Monovalent Cation/Sodium: Proton Antiporter Proteins of *Ehrlichia chaffeensis*

by

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Abstract

Anaplasmataceae family rickettsial bacteria are mostly vector-transmitted pathogens causing important diseases in several vertebrates, including humans, canines, and ruminants. *Ehrlichia chaffeensis*, a tick-transmitted intraphagosomal rickettsial bacterium, is the causative agent of human monocytic ehrlichiosis (HME). Little is known about how this and other related rickettsial organisms are able to reside and replicate within an acidified phagosome environment. Similarly, it is unclear how the infectious form of the bacterium maintains pH homeostasis in the extracellular milieu where the pH is about 7.35-7.45, before its infection to a naïve host cell. Sodium/cation: proton antiporters are integral membrane proteins reported from a wide range of species. They exchange sodium or other monovalent cations against protons across a plasma membrane in maintaining the cytoplasmic pH of a cell. We recently described a mutation within the Ech_0379 gene of *E. chaffeensis* that is predicted to encode for a Na^+/H^+ antiporter protein. The mutation caused the attenuated growth of the organism in vertebrate hosts, resulting in a reduced level of the bacterial presence in the circulation. In this study, we evaluated several antiporter protein genes of *E. chaffeensis*. Its genome contains 10 coding sequences encoding for polypeptides which may form at least six functional proteins. To define their function, a sodium sensitive *Escherichia coli* strain having a mutation in two of its three antiporter protein genes (EP432) is used to carry out the functional complementation assay with *E. chaffeensis* genes from their respective promoters. The EP432 strain has a growth defect during its replication in the presence of NaCl that can be restored with functional complementation. All six *E. chaffeensis* genes could complement the growth defect of EP432 under acidic pH, while Ech_0379 and Ech_0179 also complemented at basic pH. Ech_0179 complemented at neutral pH as well. The complementation of all genes at

neutral and basic pHs, except Ech_0179 and Ech_0379, made EP432 *E. coli* strain be more sensitive to the presence of 200 mM NaCl.

The channeling activity is verified independently by constructing a proteoliposome in vitro with the recombinant protein Ech_0379. The recombinant protein showed antiporter activity at all three pHs in the presence of 100 or 200 mM NaCl when assessed using the recombinant proteoliposome. This research is the first description of antiporter proteins of *E. chaffeensis*.

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Dedication

This dissertation is dedicated to my family, teachers, and friends. Thanks to their love, encouragement, trust, and support, I can complete my graduate study and enjoy the research finally.

Chapter 1 - Literature Review

1.1 *Ehrlichia chaffeensis*

Discovery

Human monocytic ehrlichiosis (HME) is discovered in the year 1986 from a patient with the clinical symptoms similar to Rocky Mountain spotted fever without rash [1]. From the peripheral-blood smear, blue-staining inclusions in leukocytes were found [1]. Serologic cross-reaction to another pathogen *Ehrlichia canis* of the sample was also observed [1]. *Ehrlichia chaffeensis* was later identified as the causative agent of HME, an obligatory intracellular bacterium, belonging to the family *Anaplasmataceae*. This organism replicates in phagosomes (pH~5.0) of infected host monocytes and macrophages[2]. *E. chaffeensis* was first isolated from a patient with HME at Fort Chaffee, Arkansas in 1990 [1].

Classification

According to the *Bergey's Manual of Systematic Bacteriology*, the order *Rickettsiales* contains three families: *Rickettsiaceae, Anaplasmataceae, and Holosporaceae* [3]. However, the family *Rickettsiaceae* contains two genera: *Rickettsia* and *Orientia* [4]. They are obligately intracellular Gram-negative bacteria, living in the host cell cytosol. The ATP/ADP translocase can transport the host adenosine triphosphate (ATP) to the bacteria, though *Rickettsia* can produce ATP by itself [4]. Based on the lipopolysaccharide (LPS), *Rickettsia* is classified into different groups: the typhus group (TG), and the spotted fever group (SFG) [5]. The family *Anaplasmataceae* contains several genera: *Ehrlichia, Anaplasma*, *Wolbachia*, and *Neorickettsia* [6]. The genus *Ehrlichia* contains several species: *E. canis*, *E. phagocytophila*, *E. sennetsu*, *E. equi*, *E. risticii*, *E. chaffeensis*, *E. ewingii*, and *E. muris* [7]. Bacteria from the family *Anaplasmataceae*

are all obligate intracellular microorganisms, which reside and replicate in the host cell membrane-derived vacuole [8]. The family *Holosporaceae* contains the genus *Holospora* [9], which includes three species: *Holospora obtusa*, *Holospora undulata*, and *Holospora elegans* [10]. *Ehrlichia chaffeensis* is an obligatory intracellular bacterium, classified in the family *Anaplasmataceae*, order *Rickettsiales*, class *Alphaproteobacteria* [6]. It is a Gram-negative bacterium without components like lipopolysaccharide and peptidoglycan due to the lack of the genes for the biosynthesis of lipid A and peptidoglycan [11].

Life cycle and reserve host

The lone star tick (*Amblyomma americanum*) has been identified as the biological vector of *E. chaffeensis* [12], and the white-tailed deer is regarded as its major reservoir host in maintaining the transmission cycle [13]. Infected nymphs and adult ticks can transmit the pathogen to susceptible hosts, such as humans, deer, dogs, and other vertebrate species while taking a blood meal; eggs and larvae of the lone star tick are considered to be non-infectious [13] (figure 1.1).

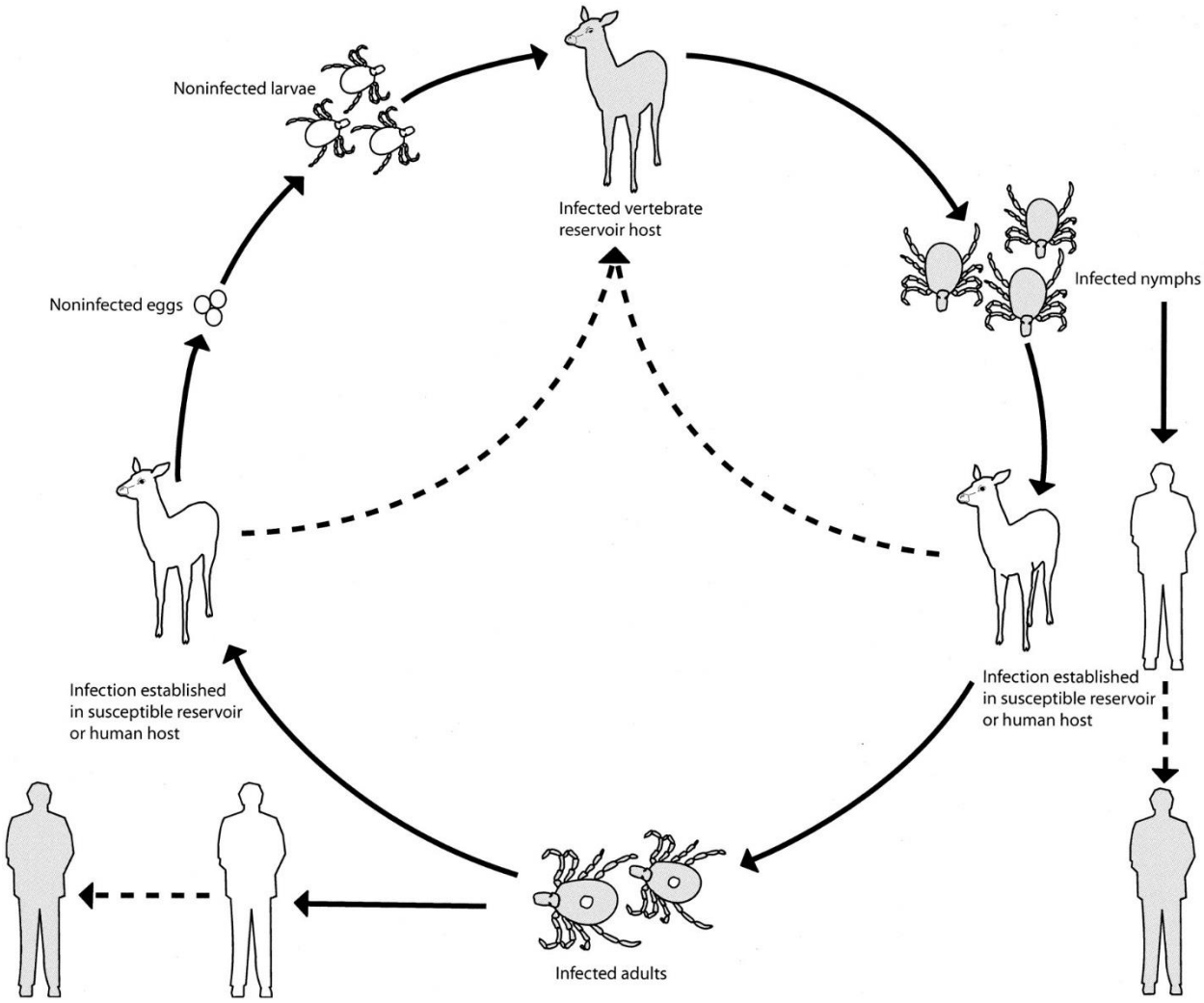


Figure 1.1 The life cycle of *E. chaffeensis*[7]

Development cycles (DC/RC)

In mammalian cells, *E. chaffeensis* has two different forms based on the electron microscopy: the dense-core cells (DC, 0.4-0.6 μm) and the reticulate cells (RC, 0.4-0.6 by 0.7-1.9 μm) [14]. DC are smaller than RC and possess dense nucleoid, and RC have uniformly nucleoid filaments and ribosomes [15]. The DC are the infectious form, which enters into the host cells, transforms to RC, and multiply within the host cell phagosomes [14]. The development cycle of *E. chaffeensis* in vertebrate cells starts from the form of DC. The DC attach to the host cell membrane, enter into

the host cells through phagocytosis, and transform to RC and multiply for about three days before converting back to DC [14]. The matured DC are released through the lysis of host cells or exocytosis to initiate new infection in naïve cells.

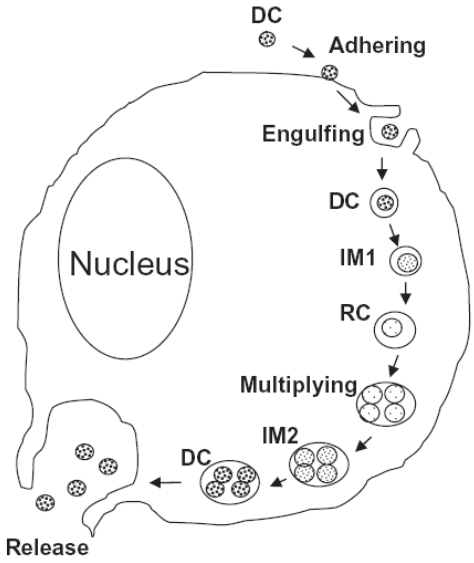


Figure 1.2 The development cycle of *E. chaffeensis* in vertebrate cells [14]

Human Monocytic Ehrlichiosis

Clinical symptoms of HME are from moderate to severe with 41-63% of patients needing the hospitalization, and the fatality rate of 2.7% [16, 17]. The flu-like symptoms with fever, headache is the initial symptom and myalgia, followed by occasionally nausea, dizziness, cough, pharyngitis, regional lymphadenopathy, abdominal tenderness, rash, photophobia, confusion, and stiff neck [17-19]. However, for immunocompromised patients, HME can cause severe illness with leukopenia, neutropenia, lymphocytopenia followed by lymphocytosis, thrombocytopenia, and elevated serum hepatic transaminases [20]. In one previous reported study, organ failure occured

in some patients, and 6 of the 23 reported cases of ehrlichiosis in immunocompromised patients resulted in death [21].

1.2 Antiporter proteins

The antiporter is a secondary active transport protein and plays an important role in maintaining the balance of pH and ions of a cell. Prior research suggests a relation between changes in antiporter's activity to several diseases [22]. In the following few paragraphs, a basic introduction to antiporters is summarized with a primary focus on one of the best characterized Na^+/H^+ antiport genes, including discussion about the protein structure and pH-dependent antiport activity. So far, many studies associated with antiport activity were not conducted within intracellular organisms. *E. chaffeensis* is an obligate intracellular organism. Compared to extracellular organisms, its unique replication cycle requires it to remain pH homeostasis under different pH environments. However, no prior research reports are focused on the antiport activity of *Ehrlichia* species. Elucidation of the antiport activity of this microorganism will provide new insights for research on intracellular organisms.

Membrane transport

Cells are separated from their environment by plasma membranes. Since the biological membranes form barriers for the passage of ionic and polar substances, such substances can transport across membranes only with the help of specific transport proteins [23]. Therefore, such proteins are necessary to mediate the transmembrane movements of ions, such as Na^+, K^+, and Ca^{+2}, as well as metabolites such as pyruvate, amino acids, sugars, and nucleotides, and even water [23]. There are two important types of membrane transport mechanisms, passive transport, and active transport. Passive transport is a movement of substances across cell

membranes without the presence of energy input, while active transport requires the energy for moving substances [24].

In active transport, the molecules moving direction is from the lower concentration to the higher concentration against concentration gradients or other obstructing factors. By the use of coupling solute movement to the source of energy, active membrane transporters can help the transportation of molecules across the cell membranes [25]. There are two forms of active transports: primary active transport and secondary active transport.

Unlike primary active transport powered by the hydrolysis of ATP [26], many secondary active-transports drive the flow of ions or molecules by coupling the thermodynamically uphill flow to the downhill flow of different ions or molecules [27]. Membrane proteins that transport ions or molecules by this mean are called secondary transporters, which are classified as antiporters and symporters [27]. The secondary active transporters couple the electrochemical potential free energy of one solute to across a membrane with the movement of another [28]. For the secondary transport, the electrochemical gradient must be generated by ATP-dependent primary transporters [28]. The model of alternating access is the common foundation to discuss the mechanism of solute transport [28, 29]. Based on the model, during the transportation process, the transporter exposes the substrate binding sites to one side of the membrane or the other side. The ions or molecules move in opposite directions in antiporters, while in symporters they move in the same direction [27].

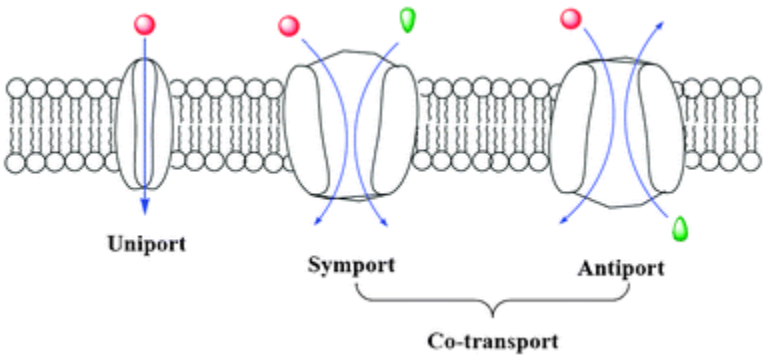


Figure 1.3 The classification of transport[30]

The Na^+/H^+ antiporters

The Na^+/H^+ antiporters are integral membrane proteins that are very important for the control of intracellular pH, cellular Na^+ concentration and cell volume in all domains of life from eukaryotic to prokaryotic organisms, including plants, animals and microorganisms [31, 32]. The entire monovalent cation proton antiporter (CPA) superfamily includes CPA1, CPA2, and NaT-DC [33]. Many structure and function studies of purified antiporter proteins have been conducted with *NhaA*, one key Na^+/H^+ antiporter of *Escherichia coli*. It is known that there are two specific Na^+/H^+ antiporters *NhaA* and *NhaB* in *E. coli* [34, 35], and one unspecific antiporter *ChaA* in *E. coli* [36]. *NhaA* helps to adapt to high salinity, endows resistance to Li^+ toxicity, and helps to grow at alkaline pH [37]. The activity of *NhaB* is not sensitive to pH in the pH6.4-8.3, while the activity of *NhaA* increase with the pH value [35]. The protein encoded by the third gene *ChaA* is not only a $Na^+(Ca^{2+})/H^+$ antiporter but also a K^+/H^+ antiporter. It antiports K^+ in addition to Na^+ and Li^+, and its ability to antiport excess K^+ is essential for cell adaptation to the high concentration of K^+ [38]. These three genes can exchange sodium ions against protons across the membrane. *NhaA* and *NhaB* are electrogenic transporters with stoichiometries of $1Na^+/2H^+$ and $2Na^+/3H^+$, respectively [39].

Although different Na^+/H^+ antiporters share some common characteristics and mechanisms, they also show distinct species-specific transport characteristics and regulatory properties. The Na^+/H^+ antiporter of *NhaA* from *E. coli* is one of the best-characterized members of the antiporter proteins. The monomer of *NhaA* can function fully, while the dimer can function better with extreme stress at alkaline pH in the presence of Na^+ or Ni^+ [40]. The Na^+/H^+ antiporters have characteristic individual pH-dependent activity profiles, critical for their homoeostatic functions [22, 41]. One previous study showed that transport activity of Na^+/H^+ antiporter *NhaA* from *Salmonella Typhimurium* was low at pH 7, and up-regulation above this pH decreased the antiport activity [39]. A study discovered that under the asymmetrical pH conditions with a high pH at the Na^+ uptake side, the transporter activity could be very active at acidic pH [42]. It is suggested that *NhaA* is organized into two functional regions [43]: (1) a cluster of amino acids responsible for pH regulation; (2) a catalytic region at the middle of the TM IV/XI assembly, which contributes to the cation binding site and helps the rapid conformational changes expected for *NhaA*. Based on this observation, a kinetic competition model was come up that Na^+ and H^+ compete for the common binding site [42].

Changed antiport activities and diseases

Altered Na^+/H^+ antiporters or exchangers have been found to be related to the pathogenesis of certain diseases: essential hypertension, congenital secretory diarrhea, diabetes, and tissue damage caused by ischemia/reperfusion [22]. In several pathologies, including cancer, cells are exposed to an extracellular acidic environment. There is a potential relation between inducible NOS induction, Na^+/H^+ exchanger expression, and activity in human ovary cancer [44].

Research on antiporter activities in *E. chaffeensis*

E. chaffeensis is an obligatory intracellular bacterium. As we discussed before, it has two forms within infected host and tick cells: DC and RC [14]. DC are the infections form, which enter into the host cells and are released from the cell by lysis [14]. While RC are the replicative form inside the acidified phagosome, which transform into RC and replicate [14]. Hence, it is necessary for the RC and DC forms of *E. chaffeensis* to be able to reside and replicate within an acidified phagosome and the DC form to maintain ion and proton homeostasis in the extracellular environment, where pH is about 7.35 to 7.45 [45].

E. chaffeensis genome contains nine predicted open reading frames encoding for proteins related to the antiport activity, as judged by computational analysis performed as per a published software (http://www.ncbi.nlm.nih.gov/nuccore/NC_007799.1). However, to the best of our knowledge, no published research has been conducted to characterize the antiporter activity of *Ehrlichia* species. In fact, there is even no published antiporter research carried out on organisms belonging to the order of *Rickettsiales*. Due to the unique replication cycle of these organisms, elucidation of the mechanism of the balance of pH homeostasis and ions will shed new light on the molecular pathogenesis of rickettsial diseases and provide new insights on the prevention and control of infections caused by *Ehrlichia* species and related *Rickettsiales* bacteria.

Chapter 2 - Material and Method

Animal intracellular Proteobacteria of the alpha subclass does not have plasmids [46]. Consistent with this, *E. chaffeensis* does not possess plasmids naturally, thus making it challenging to introduce a plasmid into this organism. Therefore, it is challenging to investigate the function of this organism's targeted proteins by directly introducing exogenous DNA by taking advantage of genetic engineering methods, such as introducing a gene using a recombinant plasmid. To study, *E. chaffeensis* antiporter proteins' activity, we established a surrogate *E. coli* complementary assay using an antiporter deficient *E. coli* strain, EP432 (Genetic Stock Center, New Haven, CT) [47]. Two of the three antiporter genes *NhaA* and *NhaB* in this *E. coli* strain are inactivated [48], thus, causing the growth of the strain sensitive to the presence of NaCl. If transformed with exogenous antiporter genes into this strain, the growth may be restored to some extent. Indeed, this strategy has been employed in studying antiporter protein function of Gram-negative [39, 49] and Gram-positive bacteria[50]. By measuring the growth curve of EP432 after transformation with a recombinant plasmid containing a gene expressing a heterologous antiporter protein, it is possible to reverse the sensitivity in the presence of NaCl. We performed such experiments to examine the antiporter protein activity of various *E. chaffeensis* genes in the current study.

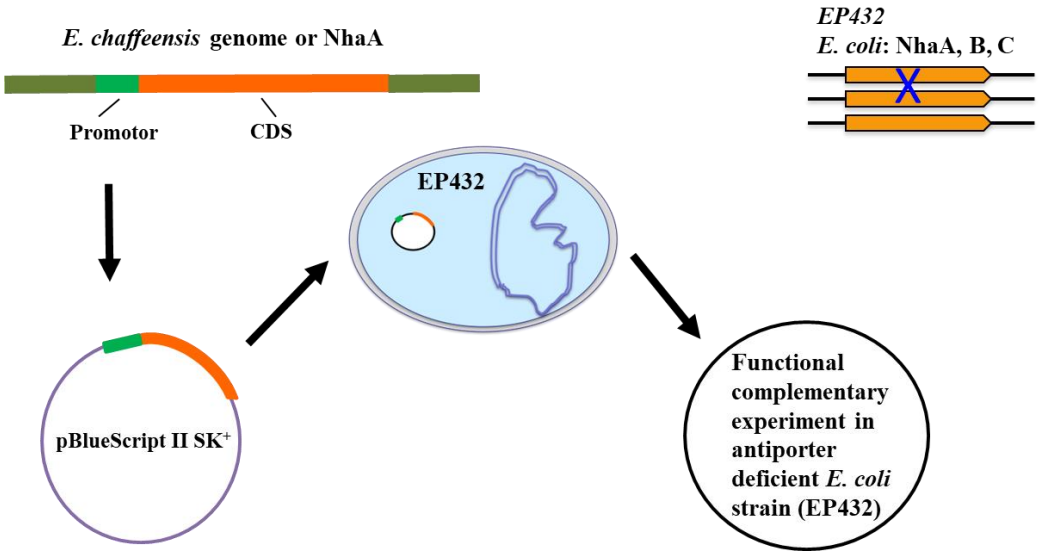


Figure 2.1 Functional complementation assay

2.1 Construct the recombinant plasmid

We predicted ten putative antiporter genes in the genome sequence of *E. chaffeensis* strain Arkansas (Genbank number CP000236.1) (Table 2.4). Based on their location on the genome, we subdivided them into six groups of genes (Figure 2.2). The coding sequence of each gene or gene group along with the respective promoter sequences were amplified by PCR using a high-fidelity DNA polymerase.

The PCR products were then inserted into the plasmid, Bluescript II SK (+) (Stratagene, San Diego, CA) by directional cloning into BamHI and SalI restriction enzyme sites. The coding sequence of *E. coli NhaA* (GenBank number, NJ74_RS08715) with its promoter sequence was also cloned into pBluescript II SK(+) plasmid to serve as a positive control. The recombinant plasmids were confirmed by sequencing. The recombinant plasmids were referred as pBSK-0179, pBSK-0328, pBSK-0379, pBSK-0466-0469, pBSK-0474, and pBSK-0944 for those containing *E.*

chaffeensis antiporter genes, while the *E. coli NhaA* gene plasmid is regarded as, pBSK-NhaA, respectively.

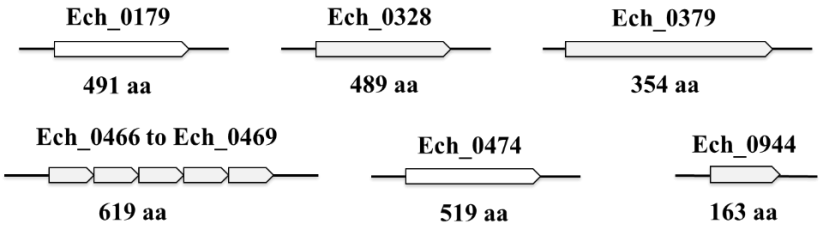


Figure 2.2 The predicted groups of antiporter genes in *E. chaffeensis*

2.1.1 PCR amplification

The coding sequence of each gene with its own promoter was amplified by high-fidelity PCR amplification kit (Platinum™ Taq DNA Polymerase High Fidelity, Invitrogen, Carlsbad, CA). Details of the PCR mix preparation are included in Table 2.1. All the primers used were synthesized in IDT (Integrated DNA Technologies, Coralville, IA). Primer sequences used for the recombination were presented in Table 2.2. To promote directional cloning into the pBlueScript plasmid, BamHI, SalI or PstI restriction enzyme sites were engineered as part of the primers (Table 2.2). PCR temperature cycles used were initial 2 min at 94℃, followed by 40 cycles of denaturation for 30 sec at 94℃, annealing for 30 sec at 49℃ or 51℃, and extension for 2.5 min at 68℃. At the end of 40 cycles, the products were maintained at 4℃ until analyzed. For the genes Ech_0179, 0328, 0466-0469, 0474, 0944, the annealing temperature used was 49℃, and for the genes Ech_0379, and *NhaA*, it was 51℃. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Germantown, MD) as per the manufacturer's protocol and concentrated by eluting in 30 µl nuclease-free water (Invitrogen, Carlsbad, CA). DNA concentration was estimated with the help of Nanodrop spectrophotometer (Thermo Fisher, Waltham, MA).

Table 2.1 PCR Reaction Mix

Reagents	volume (µl)
For each reaction	
10 x high fidelity PCR Buffer	5
50 mM MgSO4	2
10 mM dNTP	1
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Taq hifi	0.2
Template	2
Water	8.75

2.1.2 Restriction enzyme digestion and ligation

Plasmid pBluescript II SK(+) is linearized using the restriction enzymes BamHI and SalI (New England Biolabs, Ipswich, MA) following the manufacturer's protocol with a few modifications. Details of the restriction digestion method were included in Table2.3. The reaction was incubated for 1-2 hours at 37℃, and the products were purified using a QIAquick PCR Purification Kit (Qiagen, Germantown, MD). The purified digestion products were eluted in the 30 µl nuclease-free water. DNA concentrations were estimated by using the Nanodrop spectrophotometer and used for ligation step outlined below or stored at -20℃ until future use.

2.1.3 Ligation

The purified PCR products were ligated into linearized pBluescript II SK(+) plasmids using a T4 DNA ligase Kit (New England Biolabs, Ipswich, MA) as per the manufacturer's protocol with a few modifications. For each reaction, DNA insert and plasmid vector was mixed with an approximate molar ratio of 1:3.

Table 2.2 Primers used for the recombinant plasmids

Target	Primer Name	Sequence	Amplicon Size
ECH-0179	RRG2097-BamHI	Forward: 5'- tgacg-GGATCC-TAG TGC CAT TGG AGT ATA TGT AAG -3'	1976 bp
	RRG2098-SalI	Reverse: 5'- tgacg- GTCGAC -TTT ACT TTA ATT TTA ATA AAG CTG CTG -3'	
ECH_0328	RRG2099-SalI	Forward: 5'- tgacg- GTCGAC-TCT TAA TCT ATA AGC GGC ATA TGC -3'	1893 bp
	RRG2100-BamHI	Reverse: 5'- tgacg-GGATCC- AAT ATA GGA ACA ACT AAA CAA ACT AC -3'	
ECH_0466, ECH-0467, ECH-1930, ECH-1935, ECH-0469	RRG2101-BamHI	Forward: 5'- tgacg-GGATCC- ATG TAG AAT TCA CAG AGC TTT AGC -3'	2291 bp
	RRG2102-SalI	Reverse: 5'- tgacg- GTCGAC-ATT ATG ATT TGA CAC TAG ACT TAC AC -3'	
ECH_0474	RRG2103-BamHI	Forward: 5'- tgacg-GGATCC- TGT TGT TGA TAT ATA TGT TCA GTA TG -3'	1883 bp
	RRG2104-SalI	Reverse: 5'- tgacg- GTCGAC -TTA CTC TGC ATC TTT TGG ATT ACA -3'	
ECH-0944	RRG2105-BamHI	Forward: 5'- tgacg-GGATCC- GTA ATA CAA CCC CAG TTA TAC AGA -3'	828 bp
	RRG2106-SalI	Reverse: 5'- tgacg- GTCGAC -TAA TCA CTT GTC AGA TTT AAT GAC A -3'	
ECH_0379	PRG2131-BamHI	Forward: 5'- tgacg-GGATCC- GTT TTT TAG CAT CCT TTG TGT TAA AAG -3'	1543 bp
	PRG2132-SalI	Reverse: 5'- tgacg- GTCGAC - ATA TCG ACA AGC AAT TGA TAC AGA G -3'	
NhaA	PRG2158-SalI	Forward: 5'- tgacg- GTCGAC- GCT CAT TTC TCT CCC TGA TAA CA -3'	1817 bp
	PRG2159-PstI	Reverse: 5'- tgacg- CTGCAG - TGC TCT CTT CTC CTT GAC CTT AC -3'	

Table 2.3 Restriction Enzyme Digestion-1

Reagents	Volume (µl)
DNA	5
10x NEBuffer 3.1	2
BamHI	1
SalI	1
Nuclease-Free Water	12.5

Each ligation reaction consisted of 50 ng of plasmid, 1 µl T4 ligase, and 2 µl 10x ligation buffer and three times the molar concentration of insert DNA. Nuclease-free-water was added to make up the volume to 20 µl. The reaction was incubated at 22^{o}C for 2h. Then changed the incubation temperature to 16^{o}C for another 12-14 hours. At the end of incubation, 2 µl of ligation products or control plasmid was used to perform the transformation experiment using 40 µl *E. coli* TOP10 competent cells (Invitrogen™ One Shot™ TOP10 Chemically Competent cells). The ligation products and competent cells were mixed gently and incubated on ice for 30 min, then transferred to 42^{o}C for 30 sec followed by 4^{o}C incubation for 2 min. To the mixture, 300 µl of SOC medium was added at room temperature and transferred to a shaker incubator set at 220 rpm at 37^{o}C for 1 h. Fifty µl of the transformed culture was then spread onto LB plates containing ampicillin at 100 µg/ml concentration. The plates were then incubated at 37^{o}C overnight. Isolated recombinant white colored colonies were picked from each plate, transferred to sterile glass test tubes containing 3 ml LB medium containing ampicillin at 100 µg/ml. The cultures were then incubated at 37^{o}C overnight in a 220 rpm shaking incubator.

Plasmid DNAs from overnight cultures were isolated using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Briefly, 1.5 ml of each culture was transferred to a microcentrifuge tube, centrifuged at 13,000 rpm for 5 min. Supernatants were then discarded, the pellets were resuspended in 250 µl of Resuspension Buffer

(R3) with RNase A by vigorous vortexing until the solution turned homogeneous. Subsequently, 250 µl of lysis buffer was added to each tube, mixed by inverting the tubes for several times until the solution turned homogeneous and incubated for 5 min at room temperature. DNA from each solution was then precipitated by adding 350 µl precipitation buffer (N4). The mixtures were centrifuged for 10 min at 13,000 rpm. Transferred the supernatant to the spin column provided. Binding the plasmids DNA on the column by centrifugation at 13,000 rpm for 1 min, and flow through was discarded. Five hundred µl of wash buffer (W10) was added to the column, centrifuged at 13,000 rpm for 1 min. Discarded the flow-through, and 700 µl wash buffer (W9) was then added. The column was subjected to centrifugation at 13,000 rpm for 1 min, and the collected flow-through solutions were then discarded. Spin column was transferred to a clean 1.5 ml Eppendorf tube to which 70 µl of elution buffer was added to the column, incubated for 5min at room temperature, and then centrifuged at 13,000 rpm for 2min. The final recovered plasmid DNAs were stored at -20 or -80^{o}C until use.

The presence of insert in the recombinant plasmids was verified by restriction enzyme digestions following the protocol described previously (Table 2.2). The presence of specific sized inserts was confirmed by agarose gel analysis of the digestion products. Typically 5 or 10 µl each of the restriction-digested samples were resolved in a 1% or 1.5% agarose gel prepared in 1 x TAE buffer and images of the resolved DNAs were captured following staining with ethidium bromide and using the Kodak Gel Logic 200 imaging system. The presence of specific inserts in the recombinant plasmids was verified by sequencing analysis (Molecular Cloning Laboratories, South San Francisco, CA).

2.2 Cultivation of *E. chaffeensis*

Ehrlichia species are obligate intracellular bacteria and so require host cells for the essential nutrients to support the growth under in vitro culture conditions. *E. chaffeensis* Arkansas isolate (ATCC # CRL-10389) [51, 52] or in the *Ixodes scapularis* embryonic cell line (ISE6) as previously described [47, 53]. Cultured the canine macrophages (DH82) cells in sterile 6-well cell culture plates or T25, T75 or T150 flasks to 80-90% confluence. Inoculated the DH82 cells with 10% to 20% of the infected culture to promote the growth to about 80-90% infectivity which requires one week to 10 days. For RNA expression analysis experiment, infected cultures were recovered at the following post-infection time points 0h, 6h, 12h, 24h, 30h, 36h, 48h, 54h, 60h, 72h, 84h, 96h, and 108h. The harvested cells from each flask were concentrated by centrifugation at 12,000 g for 10 min. Supernatants were discarded, and the final pellets were re-suspended in 1 ml of TRI-Reagent (Sigma-Aldrich, Sigma-Aldrich, St. Louis, MO) and stored at -80^{o}C until RNA isolation protocol was employed. The *E. chaffeensis* culture was similarly grown in ISE6 cell line as per the protocols outlined in the reference [52].

Table 2.4 Primers and probes used for the RT-qPCR

Gene	product	protein size	Primer Name	Sequence	Size
ECH-0179	cation:proton antiporter	491	RRG2107	Forward: 5'- GGC TAT ACA AGT TGG GTT GTT GT -3'	672 bp
			RRG2108	Reverse: 5'- CAC ACA TAC ACC ACA GAT AGA CCT -3'	
ECH_0328	cation:proton antiporter	489	RRG2068	Forward: 5'-GCA TGC GAT ATC ATT TGG AA-3'	370 bp
			RRG2069	Reverse: 5'-GAA TTG GAA AAG CCG CAT TA-3'	
ECH_0466	monovalent cation/H+ antiporter subunit F	89	RRG2054	Forward: 5'-TGC TGC AAA TTT GTT TGG AA-3'	165 bp
			RRG2055	Reverse: 5'-TCT CCA AAA GAA CCA TGA AGA -3'	
ECH_0467	cation:proton antiporter	99	RRG2056	Forward: 5'-TGG ACT TGC TAT GCG ATC TG-3'	151 bp
			RRG2057	Reverse: 5'-TCA GTC AGC ATC ATC TTT ACC TTT -3'	
ECH_1930	cation:proton antiporter	181	RRG2062	Forward: 5'-TTT GAT GGC ATT GTT GCA TT-3'	332 bp
			RRG2063	Reverse: 5'-CTC GAA AAC TTG CTA AAA TTG C-3'	
ECH_1935	monovalent cation/H+ antiporter subunit B	139	RRG2060	Forward: 5'-CAG GCT GGT GTT ATT GTT GC-3'	259 bp
			RRG2061	Reverse: 5'-TAC ACA CAG TCA TGC CCA CA-3'	
ECH_0469	cation:proton antiporter	111	RRG2058	Forward: 5'-GGT TAT AGG TTT GTA TGT TAC TAC TGC -3'	213 bp
			RRG2059	Reverse: 5'-ATT GCA ACC CCA ACA ACA AT-3'	
ECH_0474	monovalent cation/H+ antiporter subunit D	519	RRG2109	Forward: 5'- GGC ATC TGG TGG GTT TTT AGG -3'	525 bp
			RRG2110	Reverse: 5'- GCA GAA CAT ACT GCC TCT ACT G -3'	
ECH-0944	sodium:proton antiporter	163	RRG2064	Forward: 5'-GGT TTG CCC TAT CAG GGT ATC -3'	426bp
			RRG2065	Reverse: 5'-CAC CAG ACA TTG ACT CTT CAT CT-3'	
ECH_0379	hypothetical protein	353	RRG1276	Forward: 5'-CTA AGG TTG TAG GGA ATG CAA CC -3'	374 bp
			RRG1277	Reverse: 5'-ACA AGG TAA GTA CCT TGC TTG CTC -3'	
E. chaffeensis 16S rRNA q-PCR*	NA	NA	TaqMan-forward	Forward: 5'- CTC AGA ACG AAC GCT GG -3'	148 bp
			TaqMan-reverse	Reverse: 5'- CAT TTC TAA TGG CTA TTC C -3'	
			TaqMan Probe	Probe: 5'-TET-CTT ATA ACC TTT TGG TTA TAA ATA ATT GTT AG-TAMRA-3'	

Note: * the sequences were first used in one previous published paper [54].

2.3 RNA isolation

Total RNA was extracted using the TRI-Reagent method (Sigma-Aldrich, St. Louis, MO) following the manufacturer's recommended protocol with a few modifications. The cell pellet dissolved in TRI-Reagent was thawed at room temperature, to which 0.2 ml of chloroform was added per 1 ml each of the TRI-Reagent solutions. Then the mixture was shaken vigorously for 15 sec, stand for 15 min at room temperature, and then centrifuged for 15 min at 12,000 rpm at 4^{o}C. Upper aqueous phase was transferred to a clean RNase-free tube and to which 0.5 ml of pre-cooled 2-propanol was added per ml of TRI reagent, then incubated the solution for 10 min. RNA was recovered by centrifugation for 10 min at 12000 rpm at 4℃. The supernatant was discarded, and the pellets were rinsed by adding 1 ml of 75% pre-cooled ethanol per every ml of TRI reagent solution containing RNA. The mixtures were gently mixed by pipetting up and down and then centrifuged at 12000 rpm for 5 min at 4℃. Supernatants were discarded; the RNA pellets were air dried and resuspended in 50 or 100 µl of nuclease-free water.

Residual genomic DNAs from each RNA were eliminated by adding RQ1 RNase-Free DNase (Promega, Madison, WI) as per the manufacturer's protocol. The reaction mixture consists 2 µl RQ1 DNase 10X Reaction Buffer, 1 µl RQ1 RNase-Free DNase, and 17 µl RNA sample. The mixture was incubated for 1 hour at 37℃. Then the reaction was stopped by adding 2 µl stop solution and incubating for 15 min at 65℃. Aliquoted the DNase treated RNA samples and stored at -80℃ until use.

2.4 Gene expression levels determined by Semi-quantitative PCR

The Total RNA samples from the different time points were normalized by performing the One-Step reverse transcription quantitative PCR (RT-qPCR) targeted on the 16 S rRNA [54] using

the commercial kit SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA). The primer and probe sequences are presented in Table 2.4. The preparation for the PCR reaction mix please refer to Table 2.5. The amplification protocol is: reverse transcription, 30 min at 50℃, 5 min at 95℃; followed by 40 cycles of amplification of 15 sec at 95℃, 40 sec at 50℃, 45 sec at 60℃ with optics on.

Table 2.5 One-step RT-PCR reaction mix with probe

Reagents	volume (μl)
2X Reaction Mix	12.5
Forward primer (10 μM)	0.5
Reverse primer (10 μM)	0.5
SuperScript® III RT/Platinum Taq Mix	0.5
Taqman® Probe (10 μM)	0.25
Template	2
Nuclease-Free Water	8.75

Based on the 16S RT-qPCR results, the RNA concentrations were adjusted to the equal copy number of 16 S rRNA per unit volume. The nucleotide sequences of the putative antiporter genes were obtained from NCBI GenBank (https://www.ncbi.nlm.nih.gov/). Primers for the one-step RT-qPCR were designed from within the coding sequences and synthesized by Integrated DNA Technologies (Coralville, IA, USA). The nucleotide sequences of the primers are listed in Table 2.4. Primers targeted to putative antiporter genes were used to perform one-step reverse transcription PCR (RT-PCR) using 2 μl each of normalized RNA templates with the commercial kit SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA). The RT-PCR reaction mix was prepared as outlined in Table 2.6. The amplification condition was a reverse transcription, 1 h at 50℃; amplification cycles set at 30 sec at 94℃, 30 sec at 51℃ or 54℃, 30 sec at 72℃ followed by a final extension step for 5 min at 72℃, and then stored at 4℃

until analyzed. The amplification cycles varied from 25 to 45 cycles to estimate the variations of gene transcription levels. The amplicons were resolved in 1.5% agarose gel containing EtBr, and images of the DNA resolved in the gels were captured using a Kodak Gel Logic 200 imaging system.

Table 2.6 One-step RT-PCR reaction mix without probe

Reagents	volume (μl)
2X Reaction Mix	12.5
Forward primer (10 μM)	0.5
Reverse primer (10 μM)	0.5
SuperScript® III RT/Platinum Taq Mix	0.5
Template	2
Nuclease-Free Water	9

2.5 Growth complementation under NaCl stress

Antiporter activities of the putative antiporter proteins were assessed by functional complementation assay using the EP432 *E. coli* strain. The verified recombinant plasmids generated in 2.1.3 or the control plasmid were transformed into EP432 by following the protocols outlined above and used in this experiment.

The plasmid pBSK (vector only) alone served as the negative control and the plasmid with *E. coli NhaA* gene served as the positive control. Prior to investigating the antiporter activities of the *E. chaffeensis* genes, we verified the RNA expression from each of the genes in EP432 strain. Total RNA was recovered from 3 ml each of overnight cultures of the recombinant *E. coli* strain (cultured in LB medium in the presence of 200 mM NaCl). After the DNase treatment, RNAs were assessed by one-step RT-PCR following the same protocol described previous section but set the

amplification cycles to 40. The PCR products were analyzed in a 1.5% agarose gel and the DNA images were captured.

Upon verification that all *E. chaffeensis* putative antiporter genes and the *E. coli* antiporter genes were making the transcripts, but not the non-recombinant plasmids, all EP432 transformed cultures were regrown to test the antiporter activities. Overnight cultures of EP432 with recombinant plasmids were regrown in LBK medium (10 g/L tryptone, 5 g/L yeast extract, and 6.5 g/L KCl). The cultures were then diluted to 0.02 units of OD_{600nm} in three different buffered media having different pH conditions: 1) 100 mM MES pH 5.5, 2) 100 mM Tris-HCl pH 7.0, or 3) 100 mM Tris-HCl pH 8.0 in LB medium (10 g/L tryptone, 5 g/L yeast extract) containing 200 mM NaCl or 800 mM NaCl. Two hundred μl of the diluted culture was transferred into wells of a 100-well microplate. The plate was incubated in a Microbiology Reader Bioscreen C (Oy Growth Curves Ab Ltd, Helsinki, Finland) at 37^{o}C with continuous shaking. The growth in the wells of the culture plate was monitored once every 15 min at OD_{600nm} for up to 24 h. The experiment was conducted for three independent times.

2.6 Purification of the recombinant protein Ech_0379

2.6.1 Construction of the recombinant expression plasmid

The pET-28a-c(+) vector (Novagen, Darmstadt, Germany) encoding Ech_0379 was prepared and used to overproduce Ech_0379 protein for purification. The entire protein coding sequence of Ech_0379 was amplified by PCR from *E. chaffeensis* genomic DNA using pfu DNA polymerase (Promega, Madison, WI) with the gene-specific PCR primers designed to include NheI and XhoI sites on the forward and reverse primers, respectively. The PCR product was subsequently inserted into pET28 plasmid at the above restriction sites after digesting both plasmid and inserts and

ligating using T4 DNA ligase following the similar procedure described before (refer to 2.1.3). The presence of the insert in the recombinant plasmid (pET28-Ech_0379) for expression as an N-terminal His-tag protein after transforming into a TOP10 strain of *E. coli* was verified by restriction enzyme digestion analysis (Table 2.7) and further confirmed the gene sequence by DNA sequence analysis. Subsequently, the recombinant plasmid was then chemically transformed into the host strain *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA) following the standard molecular cloning protocols as outlined previously under the section 2.1.3.

Table 2.7 Restriction Enzyme Digestion-2

Reagents	Volume (µl)
DNA	5
10x NEBuffer 3.1	2
NheI	1
XhoI	1
Nuclease-Free Water	12.5

Histidine-tagged Ech_0379 protein was purified using Ni-NTA-agarose (Qiagen, Germany). The other reagents used in this procedure please refer to Table 5.1. (1) The cell pellet was suspended in 15 ml of lysis buffer I and incubated 30 min on ice with occasional gentle mixing. Before using added 5 µl /ml lysozyme (100 mg/ml) and 2 µl /ml protease inhibitor into lysis buffer I. (2) An equal volume of lysis buffer II was added and incubated on ice for 30 min with occasional shaking. (3) To entirely disrupt the cell wall and release the interior content, a Sonic Dismembrane sonicator was used (Fisher Scientific, Pittsburgh, Pennsylvania) at the setting of 12.5 for 15 sec with a 15 sec break each time until the solution turned translucent. Kept the sample on ice all the time during the sonication. (4) Spun down the lysate at 10,000 g for 15 min at 4°C. Transferred the supernatant to a new tube. (5) Added 2 ml Ni-NTA-agarose slurry (Qiagen, Germany), 2 ml

lysis buffer I and lysis buffer II to equilibrate. Mixed and stand until the agarose set down. (6) Discarded the supernatant and added the supernatant in step 4 into the pellet. Incubated the mixture for 1h at 4°C with gentle rotation. (7) Loaded the mixture on a poly-prep chromatography column (Bio-Rad, Hercules, CA). (8) Washed the column with 10 ml washing buffer A. (9) Washed the column with 5, 5 ml washing buffer B twice. (10) The desired protein was eluted by 1x elute buffer, collected in 1 ml fractions, and analyzed in 12% SDS–PAGE. The concentration of protein was evaluated by the Bio-Rad protein assay kit (Hercules, CA) following the manufacturer's recommended protocol.

2.6.2 SDS-PAGE and Western-blot analysis

Ten µl of purified protein was boiled for 5 min before mixing with 5x sample loading buffer to the final concentration of 1x. Then the mixture was analyzed on an SDS-PAGE to identify the protein expression and the level of expression. The electrophoresis parameters are 120 v for about 20 min and then switched to 90 v for about 2 hours. After electrophoresis, the gel was stained using the reagents of Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA) as per manufacturer's recommended protocol. Western-blotting targeting the N-terminal His6-tag of the recombinant protein was carried out as per the manufacturer's protocol. This involved transferring the proteins from the PAGE gel to a nitrocellulose membrane, blocking the membrane with 5% milk at room temperature for 1 h, washing the membrane with PBST buffer for five times after adding 5% milk per 100 ml of buffer, and incubating with the 6x-His Tag Monoclonal Antibody MA1-21315 (Thermo Fisher, Rockford, IL). The antibody was diluted 1:1,000 and incubated at 4^{o}C overnight. Then washed the membrane as previously described, and final incubation was carried out with the secondary antibody (goat anti-Mouse IgG, Temecula, CA), diluted the antibody 1: 5,000 at room

temperature for 1 h. Finally, the membrane was washed as outlined above and subjected protein detection analysis by the medical film processor (Konica Minolta, Japan)

2.7 Swelling assay

To determine the antiporter activity of the putative antiporter protein, the proteoliposome was constructed to perform the swelling assay as described previously with some small modifications [55-58]. Two hundred forty µl of egg phosphatidylcholine (10 µmol/ml) (Avanti Polar Lipids, Alabaster, AL) and 20 µl dicetylphosphate (10 µmol) (Sigma-Aldrich, St. Louis, MO) dissolved in chloroform-methanol (2:1 [vol/vol]) was mixed, and dried under a stream of N_2 gas for about 5 min in the round bottom of the 5-SV borosilicate 5 ml tube (Thermo Fisher, Waltham, MA). Resuspended the lipid film with 200 µl of 20 mM Tris-HCl buffer (pH 7.0) containing 2 µg of purified recombinant protein. The mixture was vortexed and homogenized by Branson water-bath M1800 sonicator (Branson Ultrasonic, Danbury, CT) at room temperature until it became translucent and then dried the lipid-protein mixture. Carefully added 300 µl suspension buffer (10 Mm Tris-HCl, pH 7.5, 15% (wt/vol) dextran 40 [Sigma-Aldrich, St. Louis, MO]) to the mixture. Rotated the tube gently to wet the film and by incubating contents for 60 min. The mixture was then resuspended by gently shaking the tube. Pipetted 17 µl proteoliposome suspension into 600 µl testing solutions (100 mM MES pH 5.5, 100 mM Tris-HCl pH 7.0 or 100 mM Tris-HCl pH 8.0) containing 100 mM NaCl or 200 mM NaCl in a 0.1 cm quartz cuvette. Immediately following gentle mixing the contents by pipetting up and down for 5 times without producing air bubbles, recording was taken at the optical density at 400 nm every 0.03 min (1.8 sec) continuously for 12 min by using a spectrophotometer (Varian, Palo Alto, CA). Liposome was made with only 200 µl

of 20 mM Tris-HCl buffer (pH 7.0) (with no purified recombinant protein added) and tested following the same protocol as proteoliposome, serving as the negative control.

2.8 The secondary structure prediction

Amino acid sequences of the 10 putative antiporter proteins of *E. chaffeensis* were aligned using the online multiple sequences alignment tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The online sequence analysis tool, Smart (http://smart.embl-heidelberg.de/), or TMpred were used to predict the secondary structure and the transmembrane domain (https://www.ch.embnet.org/software/TMPRED_form.html). To draw the prediction transmembrane domain, the software TMR Pre2D was used. For motif or subunit prediction, the NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the database KEGG (http://www.genome.jp/kegg/) were used. For the function prediction, the online tool SIB BLAST+ Network Service was used (https://web.expasy.org/blast/).

Chapter 3 - Results

3.1 The antiporter gene expression of *E. chaffeensis* during the development

Ten genomic regions were identified as having protein coding sequence similar to antiporter genes found in other bacteria (Table 2.4). To assess if the predicted genes are transcriptionally active, *E. chaffeensis* RNA recovered from various time points following infecting canine macrophage cultures (DH82) were evaluated by semi-quantitative RT-PCR (Figure 3.1). Nine of the 10 putative antiporter genes were tested to be positive for the presence of mRNA, except the gene Ech_0179. Three genes Ech_0466, Ech_0467, Ech_0469 had transcripts being expressed at similar levels during the course of infection when assessed from 6 to 108 hours post infection of DH82 cultures. The remaining genes had varying levels of expression, and the expression levels were increased as time progressed; at later times, the expression levels were declined. For Ech_0379 gene, the highest expression was observed for 84 hours post-infection time, while for other genes the expression levels were higher near 48 to 54 hours. As the replicating forms peak at mid infection stage, we reasoned that the peak expression for the majority antiporter genes might represent the active demand for the proteins made from these transcripts during the replicating stage of the pathogen within an infected phagosome. The bacterial RNA was also assessed when the organism was cultured in the ISE6 tick cell line. The gene expression patterns appeared similar for the genes; Ech_0467, Ech_0469, Ech_1930 and Ech_0328 as in DH82 culture, although the expression levels were variable. Expression of the remaining six genes in tick cell cultures was too low to be detected by the semi quantitate RT-PCR assay.

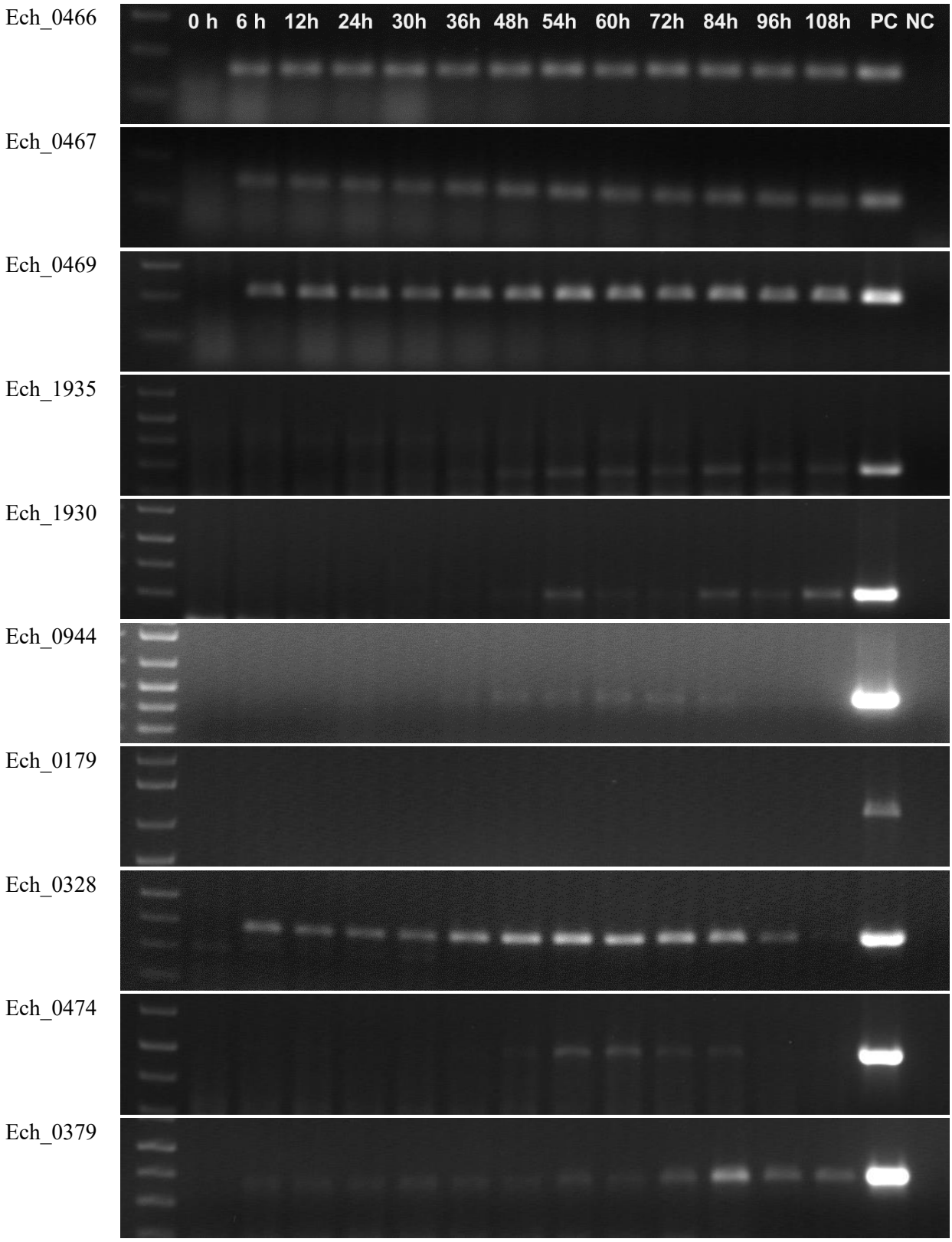


Figure 3.1 Messenger-RNA levels of the putative antiporter genes in *E. chaffeensis* during infection of canine macrophages DH82

RNA concentration was measured by RT-qPCR targeted on the 16 S rRNA and normalized based on the PCR results. The transcript levels of the targeted gene were compared by semi-quantitative PCR with different amplification cycles. The transcript levels were measured after the post infection time 0, 6, 12, 24, 30, 36, 48, 54, 60, 72, 84, 96, and 108 h. PC refers to the positive control used the *E. chaffeensis* genomic DNA as the template. NC refers to the negative control using the nuclease-free water as the template. Line one to line ten: Ech_0466 (35 cycles), Ech_0467 (35 cycles), Ech_0469 (30 cycles), Ech_1935 (35 cycles), Ech_1930 (45 cycles); 6-10: Ech_0944 (45 cycles), Ech_0179 (45 cycles), Ech_0328 (35 cycles), Ech_0474 (35 cycles), Ech_0379 (35 cycles).

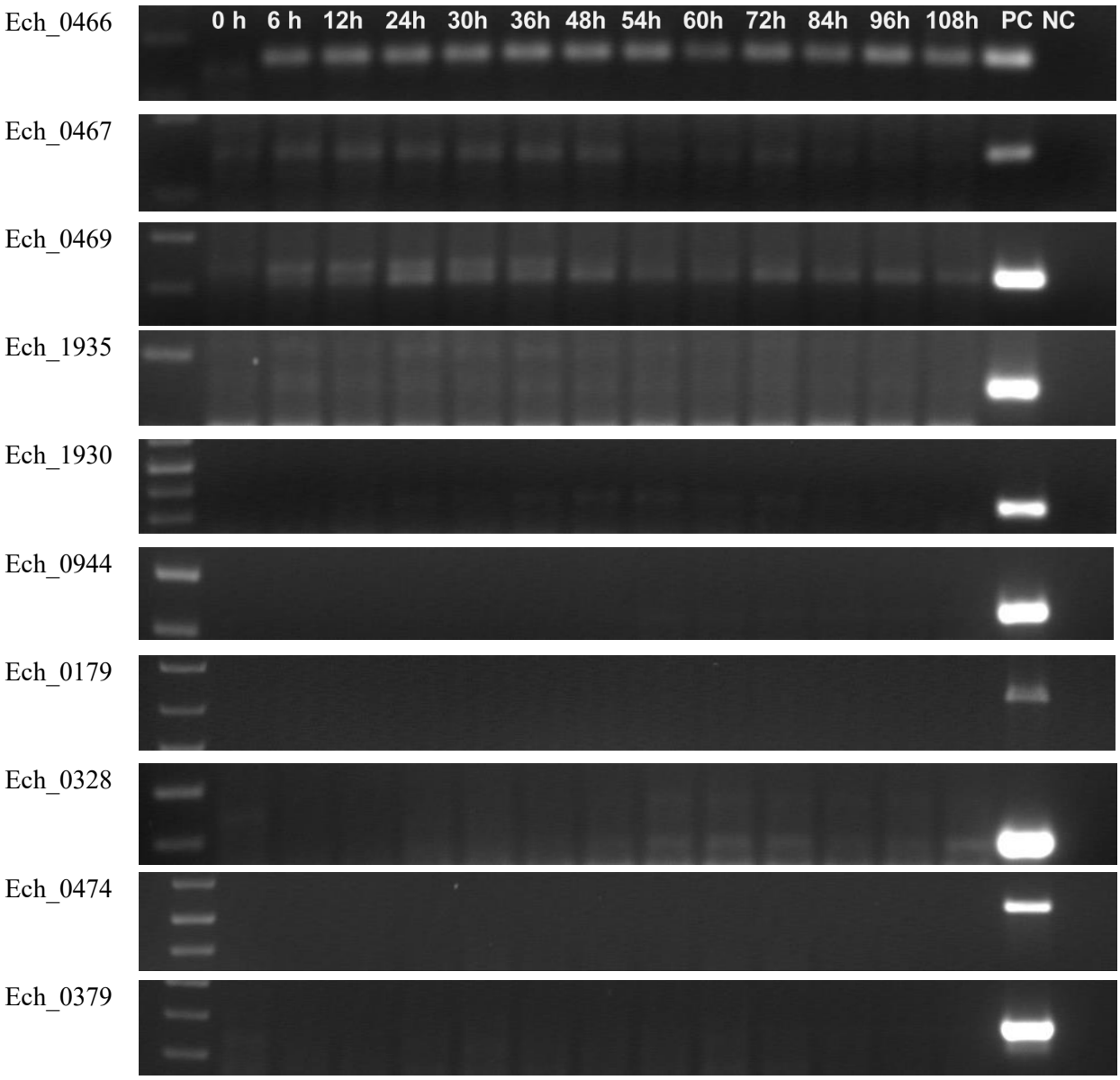


Figure 3.2 Messenger-RNA levels of the putative antiporter genes in *E. chaffeensis* during infection of tick cell line (ISE6)

The transcript levels were measured after the post infection time 0, 6, 12, 24, 30, 36, 48, 54, 60, 72, 84, 96, and 108 h. PC refers to the positive control used the *E. chaffeensis* genomic DNA as the template. NC refers to the negative control using the nuclease-free water as the template. Line one to line ten: 1-5: Ech_0466 (40 cycles), Ech_0467 (45 cycles), Ech_0469 (45 cycles), Ech_1935 (45 cycles), Ech_1930 (35 cycles); 6-10: Ech_0944 (45 cycles), Ech_0179 (45 cycles), Ech_0328 (45 cycles), Ech_0474 (45 cycles), Ech_0379 (45 cycles)

3.2 The expression of the recombinant plasmid in the host cell

Ehrlichia species, including *E. chaffeensis*, do not harbor plasmids, thus making it a challenge to study a gene function in these pathogenic bacteria in vivo. Furthermore, their dependence on a host cell for continued replication is an added limitation to investigate the function of *Ehrlichia* proteins. To overcome this limitation, we have been exploring the *E. coli*-based surrogate systems to study proteins and genes of *E. chaffeensis* [47, 51]. In the current study, we similarly used an *E. coli* system to identify the antiporter function of the putative antiporter genes. All ten putative antiporter gene open reading frames (ORFs) were grouped as six sub-groups based on their location in the genome (Figure 2.2). We considered the genes Ech_0466, Ech_0467, Ech_1930, Ech_1935, and Ech_0469 as one group because the entire region spans the five ORFs. The upstream promoter was also cloned into the plasmid vector, pBlueScript, and transformed into an antiporter gene-deficient *E. coli* strain, EP432 with two of its three antiporter genes inactivated [75]. The remaining five genes were also cloned similarly into the *E. coli* strain, EP432. RT-PCR analysis was performed on RNA recovered from all six-transformed EP432 *E. coli* culture. For each recombinant plasmid containing *E. coli*, four different amplifications were performed to confirm expression data only from RNA: RNA sample containing reverse transcriptase and Taq polymerase, one negative control with no RNA template added, a second negative control where reverse transcriptase was not included (a control for monitoring the presence of plasmid DNA contamination), and test positive control where *E. chaffeensis* genomic DNA was used in place of bacterial RNA to serve as the reaction positive control. The *E. chaffeensis* RNA expression from the predicted antiporter genes in *E. coli* was observed for all six recombinant plasmids, suggesting that the *E. coli* system supported the transcription of the pathogen genes using their respective native promoters (Figure 3.3).

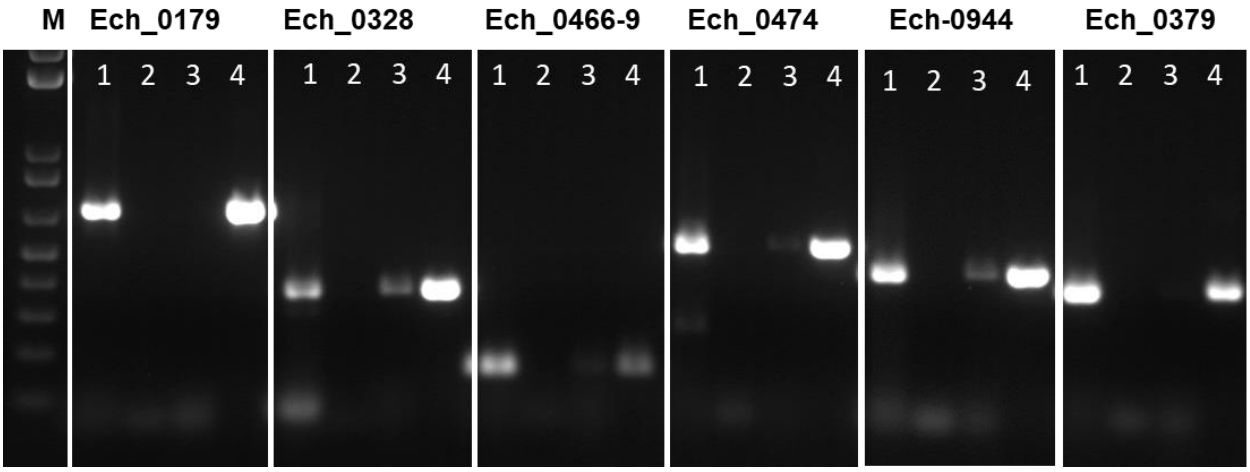


Figure 3.3 One-step RT-PCR on the expression of recombined plasmids

For each group, there were four sample sets. The first lane was the RNA sample with Reverse transcriptase and Taq polymerase. The second lane was nuclease-free water with reverse transcriptase and Taq polymerase, serving as the negative control. The third lane was the RNA sample with Taq polymerase, serving as negative control. This group was set to eliminate the false positive caused by the residual plasmid DNA. The fourth lane was the *E. chaffeensis* genomic DNA with Taq polymerase, serving as the positive control. For some groups, the lane three had some weaker band than lane one, due to the incomplete digestion of DNA.

3.3 Functional complementation assay

Antiporter activities of all six genes in *E. coli* were assessed by conducting the real-time monitoring of the growth of the transformed EP432 containing the individual recombinant plasmids. The *E. coli NhaA* gene (one of the two *E. coli* antiporter genes) was cloned along with its native promoter to serve as a positive control for the studies in the antiporter deficient *E. coli* strain. The expression of the antiporter gene *NhaA* resulted in the enhanced growth of the antiporter deficient *E. coli* strain in the media containing 200 mM NaCl at pH 5.5 compared to the non-recombinant plasmid containing *E. coli* (negative control). Gene expression in EP432 from Ech_0179, Ech_0328, and Ech_0944 genes under the same experimental conditions also resulted

in similar growth restorations as observed for the positive control. Functional complementation assays with Ech_0466-0469 also resulted in improved growth, but slightly less than that observed for the above listed three genes. Likewise, Ech_0474 and Ech_0379 gene expressions resulted in weaker growth restorations at 200 mM NaCl at pH 5.5. We then investigated the impact of gene expression from the six plasmids at neutral pH and basic pH conditions (pH 7.0 and pH 8.0, respectively). The *E. coli* growth restoration is the greatest with the Ech_0179 gene at both neutral and basic pHs, similar to acidic pH. Ech_0379 gene expression complemented the greatest at pH 8.0 with no major impact at neutral pH. *E. coli NhaA* gene expression similarly restored the growth at all three pHs. On the contrary, expression from the remaining four recombinant plasmids having the *E. chaffeensis* antiporter genes at neutral and basic pHs resulted in the negative outcome, i.e., their expression resulted in the greater inhibition of the growth of the antiporter mutant *E. coli* EP 432.

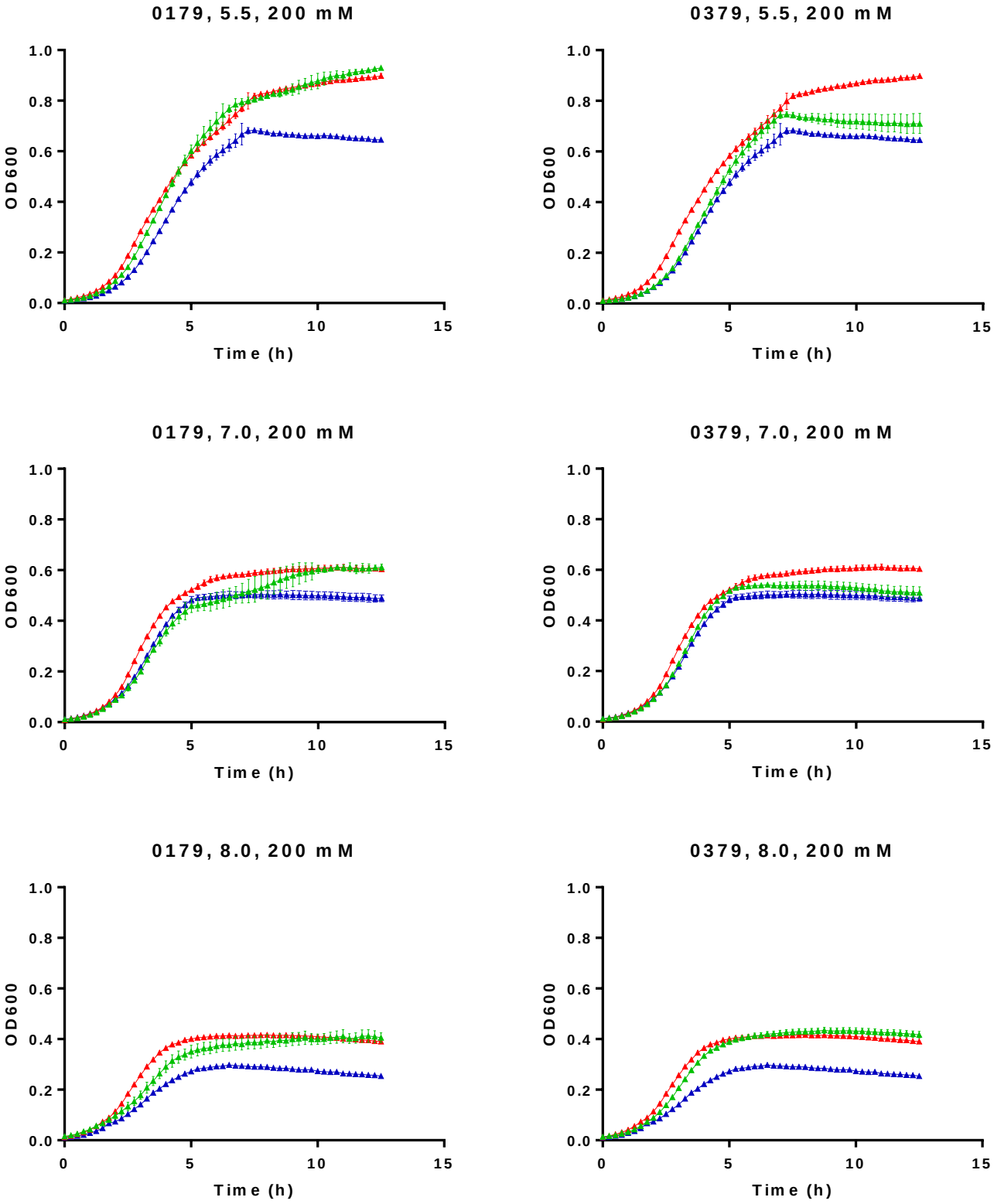

0179, 5.5, 200 mM
0379, 5.5, 200 mM
0179, 7.0, 200 mM
0379, 7.0, 200 mM
0179, 8.0, 200 mM
0379, 8.0, 200 mM
OD600
Time (h)
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15

Figure 3.4 a

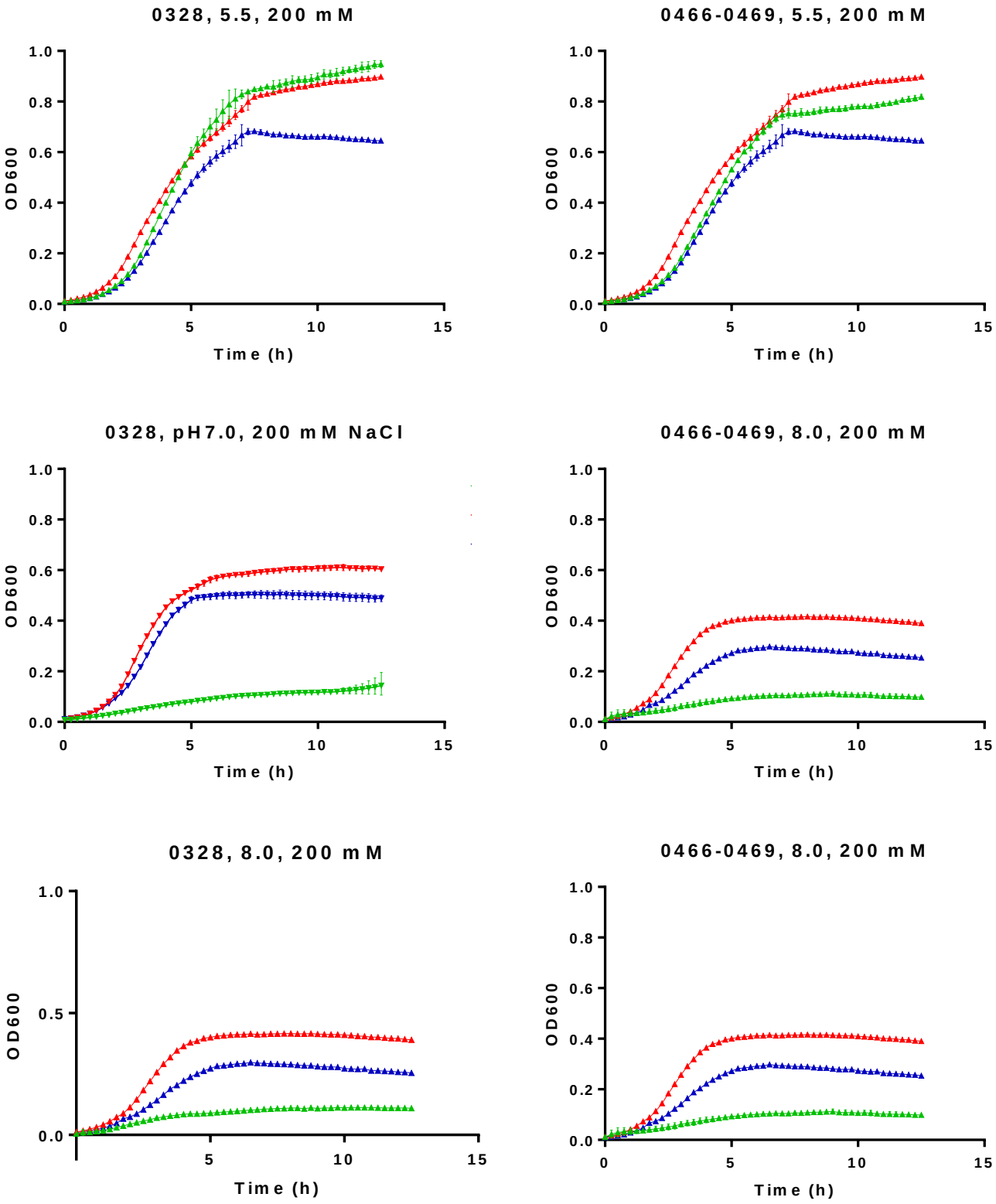

0328, 5.5, 200 mM
0466-0469, 5.5, 200 mM
0328, pH7.0, 200 mM NaCl
0466-0469, 8.0, 200 mM
0328, 8.0, 200 mM
0466-0469, 8.0, 200 mM
OD600
Time (h)

Figure 3.4 b

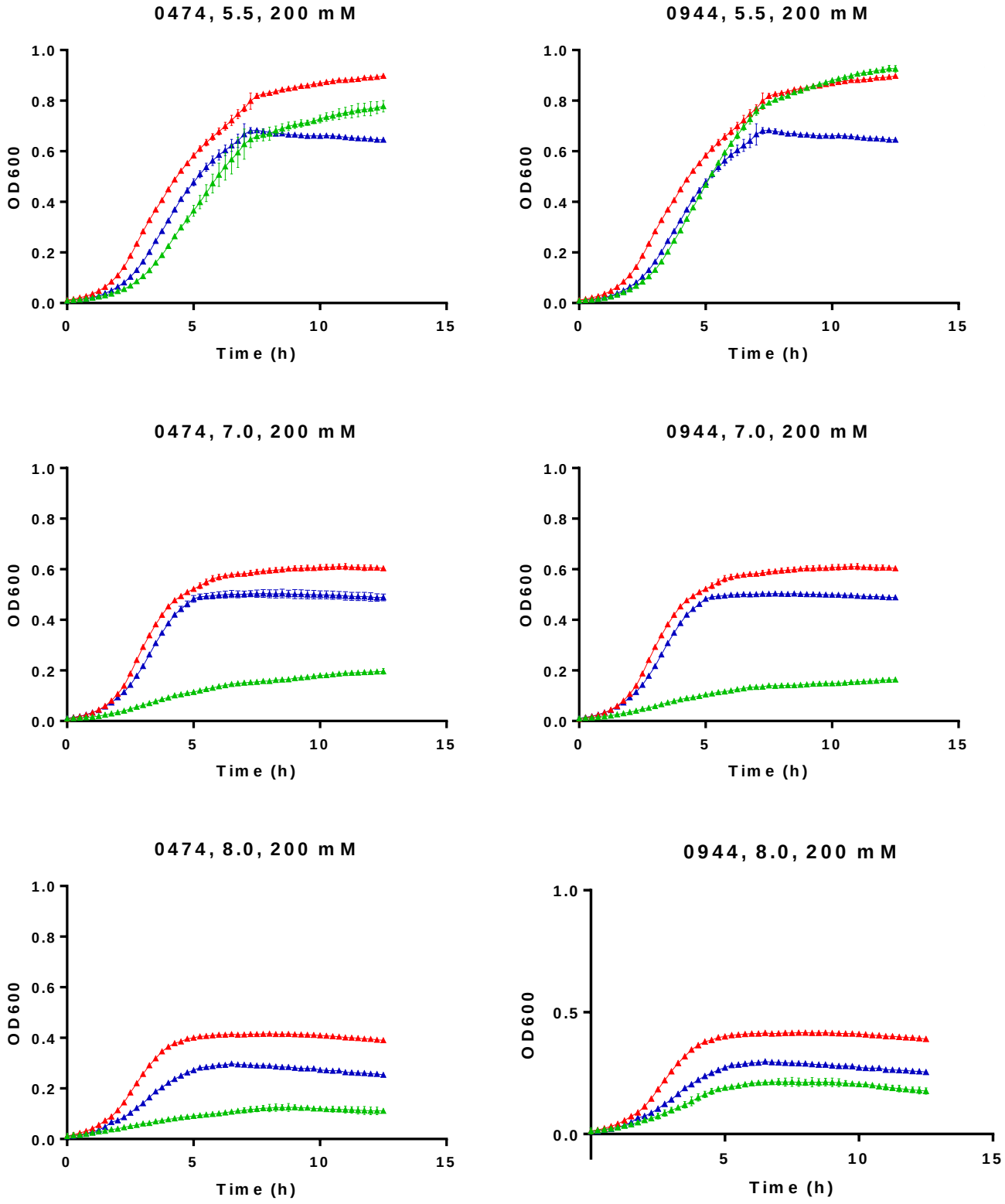

0474, 5.5, 200 mM
0944, 5.5, 200 mM
0474, 7.0, 200 mM
0944, 7.0, 200 mM
0474, 8.0, 200 mM
0944, 8.0, 200 mM
OD600
Time (h)
0
5
10
15
0.0
0.2
0.4
0.6
0.8
1.0
0.5

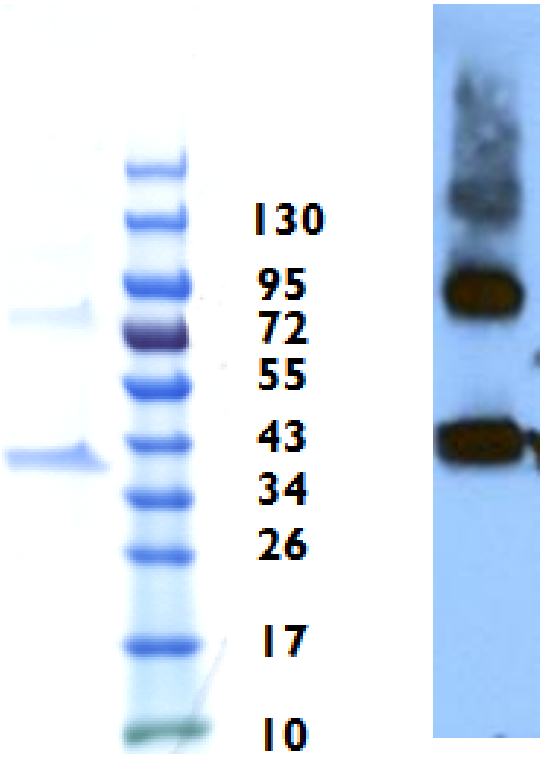
Figure 3.4 c

Figure 3.4 Expression of *E. chaffeensis* putative antiporter genes restored the growth of salt-sensitive Na^+/H^+ antiporter deficient *E. coli* strain.

The growth curves were measured by monitoring the OD_{600nm} each 15 min for up to 18 h. The salt-stress was induced by 200 mM NaCl in buffered LB media (pH5.5, pH7.0, or pH8.0). EP432 transformed with pBSK or pBSK-*NhaA* served as negative or positive control respectively. The red line (—) indicates the positive control, the blue line (—) indicated the negative control, and the green line (—) indicates the sample. (a) The growth curves in the presence of pBSK-0179 or pBSK-0379. (b) The growth curves in the presence of pBSK-0328 or pBSK-0944. (c) The growth curves in the presence of pBSK-0474 or pBSK-0466-0469.

3.4 The swelling assay

Ech_0379 protein was recombinantly expressed and purified (Figure 3.5). The recombinant protein appeared on a polyacrylamide gel at two different locations with the calculated molecular masses of 40 kDa and 80 kDa. Based on the amino acid sequence, the estimated molecular mass of the recombinant protein should have been 39 kDa. The presence of the second larger band with double size of the predicted molecular mass suggests that the protein also aggregated as a dimer. Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) analysis were performed to confirm that both the bands represented the same protein (not shown). We then used the purified recombinant Ech_0379 protein to construct a proteoliposome. The antiporter activity in the proteoliposome was evaluated by subjecting to swelling assay in the presence of 100 or 200 mM NaCl solution. The swelling assay was measured real time using a spectrophotometer to monitor the decrease in optical density at 400 nm (Figure 3.6). Liposome alone served as the negative control where we did not observe any change in OD_{400nm}. While the proteoliposome caused a continuous decline in the absorbance in the presence of NaCl solution measured at three different pH conditions (pH 5.5, pH 7.0, and pH 8.0), suggesting that the Ech_0379 recombinant protein facilitated the movement of salt into the liposome.



a b

Figure 3.5 Purified recombinant protein Ech_0379

(a) The purified recombinant protein was subjected to 12% SDS-PAGE analysis and stained with Coomassie Blue. (b) The western-blot analysis targeted on the N-terminal His-tag of the purified protein were performed after the SDS-PAGE.

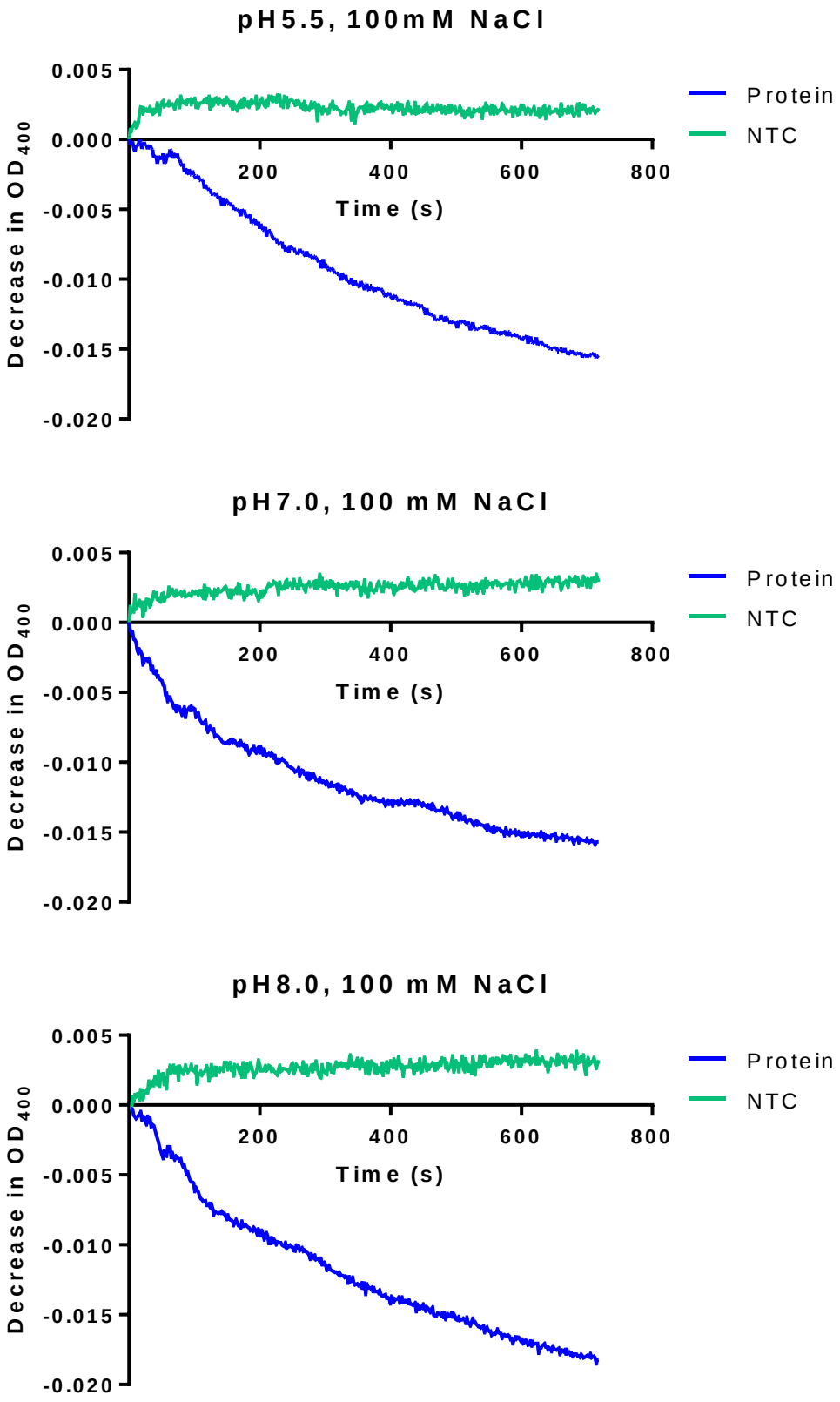

pH5.5, 100mM NaCl
Decrease in OD400
0.005
0.000
-0.005
-0.010
-0.015
-0.020
200
400
600
800
Time (s)
Protein
NTC
pH7.0, 100 mM NaCl
Decrease in OD400
0.005
0.000
-0.005
-0.010
-0.015
-0.020
200
400
600
800
Time (s)
Protein
NTC
pH8.0, 100 mM NaCl
Decrease in OD400
0.005
0.000
-0.005
-0.010
-0.015
-0.020
200
400
600
800
Time (s)
Protein
NTC

Figure 3.6 a

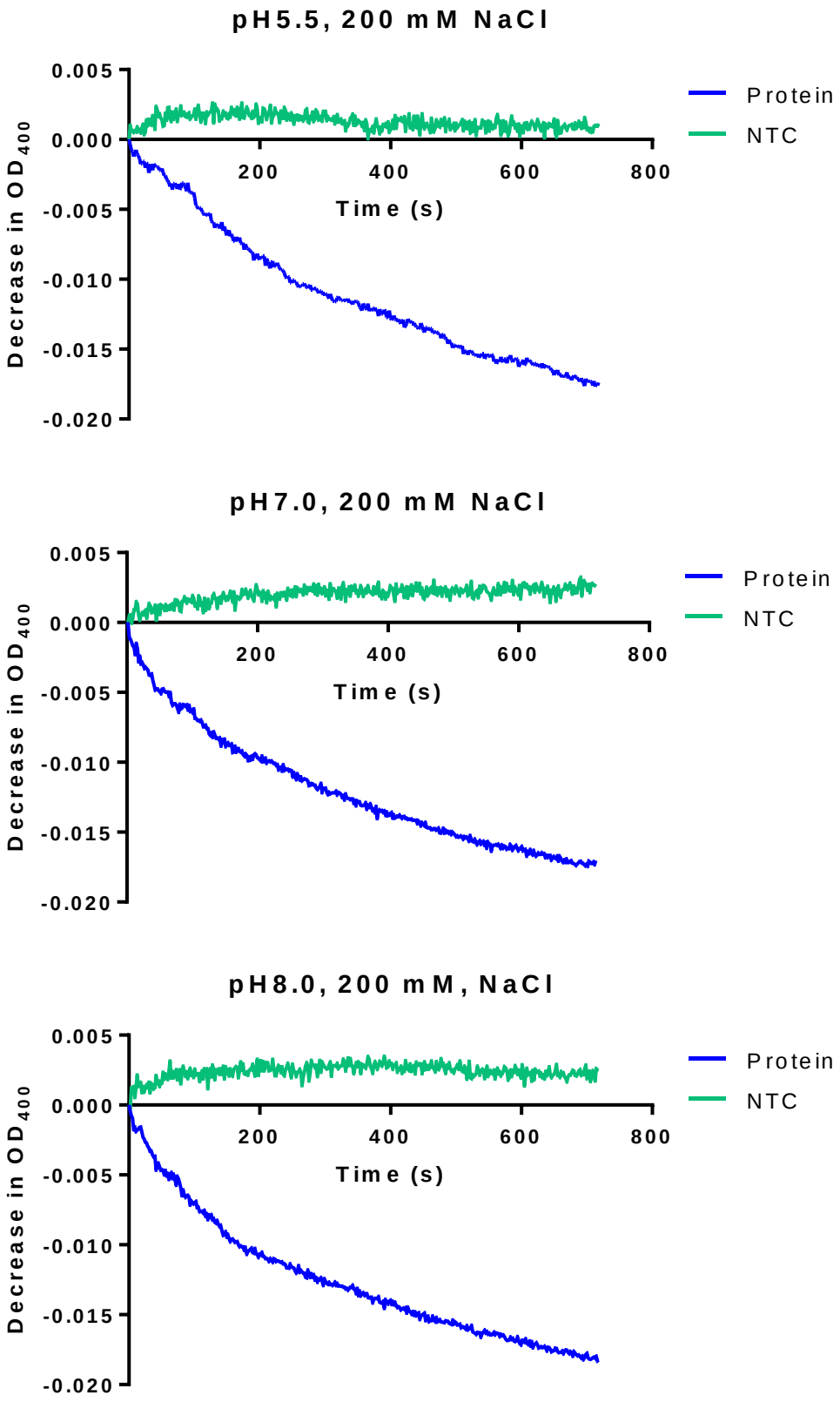

pH5.5, 200 mM NaCl
Decrease in OD400
0.005
0.000
-0.005
-0.010
-0.015
-0.020
200
400
600
800
Time (s)
Protein
NTC
pH7.0, 200 mM NaCl
Decrease in OD400
0.005
0.000
-0.005
-0.010
-0.015
-0.020
200
400
600
800
Time (s)
Protein
NTC
pH8.0, 200 mM, NaCl
Decrease in OD400
0.005
0.000
-0.005
-0.010
-0.015
-0.020
200
400
600
800
Time (s)
Protein
NTC

Figure 3.6 b

Figure 3.6 The porin activity of the *E. chaffeensis* Ech_0379 protein

The diffusion rates of the solutes into the proteoliposome were measured by monitoring the decrease in OD_{400nm} in the presence of different concentration of NaCl at three pHs.
(a) The OD_{400nm} decrease in the presence of 100 mM NaCl under pH5.5, pH7.0, and pH8.0 respectively. The blue line indicates the proteoliposome, and the green line indicates the negative control. (b) The OD_{400nm} decrease in the presence of 200 mM NaCl under pH5.5, pH7.0, and pH8.0 respectively. The blue line (—) indicates the proteoliposome, and the green line (—) indicates the negative control. Results showed the average of three independent experiments.

3.5 The secondary structure prediction

The amino acid sequences of the 10 putative antiporter proteins coding sequences of *E. chaffeensis* were aligned using the online multiple sequences alignment tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). For the secondary structure of the proteins, the online sequence analysis Smart (http://smart.embl-heidelberg.de/), or TMpred (https://www.ch.embnet.org/software/TMPRED_form.html) were used to predict the secondary structure and the transmembrane domain. The transmembrane domain prediction of the target protein showed that all the proteins contain transmembrane domains which varied from three to 15. To draw the prediction transmembrane domain, the software TMR Pre2D was used. For motif or subunit prediction, the NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) or the database KEGG (http://www.genome.jp/kegg/) was used. The targeted proteins were predicted to contain several motifs associated with the antiporter activity (Table 3.1) The putative antiporter proteins were predicted to be different subunits of the antiporter protein (Table 3.1). The transmembrane domain predictions were shown in the supplementary material Figure 5.1 to Figure 5.10.

Table 3.1 The predicted motif and subunit of the six group antiporter genes in *E. chaffeensis*

Subunit	Motif	Function	Identity (%)								
			179	328	379	466	467	469	474	944	1930& 1935
MrpD	pf:NADHdeh_related	NADH dehydrogenase I, subunit N related protein	32.08								
MrpD	pf:Proton_antipo_M	Proton-conducting membrane transporter	30.11	30.65					24.26		
MrpA	pf:Proton_antipo_N	NADH-Ubiquinone oxidoreductase (complex I), chain 5 N-terminus			24.49						
MrpF	pf:MrpF_PhaF	Multiple resistance and pH regulation protein F (MrpF / PhaF)				33.33					
MrpG	pf:PhaG_MnhG_YufB	Na+/H+ antiporter subunit					34.18				
MrpC	pf:Oxidored_q2	NADH-ubiquinone/plastoquinone oxidoreductase chain 4L						24.49			
MrpE	pf:MNHE	Na+/H+ ion antiporter subunit								23.39	
MrpB	pf:MNHB	NA									32.74

Chapter 4 - Conclusion and Discussion

RNA transcripts of the putative antiporter protein genes of *E. chaffeensis* were detected when cultured in the canine macrophage cell line, DH82, suggesting that they are functional genes and that the translated proteins of the antiporters may have a biological function during the development cycle. The expression levels determined by the semi-quantitative RT-PCR varied during different post-infection times in the DH82 cells, suggesting that the genes may also play differential function during the development of the bacterium. For the gene Ech_0379, the transcript level started to increase after around 72h post infection time, when the bacterium transformed from the replication form (RC) to the infectious form (DC). The DC release from the infected host cells by whole cell lysis or by exocytosis to continue the infectious cycle. Once released into a host cell free environment, DC form of the bacteria are at higher pH environment compared to RC form which resides in the acidic vacuole. Thus, the pH homeostasis must be managed differently by the bacterium. Ech_0179 and Ech_0379 may play functional role in acidic, neutral and basic pH conditions as our studies using surrogate system suggested that the proteins made from these genes support in relieving the NaCl sensitivity at all three pHs tested which ranged from acidic to basic. However, our *E. chaffeensis* RNA analysis from the cultured organisms suggested that the Ech_0179 expression is undetectable, while the Ech_0379 expression was variable with higher expression detected at later time points when it was closer to the transformation of RC to DC forms. Ech_0379 complemented the greatest at pH8.0 with minor impact at acidic pH and no major impact at neutral pH (Figure 3.4 a). These data suggest that the Ech_0379 protein may be critical for the bacterium during its growth within the phagosomes and after their release as DC. The lack of detectable RNA transcripts for the gene Ech_0179 in the time

course experiment may indicate that it may be expressed low under in vitro culture conditions and its expression in vivo remains to be determined. As this protein worked well in relieving the salt sensitivity at all three pH conditions for EP432 strain of *E. coli*, we predicted that its function might be critical for the organism in vivo. Together, Ech_0379 and Ech_0179 gene products may help the bacterium to maintain the pH homeostasis both within the phagosomal conditions and during its presence outside the host cell.

The levels of RNA transcripts for Ech_0466, Ech_0467, Ech_0469 genes were constant throughout the time course of infection starting from 6h to 108h post-infection, while the transcription level of the two genes Ech_1935, and Ech_1930 was variable. In the *E. coli* surrogate system, recombinant proteins made from these gene open reading frames functionally complemented the growth of the antiporter deficient *E. coli* in the acidic pH, while their expression at neutral pH had an adverse effect, thus making the EP432 more sensitive to NaCl. This group of genes may only help the bacterium within the phagosomes when *E. chaffeensis* exists in both RC and DC forms. Our studies are consistent with prior reports on antiporter proteins of other Gram-negative bacteria, where researchers reported that the Na^+/H^+ antiporters have characteristic individual pH-dependent activity profiles, critical for their homoeostatic functions [65, 66].

The expression patterns of some genes are also host cell-dependent. Though the gene expression patterns appeared similar for some genes; Ech_0466, Ech_0467, Ech_0469 and Ech_0328 as in DH82 culture, expression of the remaining six open reading frame transcripts in tick cell cultures were too low to be detected by the semi quantitate RT-PCR assay.

SDS-PAGE and the Western-blot analysis of the purified recombinant protein Ech_0379 revealed two resolved proteins having different molecular weights. The fast migration protein was about 40 kDa, that is similar to the estimated molecular weight (39 kDa) based on the weight

calculated from the primary amino acid sequence. The slow migration protein band was nearly 80 kDa, which is twice the size of the predicted molecular weight, suggesting that it represents a dimerized protein. Similar results were reported in *E. coli* [59], and *Salmonella* [39], previously. The purified recombinant protein Ech_0379 reconstituted in liposome with a lipid bilayer, showed the antiporter activities under the three different pHs tested and at two different NaCl concentrations. These data validate that the ech_0379 indeed has the antiporter function.

Our working hypothesis is that there are two groups of antiporter proteins in *E. chaffeensis* (Figure 4.1). When *E. chaffeensis* resides inside a host cell, where the pH value is acidic, expression of all proteins are critical in supporting the bacterial ion balance to regulate its internal pH, while its release out of a host cell where pH is likely to be close to 7.35 to 7.45 [45], Ech_0179, Ech_0379 may also aid in pH homeostasis of the organism.

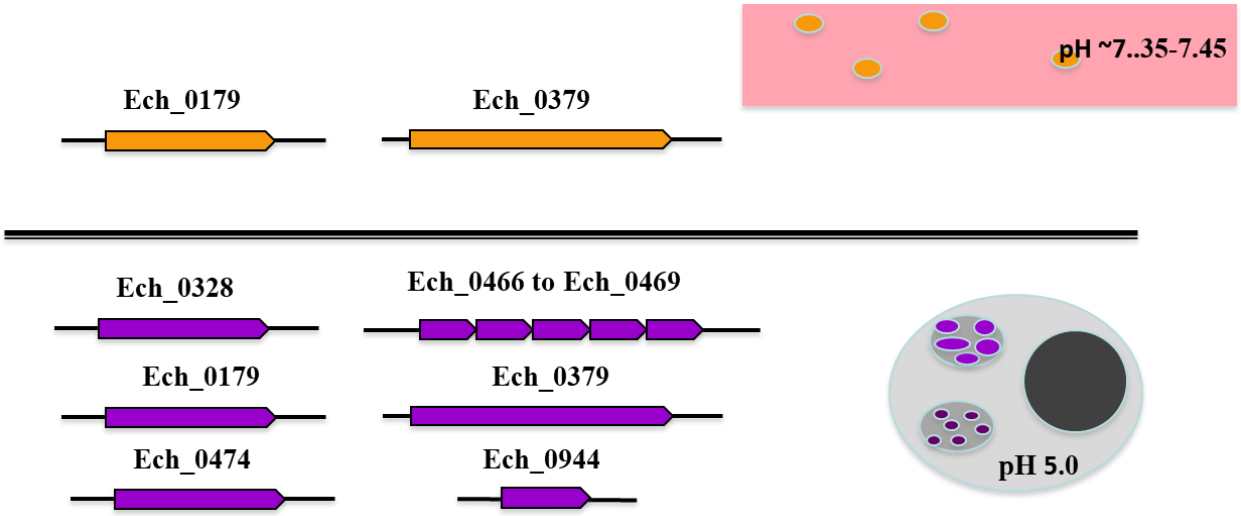


Figure 4.1 Predicted two group of antiporter protein in acidified phagosome and extracellular tissue

Chapter 5 - Supplementary Material

5.1 Transmembrane domain prediction

The online sequence analysis Smart (http://smart.embl-heidelberg.de/), were used to predict the secondary structure and the transmembrane domain https://www.ch.embnet.org/software/TMPRED_form.html). The software TMR Pre2D was used to draw the prediction transmembrane domain.

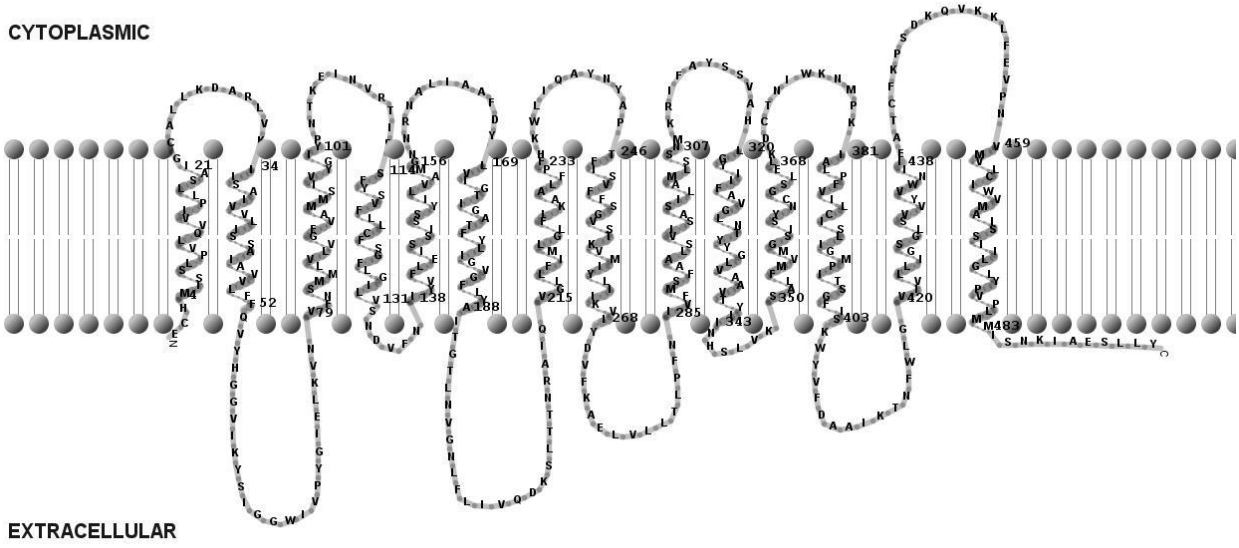


Figure 5.1 The predicted transmembrane domain of Ech_0179

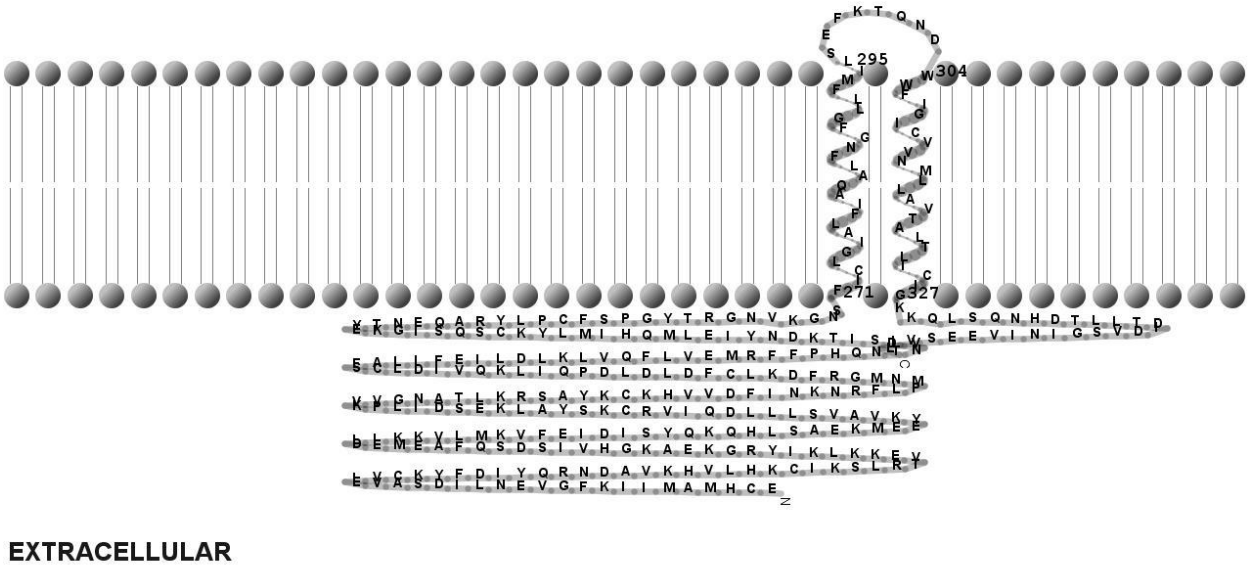


Figure 5.2 The predicted transmembrane domain of Ech_0379

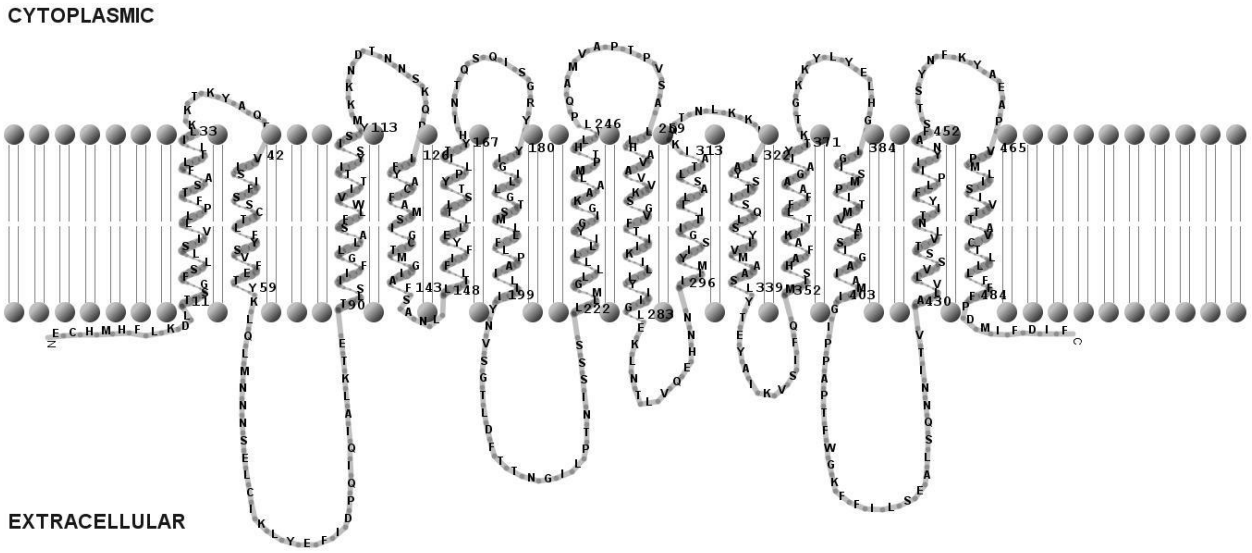


Figure 5.3 The predicted transmembrane domain of Ech_0328

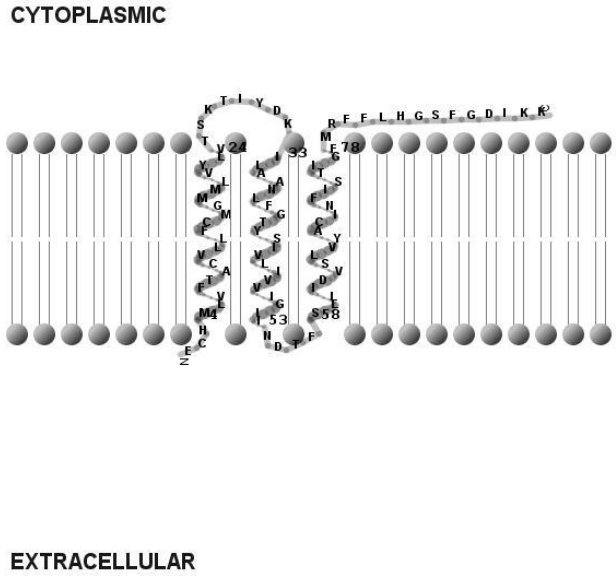


Figure 5.4 The predicted transmembrane domain of Ech_0466

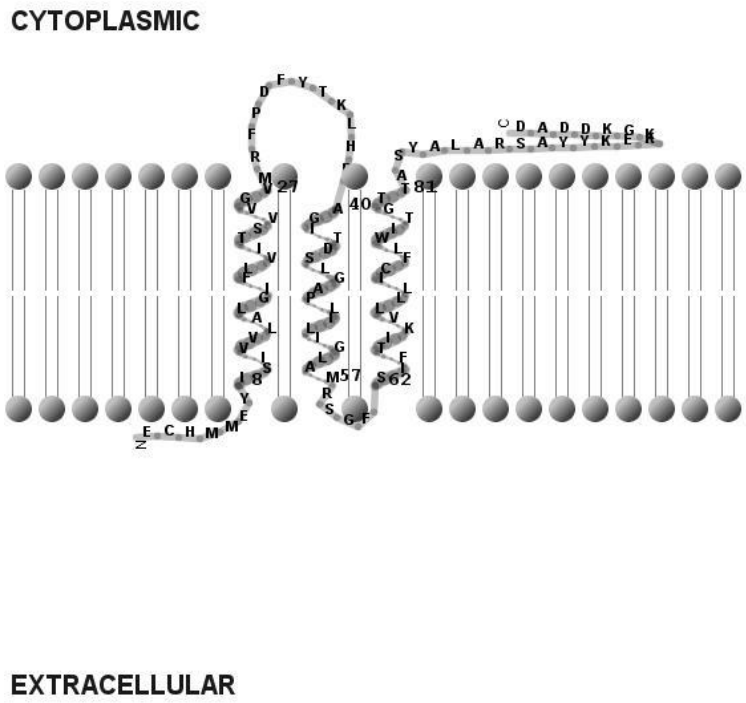


Figure 5.5 The predicted transmembrane domain of Ech_0467

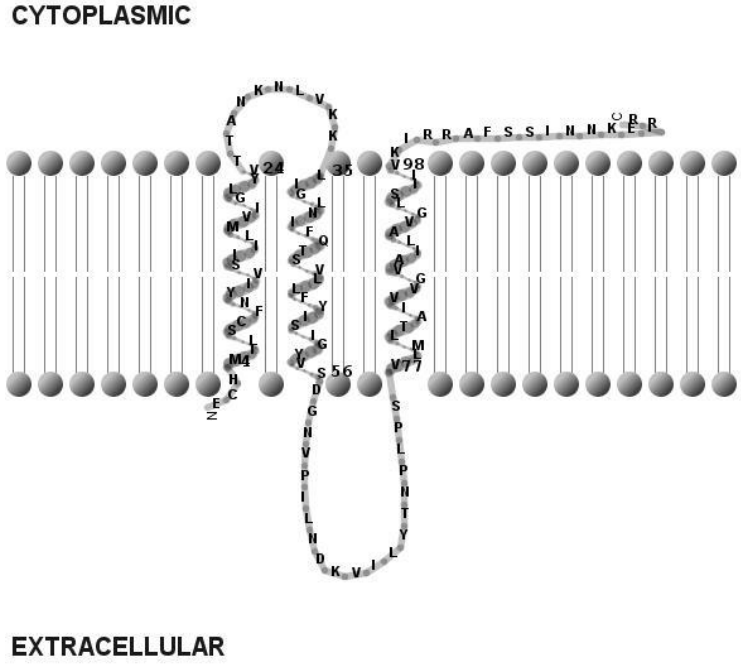


Figure 5.6 The predicted transmembrane domain of Ech_0469

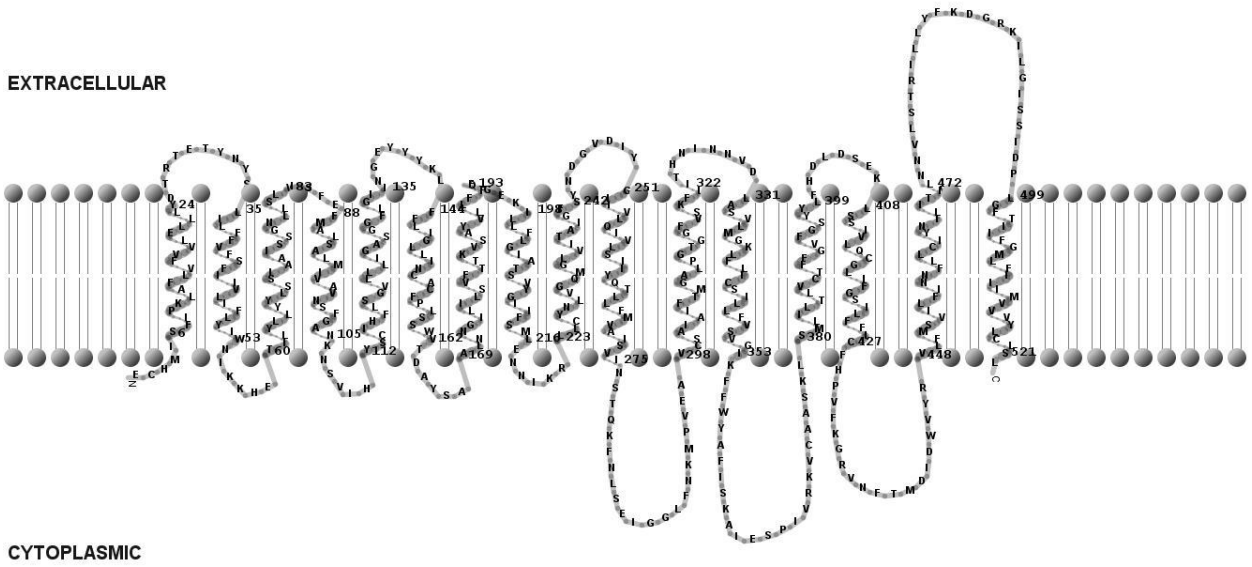


Figure 5.7 The predicted transmembrane domain of Ech_0474

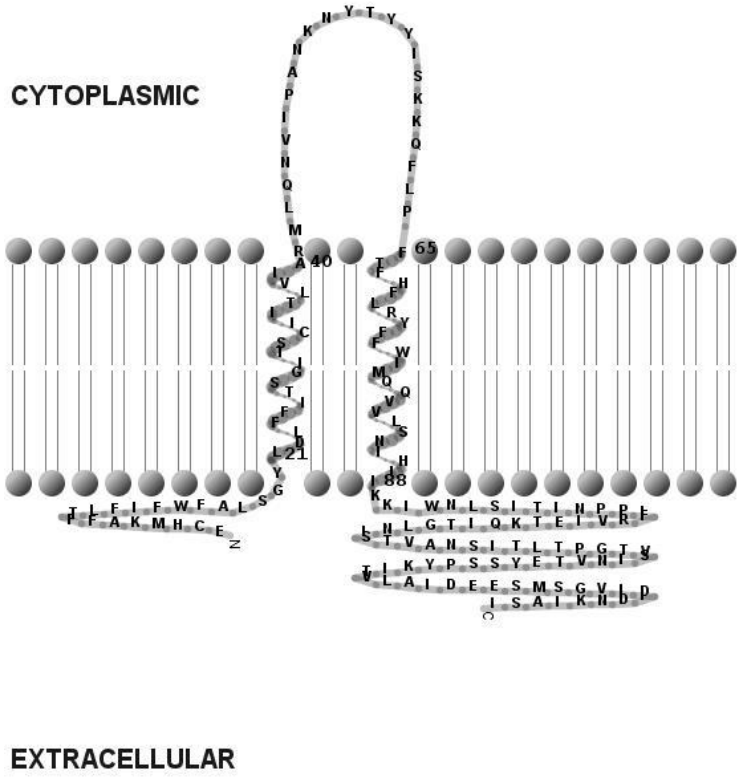


Figure 5.8 The predicted transmembrane domain of Ech_0944

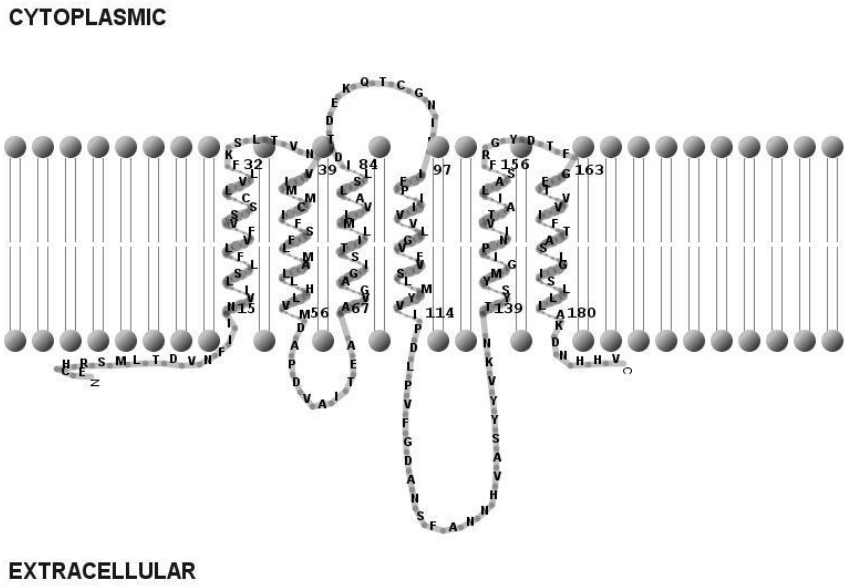


Figure 5.9 The predicted transmembrane domain of Ech_1930

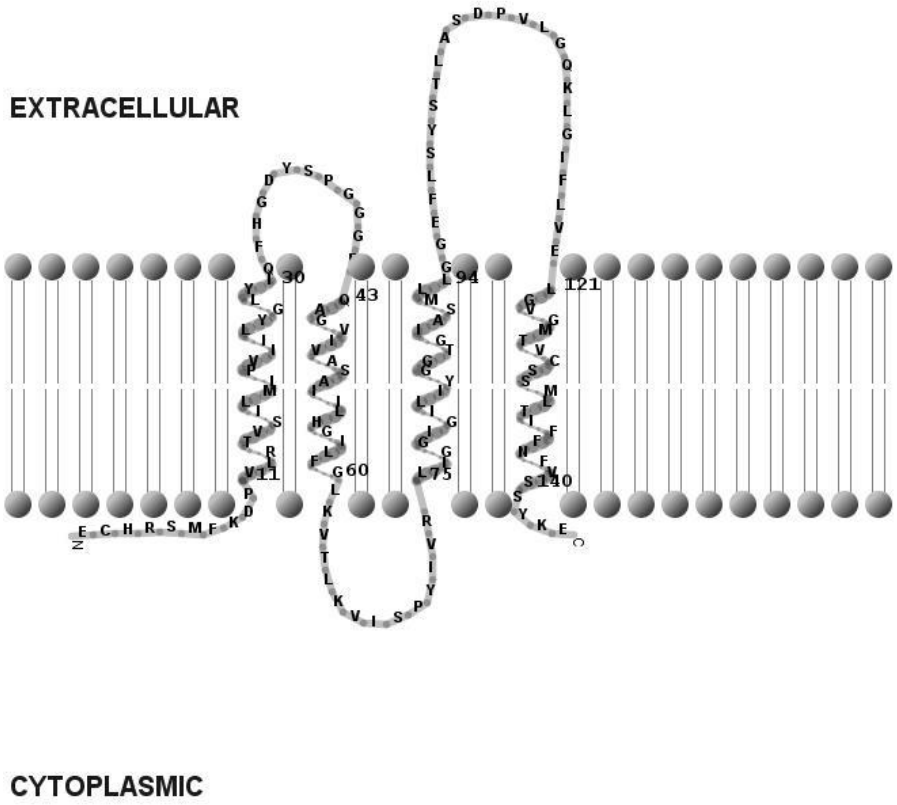


Figure 5.10 The predicted transmembrane domain of Ech_1935

Table 5.1 Reagents used for the protein purification

Lysis Buffer I	
Sodium phosphate pH 8.0	50 mM
lysozyme	0.5 mg/ml (added before used)
Lysis buffet II	
Sodium phosphate pH 8.0	50 mM
NaCl	2 M
Imidazole	10 mM
Glycerol	20% (v/v)
Triton-100	1% (v/v)
Washing buffer A	
Sodium phosphate pH 8.0	50 mM
Imidazole	60 mM
NaCl	600 mM
Glycerol	10% (v/v)
Washing buffer B	
Sodium phosphate pH 8.0	50 mM
Imidazole	80 mM
NaCl	600 mM
Glycerol	10% (v/v)
Elution buffer	
Sodium phosphate pH 8.0	50 mM
Imidazole	80 mM
NaCl	137 mM
Glycerol	10% (v/v)

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