Comparative proteomic analysis of outer membrane protein 43 (omp43)-deficient Bartonella henselae

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Outer membrane proteins (OMPs) of Gram-negative bacteria constitute the first line of defense protecting cells against environmental stresses including chemical, biophysical, and biological attacks. Although the 43-kDa OMP (OMP43) is major porin protein among Bartonella henselae-derived OMPs, its function remains unreported. In this study, OMP43-deficient mutant B. henselae (Δomp43) was generated to investigate OMP43 function. Interestingly, Δomp43 exhibited weaker proliferative ability than that of wild-type (WT) B. henselae. To study the differences in proteomic expression between WT and Δomp43, two-dimensional gel electrophoresis-based proteomic analysis was performed. Based on Clusters of Orthologous Groups functional assignments, 12 proteins were associated with metabolism, 7 proteins associated with information storage and processing, and 3 proteins associated with cellular processing and signaling. By semi-quantitative reverse transcriptase polymerase chain reaction, increases in tld, omp, ntr, pdh, purB, and atpA mRNA expression and decreases in Rho and yfeA mRNA expression were confirmed in Δomp43. In conclusion, this is the first report showing that a loss of OMP43 expression in B. henselae leads to retarded proliferation. Furthermore, our proteomic data provide useful information for the further investigation of mechanisms related to the growth of B. henselae.

Keywords: Bartonella, omp43, outer membrane protein, proliferation, proteomics

Introduction

Bartonella henselae is a widely distributed, Gram-negative, slow-growing, fastidious, facultative intracellular bacterium causing various diseases including bacillary angiomatosis, bacillary peliosis, and cat scratch disease (CSD) [2]. The transmission of B. henselae to humans is associated with exposure to B. henselae-infected cats and fleas [22]. CSD is usually a self-limiting inflammation of the lymph nodes near the scratch site. In immunocompromized patients, B. henselae causes tumorous proliferation of endothelial cells in internal organs, as well as a recurrent infection that can persist for a prolonged period [22]. Exposure of primary human umbilical vein cells (HUVEC) to B. henselae has been shown to result in bacterial aggregation on the cell surface, and subsequent engulfment and internalization of the bacterial aggregate by the formation of invasomes [13]. One of the most commonly identified pathogenic factors of B. henselae is the Bartonella adhesin A (BadA) protein located in the outer membrane of the bacterium. BadA mediates the mechanism underlying the binding of B. henselae to extracellular matrix proteins and endothelial cells, and it activates hypoxia-inducible factor-1. Moreover, the BadA neck is a major functional domain related to host adhesion, auto-agglutination, and angiogenic reprogramming [20]. On the other hand, B. henselae outer membrane proteins (OMPs), as well as B. henselae itself, can induce adhesion molecule expression in endothelial cells [15]. In the sarcosyl-insoluble fraction of B. henselae lysates, nine proteins were detected, five of which (28, 32, 43, 52, and 58 kDa) were attached to HUVECs [6]. Moreover, Dehio et al. [13] have suggested that the 43-kDa OMP (OMP43) is the major adhesin among B. henselae-derived OMPs that interacts with HUVEC.

The outer membranes of Gram-negative bacteria determine the molecules to be taken in or excreted by the cells. Moreover, in many bacteria, the outer membrane is the predominant layer that interacts with antibodies and other proteins. Porins, which were discovered in 1976, are the major proteins of the outer membrane and are found in every Gram-negative species [26]. Nonspecific diffusion of hydrophilic solutes across the outer
membrane usually occurs through porin channels with distinctive diameters. Consequently, porins are the major uptake/excretory route for nutrients, toxins, antibiotics, hydrolytic enzymes, etc. [28]. On the other hand, some researchers were reported that the OMP43 sequence of B. henselae showed 38% identity and 53% similarity to the Omp2b porin of Brucella species [7]. In addition, B. henselae OMP43 showed homology to the proteins of Rhizobium leguminosarum that may possess pore-forming abilities [12].

These data suggest that B. henselae omp43 could be a porin-coding gene and that the OMP43 protein interacts with other cells and molecules. This study was aimed at characterizing the proteome of Δomp43 and comparing it to that of the wild-type (WT) strain by applying proteomic methods, which can help elucidate the pathogenesis of B. henselae. In addition, we performed semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) to confirm the proteomic data.

Materials and Methods

Bacterial strains and growth conditions

B. henselae strain Houston-1 (ATCC 49882) was cultured on Columbia blood agar plates containing 5% defibrinated sheep blood (BAP-agar plates) in a humidified atmosphere at 37°C and 5% CO2. Escherichia coli was grown in Luria-Bertani (LB) broth at 37°C.

Construction of pΔomp43

Primers, plasmids, and bacterial strains used in this study are listed in Table 1 [7,31]. DNA extraction was performed according to standard protocols. Chromosomal DNA was extracted from B. henselae (Houston-1) by using DNaseasy Blood & Tissue Kits (Qiagen, Germany) according to the manufacturer’s instructions.

For construction of the omp43 plasmid (pomp43), the omp43 gene was amplified by using BamH-omp43 and omp43-Hind primers. The amplicon was cloned with pGEM-T Easy Vectors (Promega, USA), followed by transformation into E. coli DH5α. Purification of plasmid DNA was performed by using the Wizard Plus SV Miniprep DNA Purification System (Promega) according to the manufacturer’s instructions. The pBluescript II KS plasmid and T vector containing omp43 sequences were digested with BamH I and Hind III. The insert (containing the omp43 gene) was ligated into the pBluescript II KS vector and transferred into E. coli DH5α.

A kanamycin resistance gene (Km’) was amplified from pET-28α by using Sph I-km-F and Sph I-km-R primers, cloned

Table 1. Bacterial strains, plasmids, and primers used in this study

| Strains                  | Characteristic or sequence | Reference or source |
|--------------------------|----------------------------|---------------------|
| **Bartonella henselae**  |                            |                     |
| Wild-type                | B. henselae Houston-1, ATCC 49882 | [31]                |
| Δomp43                   | B. henselae omp43 deficient mutant, Km’ | This study          |
| **Escherichia coli**     |                            |                     |
| DH 5α                    | Host strain used for cloning | Invitrogen          |
| Top 10                   | Host strain used for cloning | Invitrogen          |
| BL21 (DE3)               | Host strain used for protein expression | Stratagene         |
| **Plasmids**             |                            |                     |
| pGEM-T easy              | Vector for cloning, Ap’     | Promega             |
| pET-28a                  | Vector for protein expression, Km’ | Novagen             |
| pBAD/His                 | Vector for protein expression, Ap’ | Invitrogen          |
| pBluescript II KS        | Vector for homologous recombination, Ap’ | Stratagene         |
| pomp43                   | pBluescript II KS containing a 1.2 kb of B. henselae omp43 fragment, Ap’ | This study          |
| pΔomp43                  | pomp43 containing a kanamycin cassette in the middle site ofomp43 gene sequence, Ap’, Km’ | This study          |
| **Primers**              |                            |                     |
| Bgl-omp43                | ctgagatcttgtctcaagttatttgca | [7]                 |
| omp43-EcoR               | cggagatcttaaagcgttggaagcg | [7]                 |
| BamH-omp43               | gagagattcaaatgagcttggaagcg | This study          |
| omp43-Hind               | gagagattcatttaaaatgagcttggaagcg | This study  |
| Sph1-km-F                | gagagatgcaaatgagccatattca | This study          |
| Sph1-km-R                | gagagatgcatcttataaaactcatgca | This study          |
with pGEM-T Easy Vector as previous described. After purifying, the T vector containing Km together with omp43 were digested with Sph I and then the cut Km was ligated with pomp43 (middle region of omp43 gene sequences). As a result, the pΔomp43 plasmid was acquired. Additionally, the pΔomp43 sequence was confirmed by dideoxy termination with an automatic sequencer (ABI 3730xl capillary DNA sequencer, Applied Biosystems, USA).

Expression of OMP43

The OMP43 of B. henselae was prepared as described previously [7]. Briefly, omp43 without the signal peptide was amplified from B. henselae (Houston-1) by using Bgl-omp43 and omp43-EcoR primers. The pBAD/His B plasmid and the omp43 PCR product were digested using restriction enzymes (Bgl II and EcoR I). The digested omp43 amplicon was cloned with the digested pBAD/His B vector, followed by transformation into E. coli TOP10. OMP43 was expressed by induction with 0.02% arabinose for 6 h at 37°C in LB containing 50 mg/mL ampicillin. The denatured samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. OMP43 protein was detected by western blotting using the anti-Xpress antibody (Invitrogen, USA) and anti-mouse IgG secondary antibody (Cell Signaling, USA) (data not shown).

Purifying and polyclonal antibody production of OMP43

Purified recombinant OMP43 was acquired by Ni²⁺ affinity chromatography (via request to Bio Basic, USA). Briefly, induced bacteria were harvested by centrifugation and then sonicated in ice in lysis buffer. The OMP43 fusion protein inclusion bodies were diluted into refolding buffer, 1 mM GSSG (oxidized glutathione), and stirred at 4°C for 24 h. The dissolved OMP43 fusion protein solution underwent dialysis against a buffer solution. The refolded protein was harvested by sonification (Thermo Fisher Scientific, USA) until the solution turned an opaque yellow color. By centrifugation at 30,000 × g for 40 min, the debris was pelleted. The supernatant contained solubilized whole cell lysate, which was quantified using the RC/DC Protein Assay kit (BioRad) and the results converted into electronic files and analyzed by using the Image Master Platinum 5.0 image analysis program (Amersham Biosciences). Analysis was conducted to identify spots with a minimum 2-fold increased or decreased difference between B. henselae Houston-1 (ATCC49882) and kanamycin-resistant omp43 deficient B. henselae (Δomp43).

Electroporation of B. henselae

Five-day-old B. henselae were harvested from 2 BAP-agar plates with a sterile cotton swab into ice-cold distilled water (DW) containing 10% glycerol. Competent cells were prepared by washing three times with ice-cold DW containing 10% glycerol. The pellet was resuspended in 100 μL of ice cold DW containing 10% glycerol in a cooled electroporation 0.1-cm-gap cuvette (BioRad, USA). Subsequently, 10 μL of the pΔomp43 plasmid solution (2 μg/μL) was added into the cuvette and gently mixed before being allowed to stabilize on ice for 15 min. Electroporation was conducted with a field strength of 1.2 kV/cm and a constant capacitance of 25 μF at 200 Ω. Electroporated cells were immediately transferred into 1 mL super broth (SB) broth at room temperature. Subsequently, cells were incubated for 4 h at 37°C in 5% CO₂ and then seeded on BAP-agar plates containing kanamycin.

Growth curve

For the comparison of growth abilities of WT and Δomp43, counted bacteria (100,000 colony-forming unit [CFU]) were seeded on BAP-agar plates and incubated in a humidified atmosphere at 37°C and 5% CO₂. After harvesting from BAP-agar plates with a sterile cotton swab, the bacteria were resuspended in phosphate buffered saline (PBS). Optical density (OD) was estimated on 600 nm.

Two-dimensional proteomics

Whole cell protein extractions: For whole cell protein extraction, cells were harvested from BAP-agar plates and resuspended in iced PBS. Bacteria were washed three times with iced PBS, centrifuged, and resuspended in lysis buffer. Subsequently, cells were broken by sonication using a Branson sonifier (Thermo Fisher Scientific, USA) until the solution turned an opaque yellow color. By centrifugation at 30,000 × g at 4°C for 40 min, the debris was pelleted. The supernatant contained solubilized whole cell lysate, which was quantified using the RC/DC Protein Assay kit (BioRad) according to the manufacturer’s protocol.

Two-dimensional SDS-PAGE: Whole cell proteins were separated in the first dimension by using immobilized pH 3 to 10 nonlinear gradient strips (Amersham Biosciences, UK). Isoelectric focusing of the protein-containing samples was performed in a protein IEF cell (BioRad). Afterward, second-dimension analysis was performed on 9% to 16% linear gradient polyacrylamide gels, and protein fixation was performed. They were scanned in a Biorad GS710 densitometer (BioRad) and the results converted into electronic files and were analyzed by using the Image Master Platinum 5.0 image analysis program (Amersham Biosciences). Analysis was conducted to identify spots with a minimum 2-fold increased or decreased difference between B. henselae Houston-1 (ATCC49882) and kanamycin-resistant omp43 deficient B. henselae (Δomp43).

Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS):

Protein processing

Selected spots were excised from stained two-dimensional (2D) gels by using an Ettan spot picker (GE Healthcare, UK) and then transferred into 96-well plates. Tryptic digestion with subsequent spotting on a MALDI-target was carried out automatically with the Ettan Spot Handling Workstation (GE
Table 2. Primers for reverse transcriptase polymerase chain reaction in this study

| Gene | Forward primer | Reverse primer | Expected bp |
|------|----------------|----------------|-------------|
| tldD | CAAAAAGCCCAAGAAAAAGCAG | TGGGAAATCTGTCGATGTA | 328 |
| Rho  | CTTCTCTGCTCTTCATCAAG | CTTCTCTGCTCTTCATCAAG | 400 |
| efp  | AGGTGGCGCATTTAATCAAG | ATTTCGCCGAGCACACATTTA | 463 |
| ntrX | AAATGCTGCAACGATTACCC | ACAAAATGGCGCGAGAACGAT | 381 |
| pdhA | AGGGGCAGGTTTACGAAATG | TCAATCCGATCTCTTGTTCTTC | 355 |
| purB | CTTTAAACCGCTGTGCTTTAATG | AGTGAGCCGAGGACAAAGTTC | 315 |
| ATPA | ACGTGGAGAATATGCTGGGAG | CTCCGTTATGTTGTTGAT | 302 |
| yfeA | TGTCTCCGACTTCAGCATTG | TCCGAACTTGTTTAGCAGGA | 375 |

After calibration, peak lists were obtained by using the ‘Peak to MASCOT’ script of 4700 Explorer Software (Applied Biosystems). For protein identification, the peptide mass lists were matched against databases proposed by the search engine Mascot (Matrix Science, UK). To select candidate antigens, the highest sequence coverages and Mascot scores were used. According to the Clusters of Orthologous Groups (COGs) classification, the identified proteins were functionally categorized (National Center for Biotechnology Information, USA).

Semi-quantitative RT-PCR

The WT and Δomp43 B. henselae were cultured for 6 days and harvested from BAP. The RNA was extracted from them by using the Ribopure-bacteria kit (Ambion, USA) according to the manufacturer’s instructions. The cDNA synthesis was carried out with the Primerscript 1st strand cDNA synthesis kit (Takara, Japan) according to the manufacturer’s instructions. The primers used for RT-PCR are listed in Table 2. The PCR conditions were 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C for 20 cycles (16S gene) or 28 cycles (tldD, efp, ntrX, pdhA, and ATPA genes) or 29 cycles (Rho, purB, and yfeA genes). PCR amplicons were analyzed by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. RT-PCR band intensities were measured by performing scanning densitometry with the Kodak 1D image analysis software (Eastman Kodak, USA) and analyzed by normalization to 16S rRNA obtained over the same period. Significance of differences was determined by using a Student’s t-test, and p values less than 0.05 were regarded as statistically significant. The data are presented as means ± SDs.

Results

omp43-targeted mutagenesis using homologous recombination in B. henselae

Western blots of OMP43 in E. coli revealed a fusion protein of approximately 45 kDa containing a 3 kDa region resulting

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from an N-terminal Xpress tag (data not shown). The OMP43 protein was purified using Ni\textsuperscript{2+} affinity chromatography and the purity was confirmed by using SDS-PAGE (Fig. 1).

Kanamycin-resistant \textit{B. henselae} grew to form visible single colonies 2 weeks after electroporation. Several PCRs using primers specific for \textit{omp43}, kanamycin resistance, internal transcribed spacer [34], and 16S rRNA gene sequences were performed to confirm \textit{Δomp43} expression (Table 1). Two kanamycin-resistant \textit{B. henselae} mutants were acquired and the \textit{omp43} gene sequences were confirmed by PCR and sequencing (data not shown).

Additionally, to confirm the expression of OMP43, SDS-PAGE gel staining and western blotting were performed. OMP43 expression in the WT cell lysate was detected by using SDS-PAGE gel staining and western blots, but OMP43 expression in the \textit{Δomp43} lysate could not be detected (Fig. 1). This result showed that there was a complete loss of OMP43 protein expression in \textit{Δomp43}.

\textbf{Δomp43 grows at slower rate compared to the WT bacterium}

On BAP, \textit{Δomp43} grew to form visible single colonies, but at a significantly lower growth rate than that of the WT bacterium. To analyze the difference in the growth rates between \textit{Δomp43} and the WT, we cultured the bacteria on BAP and determined the OD\textsubscript{600} at 5, 6, 7, 8, and 9 days after seeding. The \textit{Δomp43} showed significantly lower OD than the WT at 6 days (Fig. 2). Additionally, over-grown colonies of \textit{Δomp43} were slightly smaller than those of the WT. This result indicated that \textit{omp43} affected the growth of the \textit{B. henselae} Houston-1 strain.

\textbf{Fig. 2.} Growth curve of \textit{Bartonella henselae}. Viable cell counts (reported as OD\textsubscript{600}) on blood agar plates were determined at 24 h intervals after plating of individual cultures. OD, optical density; WT, wild-type strain; \textit{Δomp43}, mutant strain. Data significance was evaluated with a Student’s t-test; * \textit{p} < 0.01, ** \textit{p} = 0.0562.

\textbf{Fig. 3.} Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of \textit{Bartonella henselae} wild-type strain (WT, left) and mutant strain (\textit{Δomp43}, right). The pH gradient is indicated at the bottom, and molecular mass standard is indicated to the left. The analyzed target proteins by reverse transcriptase polymerase chain reaction are labeled on the panels. Three independent sets of cultures were analyzed, but only one representative set grown on BAP is shown here. MW, molecular weight.
Two-dimensional gel electrophoresis profiles of *B. henselae*

To find new targets associated with *omp*43, total proteins of Δomp43 and the WT were extracted for 2D gel electrophoresis (2-DE). Representative 2-DE gel images are shown in Fig. 3. In the 2-DE analysis, 422 and 375 protein spots were detected on the 2-DE WT and Δomp43 gels, respectively. Additionally, 282 paired and 233 non-paired protein spots were identified (Fig. 3). Forty-six protein spots showed a 2-fold change in expression levels. Of the 46 protein spots, 26 showed lower protein expression and 20 showed higher protein expression in Δomp43 than in the WT.

Analysis of the expressed proteins in Δomp43

To identify the differentially expressed proteins, 46 of the protein spots selected from the 2-DE gels were excised and subjected to trypsin digestion and subsequent analysis by MALDI-TOF-MS. The 46 protein spots representing 28 different proteins were successfully identified by MALDI-TOF-MS and by MASCOT database searches. Of the identified proteins, 20 showed lower expression and 8 showed higher expression in Δomp43 than in the WT (Tables 3 and 4). Of these proteins, only the *tldD*-encoded protein displayed both higher and lower expressions, and these two spots were distributed in different parts of immobilized pH gradient (IPG) strips (pH 4.0–5.0 and pH 6.5–7.0, respectively). However, the spot that displayed lower expression and was observed in the part of IPG strip corresponding to pH 6.5–7.0 was more credible than the other spot because of the numerical value of the MASCOT score, the coverage, and the masses matched. This credibility supposition was confirmed by RT-PCR (Fig. 4).

Using categories designated based on the COG database, the differentially expressed proteins could be divided into 4 groups. We found 12 proteins to be associated with metabolism, 7 proteins associated with information storage and processing, 3 proteins associated with cellular processing and signaling, and 6 poorly characterized proteins (Tables 3 and 4). In particular, the number of differentially expressed proteins associated with metabolism indicated that various metabolic processes were affected by the loss of OMP43 expression. Additionally, this phenomenon reflected the changes in the proteins involved in information processing such as replication, translation, and transcription. In addition, the 60 kDa heat-shock protein and protein-L-isoaspartate (D-aspartate)-O-methyltransferase (*pcm2*), which are classified in a cellular processing and signaling group involved in maintaining protein structure and integrity, were also affected. These data indicate global changes in the metabolic pathways in the Δomp43 mutant.

RNA expression analysis of the identified proteins by RT-PCR

To verify the proteomics data, semi-quantitative RT-PCR analysis was performed to correlate gene expression with protein expression. Fourteen of the 27 genes (*tld/D* was duplicated) whose encoded proteins were found in the 2-DE analysis were selected for further investigation (Table 2). Although the mRNA expression of 6 genes was unchanged, that of the other 8 genes showed significant changes (Fig. 4). In RT-PCR analysis, the mRNA expressions of 6 targeted genes (*tldD, efp, ntrX, pdhA, purB, and ATPA*) were lower, while those of 2 targeted genes (*Rho* and *yfeA*) were higher in Δomp43 than in the WT (panel A in Fig. 4). The density of each band was quantified by using scanning densitometry, and the expression was subsequently normalized to 16S mRNA expression (panel B in Fig. 4). The results were consistent with those obtained from the proteins identified in the MALDI-TOF-MS assay.

Discussion

The loss or decrease in OMP expression in several Gram-negative bacteria occasionally results in decreased proliferation and fitness *in vitro* and *in vivo*. For example, in *Haemophilus* species, *ompA*, which maintains cell structure and functions as a porin regulating the entry of nutrients into the bacterium, has been well established [37]. Additionally, OMP- or porin-deficient mutant strains have shown reduced growth or loss of viability in *Mycobacterium* species [24], *Salmonella enterica* [5], *Haemophilus ducreyi* [10], and *E. coli* [9].

However, the roles of OMPs in *B. henselae* have not yet been elucidated. Studies on OMPs in *B. henselae* have mainly focused on their primary role in host-bacterial interactions, and OMP43 has been suggested as the major adhesion protein in the outer membrane [6,7]. Burgess *et al.* [7] produced recombinant *E. coli* expressing the *B. henselae* OMP43 as a fusion protein for use in identifying the features of OMP43. Although they successfully determined the amino sequences and characterized the membrane topology of OMP43, as well as the attachment of OMP43 to HUVECs, the function of OMP43 has not yet been described [7]. Therefore, in this study, we established a Δomp43 mutant in *B. henselae*. In order to confirm that proliferation in Δomp43 was significantly lower than that of the WT, protein expression in Δomp43 was investigated by undertaking proteomic analysis and semi-quantitative RT-PCR.

Among the 20 protein spots that showed decreased expression in 2-DE, the mRNA expressions of the ATPA, *efp, ntrX, pdhA, purB,* and *tld/D* genes decreased in Δomp43. These proteins were mainly categorized based on their role in metabolism, based on their COG assignment (Table 3). This result suggested that the loss of OMP43 expression in Δomp43 disrupted energy metabolism, which might have affected cell growth.

Elongation factor P (*efp*) is a translation factor that can stimulate ribosomal peptidyl transferase activity and is homologous to the eukaryotic translation factors elf5A and elf5A [17]. Although elf5A may not be absolutely essential for general protein synthesis, several studies have shown that *efp* is
Table 3. Decreased protein expression levels in *Bartonella henselae* Δomp43 compared with *B. henselae* wild-type strain (Houston-1) by MALDI-TOF-MS

| Spot No. | Mascot score | Coverage (%) | Locus (primary annotation) | Protein identification by MALDI-TOF-MS (gene) | COG assignment | Predicted mass | Masses matched | Wild-type/Δomp43 | pI |
|----------|--------------|--------------|-----------------------------|---------------------------------------------|----------------|---------------|----------------|-----------------|-----|
| 386      | 151          | 39           | gi|49476233 Succinate dehydrogenase flavoprotein subunit (sdhA) 60 kDa heat-shock protein | Metabolism-energy production and conversion | 67,387 | 23 | 2.0 | 6.04 |
| 467      | 128          | 46           | gi|3603159 ATP synthase F0F1 subunit alpha (ATPα) | Metabolism-energy production and conversion | 55,648 | 24 | 2.4 | 5.91 |
| 483      | 183          | 51           | gi|49476189 Nitrogen regulation protein (ntRX) | Function unknown | 50,597 | 24 | 2.9 | 5.62 |
| 498      | 100          | 49           | gi|49476301 Dihydrolipoamide dehydrogenase (phD2) | Metabolism-energy production and conversion | 49,616 | 14 | 2.2 | 6.05 |
| 570      | 165          | 56           | gi|49475235 Putative modulator of DNA gyrase (tldD) | Information storage and processing-replication, recombination and repair | 50,933 | 22 | 2.7 | 6.33 |
| 655      | 84           | 49           | gi|49475961 Outer membrane protein 43 | Cellular processes and signaling-cell wall/membrane/envelope biogenesis | 44,311 | 12 | * | 8.52 |
| 665      | 84           | 43           | gi|49475961 Outer membrane protein 43 | Cellular processes and signaling-cell wall/membrane/envelope biogenesis | 44,311 | 12 | 4.1 | 8.52 |
| 665      | 117          | 69           | gi|49476065 Hypothetical protein BH14010 | Not present in COG | 41,120 | 18 | 4.1 | 6.99 |
| 760      | 84           | 32           | gi|49475823 Ketol-acid reductoisomerase | Metabolism-amino acid and coenzyme transport, and metabolism | 37,725 | 14 | 2.2 | 6.08 |
| 871      | 67           | 39           | gi|49475067 Hemin binding protein A (hbpA) | Metabolism-inorganic ion transport and metabolism | 29,898 | 11 | 2.9 | 5.37 |
| 935      | 69           | 39           | gi|49475425 ABC transporter periplasmic amino acid-binding protein | General function prediction only | 28,390 | 8 | 2.7 | 5.54 |
| 1035     | 93           | 48           | gi|49475323 50S ribosomal protein L9 | Information storage and processing-translation, ribosomal structure and biogenesis | 22,900 | 15 | 3.0 | 5.17 |
### Table 3. Continued

| Spot No. | Mascot score | Coverage (%) | Locus (primary annotation) | Protein identification by MALDI-TOF-MS (gene) | COG assignment | Predicted mass | Masses matched | Δomp43 | pl |
|----------|--------------|--------------|-----------------------------|-----------------------------------------------|----------------|---------------|---------------|---------|----|
| 1041     | 109          | 67           | gi|49475323  | 50S ribosomal protein L9 | **Information storage and processing**-translation, ribosomal structure and biogenesis | 22,900         | 14            | 2.2       | 5.17 |
| 1041     | 99           | 58           | gi|49476159 | Elongation factor P (efp) | **Information storage and processing**-translation, ribosomal structure and biogenesis | 21,456         | 11            | 2.2       | 5.21 |
| 1173     | 70           | 40           | gi|49475636 | Hypothetical protein BH08730 | Not present in COG | 22,659         | 12            | 2.2       | 8.99 |
| 1212     | 96           | 50           | gi|49474947 | 3-Hydroxydecanoyl-(acyl-carrier-protein) dehydratase (fabA) | Metabolism-lipid transport and metabolism | 19,017         | 17            | 2.2       | 5.28 |
| 1254     | 84           | 84           | gi|49476168 | Hypothetical protein BH15110 | Not present in COG | 14,749         | 12            | 2.2       | 5.75 |
| 1283     | 76           | 52           | gi|49475926 | Hypothetical protein BH12070 | Not present in COG | 21,747         | 10            | *         | 9.41 |
| 1373     | 86           | 74           | gi|49475397 | 50S ribosomal protein L7/L12 | **Information storage and processing**-translation, ribosomal structure and biogenesis | 12,707         | 9             | 6.8       | 4.81 |

Words in bold represent the name of four functional categories. MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight-mass spectrometry; COG, Clusters of Orthologous Groups; pi, isoelectric point; ATP, adenosine triphosphate; ABC, ATP-binding cassette. *Not expressed proteins in mutant strain.
Table 4. Increased protein expression levels in *Bartonella henselae* Δomp43 compared with *B. henselae* wild-type strain (Houston-1) by MALDI-TOF-MS

| Spot No. | Mascot score | Coverage (%) | Locus (primary annotation) | Protein description (gene) | COG assignment | Predicted mass | Masses matched | Δomp43/wild-type | pI |
|----------|--------------|--------------|-----------------------------|----------------------------|----------------|----------------|----------------|------------------|----|
| 327      | 264          | 65           | gi|49475797 | Elongation factor G (*fusA*) | Information storage and processing-translation, ribosomal structure and biogenesis | 76,456 | 33 | 2.1 | 5.12 |
| 556      | 81           | 36           | gi|49475235 | Putative modulator of DNA gyrase (*tdrI*) | Information storage and processing-replication, recombination and repair | 50,993 | 13 | 2.0 | 6.33 |
| 575      | 255          | 66           | gi|49476319 | Transcription termination factor *Rho* | Information storage and processing-transcription | 47,429 | 33 | 2.1 | 5.47 |
| 755      | 130          | 49           | gi|49476165 | Glyceraldehyde-3-phosphate dehydrogenase (*gap*) | Metabolism-carbohydrate transport and metabolism | 36,554 | 17 | 2.0 | 6.04 |
| 781      | 62           | 36           | gi|49475018 | L-asparaginase | Metabolism-amino acid transport and metabolism | 36,042 | 9 | 2.2 | 5.83 |
| 886      | 133          | 52           | gi|49475276 | Hemin binding protein D (*hbpD*) | Metabolism-inorganic ion transport and metabolism | 30,251 | 20 | 2.1 | 9.06 |
| 915      | 123          | 51           | gi|49474908 | Iron transport protein *yfeA* | Metabolism-inorganic ion transport and metabolism | 33,766 | 15 | 2.1 | 6.01 |
| 995      | 97           | 49           | gi|49475809 | Protein-L-isoaspartate(D-aspartate) O-methyltransferase (*pcm2*) | Cellular processes and signaling-posttranslational modification, protein turnover, chaperones | 24,570 | 13 | 2.5 | 5.14 |

Words in bold represent the name of four functional categories. MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight-mass spectrometry; COG, Clusters of Orthologous Groups; pI, isoelectric point.
**Fig. 4.** Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of target gene mRNAs (tldD, efp, ntrX, pdhA, purB, ATPA, Rho, and yfeA) between the wild-type (WT) and mutant (Δomp43) strains. (A) Agarose gel electrophoresis showing representative RT-PCR results. (B) Relative intensities of the PCR bands were quantified by scanning densitometry. Each datum represents the mean ± SD (error bars) obtained from triplicate cultures of a representative experiment out of three performed. Data are expressed as ratios of target mRNA normalized to 16S mRNA. Data significance was evaluated with a Student’s t-test; *p < 0.05, **p < 0.01.

F<sub>0</sub>F<sub>1</sub>-ATP synthase, might affect ATP synthesis.

The tldD gene is associated with the activity of DNA gyrase in *E. coli*. Additionally, the protein products of the tldD and tldE genes regulate the stability of the ccdA and ccdA41 antidotes and are essential for MccB17 maturation in *E. coli* [1]. These results suggest that tldD gene products are involved in protein processing and degradation.

Decreased expression of the above-mentioned genes in Δomp43 might be affected by the loss of OMP43 expression. Therefore, the retarded growth in Δomp43 was possibly induced by metabolic pathways altered as a result of the decrease in the expressions of these genes. In contrast, eight protein spots showed increased expression in the 2-DE analysis, and among those, the mRNA expression levels of Rho and yfeA were increased in Δomp43. Additionally, based on COG assignment, Rho and yfeA are categorized as being important in transcription and metabolism, respectively (Table 4). The increased expression of these genes might contribute to functional compensation for the absence of OMP43 expression in Δomp43.

The colonization and growth of many bacterial pathogens is affected by their ability to invade mammalian hosts to obtain iron. However, the iron in host cells is strongly bound by ferritin, transferrin, and lactoferrin [18]. The ATP-binding cassette (ABC) transport system is a well-characterized uptake system. In *Yersinia pestis*, the yfe locus is composed of five genes (yfeA-E) and forms a system similar to the ABC transport system associated with the acquisition of inorganic iron and other ions. Moreover, it has been reported that yfe affects the growth of *Y. pestis* [4]. Although a detailed study on the ABC transport system of *B. henselae* has not been undertaken, it has been reported that the acquisition of heme compounds such as hemin, erythrocyte membrane fractions, and hemoglobin is essential for the growth and survival of the bacterium [32]. Hence, an upregulated yfeA expression in Δomp43 might be a mechanism to compensate for energy loss due to alterations in the metabolic pathways.

The bacterial transcriptional terminators have two types of pathways: factor-independent (intrinsic) and factor-dependent (Rho-dependent) [30]. The Rho-dependent pathway serves to terminate the synthesis of transcripts. Recently, Leela et al. [23] reported that Rho-dependent transcription termination is necessary to avoid the excessive genome-wide R-loops in *E. coli*. The observation that Rho expression was increased in Δomp43 suggests that Rho-dependent transcription termination is associated with repair of disrupted transcription occurring because of gene manipulation or other changes. However, further studies are needed to confirm this hypothesis.

In conclusion, the Δomp43 strain, which is the first omp43-deficient bacterial strain generated by using electrophoresis, showed significantly decreased proliferation. The changes in the mRNA expressions of genes in Δomp43 were mainly associated with metabolic processes. Although the possibility that other undetected proteins or genes may display altered expression or function to compensate for the loss of OMP43 cannot be ruled out, our data suggest that the growth retardation in Δomp43 is induced by altered metabolic pathways and will provide useful information for further investigation of the mechanisms underlying this effect.
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Conflict of Interest

The authors declare no conflicts of interest.

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