae TIGR4 was grown in THB, in which the iron concentration available for the bacteria, measured by ICP-MS, was 734 μg/L. In order to perform the further transcriptomic, proteomic and metabolomic analyses, we firstly monitored the growth of this strain in presence of 100 μM DFO. As observed for other microorganisms, iron depletion caused a slight lag phase in the growth of pneumococcus (control growth rate: 1.93 h^{-1}; DFO-treated culture growth rate: 1.39 h^{-1}), and instead lead to a decrease in the OD at the plateau phase (Fig. 1). We chose the mid-exponential phase for sampling RNA, proteins and metabolites in curve points such that almost no delays in the time points of collecting cultures took place. These corresponded to OD = 0.3.

Transcriptomics

Limma analysis of the signals obtained after hybridizing the RNAs with the arrays revealed a number of differentially expressed genes when comparing the DFO treatment with its control (Supplemental Dataset 1): 338 genes changed significantly after DFO exposure, of which 118 genes increased their expression and 220 were down-regulated. Fig. S3 shows the differentially expressed genes as a Volcano plot.

The $\log_2$ fold change values, resulting from averaging four independent biological replicates for each condition, ranged between 1.78 and -2.13. We noticed a consistent differential expression in which all of the genes pertaining to a same operon (according to the DOOR annotation database) changed in the same trend and even experienced almost identical $\log_2$ fold change values. We grouped the genes into operons according to the DOOR database (Supplemental Dataset 1). In total, 29 operons were clearly up-regulated, including those

coding for the iron-compound ABC transporter system (genes SP_1869, SP_1870 and SP_1871), the closely neighbour gene SP_1872 coding for the iron-compound ABC transporter, the manganese ABC transporter system (genes SP_1648, SP_1649 and SP_1650), the ATP synthase complex (genes SP_1509 to SP_1513), and others including several transporter systems. Also, an operon containing genes for the riboflavin biosynthetic system (genes SP_0175 to SP_0178) was clearly up-regulated. Forty-four operons were clearly down-regulated, including the branched-chain amino acid transporter system (genes SP_0750 to SP_0753) and the large operon of 13 genes (SP_0419 to SP_0431) involved in fatty acid biosynthesis, as well as two operons involved in competence (genes SP_0042 and SP_0043, and genes SP_2235 and SP_2236). In all the cases, the $\log_2$ FC values were almost identical for all the genes of the same operon. Table 1 shows the main operons that were differentially regulated.

The differentially expressed genes were annotated with Gene Ontology terms, using Blast2GO (Fig. 2). According to the molecular function level, the most enriched term among the over-expressed genes corresponded to "ion binding" (GO:0043167), representing around 25% of the genes. At the biological process level, the most enriched term was "cell metabolic process" (GO:0044237). For down-expressed genes, the most prevalent term at the biological process level was the same as for over-expressed genes, i.e. "cell metabolic process", and at the molecular function level, 3 different terms, including also that of "ion binding" (GO:0043167), represented around 13% each.

We validated the results obtained in the microarrays with RT-q-PCR on a subset of 16 differentially expressed genes of the TIGR4 strain (Table S1). For all the genes, there was the same trend (either over- or down-expression) and a high correlation (r^2 = 0.93) in the fold changes measured both at the microarray and the RT-q-PCR, thus confirming that the microarray data were reliable.

292 **Proteomics**

293 We compared the proteomes of two different protein fractions, cell extracts and secreted
294 proteins, by 2-DE under iron deprivation conditions (Fig. S4). We then analyzed both the
295 qualitative (i.e. absolute appearance or disappearance of a protein in a condition compared to
296 the other one) and quantitative changes (i.e. changes in protein abundance on spots present in
297 both conditions) when comparing for each protein fraction the iron starvation condition with
298 its non-iron deprived control (Tables 2 and 3).

299 For the two analyzed protein fractions, most of the changes corresponded to predicted
300 cytoplasmic proteins, mainly affecting enzymes of the primary metabolism. Glyceraldehyde-
301 3-phosphate dehydrogenase was less abundant under iron deprivation. This enzyme was
302 strongly reduced in total extracts after DFO treatment (fold-change decrease >4), as well as
303 not detected in the secretome fraction of DFO-exposed culture (but detected in the control
304 secretome). Another enzyme of the glycolysis pathway that also decreased its abundance was
305 the phosphoglycerate kinase. Other two Mg^{2+}-dependent enzymes of the glycolysis pathway,
306 enolase and pyruvate kinase, also decreased in secretomes of DFO-exposed cells. We also
307 found that one enzyme of the fatty acid biosynthesis pathway was more abundant under iron
308 deprivation: 3-oxoacyl-[acyl-carrier protein] reductase in total extracts (fold change around
309 7). Also, two major and very abundant extracellular proteins were detected to decrease in
310 DFO-treated cultures: the choline-binding protein A (SP_2190) and the PcsB protein
311 (SP_2216).

312 We also found a decrease in the abundance levels of an enzyme taking part in the recycling
313 pathway of amino sugar compounds: the divalent cation-dependent *N*-acetylglucosamine-6-
314 phosphate deacetylase, SP_2056 (NagA), which was found to be almost 9-fold less abundant
315 in DFO-exposed secretomes.

316 Other cation-containing or dependent enzymes decreased their abundances under iron
317 deprivation conditions. Thus, lower levels of zinc-containing alcohol dehydrogenase and
318 manganese-dependent inorganic pyrophosphatase were detected, as well as of hypoxanthine-
319 guanine phosphoribosyltransferase, which requires Mg^{2+} as a cofactor.

320 **Metabolomics**

321 Finally, we studied the changes in the metabolic profile of intracellular metabolite fractions
322 from both pneumococcal strains, by using LC–MS/MS, which allowed detection of 719 and
323 826 different chromatographic peaks, in negative and positive ionization mode, respectively.
324 Statistical analysis by Volcano plot revealed that 64 entities presented a *p*-value below 0.05
325 and a fold change, in terms of relative concentrations, higher than 2 for discrimination
326 between treated and non-treated TIGR4 samples (data not shown).

327 A multivariate statistical analysis was carried out with significant entities to evaluate whether
328 the iron deprivation treatment had an effect on the metabolite profile. Fig. S5 shows the
329 principal component analysis (PCA) of identified metabolites when comparing iron-depleted
330 and non-depleted cultures, revealing that the first principal component (X-axis) clearly
331 grouped the three control biological replicates, which were clearly separated from two out of
332 the three DFO-treated samples. However, there was dispersion in the three biological
333 replicates of the DFO treatment, as the first principal component did not group them.

334 The tentative identification of significantly changing entities led to a panel of 17 compounds
335 (Table 4). Although the number of changing metabolites positively identified was low, we
336 clearly found an increase in the concentration of intermediate amino sugar metabolites
337 involved in the cell wall peptidoglycan metabolism: there was an increase in uridine-5'-
338 diphosphoglucuronic acid (2.2-fold), UDP-*N*-acetylmuraminate (>20-fold), *N*-
339 acetylglucosamine (3.8-fold), and UDP-*N*-acetylglucosamine (2.4-fold).

Discussion

There is little knowledge about the mechanisms of iron intake by the pneumococcus (Hoyer et al. 2018). In this work, we have approached a multi-omics strategy to understand the changes at the molecular level occurring in the pneumococcus during iron deprivation, similarly to what theoretically happens during an *in vivo* infection. To our knowledge, this is the unique study carried out so far for iron deprivation in this human pathogen using three different "omics". Very recently, a combined translatomics/proteomics approach has been applied to identify novel iron-transporting proteins in the pneumococcus (Yang et al. 2016). Previously, it had been approached using only proteomics (Nanduri et al. 2008; Yang et al. 2015).

We selected as iron chelator the DFO, which has been described to have high iron chelating specificity, although it might also sequester other divalent cations (Eichenbaum et al. 1996). We chose the culture points at which almost no alterations in growth were observed, as described for other studies in bacteria in which this iron chelator was used at the same or very similar concentration (Basler et al. 2006; Smith et al. 2001; Trappetti et al. 2011). These conditions were applied to obtain transcriptome, proteome and metabolome preparations of the reference pneumococcal strain TIGR4.

Our transcriptomic analysis showed very consistent and reproducible results between biological replicates. The range of $\log_2$ fold changes was apparently low, ranging between 1.78 and -2.13, but the numbers obtained were in general quite similar to those observed for transcriptomic analyses of iron starvation in other microorganisms (Allen et al. 2010; Basler et al. 2006; Brickman et al. 2011; Klitgaard et al. 2010; Madsen et al. 2006). However, very interestingly the changes observed in our work were in most cases for genes grouped in operons, and genes belonging to the same operon underwent, as expected, almost identical fold change values. This is another argument that confirms the validity of our microarray

results, as already described in similar works (Allen et al. 2010; Klitgaard et al. 2010; Vasileva et al. 2012).

In TIGR4, we found an up-regulation of 29 operons, and a down-regulation of 44 operons. Among those being over-expressed, we found two operons coding for iron-compound ABC transport systems, both of them localized together in the genome: the operon 38857 (genes SP_1869 to SP_1871) and the operon 1446903 (containing only one gene, SP_1872). The TIGR4 genome has other operon (no. 38677) containing four genes (SP_1032 to SP_1035) participating in a third iron-compound ABC system, but this operon was not found differentially expressed in our study. The up-regulation of the manganese ABC transporter system (operon 38805) could indicate that the chelator used is not completely specific for iron, as already known, but it cannot be ruled out that this operon might have a function related to iron uptake. Actually, recently Yang et al have reported the over-expression in the *S. pneumoniae* D39 strain of genes coding for sugar and other substrate-ABC transporters (Yang et al. 2016) and validated one of them at the protein level, thus indicating that iron deprivation may affect other transporter systems which are not annotated in databases as "iron transporters". We also found the up-regulation of the operon 38501, responsible for riboflavin biosynthesis. Very recently, it has been described in *Vibrio cholerae* the cross-modulatory effect between riboflavin and iron (Sepulveda-Cisternas et al. 2018). Among the down-expressed operons, we found some transporter systems, as the branched-chain amino acid ABC transporter system (operon 38620, genes SP_0750 to SP_0753), a fluoride ion transporter system (genes SP_1294 and SP_1295) or a phosphate ABC transporter system (genes SP_2085 to SP_2088). We ignore the meaning of these changes, but this work opens new possibilities to explore the role of these genes in the iron uptake.

In previous works, we have analyzed the surface proteome ("surfome") of the pneumococcus specifically targeting the discovery of vaccine or diagnostic candidates (Jimenez-Munguia et

al. 2015; Olaya-Abril et al. 2012; Olaya-Abril et al. 2013; Olaya-Abril et al. 2015). In this study, we searched for proteome changes using 2-D gel-based analysis on total cell extract and secretome fractions. As expected, most changes were in cytoplasmic proteins, as these are the most abundant ones in the bacterial cells. This class of proteins was also found in the secretome fractions, as extensively reported for numerous works in a wide variety of microorganisms (for an extensive review about the presence and role of cytoplasmic proteins in extracellular and/or surface protein preparations, see (Olaya-Abril et al. 2014a)).

Many of the changes observed in our work are coinciding with other results already described in the pneumococcus and other microorganisms. In the present study, the enzyme GAPDH was less abundant in iron-depleted protein fractions, as reported for pneumococcus (Nanduri et al. 2008) and *Staphylococcus aureus* (Friedman et al. 2006). This protein has been described as a moonlighting protein with different functions, including an important role in iron metabolism (Boradia et al. 2014). The zinc-containing alcohol dehydrogenase has been also described to be less abundant in iron-depleted *S. aureus* protein fractions (Friedman et al. 2006). We found a decrease in some Mg^{2+}-dependent glycolytic enzymes (phosphoglycerate kinase, enolase, pyruvate kinase), which might be due to the partially non-specific sequestration of this cation by DFO. Very similar results have been obtained in pneumococcal biofilms, where iron is less available than in planktonic cultures (Allan et al. 2014; Trappetti et al. 2011).

In a very recent paper, Hoyer and colleagues have studied the changes in the proteome of *Streptococcus pneumoniae* D39 under iron deprivation using 2,2'-bipyridine as chelator, in two different culture media (Hoyer et al. 2018). They have performed the proteomic analysis by LC-MS/MS, which is much more sensitive than our 2-DE/MALDI-TOF approach for detecting both high numbers of proteins and changes in their abundances. However, in spite of using different strains, growth media and proteomic approaches, by comparing our results

with those obtained in the THY medium in this cited work, there is a strong coincidence in the protein changes: 16 out of our 38 changing different proteins (namely SP_1284, SP_2190, SP_1456, SP_0459, 1534, SP_2012, SP_0236, SP_1508, SP_0421, SP_2055, SP_0499, SP_1489, SP_0897, SP_2056, and SP_1128) changed accordingly in both works. Many of the proteins are cation-dependent, as already described above. Interestingly, one of the common changes is the NagA.

Perhaps metabolomics data are generally the most difficult ones to integrate with the other "omes", as it is still a step backwards compared to transcriptomics and proteomics. There are very few studies on metabolomics in bacteria. We have recently published a work of proteomics and metabolomics integration in the pneumococcus to study the effect of an antimicrobial compound (Mitsuwan et al. 2017). Very recently, Leonard and colleagues have described the metabolome inventory of a non-encapsulated *S. pneumoniae* TIGR4 in a chemically-defined medium, and using three techniques: ^{1}H-NMR, HPLC-MS and GC-MS (Leonard et al. 2018). Although these results are not comparable to ours (different growth medium, different methods and purpose), the cited work reveals the identification of some tens of metabolites, with a predominance of precursors of peptidoglycan synthesis (UDP-MurNAc, UDP-GlcNAc) as in our study. In this present work, we unambiguously detected only a few compounds changing after DFO treatment. Partially, this could be due to the variability in the three DFO-treated metabolome samples, as observed in Fig. S5. In addition, we could not calculate the adenylate energy charge, a well accepted quality control in metabolomic preparations, as we did not unambiguously identified ATP in our samples. These two issues make that our metabolomic data should be interpreted with caution. Nevertheless, some of the changes detected were in metabolites related to peptidoglycan metabolism. Very interestingly, the enzyme NagA, taking part in the amino sugar metabolism, was clearly depleted. Actually, according to the UniProtKB database, NagA has divalent cations as

Luciana Leite 23/5/2018 10:58
Comment [1]: Suggested correction: identify

Luciana Leite 23/5/2018 10:59
Comment [2]: Suggested correction: indicate

cofactors, including Fe^{2+}, as it posseses a conserved domain belonging to the metallo-dependent hydrolases superfamily. NagA deacetylates GlcNAc-6P to GlcN-6P. The increase observed in our work in GlcNAc, a precursor of GlcNAc-6P, might be due to the decrease in enzymes like NagA that avoid their by-products conversions. This protein has been recently detected in the pneumococcal secreted fraction as in our work, and has been proposed as a pneumococcal diagnostic marker because of its high immunogenicity (Choi et al. 2013).

Luciana Leite 23/5/2018 11:00
Comment [3]: Suggested correction: possesses

In our opinion this work shows a possible unknown effect of iron deprivation on the global physiology of the bacterium, as it seems to be a relationship among iron starvation, the depletion of this enzyme and the alteration of the above discussed metabolites. Further research will be needed to go in depth in this aspect. This may help to identify pathways or biomolecules that can be used as targets for therapies against pneumococcal infection.

Conclusions

A global multi-omic analysis has been carried out to study the effect of iron limitation in the pneumococcus, similarly to what occurs during *in vivo* infection within the host. A significant number of genes changed in their expression, many of them involved in ion binding functions. Proteomics revealed changes in enzymes participating in the primary and peptidoglycan metabolism, many of them being cation-dependent. The metabolomic analysis revealed some changes in the levels of intermediates involved in the peptidoglycan biosynthesis. The different "omics" show sets of changing biomolecules that can complement themselves to provide a global insight into the adaptation of pneumococcus to iron starvation.

Availability of microarray data

The complete design of the microarray was deposited at the Gene Expression Omnibus NCBI database, with accession number GSE109693 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109693).

Acknowledgements

MALDI-TOF/TOF and ICP-MS analyses were performed at the Proteomics and the Mass Spectrometry Facilities, respectively (SCAI, University of Córdoba). We are indebted to members of the AGR-164 group, University of Córdoba, for lab support.

References

Allan RN, Skipp P, Jefferies J, Clarke SC, Faust SN, Hall-Stoodley L, and Webb J. 2014. Pronounced metabolic changes in adaptation to biofilm growth by Streptococcus pneumoniae. *PLoS One* 9:e107015. 10.1371/journal.pone.0107015

Allen KJ, Lepp D, McKellar RC, and Griffiths MW. 2010. Targeted microarray analysis of stationary phase Escherichia coli O157:H7 subjected to disparate nutrient conditions. *J Appl Microbiol* 109:2118-2127. 10.1111/j.1365-2672.2010.04843.x

Basler M, Linhartova I, Halada P, Novotna J, Bezouskova S, Osicka R, Weiser J, Vohradsky J, and Sebo P. 2006. The iron-regulated transcriptome and proteome of Neisseria meningitidis serogroup C. *Proteomics* 6:6194-6206. 10.1002/pmic.200600312

Blasi F, Mantero M, Santus P, and Tarsia P. 2012. Understanding the burden of pneumococcal disease in adults. *Clin Microbiol Infect* 18 Suppl 5:7-14. 10.1111/j.1469-0691.2012.03937.x

Boradia VM, Raje M, and Raje CI. 2014. Protein moonlighting in iron metabolism: glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Biochem Soc Trans* 42:1796-1801. 10.1042/BST20140220

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254.

Brickman TJ, Cummings CA, Liew SY, Relman DA, and Armstrong SK. 2011. Transcriptional profiling of the iron starvation response in Bordetella pertussis provides new insights into siderophore utilization and virulence gene expression. *J Bacteriol* 193:4798-4812. 10.1128/JB.05136-11

Choi CW, An HY, Lee YJ, Lee YG, Yun SH, Park EC, Hong Y, Kim GH, Park JE, Baek SJ, Kim HS, and Kim SI. 2013. Characterization of Streptococcus pneumoniae N-acetylglucosamine-6-phosphate deacetylase as a novel diagnostic marker. *J Microbiol* 51:659-664. 10.1007/s12275-013-3451-8

Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, and Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21:3674-3676. 10.1093/bioinformatics/bti610

Dall'Agnol HP, Barauna RA, de Sa PH, Ramos RT, Nobrega F, Nunes CI, das Gracas DA, Carneiro AR, Santos DM, Pimenta AM, Carepo MS, Azevedo V, Pellizari VH, Schneider MP, and Silva A. 2014. Omics profiles used to evaluate the gene expression of Exiguobacterium antarcticum B7 during cold adaptation. *BMC Genomics* 15:986. 10.1186/1471-2164-15-986

Dam P, Olman V, Harris K, Su Z, and Xu Y. 2007. Operon prediction using both genome-specific and general genomic information. *Nucleic Acids Res* 35:288-298. 10.1093/nar/gkl1018

Eichenbaum Z, Green BD, and Scott JR. 1996. Iron starvation causes release from the group A streptococcus of the ADP-ribosylating protein called plasmin receptor or surface glyceraldehyde-3-phosphate-dehydrogenase. *Infect Immun* 64:1956-1960.

Feng J, Billal DS, Lupien A, Racine G, Winstall E, Legare D, Leprohon P, and Ouellette M. 2011. Proteomic and transcriptomic analysis of linezolid resistance in Streptococcus pneumoniae. *J Proteome Res* 10:4439-4452. 10.1021/pr200221s

Fondi M, and Lio P. 2015. Multi -omics and metabolic modelling pipelines: challenges and tools for systems microbiology. *Microbiol Res* 171:52-64. 10.1016/j.micres.2015.01.003

Friedman DB, Stauff DL, Pishchany G, Whitwell CW, Torres VJ, and Skaar EP. 2006. Staphylococcus aureus redirects central metabolism to increase iron availability. *PLoS Pathog* 2:e87. 10.1371/journal.ppat.0020087

Froehlich BJ, Bates C, and Scott JR. 2009. Streptococcus pyogenes CovRS mediates growth in iron starvation and in the presence of the human cationic antimicrobial peptide LL-37. *J Bacteriol* 191:673-677. 10.1128/JB.01256-08

Fu F, Cheng VW, Wu Y, Tang Y, Weiner JH, and Li L. 2013. Comparative proteomic and metabolomic analysis of Staphylococcus warneri SG1 cultured in the presence and absence of butanol. *J Proteome Res* 12:4478-4489. 10.1021/pr400533m

Ge R, and Sun X. 2014. Iron acquisition and regulation systems in Streptococcus species. *Metallomics* 6:996-1003. 10.1039/c4mt00011k

Grady SL, Malfatti SA, Gunasekera TS, Dalley BK, Lyman MG, Striebich RC, Mayhew MB, Zhou CL, Ruiz ON, and Dugan LC. 2017. A comprehensive multi-omics approach uncovers adaptations for growth and survival of Pseudomonas aeruginosa on n-alkanes. *BMC Genomics* 18:334. 10.1186/s12864-017-3708-4

Hoyer J, Bartel J, Gomez-Mejia A, Rohde M, Hirschfeld C, Hess N, Sura T, Maass S, Hammerschmidt S, and Becher D. 2018. Proteomic response of Streptococcus pneumoniae to iron limitation. *Int J Med Microbiol*. S1438-4221(17)30471-X. 10.1016/j.ijmm.2018.02.001

Jimenez-Munguia I, van Wamel WJ, Olaya-Abril A, Garcia-Cabrera E, Rodriguez-Ortega MJ, and Obando I. 2015. Proteomics-driven design of a multiplex bead-based platform to assess natural IgG antibodies to pneumococcal protein antigens in children. *J Proteomics* 126:228-233. 10.1016/j.jprot.2015.06.011

Johnson HL, Deloria-Knoll M, Levine OS, Stoszek SK, Freimanis Hance L, Reithinger R, Muenz LR, and O'Brien KL. 2010. Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project. *PLoS Med* 7: e1000348. 10.1371/journal.pmed.1000348

Kanaujia PK, Bajaj P, Kumar S, Singhal N, and Virdi JS. 2015. Proteomic analysis of Yersinia enterocolitica biovar 1A under iron-rich and iron-poor conditions indicate existence of efficiently regulated mechanisms of iron homeostasis. *J Proteomics* 124:39-49. S1874-3919. 10.1016/j.jprot.2015.04.015

Klitgaard K, Friis C, Angen O, and Boye M. 2010. Comparative profiling of the transcriptional response to iron restriction in six serotypes of Actinobacillus pleuropneumoniae with different virulence potential. *BMC Genomics* 11:698. 10.1186/1471-2164-11-698

Kohlstedt M, Sappa PK, Meyer H, Maass S, Zaprasis A, Hoffmann T, Becker J, Steil L, Hecker M, van Dijl JM, Lalk M, Mader U, Stulke J, Bremer E, Volker U, and Wittmann C. 2014. Adaptation of Bacillus subtilis carbon core metabolism to simultaneous nutrient limitation and osmotic challenge: a multi-omics perspective. *Environ Microbiol* 16:1898-1917. 10.1111/1462-2920.12438

Leonard A, Gierok P, Methling K, Gomez-Mejia A, Hammerschmidt S, and Lalk M. 2018. Metabolic inventory of Streptococcus pneumoniae growing in a chemical defined environment. *Int J Med Microbiol*. S1438-4221. 10.1016/j.ijmm.2018.01.001

Madsen ML, Nettleton D, Thacker EL, and Minion FC. 2006. Transcriptional profiling of Mycoplasma hyopneumoniae during iron depletion using microarrays. *Microbiology* 152:937-944. 10.1099/mic.0.28674-0

Mao F, Dam P, Chou J, Olman V, and Xu Y. 2009. DOOR: a database for prokaryotic operons. *Nucleic Acids Res* 37:D459-463. 10.1093/nar/gkn757

Mitsuwan W, Olaya-Abril A, Calderon-Santiago M, Jimenez-Munguia I, Gonzalez-Reyes JA, Priego-Capote F, Voravuthikunchai SP, and Rodriguez-Ortega MJ. 2017. Integrated proteomic and metabolomic analysis reveals that rhodomyrtone reduces the capsule in Streptococcus pneumoniae. *Sci Rep* 7:2715. 10.1038/s41598-017-02996-3

Nanduri B, Shah P, Ramkumar M, Allen EB, Swiatlo E, Burgess SC, and Lawrence ML. 2008. Quantitative analysis of Streptococcus pneumoniae TIGR4 response to in vitro iron restriction by 2-D LC ESI MS/MS. *Proteomics* 8:2104-2114. 10.1002/pmic.200701048

O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, Lee E, Mulholland K, Levine OS, Cherian T, Hib, and Pneumococcal Global Burden of Disease Study T. 2009. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *Lancet* 374:893-902. 10.1016/S0140-6736(09)61204-6

Olaya-Abril A, Gomez-Gascon L, Jimenez-Munguia I, Obando I, and Rodriguez-Ortega MJ. 2012. Another turn of the screw in shaving Gram-positive bacteria: Optimization of proteomics surface protein identification in Streptococcus pneumoniae. *J Proteomics* 75:3733-3746. 10.1016/j.jprot.2012.04.037

Olaya-Abril A, Jimenez-Munguia I, Gomez-Gascon L, Obando I, and Rodriguez-Ortega MJ. 2013. Identification of potential new protein vaccine candidates through pan-surfomic analysis of pneumococcal clinical isolates from adults. *PLoS One* 8:e70365. 10.1371/journal.pone.0070365

Olaya-Abril A, Jimenez-Munguia I, Gomez-Gascon L, Obando I, and Rodriguez-Ortega MJ. 2015. A Pneumococcal Protein Array as a Platform to Discover Serodiagnostic Antigens Against Infection. *Mol Cell Proteomics* 14:2591-2608. 10.1074/mcp.M115.049544

Olaya-Abril A, Jimenez-Munguia I, Gomez-Gascon L, and Rodriguez-Ortega MJ. 2014a. Surfomics: shaving live organisms for a fast proteomic identification of surface proteins. *J Proteomics* 97:164-176. 10.1016/j.jprot.2013.03.035

589 Olaya-Abril A, Prados-Rosales R, McConnell MJ, Martin-Pena R, Gonzalez-Reyes JA,
590 Jimenez-Munguia I, Gomez-Gascon L, Fernandez J, Luque-Garcia JL, Garcia-Lidon
591 C, Estevez H, Pachon J, Obando I, Casadevall A, Pirofski LA, and Rodriguez-Ortega
592 MJ. 2014b. Characterization of protective extracellular membrane-derived vesicles
593 produced by Streptococcus pneumoniae. *J Proteomics* 106:46-60.
594 10.1016/j.jprot.2014.04.023

595 Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, and Smyth GK. 2015. limma powers
596 differential expression analyses for RNA-sequencing and microarray studies. *Nucleic*
597 *Acids Res* 43:e47. 10.1093/nar/gkv007

598 Sepulveda-Cisternas I, Lozano Aguirre L, Fuentes Flores A, Vasquez Solis de Ovando I, and
599 Garcia-Angulo VA. 2018. Transcriptomics reveals a cross-modulatory effect between
600 riboflavin and iron and outlines responses to riboflavin biosynthesis and uptake in
601 Vibrio cholerae. *Sci Rep* 8:3149. 10.1038/s41598-018-21302-3

602 Sharov AA, Dudekula DB, and Ko MS. 2005. A web-based tool for principal component and
603 significance analysis of microarray data. *Bioinformatics* 21:2548-2549.
604 10.1093/bioinformatics/bti343

605 Smith HE, Buijs H, de Vries RR, Wisselink HJ, Stockhofe-Zurwieden N, and Smits MA.
606 2001. Environmentally regulated genes of Streptococcus suis: identification by the use
607 of iron-restricted conditions in vitro and by experimental infection of piglets.
608 *Microbiology* 147:271-280. 10.1099/00221287-147-2-271
609 Trappetti C, Potter AJ, Paton AW, Oggioni MR, and Paton JC. 2011. LuxS mediates iron-
610 dependent biofilm formation, competence, and fratricide in Streptococcus
611 pneumoniae. *Infect Immun* 79:4550-4558. 10.1128/IAI.05644-11

612 Vasileva D, Janssen H, Honicke D, Ehrenreich A, and Bahl H. 2012. Effect of iron limitation
613 and fur gene inactivation on the transcriptional profile of the strict anaerobe
614 Clostridium acetobutylicum. *Microbiology* 158:1918-1929. 10.1099/mic.0.056978-0

615 Weycker D, Strutton D, Edelsberg J, Sato R, and Jackson LA. 2010. Clinical and economic
616 burden of pneumococcal disease in older US adults. *Vaccine* 28:4955-4960.
617 10.1016/j.vaccine.2010.05.030

618 Yang S, Giannone RJ, Dice L, Yang ZK, Engle NL, Tschaplinski TJ, Hettich RL, and Brown
619 SD. 2012. Clostridium thermocellum ATCC27405 transcriptomic, metabolomic and
620 proteomic profiles after ethanol stress. *BMC Genomics* 13:336. 10.1186/1471-2164-
621 13-336

622 Yang XY, He K, Du G, Wu X, Yu G, Pan Y, Zhang G, Sun X, and He QY. 2016. Integrated
623 Translatomics with Proteomics to Identify Novel Iron-Transporting Proteins in
624 Streptococcus pneumoniae. *Front Microbiol* 7:78. 10.3389/fmicb.2016.00078
625 Yang XY, Sun B, Zhang L, Li N, Han J, Zhang J, Sun X, and He QY. 2014. Chemical
626 interference with iron transport systems to suppress bacterial growth of Streptococcus
627 pneumoniae. *PLoS One* 9:e105953. 10.1371/journal.pone.0105953

628 Yang XY, Zhang L, Liu J, Li N, Yu G, Cao K, Han J, Zeng G, Pan Y, Sun X, and He QY.
629 2015. Proteomic analysis on the antibacterial activity of a Ru(II) complex against
630 Streptococcus pneumoniae. *J Proteomics* 115:107-116. 10.1016/j.jprot.2014.11.018

631

632 **Figure captions**

633 **Figure 1. Effect of deferoxamine (DFO) on the growth of *Streptococcus pneumoniae***
634 **TIGR4.** Each determination represents the mean of three different biological replicates. Solid
635 circles indicate the mean ± standard deviation (SD) of untreated cultures; solid squares
636 indicate the mean ± SD of DFO-treated cultures.

637 **Figure 2. Gene Ontology analysis of the differentially expressed genes of *Streptococcus***
638 ***pneumoniae* TIGR4 after deferoxamine treatment.** Gene Ontology terms were retrieved
639 from annotated genes or their orthologs in case of non-annotated genes using BLAST2GO.
640 Terms are indicated in the GO nomenclature. BP: biological process; MF: molecular function.