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Shotgun proteomic analysis of *Yersinia ruckeri* strains under normal and iron-limited conditions

Gokhlesh Kumar1*, Karin Hummel2, Maike Ahrens3, Simon Menanteau-Ledouble1, Timothy J. Welch4, Martin Eisenacher3, Ebrahim Razzazi-Fazeli2 and Mansour El-Matbouli1

Abstract

*Yersinia ruckeri* is the causative agent of enteric redmouth disease of fish that causes significant economic losses, particularly in salmonids. Bacterial pathogens differentially express proteins in the host during the infection process, and under certain environmental conditions. Iron is an essential nutrient for many cellular processes and is involved in host sensing and virulence regulation in many bacteria. Little is known about proteomics expression of *Y. ruckeri* in response to iron-limited conditions. Here, we present whole cell protein identification and quantification for two motile and two non-motile strains of *Y. ruckeri* cultured in vitro under iron-sufficient and iron-limited conditions, using a shotgun proteomic approach. Label-free, gel-free quantification was performed using a nanoLC-ESI and high resolution mass spectrometry. SWATH technology was used to distinguish between different strains and their responses to iron limitation. Sixty-one differentially expressed proteins were identified in four *Y. ruckeri* strains. These proteins were confirmed to be differentially expressed at the transcriptional level using quantitative real time PCR. Our study provides the first detailed proteome analysis of *Y. ruckeri* strains, which contributes to our understanding of virulence mechanisms of *Y. ruckeri*, and informs development of novel control methods for enteric redmouth disease.

Introduction

Enteric redmouth disease (ERM) is one of the most important diseases of salmonids, and causes significant economic losses in commercial aquaculture worldwide [1]. The disease is caused by *Yersinia ruckeri*, a Gram-negative rod-shaped enterobacterium. This bacterium has been reported in Europe, North and South America, Middle East and China [1–4]. *Yersinia ruckeri* strains are divided into two biotypes: biotype 1 strains are motile and lipase secretion positive, whereas strains of biotype 2 are non-motile and test negative for lipase [5]. In the past, the majority of epizootic outbreaks in salmonids were caused by biotype 1 strains, against which an effective vaccine was developed [6]. However, biotype 2 strains have recently been responsible for outbreaks in both native and vaccinated rainbow trout (*Oncorhynchus mykiss*) in Europe and North America, suggesting that this biotype was able to overcome the protection granted by motile strains vaccines [3, 7, 8].

Iron is an essential nutrient for microorganisms and occurs as ferrous and ferric oxidation states. It influences cell composition, secondary metabolism, enzyme activity, host cell interactions and pathogenicity of bacteria [9]. During reduced availability of iron pathogens express differentially regulated proteins, several of which are involved in invasion and pathogenicity activities. Iron homeostasis systems and virulence mechanisms have not been thoroughly investigated in *Y. ruckeri* and little is known about its protein expression. The expression of outer membrane proteins (OMPs) of *Y. ruckeri* isolates was examined by Davies [10] using SDS-PAGE, who observed four OMP bands at 72, 69.5, 68 and 66 kDa under iron-limited conditions. Tinsley et al. [11] also used...
SDS-PAGE to show induction in OMPs of *Y. ruckeri* isolates at 90 and 100 kDa, under iron-restricted conditions; these proteins were not identified by mass spectrometry. An alternative protein analysis technology has emerged: label-free, gel-free shotgun proteomics, and this has been used in *Y. pestis* to identify whole cell proteins [12, 13]. Recently, a reference proteome map of *Y. pestis* has been created, and is leading to an understanding of the pathogenesis of this bacterium [12]. Additionally, differentially expressed proteins of *Y. pestis* and *Y. enterocolitica* have been identified and quantified under iron-limited conditions using both 2D-PAGE and MALDI TOF/TOF MS [14, 15].

The aim of the present study was to identify and quantify the whole cell proteomic expression profiles of biotype 1 and biotype 2 strains of *Y. ruckeri* grown in vitro, under normal and iron-limited conditions using a label-free, gel-free shotgun proteomics approach. This study represents one of the first descriptive and comparative proteomic approaches of motile and non-motile *Y. ruckeri* strains in response to iron-limited conditions.

**Materials and methods**

*Yersinia ruckeri* strains

Four strains (SP-05, CSF007-82, 7959-11 and YRNC-10) of *Y. ruckeri* were used for deep proteomic analysis. Strains SP-05 and 7959-11 were obtained from our bacterial repository at the University of Veterinary Medicine, Vienna, Austria. The other two strains, CSF007-82 and YRNC-10, were obtained from the National Center for Cool and Cold Water Aquaculture, Kearneysville, West Virginia, USA. These strains were tested for serotype, flagella motility and phospholipase activity [3] and their identity confirmed by PCR [16]. Strains, SP-05 and CSF007-82, belong to serotype 1 and biotype 1 (motile and lipase positive, Figure 1A), while strains 7959-11 and YRNC-10 belong to serotype 1 and biotype 2 (non-motile and lipase negative, Figure 1B).

Culture conditions

Bacterial strains were grown on tryptic soy agar for 48 h at 22 °C. A single colony of each strain was inoculated into 10 mL of tryptic soy broth [Casein peptone (17 g), dipotassium hydrogen phosphate (2.5 g), glucose (2.5 g), sodium chloride (5 g), soya peptone (3 g) per liter, Sigma, Germany] and incubated for 17 h at 22 °C with shaking (150 rpm). These starter cultures were then diluted with fresh sterile tryptic soy broth to an optical density (OD 600) of 0.10 ± 0.05. Five hundred microlitres of the diluted starter cultures were inoculated in duplicates, into 25 mL of tryptic soy broth with or without iron chelator, 2,2′-bipyridyl, MW 156.18 g/mol (Sigma, Germany). Iron depletion was attained by the addition of 100 µM of 2,2′-bipyridyl to the broth. Cultures were grown overnight at 22 °C with shaking (150 rpm) until the late log phase. Cells were harvested by centrifugation at 4000 rpm for 10 min at 4 °C, then washed three times with phosphate buffered saline containing bacterial protease inhibitor cocktail (Sigma, Germany) and stored at −80 °C.

**Protein extraction and digestion**

Bacterial pellets were resuspended in 800 µL pre-cooled denaturing lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS and 1% DTT) containing bacterial protease inhibitor cocktail. Bacterial cell suspensions were disrupted by sonication on ice for 20 cycles of 10 s pulse-on and 30 s pulse-off. The lysates were centrifuged at 14 000 rpm for 30 min at 4 °C and supernatants were collected. Total protein concentration of each lysate was determined colorimetrically with a NanoDrop 2000c (Thermo Fisher Scientific, USA) spectrophotometer using a Pierce 660 nm Protein Assay according to the manufacturer’s instructions.

Protein digestion was performed using the standard two-step in-solution digestion protocol for Trypsin/ LysC-Mix according to the user manual (Promega, USA). In summary, 10 µg of proteins was diluted with 8 M urea in 50 mM Tris–HCl (pH 8.0) to a total volume of 10 µL. Proteins were reduced in 5 M dithiothreitol (final concentration) for 30 min at 37 °C and alkylated with 15 mM iodoacetamide for 30 min at 25 °C. Trypsin/LysC mix was added in a ratio of 25:1 protein to protease (w/w). The first digestion step was performed for 4 h at 37 °C under high urea concentration. To enable protease activity of trypsin, urea was diluted afterwards with 50 mM Tris–HCl (pH 8.0) to a concentration below 1 mol/L. The second digestion step was performed for 8 h at 37 °C. Finally, digested samples were acidified with concentrated trifluoroacetic acid.

**NanoLC-MS/MS analysis**

Tryptic peptides were separated by a nano liquid chromatography system (Dionex Ultimate 3000 RSLC, Thermo Fisher Scientific, USA) and analyzed with a high-resolution hybrid triple quadrupole time of flight mass spectrometer (TripleTOF 5600+, Sciex, USA) coupled via a nano-ESI interface.

Samples were pre-concentrated and desalted with a 5 mm Acclaim PepMap µ-Precolumn (300 µm inner diameter, 5 µm particle size, and 100 Å pore size) (Dionex, Thermo Fisher Scientific, USA). Samples were loaded and desalted using 2% acetonitrile (ACN, Chem-Lab, Belgium) in LC–MS grade water (Optima, Thermo Fisher Scientific, USA). 0.05% TFA was used as a mobile phase with a flow rate of 5 µL/min. Three microliter
injection volumes (corresponding to 370 ng digested protein absolute on column) were used per injection.

Peptide separation was performed on a 25 cm Acclaim PepMap C18 column (75 µm inner diameter, 2 µm particle size, and 100 Å pore size) with a flow rate of 300 nL/min. The gradient started with 4% B (80% ACN with 0.1% formic acid) and increased to 35% B in 120 min. It was followed by a washing step with 90% B. Mobile phase A consisted of LC-MS grade water with 0.1% formic acid.

For information dependent data acquisition (IDA runs), MS1 survey scans were collected in the range of 400–1500 m/z. The 25 most intense precursors with charge state 2–4 that exceeded 100 counts per second, were selected for fragmentation for 250 ms. MS2 product ion scans were collected in the range of 100–1800 m/z for 110 ms. Precursor ions were dynamically excluded from reselection for 12 s.

For quantitative measurements, data independent SWATH (Sequential Window Acquisition of all Theoretical spectra) technology based on MS2 quantification was used [17, 18]. Peptides were fragmented in 35 fixed fragmentation windows of 20 Da in the range of 400–1100 Da with an accumulation time of 50 ms in TOF MS mode and 80 ms in product ion mode. The nano-HPLC system was operated by Chromeleon 6.8 (Dionex, USA) and the MS by Analyst Software 1.6 (Sciex, USA).

**Data processing, quantification and statistical evaluation**

IDA raw data was processed with ProteinPilot Software version 5.0 (Sciex, USA) for re-calibration and database searches. The UniProt database (Release 04_2015) was restricted to *Yersinia ruckeri*, but contained possible contaminants from the growth medium, e.g. *Glycine max* (Soybean) and bovine caseins. Mass tolerance in MS mode was set with 0.05 and 0.1 Da in MSMS mode for the rapid recalibration search, and 0.0011 Da in MS and 0.01 Da in MSMS mode for the final search. The following sample parameters were applied: trypsin digestion, cysteine alkylation set to iodoacetamide, search effort set to rapid ID. False discovery rate analysis (FDR) was performed using the integrated tools in ProteinPilot. Global false discovery rate was set to <1% on protein level.

IDA identification results were used to create the SWATH ion library with the MS/MS (ALL) with SWATH Acquisition MicroApp 2.0 in PeakView 2.2 (both Sciex, USA). Peptides were chosen based on a FDR rate <1%.

**Figure 1** Flagellar motility and lipase secretion of *Y. ruckeri* biotype 1 and 2 strains. Motility and lipase examinations were performed at 24 and 48 h, respectively. A Biotype 1 strain shows flagellar motility and lipase secretion, B Biotype 2 strain does not show flagella and lipase secretion. Flagella were examined by staining using a Flagella Stain (BD) and phospholipase activity was determined on tween 80-tryptic soy agar plates. Scale bar 10 µm.
excluding shared and modified peptides. Up to 6 peptides per protein and up to 6 transitions per peptide were used.

MarkerView 1.2.1 (Sciex, USA) was used for calculation of peak areas of SWATH samples after retention time alignment and normalization using total area sums. Resulting protein lists were then used for visualization of data after principal component analysis (PCA) in form of loadings plots and score plots to get a first impression of the overall data structure, and to assess variability between technical and biological replicates.

To determine differentially regulated proteins, statistical evaluation was performed in R programming language [19]. Raw peak areas after normalization to total area sums were log2-transformed to approach a normal distribution. On a logarithmic scale, technical replicates were aggregated by arithmetic mean before application of statistical tests. This procedure is equivalent to the application of a hierarchical model in the subsequent ANOVA, as the same number of technical replicates was measured per biological replicate.

For each growing condition, differential expression of proteins in each Y. ruckeri strain was assessed using one-way ANOVA for each protein. To adjust for multiple testing, the method of Benjamini and Hochberg [20] was used to control the FDR. Differences were considered significant if adjusted \( p \)-values were smaller than the significance level of \( \alpha = 0.001 \). For those proteins, Tukey’s honest significant difference (HSD) method was applied as post hoc test to assess the significance of the pairwise comparisons. Protein expression was considered differential if the adjusted \( p \)-value was below \( \alpha \) and the absolute fold change was at least three (fold change \( \leq -3 \) or \( > +3 \)).

In a second approach, significant differences between the two growing conditions were examined separately for each strain with unpaired two-sample \( t \) tests. All resulting \( p \)-values were adjusted for multiple comparisons by FDR [20]. Candidates of interest were chosen based on a \( p \)-value of lower than 0.05 (corresponding to \(-\log_{10} (p)\) of 1.3) and a fold change \( < -3 \) or \( > +3 \) (corresponding to \( \log_2 \) (rm) of \( < -1.6 \) or \( > 1.6 \)). Results were summarized as Volcano plots (ratio of means vs. adjusted \( p \)-value). Fold changes of all significant candidates were listed as tables for each comparison. Computations related to the statistical test procedures were performed in R. Afterwards, venn diagrams were used as a tool for data visualization.

GO annotation of differentially expressed proteins
We used software tool for rapid annotation of proteins (STRAP version 1.5) for classification of biological process, cellular component and molecular function of differentially expressed proteins of Y. ruckeri [21].

qRT-PCR analysis
Total RNA was extracted from Y. ruckeri strains grown under normal and iron-limited conditions using an RNeasy Mini Kit (Qiagen) and included an on-column DNase digestion step. cDNA was synthesized using an iScript cDNA Synthesis Kit (BIO-RAD) with 1 µg total RNA according to the user’s manual.

PCR primers specific for the six selected genes were designed using NCBI Primer-BLAST software (Additional file 1) and optimized using gradient PCRs to determine the annealing temperature and primer concentration. qPCRs were performed in a final volume of 20 µL, which contained 4 µL of 1:20 fold diluted cDNA, 0.4 µM of each primer, 1X SYBR Green Supermix and sterile distilled water. After 5 min of cDNA denaturation at 95 °C, 38 cycles were performed at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s. Each qPCR was performed in biological replicates with three technical reactions using CFX96 Touch Real-Time PCR detection system (BIO-RAD).

Recombination protein A, signal recognition particle protein A and 16S rRNA genes were tested to determine the suitable stability of them as reference genes. All raw Cq values from qPCR were used to assess the output of the four computational programs (geNorm, Normfinder, BestKeeper, and delta Ct method) using web-based RefFinder software tools [22]. Based on RefFinder, recombination protein A and signal recognition particle protein were found to be the most stable reference genes across normal and iron-limited samples, and thus were used for normalization of target genes. Relative quantity values of each sample of each target gene were normalized with values of two reference genes (recombination protein A and signal recognition particle protein) using CFX Manager Software version 3.1 (BIO-RAD) in normalized expression mode \((\Delta\Delta Cq)\). Then, normalized values of each sample were used for statistical analysis and differences between both growing conditions were analyzed using \( t \)-tests for samples with Bonferroni \( \alpha \)-correction. All statistical analyses were conducted with SPSS version-20 software. The expression levels were considered statistically significant if \( p \)-values were <0.05.

Results
Bacterial growth under iron-limited condition
Growth curves of Y. ruckeri strains were determined in tryptic soy broth. Three strains (CSF007-82, 7959-11 and YRNC-10) were shown to have growth culture patterns similar to each other. However, the yield of strain SP-05 was slightly lower compared to the other three strains. The iron chelator, 2,2'-bipyridyl reduced the growth and yield of all strains as the final optical density reading of
Iron-limited strains was always lower than under iron-sufficient conditions (Additional file 2).

**Label-free quantification of proteins by SWATH technology**

Identified proteins at FDR 1% with more than one peptide of each strain under normal and iron-limited conditions are given in Additional file 3. Results of \( t \) test comparisons (\( p < 0.05 \)) between normal and iron-limited conditions for each strain are provided in Additional file 4. Proteins determined to be expressed differently among the four *Y. ruckeri* strains according to ANOVA and Tukey HSD analysis (\( p < 0.001 \)) with a fold change of \(< -3 \) or \( > +3 \) are listed in supplementary data (Additional files 5 and 6).

Additional file 7 shows a PCA graphical overview of the complex proteomics data of strains. PCA score plots confirmed high reproducibility of biological replicates by grouping the replicates together. The score plots of all strains under both growth conditions suggested that strain SP-05 in both conditions differs from the three strains (CSF007-82, 7959-11 and YRNC-10). However, these three strains showed minor proteomic differences between each other. Principal component scores for PC1 and PC2 were 63.5 and 14.7% (Additional file 7A) under normal condition and 60.4 and 9.8% (Additional file 7B) under iron-limited condition, respectively, showed that a major part of the overall variation in the data was captured in the first two principal components. Volcano plots (Additional file 8) were used for joint visualization of effect size and corresponding significance of the differentially expressed proteins.

**Differentially expressed proteins of *Y. ruckeri* strains under iron-limited condition**

In *Y. ruckeri*, we identified 1395 proteins under normal and 1447 proteins under iron-limited growth conditions (Additional file 3). Sophisticated statistical evaluation revealed a total number of 61 differentially expressed proteins within the four analyzed *Y. ruckeri* strains (Tables 1, 2, 3). Of these, 35 proteins were upregulated and 26 proteins were downregulated. We found one uncharacterized (K7M2F1_SOYBN) soybean protein as a contaminant in our protein samples because of tryptic soy broth medium, which contains soya peptone.

As can be seen in Tables 1, 2, 3, most of the upregulated proteins were related to iron ion binding and transport and in particular to siderophore, oxidoreductase and glycolysis activities. The rest of the proteins were related to copper, nonribosomal peptide formation, protein folding and lipoprotein. Similarly, most of the downregulated proteins were related to transport, flagellar motility, oxidoreductase, iron binding, translation and glycolysis activities.

Fifteen proteins in SP-05, nine proteins in CSF007-82, four proteins in 7959-11 and nine proteins in YRNC-10 were uniquely differentially expressed (Figure 2). When comparing all strains, we noted some proteins that were upregulated by fold changes between 10 and 96: iron(III)-binding periplasmic protein (YfuA), iron ABC transporter substrate-binding protein (YiuA), periplasmic chelated iron-binding protein (YfeA), hemin transport protein (HemS), ferrous iron transport protein A (FeoA), ferrichryso- bacterin receptor, enterobactin synthetase component B & C (EntB and EntC) and 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase of siderophore biosynthesis (EntA) proteins, which were highly expressed in both motile and non-motile *Y. ruckeri* strains.

**GO annotation of differentially expressed proteins**

Differentially expressed proteins of *Y. ruckeri* were classified by Gene Ontology terms for biological processes, cellular components and molecular functions using the STRAP software. The identified proteins were associated with cellular process (50%), localization (17%), metabolic process (12%), regulation (10%), response to stimulus (5%), and interaction with cells and organisms (2%) (Figure 3A). Most of the differentially expressed proteins were localized in the plasma membrane (25%), extracellular (6%) and cytoplasm (6%) (Figure 3B). Differentially expressed proteins were involved in catalytic (43%), binding (34%), structural molecule (5%) and molecular transducer activities; functions of 10% of the proteins remained others (Figure 3C).

**qRT-PCR**

Differentially expressed proteins identified by shotgun proteomics analysis were validated at the transcriptional level by qRT-PCR (Figure 4). We selected six genes coding for differentially expressed proteins. The candidate proteins were selected on the basis of their differential expression as representative of iron binding protein, superoxide metabolic process, copper-exporting P-type ATPase, iron ion transport and siderophore biosynthesis. Selected genes were overexpressed (\( p < 0.0001 \)) in the iron-limited samples, relative to the control samples (normal growth condition).

**Discussion**

Label-free shotgun proteomics is a straightforward, fast, cost effective and broadly applicable approach to quantify proteins [23]. This approach has been used successfully for the identification and quantification of proteins in *Y. pestis*, the causative agents of bubonic plague [12, 13]. In the present study, we identified and quantified whole cell proteins of biotype 1 and biotype
Y. ruckeri strains using a label-free, gel-free shot-gun proteomic method. We identified 1395 proteins in Y. ruckeri, with strains having between 1077 and 1161 proteins under normal growth conditions. This number of proteins is consistent with that reported in other members of the genus: 1074 proteins were identified in Y. pestis KIM5 isolated from human [12]; 1421 in Y. pestis 91 001 isolated from mice [13]; 1074–1078 in Y. pestis Yp1945/Yp2126 isolated from fleas [24]. In the present study, 61 differentially expressed proteins (35 up- and
26 down-regulated) were identified in vitro in *Y. ruckeri* strains (FDR-adjusted *p* value <0.05 and fold change < −3 or > +3) in response to iron-restriction conditions. The function of these proteins included iron binding, molecular transduction, as well as catalytic and structural molecule activities.

In both motile and non-motile *Y. ruckeri* strains, we observed strong upregulation of iron-binding proteins
(YfuA, YiuA and YfeA; 3.3- to 35-fold) and iron-binding transporters (HemS and Feo: 6.6- to 96.2-fold) (Tables 1, 2, 3). Previous work has shown in Y. pestis in a mouse model that Yfu and Yiu function as part of an iron uptake system, but are not involved in virulence [25]. The YfeA system, which transports iron and manganese, is required for the full virulent phenotype of Y. pestis [26]. Upregulation (1.6- to 4.5-fold) of iron-binding proteins YfuA, YiuA and YfeA, has been reported in Y. pestis and Y. enterocolitica in response to iron starvations [14, 15]. This is consistent with the results from our in vitro Y. ruckeri. Hemin transport protein HemS is involved in the

### Table 3 Differentially expressed proteins of Y. ruckeri strains (SP-05, CSF007-82, 7959-11 and YRNC-10)

| UniProt accession number | Ambiguous accession number | Protein Function | Function | SP-05 | CSF007-82 | 7959-11 | YRNC-10 |
|--------------------------|----------------------------|------------------|----------|-------|-----------|---------|---------|
| C4UF95_YERRU             | A0A085U413_YERRU           | 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase | Glycolytic process | 3.8* | 3.6* | 3.2* | 3.1* |
| C4UMV0_YERRU             | A0A085U9H2_YERRU           | Succinate dehydrogenase iron-sulfur subunit | Glycolytic process/iron-sulfur cluster binding | -3.3* | -1.9 | -1.9 | -1.9 |
| C4UH2S_YERRU             | A0A085U3P6_YERRU           | Phosphoenolpyruvate carboxykinase | Gluconeogenesis | -3.2* | 1.3 | -4.0 | -3.0 |
| C4UIX8_YERRU             | A0A085U3P6_YERRU           | 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | Glycolytic process | -4.0* | -3.5* | -3.7* | -3.6* |
| C4UN43_YERRU             | A0A085U407_YERRU           | Fumarate hydratase class I, aerobic | Fumarate hydratase activity | -4.6* | -2.5 | -2.4 | -2.7 |
| A0A0ASFIV0_YERRU         | A0A085U4U1_YERRU           | Catalase | Peroxidase activity | -6.2* | -2.7 | -2.4 | -2.7 |
| C4UIX0_YERRU             | A0A085U3N7_YERRU           | Aromatic-L-amino-acid decarboxylase | Amino acid metabolic process | 1.3 | 1.9 | 4.4* | 3.4* |
| C4UM91_YERRU             | A0A085U3N7_YERRU           | Periplasmic protein CpxP | Protein misfolding | 4.4* | 1.8 | 2.6 | 1.9 |
| C4UL33_YERRU             | A0A085UBI2_YERRU           | Amino acid adenylation domain protein | Amino acid activation for nonribosomal peptide biosynthetic process | 1.1 | 2.0 | 3.9* | 2.4 |
| C4UL33_YERRU             | A0A085UBI2_YERRU           | FKBP-type 16 kDa peptidyl-prolyl cis–trans isomerase | Protein folding | -1.7 | -1.0 | 3.3 | 5.0* |
| A0A094SGT7_YERRU         | A0A085U807_YERRU           | 50S ribosomal protein L35 | rRNA binding | -2.3 | -3.4 | -1.9 | -3.6* |
| C4UFWS_YERRU             | A0A085U806_YERRU           | 50S ribosomal protein L20 | rRNA binding | -4.1* | -1.9 | 1.0 | -1.6 |
| A0A094SMR6_YERRU         | A0A085U3C8_YERRU           | Mbth1-like family protein | Nonribosomal peptide formation | 14.6 | 5.6* | 12.5 | 7.7 |
| C4UX4_YERRU              | A0A085U549_YERRU           | Uncharacterized protein | Putative exported protein | 4.9* | 1.4 | 1.3 | 1.5 |
| A0A094SSK4_YERRU         | A0A085UBK3_YERRU           | Probable phosphoglycerate mutase GpmB | Phosphoglycerate mutase activity | -3.1* | -1.1 | -2.0 | -1.4 |
| C4UF96_YERRU             | A0A085U4I2_YERRU           | PsF repeat protein | None predicted | -1.9 | -5.2* | -4.5 | -1.1 |
| C4UEV3_YERRU             | A0A085U4A9_YERRU           | Molybdopterin biosynthesis protein moeB | Molybdopterin cofactor biosynthetic process | 1.1 | -1.2 | -2.3 | -4.1* |
| C4UP53_YERRU             | A0A085U2Q2_YERRU           | Phosphopantetheine attachment site family protein | Phosphopantetheine binding | -1.8 | -1.2 | -1.7 | -3.2* |
| C4UF99_YERRU             | A0A085U7Q2_YERRU           | Peptidase S49 family protein | Serine-type endopeptidase activity | -1.0 | -2.3 | -2.9 | -4.1* |
| A0A085U7Q2_YERRU         | A0A085U7Q2_YERRU           | Uncharacterized protein | Putative lipoprotein | -1.2 | 1.1 | -1.0 | 4.8* |

Differentially expressed proteins details as in Table 1.
uptake of hemin-containing iron. Hemolysin activator protein was identified as an in vivo induced extracellular cytolysin in *Y. ruckeri* [27] and was implicated in the virulence and cytotoxicity of *Y. ruckeri* [28]. A ferrous iron transport system plays a role in the acquisition of ferrous iron during intracellular growth of the bacterium and is composed of three proteins, FeoA, FeoB and FeoC [29]. Both FeoA and FeoB are essential components of the iron acquisition system in *Y. pestis* [30]. Upregulation of FeoA and FeoB proteins under iron-starvation conditions suggests that the Feo system is involved in the iron acquisition system of *Y. ruckeri* during the infection process, however, its significance for pathogenesis is unknown. Based on the results of the present study, it appears that the iron binding proteins/transporters are upregulated in response to iron-scarcity. Because iron is often the limiting factor during the infectious process, this has likely clinical implications regarding the survival of the bacterium inside the host and the establishment of disease by both motile and non-motile *Y. ruckeri* strains.

We found that ferrichrysobactin and TonB-dependent iron-binding receptors were upregulated 4.5- and 14.9-fold, respectively, in *Y. ruckeri* strains under iron-limited conditions. Notably, in *Y. ruckeri*, both of these receptors have been shown to be upregulated during the infection process in fish [27]. TonB-dependent receptors are outer membrane receptors of Gram-negative bacteria involved in active transport of a number of molecules, including the uptake of siderophores [9, 31]. The role of the *tonB* gene has been examined in *Y. pestis*, where utilization of siderophore and uptake of hemin are TonB-dependent.

![Figure 2](https://example.com/f2.png) **Figure 2** Venn diagram showing the number of differentially expressed proteins identified in *Y. ruckeri* strains. The number of unique or shared proteins of each strain is indicated in each set or subset.

![Figure 3](https://example.com/f3.png) **Figure 3** Classification of differentially expressed proteins of *Y. ruckeri*. Proteins were classified by GO terms for biological processes, cellular components and molecular functions using STRAP. **A** Biological process, **B** cellular component, and **C** molecular function.
Siderophores are small, high-affinity iron chelating molecules secreted by bacteria, and are transported into the periplasm of cells by TonB-dependent receptors [9, 31]. We found for the first time, that the siderophore biosynthesis proteins EntA and EntB are upregulated in Yersinia species in response to iron-limited conditions. Upregulation (17.95-fold) of TonB has been observed in Edwardsiella ictaluri during in vitro iron restriction [33] and in Aeromonas salmonicida during in vivo infection [34]. Similarly, strong upregulation (4.4- or 23.4-fold) of siderophore biosynthesis proteins (EntA, EntB and EntC) were observed in iron-starved Y. ruckeri cells. This suggests that iron is a limiting factor and that iron-binding receptors are required for the survival of Y. ruckeri during the infection in the fish host.

Superoxide dismutases (SODs) represent one of the major defense mechanisms of cells against oxidative stress, by catalyzing conversion of superoxide radicals [35]. SODs are classified based on the bound metal ion co-factor, for example: iron (Fe-SOD), manganese (Mn-SOD), copper/zinc (Cu–Zn-SOD) and nickel (Ni-SOD) [36, 37]. We found 3.5- to 5.6-fold upregulation of Mn-SOD (SodA), and 12.3- to 28.6-fold downregulation of Fe-SOD (SodB) in Y. ruckeri strains under iron-limited conditions. The regulation of superoxide dismutases has been linked to iron metabolism in another important fish pathogen, A. salmonicida subsp. salmonicida: Ebanks et al. [38] observed expression of SodA under both iron restriction and fur::KO mutant growth conditions, and demonstrated involvement of the ferric uptake regulator in the pathogenicity of the bacterium in fish. The sodA gene has been shown to contribute to the pathogenicity of many other bacteria, including Y. enterocolitica and Streptococcus agalactiae, during host invasion, using a mouse model [35, 37].

We observed downregulation of catalase in iron-depleted Y. ruckeri cells, but the magnitude of the change was not significant (< −threefold). Catalase contains four porphyrin heme groups and catalyzes hydrogen peroxide into water and oxygen, thereby protecting the bacterium from this reactive oxygen species [39]. Downregulation of catalase may be due the depletion of porphyrin heme groups during iron-limited conditions.

We observed upregulation of copper-exporting P-type ATPase (>3.5-fold) in motile Y. ruckeri strains SP-05 and CSF007-82, but saw no significant expression in non-motile strains under iron-limited conditions. This expression was validated by qRT-PCR to confirm the results of shotgun proteomic analysis. Copper-exporting P-type ATPase A is involved in copper export from the cytoplasm to the periplasm, and it is known that copper metabolism in bacteria is linked to that of iron, and
that some siderophores interact with copper [40]. A link between copper and siderophores has been shown in Escherichia coli [41]. We are the first to report the upregulation of copper-exporting P-type ATPase in Yersinia species in response to iron-limited conditions, however the relevance of this to pathogenicity is unknown as there is a paucity of knowledge on copper metabolism in Yersinia species.

Under iron-limited conditions, we observed 3.1- to 5.8-fold upregulation of enzymes involved in glycolysis (2,3-bisphosphoglycerate-dependent phosphoglycerate mutase) and oxidoreduction (glutaredoxin and FAD-binding 9 siderophore-interacting domain protein), suggesting an increased role of these enzymes in metabolic processes when the cells are starved of iron. Furthermore, we saw down-regulation other metabolic enzymes (fumarate reductase, fumarate hydratase, phosphoenolpyruvate carboxykinase and molybdoenzyme). This was expected, as these enzymes are dependent on iron-sulfur clusters or other iron cofactors, and is consistent with previous observations of downregulation of metabolic proteins in iron-starved Y. pestis cells [14].

Cellular iron depletion can suppress expression levels of iron-dependent proteins [9, 31]. We observed strong downregulation of iron-dependent proteins, including bacterioferritin (−3 to −12.5-fold) and iron-sulfur cluster assembly scaffold protein IscU (−3.6 to −3.8-fold) in Y. ruckeri strains under iron starvation conditions. Bacterioferritin is a main cytoplasmic iron storage protein and IscU provides iron for iron-sulfur cluster assembly [9, 31]. These observations are consistent with a shift in bacterial metabolic activities to iron-independent biochemical pathways when the supply of iron is limited.

We observed −3.6 to −5.3-fold downregulation of flagellar proteins including FlIC, FlgH and FlIN in Y. ruckeri strains under iron-limited conditions. Previous mutational research conducted by Evenhuis et al. [42] showed that the absence of flagellar motility does not affect Y. ruckeri virulence, and that flagellin expression is not required for production of innate immune response in fish [43], as suggested by the clinical importance of non-motile isolates of the bacterium [44].

Shotgun proteomics revealed minor protein differences among motile and non-motile Y. ruckeri strains under normal and iron-limited growth conditions, consistent with observations by Huang et al. [45, 46] who used pulsed-field gel electrophoresis and fatty acid profiles. In the present study, PCA score plots (Additional file 7) highlight major protein differences between the European motile strain (SP-05) and USA motile strain (CSF007-82). These differences may be due to high genetic diversity and epidemic population structure as seen by multilocus sequence typing [47].

In conclusion, this is the first detailed description of differentially expressed proteins in Y. ruckeri strains cultured in iron limited conditions, as revealed by shotgun proteomic analysis. It is likely that the changes in expression of iron-binding proteins, iron ion transporters/receptors, siderophore biosynthesis proteins and manganese-binding superoxide dismutase play an important role in the survival of Y. ruckeri inside the host. These findings might provide the basis for the development of novel control methods for enteric redmouth disease in fish.

Additional files

Additional file 1. List of quantitative real-time PCR primers. PCR primers specific for the selected genes were designed using NCBI Primer-BLAST software and used in this study.

Additional file 2. Growth curves of Yersinia ruckeri strains. Duplicate bacterial strains were grown in tryptic soy broth under normal and iron-limited conditions at 22 °C. Growth was monitored at different time points by determining the optical density at 600 nm. Error bars indicate standard deviation. ILC: Iron limited culture.

Additional file 3. Total number of identified proteins of Y. ruckeri strains. Number of proteins was identified at FDR 1% with more than one peptide.

Additional file 4. Total number of differentially expressed proteins of Y. ruckeri strains (strain versus strain). Differentially expressed proteins were assessed according to ANOVA and Tukey HSD (p < 0.001) with a fold change < −3 or > +3.

Additional file 5. Fold changes of differentially expressed proteins of Y. ruckeri strains compared to each other under normal culture conditions. ANOVA was performed for UniProt database searches. * denotes statistically significant difference according to t-test with FDR-adjusted p value < 0.05 and fold change < −3 or > +3.

Additional file 6. Fold changes of differentially expressed proteins of Y. ruckeri strains compared to each other under iron-limited conditions. ANOVA was performed for UniProt database searches. * denotes statistically significant difference according to t-test with FDR-adjusted p value < 0.05 and fold change < −3 or > +3.

Additional file 7. Principal component analysis score plots of Yersinia ruckeri strains. Grouping of biological replicates shows good reproducibility of technical replicates for each sample. Three score plots show that strain SP-05 differs from the three strains under both normal and iron-limited conditions, which show minor protein differences to each other. (A) PCA score plot under normal condition, (B) PCA score plot under iron-limited condition.

Additional file 8. Volcano plots of Y. ruckeri strains. Volcano plots showing the distribution of the ratios (log2) versus the FDR adjusted p value (−log10) for all proteins identified under iron-limited condition. Differentially expressed proteins are shown as black circles. (A) Motile strain CSF007-82, (B) Non-motile strain 7959-11.

Abbreviations

ERM: enteric redmouth disease; OMPs: outer membrane proteins; 2D-PAGE: two-dimensional gel electrophoresis; MALDI-TOF-MS: matrix-assisted laser desorption/ionization mass spectrometry; LC-MS: liquid chromatography–mass spectrometry; FDR: false discovery rate; SWATH: sequential window acquisition of all theoretical spectra; IDA: information dependent data acquisition; PCA: principal component analysis; Feo: ferrous iron transport; SOD: superoxide dismutase.

Competing interests

The authors declare that they have no competing interests.
Authors’ contributions

GK and MEM conceived and designed the experiment. GK performed the experiment, extracted the whole cell proteins, synthesized the cDNA, designed and optimized the primers, performed the real time PCR, analyzed the data and drafted the manuscript. KH and ERF contributed in the LCM–AGE MS and helped in quantification of proteins. NA and ME contributed in the R statistics analysis. SML, TW, ERF and MEM revised the manuscript. All authors read and approved the final manuscript.

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