Journal of proteome research

Subscriber access provided by DigiTop | USDA's Digital Desktop Library

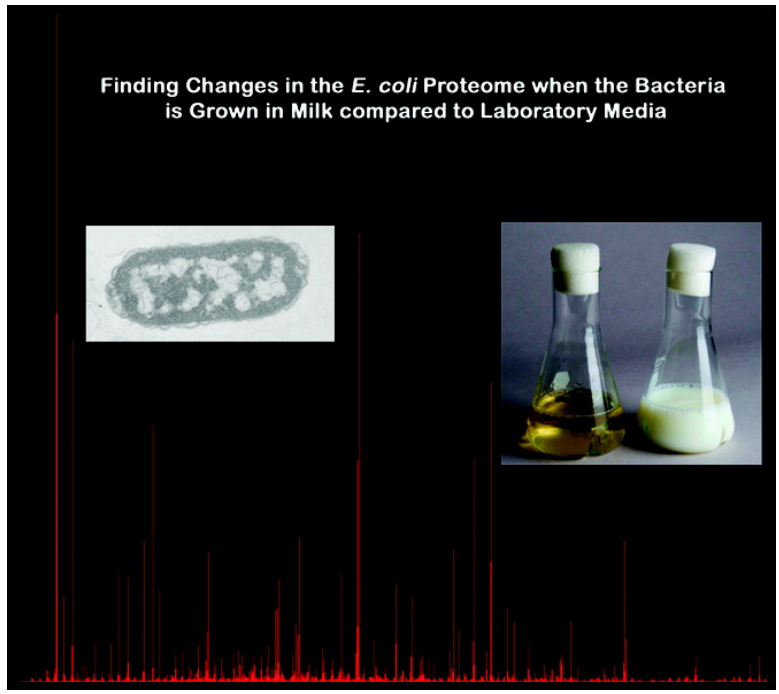
Article

Proteomic Changes in *Escherichia coli* When Grown in Fresh Milk versus Laboratory Media

John D. Lippolis, Darrell O. Bayles, and Timothy A. Reinhardt

J. Proteome Res., **2009**, 8 (1), 149-158• DOI: 10.1021/pr800458v • Publication Date (Web): 18 November 2008

Downloaded from http://pubs.acs.org on March 20, 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

Journal of Proteome Research is published by the American Chemical Society.
1155 Sixteenth Street N.W., Washington, DC 20036

research articles Journal of proteome research

Proteomic Changes in *Escherichia coli* When Grown in Fresh Milk versus Laboratory Media

John D. Lippolis,*,† Darrell O. Bayles,‡ and Timothy A. Reinhardt†

Periparturient Diseases of Cattle Research Unit, and Bacterial Diseases of Livestock Research Unit, USDA-ARS, National Animal Disease Center, Ames, Iowa 50010

Received June 20, 2008

To establish an infection, bacteria must first adapt to the host environment and grow. For mastitis, the media in which bacteria must adapt to is milk. Mammary pathogenic *Escherichia coli* may express factors that are important for these bacteria to grow in milk and establish both acute and chronic infections of the mammary gland. We have used shotgun expression proteomics to determine the changes in protein expression when *E. coli* were grown in laboratory media compared to bacteria grown in whole fresh bovine milk. We report the expression data of approximately 1000 proteins. We found many proteins involved in the metabolism of lactose and various amino acids were up-regulated when bacteria were grown in milk. In addition, the LuxS protein was up-regulated when bacteria were grown in milk; this protein is associated bacterial quorum sensing as well as the expression of mobility proteins, such as flagellar components, and a number of genes associated with virulence. Growth of a mammary pathogenic *E. coli* in milk results in the up- and down-regulation of hundreds of proteins, some of which may be important to the pathogenesis of this organism.

Keywords: Mastitis • *E. coli* • Infection • Dairy cow

Introduction

Analysis of changes in bacterial proteomes has been an area of intense research in the last several years. Changes in bacterial growth media or conditions can have significant effects on protein expression.[1−6] For example, *Pseudomonas aeruginosa* is an environmental bacterium that is a significant opportunistic pathogen in cystic fibrosis patients. This bacterium can grow in either aerobic or anaerobic conditions and is thought to grow anaerobically within mucopurulent masses in the lung. Proteomic analysis of *P. aeruginosa* grown in aerobic versus anaerobic conditions showed the differential expression of over 150 proteins giving clues to the metabolic pathways needed to maintain an infection in the host's tissues.[6] Understanding in vivo changes in protein expression by bacteria as they adapt to their environment and the pressures exerted by the host immune system is fundamental to finding better therapeutics for treatment of bacterial infections. A critical factor that affects bacterial protein expression changes is the environment in which the bacteria exist. How bacteria adapt to their environment may determine their ability cause an infection and possibly their ability to evade the immune system.

Infection of the mammary gland is a health concern for humans and animals. Depending on the type of delivery, 1.7−3.0% of women suffer from mastitis.[7] In the dairy industry, this disease alone accounts for $2 billion a year in losses to the industry.[8] Milk is the environment that bacteria must grow in to establish an infection of the mammary gland. However, milk is not a rich growth media for bacteria. In fact, milk naturally contains many mechanisms to inhibit bacterial growth, such as lactoferrin to sequester iron, making it unavailable for bacterial use.[9] In addition, the immune system has numerous mechanisms that detect, signal, and respond to a bacterial infection. The major and first immune response is the infiltration of neutrophils into the infected gland.[10] However, this response requires 6−12 h to occur in an infected dairy cow and growth rates of various stains of *Escherichia coli* in an infected gland prior to the immune response have been correlated with final severity of the infection.[11] The ability of an *E. coli* strain to grow in milk may be linked to the pathogenesis of the organism, and therefore, understanding the proteins expressed when the bacteria is grown in milk may elucidate cellular pathways important for the initial steps in pathogenesis.

Bacteria have evolved to grow in inhibitory media, such as milk, and comparing levels of various proteins expressed when grown in milk versus growth in a rich culture media may reveal metabolic pathways necessary for adaptive growth in milk as well as adaptations important to an intramammary infection.[12] It has been suggested that virulence factors specific for mammary pathogenic *E. coli* (MPEC) exist and that these factors may be important for persistent infections.[13] Some of these factors may simply be those that allow the bacteria to grow in milk.

Understanding bacterial adaptions to growth in milk by determining which proteins are differentially expressed in milk may provide critical information on how bacteria live in the

* To whom correspondence should be addressed. E-mail: john.lippolis@ars.usda.gov.
† Periparturient Diseases of Cattle Research Unit.
‡ Bacterial Diseases of Livestock Research Unit.

10.1021/pr800458v This article not subject to U.S. Copyright. Published 2009 by the American Chemical Society
Published on Web 11/18/2008

mammary gland and how to block them from adapting to life in the mammary gland. This work compares the proteome of a MPEC *E. coli* strain grown in laboratory media to the same strain grown in fresh unprocessed bovine milk.

Materials and Methods

Bacterial Strain, Media and Growth. The strain used for these experiments was *E. coli* strain P-4, serotype O32:H37, which was originally isolated from a clinical case of bovine mastitis (kind gift from M. J. Paape, Immunology and Disease Resistance Laboratory, ARS, USDA, Beltsville, MD). Fresh unprocessed milk, collected by hand, from three midlactation Holstein cows, was combined and used as a bacterial growth media. The milk pool was checked for bacterial contamination by plating on TSA + 5% Sheep RBC (Blood Agar) (BD, Sparks, MD) plates and incubating overnight at 39 °C. Inoculum stocks of bacteria were grown in Luria−Bertani (LB) (BD, Sparks, MD) or fresh pooled milk to midlog phase grown at 37 °C and shaking at 225 rpm and stored at 4 °C until use. Bacteria growth in LB was monitored by optical density at 600 nm and bacterial growth in milk was determined by colony count on blood agar plates. Multiple 2-L flasks with 500 mL of clean freshly collected milk or LB culture media were inoculated with bacteria grown in matching media. Multiple flasks were used for each media. The cultures were grown to late midlog phase at 37 °C and shaking at 225 rpm. One flask was used to gather additional time points after the others were removed to isolate bacteria. Two independent experiments were run, consisting of bacteria grown in LB and milk. These experiments are denoted in the supplementary tables in Supporting Information as Sample A and Sample B.

Isolation of Bacteria from Milk. Bacteria were pelleted by centrifugation at 10 000*g* for 20 min at 4 °C. Milk whey and as much as the fat as possible were removed from the pelleted bacteria that were grown in milk. All bacterial pellets were resuspended in cold DPBS without calcium or magnesium (Sigma, St. Louis, MO) and transferred to a new centrifuge bottle and washed twice in cold DPBS. Pellets were resuspended in 25 mL of cold DPBS and were overlaid on 15 mL of 45% sucrose (w/v) prepared in deionized H_2O in a 50 mL conical centrifuge tube. The gradients were centrifuged for 40 min at 10 000*g* at 4 °C. Pellets were again resuspended in cold DPBS and a second sucrose gradient performed. After the second gradient spin, the pellets were washed six times with 45 mL of cold DPBS. All supernatant was removed and the pellets were snap-frozen in liquid nitrogen and were stored at −80 °C until processed for MS analysis.

Sample Processing. All procedures were done at 4 °C. Bacterial pellets were sonicated in 4 mL of 10 mM triethylammonium bicarbonate, pH 8.5, 50% duty cycle, 20 cycles, 3 times. The homogenate was centrifuged at 1000*g* for 10 min to remove cellular debris. The pellet was discarded and the supernatant was then centrifuged at 100 000*g* for 1 h. This provided the crude membrane (pellet) and the cytosol (supernatant) protein fractions. These were stored at −20 °C until used. Total protein concentrations were determined using the Protein Assay Kit (BioRad, Hercules CA), using a BSA standard. For experimental workflow, please see Figure 1.

Briefly, 100 μg of each sample was resuspend in 60 μL of 25 mM triethyl-ammonium bicarbonate (pH 8) and heat denatured at 95 °C for 30 min.[14] Samples were cooled on ice for 10 min and 120 μL of methanol was added to each tube.[15] For trypsin digestion, 25 μL of proteomic grade trypsin (20 μg /mL in 25 mM triethyl-ammonium bicarbonate) was added to each sample and incubated overnight at 37 °C. Samples were dried in a vacuum centrifuge. Samples were resuspended, denatured, reduced and then cysteine residues were blocked with MMTS and finally labeled with various iTRAQ reagents as described in the manufacturer's instructions for the iTRAQ kit (Amine-Modifying Labeling Reagents for Multiplexed Relative and Absolute Protein Quantitation (Applied Biosystems, Foster City CA)).

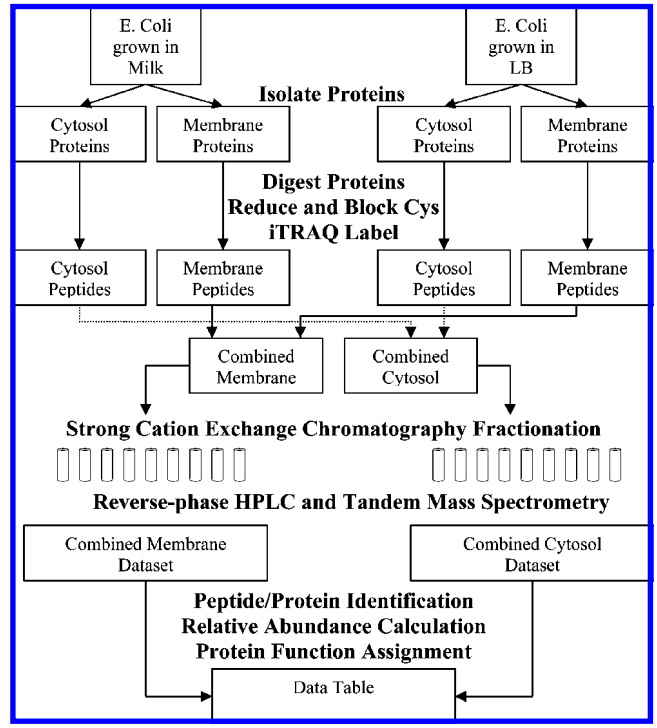


Figure 1. Workflow of proteomic experiments in milk and LB *E. coli* strain P4 was grown in fresh whole bovine milk or LB. Bacteria were isolated from media by centrifugation and multiple sucrose gradients. Bacteria were lysed and cytosol and membrane proteins recovered. Proteins samples were digested and reduced, and free cysteines were blocked with MMTS and finally iTRAQ labeled. Cytosol peptides from milk and LB were combined into one tube as was the membrane peptides. Each combined sample was run over a strong-cation exchange column and approximately 30 fractions were collected. Each fraction was run on a reverse-phase HPLC column in-line with a tandem mass spectrometer (qTOF). All data from one group (e.g., Combined Membrane) were combined into one.pkl file and submitted to Mascot for peptide/protein identification and relative abundance calculation. We generated a computer script to align TIGR functions with our protein matches. This whole procedure is repeated to generate a duplicate data set (B).

Peptides labeled with iTRAQ were dried and resuspended in 300 μL of 20 mM formic acid and 20% acetonitrile (ACN). Samples were run on a strong cation exchange (SCX) column (Mono S PC 1.6/5, Amersham, Piscataway, NJ) with a gradient of solution A (20 mM formic acid; 20% ACN; pH 2.7) and solution B (20 mM formic acid; 20% ACN; 350 mM ammonium bicarbonate; pH 4.7). Sample fractions (0.5 mL) were collected over a 75-min gradient of 0−100% solution B. Sample fractions were dried down and resuspended in 30 μL of 0.1% formic acid in 5% ACN.

High Performance Chromatography and Tandem Mass Spectroscopy of the Samples. Each SCX fraction was analyzed by capillary high-pressure liquid chromatography (CapLC,

Waters, Milford MA) in-line with a Q-TOF Ultima API mass spectrometer (Waters, Milford MA). An Altantis C_{18} NanoEase column (75 mm × 100 mm) was used for peptide separation. The system was configured to concentrate and wash the injected sample on a Symmetry 300 C_{18} precolumn. Seven minutes after the start of sample loading, the precolumn was switched in-line with the analytical column to allow the trapped peptides to be eluted onto the analytical column. Mobil phase A was 0.1% formic acid in 5% ACN. Mobil phase B was 0.1% formic acid in 95% ACN. The gradient was 95% A for 5 min and then ramped linearly to 60% A over 85 min. Over the next 2.5 min, it was ramped to 10% A and held an additional 10 min before re-equilibration of the column. The flow rate was approximately 300 nL/min. The analytical column was connected to Waters Lockspray-nanospray interface on the front of the mass spectrometer. The lockspray used the peptides [Glu[1]]-Fibrinopeptide B and Leucine Enkephalin as standards (Sigma, St. Louis, MO). The capillary voltage was 3700 V and was tuned for signal intensity. The 5 most intense ions with charge states between 2 and 4 were selected in each survey scan if they met the abundance threshold to sequence the peptide. Three collision energies were used to fragment each peptide ion based on its mass to charge (m/z) values. Each fraction was run multiple times, collecting MS/MS data on parent masses in different mass ranges.[16]

Protein Identification and Quantitation. All MS data files were processed into.pkl files using ProteinLynx Global Server 2.0 (Waters, Milford, MA). Data was calibrated throughout the run by using lock-spray correction of MS data with [Glu[1]]-Fibrinopeptide B and MS/MS with Leucine Enkephalin. The.pkl files from a sample group were merged into a single file using the program Merge (Matrix Science, London, U.K.). The data was then analyzed with Mascot version 2.2.1 (Matrix Science, London U.K.) using the Swiss-Prot protein database (51.6) with *E. coli* taxonomy, MMTS (C), iTRAQ (K) and iTRAQ (N-term) as fixed modifications. The peptide tolerance was 0.1 Da and the MS/MS tolerance was 0.1 Da.[17,18] Protein quantitation data was obtained from the Mascot results. Quantitation data along with other protein data such as Protein Description, Gene Name, Protein Mass, Protein Score, Number of Peptide Matches, Coverage of Protein, and emPAI score were exported to an Excel spreadsheet (see Supplementary Tables 1−5 in Supporting Information). When the quantitation data for a protein was less than 1, meaning that the protein was down-regulated, we took the reciprocal to that number and labeled the protein as down-regulated. If the quantitation data for a protein was greater than 1, it was labeled as up-regulated.

Data Mapping to Functional Categories. The expression proteomics data set was annotated by populating the Mascot output data with The Institute for Genomic Research (TIGR) role and subrole categories via relational databases. The Mascot output contained the Swiss-Prot IDs (entry names) for matches found to the Swiss-Prot database. A relational database indexing all Swiss-Prot IDs to corresponding Swiss-Prot accession numbers was built from an index flat file obtained from Expasy via ftp (ftp.ebi.ac.uk/pub/databases/uniprot/current_release/knowledgebase/complete/docs/acindex.txt). This first relational database allowed the Swiss-Prot accession numbers to be obtained for entries in the expression proteomics data set. A second relational database indexing Swiss-Prot accession numbers to TIGR role and subrole definitions was created from information obtained from the Comprehensive Microbial Resource (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi). The second relational database allowed the penultimate mapping of the TIGR roles and subroles to the expression proteomics data set.

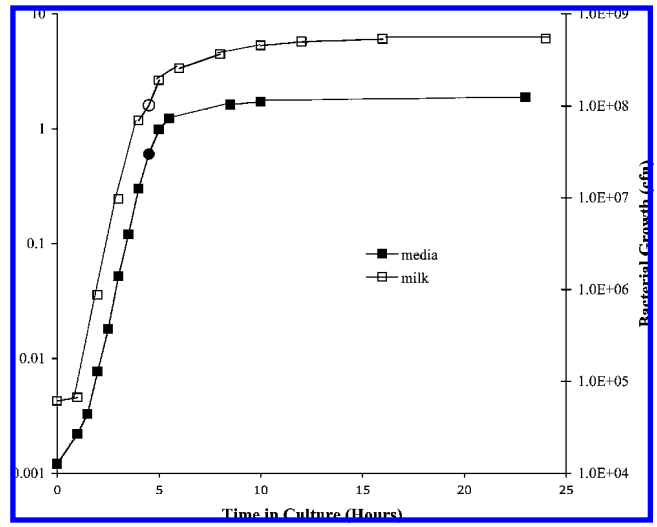


Figure 2. Growth of *E. coli* in milk and LB. *E. coli* was grown in LB or fresh unprocessed bovine milk. Growth of bacteria in LB (filled symbols) was monitored by optical density (OD_{600}), whereas, growth of bacteria in milk (open symbols) was determined by colony counts of plated samples on bacteria plates (cfu). The squares (filled and open) were the time points that the bacterial samples were removed for processing (4.5 h). A duplicate sample was permitted to continue to grow to gather additional time points.

Western Blots. Fresh bovine milk and whey samples were obtained and the protein concentration for all the samples (milk, whey, and membrane sample for *E. coli* grown in milk of LB) were determined by BCA assay (Pierce, Rockford, IL) according to manufacturer's instructions. The same amount of protein (5 μg) from each sample was prepared and run on 4−12% Bis-Tris Gradient Mini Gel (Invitrogen, Carlsbad, CA) using the instructions for running denaturing NuPage gels. Gels were either stained or used in a Western blot. Staining was done using Imperial Blue Stain (Pierce, Rockford, IL) according to manufacturer's instructions and subsequently imaged using a VersaDoc imager (BioRad, Hercules, CA) using the Coomassie Blue setting. The gels that were transferred for Western Blot were done using the iBlot apparatus (Invitrogen, Carlsbad, CA) according to manufacturer's directions. The membranes were then blocked overnight at 4 °C in Starting Block (Pierce, Rockford, IL). The membrane were probed with a 1:10 000 dilution of Rabbit anti-Bovine Casein HRP conjugated antibody (ICL, Newberg, OR) in Starting Block and 0.05% Tween 20 (Pierce, Rockford, IL). The membrane was washed 6 × 50 mL/wash with DPBS and 0.05% Tween 20 (Sigma, St. Louis, MO). After the last wash, the blot was stained with WEST Dura (Pierce, Rockford, IL) and imaged using a VersaDoc imager (BioRad, Hercules, CA) using the Chemoluminence Hi Sensitive setting.

Results

Bacterial Growth and Isolation. *E. coli* was grown in fresh unprocessed bovine milk or LB and harvested in late log-phase growth (Figure 2). Bacterial densities were similar between the two media at the time of harvest, 1.0×10^8 cfu/mL in milk and 2.9×10^8 cfu/mL in LB (data not shown). To remove the

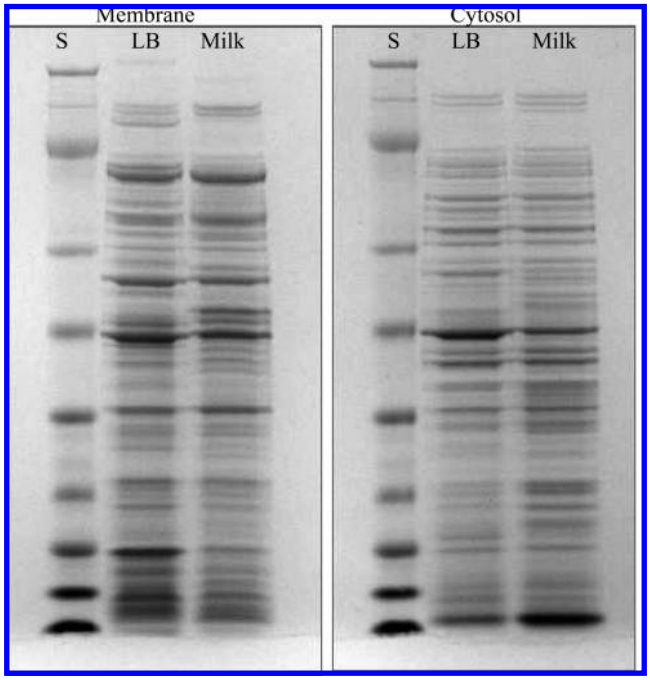


Figure 3. Membrane and cytosol protein preparations from *E. coli* grown in LB or milk. A total of 7.5 μg of protein from the membrane or cytosol fractions of *E. coli* grown in LB or milk was run on a SDS-PAGE gel and stained with Imperial Blue (Pierce, Rockford, IL). Lane S is SeeBluePlus2 prestained molecular weight standards (Invitrogen, Carlsbad, CA), Lane LB is the proteins isolated from the sample grown in LB, and Lane Milk is the proteins isolated from the sample grown in fresh unprocessed bovine milk.

milk proteins, bacterial pellets were subjected to sucrose-gradient centrifugation. SDS-PAGE of protein extracts from bacteria grown in milk or LB did not show any overwhelming protein bands in the milk sample compared to the media sample, suggesting that a majority of the milk proteins were removed (Figure 3). For comparison, milk, whey, and the membrane samples of *E. coli* grown in milk or LB were run on a SDS-PAGE gel (Figure 4). Figure 4A shows that both milk and whey samples contained the typical high abundance proteins, which include β-casein and α-casein (between 32 and 25 kDa), whereas the *E. coli* protein sample grown in milk does not appear to have these bands in large abundance. Figure 4B shows a Western blot of these same samples using an anti-casein antibody as a probe. Again, clear β-casein and α-casein bands are observed in the milk and whey samples and no bands are detected in the *E. coli* sample preparations. When the mass spectrometry data was analyzed using the protein identification software with no taxonomy restriction, we did identify some proteins of bovine origin. However, using the emPAI number as an indicator of protein abundance,[19] we estimate that there are over 100 more abundant proteins than the most abundant contaminating milk protein (alpha casein, emPAI score 1.52) in the cytosol sample. Interestingly, in the membrane sample, the 24th most abundant protein (using emPAI score) was bovine Complement C3 precursor (emPAI score 8.1) (Supplementary Table 5 in Supporting Information). This observation is consistent with the known function of complement C3b, which is to opsonize pathogens. The fact that the majority of proteins identified did not show any expression change, again strongly suggest that most of the bovine proteins were removed from our samples.

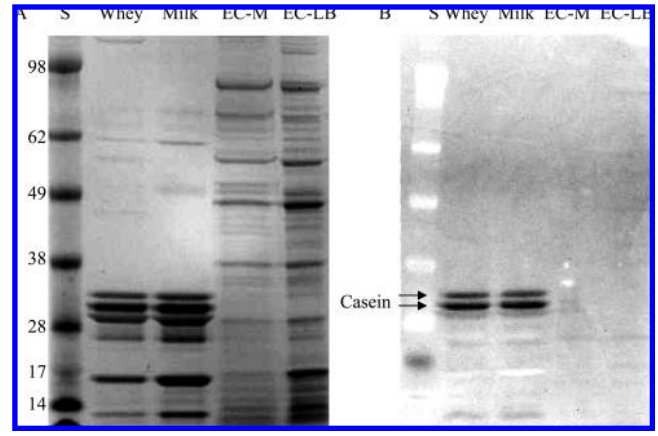


Figure 4. SDS-PAGE and Western blot of protein in milk and bacterial samples. A 5 μg aliquot of total protein isolated from fresh bovine whey, fresh bovine milk, and the membrane samples of *E. coli* grown in milk (EC-M) or LB (EC-LB) were run on two SDS-PAGE gels. The first gel (A) was stained with Imperial Blue (Pierce, Rockford, IL). Lane S is SeeBluePlus2 prestained molecular weight standards (Invitrogen, Carlsbad, CA). Numbers on the left side of gel A indicate molecular weights of standards (kDa). The second gel (B) was used in a Western blot and probes with a bovine casein specific antibody.

Table 1. Number of Peptide Matches and False Discovery Rate

peptide matches above identity threshold	membrane A	cytosol A	membrane B	cytosol B
Swiss-Prot	6466	7794	2559	2339
Decoy	200	114	62	42
False Discovery Rate	3.09%	1.46%	2.42%	1.80%

Protein Expression Changes in Milk. Membrane and cytosolic fractions were generated for each sample as an initial means of protein separation. Further separation was achieved after the proteins were digested by SCX and reverse-phase chromatography. Bacterial protein samples were trypsin-digested and iTRAQ-labeled, resulting in four samples labeled membrane A, cytosol A, membrane B, and cytosol B. Each sample was run over a SCX column resulting in ~30 fractions. Each SCX fraction was run on the LC-MS/MS and the data files for each sample (e.g., all 30 SCX fractions for the membrane A sample) were combined into one data set and analyzed with Mascot (Figure 1). Protein identification and relative abundance information was exported from Mascot into a spreadsheet (see supplementary Table 1 in Supporting Information). Table 1 contains the number of peptide matches above the identity threshold along with the false discovery rate for each of the four data sets. Protein identification and quantitation data from all four data sets were exported from Mascot and combined into a single spreadsheet (see supplementary Table 1 in Supporting Information). This spreadsheet is divided up into several worksheets that split the data into proteins whose expression went up in milk, went down in milk, did not change, or all proteins (see supplementary Tables 1−4 in Supporting Information). The numbers of proteins identified are summarized in Table 2. All together, we were able to identify 1000 proteins for which there was expression data. Replicate of this experiment (Sample A and Sample B) yielded similar data; however, for an unknown reason, Sample B yielded approximately one-third the number of peptide matches. The

Table 2. Number of Protein Matches with Expression Data or with Significant Expression Data

	membrane	cytosol	both	total
All Hits	336	356	308	1000
Significant	256	244	133	633

result of this is that some proteins are identified only in sample A. Of those 1000 proteins, 633 had expression data that was determined by Mascot to be significant (see supplementary Table 1 in Supporting Information). Mascot significance is independent of changes in expression and only indicates that the quality and quantity of the expression data is sufficient to have confidence in the data (www.matrixscience.com/help/quant_statistics_help.html).

Identified proteins were associated with functions as categorized by TIGR. We associated gene products with TIGR main roles and subroles (see supplementary Table 1 in Supporting Information). The functional categories for all the proteins observed are summarized in Table 3. Proteins within each functional category are divided into those proteins up-regulated and those down-regulated when bacteria were grown in milk. For example, under the main role of "Amino acid biosynthesis" is the subrole "Aromatic amino acid family", wherein 16 proteins were identified as belonging to this functional group. Of the 16 identified proteins of that group, 12 were up-regulated and 0 were down-regulated when grown in milk (Table 3). This pattern of a majority of proteins being up-regulated is consistent for most of the subroles in the "Amino acid biosynthesis main role". Not all functional groups showed protein expression up-regulation in milk. Of the 19 proteins identified to the subrole "Chemotaxis and motility", 0 were up-regulated and 8 were down-regulated when bacteria were grown in milk. A list of the proteins that were down-regulated in the "Chemotaxis and motility" subrole is shown in Table 4, of which two proteins are part of the flagellar complex (FlgE and FlgG) and six proteins that are involved in chemotaxis (Mcp1, Mcp2, Mcp3, CheR, MotA and MotB). Table 4 also includes other groups of proteins whose expression was altered by the bacteria's change in environment. In addition, proteins that regulated use of galactose are included in Table 4. This is consistent with the different primary carbon source in LB (glucose) and milk (lactose).

Tables 5, 6 and 7 have a summary of proteins up-regulated, down-regulated, and not changed, respectively. The supplementary tables contain all of the proteins we identified and their associated expression data. The four supplementary tables contain a list of all proteins (Supplementary Table 1), those proteins up-regulated in milk (Supplementary Table 2), only those proteins down-regulated in milk (Supplementary Table 3), and only those protein with no expression change (Supplementary Table 4 in Supporting Information). Supplementary Table 5 in Supporting Informationcontains proteins of bovine origin found in the membrane sample.

Discussion

Milk is the media in which bacteria must be able to initially grow in order to cause mastitis. However, milk is not a rich growth media for bacteria. In fact, milk contains a number of proteins, lipids, carbohydrates, and various cells that are antimicrobial.[20−22] These components not only serve to protect from and clear any infections of the lactating animal, but also are passively transferred to the neonate for protection against infections. Bacteria have evolved to grow in inhibitory media, such as milk, and comparing levels of various proteins expressed in bacteria when grown in milk versus bacteria grown in a laboratory media revealed bacterial protein pathways activated for adaptive growth in milk. In this study, we focus only on protein changes by a mastitis-causing *E. coli* strain that occurs when it is grown in freshly isolated bovine milk compared to a laboratory bacterial medium (LB).

With the use of isotopic peptide labeling in shotgun proteomic experiments, we have identified 1000 proteins and their relative expression levels comparing *E. coli* grown in milk versus grown in LB. A number of protein expression changes are easily attributable to nutrient changes that the bacteria must react to with the change of environment. For example, β-galactosidase is up-regulated nearly 4-fold when the bacteria are grown in milk. Additionally, three other proteins involved in galactose utilization were up-regulated when that bacteria was grown in milk (Table 4). These observations are consistent with the fact that these enzymes are involved in lactose metabolism and lactose is the major sugar in milk. One of the genes involved in galactose utilization whose product was up-regulated was UDP-glucose 4-epimerase (GalE) (Table 4). An *E. coli* strain (J5) that lacks this protein (GalE) has been shown to have an altered cell wall lipopolysaccharide.[23] Interestingly, the J5 strain *E. coli* is used in the dairy industry as a vaccine against coliform mastitis.[24] Our data showed the GalE expression was up-regulated greater than 5-fold when our pathogenic *E. coli* strain was grown in milk. It is possible that altered GalE expression may alter the lipopolysaccharide of the pathogenic strain making the pathogen less detectable by the immune system. In addition, nearly one-quarter of the detected proteins involved in amino acid biosynthesis are up-regulated in milk. This is consistent with the fact the LB contains a rich supply of free amino acids (yeast extract) that are not available in milk. Because these observations are consistent with known biological pathways that should be affected during growth in milk, this gives us greater confidence in our data set.

Of particular interest in our data set are proteins that are involved in the pathogenesis of the bacteria. One protein of interest is *S*-ribosylhomocysteine lyase or the LuxS gene product that was up-regulated 3.6-fold in milk. This enzyme is critical for the synthesis of a bacterial hormone-like compound called autoinducer 2 (AI-2).[25] AI-2 is involved in interbacterial communications also called quorum sensing. AI-2 is also involved in the regulation of a number of genes. In microarray experiments, the expression of 242 genes were significantly altered in response to AI-2.[26] We do not know if the increase of the LuxS protein seen in this study resulted in a increase of AI-2.

Flagella have been long associated with bacterial pathogenesis. For example, in mucosal infections, pathogenic bacteria must express flagella for motility, adhesion, invasion and secretion of virulence factors.[27] However, in this study, of the five structural flagellar proteins identified, all were down-regulated (1.5- to 4.3-fold; see Supporting Information). Interestingly, a flagellar protein called flagellin (FliC) has been identified as a ligand for toll-like receptor 5.[28] Toll-like receptors are a family of cell surface receptors that bind to various molecules that are specific to pathogens. When they bind to their ligands, these receptors initiate the activation of genes encoding various cytokines and chemokines that are central to an immune response.[29] Therefore, the reduced expression of flagellin (1.8-fold) may provide less ligand to initiate an

Table 3. Categorization of Proteins by TIGR Main Roles and Subroles

TIGR main roles and subroles	total	up[a]	down[a]
Amino acid biosynthesis			
Aromatic amino acid family	16	12	0
Aspartate family	16	11	0
Glutamate family	15	11	0
Histidine family	6	5	0
Other	1	0	0
Pyruvate family	11	10	0
Serine family	11	5	0
Biosynthesis of cofactors, prosthetic groups, and carriers	44	10	1
Cell envelope			
Biosynthesis/degradation of murein sacculus and peptidoglycan	18	2	0
Biosynthesis/degradation of surface polysaccharides and lipop	22	2	0
Other	31	5	4
Surface structures	4	3	0
Cellular processes			
Adaptations to atypical conditions	11	1	1
Chemotaxis and motility	19	0	8
Detoxification	11	3	0
Other	23	4	0
Pathogenesis	7	3	1
Toxin production and resistance	10	1	0
Central intermediary metabolism			
Other	32	11	2
Sulfur metabolism	7	5	0
DNA metabolism	29	4	2
Energy metabolism			
Amino acids and amines	7	3	3
Anaerobic	6	0	5
Biosynthesis and degradation of polysaccharides	5	2	1
Electron transport	32	3	6
Glycolysis/gluconeogenesis	15	1	0
Other	32	3	6
Pentose phosphate pathway	8	3	0
Sugars	9	6	0
TCA cycle	16	3	5
Fatty acid and phospholipid metabolism	21	1	0
Hypothetical proteins	115	20	6
Mobile and extrachromosomal element functions	1	1	0
Protein fate	46	4	0
Protein synthesis			
Other	42	1	0
Ribosomal proteins: synthesis and modification	54	0	15
Purines, pyrimidines, nucleosides, and nucleotides	38	9	1
Regulatory functions	18	0	3
Transcription	18	1	0
Transport and binding proteins			
Amino acids, peptides and amines	28	22	3
Anions	5	2	1
Carbohydrates, organic alcohols, and acids	23	11	2
Cations and iron carrying compounds	13	6	4
Other	11	4	2
Unclassified	51	17	6

[a] Protein expression up in bacteria grown in milk compared to grown in LB. [b] Protein expression down in bacteria grown in milk compared to grown in LB.

immune response and provide a means for the bacteria to escape immune surveillance. Whether down-regulation of the flagellar proteins is the result of a host factor to make the bacteria less virulent or is the result of bacterial immune detection escape mechanism is unknown.

Iron is an essential element for the growth of bacteria and the availability of iron has been linked to the pathogenicity of the bacteria.[30] The innate immune system has evolved mechanisms to sequester iron to deprive invading pathogens of this necessary nutrient.[31] Lactoferrin is such a protein that binds and sequesters iron and is found in milk.[9] However, bacteria have high affinity iron chelators called siderophores and surface receptors that compete for the available iron in the host.[32] Our data showed that four outer membrane siderophore receptors (FecA, FepA, FhuA and Fiu) were up-regulated when the bacteria were grown in milk (Table 4). These data suggest that the bacteria are reacting to the milk environment by increasing their ability to remove iron bound to lactoferrin. Attempts to

Table 4. Representative TIGR Roles and Proteins that Showed Expression Changes

gene	protein	change	up/down
	Cellular Processes, Chemotaxis and Motility		
FLGE	Flagellar hook protein flgE	4.29	Down
FLIC	Flagellin	1.76	Down
FLIG	Flagellar motor switch protein fliG	2.26	Down
MOTA	Chemotaxis protein motA	2.59	Down
MOTB	Chemotaxis protein motB	2.35	Down
CHER	Chemotaxis protein methyltransferase	2.13	Down
MCP1	Methyl-accepting chemotaxis protein I	2.42	Down
MCP2	Methyl-accepting chemotaxis protein II	2.36	Down
MCP3	Methyl-accepting chemotaxis protein III	2.89	Down
	Transport, Cations and Iron Carrying Compounds		
ATMA	Magnesium-transporting ATPase, P-type 1	5.00	Down
ATZN	Lead, cadmium, zinc and mercury-transporting ATPase	2.22	Down
BCP	Putative peroxiredoxin bcp	2.59	Up
CORA	Magnesium transport protein corA	2.94	Down
EXBD	Biopolymer transport protein exbD	2.79	Up
FECA	Iron(III) dicitrate transport protein fecA precursor	3.83	Up
FECB	Iron(III) dicitrate-binding periplasmic protein precursor	5.00	Up
FECE	Iron(III) dicitrate transport ATP-binding protein fecE	3.33	Up
FEOB	Ferrous iron transport protein B	2.03	Up
FEPA	Ferrienterobactin receptor precursor	8.20	Up
FHUA	Ferrichrome-iron receptor precursor	2.47	Up
FIU	Catecholate siderophore receptor fiu precursor	6.44	Up
FTNA	Ferritin-1	3.58	Down
TONB	Protein tonB	3.68	Up
	Detoxification		
FADB	Fatty acid oxidation complex subunit alpha	2.02	Up
TPX	Thiol peroxidase	2.19	Up
SODM	Superoxide dismutase [Mn]	2.21	Up
	Pathogenesis		
TOLB	Protein tolB precursor	2.06	Up
FKBB	FKBP-type 22 kDa peptidyl-prolyl cis−trans isomerase	2.21	Up
CIRA	Colicin I receptor precursor	7.44	Up
	Energy Metabolism		
BGAL	Beta-galactosidase	3.84	Up
GALP	Galactose-proton symporter	6.59	Up
GALE	UDP-glucose 4-epimerase	5.88	Up
DGAL	D-galactose binding periplasmic protein	3.71	Up
	Other		
THIO	Thioredoxin-1	2.09	Up
GLRX2	Glutaredoxin-2	4.01	Up
OSMY	Osmotically inducible protein Y precursor	2.14	Up
LUXS	*S*-ribosylhomocysteine lyase	3.60	Up
AER	Aerotaxis receptor	2.24	Down
BORD	Lipoprotein bor homologue from lambdoid prophage DLP12 precursor	18.02	Down
OMPP	Outer membrane protease ompP precursor	5.25	Down
NMPC	Outer membrane porin protein nmpC precursor	2.58	Down
BASS	Sensor protein basS/pmrB	2.53	Down

block iron uptake by *E. coli* by generating an immune response to FecA were successful in generating antibody but unsuccessful in changing the clinical severity of an infection.[33] Our data shows the up-regulation of multiple siderophore receptors, indicating that any successful blocking of iron uptake would require blocking multiple receptors.

Supplementary Table 5 in Supporting Information contains a list of proteins that were identified to be of bovine origin. Proteins such as lactophorin, casein, and butyrophilin are major protein components of milk and it is not surprising to find them in the membrane sample of the bacterial proteome. The most abundant bovine protein found associated with the bacteria is the complement C3 precursor. This observation is consistent with the known function of complement C3b, which is to specifically bind to pathogens in order to opsonize them for subsequent clearance by immune cells. The abundance of complement C3 in uninfected milk is very low and rises in cases of clinical mastitis.[34] Although the abundance of C3 in uninfected milk is very low, our data identified C3 as the most abundant bovine protein tightly associated with bacteria grown in milk. This could be due to specificity and affinity for bacteria of a naturally low level of C3 found in milk. Alternatively, fresh unpasteurized milk contains cells, many of which are neutrophils, and these cells may be stimulated to release C3 by bacterial growth. This illustrates the immediate battle between

Table 5. Proteins Up-Regulated When *E. coli* Was Grown in Milk[a]

6PGL	2.5↑	CAPP	2.2↑	DPPA	3.0↑	GAL7	2.2↑	HISJ	4.8↑	METE	8.3↑	PSPA	2.0↑	TALA	2.1↑	YCIH	3.6↑
AAT	2.9↑	CAPP	2.2↑	DPPF	6.4↑	GALE	5.8↑	HMP	2.2↑	METF	2.7↑	PTGA	2.6↑	THD1	3.3↑	YCII	2.8↑
ADHE	2.8↑	CARB	3.2↑	ELAB	2.5↑	GALM	2.2↑	HYFG	3.3↑	METI	2.5↑	PTGCB	3.4↑	THIO	2.0↑	YDEN	2.8↑
AK1H	2.9↑	CATE	2.9↑	ELBB	3.8↑	GALP	6.5↑	IHFA	2.0↑	METK	2.5↑	PTHC	2.2↑	THRC	3.2↑	YDHR	2.5↑
AK1H	9.2↑	CIRA	10.6↑	ENTA	3.8↑	GCH1	2.8↑	ILVC	3.0↑	METN	5.5↑	PTHP	3.1↑	TOLB	2.0↑	YDJN	4.3↑
AK3	2.9↑	CIRA	7.4↑	ENTB	4.0↑	GCSH	3.7↑	ILVC	3.0↑	METQ	2.7↑	PTW3C	3.2↑	TONB	3.6↑	YEAD	2.6↑
AK3	4.0↑	CISY	2.4↑	ENTB	5.0↑	GCST	2.0↑	ILVC	7.5↑	METQ	3.9↑	PUR2	3.4↑	TPX	2.1↑	YEAG	3.5↑
ALF1	2.8↑	CITT	5.3↑	ENTC	2.4↑	GLGB	2.1↑	ILVD	3.0↑	MSCS	2.9↑	PUR4	2.0↑	TRAJ4	2.5↑	YECS	2.2↑
AMPA	2.1↑	CODA	2.3↑	ENTC	9.5↑	GLNA	3.5↑	ILVD	3.4↑	MTE8	5.8↑	PUR4	2.8↑	TRPA	6.3↑	YGAU	2.1↑
ARGA	5.6↑	CODB	2.4↑	ENTE	2.0↑	GLNA	4.4↑	ILVE	11.6↑	MTOX	2.0↑	PUR4	3.0↑	TRPA	9.0↑	YGBJ	5.3↑
ARGB	6.2↑	CRL	2.3↑	ENTF	3.4↑	GLNH	3.1↑	ILVI	3.5↑	MURC	3.5↑	PUR5	4.8↑	TRPB	2.5↑	YHBS	2.2↑
ARGT	3.0↑	CRL	2.5↑	EXBB	2.3↑	GLNK	13.0↑	KDSB	2.9↑	NADE	2.0↑	PUR7	2.5↑	TRPB	3.6↑	YHDJ	4.2↑
ARLY	3.4↑	CSPD	2.1↑	EXBD	2.7↑	GLNP	2.1↑	KHSE	3.9↑	NAGA	3.6↑	PUR7	4.8↑	TRPC	3.8↑	YHDW	2.4↑
AROA	2.6↑	CYAY	2.2↑	FABZ	2.2↑	GLNQ	2.4↑	LEU1	3.1↑	NAGB	3.9↑	PUR9	5.7↑	TRPE	2.2↑	YHDZ	2.6↑
AROF	2.7↑	CYSA	8.8↑	FADB	2.0↑	GLRX2	4.0↑	LEU1	3.3↑	NANA	3.3↑	PURK	2.8↑	TRPE	2.5↑	YHJE	2.9↑
AROF	5.9↑	CYSD	2.0↑	FADE	2.0↑	GLTB	2.7↑	LEU2	3.3↑	NANC	10.7↑	PURT	2.6↑	TRPG	3.4↑	YHJS	2.4↑
AROG	2.5↑	CYSD	7.4↑	FECA	3.8↑	GLTB	3.8↑	LEU3	4.4↑	NANE	5.6↑	PUTA	2.4↑	TRPR	2.6↑	YIFK	2.8↑
AROP	9.3↑	CYSH	20.5↑	FECA	4.6↑	GLTI	17.9↑	LEU3	7.1↑	NANT1	4.6↑	PYRC	2.1↑	TYRA	3.6↑	YJCD	3.1↑
ARTI	4.4↑	CYSI	5.2↑	FECB	5.0↑	GLTJ	2.3↑	LEUD	9.5↑	NLPA	5.1↑	RAIA	2.0↑	TYRA	4.9↑	YJHT	2.7↑
ARTJ	3.6↑	CYSJ	3.2↑	FECB	6.8↑	GLTL	8.5↑	LIVF	5.8↑	NTRB	2.9↑	RBFA	2.1↑	TYRB	4.0↑	YLIJ	2.1↑
ASNA	5.1↑	CYSJ	4.7↑	FECE	3.3↑	GLYA	2.0↑	LIVJ	4.6↑	OPPA	2.6↑	RBSA	2.8↑	UBIG	2.4↑	YLIJ	2.6↑
ASNA	6.8↑	CYSK	4.4↑	FEOB	2.0↑	GNTT	2.1↑	LIVJ	4.6↑	ORN	2.0↑	RBSB	2.9↑	UHPT	8.2↑	YNCE	2.1↑
ASNB	3.3↑	CYSK	4.4↑	FEPA	8.2↑	GPMA	2.9↑	LIVK	6.3↑	OSMY	2.1↑	RL27	2.1↑	UXUA	2.0↑	YNCE	3.4↑
ASNB	4.4↑	CYSN	5.7↑	FHUA	2.4↑	GSIA	4.2↑	LRP	2.4↑	PAND	2.3↑	RPIA	2.2↑	YAGU	3.7↑	YOJI	2.5↑
ASSY	3.1↑	CYSP	5.4↑	FHUE	6.5↑	GUAC	2.0↑	LRP	3.6↑	PHEA	2.3↑	SERA	3.5↑	YBDB	5.6↑	YRAL	2.3↑
ASSY	3.9↑	DAPD	2.4↑	FIU	6.4↑	HELD	4.4↑	LUXS	3.6↑	PHEA	4.0↑	SERA	5.3↑	YBGI	2.2↑	YRBC	2.2↑
ASTC	3.0↑	DCP	2.3↑	FKBB	2.2↑	HIS1	3.9↑	MALE	3.1↑	PNTB	2.3↑	SERC	3.6↑	YBIC	2.2↑	YTFB	2.7↑
AVTA	3.0↑	DGAL	3.7↑	FLAV	2.2↑	HIS5	2.8↑	MANA	2.0↑	POTD	4.9↑	SKP	2.8↑	YBIS	2.2↑		
BCP	2.2↑	DHAS	3.9↑	FLIY	4.8↑	HIS6	4.3↑	MDH	2.2↑	POTF	4.2↑	SODM	2.2↑	YBJQ	2.3↑		
BCP	2.5↑	DHE4	4.8↑	GABD	4.1↑	HIS7	2.8↑	MDH	2.4↑	POTG	3.0↑	SRP54	8.6↑	YCDO	3.8↑		
BGAL	3.8↑	DHE4	4.9↑	GABT	2.8↑	HIS7	4.0↑	META	2.5↑	PPIB	2.7↑	SUFC	2.7↑	YCDO	5.3↑		
BGAL	4.9↑	DLD	2.2↑	GAL1	2.5↑	HIS8	2.4↑	METE	6.5↑	PQQL	2.5↑	T341	3.6↑	YCGM	4.5↑		

[a] For complete MS/MS data for proteins up-regulated when bacteria were grown in milk, please see Supplementary Table 2 in Supporting Information.

Table 6. Proteins Down-Regulated When *E. coli* Was Grown in Milk[a]

ACKA	2.0↓	CYDB	2.2↓	FRDB	4.2↓	GLPT	5.8↓	NARJ	3.5↓	PROW	4.1↓	RL31	2.0↓	YEDE	13.3↓
AER	2.2↓	DEOD	2.2↓	FRDC	3.6↓	LAMB	3.7↓	NARK	2.1↓	PTHB	2.2↓	RL33	2.6↓	YEIU	3.0↓
ASPA	3.0↓	DHSA	2.6↓	FRDD	5.9↓	LDCI	3.1↓	NARY	7.5↓	PTTBC	6.4↓	RL34	2.1↓	YJDB	5.4↓
ASPA	3.0↓	DHSB	2.7↓	FTNA	3.5↓	LIPA	2.9↓	NARY	8.0↓	RAIA	3.3↓	RS19	3.2↓	YJIY	2.6↓
ATMA	5.0↓	DHSC	2.0↓	FTNA	6.1↓	MBHM	2.4↓	NMPC	2.5↓	RL11	2.0↓	RS21	3.0↓	YLEA	2.2↓
ATZN	2.2↓	DHSD	4.1↓	FUMA	2.0↓	MCP1	2.4↓	NRDD	10.3↓	RL16	2.4↓	RS3	2.1↓	YQHD	2.2↓
BASS	2.5↓	DMSA	12.5↓	GADC	2.6↓	MCP2	2.3↓	NUOCD	2.0↓	RL19	2.3↓	RS9	2.1↓	YQJD	2.1↓
BORD	18.0↓	ENDPH	3.3↓	GARP	9.6↓	MCP3	2.8↓	NUOG	2.0↓	RL24	2.0↓	SDAC	7.1↓	ZNUC	2.9↓
BORD	5.7↓	FADL	3.7↓	GIDB	4.6↓	MOTA	2.5↓	OMPP	5.2↓	RL25	2.2↓	SOPB	3.8↓		
CHER	2.1↓	FDNG	2.7↓	GLMS	2.3↓	MOTB	2.3↓	OMPX	2.3↓	RL25	2.3↓	SYK2	2.0↓		
CORA	2.9↓	FLGE	4.2↓	GLPD	5.0↓	NARG	3.6↓	PHOE	2.0↓	RL25	2.4↓	TDH	2.9↓		
CSPC	2.2↓	FLIG	2.2↓	GLPK	2.3↓	NARG	3.8↓	PITA	2.2↓	RL25	2.8↓	TREC	2.5↓		
CYDA	2.1↓	FRDA	2.8↓	GLPK	6.7↓	NARH	5.0↓	PROV	2.3↓	RL28	2.1↓	YBJX	2.7↓		

[a] For complete MS/MS data for proteins down-regulated when bacteria were grown in milk, please see Supplementary Table 3 in Supporting Information.

the invading pathogen and the host's immune defenses, which must be overcome in order for the pathogen to establish an infection.

Two expression proteomic studies have recently been published that have studied the changes in protein expression of two bacteria species, used in the manufacture of fermented dairy products, grown in laboratory culture media or milk.[3,35] With the use of two-dimensional gel electrophoresis, the authors of one study estimated 10−20% of the observed proteins from *Lactococcus lactis* were up-regulated when the bacteria were grown in milk or milk microfiltrate as compared to synthetic media.[35] This is consistent with our observations, where we estimate 20% of the identified proteins are up-regulated when bacteria are grown in milk and another 10% of the proteins are down-regulated. Many of the changes in protein expression are likely a response to changes in nutrient availability in milk versus LB (e.g., lactose as the primary sugar in milk). Although this type of response is not directly linked to the pathogenesis of an organism, it is a necessity for any pathogen to be able to adapt to its environment. Hence, any substance that would inhibit bacterial utilization of lactose might be an effective therapeutic against mastitis-causing pathogens.

Mastitis-causing pathogens must grow in the milk environment. Despite the innate antibacterial properties of milk, *E. coli* can grow from an inoculum of less than 100 cfu/mL to several thousand cfu/mL in 8 h.[36] During this early phase of the infection, the bacteria do not contend with cells of the immune system, the majority of which arrive in the gland 6−12 h post-infection.[11] In these first few hours, the bacteria must adapt to their new environment by altering their proteomes to take advantage of the energy sources available (e.g., lactose), to synthesize any missing metabolites (e.g., amino acids) and to express factors to allow persistence of the bacteria (e.g., virulence factors). Would a substance that specifically inhibited a bacterial function required for the growth of the microorganism in the first hours of the infection be an effective therapeutic agent? For example, would the specific blockage of bacterial utilization of lactose block an infection?

The degree to which growth in milk affects the virulence of *E. coli* is unknown. However, experiments with *Staphylococcus aureus* have shown that passage of bacteria in milk whey increased the virulence of a strain. Mamo and co-workers showed that passage of *S. aureus* in laboratory media or milk whey resulted in a mouse mortality rate of 5% or 85%, respectively.[37] Thus, simple adaptation of bacteria to milk may

Table 7. Proteins Not Affected When *E. coli* Was Grown in Milk[a]

6PGD	ATPL	DACC	EFTS	FTSH	GYRA	KUP	METK	NUOG	PFLA	PUTP	RL23	RS13	SPPA	TDCE	WECA	YCIK	YGIM
AAT	BCCP	DADA	EFTU	FTSI	GYRB	K1PF	MFD	NUOH	PFLB	PYRD	RL24	RS14	SRMB	THD1	WECB	YCJF	YHAH
ACCA	BCSB	DAMX	EMRA	FTSK	HCHA	K6PF1	MGLA	NUOI	PGK	PYRE	RL25	RS15	SRP54	THIE	WECF	YDGA	YHBY
ACCC	BGLX	DAPA	ENGA	FTSL	HEM2	KAD	MGSA	NUOL	PGM	PYRG	RL27	RS16	SSB	TIG	WRBA	YDGH	YHCB
ACCD	BTUB	DAPB	ENGB	FTSQ	HEM3	KDGR	MINC	NUOM	PHOQ	QUEF	RL28	RS17	SSB7	TKRA	WZZB3	YDGJ	YHCG
ACEA	CAN	DAPD	ENGC	FTSX	HEMG	KDSA	MIND	NUSA	PHSM	RAPA	RL29	RS18	SSBF	TKT1	WZZE	YDGK	YHDH
ACKA	CARA	DBHA	ENGD	FTSY	HEMH	KDSD	MINE	NUSB	PLSB	RBFA	RL3	RS19	SSPA	TKT2	XGPT	YDJA	YHDP
ACON2	CATA	DBHB	ENO	FTSZ	HEMX	KDTA	MLTA	NUSG	PLSC	RCSC	RL30	RS2	STHA	TLDD	YAAA	YEBC	YHES
ACP	CDH	DCUP	ERA	FUR	HEMY	KEFA	MLTB	ODO1	PNP	RCSF	RL31	RS20	STPA	TNPA	YACF	YEBT	YHGF
ACRA	CFA	DCRB	EX1	G3P1	HFLC	KGUA	MOAB	ODO2	PNTA	RDGC	RL33	RS21	SUCC	TOLA	YADG	YECJ	YHHX
ACRB	CH10	DCTA	EX3	G6PD	HFLK	KPRS	MOAC	ODP1	POTB	RECA	RL34	RS3	SUCD	TOLB	YAEH	YECR	YHII
ACSA	CH60	DEAD	F16P	G6PI	HFLX	KPYK1	MOEA	ODP2	PPCK	RECG	RL35	RS4	SUHB	TOLC	YAET	YEDD	YHJC
ADD	CHEA	DEDA	FABA	GALF	HISX	KPYK2	MPL	OMPA	PPIC	RF2	RL4	RS5	SURA	TOLQ	YAIW	YEEF	YHJK
ADHE	CHEB	DEDD	FABB	GALU	HLDD	KSGA	MQO	OMPC	PPID	RFAC	RL5	RS6	SYA	TOLR	YAJC	YEEX	YHJU
AHPC	CHEW	DEF	FABD	GCH1	HLDE	LCFA	MREB	OMPF	PPSA	RFAJ	RL6	RS7	SYC	TOP1	YAJG	YEEZ	YIAD
AHPF	CHEZ	DEGP	FABF	GCSP	HNS	LEP	MRP	OMPN	PPX	RFAL	RL7	RS8	SYD	TPIS	YAJQ	YEGQ	YIAF
AK2H	CISY	DEGQ	FABG	GCST	HPRT	LEPA	MSBA	OMPR	PQIB	RFAZ	RL9	RS9	SYE	TPPB	YBAB	YEJL	YIBF
ALDA	CLPA	DEOB	FABH	GIDA	HSCA	LEU2	MSBB	OMPT	PRC	RHLB	RLMG	RSEP	SYFA	TPX	YBAL	YFBQ	YIBN
ALDB	CLPB	DEOC	FABI	GIDB	HSLJ	LEUD	MUKB	OPDA	PROB	RHO	RLPA	SDAC	SYFB	TRAC	YBBN	YFBU	YIBP
ALF	CLPP	DEOD	FABZ	GLGA	HSLU	LIPA	MURA	OPGH	PROP	RIBD	RLUB	SECA	SYGA	TRAI2	YBBO	YFCZ	YIDA
ALKH	CLPX	DHAM	FAPR	GLMM	HTPG	LLDD	MURC	OSME	PROQ	RIHA	RLUC	SECB	SYGB	TRAT1	YBDG	YFEX	YIFE
AMPA	COABC	DHG	FCTA	GLMS	HTPX	LLDP	MURD	OSTA	PSD	RIMM	RLUD	SECD	SYH	TRMB	YBEB	YFGA	YIIU
AMPH	CORC	DHNA	FDHE	GLMU	HTRB	LNT	MURE	OTC1	PSS	RIR1	RNB	SECE	SYI	TRME	YBED	YFGB	YIJP
AMPM	CRP	DHSA	FDOG	GLPK	IADA	LOLB	MURG	OXAA	PSTB	RIR2	RNC	SECF	SYK1	TRUD	YBFA	YFGM	YJGA
AMPN	CSPA	DHSB	FDOH	GLRX4	IBPA	LON	MUTS	OXYR	PSTC	RISA	RNE	SECG	SYK2	TRXB	YBFF	YFHM	YJGF
APHA	CSPC	DJLA	FIEF	GLYA	IDH	LONH	MVIN	PA1	PT1	RISB	RNFC	SECY	SYL	TSX	YBGJ	YFIF	YJGQ
APT	CSPE	DKSA	FIS	GNSA	IF2	LPP	NUPC	PABC	PTA	RL1	RNFG	SELB	SYM	TYPA	YBHC	YFIO	YJJK
ARCA	CSPG	DLDH	FKBA	GNSB	IF3	LPXA	NARZ	PAL	PTFBC	RL10	RNPA	SELD	SYN	TYPH	YBIB	YGAM	YLEA
ARCB	CVRA	DNAA	FLGM	GNTY	IGAA	LPXC	NDK	PANB	PTGA	RL11	RNR	SEQA	SYP	TYSY	YBIX	YGAP	YLIG
ARGR	CYCA	DNAJ	FLIC	GPH	IHFA	LTAE	NFNB	PANC	PTGCB	RL13	ROF	SKP	SYQ	UBIB	YBJP	YGDH	YMBA
AROB	CYDC	DNAK	FLIF	GPMA	IHFB	MALK	NFSA	PARC	PTH	RL14	RPOA	SLMA	SYR	UBIE	YCAO	YGER	YNEI
AROK	CYDD	DNLJ	FLIL	GPMI	ILVB	MALT	NIFU	PBP2	PTM3C	RL15	RPOB	SLYD	SYS	UBIG	YCBB	YGFZ	YOJN
ARTP	CYOA	DPO1	FLIM	GRCA	ILVE	MAO2	NLPB	PBPA	PTNAB	RL16	RPOC	SMPA	SYT	UDG	YCCW	YGGE	YQJH
ASMA	CYOB	DPO3A	FLIP	GREA	IMDH	MASZ	NMPC	PBPB	PTND	RL17	RPOD	SODF	SYV	UDG8	YCDX	YGGM	YRAM
ATPA	CYSB	DPPA	FNR	GRPE	INGK	MBHT	NPD	PDXB	PUR1	RL18	RPOZ	SOHB	SYW	UDP	YCDY	YGGS	YRAP
ATPB	CYSC	DPS	FOLC	GSA	IPYR	MCP4	NUOA	PDXJ	PUR3	RL19	RRF	SOPA	SYY	UPP	YCED	YGGT	YRBC
ATPD	CYSE	ECNB	FOLD	GSHB	ISCR	MDAB	NUOB	PEPB	PUR8	RL2	RS1	SOPB	T1MK	URK	YCEG	YGHQ	YRBD
ATPE	CYSM	EFG	FRSA	GUAA	ISCS	MENB	NUOCD	PEPD	PURA	RL20	RS10	SPEA	T1RK	USPF	YCFC	YGIB	YRBK
ATPF	DACA	EFP	FTSA	GUAC	ISPB	METH	NUOE	PEPE	PURR	RL21	RS11	SPED	TALB	USPG	YCFP	YGIC	ZIPA
ATPG	DACB	EFPL	FTSE	GUTQ	IVY	METJ	NUOF	PEPQ	PUTA	RL22	RS12	SPEE	TATA	VISC	YCGR	YGIF	

[a] For complete MS/MS data for proteins with no expression change when bacteria were grown in milk, please see Supplementary Table 5 in Supporting Information.

affect the virulence of that organism. It is has been shown that, in the majority of cases of reoccurring *E. coli* infections, the primary and secondary infection were caused by the same genotype. Once thought to only cause an acute infection, *E. coli* is now thought to have adapted into a chronic pathogen.[38] It is unknown whether growth in milk may affect the ability of *E. coli* to establish a persistent infection.

Proteomics allows for the search for potentially new and unexpected connections between a change in treatment and change in protein expression, which may affect the virulence of a pathogen. *E. coli* mastitis is considered an environmental pathogen that is spread primarily by fecal contamination. It is often rapidly cleared by the immune system of the cow but can become septic and rapidly cause death in some animals. The proteomic data presented here demonstrated mark changes and adaptation of *E. coli* grown in milk. We showed that the expression levels of hundreds of *E. coli* proteins were affected by the change in the growth environment from LB to milk. This raises questions about which genes are necessary for an *E. coli* strain to be categorized as a MPEC strain. It is hoped that some of these protein changes required for bacteria growth in milk might one day be targets for therapeutic interventions. Furthermore, it is clear that some MPEC strains show species specificity in whether an infection will result in a mild or severe infection.[12] These results point to a need for future research efforts to understand the potential significance of milk adapted mastitis pathogens on the severity and duration of infections and their outcomes for this economically important disease in cattle. Clearly, a proteomic analysis of MPEC stains of *E. coli* versus nonvirulent stains of *E. coli* is needed as a continuation of this work. Since growth in milk has such a dramatic affect on the *E. coli* proteome, we would argue that expression proteomics comparing MPEC and nonvirulent *E. coli* should be done with milk as the growth media. We speculated that not using milk as a growth media would alter the comparison of MPEC and nonvirulent bacteria. In the case of mastitis, we have the advantage of having the ability to obtain large quantities of the biological fluid in which an infection occurs. It is very possible that proteomes are as dramatically altered in bacteria that infect other sites in the body as they adapt to whatever biological fluid become their growth media as we have seen in milk.

Acknowledgment. Authors thank Randy Atchison and Duane Zimmerman for their technical assistance.

Supporting Information Available: Worksheet of all proteins found, tables of proteins that were up- or down-regulated greater than 1.7-fold in milk compared to media, proteins that had no change (were up- or down-regulated less than 1.7-fold in milk compared to media), bovine proteins associated with *E. coli* when grown in milk. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Guina, T.; Wu, M.; Miller, S. I.; Purvine, S. O.; Yi, E. C.; Eng, J.; Goodlett, D. R.; Aebersold, R.; Ernst, R. K.; Lee, K. A. Proteomic analysis of Pseudomonas aeruginosa grown under magnesium limitation. *J. Am. Soc. Mass Spectrom.* **2003**, *14* (7), 742–51.

(2) Hughes, M. A.; Silva, J. C.; Geromanos, S. J.; Townsend, C. A. Quantitative proteomic analysis of drug-induced changes in mycobacteria. *J. Proteome Res.* **2006**, *5* (1), 54–63.
(3) Derzelle, S.; Bolotin, A.; Mistou, M. Y.; Rul, F. Proteome analysis of Streptococcus thermophilus grown in milk reveals pyruvate formate-lyase as the major upregulated protein. *Appl. Environ. Microbiol.* **2005**, *71* (12), 8597–605.
(4) Chromy, B. A.; Choi, M. W.; Murphy, G. A.; Gonzales, A. D.; Corzett, C. H.; Chang, B. C.; Fitch, J. P.; McCutchen-Maloney, S. L. Proteomic characterization of Yersinia pestis virulence. *J. Bacteriol.* **2005**, *187* (23), 8172–80.
(5) Lamonica, J. M.; Wagner, M.; Eschenbrenner, M.; Williams, L. E.; Miller, T. L.; Patra, G.; DelVecchio, V. G. Comparative secretome analyses of three Bacillus anthracis strains with variant plasmid contents. *Infect. Immun.* **2005**, *73* (6), 3646–58.
(6) Wu, M.; Guina, T.; Brittnacher, M.; Nguyen, H.; Eng, J.; Miller, S. I. The Pseudomonas aeruginosa proteome during anaerobic growth. *J. Bacteriol.* **2005**, *187* (23), 8185–90.
(7) Yokoe, D. S.; Christiansen, C. L.; Johnson, R.; Sands, K. E.; Livingston, J.; Shtatland, E. S.; Platt, R. Epidemiology of and surveillance for postpartum infections. *Emerg. Infect. Dis.* **2001**, *7* (5), 837–41.
(8) USDA *Part I: Reference of Dairy Health and Management in the United States, 2002*; USDA: APHIS, VS, CEAH, National Animal Health Monitoring System: Fort Collins, CO, 2002.
(9) Legrand, D.; Elass, E.; Carpentier, M.; Mazurier, J. Lactoferrin: a modulator of immune and inflammatory responses. *Cell. Mol. Life Sci.* **2005**, *62* (22), 2549–59.
(10) Shuster, D. E.; Lee, E. K.; Kehrli, M. E., Jr. Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at midlactation. *Am. J. Vet. Res.* **1996**, *57* (11), 1569–75.
(11) Kornalijnslijper, J. E.; Daemen, A. J.; van Werven, T.; Niewold, T. A.; Rutten, V. P.; Noordhuizen-Stassen, E. N. Bacterial growth during the early phase of infection determines the severity of experimental *Escherichia coli* mastitis in dairy cows. *Vet. Microbiol.* **2004**, *101* (3), 177–86.
(12) Shpigel, N. Y.; Elazar, S.; Rosenshine, I. Mammary pathogenic *Escherichia coli*. *Curr. Opin. Microbiol.* **2008**, *11* (1), 60–5.
(13) Gonen, E.; Vallon-Eberhard, A.; Elazar, S.; Harmelin, A.; Brenner, O.; Rosenshine, I.; Jung, S.; Shpigel, N. Y. Toll-like receptor 4 is needed to restrict the invasion of Escherichia coli P4 into mammary gland epithelial cells in a murine model of acute mastitis. *Cell Microbiol.* **2007**, *9* (12), 2826–38.
(14) Park, Z. Y.; Russell, D. H. Thermal denaturation: a useful technique in peptide mass mapping. *Anal. Chem.* **2000**, *72* (11), 2667–70.
(15) Russell, W. K.; Park, Z. Y.; Russell, D. H. Proteolysis in mixed organic-aqueous solvent systems: applications for peptide mass mapping using mass spectrometry. *Anal. Chem.* **2001**, *73* (11), 2682–5.
(16) Spahr, C. S.; Davis, M. T.; McGinley, M. D.; Robinson, J. H.; Bures, E. J.; Beierle, J.; Mort, J.; Courchesne, P. L.; Chen, K.; Wahl, R. C.; Yu, W.; Luethy, R.; Patterson, S. D. Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. I. Profiling an unfractionated tryptic digest. *Proteomics* **2001**, *1* (1), 93–107.
(17) Lippolis, J. D.; Peterson-Burch, B. D.; Reinhardt, T. A. Differential expression analysis of proteins from neutrophils in the periparturient period and neutrophils from dexamethasone-treated dairy cows. *Vet. Immunol. Immunopathol.* **2006**, *111* (3−4), 149–64.
(18) Reinhardt, T. A.; Lippolis, J. D. Developmental changes in the milk fat globule membrane proteome during the transition from colostrum to milk. *J. Dairy Sci.* **2008**, *91* (6), 2307–18.
(19) Ishihama, Y.; Oda, Y.; Tabata, T.; Sato, T.; Nagasu, T.; Rappsilber, J.; Mann, M. Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol. Cell. Proteomics* **2005**, *4* (9), 1265–72.
(20) Isaacs, C. E. Human milk inactivates pathogens individually, additively, and synergistically. *J. Nutr.* **2005**, *135* (5), 1286–8.
(21) Newburg, D. S. Innate immunity and human milk. *J. Nutr.* **2005**, *135* (5), 1308–12.
(22) Wheeler, T. T.; Hodgkinson, A. J.; Prosser, C. G.; Davis, S. R. Immune components of colostrum and milk--a historical perspective. *J. Mammary Gland Biol. Neoplasia* **2007**, *12* (4), 237–47.
(23) Elbein, A. D.; Heath, E. C. The biosynthesis of cell wall lipopolysaccharide in Escherichia coli. I. The biochemical properties of a uridine diphosphate galactose 4-epimeraseless mutant. *J. Biol. Chem.* **1965**, *240*, 1919–25.
(24) Dosogne, H.; Vangroenweghe, F.; Burvenich, C. Potential mechanism of action of J5 vaccine in protection against severe bovine coliform mastitis. *Vet. Res.* **2002**, *33* (1), 1–12.
(25) Sperandio, V.; Torres, A. G.; Jarvis, B.; Nataro, J. P.; Kaper, J. B. Bacteria-host communication: the language of hormones. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (15), 8951–6.
(26) DeLisa, M. P.; Wu, C. F.; Wang, L.; Valdes, J. J.; Bentley, W. E. DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in Escherichia coli. *J. Bacteriol.* **2001**, *183* (18), 5239–47.
(27) Ramos, H. C.; Rumbo, M.; Sirard, J. C. Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. *Trends Microbiol.* **2004**, *12* (11), 509–17.
(28) Smith, K. D.; Andersen-Nissen, E.; Hayashi, F.; Strobe, K.; Bergman, M. A.; Barrett, S. L.; Cookson, B. T.; Aderem, A. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nat. Immunol.* **2003**, *4* (12), 1247–53.
(29) Lippolis, J. D. Immunological signaling networks: integrating the body's immune response. *J. Anim. Sci.* **2008**, *86* (14 Suppl), E53−63.
(30) Klebba, P. E. Three paradoxes of ferric enterobactin uptake. *Front. Biosci.* **2003**, *8*, s1422−36.
(31) Ong, S. T.; Ho, J. Z.; Ho, B.; Ding, J. L. Iron-withholding strategy in innate immunity. *Immunobiology* **2006**, *211* (4), 295–314.
(32) Andrews, S. C.; Robinson, A. K.; Rodriguez-Quinones, F. Bacterial iron homeostasis. *FEMS Microbiol. Rev.* **2003**, *27* (2−3), 215–37.
(33) Takemura, K.; Hogan, J. S.; Lin, J.; Smith, K. L. Efficacy of immunization with ferric citrate receptor FecA from Escherichia coli on induced coliform mastitis. *J. Dairy Sci.* **2002**, *85* (4), 774–81.
(34) Rainard, P. The complement in milk and defense of the bovine mammary gland against infections. *Vet. Res.* **2003**, *34* (5), 647–70.
(35) Gitton, C.; Meyrand, M.; Wang, J.; Caron, C.; Trubuil, A.; Guillot, A.; Mistou, M. Y. Proteomic signature of Lactococcus lactis NCDO763 cultivated in milk. *Appl. Environ. Microbiol.* **2005**, *71* (11), 7152–63.
(36) Bannerman, D. D.; Paape, M. J.; Lee, J. W.; Zhao, X.; Hope, J. C.; Rainard, P. Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection. *Clin. Diagn. Lab. Immunol.* **2004**, *11* (3), 463–72.
(37) Mamo, W.; Lindahl, M.; Jonsson, P. Enhanced virulence of Staphylococcus aureus from bovine mastitis induced by growth in milk whey. *Vet. Microbiol.* **1991**, *27* (3−4), 371–84.
(38) Bradley, A. J.; Green, M. J. Adaptation of Escherichia coli to the bovine mammary gland. *J. Clin. Microbiol.* **2001**, *39* (5), 1845–9.

PR800458V