Zinc limitation in Klebsiella pneumoniae profiled by quantitative proteomics influences transcriptional regulation and cation transporter-associated capsule production

CURRENT STATUS: UNDER REVISION

Arjun Sukumaran
University of Guelph

Jennifer Geddes-McAlister
University of Guelph

jgeddesm@uoguelph.ca Corresponding Author

DOI: 10.21203/rs.2.18241/v1

SUBJECT AREAS
Applied & Industrial Microbiology

KEYWORDS
Klebsiella pneumoniae, zinc limitation, cation transporter, polysaccharide capsule, quantitative proteomics, secretome, nutritional immunity
Abstract

Background: Microbial organisms encounter a variety of environmental conditions, including changes to metal ion availability. Metals ions play an important role in many biological processes for growth and survival. As such, microbes alter their cellular protein regulation and secretion patterns in adaptation to changing environmental conditions. This study focuses on Klebsiella pneumoniae, an opportunistic bacterium responsible for nosocomial infections and by using K. pneumoniae, we aim to determine how a nutrient-limited environment (e.g., zinc) modulates the cellular proteome and secretome of the bacteria. This information will inform on protein-level regulation of bacterial responses to nutritional immunity within the host and improve our understanding of the dynamic and complex relationship between host and pathogen during infection.

Results: Analysis of intra- and extracellular changes identified 2,380 proteins from the total cellular proteome (cell pellet) and 246 secreted proteins (supernatant). Specifically, hutC, a repressor of the histidine utilization operon, showed significantl increases abundance under replete conditions, which coincided with an expected reduction in expression of genes within the hut operon from our validation qRT-PCR analysis. Additionally, we characterized a putative cation transport regulator, chaB that was significantly abundant under zinc-replete conditions. Phenotypic analysis of a chaBdeletion strain observe a reduction in capsule production, greater tolerance to high extracellular zinc concentrations, and unimpaired virulence when compared to the WT strain.

Conclusions: This is first study to comprehensively profile the impact of zinc availability on the proteome and secretome of K. pneumoniae and uncover a novel connection between zinc transport and capsule production in the bacterial system.

1. Background

Klebsiella pneumoniae is an opportunistic, Gram-negative bacterium that primarily causes nosocomial infections. It is found ubiquitously in the environment, commonly in soil and surface water, but is also very prevalent in medical settings. Infections of K. pneumoniae primarily result in pneumonia and urinary tract infections but can lead to liver abscesses or soft tissue infections in more serious cases. Understanding the mechanisms used by the bacteria to adapt and survive within changing
environments, including the host, may uncover novel treatment strategies.

Transition metals play a crucial role in numerous functions within all living organisms. Their acquisition is essential to microbial survival, but similarly, the maintenance of intracellular concentration is important. Elevated intracellular levels of metals have negative effects on microbial fitness\(^2\). The most common transition metals include redox (e.g., iron or copper) and non-redox (e.g., zinc) metals. Previous studies investigating the relationship between \(K.\ pneumoniae\) and transition metal availability focused primarily on iron limitation. These studies found iron-associated impact on bacterial growth and capsule development\(^3,4\). Moreover, \(K.\ pneumoniae\) produces siderophores, iron-chelating molecules that can acquire free iron from the environment or steal it from host proteins, to regulate intracellular iron levels during infection\(^5\)–\(^7\).

Next to iron, zinc is the second most abundant transition metal\(^8\). As a non-redox metal, zinc is stable and can serve as an electrophilic catalyst, which allows it to either participate in catalysis or maintain stability\(^9\). In bacteria, zinc is essential for enzyme activity, DNA replication and repair, and regulation of transcription factors\(^10\). The maintenance of zinc levels is primarily controlled by the zinc uptake regulator (Zur)\(^11\). Microarray analysis of zinc depletion in \(Escherichia\ coli\) identified upregulated genes proposed to interact with Zur to regulate zinc uptake\(^12\). Zur also interacts with zitB, a zinc exporter, hinting at a possible role in both sides of zinc homeostasis\(^13\). Under zinc limited conditions, \(Mycobacterium\) expresses four zinc-independent alternative ribosomal proteins (altRP) proposed to interact with the Zur regulon\(^14\). Analysis of \(Mycobacterium\ smegmatis\) identified that these proteins are essential for survival under zinc limited conditions, and mutations to the altRP operon affected cell morphology\(^15\).

The acquisition of zinc is important for microbial survival within the host. For example, during invasion by pathogenic microbes the host will sequester transition metals from the environment thereby, restricting nutrients from the pathogen, a term coined nutritional immunity\(^10\). Conversely, elevated intracellular levels also have toxic effects\(^16\). Alongside limitation of free zinc ions, host
immune cells increase intracellular zinc concentrations upon phagocytosis to induce zinc toxicity in response to invasion of *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, and *E. coli*\(^{17-19}\). To date, the acquisition and regulation of zinc has been described at the transcriptional level in multiple bacterial species, which provides valuable insight into bacterial adaptability and the relevance of zinc within the host. However, investigation of the impact of zinc limitation in *K. pneumoniae*, a clinically-relevant bacterium, and defining the influence of zinc at the protein-level, which provides novel insight into protein production in response to environmental stresses, has yet to be explored. Information on protein production, abundances, and pathway involvement can contribute to our mechanistic understanding of bacterial adaptation from a systems perspective.

The present study aims to understand the relationship between zinc and *K. pneumoniae* using quantitative proteomics to tether apart the impact of zinc availability on intra- and extracellular processes. Using mass spectrometry-based proteomics, we identified differentially abundant proteins under zinc-limited and -replete conditions in the cellular proteome (cell pellet) and secretome (culture supernatant) of *K. pneumoniae*. We identified a previously uncharacterized protein, ChaB whose abundance increased significantly under replete conditions. *In silico* analysis putatively identified ChaB as a cation transport regulator. Deletion of *chaB* resulted in *K. pneumoniae* with reduced capsule production compared to wild-type, however, virulence was not impacted by this phenotype. We validated our proteomic results by quantifying the impact of a histidine utilization repressor (HutC) on its target genes at the transcriptional level. Overall, this study provides new insight into the global impact of zinc in *K. pneumoniae* and characterizes a cation transport regulator, uncovering a novel connection between zinc availability and capsule production.

2. Results

*Profiling of the K. pneumoniae cellular proteome influenced by zinc availability impacts transcriptional and transport processes*

Under infection conditions, the host will sequester free zinc ions to deprive the invading pathogen of this vital micronutrient\(^29\). Free zinc concentration in the serum is expected in the micromolar range, in agreement with a recent study in *E. coli*, here, we evaluate the impact of zinc availability by
quantitative proteomic profiling of *K. pneumoniae* WT cells grown in zinc-limited and -replete (10 µM) media (Fig 1)\(^30\). We identified a total of 2,380 proteins (representing 46.4% of encoding regions) in the cellular proteome. Upon filtering for proteins identified in three of four biological replicates, we further analyzed 2,002 proteins. Notably, we identified proteins unique to each growth condition: 15 proteins in limited and 99 proteins in replete (Fig 2a). A principal component analysis (PCA) defined separation of conditions (limited vs. replete media) (component 2, 30.4%) and separation of biological factors (component 1, 40.7%) (Fig 2b). Replicate reproducibility was 92.7% amongst the limited biological replicates and 96.7% amongst the replete biological replicates.

Using a 1D-annotation enrichment analysis, which demonstrates a global overview of changes to protein abundance by testing every annotation term if the corresponding numerical values have a preference to be systematically larger or smaller than the global distribution of the values for all proteins, we aimed to identify categories of proteins based on Uniprot Keywords that were enriched or de-enriched under limited or replete conditions\(^31\). We identified three categories enriched under replete conditions, including proteins associated with lipoproteins and ribosomal-associated proteins (Fig 2c). Conversely, we observed an enrichment of 4Fe-4S, iron-sulfur, metal-binding and transferase under limited conditions. These correlate to a reduction in metal ion acquisition, an expected result when analysing the limited conditions where zinc would be less bioavailable.

Next, to define proteins with significant changes in protein abundance between zinc-limited vs. -replete conditions in the cellular proteome, we performed false discovery rate (FDR)-corrected Student’s t-test. We identified 19 proteins whose abundance was significantly different between replete and limited conditions: 17 proteins significantly increased in abundance in replete media vs. two proteins significantly increased in abundance in limited media (Fig. 2d; Table 1). Under replete conditions, the most differentially abundant protein (>13-fold change) was the histidine utilization repressor (HutC), responsible for regulating the hut operon, which is involved in degradation of histidine to glutamate and ammonia\(^32\). Other proteins highly abundant under replete conditions include phage shock proteins (PspB and PspC) and a thiosulfate transport system permease T protein
(CysU), hinting at induction of a stress response to zinc availability. We also observed a significant change in abundance (>5-fold change) of a putative cation transporter (ChaB). Conversely, the two proteins significantly increased under limited conditions, include acetylacetate synthase small subunit (IlvN) and a Polycystic kidney and hepatic disease-type hydroxylase (KPN78578_12210).

**Secretome profiling of zinc availability defines changes in protein secretion patterns**

Acquisition of zinc from the environment may result in the secretion of proteins associated with sequestering and transport into the extracellular space. To identify if *K. pneumoniae* secrete proteins into the extracellular environment, we performed proteomic profiling of the supernatant (secretome) of *K. pneumoniae* cultures. We identified 246 proteins prior to filtering and performed subsequent analyses on 130 secreted proteins (present in three of four replicates), 56 of which were uniquely present under limited conditions and eight under replete conditions (Fig. 3a). Visualizing the variance of the biological replicates using a PCA plot identified the largest separating component (component 1, 38.7%) distinguishing the limited and replete samples (Fig. 3b). The second component (component 2, 16.9%) separated by biological variance. Amongst the conditions, we found a reproducibility of 80.8% and 78.6% for the limited and replete samples, respectively. Enrichment analysis of the secretome based on Uniprot Keywords did not show category enrichment in either limited or replete conditions. However, analysis of the secreted proteins based on Gene Ontology cellular compartment identified 33 proteins to be associated with conventional secretion patterns (e.g., transmembrane, signal peptide) (Fig. 3c). In addition, we compared our secretome data set to previously proteome profiling of *K. pneumoniae* vesicles and found overlap of 41 (31.5%) protein identifications, supporting our detection of traditionally intracellular proteins within the extracellular environment (Supp. Table 2)

Statistical analysis (FDR-corrected Student’s t-test) of the secretome dataset identified 23 significantly different proteins, including seven proteins with higher abundance in replete media and 14 proteins with higher abundance in limited media (Fig. 3d; Table 2). Under replete conditions, we identified proteins involved with transport such as a putative periplasmic binding protein (KPN_00624), a taurine transport protein (TauA), and a periplasmic chaperone (HlpA). The most
differentially abundant protein (>15-fold change) under replete conditions was a repressor of methionine biosynthesis, (MetJ). Conversely, there were 14 proteins that displayed an increased abundance under limited conditions, including two chaperones: a heat shock protein (DnaK) and a cold shock protein (CspC), a lipoprotein (MetQ), and an outer membrane protein (OmpX). Proteins displaying the largest fold-difference (>5-fold change) were two dehydrogenases: dihydrolipoyl dehydrogenase (LpdA) and pyruvate dehydrogenase E1 component (AceE).

qRT-PCR analysis of hutC validates protein changes in the presence of zinc and supports its role as a transcriptional repressor

Under zinc-replete conditions we observed a significant increase in abundance of HutC, a repressor of the histidine utilization (hut) operon, involved in the degradation of histidine to glutamate and ammonia\textsuperscript{32}. Under high zinc conditions, the abundance of HutC increases significantly and therefore, to validate our proteomic results, we aimed to quantify changes in transcript levels of its target genes (e.g., hutG, hutH, hutI, hutT, and hutU). We performed a gene expression analysis using qRT-PCR on the hut operon under limited and replete conditions. Under replete conditions, we report a down-regulation of the hut operon genes relative to limited conditions, as expected in the presence of high HutC production (Fig. 4). These results suggest an increase in histidine degradation under zinc limited conditions and support a role of zinc in influencing transcriptional repression.

Characterization of chaB and its role in zinc homeostasis

Under zinc replete conditions, we report the increased abundance (>5-fold change) of a putative cation transporter, ChaB. To investigate the role of this protein, we produced a deletion strain using Lambda Red recombination and evaluated an impact on growth between rchaB and WT K. pneumoniae under zinc-limited and -replete conditions. The WT strain reached stationary phase at a faster rate under limited conditions (6.5 h) compared to replete (9.5 h), suggesting delayed growth and possibly reflecting a negative response to the presence of 10 µM zinc (Fig. 5a). In contrast, both independent mutants of rchaB reached stationary phase of growth faster in replete conditions (8 h) compared to limited (9.5 h), suggesting an inability of the mutant strain to transport excess levels of zinc, which may impair bacterial growth. We also observe a lower growth rate of rchaB strains under
limited conditions, compared to WT, suggesting that *chaB* may also regulate the transport of additional cations (e.g., Ca\(^{2+}\) or Na\(^{+}\)) under limited conditions. These time- and nutrient-dependent data were supported by growth of the WT and *rchaB* strains on zinc-limited and -replete agar plates (Fig. 5b).

We also set out to phenotypically characterize *rchaB* through visualization of differences in capsule production between the WT and mutant strains. The combination of India ink and differential interference contrast (DIC) microscopy allowed for visualization of the capsule, from which, based on comparison of a representative number of *K. pneumoniae* cells (100 cells per condition), we report that deletion of *chaB* resulted in a reduction in capsule size (Fig. 5c). We do not report any additional morphological differences associated with zinc availability, furthermore, the *rchaB* strain, similarly, did not display any morphological differences between either zinc condition in the outlined experiments. The reduction in capsule size may reflect our observation of a slower growth rate by OD\(_{600}\) measurements of *rchaB* *K. pneumoniae* under zinc limited conditions.

The capsule is a known virulence factor for *K. pneumoniae* and to test impact of the gene deletion on virulence, we infected macrophages with WT or *rchaB* strains. Using a cytotoxicity assay, we measured the release of lactate dehydrogenase (LDH) from macrophages across four time points. Comparison of the two strains indicated that the reduction in capsule size reported with the *rchaB* mutant did not impair the virulence of the bacteria (Fig. 5d). We identified a steady increase in macrophage death from 5% at 1 h to ~65% at 18 h post-infection. Uninfected macrophages displayed a gradual increase in cell death, with 30% cell death at 18 h post infection, representative of natural cell death over time. The similar profile of the WT and *rchaB* strain indicate that the ability of *K. pneumoniae* to evade phagocytosis or to invade macrophages was not affected by the gene deletion.

3. Discussions
Zinc availability is regulated by hosts to both restrict bacterial growth (nutritional immunity) and to promote metal toxicity as defense mechanisms during pathogen infection. Therefore, maintenance of intracellular levels of zinc for pathogenic bacteria such as *K. pneumoniae* are important for bacterial growth and survival under infection conditions. In this study, we present the first in-depth proteomic
profile of the impact of zinc availability on *K. pneumoniae*. Our study identified cellular and secreted proteins whose abundance was altered by the presence or absence of zinc. We analyzed the hut operon to validate our proteomics data and characterized the ChaB protein to define a novel role in zinc homeostasis and capsule regulation.

We identified HutC (transcriptional repressor) with the largest increase in fold difference under replete conditions in our cellular proteome profiling. In the Gram-negative bacterium, *Acinetobacter baumannii*, histidine can chelate free zinc, and within nutrient-limited environments, the bacteria express components of the hut operon to promote histidine degradation, thereby releasing free zinc into the environment. Given our observation of the altered abundance of HutC in the presence of zinc, our data supports a role of HutC in *K. pneumoniae* associated with zinc acquisition. This is further supported through our gene expression analysis, where we discovered that members of the hut operon (*hutG, hut H, hutl, hutT, and hutU*) exhibited reduced transcript expression under replete conditions associated with an increase in abundance of HutC and zinc availability. This mimics the observation in *A. baumannii*, where the hut operon was active under limited conditions to possibly promote zinc release from histidine. This proposes a putative mechanism to combat host defense responses that was identified in *A. baumannii* and may also exist in *K. pneumoniae*.

Characterization of the rchaB strain demonstrated a growth curve profile under replete conditions that mimicked the WT strain under limited conditions. We propose that ChaB may play a role in the import of zinc and other cations into the cells. Whereupon, under replete conditions, WT *K. pneumoniae* displays delayed growth when the transport regulator is present due to excess import of zinc, which may have a slight cytotoxic effect on the cells. However, rchaB strain is not affected by the extracellular concentration of zinc, but demonstrates a delayed growth rate under limited conditions, supporting the role of ChaB in transporting zinc and additional cations into the cell for optimal growth. Zinc can be imported into the cells by producing an ion gradient such as calcium or sodium, to generate zinc gradients. Moreover, the reduction in capsule we identified through DIC microscopy in rchaB in comparison to WT may explain why the growth curve under limited conditions
showed slower growth in rchaB. The early onset and increased production of capsule in the WT could lead to the appearance of rapid growth when using OD_{600} values as an indicator of growth. Acapsular mutants have previously been reported to display slower growth compared to WT strains in \textit{Streptococcus pneumoniae}^{38,39}. Taken together, ChaB has an important role in maintaining zinc homeostasis and regulation of capsule production, which may influence cellular growth within \textit{K. pneumoniae}.

Macrophage cytotoxicity assays showed that deletion of \textit{chaB}, resulting in a reduced capsule size did not impact bacterial virulence. Analysis of the assay identified that both the WT and rchaB had similar cytotoxic effects on macrophage cells over the course of 18 hours. These results suggest that capsule presence associated with \textit{chaB} is not essential to the virulence of the bacteria upon phagocytosis. Previous findings have reported that upon phagocytosis, \textit{K. pneumoniae} survives in a vacuolar compartment where it triggers programmed cell death of macrophages\textsuperscript{28}. Furthermore, once internalized, \textit{K. pneumoniae} reduced the expression of capsule biosynthesis genes. While survival of macrophage is unaffected, the rchaB strain may be more susceptible to phagocytosis, due to its reduced size in the absence of capsule compared to WT, as the capsule is an important virulence factor protecting the bacteria from phagocytosis\textsuperscript{40}. Moreover, the difference in size between WT cells producing a capsule and rchaB, which lacks a capsule, may influence the number of phagocytosed cells, suggesting that virulence of \textit{K. pneumoniae} is influenced by both capsule production and number of internalized cells.

With the secretome analysis, the protein that displayed the greatest fold change was MetJ, a repressor of methionine biosynthesis. Zinc has been previously shown to bind to methionine synthase proteins and is able to form complexes with methionine\textsuperscript{41}. We hypothesize that under elevated zinc conditions, bacteria regulate high intracellular zinc levels by promoting methionine synthesis. Therefore, the elevated levels of MetJ found in the extracellular space may be due to the export of this protein to thereby promote methionine biosynthesis or represent a signaling factor among bacterial cells in the presence of excess zinc. In addition, the abundance of MetJ in the cellular proteome under
both limited and replete conditions were not significantly different, suggesting the bacteria may secrete the protein as a method of regulation.

Conclusions
In summary, we used quantitative proteomics to investigate the first analysis of zinc availability on the proteome and secretome of *K. pneumoniae*. We identified proteins that display a significant change in abundance in the presence or absence of zinc, including a transcriptional repressor and putative cation transport regulator. We validate our proteomic results by performing gene expression analysis on the hut operon, confirming repression of its target genes under replete conditions. In addition, we identified a cation transport regulator (ChaB) under replete conditions and characterized its role in growth, capsule production, and virulence of *K. pneumoniae* in a deletion strain. Moreover, we identified a novel morphological difference – a reduction in capsule size – associated with the absence of the chaB that may provide insight into a novel role in zinc homeostasis. Further studies are required to identify either a direct or indirect relationship between ChaB and capsule production.

4. Methods
*Bacterial cells and growth conditions*

2. *pneumoniae* K52145, a hypervirulent wild-type strain (generously provided by Dr. Arturo Zychlinsky, Max Planck Institute for Infection Biology), was used in all experiments. The bacterial strain was cultured in Tryptic Soy broth (TSB) (Sigma-Aldrich) or grown on Tryptic Soy agar (TSA). Zinc-limited media was produced by first passing milliQ water through Chelex-100 resin (Bio-Rad). Chelex-treated milliQ water was used to prepare m9 minimal media. M9 minimal salts (5X) (Fisher Scientific) were prepared according to manufacturer’s instructions, followed by supplementation with 20% glucose, 1M MgSO_4_ and 1M CaCl_2_. Zinc-replete media was produced by supplementing the limited media with ZnSO_4_ to a final concentration of 10 µM. *K.*
*pneumoniae* was grown at 37°C with 200 rpm shaking.

**Sample preparation for mass spectrometry analysis**

WT *K. pneumoniae* was cultured in TSB overnight, cells were collected, washed with limited-media, and sub-cultured into either zinc-limited or -replete media. Each condition included four biological replicates. Samples were grown until cells reached mid-log phase, upon which, for total proteome analysis cells were collected, washed twice with phosphate-buffered saline (PBS), and resuspended in cold 100 mM Tris-HCl (pH 8.5). Cells were lysed using a probe sonicator set to five cycles of 30 sec on/off at an amplitude of 30%. Samples were treated with 2% sodium dodecyl sulphate and 10 mM dithiothreitol (DTT). Samples were heated to 95°C, prior to treatment with 55 mM iodoacetamide (IAA). Proteins were submitted to acetone precipitation, followed by digestion of proteins using LysC and trypsin. Digested peptides were purified and desalted using STop And Go Extraction (STAGE) tips.

For secretome analysis, the culture supernatant was filtered through 0.22 μM syringe filters. For each sample, 500 µl of filtered supernatant was treated with DTT, IAA, followed by digestion using LysC and Trypsin. Digested peptides were desalted and purified as described above.

**Mass spectrometry**

Digested peptides were resuspended in 12 µl buffer A (0.1% TFA). Six µl of each sample was analyzed on a Q Exactive™ HF-X hybrid quadrupole-orbitrap mass spectrometer (ThermoFisher Scientific) coupled to an Easy-nLC™ 1200 High-Performance Liquid Chromatography (ThermoFisher Scientific). Samples were loaded onto an in-line 75 μm x 50 cm PepMap RSLC EASY-Spray column filled with 2 μm C18 reverse-phase silica beads (ThermoFisher Scientific). Peptides were separated and directly electrospayed into the mass spectrometer using a linear gradient from 3% to 20% Buffer B over 18 min, from 20% to 35% Buffer B over 31 mins, followed by a steep 2 minute ramp to 100% Buffer B for 9 minutes in 0.1% Formic acid at a constant flow of 250 nl/min. The mass spectrometer was operated in data-dependent mode, switching automatically between one fill scan and subsequent MS/MS scans of 30 most abundant peaks, with full-scans (m/z 400-1600) acquired in the Orbitrap.
analyzer with a resolution of 60,000 at 400 m/z.

Data analysis and bioinformatic processing

Raw mass spectrometry files were analyzed using MaxQuant (ver. 1.6.0.26)\textsuperscript{21}. The spectra were searched using the Andromeda search engine against the UniProt \textit{K. pneumoniae} proteome (\textit{Klebsiella pneumoniae} subsp. \textit{pneumoniae} (strain ATCC 700721 / MGH 78578), accessed Dec. 2018 with 5127 sequences)\textsuperscript{22}. For the search, cysteine carbomethylation was set as a fixed modification, while N-acetylation of proteins and oxidation of methionine were set as variable modifications. Additional parameters include: trypsin digestion, a minimum peptide length of seven amino acids, and allowing up to two missed cleavages. For protein identification, we required a minimum of two peptides and set the FDR to 1%. The ‘match between runs’ feature of MaxQuant was used. Quantification was performed by label-free quantification using the MaxLFQ algorithm\textsuperscript{23}. The raw data is deposited in the PRIDE partner repository for the ProteomeXchange Consortium (Data set identifier = PXD016042). All mass spectrometry experiments were performed in biological quadruplicate and technical duplicate.

Downstream statistical analysis and data processing was performed using Perseus (ver. 1.6.2.2)\textsuperscript{24}. Further statistical analysis was only performed on proteins that were present in at least three of four replicates within each sample condition. Upon filtering, missing values were imputed based on a normal distribution. A Student’s \textit{t}-test was performed to identify significantly different proteins (\textit{p}-value \(\leq 0.05\)). The Benjamini-Hochberg multiple hypothesis correction testing (FDR = 0.05) was applied. For 1D annotation enrichment, a two-sample test was performed using a Student’s \textit{t}-test with FDR = 0.05 and score < -0.5, < 0.5. Data visualization was produced using RStudio.

Gene expression analysis

Total RNA was extracted from WT \textit{K. pneumoniae} cultured in zinc-limited or -replete media using the PureLink™ RNA Mini Kit (Invitrogen). Extracted RNA was treated with DNaseI (ThermoFisher Scientific). DNase-treated RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied BioSystem). For qRT-PCR analysis, 2X PerfeCta SYBR Green FastMix, ROX
(Quanta BioScience) was combined with gene-specific primers (Supp. Table 1). Reactions were analyzed using StepOnePlus Real Time PCR system (ThermoFisher Scientific). All reactions were performed in three biological replicates and two technical replicates, and expression was normalized to the reference genes, \textit{recA} and \textit{rho}^{25}.

\textit{Construction of rchaB deletion mutant}

Mutation of \textit{K. pneumoniae} was produced following the lambda red recombination protocol\textsuperscript{26}. To summarize, electrocompetent WT \textit{K. pneumoniae} was transformed with the pSim6 plasmid, which contains the lambda red machinery\textsuperscript{27}. Primers were designed (Supp. Table 1) homologous to the regions upstream and downstream of \textit{chaB} to amplify the chloramphenicol resistance marker from the pKD3 plasmid. Subsequently, \textit{K. pneumoniae}-pSim6 was transformed with the PCR product and plated on TSA containing 15 µg/ml of chloramphenicol. Deletion strains were confirmed by PCR, followed by incubation at 37°C to cure the pSim6 plasmid.

\textit{Growth curve analysis}

To determine the impact of deleting \textit{chaB} on growth, WT and mutant strains of \textit{K. pneumoniae} were cultured overnight in TSB prior to sub-culturing into zinc-limited or -replete media. Cultures were grown at 37°C with 200 rpm shaking. Growth was monitored through OD\textsubscript{600} measurements taken every 15 minutes in a Synergy H1 microplate reader (BioTek). Two individual mutants of \textit{rchaB} were monitored in five biological replicates and two technical replicates. Growth curves were confirmed by growth in shaker flasks at 37°C with 200 rpm shaking.

\textit{Capsule morphology}

To visualize changes in capsule production between the WT and \textit{rchaB} strains of \textit{K. pneumoniae}, cultures were set as above, and at mid-log phase of growth, culture aliquots were collected and stained with India Ink. Cells were visualized on a Zeiss Axiovert 200M microscope using DIC and images were captured using a Hamamatsu ORCA-R2 digital camera. Volocity (ver. 6.3) software was used for image capture and processing.

\textit{Zinc utilization plates}
To examine the impact of zinc on colony morphology and growth, WT and rchaB strains of *K. pneumoniae* were initially grown in TSB overnight. Cells were collected, washed, and resuspended in zinc-limited media. Cells were plated on Chelex-treated m9 minimal media plates supplemented with 10 µM ZnSO$_4$ or plates without zinc. Cells were plated as a serial dilution starting from $1 \times 10^7$ to $1 \times 10^2$. Each dilution series was set with two biological and two technical replicates and performed twice. Two individual mutants of rchaB were plated. Plates were incubated at 37°C.

**Macrophage cell culture**

Immortalized murine macrophages originally derived from WT Balb/C mice (generously provided by Dr. Felix Meissner, Max Planck Institute of Biochemistry) were grown in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and Pen/Strep. Cells were grown at 37°C in a humidified 5% CO$_2$ atmosphere.

**Infection of macrophage**

To test the impact of deleting chaB on virulence, infection of Balb/C macrophages were set following an adapted protocol. Macrophages were seeded into 12-well plates at $0.1 \times 10^6$ in DMEM complete medium and grown as described above. At confluence ($0.5 \times 10^6$), media was exchanged with DMEM complete media without Pen/Strep. WT and rchaB *K. pneumoniae* were cultured in TSB, harvested, washed with PBS, and $2.5 \times 10^7$ cells were added to the macrophage cells to achieve multiplicity of infection (MOI) of 50:1. After a 90-minute incubation, media was removed, cells were washed with PBS, and incubated in Pen/Strep-free DMEM supplemented with gentamicin (300 µg/ml). After a 90-minute gentamicin treatment, media was removed, cells were washed with PBS, and incubated in Pen/Strep-free DMEM supplemented with gentamicin (100 µg/ml). Macrophages were infected with either WT or two independent rchaB strains. Mock infections (no *K. pneumoniae*) were set following the above protocol. Both infections and mock-infections were set in biological triplicate and technical duplicate. All incubation steps were carried out at 37°C in a humidified 5% CO$_2$ atmosphere.
Cytotoxicity assay

At selected time points (1, 3, 6, and 18 hours post-infection), supernatant from *K. pneumoniae*-infected macrophages was collected. LDH release from infected and uninfected macrophages was colourimetrically measured (OD$_{490}$) using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer’s instructions. Cytotoxicity was calculated according to manufacturer’s instructions. Supernatant cell death values were normalized to total cell lysate OD$_{490}$ measurements.

Abbreviations

WT wild type
PCA principal component analysis
FDR false discovery rate
DIC differential interference contrast
LDH Lactate dehydrogenase
TSB tryptic soy broth
TSA tryptic soy agar
PBS phosphate-buffered saline
DTT dithiothreitol
IAA iodoacetamide
STAGE STop And Go Extraction
nLC nano liquid chromatography
MS/MS mass spectrometry/mass spectrometry
LFQ label-free quantification
DMEM Dulbecco's Modified Eagle Medium
FBS fetal bovine serum
MOI multiplicity of infection

Declarations
Ethics approval and consent to participate

Not applicable

Consent for publication

The authors consent this manuscript for publication

Availability of data and materials

The mass spectrometry proteomics data have been deposited in the PRIDE partner repository for the ProteomeXchange Consortium with the data set identifier: PXD016042

Reviewer account username: reviewer79615@ebi.ac.uk

Password: 0xZA4mvc

Competing interests

The authors declare they have no competing interests

Funding

This work was supported, in part, by the University of Guelph for graduate student funding and mass spectrometry fees and the Canadian Foundation of Innovation (JELF 38798) for equipment and infrastructure for J.G.-M. Funding bodies had no role in study design, collected data, analysis, or writing.

Author Contributions

J.G.-M. & A.S. conceived the project, planned the experiments, performed the data analysis and interpretation, generated the figures, and wrote and edited the manuscript. A.S. performed the experiments. All authors have read and approved the manuscript.
Acknowledgments

The authors wish to thank Mr. Duncan Carruthers-Lay for preliminary optimization of qRT-PCR primers, Ms. Lilianne Gee and Mr. Benjamin Muselius for technical assistance, Dr. Jonathan Krieger of Bioinformatics Solutions Inc. for operating the mass spectrometer, and members of the Geddes-McAlister lab for their critical reading and insightful comments during manuscript preparation.

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Tables
Table 1: Significantly different proteins identified in the cellular proteome of *K. pneumoniae* relative to zinc replete conditions.
| Protein IDs | Fold difference | Gene names | Protein names |
|------------|----------------|------------|---------------|
| A6TFX2     | -5.522         | ilvN       | Acetolactate synthase small subunit |
| A6T7W1     | -3.923         | KPN78578_12210 | PKHD-type hydroxylase |
| A6THZ7     | 3.587          | deoA       | Thymidine phosphorylase |
| A6TGG0     | 3.783          | ilvY       | Positive regulator for ilvC |
| A6TFL0     | 3.945          | yibQ       | Uncharacterized protein |
| A6T709     | 4.210          | KPN78578_09190 | UPF0434 protein |
| A6T7L3     | 4.406          | yciE       | Uncharacterized protein |
| A6TBM1     | 4.507          | yohC       | Putative transport protein |
| A6THH0     | 5.221          | treR       | Trehalose repressor |
| A6T810     | 5.363          | pspB       | Phage shock protein B |
| A6TAN3     | 5.442          | chaB       | Cation transport regulator |
| A6T811     | 5.691          | pspC       | Phage shock protein |
| A6TI69     | 5.862          | KPN_pKPN3p05911 | Uncharacterized protein |
| A6TC61     | 6.434          | cysU       | Sulfate, thiosulfate transport system permease T protein |
| A6T9K1     | 7.243          | KPN_01841  | Uncharacterized protein |
| A6TF61     | 7.816          | yhhA       | Uncharacterized protein |
| A6TAE9     | 12.359         | KPN_02142  | Uncharacterized protein |
| A6T769     | 13.369         | acyP       | Acylphosphatase |
| A6T6K9     | 13.487         | hutC       | Histidine utilization repressor |

Table 2: Significantly different proteins identified in the secretome of *K. pneumoniae* relative to zinc replete conditions.

| Protein IDs | Fold difference | Gene name | Protein name |
|-------------|-----------------|-----------|--------------|
| A6T9K1      | 5.672           | lpdA      | Dihydrolipoyl dehydrogenase |
| Rank | Gene | Description                                      |
|------|------|--------------------------------------------------|
| 5.271 | aceE | Pyruvate dehydrogenase E1 component              |
| 5.135 | rpsT | 30S ribosomal protein S20                        |
| 5.043 | pgi  | Glucose-6-phosphate isomerase                     |
| 4.366 | ompX | Outer membrane protein X                          |
| 4.238 | adhP | Alcohol dehydrogenase                            |
| 4.018 | atpD | ATP synthase subunit beta                        |
| 4.017 | metQ | Lipoprotein                                       |
| 3.970 | rplV | 50S ribosomal protein L22                        |
| 3.743 | rplP | 50S ribosomal protein L16                        |
| 3.615 | thiE | Thiamine-phosphate synthase                      |
| Gene ID | log2 Fold Change | Description |
|--------|------------------|-------------|
| dnaK   | 3.306            | Chaperone protein DnaK (HSP70) |
| fusA   | 3.244            | Elongation factor G |
| cspC   | 3.239            | Cold shock protein |
| hlpA   | -2.361           | Periplasmic molecular chaperone for outer membrane proteins |
| KPN78578_01770 | -2.743         | UPF0325 protein |
| KPN_00624 | -3.185         | Putative periplasmic binding protein/LacI transcriptional regulator |
| tauA   | -3.396           | Taurine transport protein |
| ihfA   | -6.877           | Integration host factor subunit alpha |
| rpoZ   | -14.425          | DNA-directed RNA polymerase subunit omega |
Figures

Mass spectrometry-based proteomics workflow of K. pneumoniae during zinc limitation.

Bottom-up proteomics workflow for profiling the cellular proteome and secretome of K. pneumoniae under zinc-limited (M9 minimal media with 0 µM zinc) and zinc replete (M9 minimal media supplemented with 10 µM zinc) conditions. Proteins were extracted from cells and supernatant followed by enzymatic digestion and purification on C18 resin tips. The purified peptides are measured in the first MS scan and peptide fragmentation patterns are observed in the second MS scan (MS/MS). The data is processed and analyzed using the publicly available MaxQuant and Perseus platforms. Figure generated with Biorender.com.
Cellular proteome profiling of K. pneumoniae cultured in zinc-limited and -replete media. A) Venn diagram depicting total unique proteins identified in limited (red) and replete (blue) conditions. B) Principal component analysis. Variance amongst biological replicates of cells grown in limited (red) and replete (blue) conditions separated based on component 1 (40.7%) and component 2 (30.4%). C) 1D-annotation enrichment. UniProt keywords enriched in the replete condition relative to the limited. Statistical significance confirmed by Student’s t-test (p-value <0.05, FDR = 0.05, score < -0.5, <0.5). D) Volcano plot depicting proteins present in cellular proteome. Proteins in blue are significantly abundant under replete conditions. Proteins in red are significantly abundant under limited conditions. Statistical analysis was performed using a Student’s t-test (p-value <0.05; FDR = 0.05; S0=1). Experiment performed in biological quadruplicate and technical duplicate.
Secretome profiling of K. pneumoniae cultured in zinc-limited and -replete media. A) Venn diagram depicting total proteins identified in limited (red) and replete (blue) conditions. B) Principal component analysis. Variance amongst biological replicates of cells grown in limited (red) and replete (blue) conditions separated based on component 1 (38.7%) and component 2 (16.9%). C) Pie chart depicting Gene Ontology Cellular Compartment categories and presence of signal peptide. D) Volcano plot depicting proteins present in secretome. Proteins in blue are significantly abundant under replete conditions. Proteins in red are significantly abundant under limited conditions. Statistical analysis was performed using a Student’s t-test (p-value <0.05; FDR = 0.05; S0=1). Experiment performed in biological quadruplicate and technical duplicate.
Gene expression analysis of the hut operon. Total RNA was extracted from K. pneumoniae grown in zinc-limited and -replete media. Values are normalized against the expression of the reference genes, rho and recA. Values reflect the expression of each gene in the replete condition relative to the limited condition. The asterisk (*) denotes statistically significant expression (one-tail Student’s t-test, p-value < 0.05). Data is reflective of three biological replicates and two technical replicates per biological replicate. Ratio of protein abundance of HutC under zinc-replete conditions relative to -limited is denoted by the bar with the red dotted border.
Characterization of growth, morphology, and virulence of \( \Delta \text{chaB} \) K. pneumoniae. A) Growth curve analysis of WT and \( \Delta \text{chaB} \) strains cultured in zinc-limited and -replete media. Experiment was performed in biological triplicate and two technical replicates. B) Zinc utilization plates. WT and \( \Delta \text{chaB} \) grown on either 0 or 10 \( \mu \text{M} \) ZnSO\(_4\) plates at 24, 48, and 72 hpi. C) DIC microscopy at 100x magnification of WT and \( \Delta \text{chaB} \) strain at mid-log phase of growth. Scale bar = 100 \( \mu \text{m} \). D) Cytotoxicity assay of Balb/C macrophages infected with WT and \( \Delta \text{chaB} \) K. pneumoniae (MOI 50:1). Results reflect the average of three biological replicates and technical duplicates. Error bars depict standard deviation. Two independent deletion mutants of chaB were characterized across all experiments.

**Supplementary Files**

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S-TABLES.docx