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The AAX system from *Chlamydia pneumoniae*

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The AAX system from *Chlamydia pneumoniae*

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To Uncle Phil, Uncle Terry, and Grandma Smith

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The AAX system from *Chlamydia pneumoniae*

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Arginine uptake and degradation systems are common throughout bacteria and archaea. The genome of human pathogen *Chlamydia pneumoniae* encodes three proteins now called AaxA, AaxB, and AaxC which function together to take up arginine, decarboxylate it, and expel the decarboxylation product, agmatine. AaxB is the previously characterized pyruvoyl-dependent arginine decarboxylase, AaxC is an inner membrane amino acid transport protein that functions as an arginine-agmatine antiporter, and AaxA is an outer membrane porin, which facilitates the uptake of arginine and also functions as a general porin with broad specificity. *C. pneumoniae* is a non-typical Gram negative bacteria and an obligate intracellular parasite with a unique 2-phase life cycle. The role of this system for arginine-agmatine exchange has yet to be determined but it may function to deplete host cell arginine as a means of inactivating host inducible nitric oxide synthase (iNOS), a molecule used in the innate immune response that has been

shown to have an inhibitory affect on the growth of *C. pneumoniae* in cell culture. AaxB and AaxC are able to complement the loss of extreme acid-resistance in *E. coli* mutants that lack their own system for arginine-agmatine exchange, making pH homeostasis another possible role for this system. The porin AaxA is able to enhance arginine-agmatine exchange by AaxB and AaxC in *E. coli* mutants as well as by the native arginine decarboxylase AdiA and the native arginine-agmatine antiporter AdiC in wild type *E. coli*. AaxA is not an arginine-specific porin and instead acts as a general porin with a broad specificity. AaxA discriminates only against large and negatively charged solute molecules, and therefore it may have a broad role in the uptake of various biomolecules essential for chlamydial growth in addition to its role as part of a system for arginine-agmatine exchange.

Table of Contents

List of Tables……...x
List of Figures……xi

CHAPTER 1 **1**

Introduction……..1
1.1. The Chlamydial life cycle……3
1.2. Chlamydial Disease……..…5
1.3. Arginine uptake and degradation……....…7
1.4. Bacterial porins……9
1.5. Arginine agmatine exchange…….....11
1.6. The Chlamydial arginine decarboxylase……...…12
1.7. *C. pneumoniae* and nitric oxide…….13

CHAPTER 2 **15**

Arginine uptake by AaxC……..15
2.1. Overview……15
2.2. Experimental procedures……...……18
2.2.1. Strains and DNA……18
2.2.2. Cloning……...……19
2.2.3. Protein Expression…….21
2.2.4. Membrane fractionation…….22
2.2.5. Western blotting…….22
2.2.6. Cellular transport assays……23
2.2.7. Arginine-dependent acid resistance assay…….24
2.3. Results……..…….25
2.3.1. Expression of AaxC and localization to the cytoplasmic membrane……...…25
2.3.2. Arginine uptake by AaxC in whole cells……27
2.3.3. Inhibition of arginine uptake by AaxC……...30
2.3.4. Complementation of acid resistance with AaxBC……..30
2.4. Discussion……...33

CHAPTER 3 **36**

AaxBC and AaxABC as a system for arginine uptake and decarboxylation……36
3.1. Overview……...……36
3.2. Experimental procedures……...……39
3.2.1. Cloning……..39
3.2.2. Product analysis……40
3.2.3. Arginine uptake and decarboxylation by whole cells……....41

3.2.4. Cell free decarboxylase assays……..….42
3.2.5. Inhibition of AaxBC in whole cells and B in lysates…….42
3.3. Results…...…….43
3.3.1. HPLC analysis……...43
3.3.2. Uptake and decarboxylation with AaxABC……..50
3.3.3. Comparison with lysates…….……..53
3.3.4. Inhibitors……..…….57
3.4. Discussion…….59

CHAPTER 4 **64**

The AaxA porin…….……64
4.1. Overview……...…64
4.2. Experimental procedures……...……66
4.2.1. Expression, refolding, purification of AaxA…….66
4.2.2. Arginine uptake and decarboxylation assays with whole cells….67
4.2.3. Liposome swelling assays…….67
4.3. Results……...69
4.3.1. Expression and localization to the outer membrane……...69
4.3.2. AaxA enhances uptake, DC activity by AdiAC……72
4.3.3. Oxidizing vs. reducing conditions…….76
4.3.4. Refolding and purification of AaxA……..78
4.3.5. Swelling assays using arginine as a substrate……80
4.3.6. Sugar substrates and size dependence……..…..87
4.3.7. Other amino acids and charge dependence……89
4.3.8. Polyamines……...……..89
4.3.9. Liposomes made with outer membranes……90
4.4. Discussion…...……...93

CHAPTER 5 **97**
Summary and future directions……..97
5.1. Summary……97
5.2. Future studies……...101

Appendix: List of microorganisms and plasmids……....104
Bibliography……...….105
Vita……..112

List of Tables

Table 4.1. Liposome swelling assays using refolded AaxA.……..……91

Table 4.2. Liposome swelling assays using outer membranes.……..…92

List of Figures

Figure 1.1. Aax proteins comprise a system for arginine-agmatine exchange……3

Figure 2.1. AaxC-HSV localizes to the cytoplasmic membrane……..……..27

Figure 2.2. Arginine uptake by AaxC……29

Figure 2.3. Complementation of acid resistance in *E. coli* using *aax* genes……..32

Figure 3.1. Reaction product analysis by HPLC……...……45

Figure 3.2. Conversion of arginine to agmatine in wild type *E. coli*……46

Figure 3.3. Conversion of arginine to agmatine is not detected in the DEG0147 negative control……47

Figure 3.4. AaxB and AaxC can convert arginine to agmatine in *E. coli* at pH 2.5……..48

Figure 3.5. Conversion of arginine to agmatine by AaxB and AaxC is enhanced at pH 5.0……..…….……..49

Figure 3.6. Arginine decarboxylase activity assays using whole cells…….……50

Figure 3.7. Arginine decarboxylase activity assays using cell lysates…….….55

Figure 3.8. Arginine uptake and decarboxylation by AaxBC is not affected by changes in buffer composition…….…56

Figure 3.9. L-canavanine and D-arginine inhibit arginine uptake and decarboxylation by AaxBC……....58

Figure 4.1. AaxA-HSV is localized to the outer membrane…….71

Figure 4.2. AaxA enhances whole cell decarboxylase activity in a pH-dependent fashion……...….73

Figure 4.3. AaxA and MOMP enhance whole cell decarboxylase activity……..75

Figure 4.4. AaxA porin activity is not significantly inhibited under oxidizing conditions……...……77

Figure 4.5. Expression and purification of AaxA-HSV……....79

Figure 4.6. Arginine uptake increases with increasing addition of AaxA porin……...82

Figure 4.7. Arginine is taken up by AaxA in a pH-dependent manner……...….83

Figure 4.8. Inhibition of arginine uptake using monoclonal antibodies with liposomes made with total protein refolded from inclusion bodies……85

Figure 4.9. Inhibition of arginine uptake using monoclonal antibodies with liposomes made with purified AaxA……...86

Figure 4.10. AaxA allows the uptake of sugars in a size-dependent manner…….88

CHAPTER 1

Introduction

AaxA, AaxB, and AaxC from *Chlamydia pneumoniae* comprise a system for arginine uptake and decarboxylation as well as agmatine export. AaxA is an outer membrane porin that allows arginine and agmatine, as well as other solutes, to diffuse freely across the outer membrane. AaxC is an arginine-agmatine antiporter that transports arginine into the cell across the cytoplasmic membrane while transporting agmatine, the decarboxylation product of arginine, out of the cell. AaxB is the arginine decarboxylase that converts arginine to agmatine (Figure 1.1). There are many possible roles for such a system in bacteria but its role in *C. pneumoniae* remains to be proved. One possibility that will be considered in this dissertation is that the Aax system consumes arginine for the purpose of inhibiting host nitric oxide synthesis as a means of resisting the innate immune system.

The work on this system began with the *C. pneumoniae* protein now called AaxB, a pyruvoyl-dependent arginine decarboxylase encoded by the gene Cpn1032 (Giles and Graham, 2007). This gene is flanked upstream by Cpn1033 and downstream by Cpn1031. These genes were predicted to encode an outer membrane porin and a cytoplasmic membrane transporter protein, respectively. It was hypothesized that this transporter could be an arginine-agmatine antiporter. The pairing of an arginine

decarboxylase with an arginine-agmatine antiporter is found in enteric bacteria although no such system was known in chlamydial species. The putative outer membrane porin has no homolog in enteric bacteria but it was hypothesized that it could facilitate arginine uptake into the cell.

The focus of this dissertation is the characterization of the protein encoded by Cpn1031, now known to be the arginine-agmatine antiporter AaxC, and the protein encoded by Cpn1033, now known to be the general porin AaxA. This introduction will provide an overview of arginine uptake and degradation systems in other bacteria, bacterial porins, arginine-agmatine exchange in bacteria, the arginine decarboxylase from *C. pneumoniae*, the chlamydial life cycle and the organism's role in human disease, as well as *C. pneumoniae's* relationship with nitric oxide during infection.

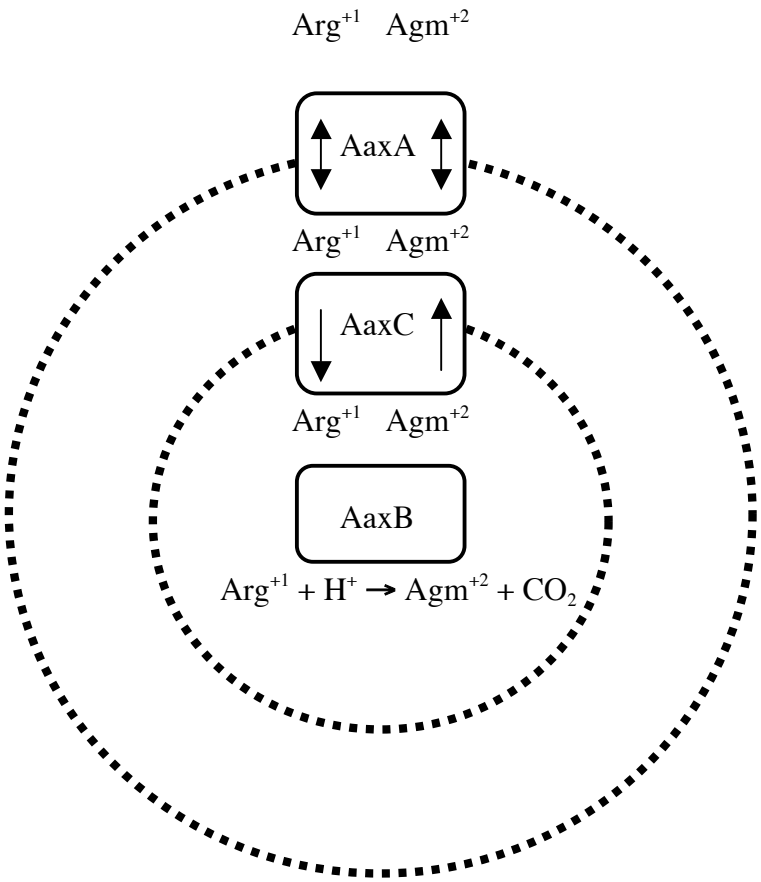

Figure 1.1. Aax proteins comprise a system for arginine-agmatine exchange.

The outer membrane porin AaxA allows both arginine and agmatine to diffuse freely across the outer membrane. AaxC exchanges extracellular arginine for intracellular agmatine across the cytoplasmic membrane. AaxB is the arginine decarboxylase, which converts arginine to agmatine.

1.1. THE CHLAMYDIAL LIFE CYCLE

Chlamydiae are aerobic Gram-negative bacterial pathogens that exist as obligate intracellular parasites with a unique two phase life cycle. The infectious form is the elementary body (EB), which enters the host cells through receptor-mediated endocytosis, pinocytosis, or phagocytosis (Wyrick, 2000). In the first 4 hours after entry

the host cytoskeleton is rearranged to aid in redistribution of EB-containing vacuoles to the perinuclear region. The vacuoles can fuse with each other but they do not fuse with lysosomes. Homotypic endosomal fusion, in which multiple chlamydial inclusions are able to fuse together, occurs only in *C. trachomatis* and provides an opportunity for genetic exchange not found in other species which present in multiple inclusions per infected cell, consistent with multiplicity of infection (Wyrick, 2000).

EBs (0.25-0.30 μm) are considered metabolically inert and they must differentiate into the non-infectious but metabolically active reticulate bodies (RBs) (0.8-1.0 μm) to reproduce. RBs, unlike the smaller and hardier EBs, are osmotically unstable and will lyse outside of the host cell. Logarithmic growth begins roughly 6 hours post infection and continues until 24 to 40 hours post infection, depending on the species, with a generation time of about 2.5 hours. RBs grow entirely within the inclusion vacuole created by the EBs' entry into the host cell and this vacuole expands as the RBs grow in number, intercepting vesicles released from the trans-golgi. Active RBs group around the periphery of the growing inclusion vacuole, presumably to take nutrients from the host, but as they fully mature and differentiate back into EBs, which are not metabolically active, they cluster in the center. RBs that adhere to the inclusion membrane may draw their nutrients from the cytoplasm using hollow structural appendages called projections, which originate in the cytoplasmic membrane and extend about 30 nm from the chlamydial surface. These projections may also be involved in secreting molecules into the host cell cytoplasm to modulate host-parasite interactions (Wyrick, 2000).

After differentiating back to EBs these infectious bodies must leave the protective inclusion vacuole to escape and infect other cells. Exit may occur by lysis or vacuolar fusion with the cytoplasmic membrane and results in the cycle of infection, differentiation, and growth starting over again from the beginning. Any cells that have not successfully differentiated back into EBs will lyse outside the host cell.

Chlamydia can only reproduce by invading the host cell, partially because of the osmotically unstable nature of their vegetative form and partially because of their fastidious nutrient requirements. Chlamydiae encode enzymes for the glycolytic pathway, the pentose phosphate pathway, the Embden-Meyerhof-Parnas pathway, and a partial TCA cycle as well as components of a functional electron transport chain (Wyrick, 2000). Chlamydia must take many nutrients from its host including glucose-6-phosphate, 2-oxoglutarate, succinate, oxaloacetate, amino acids, nucleotides, and other essential molecules that chlamydial species cannot produce themselves (McClarty, 1999).

1.2. CHLAMYDIAL DISSEASE

Chlamydial bacteria are responsible for a wide variety of diseases in humans including conjunctivitis, cervicitis, urethritis, and pneumonia. *C. trachomatis* causes genital infection as well as trachoma, which can lead to blindness. *C. pneumoniae* causes 10% of pneumonia cases and 5% of bronchitis and sinusitis cases annually (Kuo et al. 1995). Roughly 50% of the population presents detectable levels of antibody against *C.*

pneumoniae by age 20 and about 75% of the elderly population show detectable levels of antibody (Kuo et al., 1995). Although most cases of chlamydial infection can be resolved with antibiotics such as azithromycin or doxycycline, asymptomatic infection can make diagnosis difficult and *C. pneumoniae* and *C. trachomatis* can both persist to cause chronic infections that are resistant to standard treatments (Turner et al., 2002, Workowski et al. 2006). There is currently no effective vaccine against chlamydia.

Illness caused by *C. pneumoniae* most frequently manifests as bronchitis or pneumonia but asymptomatic or mildly symptomatic infections are most common (Kuo et al., 1995). Pneumonia caused by *C. pneumoniae* is usually mild, not requiring hospitalization, but recovery is often very slow with symptoms lingering for weeks. Older patients tend to suffer more severe symptoms than young adults and in cases of underlying illness and complications such as pneumococcal bacteremia fatalities can result. Severe systemic infections can occur, although they are uncommon. Other diseases associated with *C. pneumoniae* include sinusitis, otitis media, pharyngitis, endocarditis, and lumbosacral meningoradiculitis. *C. pneumoniae* has also been shown to have a roll in the development or exacerbation of asthma (Kuo et al., 1995, Hahn et al., 2006).

1.3. ARGININE UPTAKE AND DEGRADATION

Arginine uptake and degradation systems are common in many bacteria and archaea, and they are used for a variety of purposes ranging from ATP formation to defense against the innate immune system. *Pseudomonas aeruginosa* uses the membrane protein ArcD to import arginine, which is converted to ornithine by the arginine deiminase pathway (Verhoogt et al., 1992). The degradation of arginine is carried out in this case by the arginine deiminase ArcA, which catalyzes the conversion of arginine to citrulline and ammonia, the ornithine carbamoyl transferase ArcB, which produces ornithine and carbamoyl phosphate, and the carbamate kinase ArcC that converts ADP to ATP and produces CO_2 and ammonia. ArcD exports the ornithine produced by this process. Chlamydiae have no homolog for the arginine deiminase gene and there is no evidence that they are able to ferment arginine.

Many bacteria and archaea use arginine decarboxylases to produce agmatine, which is then converted by agmatine ureohydrolase to putrescine (Giles and Graham, 2008, Graham et al., 2002). Putrescine is the core polyamine used in the synthesis of spermidine and spermine. Polyamines are important regulators of cell growth and differentiation as well as cell death and they are ubiquitous in bacteria, archaea, and eukaryotes. However, the agmatine ureohydrolase enzyme is lacking in chlamydia species, ruling out this pathway for arginine catabolism as well.

Arginine decarboxylases need not be tied to polyamine biosynthesis; in many enteric bacteria, including *Escherichia coli* and *Salmonella enterica*, a pyridoxal 5'-phosphate (PLP) dependent arginine decarboxylase enzyme (AdiA) is coupled with an arginine-agmatine antiporter (AdiC) as part of a system for acid resistance and survival (Gong et al., 2003, Iyer et al., 2003, Fang et al., 2006, Kieboom and Abee, 2006). This system acts as a virtual proton pump and serves to raise the cytoplasmic pH while inverting the cell's membrane potential, creating an internal positive charge to repel protons and enhancing the rate of survival for these bacteria in the low pH environment of the stomach before they are able to colonize the gut (Richard and Foster, 2004, Fang et al., 2009). The chlamydial pyruvoyl-dependent arginine decarboxylase AaxB together with the arginine agmatine Antiporter AaxC could serve in a similar fashion, but there is no evidence of acidification during chlamydial infections, making this role unlikely (Fields and Hackstadt, 2002).

Arginine is converted to nitric oxide (NO) and citrulline by inducible nitric oxide synthase (iNOS) in macrophages. NO is an effective antimicrobial agent and is an important component of innate immunity. *Helicobacter pylori* uses an arginase (RocF) to hydrolyze arginine to urea and ornithine, a reaction that has been shown to consume host cell arginine and reduce the amount of NO released by macrophages (Gobert et al., 2001). *Chlamydia pneumoniae* infects granulocytes and alveolar macrophages in early stage acute respiratory infections, both of which use arginine to produce nitric oxide,

suggesting that *C. pneumoniae*, like *H. pylori*, may use its system for arginine uptake and degradation to protect itself from NO-mediated killing (Giles and Graham, 2009).

1.4. BACTERIAL PORINS

Outer membranes in Gram-negative bacteria are composed of an outer leaflet of lipopolysaccharide (LPS) and an inner leaflet of conventional lipids like phosphatidylethanolamine (Jap and Walian, 1996). The outer membrane is made permeable to hydrophilic solutes by the presence of channel-forming proteins called porins. The transport of solute through these porins is driven by diffusion and does not require energy from the cell. The number and type of porins in the outer membrane determine the permeability of the membrane to different kinds of solutes and can be adjusted in response to changes in the bacterial cell's environment. A porin's solute preference is determined by the size and geometry of the pore as well as the distribution of charged, polar, and aromatic amino acids. Additionally, some porins contain binding sites for specific substrates that give the porin greater specificity. It is the presence or absence of these binding sites that differentiates specific porins from general porins.

Porins are composed primarily of cylinders of β-sheets and are often made up of 3 monomers, each serving as a distinct channel. *E. coli* OmpA and *P. aeruginosa* OprF are examples of porins, which function in the membrane as monomers. Usually the porin cylinders are made up of 16 β-strands of varying lengths, although LamB from *E. coli* has 18 β-strands per cylinder. Overall topology as well as the general distribution of

hydrophobic and hydrophilic residues in bacterial porins is very similar from species to species even though sequence similarity is generally very low. The central region is made up of a belt of nonpolar residues about 25 Ångstroms in height with a ring of aromatic amino acids at the top and at the bottom positioned so that their polar portions interact with the polar headgroups of the lipids making up the membrane bilayer while the aromatic rings interact with the hydrophobic core (Jap and Walian, 1996). Inside the porin channel itself negatively charged residues are positioned across from positively charged residues in a pattern that rotates along the length of the channel. The porin channel has wide external and internal openings but constricts in the middle of the channel, creating an hourglass geometry. The specifics of these structures determines the size exclusion limits of the porin as well as the preferences for solutes, which are determined largely by the distribution of charged resides within the channel and the presence or absence of substrate binding sites (Jap and Walian, 1996).

In chlamydial species the major outer membrane protein (MOMP) and the dicarboxylate-specific porin PorB have already been characterized (Jones, et al., 2000, Sun et al., 2007, Kubo and Stephens, 2001). MOMP is a general porin with size selectivity for molecules less than 200 M. W. when expressed in *E. coli* (Jones et al. 2000). This contrasts with the general porins in *E. coli*, which have a size exclusion limit of 600 M. W. (Jap and Walian, 1996). PorB is present in chlamydial outer membranes at much lower amounts than MOMP and is specific in allowing only the uptake of 2-oxoglutarate and a few other dicarboxylates. AaxA is the newest chlamydial outer

membrane porin to be characterized and its further study will expand our understanding of the outer membrane dynamics of chlamydial bacteria (Smith and Graham, 2008).

1.5. ARGININE-AGMATINE EXCHANGE

Antiporters are transport proteins that transport substrates across the cytoplasmic membrane by coupling transport of one molecule into the cytoplasm to that of another out of the cell. The AdiC protein in *E. coli* is an amino acid transport protein localized to the cytoplasmic membrane which functions as an arginine-agmatine antiporter (Gong et al, 2003, Iyer et al., 2003, Fang et al., 2006). Together with the PLP-dependent arginine decarboxylase AdiA this antiporter allows for the exchange of extracellular arginine for the arginine decarboxylation product agmatine. The purpose of this exchange is to elevate the internal pH of the cell by using up aqueous protons with the decarboxylation of arginine and in the process to reverse the cell's membrane potential, allowing *E. coli* to survive the low pH environment of the stomach on the way to colonizing the gut. AdiC exists as a homodimer in phospholipid membranes with each subunit functioning as a transporter (Fang et al., 2009). The expression of AdiA and AdiC is induced under anaerbobic, acidic conditions and the AdiAC system for arginine agmatine exchange is most active at pH 2.5, showing only minimal activity above pH 3 (Gong et al., 2003). The *adiY* gene is located between *adiA* and *adiC* but, although over expression of AdiY reportedly increased *adiA* transcription, deletion of *adiY* was shown to have no effect on arginine-dependent acid resistance, suggesting that it may serve as a conditional regulator (Gong et al., 2003).

1.6. THE CHLAMYDIAL ARGININE DECARBOXYLASE

Arginine decarboxylation is carried out in *C. pneumoniae* by AaxB. The decarboxylation reaction is shown in the following equation:

$$R\text{-}COO^- + H^+ \rightarrow R\text{-}H + CO_2$$

While most arginine decarboxylases require pyridoxal 5'-phosphate (PLP) as a co-factor for activity, some enzymes, including AaxB, use a covalently attached pyruvoyl group. This co-factor acts as the prosthetic group for the formation of a Schiff's base with the arginine substrate while providing an electron sink to stabilize the negatively charged intermediate (Graham et al., 2002). Pyruvoyl-dependent arginine decarboxylases are translated as autocatalytic pro-enzymes that self-cleave to yield the small amino-terminal β fragment and the large carboxyl-terminal α fragment. *Methanocaldococcus jannaschii* uses such an arginine decarboxylase, and so does *Chlamydia pneumoniae* (Graham et al., 2002, Giles and Graham, 2007). These enzymes have the advantage of a co-factor that is covalently bound to the enzyme, eliminating competition with other enzymes for available PLP.

The pyruvoyl-dependent arginine decarboxylase from *C. pneumoniae*, now called AaxB, was discovered as a homolog for the arginine decarboxylase from *M. jannaschii*, a kind of archaea that lives in hydrothermal vents and uses its arginine decarboxylase in polyamine biosynthesis (Graham et al., 2002). CPn1032 (AaxB) was heterologously

expressed and purified in *E. coli* and it was found to self-cleave to give an active enzyme with an optimum activity at pH 3.4 (Giles and Graham 2007).

The arginine decarboxylases in *Chlamydia trachomatis* strains L2 and D, unlike AaxB in *C. pneumoniae*, were found to be inactive when expressed in *E. coli* (Giles and Graham, 2009). AaxB in the *C. trachomatis* L2 strain contains a nonsense mutation, which could be rescued by replacing the ochre stop codon with its ancestral tryptophan codon while AaxB from the D strain expressed as a proenzyme defective for self-cleavage that could be rescued by replacing the arginine-115 codon with an ancestral glycine codon (Giles and Graham 2009). These results suggest a relaxed selection for the maintenance of AaxB in *C. trachomatis* compared with *C. pneumoniae*, which may be a factor in tissue tropism, given that *C. trachomatis* generally infects mucosal epithelial cells, which do not express inducible nitric oxide synthase (iNOS), while *C. pneumoniae* infects granulocytes and alveolar macrophages, which do express iNOS (Giles and Graham, 2009).

1.7. *C. PNEUMONIAE* AND NITRIC OXIDE

As previously mentioned, arginine uptake and degradation can be used as a means of countering the production of antimicrobial nitric oxide (NO) by macrophages, as in the case of *H. pylori* (Gobert et al., 2001). It is possible that *C. pneumoniae*, which can infect granulocytes and alveolar macrophages, may use its arginine-agmatine exchange system for this purpose as well. Additionally, agmatine, the product of arginine

decarboxylation by AaxB, has been shown to suppress the generation of NO by inducible nitric oxide synthase (iNOS) in cell culture after being converted to an aldehyde product by host diamine oxidase (Satriano et al., 2001). The coupling of arginine degradation with the production of an iNOS inhibitor could make the chlamydial AAX system even more effective. It has already been reported that *C. trachomatis* strains, which do not generally infect cell types that express inducible nitric oxide synthase, have shown progressive inactivation of the genes for arginine-agmatine exchange by independent mutations (Giles and Graham, 2009).

It has been demonstrated that NO production stimulated by the addition of IFN-γ results in the inhibition of *C. pneumoniae* growth in macrophages but adding the nitric oxide synthase (NOS) inhibitor *N*-monomethyl-L-arginine (L-NMMA) restored growth (Carratelli et al., 2005). A similar trend was observed with the exogenous addition of an NO donor. These results showed that NO has a role in combating infection by *C. pneumoniae* and that inhibition of NOS can enhance its ability to infect and reproduce in host cells. It should be noted that the arginine concentration in the cell culture medium used for these tests was 1.1 mM while the extracellular arginine concentration in the lung is only 73-150 μM (Dweik, 2007). Therefore, it is possible that the arginine uptake and degradation system in *C. pneumoniae* was unable to significantly affect NO production due to the excessive amounts of arginine present in the media. Additional experiments using physiologically relevant concentrations of arginine will be needed to determine if NO-mediated inhibition of *C. pneumoniae* growth can be reduced by the AAX system.

CHAPTER 2

Arginine uptake by AaxC

2.1. OVERVIEW

AaxC was hypothesized to be the chlamydial arginine-agmatine antiporter that would work together with AaxB to create a system for arginine-agmatine exchange. The gene Cpn1031 was designated as *aaxC* after its role in arginine-agmatine exchange was established. AaxC's arginine-agmatine exchange function is driven by the chlamydial arginine decarboxylase AaxB (Cpn1032), a protein that was previously characterized in our lab. Cpn1033, now designated *aaxA* encodes the outer membrane porin AaxA, which will be detailed in later chapters. These 3 genes have been identified in all sequenced chlamydial genomes although functional proteins are not expressed in all strains (Giles and Graham, 2007, Giles and Graham, 2009, Smith and Graham, 2008).

AaxC and AaxB were predicted to have activity analogous to that of AdiC and AdiA from *E. coli*. AdiC is the *E. coli* arginine-agmatine antiporter and AdiA is the *E. coli* arginine decarboxylase and together with the regulatory protein AdiY they function as a system for acid-resistance (Gong et al, 2003, Iyer et al., 2003, Fang et al., 2006). AdiC is a member of the major facilitator superfamily (MFS) of transport proteins. By taking up Arg^{+} and exporting Agm^{+2} AdiC acts as a virtual proton pump. AdiA drives this exchange by decarboxylating the imported arginine, consuming protons and elevating the

intracellular pH as agmatine, with its virtual proton, is removed from the cell by AdiC. Thus, *E. coli* cells are able to survive the acidic environment in the stomach on their way to colonize the gut. Although chlamydial bacteria do not encounter this environment during their own pathogenic life cycle the ability of the Aax proteins to function as an acid resistance system when expressed in *E. coli* was investigated (Smith and Graham, 2008).

To determine if AaxC shared the same function as AdiC, *aaxC* and *aaxBC* from *Chlamydia pneumoniae* were expressed from plasmids in *E. coli* mutants unable to express *adiAYC*. Cell membranes were separated by sucrose-density gradient centrifugation and western blot analysis was used to determine that AaxC was localized to the cytoplasmic membrane, as would be predicted if AaxC shares a function with *E. coli* AdiC, which also localizes to the cytoplasmic membrane. Uptake assays using L-[^{3}H]arginine were used to measure AaxC's ability to import arginine into the cell. In the absence of any arginine decarboxylase, AaxC was observed to mediate the exchange of radiolabeled extracellular arginine for unlabeled intracellular arginine. Although arginine-agmatine exchange driven by the AaxB arginine decarboxylase was much more efficient, as detailed in chapter 3, these uptake assays verified AaxC's role in taking extracellular arginine into the cell. Together with the localization of AaxC to the cytoplasmic membrane, these results confirmed that AaxC is at least somewhat analogous to AdiC. Because AaxB is an arginine decarboxylase, identical to AdiA in function though not in mechanism, it was predicted that AaxBC would be able to rescue acid

resistance function in *E. coli* cells unable to express AdiAYC (Smith and Graham, 2007).

To address the question of whether AaxC could function together with AaxB to allow acid-resistance in *E. coli* cells defective for their native arginine-agmatine exchange system, a complementation assay was used. Cells were subjected to acid shock in media adjusted to pH 2.5 for 1 hour in the presence or absence of arginine. Cells expressing AaxBC in the presence of arginine were able to survive at wild type levels while negative controls without AaxBC showed minimal survival after the same treatment. Without arginine in the media all cells showed minimal survival (Smith and Graham, 2007).

Because *C. pneumoniae* is not known to encounter such low pH environments during normal pathogenesis, the ability of the *aax* genes to complement *E. coli* cells defective for *adiAYC* is interesting but may not be directly relevant to the chlamydial life cycle. *C. pneumoniae* does not pass through the gastrointestinal tract during infection and therefore does not need to cope with the acidic environment found in the stomach, an obstacle that enteric bacteria like *E. coli* must overcome. Additionally, while chlamydial bacterial are taken up by host cells and grow within an inclusion vacuole, this vacuole does not fuse with acidifying lysosomes (Scidmore et al., 2003). Furthermore, while AaxB was shown to have a low optimum pH of 3.4, AaxC-mediated arginine uptake was most efficient from pH 5 to 6. AaxA, which has no equivalent in enteric bacteria, has an

even higher optimum pH, as will be detailed in chapter 4. Finally, the Aax system evolved independently of the Adi system found in *E. coli*. AaxB is a pyruvoyl-dependent arginine decarboxylase while AdiA is uses PLP as a cofactor. AaxB is more closely related to the poylamine-biosynthetic arginine decarboxylase found in euryarchaea (Graham et al., 2002). AaxC is more closely related to ArcD, the arginine-ornithine antiporter found in *Pseudomonas aeruginosa* than it is to the *E. coli* arginine-agmatine antiporter AdiC (Verhoogt et al., 1992, Gong et al., 2003). While it may be possible that *C. pneumoniae* must endure low pH environments under some currently unknown circumstances during its life cycle, it is more likely that it uses its system for arginine-agmatine exchange in another way (Smith and Graham, 2007).

2.2. EXPERIMENTAL PROCEDURES

2.2.1. Strains and DNA

C. pneumoniae Kajaani 6 chromosomal DNA was a gift from Claudio Cortes Miranda and Benjamin Wizel (University of Texas Health Center at Tyler) (Ekman et al., 1993). *E. coli* EF1021 was a gift from John Foster (University of South Alabama) (Gong et al., 2003). *E. coli* MG1655 (CGSC 7740) was obtained from the *E. coli* Genetic Stock Center (Yale). *E. coli* DH5α (Invitrogen) was used as a general cloning host. Bacteriophage P1*vir* was a gift from Ian Molineux (University of Texas at Austin).

2.2.2. Cloning

The multiple cloning site of vector pBAD/HisA was amplified by PCR using primers pBADMCS2 and pBAD-Rev. The purified product was digested with NcoI and HindIII restriction enzymes and then ligated into the same sites of pBAD/HisA to produce vector pDG219. Primers 5CPn1031N and 3CPn1031H were used to amplify the CPn1031 gene from *C. pneumoniae* K6 chromosomal DNA. The PCR product was ligated between NdeI and HindIII sites in vector pET-43.1b to produce vector pDG133. Primers 5CPn1031Nc and 3CPn1031H2 were used to amplify CPn1031 from pDG133. The product was ligated between NcoI and HindIII sites of pBAD/HisA to produce pDG170 and between the same sites of pTrcHisA to produce pDG183. Primers 5CPn1031Nc and COLIDOWN-RI were used to amplify the CPn1031-HSV cassette from pDG133. The cassette encodes the CPn1031 protein fused to a carboxy-terminal herpes simplex virus (HSV) glycoprotein D epitope sequence (Novagen). This cassette was ligated between the NcoI and EcoRI sites of pTrcHisA to produce vector pDG193 and between the same sites of pBAD/HisA to produce vector pDG194. Primers 5CPn1032Nc and 3CPn1031H were used to amplify the CPn1032 and CPn1031 gene pair. This PCR product was digested with NcoI and HindIII and ligated into the same sites of plasmid pET-43.1b to produce plasmid pDG360 and in plasmid pBAD/HisA to produce pDG379. The CPn1032-CPn1031-HSV cassette was amplified from pDG360 using the primers 5CPn1032Nc and COLIDOWN. This product was digested with NcoI, phosphorylated with polynucleotide kinase, and ligated between the NcoI and SmaI sites of pDG219 to produce vector pDG366 (Smith and Graham, 2008).

See the Appendix for a table detailing strain information.

Two plasmids containing *E. coli* membrane proteins were constructed to provide markers for chlamydial membrane protein localization. LepA (EF4) is a 67-kDa cytoplasmic membrane-associated ribosomal back translocase (March et al., 1985, Qin et al., 2006). The *lepA* gene with its upstream sequence was amplified from *E. coli* DNA by using the primers 5lepAH and 3lepAX. The restriction-digested PCR product was ligated to the HindIII and XhoI sites of vector pCDFDuet-1 to produce plasmid pDG561. This multicopy plasmid encodes the LepA sequence fused to a carboxy-terminal S-Tag sequence (Novagen), under the control of the native *lepA* promoter. OmpX is an 18-kDa outer membrane protein that is overexpressed during chemical stress conditions (Dupont, et al., 2007). The *ompX* gene with its upstream sequence was amplified from *E. coli* DNA using the primers 5ompXH and 3ompXX. The product was ligated to the HindIII and XhoI sites of the multicopy vector pCOLADuet-1 to produce plasmid pDG552. The pDG552 plasmid encodes the native OmpX fused to a carboxy-terminal S-Tag sequence. Both the pDG561 and the pDG552 plasmids were transformed into *E. coli* BW25113 to create a marker strain. In addition, the pDG552 plasmid was transformed into *E. coli* BL21(DE3) to express *ompX* from a T7 RNA polymerase promoter (Smith and Graham, 2008).

An *adiAYC* deletion mutation was generated by the gene disruption method of

Datsenko and Wanner (Datsenko et al., 2000). The *adiC1*::*kan* allele from *E. coli* strain EF1021 was transduced into strain MG1655 using bacteriophage P1*vir*, producing strain DEG0100. DEG0100 cells were transformed with vector pCP20, and grown under nonpermissive conditions for plasmid replication (37°C). The kanamycin-sensitive (Kan^s) recombinant strain DEG0124 was screened by PCR using the primers EcadiCUP and EcadiCDOWN, confirming the excision of the FLP-*kan* cassette. Primers EcAdiAFwd1 and EcAdiARev1 were used to amplify the kanamycin resistance cassette from pKD13 (Datsenko et al., 2000). *E. coli* DEG0124(pKD46) was transformed with the resulting *adiA1*::*kan* PCR product. Recombinant strains were selected by growth on LB agar containing kanamycin (25 mg ml^{-1}). The *adiAYC*::*kan* allele in a Kanr recombinant (DEG0147) was confirmed by PCR using the primers EcadiAFwd2 and EcadiCDOWN. The purified 1.6-kbp PCR product was sequenced, confirming the deletion (Smith and Graham, 2008).

2.2.3. Protein Expression

Expression vectors were transformed into *E. coli* strains by electroporation. *E. coli* strains carrying pBAD/HisA or derivative plasmids were grown aerobically at 37°C for 22 h in LB Miller medium supplemented with ampicillin (100 µg ml–1) and L-arabinose (0.15% [wt/vol]). For protein expression analysis by Western blotting, cells were grown with L-arabinose for 6 h to reduce proteolytic cleavage. *E. coli* strains carrying pET-43.1a or pTrcHisA derivative plasmids were grown with ampicillin and

induced with 1 mM IPTG (isopropyl-ß-D-thiogalactopyranoside) (Smith and Graham, 2008).

2.2.4. Membrane fractionation by sucrose-density gradient centrifugation

E. coli cells were lysed by sonication, and the lysates were cleared of debris by low-speed centrifugation. Membranes were prepared by ultracentrifugation of these samples at 100,000 x *g* for 1 h at 4°C in a Beckman TLA-100.3 rotor (Ward et al., 2000). For total membrane protein analysis, the pellets were resuspended in 2% SDS with 20 mM Tris-HCl (pH 7.5). Suspensions were centrifuged at 100,000 x *g* for 1 h at 4°C. The supernatant was concentrated by using a centrifugal ultrafiltration device (10,000 molecular weight cutoff; Pall Life Sciences). For fractionation by sucrose-density gradient centrifugation, the membrane pellets were resuspended in a solution containing 25% sucrose, 20 mM Tris-HCl, and 0.5 mM EDTA (pH 7.5). This suspension was layered on top of 30, 35, 40, 45, 50, and 55% sucrose layers and centrifuged at 100,000 x *g* for 6 h at 4°C (Ward et al., 2000). The visible cytoplasmic membrane layer and the outer membrane layer (inner membrane-depleted layer) were extracted with a syringe, concentrated by centrifugation, and washed three times with 20 mM Tris-HCl (pH 7.5) to remove sucrose and EDTA. Outer membrane fractions were washed with 5 M urea to remove peripheral or aggregated proteins (Marani et al., 2006).

2.2.5. Western blotting

Protein expression was determined by immunoblotting. Samples were mixed with

sodium dodecyl sulfate (SDS) in loading dye but were not boiled. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (0.2 μm, Pall) by using a MiniVE semiwet blotter (GE Healthcare) at 100 mA for 2 h. Prestained protein marker (New England Biolabs) was used to confirm transfer and measure the apparent molecular masses of proteins detected by immunoblotting. The membrane was blocked with 3% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20. For the analysis of HSV epitope-tagged proteins, blots were incubated with HSV-tag monoclonal antibody (1:2,000 dilution; Novagen), followed by goat anti-mouse immunoglobulin G (IgG) secondary antibody conjugated to horseradish peroxidase (1:2,500 dilution; Pierce) for 1 h at room temperature. The affinity-purified NusA-HSV-His6 protein from *E. coli* BL21(DE3) (pET-43.1b) cells was used as a positive control. For the analysis of *E. coli* LepA-S-Tag or OmpX-S-Tag proteins, blots were incubated with a mouse monoclonal antibody raised against the S-Peptide (1:1,000 dilution; Affinity BioReagents), followed by detection with goat anti-mouse IgG as described above. Blots were developed by using a Super Signal West Pico mouse IgG detection kit (Pierce). Chemiluminescence was detected by using an Image Station 4000R instrument (Carestream Health) with Molecular Imaging software (version 4.0).

2.2.6. Cellular transport assays

A suspension of 3 x 10^9 *E. coli* cells was prepared in 0.1 ml of E medium containing 73 mM K_2HPO_4, 17 mM Na_2HPO_4, 0.8 mM $MgSO_4$, and 10 mM sodium

citrate at 37°C. Transport was initiated by adding 1 mM L-arginine-HCl with 2 μCi of L-[2,3,4,5-3H]arginine. After 10 min of incubation, 1 ml of 0.1 M LiCl was added to stop the reaction, and cells were collected by vacuum filtration on a polyethersulfonate membrane filter (0.2 μm, 25-mm diameter; Pall). The filters were washed with 2 ml of 0.1 M LiCl and then removed for liquid scintillation counting (Smith and Graham, 2008).

2.2.7. Arginine-dependent acid resistance assay

E. coli DEG0147 cells transformed with the indicated plasmids were grown in Luria-Bertani broth with 0.15% L-arabinose at 37°C. Approximately 3 x 10^7 *E. coli* cells in 20 μl of medium were added to 2 ml of E medium (pH 2.5) supplemented with 1.5 mM L-arginine at 37°C. E media without arginine was used as a control. Survival after 1 h of acid shock treatment was determined by drawing samples immediately after adding cells to the acid shock media and 1 hour after adding cells. Serial dilutions were made immediately in cold E media adjusted to pH 7 and the samples were plated on LB plates with ampicillin (100 μg/ml). After overnight incubation at 37°C colony-forming units (CFU) were counted and averaged from 3 plates for each time point for each sample. The survival rate is the percentage of viable cells detected after 1 h, relative to the number of viable cells detected immediately after the introduction of cells to acid shock medium (Smith and Graham, 2008).

2.3. RESULTS

2.3.1. Expression of AaxC and localization to the cytoplasmic membrane

The *E. coli* strain DEG0147 (Δ*adiAYC::kan*) was constructed by deleting the *adi* operon which encodes the arginine-agmatine antiporter AdiC, the PLP-dependent arginine decarboxylase AdiA, and the putative regulatory protein AdiY. This mutant is defective for the *E. coli* arginine-dependent acid resistance system and was used to heterologously express the chlamydial *aax* genes in the absence of any other system for arginine-agmatine exchange. DEG0147 grows normally in LB medium under aerobic conditions and is only defective for arginine-dependent acid-resistance (Smith and Graham, 2008).

The *aaxC* gene from *C. pneumoniae* was fused to a carboxy-terminal HSV epitope tag sequence in the vector pDG133 and was subcloned behind a P_{BAD} promoter in pDG194. *E. coli* DEG0147 cells transformed with either pDG194 or pBAD/HisA as a negative control were grown aerobically, harvested and lysed, and the membranes were separated by sucrose-density gradient centrifugation as described in the experimental procedures. The proteins were separated by SDS-PAGE and analyzed by Western blotting with a monoclonal HSV antibody (Figure 2.1). Compared with the pBAD/HisA control the cells expressing AaxC-HSV showed a new band with an apparent molecular mass of 41 kDa (56.1 kDa calculated). This discrepancy between the apparent and calculated molecular mass of AaxC-HSV is consistent with other integral membrane

proteins, which are known to migrate unusually fast during SDS-PAGE. The *E. coli* AdiC protein has an observed molecular mass of 34 kDa but a calculated molecular mass of 46.8 kDa (Fang et al., 2007). The AaxC-HSV band was observed only in the cytoplasmic membrane fraction of the cells' total membrane component separated by sucrose-density gradient centrifugation (Smith and Graham, 2008).

To further establish that AaxC localizes to the cytoplasmic membrane, the *E. coli* strain BW25113 (Δ*ara*BAD_{AH33}) was transformed with pDG194 as well as pDG552 and pDG561. The outer membrane marker protein OmpX and the cytoplasmic membrane marker protein LepA were expressed from these two plasmids, respectively. Both marker proteins were fused to a carboxy terminal S-Tag sequence so that they could be identified by Western blot using a monoclonal antibody raised against the S-Peptide. The AaxC-HSV protein colocalized with LepA and no AaxC was identified in the outer membrane fraction (Smith and Graham, 2008).

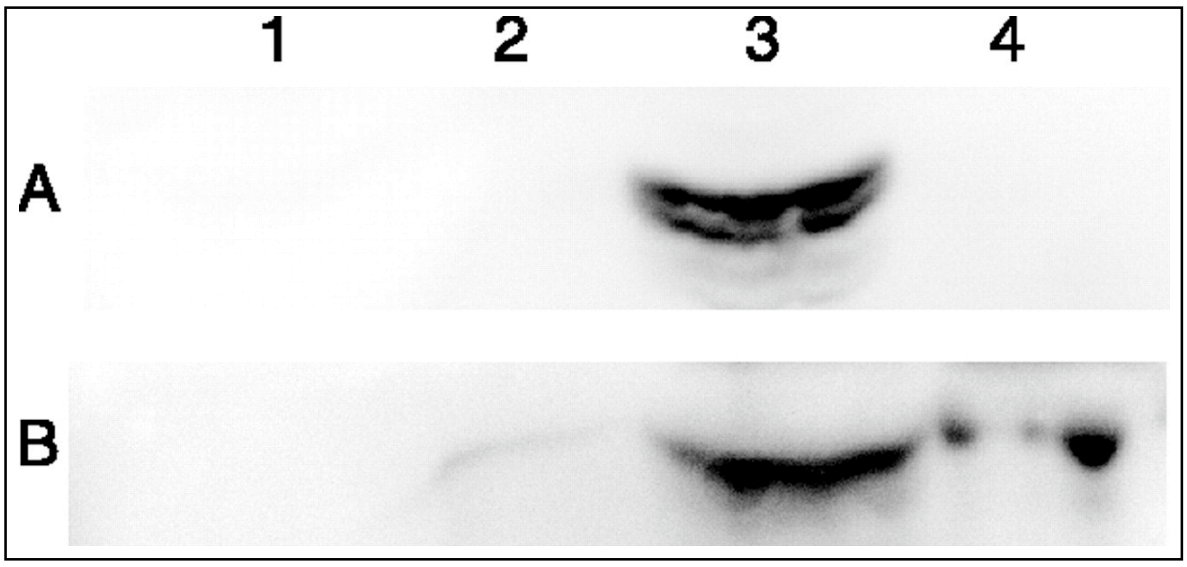

Figure 2.1. AaxC-HSV localizes to the cytoplasmic membrane

Western blots show the AaxC-HSV protein was expressed in the cytoplasmic membrane of *E. coli*. (A) A 41-kDa band corresponding to AaxC-HSV detected using an anti-HSV monoclonal antibody. (B) A 69-kDa band corresponding to the *E. coli* LepA-S-Tag protein, detected using an S-peptide monoclonal antibody. The same protein samples were used for both immunoblots. Lane 1 contains the total membrane fraction from *E. coli* BW25113(pBAD/HisA) cells. Sucrose density-gradient centrifugation was used to separate the total membrane fraction from BW25113 (pDG194, pDG552, pDG561) cells into an outer membrane fraction (lane 2), a cytoplasmic membrane fraction (lane 3), and a high-density pellet (lane 4). The LepA protein identified in the high-density pellet may be associated with ribosomes (Qin et al., 2006). The immunoblot with S-peptide monoclonal antibody also identified a 46-kDa band in the cytoplasmic membrane fraction that may represent a degradation product of LepA (data not shown).

2.3.2. Arginine uptake by AaxC in whole cells

E. coli DEG0147 cells were transformed with pDG170, which expressed the untagged AaxC from the P_{BAD} promoter and these cells were used to analyze the uptake of arginine by AaxC in the absence of arginine decarboxylase activity. The *E. coli* AdiC protein was reported to exchange intracellular arginine for extracellular arginine without

AdiA activity and therefore AaxC was expected to demonstrate similar activity (Fang et al., 2007). Washed cells mixed with 1 mM L-[^{3}H]arginine and incubated for 10 minutes at 37°C were collected by filtration and after additional washes the radiolabel on the filter was measured by liquid scintillation counting (Figure 2.2). Radiolabel retained on the filters by cells not expressing AaxC was not significantly different from the background radiolabel for reaction mixtures without cells that were used as a negative control. Cells expressing AaxC showed a significant increase in the amount of radiolabel retained on the filters with a maximum level of arginine uptake observed from pH 5 to 6. While significant uptake still occurred at pH 7, none was observed at pH 4 or lower (Smith and Graham, 2008). This contrasts with *E. coli* AdiC, which showed optimal uptake at pH 2.5 (Fang et al. 2007). It is likely that some AaxC-mediated arginine-arginine exchange may still occur at a low pH, but it was not detectable over background radiolabel on the filters.

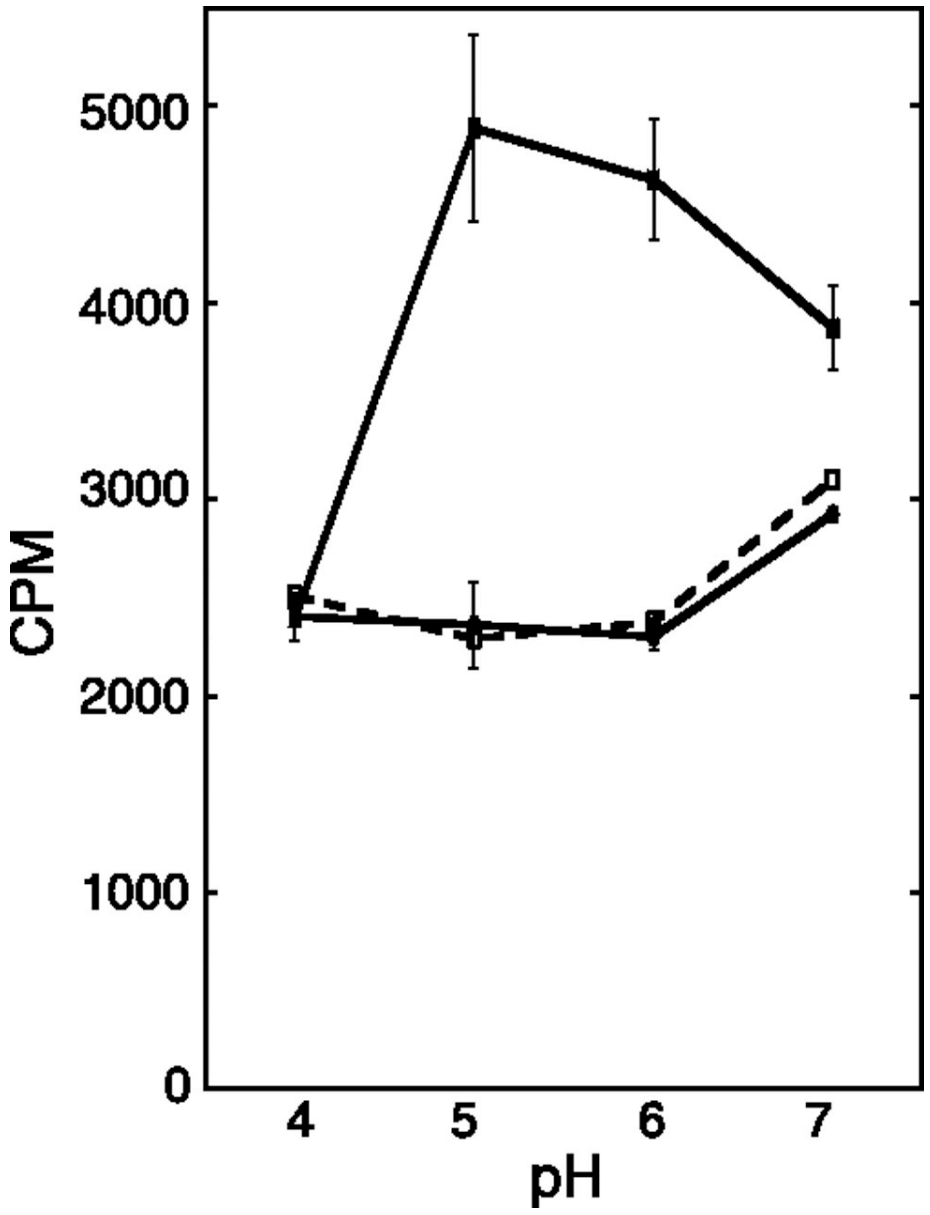

Figure 2.2. Arginine uptake by AaxC

Expression of *aaxC* promoted arginine uptake. *E. coli* DEG0147 (pDG170) cells expressing *aaxC* alone optimally transported L-[U-^{14}C]arginine from pH 5 to 6 (■). Arginine transport by *E. coli* DEG0147 (pBAD/HisA) cells (•) was not significantly different from background levels due to membrane binding of arginine in control reactions without cells (□). The net radioactivity measured in reactions containing DEG0147 (pDG170) cells at pH 5 corresponds to 0.4% arginine uptake. No net arginine transport was detected below pH 5 in these cells.

2.3.3. Inhibition of arginine uptake by AaxC

Various potential inhibitors of AaxC were tested using the assay described above. As before, AaxC was expressed from pDG170 in DEG0147 and cells were collected by filtration after incubation with radiolabeled arginine. Additionally, potential inhibitors were added to the reaction mixtures to determine their effect on arginine uptake. L-argininamide, although shown to inhibit AaxB activity (Giles and Graham, 2007), had no effect on arginine uptake by AaxC. The only tested inhibitors that significantly reduced arginine uptake were L-canavanine and D-arginine, which reduced uptake to 18% and 43%, respectively (Smith and Graham, 2008). D-arginine was not reported to inhibit AaxB activity and L-canavanine was shown to be a substrate for AaxB (Giles and Graham, 2007).

2.3.4. Complementation of acid resistance with AaxBC

E. coli cells expressing the *adiAYC* genes are able to exchange arginine for agmatine produced by the arginine decarboxylase AdiA using the AdiC arginine-agmatine antiporter. This is just one of the systems used by wild type *E. coli* to pair amino acid uptake and decarboxylation for the purpose of increasing their cytoplasmic pH and inverting their membrane potential to resist acid shock. These systems are expressed in late stationary phase under anaerobic acidic conditions and exist to allow *E. coli* to survive in the stomach before colonizing the gut (Richard and Foster, 2004).

In *E. coli* DEG0147 the deletion of *adiAYC* eliminates arginine-dependent acid resistance and in DEG0147 (pBAD/HisA) minimal survival was observed after 1 hour at pH 2.5 in the presence or absence of arginine (Figure 2.3). When AaxBC was expressed in DEG0147 (pDG379) under the same conditions, acid survival over 1 hour was observed to increase by more than 3 orders of magnitude in the presence of 1.5 mM L-arginine, yielding almost 30% survival, which is comparable to wild type *E. coli*. In the absence of arginine, this increase in survival was lost and cells expressing AaxBC fared no better than *E. coli* DEG0147 (pBAD/HisA). The complete *aaxABC* gene cluster was also expressed in *E. coli* DEG0147 (pDG484) but there was no significant increase in survival when compared with cells expressing AaxBC alone (Smith and Graham, 2008).

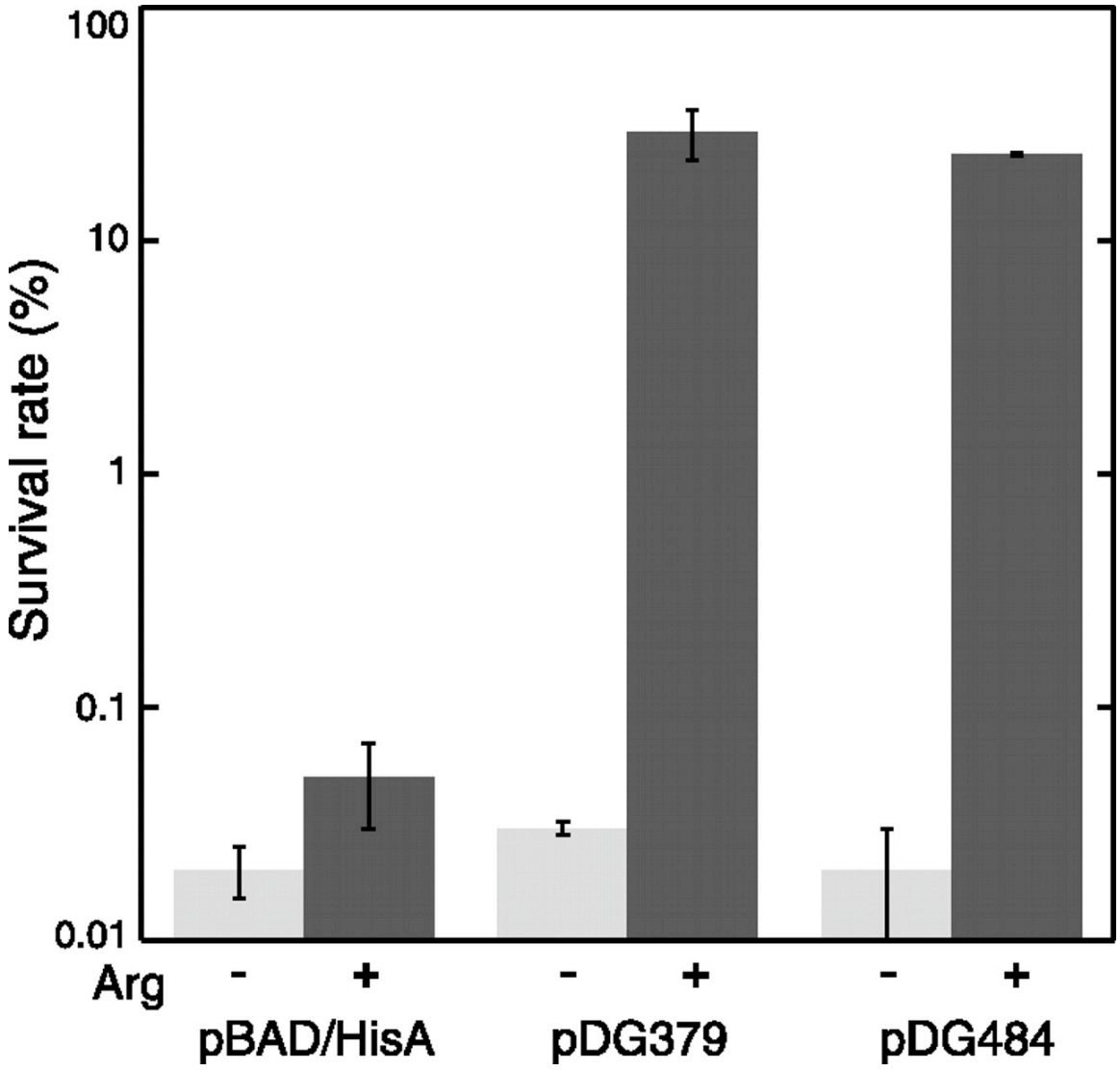

Figure 2.3. Complementation of acid resistance in *E. coli* using *aax* genes

Expression of the *C. pneumoniae aaxBC* genes restored arginine-dependent acid resistance in *E. coli* DEG0147. Cells containing pBAD/HisA (vector control), pDG379 (*aaxBC*), or pDG484 (*aaxABC*) were incubated in E medium at pH 2.5 for 1 h in the absence (light gray bars) or presence (dark gray bars) of 1.5 mM L-arginine. The mean survival rates and standard deviations are shown for each assay repeated in triplicate.

2.4. DISCUSSION

The AaxC protein from *C. pneumoniae* is involved in taking arginine into the cell, as was demonstrated by heterologously expressing AaxC in *E. coli* mutants unable to express their own equivalent arginine-agmatine antiporter AdiC. No arginine biosynthetic proteins are known to be encoded by any chlamydial genomes so chlamydial cells must take up arginine from the host cells they infect. *C. pneumoniae* has an operon regulated by the ArgR repressor that encodes a putative ABC-type arginine transporter (Schaumburg and Tan, 2006). This transporter is probably responsible for the uptake of arginine required for protein synthesis. The Aax system does not seem to be involved in taking up arginine for this purpose and instead converts arginine to agmatine, which is then exported from the cell and into the host environment (Smith and Graham, 2008, Chapter 3).

E. coli AdiA and AdiC function together as an arginine-agmatine exchange system for the purpose of enhancing survival in a low pH environment. To determine if *C. pneumoniae* AaxB and AaxC could serve the same function as AdiA and AdiC, an *E. coli* mutant was used which was unable to express *adiAYC* and was therefore defective for arginine-dependent acid resistance. In complementation experiments using these cells transformed with plasmids expressing the chlamydial *aaxBC* or *aaxABC* it was observed that the Aax system from *C. pneumoniae* was able to fully rescue acid resistance function in the presence of arginine. Without arginine in the media no such rescue was observed,

indicating that the system for acid resistance is fully dependent on arginine, as was predicted. The outer membrane porin AaxA had no significant effect on acid resistance, even though it is able to facilitate the uptake of arginine, as detailed in later chapters. However this is not a contradiction as AaxA loses activity under low pH conditions (Chapter 4). The ability of AaxB and AaxC to rescue acid-resistance function may not be relevant to the role of this system in *C. pneumoniae*, particularly if AaxA, with its higher pH optimum is an integral part of the same system. Because *C. pneumoniae* is not believed to encounter extremely low pH conditions during infection AaxABC may have a different role entirely.

The Aax system is functional for acid-resistance when expressed in *E. coli* even though arginine uptake assays using heterologously expressed AaxC showed no activity above background at pH 2.5. This suggests that arginine-agmatine exchange driven by the activity of an arginine decarboxylase should be much more efficient than arginine-arginine exchange and assays for arginine-agmatine exchange in the next chapter confirm this result. Arginine-arginine exchange can be partially inhibited by the addition of D-arginine or L-canavanine with the later being most effective. Other arginine analogs that were tested did not result in significant inhibition of arginine uptake by AaxC, suggesting that they are not taken up by AaxC and therefore cannot compete with L-arginine. The fact that D-arginine is able to partially inhibit arginine uptake suggests that AaxC does not discriminate based on stereochemistry. Since AaxC did not import any of the other arginine-analogs tested, it can be inferred that AaxC is very specific in its activity.

AaxC is an amino acid transport protein localized to the cystoplasmic membrane. It can function with the chlamydial arginine decarboxylase AaxB to rescue acid resistance function at pH 2.5 and it can exchange radiolabeled extracellular arginine for unlabeled intracellular arginine. AaxC can be partially inhibited only by L-canavanine and D-arginine. In the next chapter its function as an arginine-agmatine antipoter will be fully investigated.

CHAPTER 3

AaxBC and AaxABC as a system for arginine-agmatine exchange

3.1. OVERVIEW

If AaxC from *C. pneumoniae* functions as an arginine-agmatine antiporter like AdiC in *E. coli*, it should be possible to detect agmatine excreted from *E. coli* cells expressing AaxC and the AaxB decarboxylase together. It was shown in the last chapter that arginine can be transported by AaxC and that AaxC and AaxB can function together as a system for extreme acid-resistance in *E. coli*. However, significant inhibition of uptake by AaxC was not detected when agmatine was tested as an inhibitor. This suggests either that agmatine can only be transported by AaxC from the cytoplasm to the extracellular environment and not in the reverse direction, or else that AaxC does not transport agmatine at all and is therefore not an arginine-agmatine antiporter like AdiC. As a third option, the high background observed in the arginine uptake assays obscured the effects of agmatine as an inhibitor for arginine uptake by AaxA (Smith and Graham, 2008). There is precedence for asymmetric substrate affinity in antiporters so it would not be unexpected if agmatine is taken up more efficiently from the cytoplasm than the external environment (Goldfarb and Nord, 1987, Dierks and Krämer, 1988).

To find the answer to the question of whether or not AaxC is an arginine-agmatine antiporter two general approaches were used. In both cases Aax proteins were expressed in *E. coli* mutants unable to express their native Adi proteins for arginine-agmatine exchange. For the first method, these *E. coli* cells were incubated in media containing L-arginine spiked with L-[^{3}H]arginine. The cells were spun down and the supernatants containing radiolabeled arginine, agmatine, or a mixture of both were separated by strong cation-exchange HPLC and fractions were collected for liquid scintillation counting. UV chromatograms were compared with the distribution of radiolabel in the collected fractions to determine if arginine was being decarboxylated to produce agmatine. The production and export of agmatine in cells expressing AaxC with AaxB but not in negative controls supported the hypothesis that AaxC is an arginine-agmatine antiporter (Smith and Graham, 2008).

For the second method L-[^{14}C]arginine was used and the radiolabled CO_2 produced by the decarboxylation of arginine was collected on barium hydroxide soaked filter paper discs at the top of the reaction tubes and quantified by liquid scintillation counting. For these assays the production of CO_2 was used as a proxy for arginine uptake and decarboxylation by the Aax proteins. Unlike the arginine uptake assays used previously to examine AaxC the whole cell decarboxylase assay produces minimal background and it is much less time consuming than the HPLC method. Although arginine uptake by AaxC is not being examined directly, decarboxylation of arginine by AaxB is dependent on the uptake of arginine into the cells. Arginine decarboxylase

activity in whole cells without AaxC is minimal, although native *E. coli* transporters may allow some arginine uptake. AaxC dramatically increases whole cell decarboxylase activity over this background level, confirming its role in arginine uptake (Smith and Graham, 2008).

The dependence of this system on various ions present in the media was examined by using various buffers. Arginine uptake by AaxC was observed to be unaffected by the presence or absence of chloride, potassium, sodium, phosphate, or citrate, which contrasts with some other transport proteins which are dependent on certain ions for activity. AdiC, for example, requires the transport of chloride ions to relieve excessive buildup of positive charge caused by the decarboxylation of arginine (+1) to yield agmatine (+2). The internal positive charge created by arginine agmatine exchange is essential to acid survival but it must be possible to restore the internal negative charge to the cell after it has escaped the low pH environment. Chloride uptake may be important for doing so. However, if limiting excessive positive charge during acid challenge is a factor for arginine-agmatine exchange in *E. coli*, this does not seem to be the case for chlamydia (Fang et al., 2007).

As with the previously described AaxC arginine uptake assays, various arginine analogs were examined for their ability to inhibit arginine uptake and decarboxylase activity in whole cells. Because of the requirement for substrate uptake by a transporter for decarboxylase activity in the cell, inhibition of AaxB should only occur if the AaxC

antiporter is able to transport the inhibitor. It was previously shown that L-argininamide was an inhibitor for AaxB (Giles and Graham, 2007). However, because AaxC does not transport L-argininamide, as shown in the previous chapter, there was no significant inhibition of arginine decarboxylase activity in assays using whole cells. As expected, D-arginine and L-canavanine were able to inhibit arginine uptake and decarboxylation, which is consistent with results for arginine uptake by AaxC detailed in the previous chapter. Some inhibition by agmatine was observed using these assays, although it was small and had not been detectable using the arginine uptake assays with AaxC alone. With the AaxB arginine decarboxylase to drive arginine uptake much higher rates were observed and combined with an assay with much lower background than the one used before this result suggests that agmatine and arginine are able to compete for uptake by AaxC, even if agmatine is not a very effective inhibitor. This may be the result of a reduced affinity for agmatine at the external transport site compared to the cytoplasmic site (Smith and Graham, 2008).

AaxA is a newly discovered outer membrane porin, which will be detailed in chapter 4. As previously mentioned, it did not significantly enhance acid-survival rates in *E. coli* at pH 2.5 but it was observed to enhance whole cell arginine decarboxylase activity by a significant degree under higher pH conditions. This suggests that AaxA, AaxB, and AaxC may work together as an efficient system for arginine-agmatine exchange in *C. pneumoniae* (Smith and Graham, 2008).

3.2. EXPERIMENTAL PROCEDURES

3.2.1. Cloning

The CPn1033-CPn1032-CPn1031 cluster was amplified by PCR using the primers 5CPn1033Nc and 3CPn1031X. The product was ligated between the NcoI and XhoI sites of pBAD/HisA to produce pDG484. The CPn1033 gene was amplified using the primers 5CPn1033Nc and 3CPn1033Not and ligated between the NcoI and NotI sites of pDG366 to produce pDG512 (Smith and Graham, 2008). Recombinant DNA was sequenced at the Institute for Cellular and Molecular Biology Core Labs DNA Sequencing facility (University of Texas at Austin). Both the CPn1031 and the CPn1033 gene sequences from *C. pneumoniae* K6 were identical to their orthologous sequences reported for *C. pneumoniae* CWL029 (GenBank accession numbers AE001363.1 and AE002161.1) (Read et al., 2000).

3.2.2. Product analysis

The unlabeled agmatine produced by transport and arginine decarboxylation reactions was identified by liquid chromatography-electrospray ionization mass spectrometry (LC-MS) as the trifluoroacetyl derivative. A reaction mixture (300 µl) containing 3 x 10^9 DEG0147(pDG484) cells with 25 mM L-arginine and ammonium acetate buffer (pH 4) was incubated for 2 h at 37°C. Cells were removed by centrifugation, and the solution was evaporated to dryness under nitrogen. Trifluoroacetyl derivatives were prepared using trifluoroacetic anhydride and analyzed by LC-MS in the

positive ion mode, as described previously (Graham et al., 2008). Tandem mass spectra (MS/MS) were acquired by using collision-induced dissociation of the $[MH]^+$ ions. Peaks corresponding to the molecular ions ($[MH]^+$) are shown first, followed by characteristic ion fragments listed in decreasing order of intensity. The acyl derivative of L-arginine eluted at 2.91 min producing peaks at 271 and 251 *m/z*; MS/MS of the ion at 271 *m/z* produced peaks at 213, 255, and 230 *m/z*. The acyl derivative of agmatine eluted between 2.9 and 3.9 min, producing peaks at 227, 169, and 185 *m/z*; MS/MS of the ion at 227 *m/z* produced peaks at 169, 211, and 186 *m/z*.

For radiolabeled product analysis, reaction mixtures (2 ml) contained 10^9 cells, suspended in E medium at pH 2.5 or 5.0. Mixtures were preincubated at 37°C for 10 min before reactions were initiated by the addition of 1 mM L-arginine and 4 μCi of L-[2,3,4,5-3H]arginine (50 Ci $mmol^{-1}$; American Radiolabeled Chemicals). Samples were removed immediately after initiation and again after 2 h of incubation: these were centrifuged at 17,000 x *g* for 5 min to remove cells. Unlabeled agmatine was added to samples as a carrier. Supernatants were applied to a Luna strong cation-exchange high-pressure liquid chromatography (HPLC) column (150 by 4.6 mm, 5 μm; Phenomenex) with a guard column (4 by 3 mm); equilibrated at 35°C in mobile phase buffer containing 50 mM KH_2PO_4, 100 mM K_2SO_4, 10% CH_3CN, and water; and adjusted to pH 7.2 with phosphoric acid. Isocratic elution with this buffer was used to separate compounds at a flow rate of 1 ml min^{-1}. Fractions (1 ml) were collected, and the radioactivity was determined by liquid scintillation counting. Using this method, arginine eluted at 1.8 min

and agmatine eluted at 6.1 min.

3.2.3. Arginine uptake and decarboxylation assays by whole cells

Approximately 3 x 10^9 cells were collected by centrifugation at 18,000 x *g* for 10 min. These cells were suspended in E media adjusted to the indicated pH (Vogel et al., 1956). Suspensions were prewarmed at 37°C, and the reactions were initiated by the addition of 1 mM L-arginine and 20 nCi of L-[U-^{14}C]arginine (305 mCi mmol^{-1}; GE Healthcare) at 37°C for 15 min. Reactions were carried out in 1.5 ml polypropylene microcentrifuge tubes with filter paper discs soaked in saturated barium hydroxide at the top (Giles and Graham, 2008). Reactions were terminated by the addition of 100 µl of 4 M HCl. These solutions were heated at 70°C for 15 min, releasing $^{14}CO_2$ that was trapped by the barium hydroxide-soaked filter paper discs and the captured radioactive product was measured by liquid scintillation counting (Giles and Graham 2007, Smith and Graham, 2008).

3.2.5. Cell free decarboxylase assays

Cells were suspended in 20 mM Tris-HCl buffer (pH 7.5), and lysed by sonication on ice. Centrifugation at 18,000 x *g* for 15 min removed insoluble particles. Total protein concentrations of these cell extracts were determined using the Bradford assay (Pierce) with bovine serum albumin as a standard. ArgDC activity in cell lysates was determined by using the $^{14}CO_2$ capture assay described previously (Giles and Graham, 2007, Smith and Graham, 2008).

3.2.6. Inhibition of AaxBC in whole cells and B in lystaes

Potential inhibitors of arginine transport or decarboxylation were added to whole-cell assays containing 0.5 mM L-arginine and 20 nCi of L-[U-^{14}C]arginine. The arginine analogs screened at 2 mM concentrations were agmatine, L-arginine *O*-methyl ester, *N*-acetyl L-arginine, N^G-nitro-L-arginine methyl ester, D-arginine, L-argininamide, cadaverine, L-citrulline, L-homoarginine, L-histidine, L- lysine, and L-ornithine (Smith and Graham, 2008).

3.3. RESULTS

3.3.1. Product analysis

Whole cells expressing the arginine decarboxylase AaxB and the arginine agmatine antiporter AaxC from pDG379 were incubated at 37°C in reaction mixtures containing L-[^{3}H]arginine for 2 hours to allow for the production of agmatine. LC-MS analysis of the trifluoroacetyl derivatives of the extracellular reaction products after this incubation identified the presence of significant amounts of agmatine as well as unconverted arginine. Radiolabeled arginine and agmatine were separated from the extracellular reaction volume using strong cation exchange HPLC and the collected fractions were analyzed by liquid scintillation counting. The amount of radiolabel in each fraction was compared with the UV chromatogram showing peaks corresponding to

unlabeled carrier arginine and agmatine to determine how much arginine was converted to agmatine (Figure 3.1) (Smith and Graham, 2008).

Two strains of *E. coli* were used for this assay, DEG0147 (Δ*adiAYC::kan*) and DEG0100 (Δ*adiC1::kan*), both transformed with pDG379 to express AaxB and AaxC or with pBAD/HisA as a negative control. Because *E. coli* AdiA is not expressed from cells grown aerobically it was expected that there would be no significant difference in agmatine production whether DEG0147 or DEG0100 was used for the assay (Smith and Graham, 2008).

Although AdiA and AdiC are most efficient at an extracellular pH of 2.5 (Figure 3.2), maximum conversion of arginine to agmatine in whole cells expressing AaxB and AaxC occurred at pH 5 where *E. coli* DEG0147 (pDG379) converted 50% of arginine to agmatine (Figure 3.5). These cells converted only 20% of arginine to agmatine at pH 2.5 (Figure 3.4). No agmatine production was detected in control reactions using DEG0147 (pBAD/HisA) (Figure 3.3) and all collected radiolabel corresponded to the arginine peak (Smith and Graham, 2008).

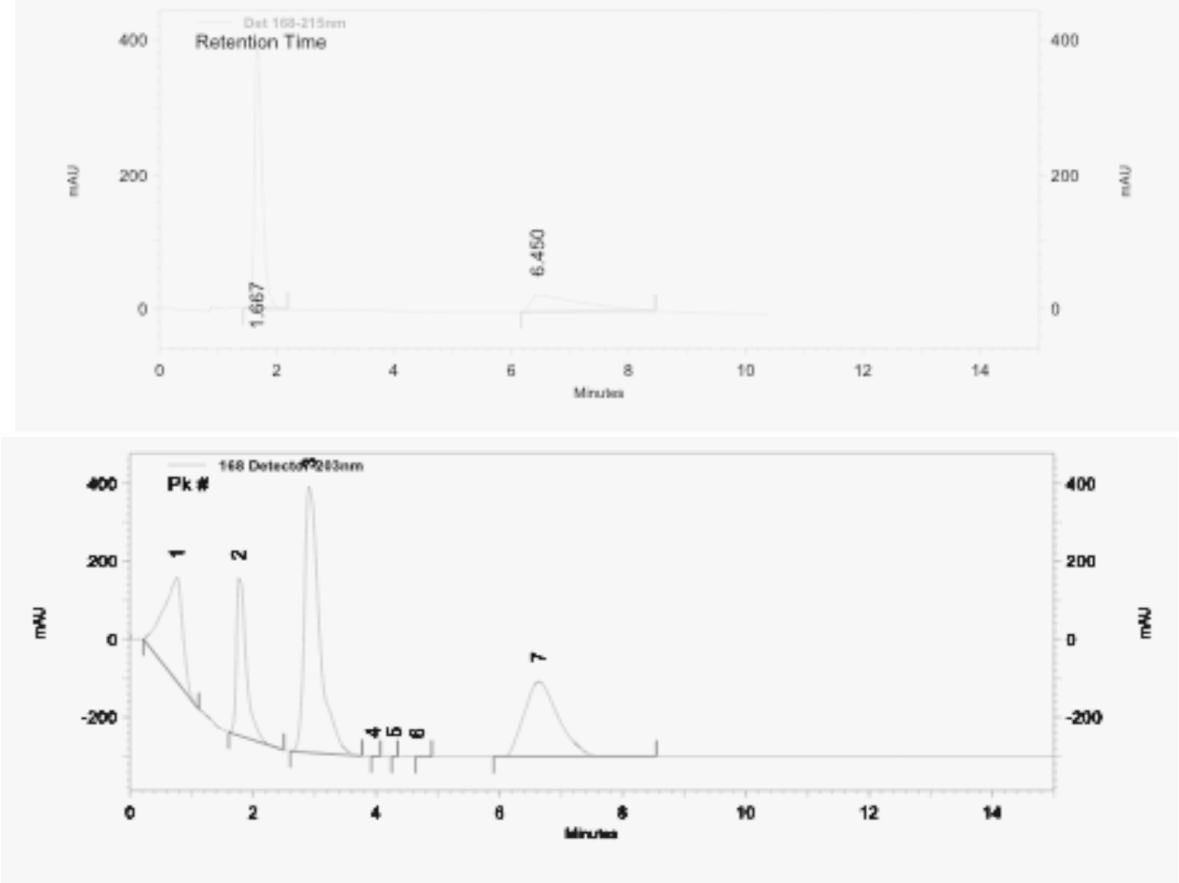

Figure 3.1. Reaction product analysis by HPLC

E. coli cells expressing *aaxBC* converted tritium-labeled L-arginine to agmatine. Separation of radiolabeled compounds by cation-exchange HPLC from a control mixture containing arginine (peak 1) and agmatine (peak 2) (top figure) is compared to a sample taken from a reaction with *E. coli* DEG0147 (pDG379) cells at pH 2.5 (bottom figure). Similar results were obtained for reactions using all other strains. Unlabeled agmatine was added as a carrier for product analysis. The second chromatogram shows the UV absorbance of arginine (peak 2) and agmatine (peak 7), which correlates with radioactivity (CPM) determined by liquid scintillation counting of 1-ml fractions as shown in the following figures.

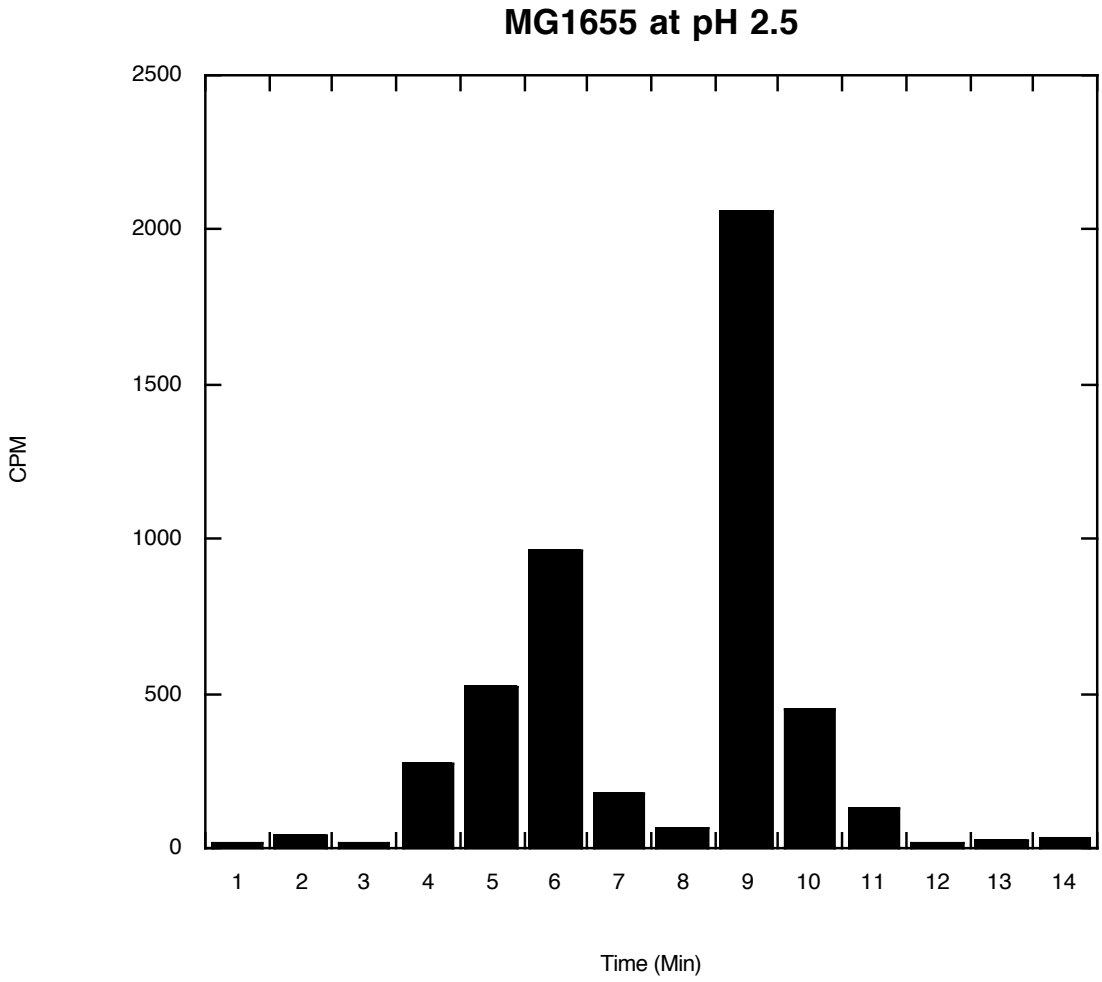

Figure 3.2. Conversion of arginine to agmatine in wild type *E. coli*.

E. coli MG1655 was used as the wild type control for the conversion of arginine to agmatine by whole cells. This strain expresses *adiAYC* and has an active AdiA arginine decarboxylase as well as an active arginine agmatine antiporter. The radiolabel in the collected samples corresponds to both the arginine peak and the agmatine peak after 2 hours. Data is presented as counts per minute (CPM).

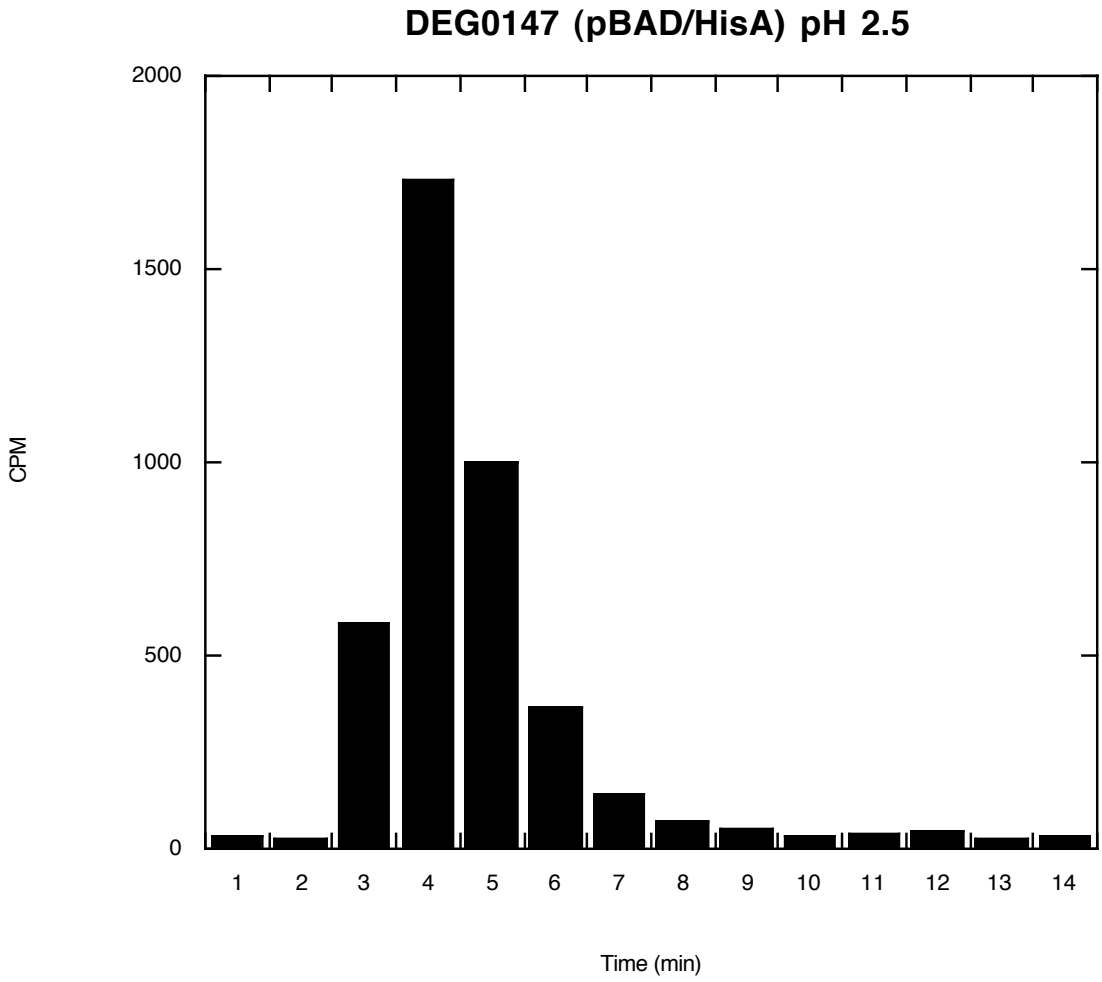

Figure 3.3. Conversion of arginine to agmatine is not detected in the DEG0147 negative control.

DEG0147 is a mutant derived from MG1655 that does not express *adiAYC*. Without AdiC and AdiA, this strain is unable to convert arginine to agmatine and as a result all radiolabel in the reaction mixture correlates with the arginine peak after a 2 hour reaction.

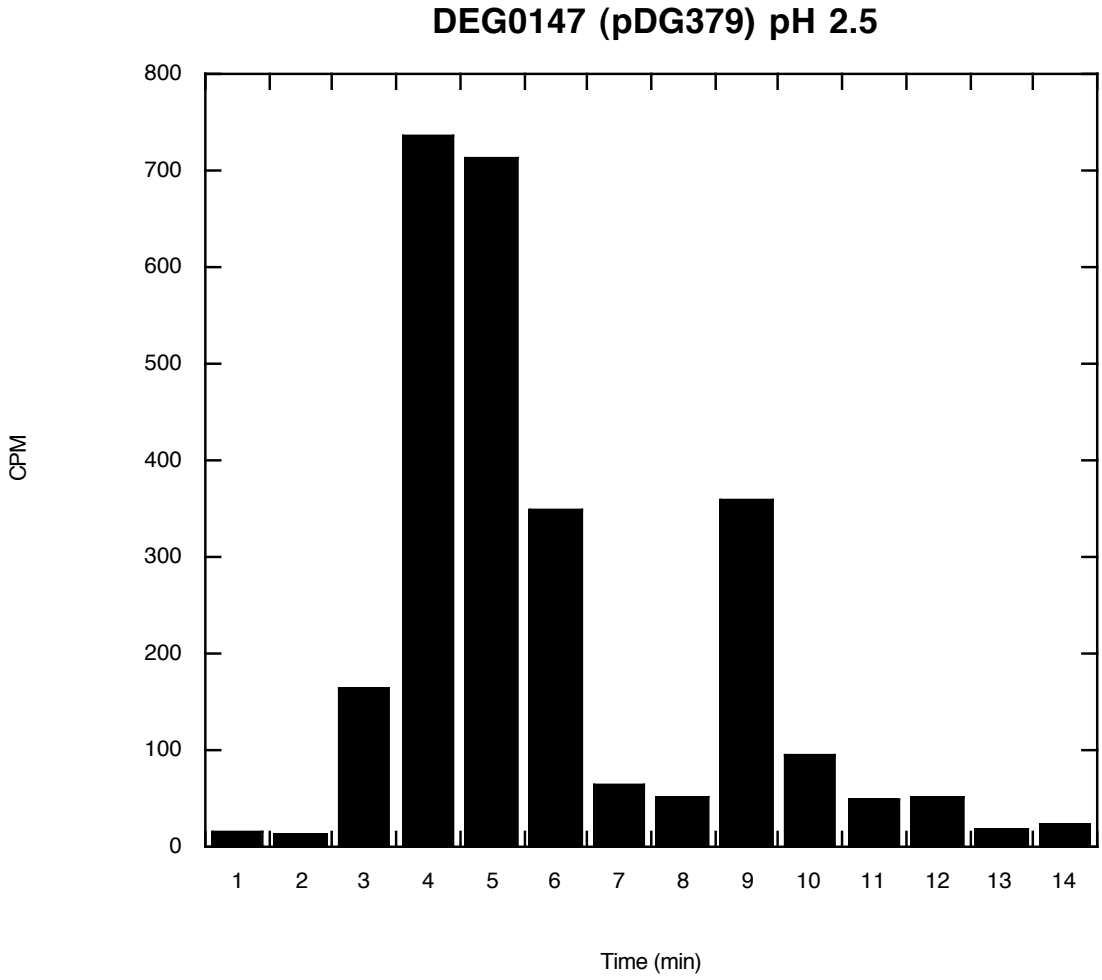

Figure 3.4. AaxB and AaxC can convert arginine to agmatine in *E. coli* at pH 2.5.

With the expression of AaxB and AaxC from pDG379 in *E. coli* DEG0147, to agmatine conversion is restored, although at levels lower than what was observed for wild type *E. coli*.

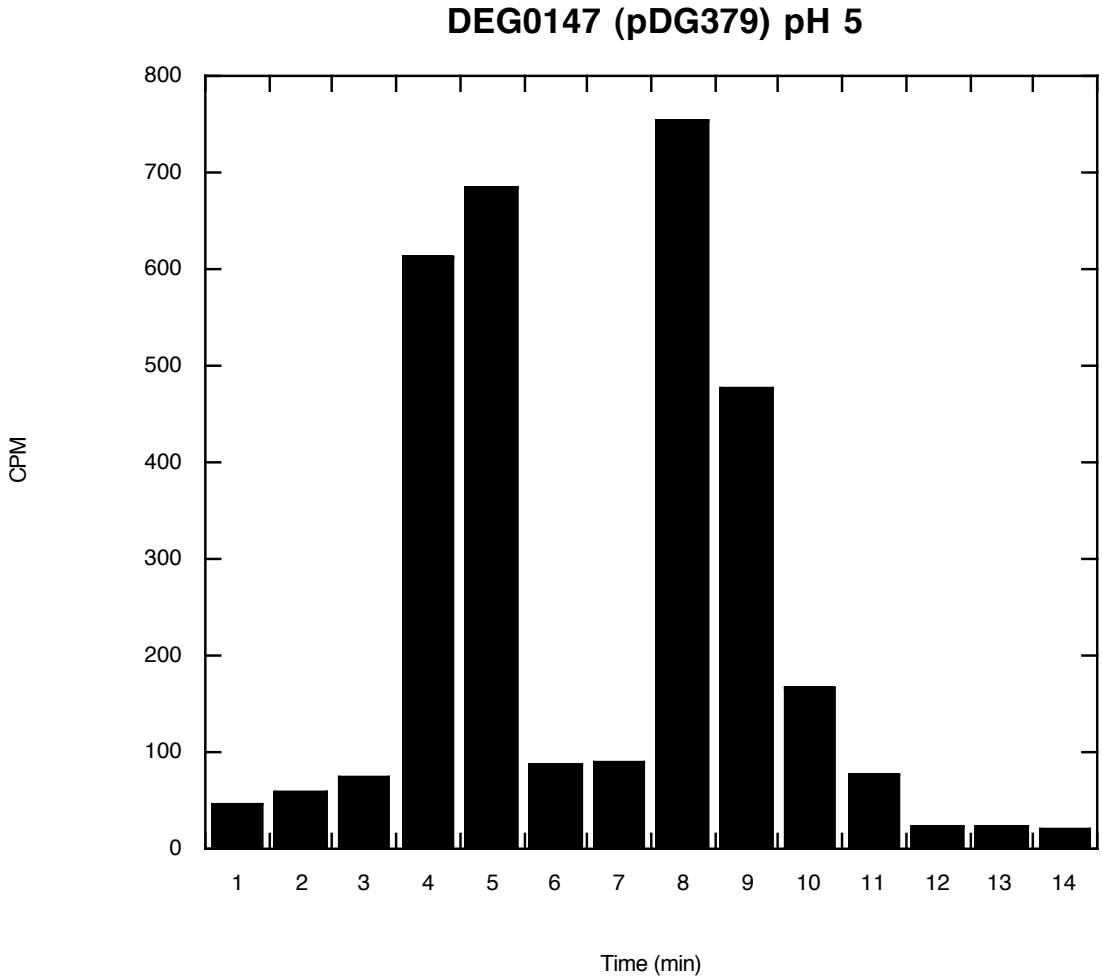

Figure 3.5. Conversion of arginine to agmatine by AaxB and AaxC is enhanced at pH 5.0.

At pH 5 conversion of arginine to agmatine by AaxB and AaxC is dramatically improved over the activity observed at pH 2.5, indicating that the chlamydial system has a higher optimal pH than that of *E. coli*.

3.3.2. Uptake and decarboxylation by Aax proteins

To simply measure arginine agmatine exchange by the Aax proteins in whole cells the decarboxylation of L-[^{14}C]arginine was used as a proxy. The decarboxylation reaction produces $^{14}CO_2$, which can be trapped on a barium hydroxide-saturated filter disc at the top of the reaction vial after acidifying and heating the reaction mixture. The radiolabel on each disc was measured using liquid scintillation counting. Reactions were carried out at a range of pH values using *E. coli* DEG0147 transformed with either the empty vector pBAD/HisA, *aaxB* in pDG339, *aaxBC* in pDG366, or *aaxABC* in pDG484 (Smith and Graham, 2008).

The negative control cells carrying pBAD/HisA showed no detectable decarboxylase activity, as expected in a background lacking the *E. coli* arginine decarboxylase AdiA (Figure 3.6). Expression of AaxB resulted in a small but significant amount of arginine decarboxylase activity but this activity was limited by the ability of the cells to import arginine using the native transport systems expressed under reaction conditions. Expression of AaxC together with AaxB resulted in a significant increase in activity over AaxB alone, approximately four times the arginine decarboxylase activity observed in the absence of the transport protein. Expression of the outer membrane protein AaxA along with AaxB and AaxC resulted in an even more impressive fifteen-fold increase in activity over AaxB alone (Smith and Graham, 2008). AaxA's role will be explored later in this dissertation (This work, Chapter 3).

In whole cell assays the arginine decarboxylase activity by AaxBC was observed to be greatest over of a pH range of 3 to 5 (Figure 3.6). This is consistent with the results for agmatine production detected by HPLC analysis. In both of these experiments arginine agmatine exchange by AaxC is driven by the decarboxylation of arginine by AaxB. In the previously discussed arginine uptake assays using AaxC in the absence of AaxB the optimum pH range was somewhat more basic with no detectable uptake at or bellow pH 4 and no significant drop in activity at pH 6 (Smith and Graham, 2008). The arginine decarboxylase AaxB was previously reported to have optimum activity at pH 3.4 which must cause the optimal pH of the AaxBC system as a whole to be lower with a significant drop in activity by pH 6 and almost no detectable activity at pH 7 (Giles and Graham, 2007, Smith and Graham, 2008).

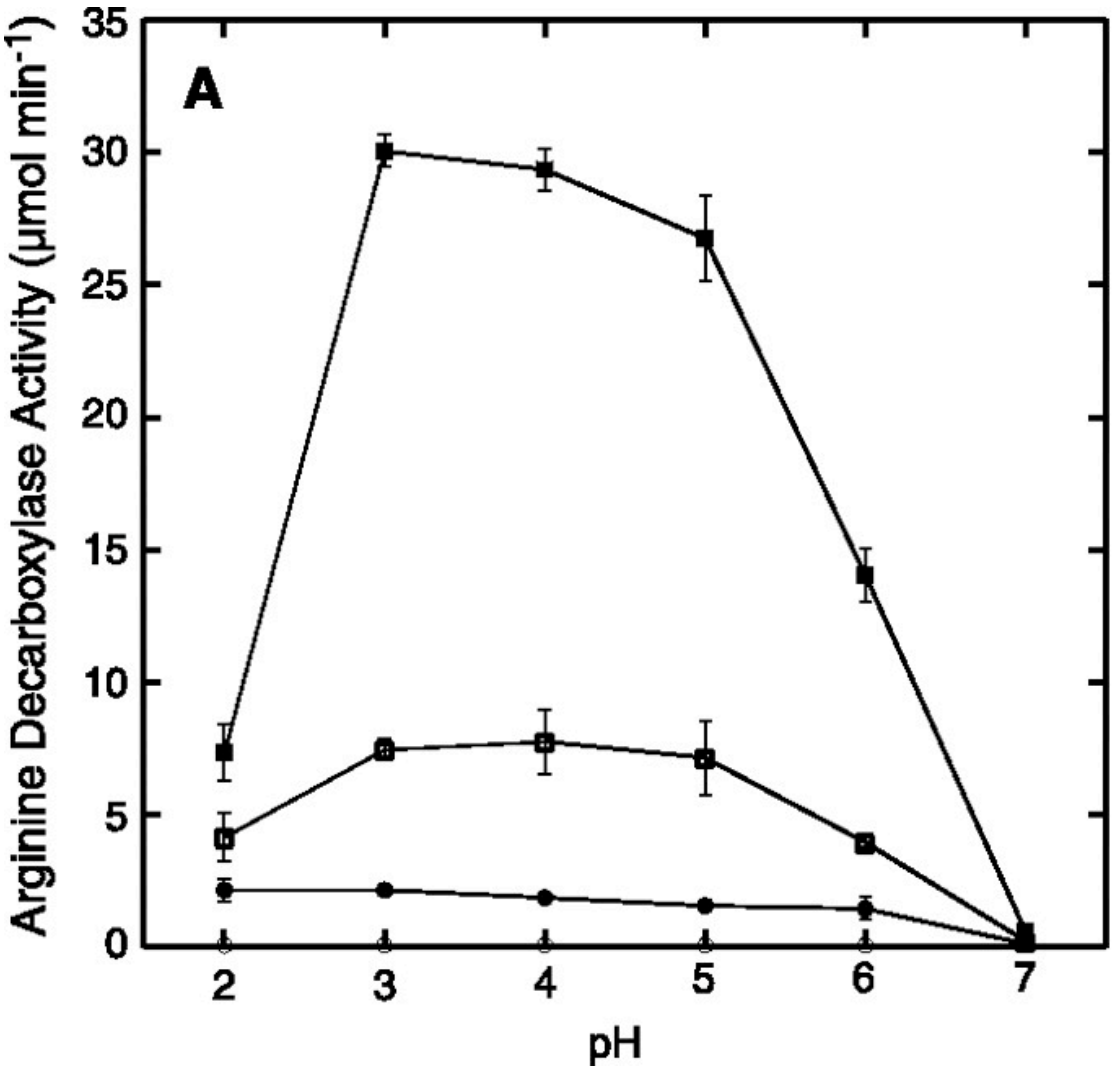

Figure 3.6. Arginine decarboxylase activity assays using whole cells

E. coli DEG0147 (Δ*adiAYC*::*kan*) cells expressing the *aaxABC* genes transport L-arginine and catalyze its decarboxylation. Reactions containing 3 x 10^9 cells were incubated with 0.5 mM [U-^{14}C]-L-arginine for 15 min at 37°C in 100 μl of E medium with buffer at the indicated pH. $^{14}CO_2$ was collected and analyzed as described in Materials and Methods. The DEG0147 strains contained empty vector pBAD/HisA (○), *aaxB* in pDG339 (•), *aaxBC* in pDG366 (□), and *aaxABC* in pDG484 (■). Error bars show the standard deviations from the mean of triplicate experiments.

3.3.3. Comparison with lysates

The whole cell decarboxylase assay results described above still leave the question of whether increased decarboxylase activity when AaxC and AaxA are expressed may only be an artifact caused by enhanced expression of AaxB from these plasmids. To examine this possibility decarboxylase assays were performed using total cell lysates, eliminating the effects of any transport proteins from the results (Figure 3.7). From these tests it was observed that there was no detectable arginine decarboxylase activity in lysates produced from DEG0147 (pBAD/HisA). The difference in decarboxylase activity between lysates from DEG0147 (pDG339) expressing AaxB and DEG0147 (pDG 366) expressing AaxBC was not statistically significant but the lysates from DEG147 (pDG484) expressing AaxABC showed activity 63% higher than that of cells expressing AaxB alone. Therefore, at least in DEG0147 (pDG484) increased AaxB expression contributes to the increase in whole cell decarboxylase activity that was observed. However, a 63% increase in decarboxylase activity cannot explain the 1500% increase in arginine uptake and decarboxylation observed in cells expressing AaxABC when compared with AaxB alone. Thus AaxA as well as AaxC clearly enhance activity in whole cells, owing to their roles in arginine uptake (Smith and Graham, 2008).

To determine if the composition of the reaction medium has any effect on the activity of AaxABC different reaction buffers were tested (Figure 3.8). Potassium and sodium salts were omitted in independent tests and citrate was replaced with tartrate

buffer. Additionally chloride ions were replaced with acetate. None of these changes resulted in any loss of activity suggesting that AaxABC is not dependent on any of these ions (Smith and Graham, 2008).

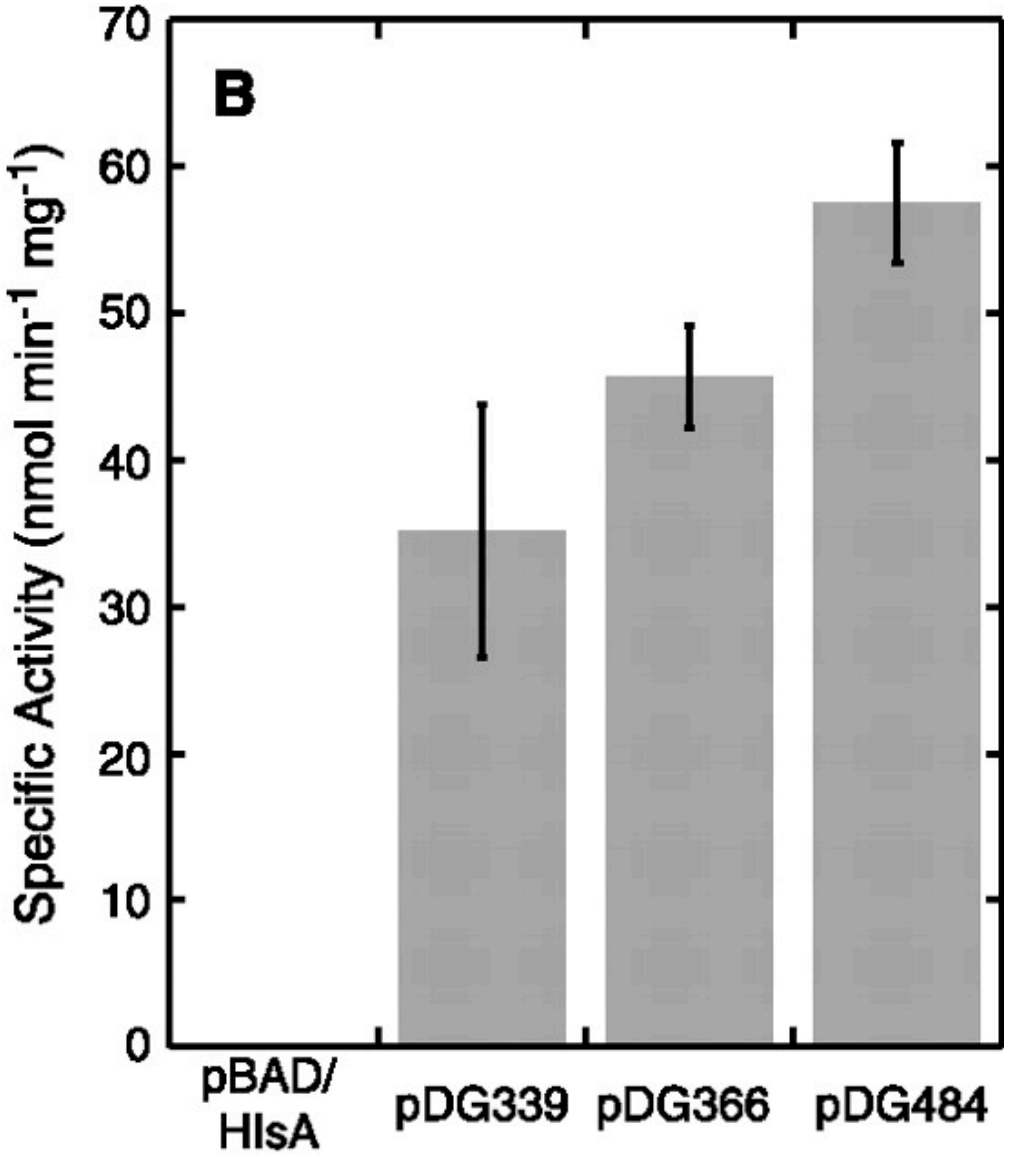

Figure 3.7. Arginine decarboxylase activity assays using cell lysates

Cell-free lysates from DEG0147 cells carrying the indicated plasmids were assayed for arginine decarboxylase activity at pH 4. No significant decarboxylase activity was detected in lysates of DEG0147 (pBAD/HisA) cells. Error bars show the standard deviations from the mean of triplicate experiments.

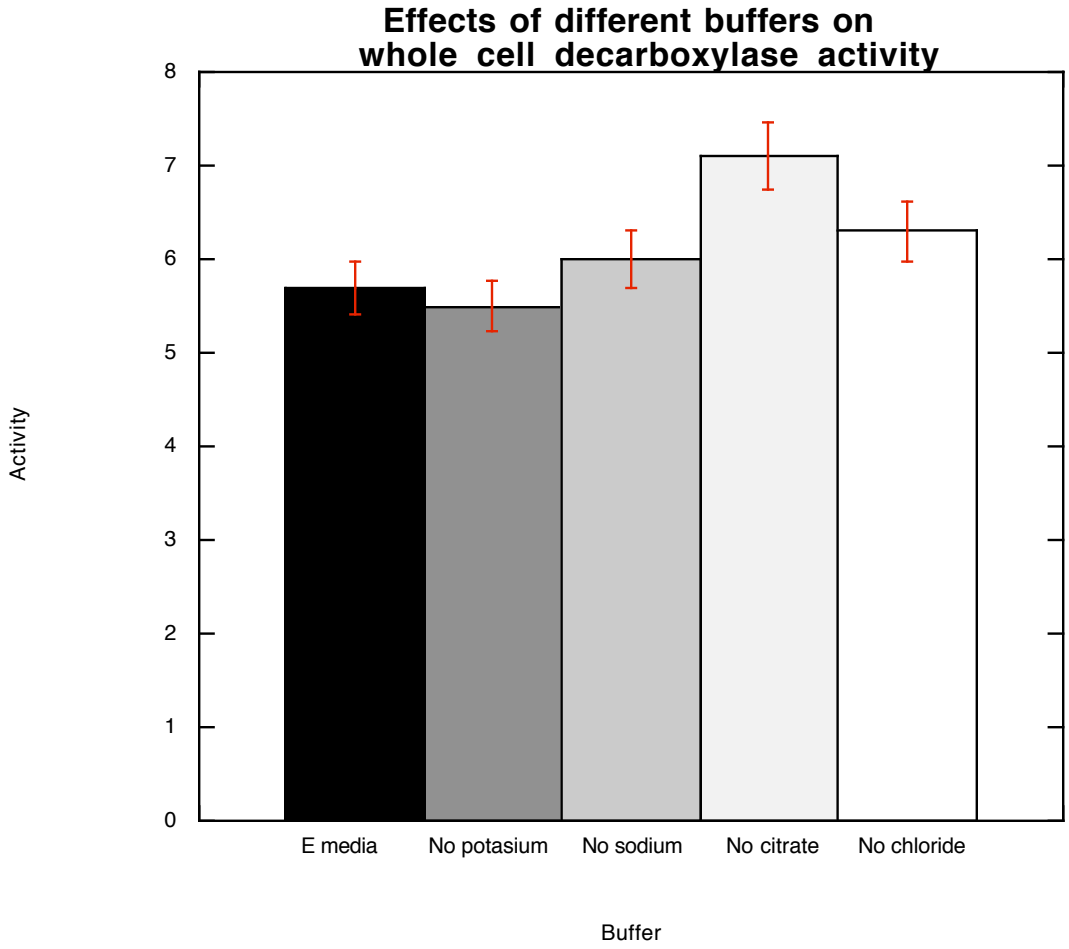

Figure 3.8. Arginine uptake and decarboxylation by AaxBC is not affected by changes in buffer composition.

E. coli DEG0147 cells carrying pDG366 containing *aaxBC* were used for decarboxylase assays in various buffers adjusted to pH 4. E media contains 73 mM K_2HPO_4, 17 mM Na_2HPO_4, 0.8 mM $MgSO_4$, and 10 mM sodium citrate. Buffer without potassium contained 90 mM Na_2HPO_4, 0.8 mM $MgSO_4$, and 10 mM sodium citrate. Buffer without sodium contained 90 mM K_2HPO_4, 0.8 mM $MgSO_4$, and 10 mM sodium citrate. Buffer without citrate contained 100 mM sodium potasium tartrate and 0.8 mM $MgSO_4$. Buffer without chloride was regular E media adjusted to the desired pH with acetate instead of HCl. Activity is given in μmol min^{-1}. Error bars show the standard deviations from the mean of triplicate experiments.

3.3.4. Inhibitors

It was previously reported that the arginine decarboxylase AaxB was inhibited by L-argininamide while L-canavanine was used as an alternative substrate (Giles and Graham, 2007). The arginine agmatine- antiporter AaxC is not inhibited by L-argininamide but it is inhibited by L-canavanine as well as D-arginine. Various arginine analogs were tested as potential inhibitors using the whole cell decarboxylase assay with *E. coli* DEG0147 (pDG366) cells at pH 6 (Figure 3.9). The arginine analogs tested were agmatine, L-arginine *O*-methyl ester, *N*-acetyl L-arginine, N^G-nitro-L-arginine methyl ester, D-arginine, L-argininamide, cadaverine, L-citrulline, L-homoarginine, L-histidine, L- lysine, and L-ornithine. Of these only L-canavanine and D-arginine were effective inhibitors, demonstrating 30% and 81% activity, respectively. These results agree with the inhibitor data for the arginine uptake assays and indicate that these compounds are imported by AaxC. Agmatine also showed some ability to inhibit arginine uptake, resulting in 86% activity. This contrasts with the results for inhibition of arginine uptake by AaxC alone in which agmatine did not significantly affect AaxC's activity.

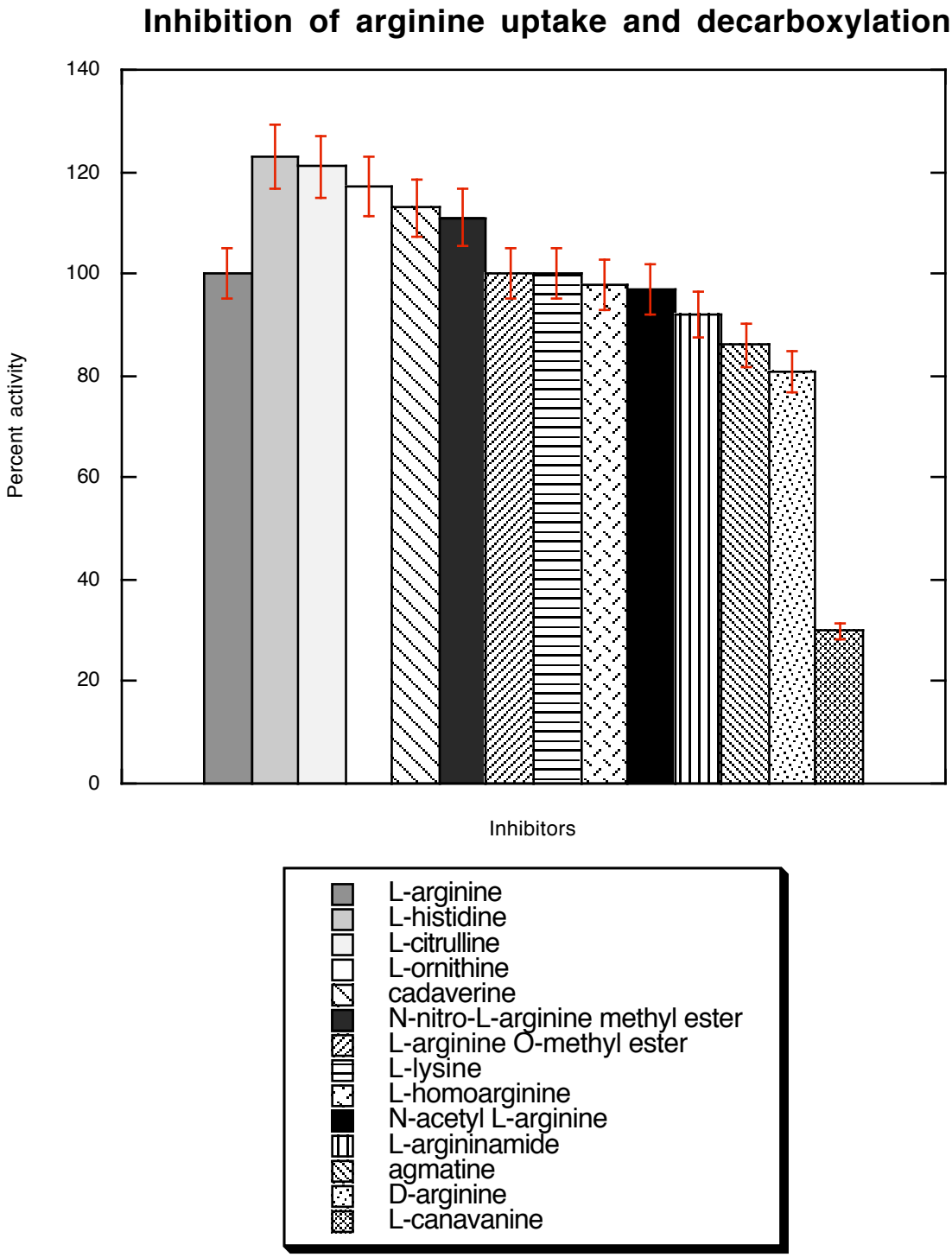
Inhibition of arginine uptake and decarboxylation
Percent activity
140
120
100
80
60
40
20
0
Inhibitors
L-arginine
L-histidine
L-citrulline
L-ornithine
cadaverine
N-nitro-L-arginine methyl ester
L-arginine O-methyl ester
L-lysine
L-homoarginine
N-acetyl L-arginine
L-argininamide
agmatine
D-arginine
L-canavanine

Figure 3.9. L-canavanine and D-arginine inhibit arginine uptake and decarboxylation by AaxBC.

E. coli DEG0147 (pDG366) cells were incubated with 2 mM concentrations of the indicated inhibitors before the addition of the arginine substrate. Whole cell decarboxylase assays were otherwise carried out as previously described. Potential inhibitors are displayed in order from least effective to most effective. L-canavanine was the most effective inhibitor tested.

3.4. DISCUSSION

AaxC is an amino acid transport protein capable of importing arginine into the cell while exporting agmatine. In assays using *E. coli* cells expressing AaxB and AaxC, separation of the radiolabeled compounds in the media by strong cation exchange HPLC after the removal of cells showed that agmatine was not only being produced, it was being exported into the extracellular environment. The production and export of agmatine as observed from the results of these assays shows that pH 2.5, the optimum pH for arginine-agmatine exchange by AdiA and AdiC, is not the optimum pH for the chlamydial proteins. AaxB has previously been shown to have a low optimal pH of 3.4 (Giles and Graham, 2007). In spite of this very low pH optimum, agmatine production by AaxBC was much higher at an extracellular pH of 5 than pH 2.5. This result is consistent with the optimum pH of AaxC for arginine uptake as detailed in the previous chapter. AaxC was able to exchange radiolabeled extracellular arginine for unlabeled intracellular arginine most efficiently at pH 5 and based on the HPLC data, which shows AaxBC operating most efficiently at pH 5, it is apparent that uptake by AaxC is the limiting step.

Arginine-agmatine exchange by AaxBC is still significant at pH 2.5, as evidenced in the previous chapter where this system was shown to rescue acid survival in *E. coli adiAYC* mutants. Arginine uptake by AaxC must be sufficient for acid-resistance even at this pH but the fact that its pH optimum is so much higher than that of AdiC suggests that AaxC did not evolve to handle extremely low pH environments. This is consistent with the current understanding of chlamydial pathogenesis in which this organism does not have to cope with such a low pH.

Whole cell decarboxylase assays showed that arginine decarboxylation by AaxB in *E. coli* is significantly enhanced by the transport activity of AaxC. This increase is most pronounced between pH 3.0 and pH 5.0, and there is no significant difference in the activity across this range. There is some observable arginine decarboxylase activity in the absence of the AaxC transporter, probably due to some background level of arginine uptake by native *E. coli* amino acid transporters. The whole cell arginine decarboxylase activity is increased 4-fold by AaxC, suggesting a large enhancement of arginine uptake across the cytoplasmic membrane by this transporter. Importantly, there is no detectable activity in control cells that do not express the chlamydial proteins. This shows that there is no background arginine decarboxylase activity by any *E. coli* enzymes under the conditions tested.

The outer membrane porin AaxA was observed to enhance the uptake and decarboxylation of arginine in whole cells by an additional 4-fold over AaxBC. By

facilitating arginine diffusion through the outer membrane AaxA seems to contribute to the chlamydial arginine-agmatine exchange system. AaxA will be investigated more thoroughly in chapter 4.

To insure that the observed effects by AaxC and AaxA were not merely the result of increased AaxB expression from the plasmids, decarboxylase assays were performed on cell lysates. In these lysates AaxB arginine decarboxylase activity could be observed without any dependence on porins or transport proteins and therefore it was expected that the activity in the lysates would not change significantly whether cell expressing *aaxABC*, *aaxBC*, or *aaxB* were used. No significant difference was observable between cells expressing AaxC and AaxB and those expressing AaxB alone, as expected. This confirms that the increase in whole cell decarboxylase activity was the result of enhanced arginine uptake rather than higher levels of AaxB being expressed. There was a small but significant difference in the activity of cells expressing AaxA with AaxB and AaxC, roughly 63% higher than cells expressing AaxB alone, but this does not account for the observed increase in activity in whole cells expressing all 3 proteins, which was 1500% when compared with AaxB alone.

Arginine uptake and decarboxylation by AaxBC in *E. coli* was observed to be unaffected by the presence or absence of the components of the E media used for the initial assays. Replacing E media with tartrate buffer containing no citrate or phosphate had no effect, nor did using acetate instead of HCl to adjust the pH of E media.

Individually removing buffers with sodium or potassium ions likewise had no effect. These results suggest that AaxC is not dependent on sodium, potassium, chloride, phosphate, or citrate for activity, although all of these were originally present in the medium used for whole cell decarboxylase assays.

Inhibition assays of arginine uptake in whole cells expressing AaxB and AaxC gave similar results to those performed with AaxC alone. L-Canavanine and D-arginine were both shown to inhibit arginine uptake while other arginine analogs did not. Agmatine, the arginine decarboxylation product, was not able to detectably inhibit AaxC alone but because of the relatively low rate of arginine-arginine exchange compared with arginine-agmatine exchange driven by AaxB and the high background of the arginine uptake assay it is possible that there was some inhibition that was too slight to be detected. Using whole cell decarboxylase assays with *E. coli* cells expressing AaxB and AaxC there was a small but significant amount of inhibition by agmatine. Since HPLC assays already showed that agmatine was exported in cells expressing AaxC, the ability of extracellular agmatine to compete with arginine for uptake by AaxC is not suprising, even if the level of inhibition is very low.

The results discussed in this chapter indicate that AaxABC is a system capable of importing and decarboxylating arginine and exporting the decarboxylation product agmatine. This process is most efficient between pH 3 and pH 5 and it is not dependent on the presence of specific ions in the medium. Suprisingly AaxA, which has no analog

in arginine-agmatine exchange systems in enterics, seems to have a large impact on the activity of the Aax system, when expressed in *E. coli*. This was unexpected, because *E. coli* outer membrane porins were predicted to take up sufficient arginine so that diffusion of arginine across the outer membrane was not expected to be limiting. AaxA will be investigated in detail in the next chapter.

CHAPTER 4

The AaxA porin

4.1. OVERVIEW

AaxA from *C. pneumoniae* is an outer membrane porin capable of enhancing the arginine uptake and decarboxylation activity by AaxB and AaxC, as shown in the previous chapter. The further characterization of this protein will be detailed in this chapter. To verify that AaxA is in fact an outer membrane porin, the localization of AaxA to the outer membrane was confirmed experimentally. This result, together with the activity shown in whole cell arginine decarboxylase assays using *E. coli* cells expressing *aaxABC* is compelling, but if AaxA is an outer membrane porin capable of enhancing arginine uptake through the outer membrane, it should not be dependent on AaxB and AaxC to do so. Therefore, wild type *E. coli* and *E. coli* porin mutants were used in whole cell arginine decarboxylase assays to show that AaxA can enhance arginine uptake and decarboxylation activity by AdiA and AdiC.

With AaxA's activity confirmed independent of the other chlamydial proteins it was next compared with the major outer membrane protein (MOMP) from *C. pneumoniae*. MOMP is a general porin and is able to enhance arginine uptake by AdiA and AdiC in a manner similar to AaxA. Although both MOMP and AaxA were active under the conditions tested, MOMP has several conserved cysteine residues that are lacking in

AaxA and it has been reported that MOMP's porin activity may be compromised under oxidizing conditions (Bavoil et al. 1984). It was therefore possible that AaxA could be active under conditions where MOMP was not. Whole cell arginine decarboxylase assays comparing oxidizing conditions to reducing conditions showed a large decrease in arginine uptake by MOMP under oxidizing conditions that was not observed for AaxA. It is not currently known when AaxA is expressed during the chlamydial life cycle but this difference between the arginine uptake activities of these two chlamydial porins is and interesting finding.

Liposome swelling assays using AaxA were used to determine the specificity of AaxA. It was originally hypothesized that AaxA might be a specific porin only involved in arginine uptake. To examine this hypothesis a variety of solutes were tested for uptake by AaxA including amino acids, polyamines, and sugars. The results of these experiments showed that AaxA is a general porin rather than a specific porin and capable of taking up a wide variety of substrates. Of the solutes tested, only large solute molecules (above about MW 260) and solute molecules with a negative charge were excluded. In general, positively charged solutes seem to be most favored with arginine and arginine analogs being the preferred substrates. As the pH is increased and arginine loses its positive charge its rate of uptake is also reduced. Solute uptake by AaxA at low pH is reduced much more sharply and agrees with whole cell arginine decarboxylase assays that show reduced or no increase in activity by AaxA at low pH values. This

result also explains why AaxA did not enhance acid survival at pH 2.5, since AaxA has no detectable activity under such acidic conditions.

4.2. EXPERIMENTAL PROCEDURES

4.2.1. Expression, refolding, and purification of AaxA

E. coli strains were grown aerobically at 37°C overnight in LB Miller broth supplemented with ampicillin (100 μg ml^{-1}) and L-arabinose (0.15% [wt/volume]) for DEG0156 carrying pBAD/HisA or derivative plasmids and with α-D-lactose (1% [wt/volume]) for BL21 (DE3) containing the pET-43.1a-derived pDG596 plasmid.

The $(His)_6$-tagged truncated AaxA was expressed from pDG596 in *E. coli* BL21 (DE3) and refolded from inclusion bodies in vitro following the method of Rutten et al. 2008. Inclusion bodies were dissolved in 8M urea, 10mM glycine pH 8.3 and AaxA was refolded by rapid 10-fold dilution into 4% *n*-dodecyl-β-D-maltoside (DDM) (wt/volume) 20 mM glycine pH 8.3. The diluted sample was then incubated at 37°C for 18 h and excess urea was removed by dialysis in 5mM Tris-HCl pH 8 containing 0.1% DDM (wt/vol). The refolded AaxA was purified using Ni-NTA agarose affinity chromatography. 1 ml of packed Ni-NTA agarose was washed 3 times with 5 ml of deionized water and twice with 5 ml of wash buffer containing 20mM Tris-HCl, 20% glycerol (vol/vol), 0.05% DDM (wt/vol) pH 8. Washes were carried out by centrifugation at 180 g for 1 min using a Beckman Coulter JS-5.3 swinging bucket rotor.

The Ni-NTA agarose was then equilibrated in wash buffer on ice for 1 h before being mixed with the refolded AaxA at 4°C for 2 h before being sedimented and washed 10 times in 5 ml wash buffer. The Ni-NTA agarose was then washed 5 times with 1 ml elution buffer containing 20 mM Tris-HCl, 200mM imidazol, 10 % glycerol (vol/vol), and 0.05% DDM (wt/vol) pH 7.5. Supernatants from the washes with elution buffer were analyzed by SDS-PAGE. The eluant was concentrated by centrifugation.

4.2.2. Arginine uptake and decarboxylation assays with whole cells

These assays were performed as described previously (Smith and Graham 2008) using DEG0156 carrying pBAD/HisA, pDG512 (AaxA), or pDG569 (MOMP). 1mM dithiothreitol (DTT) was added to reaction mixtures to determine the effects of reducing conditions on activity. All reactions were carried out at pH 4.0.

4.2.3. Liposome swelling assays

Porin function of AaxA using either extracted outer membrane protein from *E. coli* DEG0156 carrying pBAD/HisA or pDG512 or the refolded and purified AaxA expressed from BL21 (DE3) pDG596 was assessed using the liposome swelling assay of Nikaido and Rosenberg (1983). Liposomes containing 2.4 μmol of egg phosphatidyl-choline and 0.2 μmol of dicetylphosphate were made by suspending the lipids in chlorform and drying under nitrogen at 45°C for 3 hours before the film was resuspended in 0.2 ml aqueous solution containing the porin and sonicated in a bath-type sonicator for 20 min.

The solution was dried for 2 minutes by rotary evaporation in a water bath at 45°C and then additionally dried for 2 hours in a desicator over $CaSO_4$. The proteoliposomes were resuspended in 0.4 ml 15% dextran (M.W. 35,000-50,000) in 5 mM Tris-HCl, pH7.5. 20 μl of dextran-containing liposomes was diluted into 0.6 ml test solutions containing 5 mM Tris-HCl, pH7.5 with an iso-osmotic concentration of the test solute. Iso-osmotic concentrations, the concentrations that caused no net swelling or shrinking of the liposomes, were determined independently for all substrates using liposomes containing no porin. The change in absorbance of the test solution was measured at 400 nm for 3 minutes. The swelling rates were determined as $\Delta(1/A)/\Delta t$ from the absorbance changes between 0 and 30 s. To test the effect of antibodies on AaxA-mediated liposome swelling proteoliposomes were incubated with HSV-tag monoclonal antibody or T7-tag monoclonal antibody (Novagen) for 30 minutes before diluting proteoliposomes into the test buffer.

Additionally outer membranes were obtained from DEG0156 (pDG512) and DEG0156 (pBAD/HisA) by sucrose-density gradient centrifugation as previously described and the washed outer membrane preparations were reconstituted into liposomes following the above procedure (Smith and Graham, 2008).

4.3. RESULTS

4.3.1. Expression and localization to the outer membrane

The *aaxA* gene from *C. pneumoniae* was fused to a carboxy-terminal HSV tag and expressed from the plasmid pDG512 in *E. coli* DEG0147 as well as in *E. coli* BW25113 with pDG552 and pDG561 and in BL21 (DE3) with pDG552. The LepA-S-Tag cytoplasmic marker protein was expressed from pDG561 and the OmpX-S-Tag outer membrane marker protein was expressed from pDG552. The cells were grown aerobically, harvested, lysed, and the membranes were separated by sucrose density gradient centrifugation as described in the experimental procedures in chapter 3. The proteins were separated by SDS-PAGE and analyzed by Western blotting with a monoclonal HSV antibody. The cells expressing AaxA-HSV showed a new band with an apparent molecular mass of 44 kDa (49 kDa calculated). This band was identified in the lane corresponding to the outer membrane fraction but was not associated with the cytoplasmic membrane fraction (Figure 4.1). Additionally the AaxA band colocalized with the outer membrane marker OmpX-S-tag, which was detected using a monoclonal antibody against the S-Peptide (Smith and Graham, 2008).

The outer membrane fraction was washed with 5 M urea to remove protein expressed in inclusion bodies, which can colocalize with outer membrane proteins. Only

very small amounts of epitope-tagged protein were removed by this urea wash, demonstrating that AaxA is an integral outer membrane protein. Some of the heterologously expressed OmpX-S-Tag protein formed insoluble aggregates, which has been previously reported (Pautsch et al., 1999). Even so, a significant amount of the protein incorporated into the outer membrane fraction and was shown to resist removal by washing with urea (Smith and Graham, 2008).

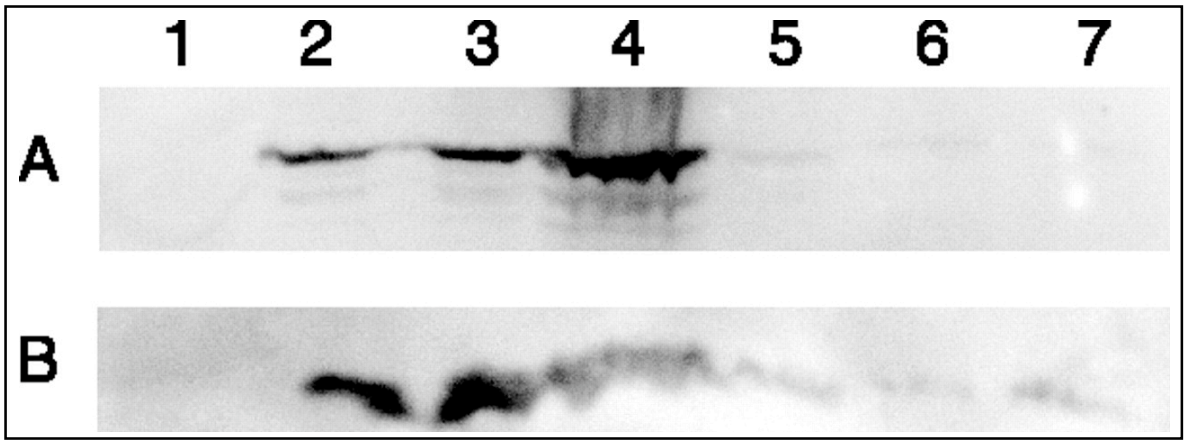

Figure 4.1. AaxA-HSV is localized to the outer membrane

Western blots show the AaxA-HSV protein was expressed in the outer membrane of *E. coli*. Lane 1 contains total membrane extract from *E. coli* BW25113(pBAD/HisA) control cells. Lane 2 contains the soluble portion of lysate from BL21(DE3)(pDG512, pDG552) cells expressing *C. pneumoniae* AaxA-HSV and *E. coli* OmpX-S-Tag proteins, after centrifugation for 15 min at 18,000 x *g*. Lane 3 contains the insoluble portion of the lysate from BL21(DE3)(pDG512, pDG552) cells. Lane 4 contains the outer membrane fraction of the same cells, purified by sucrose density-gradient centrifugation and washed with 5 M urea. Lane 5 contains urea-soluble material from washing the outer membrane fraction shown in lane 4. Lane 6 contains the cytoplasmic membrane fraction from sucrose density-gradient centrifugation. Lane 7 contains the pellet from the sucrose density-gradient centrifugation. (A) The AaxA-HSV protein was detected using an anti-HSV monoclonal antibody. (B) The OmpX-S-Tag protein was detected using an anti-S-Tag monoclonal antibody.

4.3.2. AaxA enhances uptake, DC activity by AdiAC

AaxA was able to significantly enhance arginine uptake and decarboxylation in whole cell decarboxylase assays using *E. coli* DEG0147 (pDG484) as described in chapter 3. In those assays the AaxB arginine decarboxylase and the AaxC arginine agmatine antiporter from *C. pneumoniae* were responsible for the activity and AaxA presumably enhanced this activity by acting as an outer membrane porin and increasing the rate at which arginine could enter the cell. If this is the case, AaxA was predicted to be able to enhance arginine uptake and decarboxylase activity by the arginine decarboxylase AdiA and the arginine agmatine antiporter AdiC expressed in wild type *E. coli*. *E. coli* MG1655 (pDG512) cells were tested for arginine uptake and decarboxylation along with MG1655 (pBAD/HisA) at pH 2.5, the optimum pH for the *E. coli* arginine agmatine exchange system, but there was no significant difference in activity between the cells expressing AaxA and the control cells. However, at pH 4, a compromise between the optimum pH of the *E. coli* proteins and the heterologously expressed AaxA, cells expressing AaxA showed a 4-fold increase in activity over the control strain, a result consistent with what was observed with AaxA in cells expressing AaxBC instead of AdiAYC (Figure 4.2). In chapter 2 it was shown that AaxA did not enhance acid survival at pH 2.5, this additional finding that AaxA cannot enhance arginine agmatine exchange at pH 2.5 helps explain that result, suggesting that AaxA is not effective at such low pH (Smith and Graham 2008).

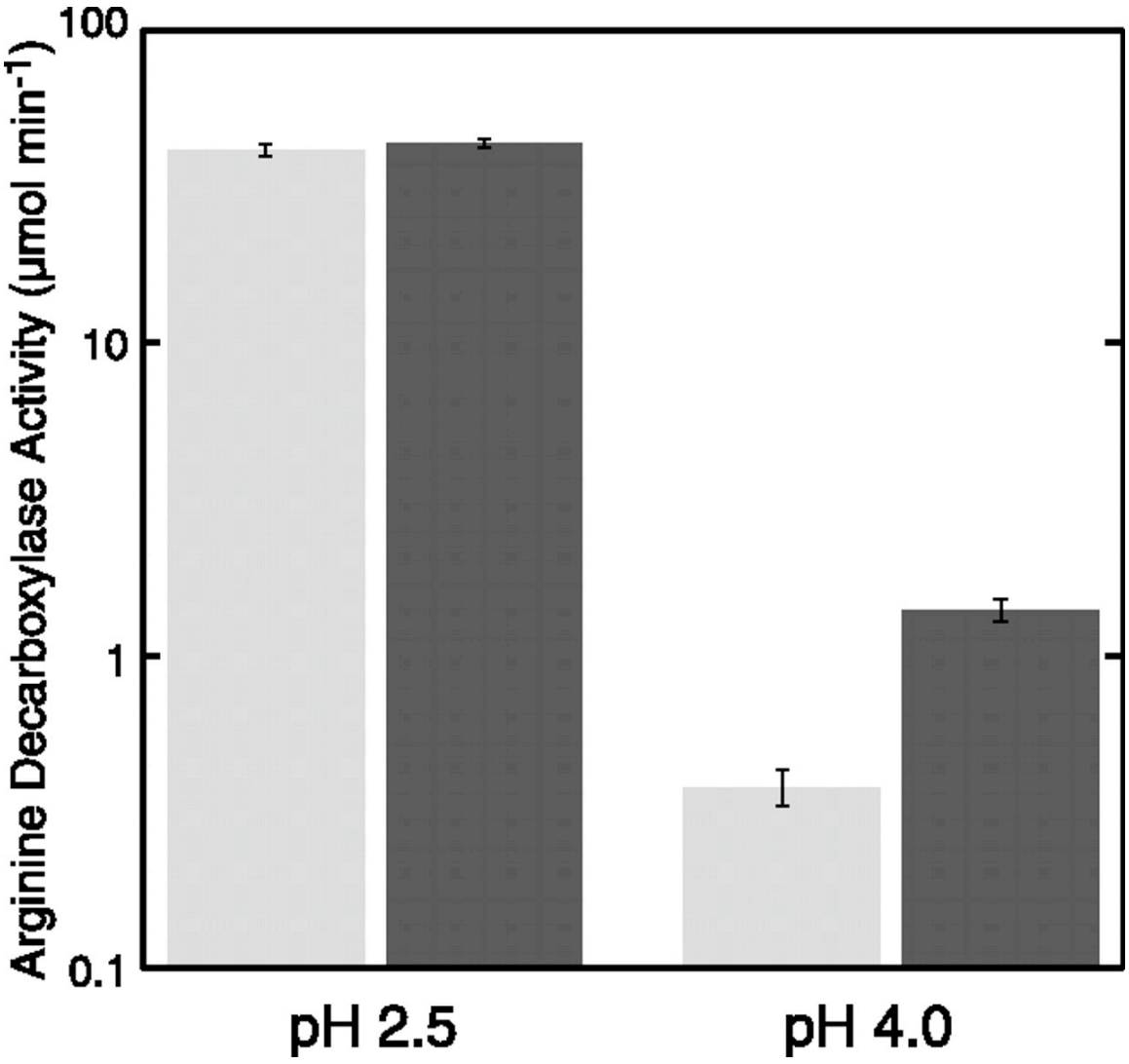

Figure 4.2. AaxA enhances whole cell decarboxylase activity in a pH-dependent fashion.
E. coli MG1655 cells expressing no chlamydial porin (light gray) and AaxA from pDG512 (dark gray) were assayed for arginine decarboxylase activity at pH 2.5 and pH 4.0. Cells were incubated with [^{14}C]-L-arginine as described previously. Arginine uptake and decarboxylation by AdiC and AdiA is enhanced by AaxA at pH 4.0, but not at pH 2.5. Error bars show the standard deviations from the mean of triplicate experiments.

To further examine the effects of AaxA on arginine uptake in these whole cell decarboxylase assays an *E. coli* strain depleted for OmpC and OmpF was constructed to reduce interference from *E. coli* porins. The *E. coli* strain DEG0156 was therefore used to further assess the activity of heterologously expressed AaxA and to compare it to the major outer membrane protein (MOMP) from *C. pneumoniae*. DEG0156 was transformed with pBAD/HisA to act as a negative control, pDG512 which expresses AaxA, or pDG569 which expresses MOMP. It was observed that in this reduced background strain both MOMP and AaxA significantly increase arginine uptake and decarboxylase activity over *E. coli* carrying pBAD/HisA under the conditions tested (Figure 4.3). Compared with the negative control, AaxA increased arginine uptake and decarboxylation 6-fold while MOMP increased activity 4-fold. Because of differences in the expression levels of MOMP and AaxA when heterologously expressed in *E. coli*, it cannot be concluded from this result alone that AaxA is more efficient at arginine uptake than MOMP but in *E. coli* depleted for OmpC and OmpF it is clear than both chlamydial porins dramatically increase arginine uptake under the conditions tested.

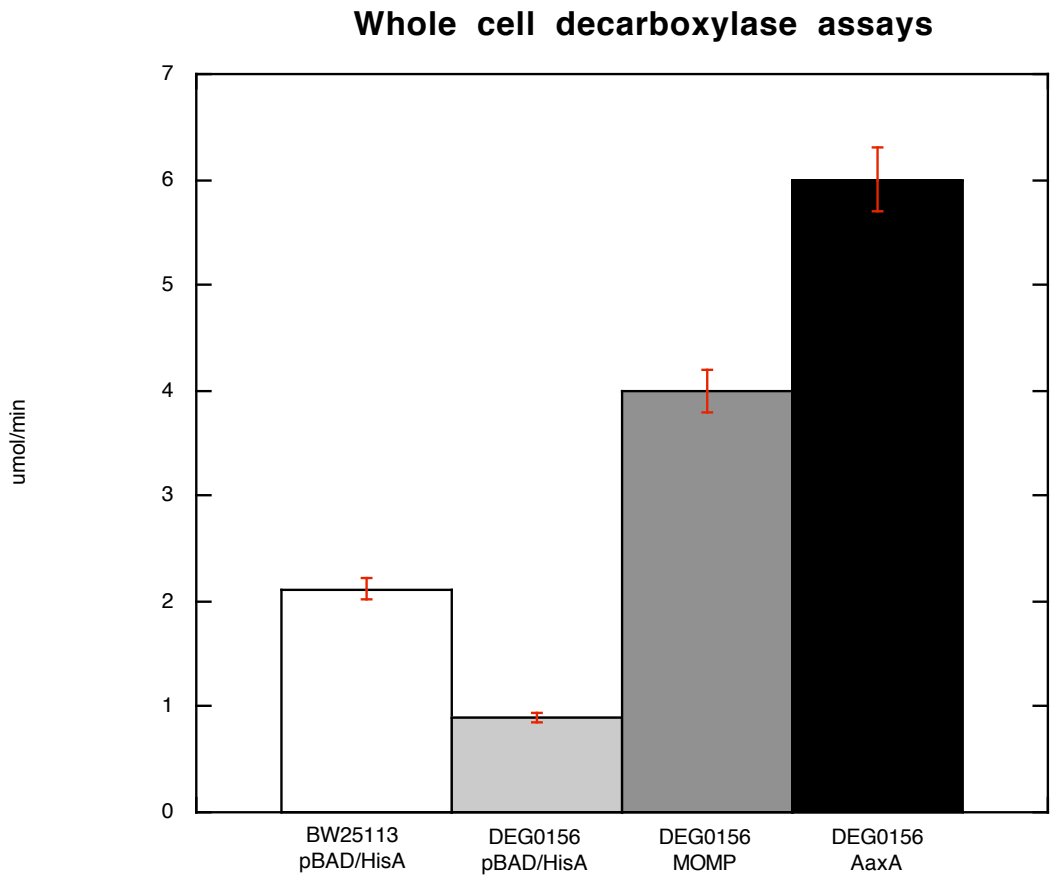

Figure 4.3. AaxA and MOMP enhance whole cell decarboxylase activity

E. coli BW25113 as well as E. *coli* DEG0156 (Δ*ompC* Δ*ompF*) cells expressing no chlamydial porin (pBAD/HisA), MOMP (pDG569), and AaxA (pDG512) were assayed for arginine decarboxylase activity at pH 4.0. Both MOMP and AaxA demonstrated an ability to significantly enhance the arginine uptake and decarboxylation activity of *E. coli* AdiA and AdiC, effectively rescuing the function lost in DEG0156 by deleting *ompC* and *ompF*. Error bars show the standard deviations from the mean of triplicate experiments.

4.3.3. Oxidizing vs. reducing conditions

While both MOMP and AaxA allow arginine to efficiently cross the outer membrane it was not clear if both would do so under other conditions than those initially tested. It has been demonstrated that MOMP activity can be enhanced in reducing environments due to a change in the crosslinking of cysteine residues (Bavoil et al., 1983). Due to the lack of conserved cysteine residues in AaxA, it was expected that there should be no significant change in activity when 10 mM DTT was added to reactions. The results for whole cell decarboxylase assays comparing reducing conditions to oxidizing conditions showed a 38% enhancement in arginine uptake and decarboxylation for cells expressing MOMP when under reducing conditions but no significant difference was observed for cells containing AaxA under the same conditions (Figure 4.4). These results were supported by the findings that the addition of DTT had no effect on the activity of AaxA using the liposome swelling assays detailed in section 4.3.5.

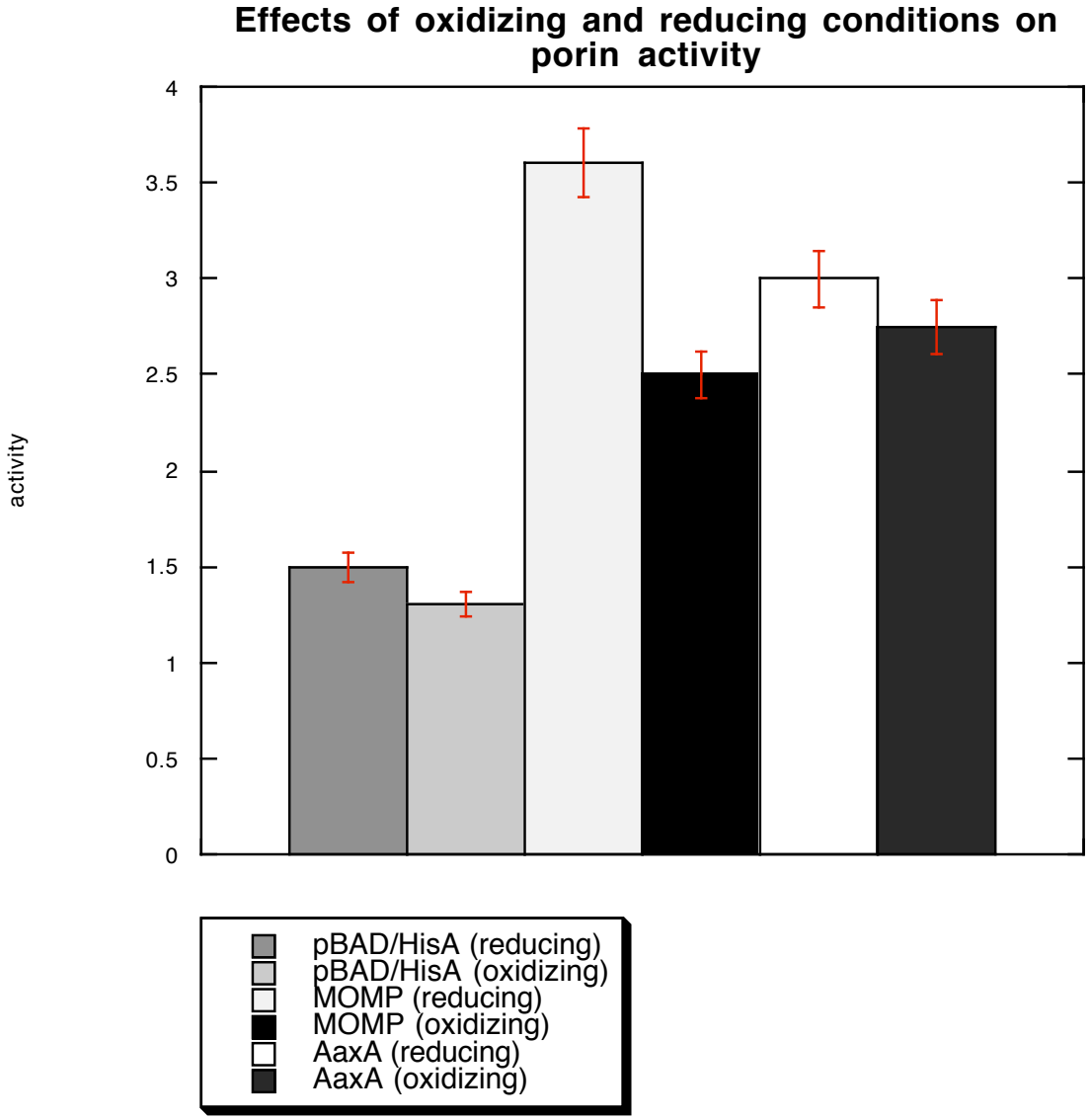

Figure 4.4. AaxA porin activity is not significantly inhibited under oxidizing conditions.

Whole cell arginine decarboxylase assays were performed using DEG0156 to reduce background activity from OmpC and OmpF. MOMP was expressed from pDG569, AaxA was expressed from pDG512, and pBAD/HisA was used as a control. Activity is given in μmol min^{-1}. Reaction mixtures were incubated with 10 mM concentrations of DTT to create reducing conditions or 10 mM iodine to create oxidizing conditions. Reactions were carried out at pH 4 and were otherwise unchanged from the whole cell decarboxylase assays described previously. These results indicate that while MOMP

from *C. pneumoniae* is partially inhibited under oxidizing conditions, AaxA is not significantly affected. The pBAD/HisA negative control also did not demonstrate a significant change in arginine uptake by remaining *E. coli* porins. Error bars show the standard deviations from the mean of triplicate experiments.

4.3.4. Refolding and purification of AaxA

Active AaxA porin, as determined by the liposome swelling assays described in the next section, was obtained both from the outer membranes of DEG0156 (pDG512) obtained by sucrose-density gradient centrifugation and from protein refolded from inclusion bodies in BL21 (DE3) (pDG596). Comparisons between OMP-containing liposomes from DEG0156 (pDG512) and DEG0156 (pBAD/HisA) were used to verify that liposome swelling in the presence of various substrates was mediated by AaxA and not *E. coli* porins such as OmpA. In spite of the notable difference in the uptake of different substrates by OM enriched for AaxA as opposed to *E. coli* OM not expressing this porin, heat modifiability, a trait of many outer-membrane proteins, was not observed by gel electrophoresis (Figure 4.5). The heat-denatured form of AaxA showed the same electrophoretic mobility as the correctly folded form, which is unusual for outer membrane porins. After purification of the refolded AaxA from BL21 (DE3) (pDG596), heat modifiability was again unobservable using SDS-PAGE and Western blot, although the liposome swelling activity did contrast significantly with that seen using liposomes containing the total protein refolded from the inclusion bodies.

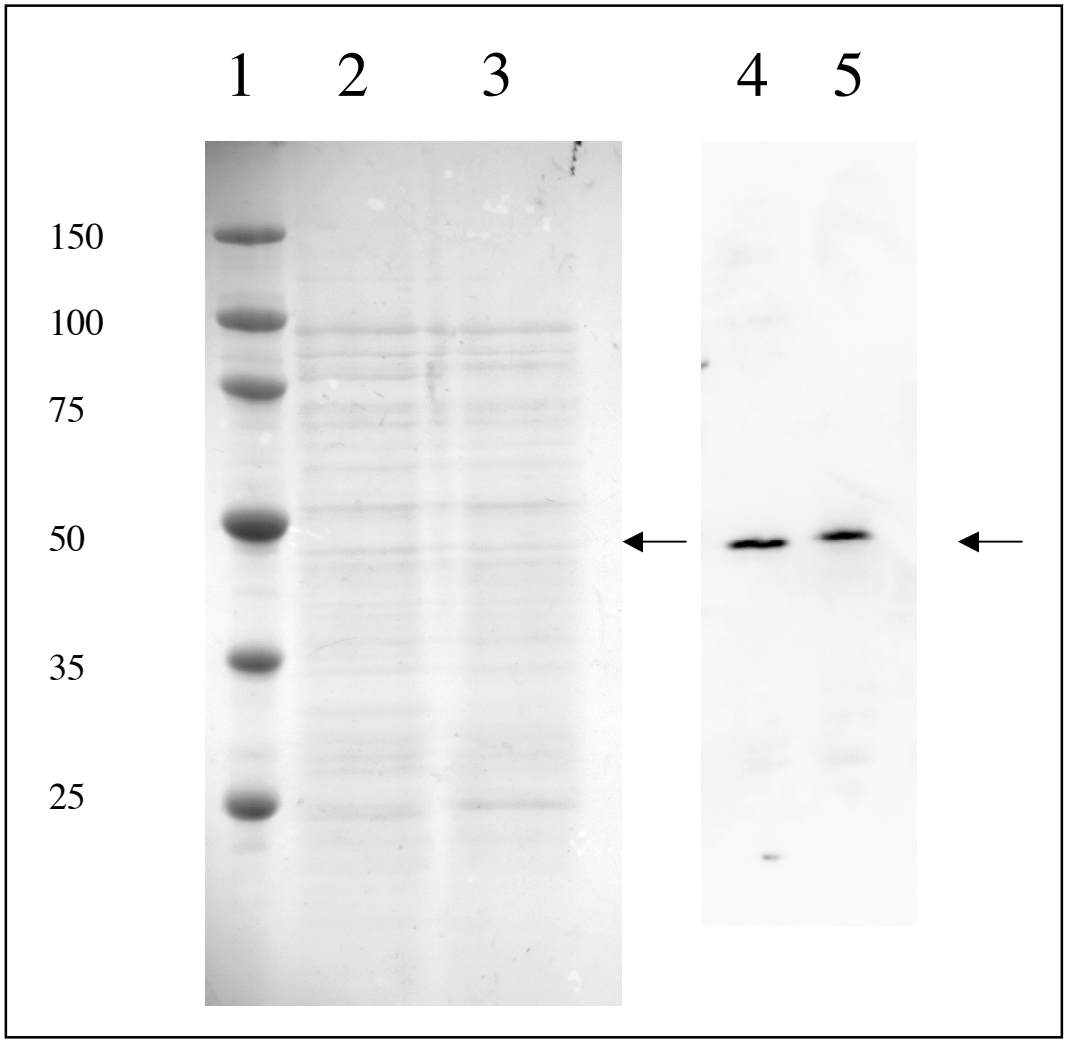

Figure 4.5. Expression and purification of AaxA-HSV

Samples containing AaxA were mixed with sodium SDS in loading dye and boiled (lanes 3 and 5) or left unboiled (lanes 2 and 4) before separation using SDS PAGE and coomassie staining (lanes 2 and 3) or immunoblotting with monoclonal antibody against the HSV epitope tag (lanes 4 and 5).

4.3.5. Liposome swelling assays

Refolded AaxA was incorporated into liposomes and swelling rates were determined using arginine as a solute. Dextran-containing liposomes were diluted into test solution containing arginine at iso-osmotic concentration in 5mM Tris-HCl, pH 8, except when testing a range of pH values. Modified E-media was used at 1/18 dilution for pH values of 6 and lower. The swelling rate was observed by a decrease in refractive index that causes a decrease in the optical density at 400 nm.

AaxA was added to liposomes at increasing concentrations with arginine as the test solute to show that porin concentration in the liposomes directly correlates with swelling rates (Figure 4.6). The results demonstrated a clear correlation between the amount of AaxA incorporated into the liposomes and the rate of arginine uptake. The results of these assays were used to identify an ideal concentration of AaxA for use in future assays, which was determined to be 12 ng of protein for each reaction.

Arginine uptake by AaxA was also examined under different pH conditions to determine the pH dependence of AaxA (Figure 4.7). Because the previous results with whole cell decarboxylase assays as well as acid survival assays suggested that AaxA was not active at pH 2.5, AaxA was expected to have a more moderate pH optimum. Swelling assays with arginine as the test solute demonstrated that AaxA does not allow for detectable uptake of arginine below a pH of 3 and the optimum pH for arginine uptake was between 6 and 8. Arginine uptake dropped and then remained constant from

pH 9 to 10, although this is likely caused by the change in the net charge of arginine, whose amino group has a pK value of 9, rather than a change in the porin itself. Arginine should begin to lose its positive charge at pH 9. If AaxA does have a preference for positively charged solute molecules, this could explain the lower rate of uptake.

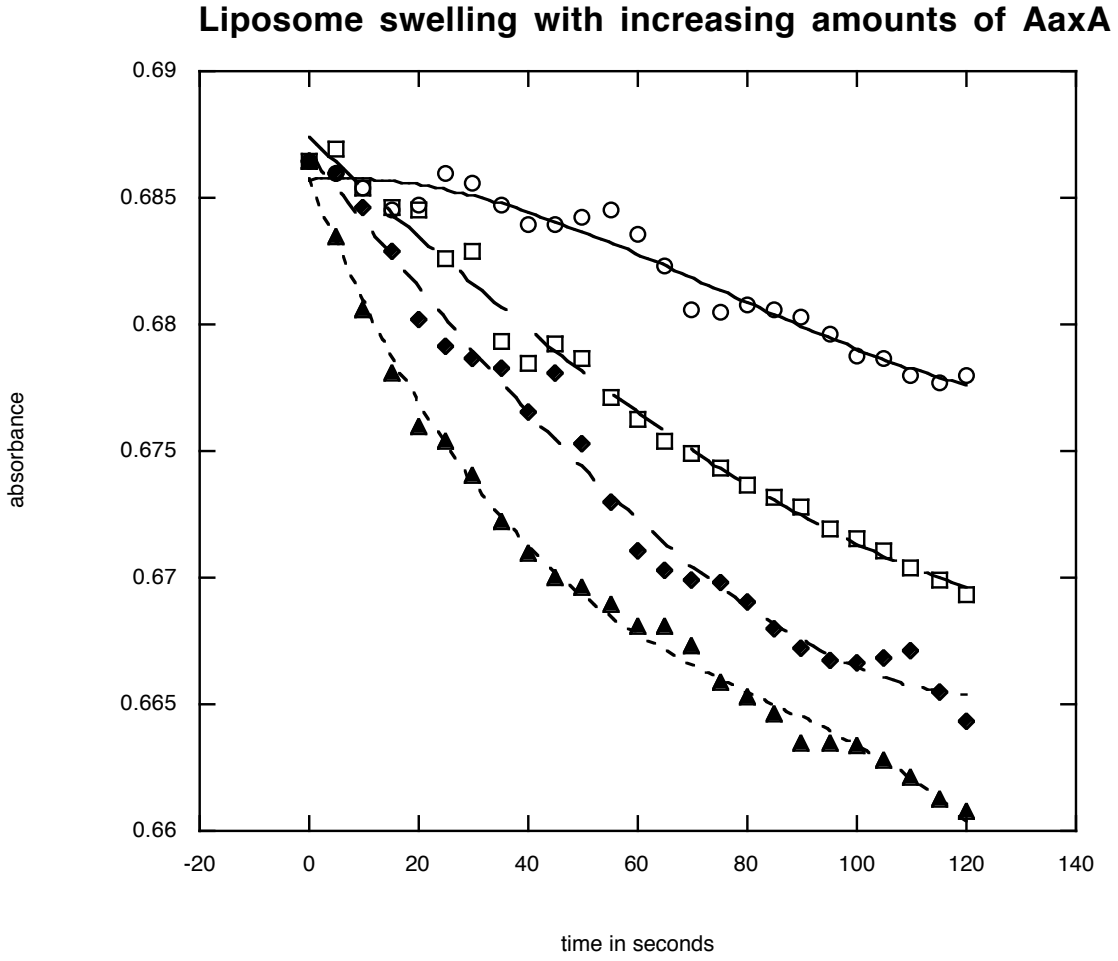

Figure 4.6. Arginine uptake increases with increasing addition of AaxA porin

Liposome swelling analysis of AaxA refolded from *E. coli* BL21 (DE3) pDG596 inclusion bodies. AaxA was added at 4 ng (empty circles), 6 ng (empty squares), 8 ng (filled diamonds), and 12 ng (filled triangles).

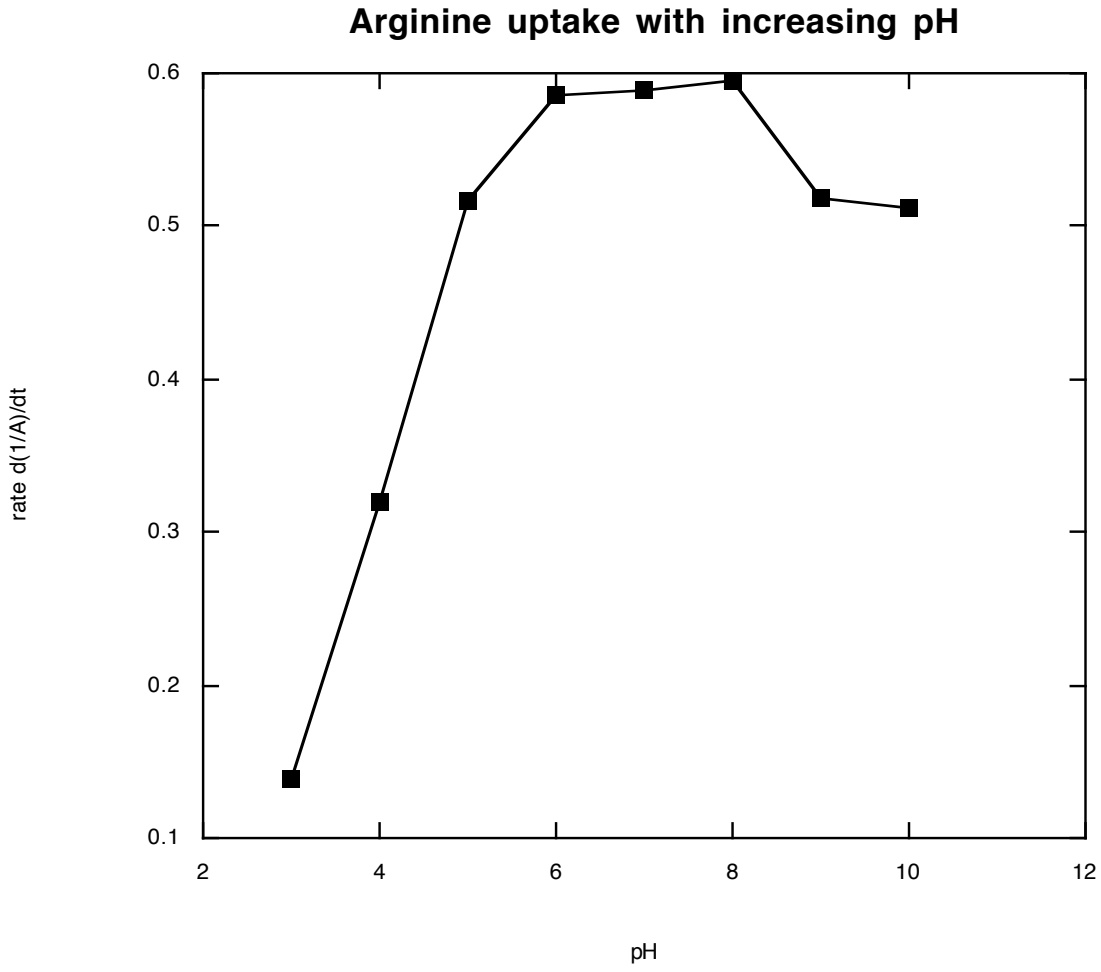

Figure 4.7. Arginine is taken up by AaxA in a pH-dependent manner.

Liposome swelling analysis of AaxA refolded from *E. coli* BL21 (DE3) pDG596 inclusion bodies was performed at pH values of 3 through 10. Swelling rates are calculated as $\Delta(1/A)/\Delta t$ from the absorbance changes between 0 and 30 s.

To address the issue of possible contamination of purified AaxA with active porins from *E. coli* two methods were used. First, assays were performed using liposomes made with porin from unpurified protein extracted from the inclusion bodies (Figure 4.8) and compared with the activity observed with the purified AaxA (Figure 4.9). Notably, swelling in the presence of sucrose and glucosamine-6-phosphate, which was significant in pre-purification samples, was reduced to insignificance with purified AaxA. The complete lack of sucrose uptake by the purified AaxA is strong evidence for the removal of *E. coli* porins, which should take up sucrose at a significant rate (Jap et al 1996). Glucose-6-phosphate, which was also taken up at very high rates in pre-purification samples showed a considerable drop in swelling, although some very limited swelling did occur. The second method used to address the purity of AaxA was to examine inhibition by monoclonal antibodies against the HSV-tag that is present on the refolded AaxA. It was observed that swelling rate in liposomes containing purified AaxA was reduced by greater than 60% in the presence of anti-HSV while antibody against the T7 antigen used as a negative control showed no significant reduction in activity. Pre-purification protein showed a drop in swelling rates with the addition of anti-HSV that was much less pronounced, roughly 17%, consistent with the presence of *E. coli* porin activity. Again, anti-T7 showed no significant reduction in swelling rates.

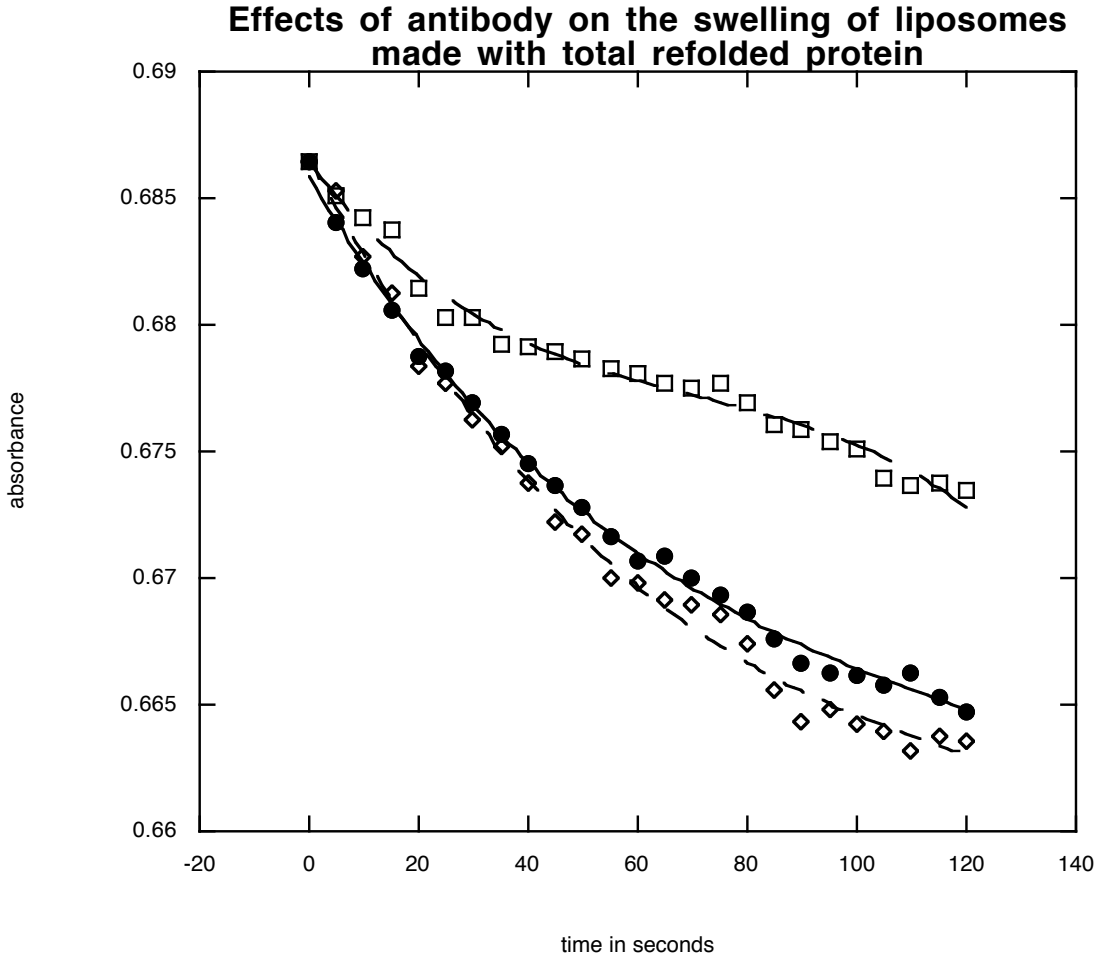

Figure 4.8. Inhibition of arginine uptake using monoclonal antibodies with liposomes made with total protein refolded from inclusion bodies.

Liposome swelling inhibition by monoclonal antibodies in liposomes containing total protein refolded from inclusion bodies. Proteoliposomes were inclubated for 30 minutes at room temperature in the absence of antibody (filled circles), in the presence of HSV-tag monoclonal antibody (empty squares), and T7-monoclonal antibody (empty diamonds) before the liposome-swelling assay was performed using arginine as the test solute.

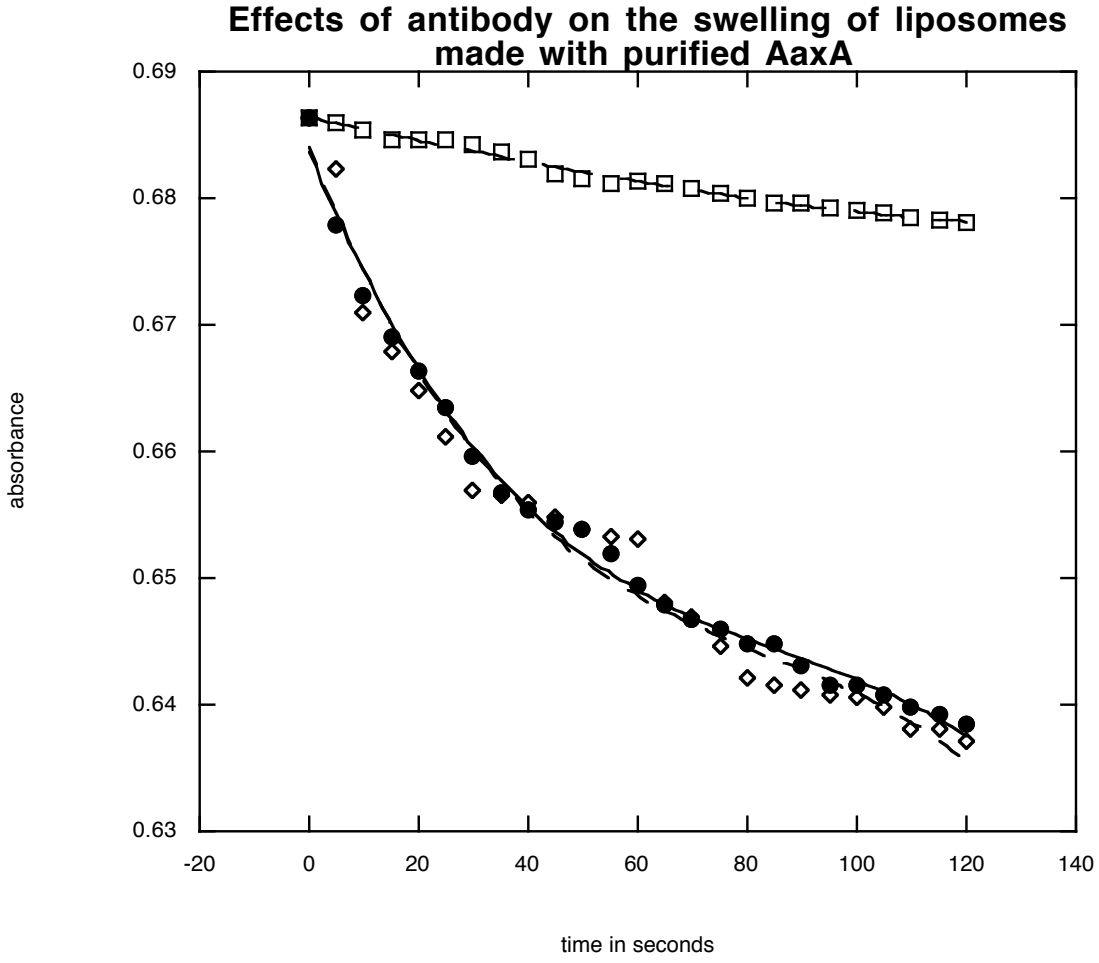

Figure 4.9. Inhibition of arginine uptake using monoclonal antibodies with liposomes made with purified AaxA.

Liposome swelling inhibition by monoclonal antibodies in liposomes containing purified AaxA. Proteoliposomes were incubated for 30 minutes at room temperature in the absence of antibody (filled circles), in the presence of HSV-tag monoclonal antibody (empty squares), and T7-monoclonal antibody (empty diamonds) before the liposome-swelling assay was performed using arginine as the test solute.

4.3.6. Sugar substrates and size dependence

The effects of solute size on liposome-swelling rate are most observable with sugars with the D-ribose taken up most quickly while the larger sucrose and stachyose, a tetrasccharide consisting of two D-galactose units, one D-glucose unit, and one D-fructose unit, caused no detectable swelling (Figure 4.10). When using most of these solutes, size seems to be the only factor affecting the rate of uptake by AaxA. This is to be expected, as size is one of the major factors that determine the ability of solute molecules to pass through a porin. However, D-ribose is taken up at a significantly higher rate than L-arabinose even though both molecules have the same molecular weight. While the differing stereochemistry may be a factor in this discrepancy, a more important factor may be that D-ribose exists more prominently in the furanose form than L-arabinose and it's possible that the 5-membered furanose ring can be taken up more readily than the 6-membered pyranose ring (Angyal and Pickles, 1972). The physiological relevance of these sugars as substrates for AaxA cannot be determined from these results alone.

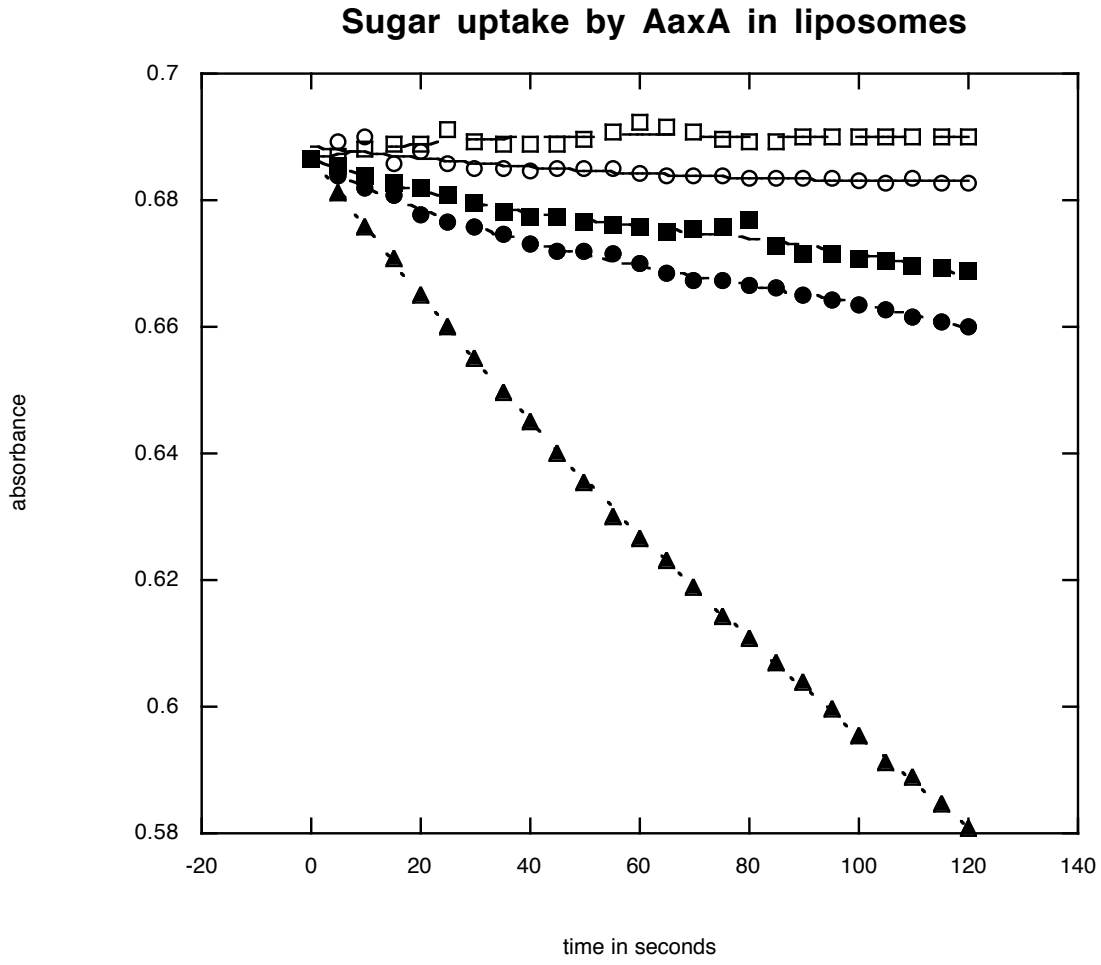

Figure 4.10. AaxA allows the uptake of sugars in a size-dependent manner

Liposome swelling in isotonic sugar solutions of stachyose (empty squares), sucrose (empty circles), glucose (filled squares), arabinose (filled circles), and ribose (filled triangles) shows a clear size-dependence with larger sugars being excluded from uptake by AaxA.

4.3.7. Other amino acids and charge dependence

In addition to arginine, many other amino acids were tested as solutes (Table 4.1). At iso-osmotic conditions it was observed that arginine was taken up most efficiently, regardless of whether D or L-arginine was used, with other positively charged amino acids also being good substrates. Of uncharged solutes, only cysteine was taken up as efficiently as arginine. Negatively charged amino acids were the worst substrates, suggesting that AaxA preferentially takes up positively charged solutes and discriminates against solutes with a net negative charge. As with sugars, size also plays a role in the uptake rates of amino acids by AaxA.

4.3.8. Polyamines

Agmatine and other polyamines were also tested as solutes for AaxA using liposome-swelling assays (Table 4.1). As with the sugars, size played the biggest role in determining uptake efficiency. Putrescine, having the lowest molecular weight of the tested polyamines, was taken up most efficiently while spermidine, the largest polyamine tested, was taken up least efficiently. Argininamide, one of the larger molecules tested, was also taken up poorly in spite of having the greatest net positive charge. Agmatine, having an intermediate molecular weight, fell in the middle of the pack, making it a good substrate, but not on par with arginine, its partner in arginine agmatine transport by AaxC.

4.3.9. Liposomes made with outer membranes

In addition to the previously described liposome swelling assays made with refolded AaxA purified from inclusion bodies, AaxA was expressed in the outer membrane of DEG0156 (pDG512) as before and the outer membrane was isolated by sucrose-density gradient centrifugation and reconstituted into liposomes. Outer membranes from DEG0156 (pBAD/HisA) were used as a control. The results from these assays showed a difference in uptake between outer membranes expressing AaxA and those that did not but there is some discrepancy between these results and those obtained using refolded and purified AaxA (Table 4.2). This can likely be attributed to the difference in relative abundance of *E. coli* porins and AaxA. Glutamic acid and aspartic acid are still very poor substrates and arginine is still taken up much more efficiently than most other amino acids. Notably sucrose is taken up at a significantly lower rate in AaxA-enriched liposomes compared with those containing only *E. coli* porins. This not only confirms that AaxA does not allow the uptake of sucrose, but that *E. coli* porins do allow sucrose uptake. The fact that there is no detectable sucrose uptake in liposomes made with refolded and purified AaxA therefore indicates that there is no contamination by *E. coli* porins contributing to the results observed in those assays. Oxaloacetate was tested in this assay, as it is a substrate for the dicarboxylate-specific chlamydial porin, PorB but it was determined not to be a substrate for AaxA.

Table 4.1. Liposome swelling assays using refolded AaxA

Test solute	MW	Rate (min^{-1})	%G
Glycine	75	0.854	100
Isoleucine	131	0.765	89.6
Alanine	89	0.437	51.1
Methionine	149	0.322	37.8
Valine	117	0.303	35.4
Phenylalanine	165	0.83	97.2
Cysteine	121	0.978	115
Proline	115	0.625	73.2
Serine	105	0.378	44
Asparagine	132	0.356	42
Glutamine	146	0.316	37
Threonine	119	0.24	28
D-Arginine	174	1.012	119
L-Arginine	174	0.992	116
Homoarginine	188	0.863	101
Citrulline	175	0.843	98.7
Lysine	146	0.756	88
Canavanine	177	0.479	56
Histidine	155	0.298	34.9
Putrescine	88	0.945	111
Cadaverine	102	0.819	95.9
Agmatine	130	0.628	73.5
Argininamide	246	0.203	23.8
Spermidine	250	0.168	6.97
Glutamic Acid	147	0.116	13.6
Aspartic Acid	133	0.018	2.1
D-Ribose	150	1.081	126
L-Arabinose	150	0.649	76
Glucose	180	0.538	63
Sucrose	342	0.0179	2.1
Stachyose	667	0.0045	0.5
Oxaloacetate	132	0.441	51.6
Isoketovalerate	132	0.169	19.8
Glucose-6-phosphate	260	0.084	9.8
Glucosamine-6-phosphate	258	0.0111	1.3

Refolded AaxA obtained from DEG0156 (pDG596) was reconstituted into liposomes. Liposome swelling assays were carried out in test solutions containing iso-osmotic concentrations of the indicated solute. Absorbance readings were taken at 400 nm and

swelling rates were determined as Δ(1/A)/ Δt from the absorbance changes between 0 and 30 s. All rates were also normalized relative to glycine (%G).

Table 4.2. Liposome swelling assays using outer membranes

Test solute	MW	OM + AaxA (%G)	OM - AaxA (%G)
Glycine	75	100 (0.579)	100 (0.582)
Alanine	89	151	98
Leucine	131	148	69
Valine	117	129	57
Methionine	149	100	81
Phenylalanine	165	113	85
Serine	115	110	52
Threonine	119	89	50
Cysteine	121	88	42
Asparagine	132	60	86
Glutamine	146	58	52
Arginine	174	137	46
Histidine	155	124	47
Aspartic acid	133	74	65
Glutamic acid	147	65	62
Agmatine	130	26	3
Sucrose	342	27	52
Oxaloacetate	132	38	156

Outer membranes obtained from *E. coli* DEG0156 were reconstituted into liposomes. Outer membranes expressing AaxA from pDG512 were compared with control membranes from cells carrying pBAD/HisA. Liposome swelling assays were carried out as before. Absorbance readings were taken at 400 nm and swelling rates were determined as Δ(1/A)/ Δt from the absorbance changes between 0 and 30 s and normalized relative to glycine (%G). The rate of uptake for glycine was 0.579 min^{-1} for outer membranes expressing AaxA and 0.582 min^{-1} for control membranes without AaxA. Oxaloacetate was tested because it is a substrate for PorB but it does not appear to be taken up by AaxA.

4.4. DISCUSSION

It was demonstrated in chapter 3 that AaxA enhances in vitro arginine uptake and decarboxylation as part of the AaxABC arginine-agmatine exchange system (Smith and Graham 2008). In this section the porin activity of AaxA was investigated using a wide assortment of substrates in addition to arginine and agmatine. While arginine is taken up more efficiently than other amino acids tested in this study, AaxA does seem to have a broader specificity than we originally hypothesized. Sugars seem to diffuse through the AaxA porin in a size-dependant manner with D-ribose resulting in the highest rate of liposome swelling while sucrose seems to be unable to diffuse through the porin at a detectable rate. This shows a marked contrast with *E. coli* porins such as OmpF, OmpC, and OmpA, which all have an approximate exclusion limit of 600 Da and therefore would be expected to allow some uptake of sucrose, which has a molecular weight of 342 Da (Jap et al 1996). The uptake of sucrose by *E. coli* porins was confirmed here in liposome-swelling assays using outer membrane preparations. It was shown that sucrose uptake was significantly lower in liposomes containing outer membrane protein enriched for AaxA than in those containing only *E. coli* outer membrane proteins. The inability of sucrose to cause swelling in liposomes made with refolded and purified AaxA is therefore a good indicator that the liposomes are not contaminated with *E. coli* porins.

Arginine diffuses through the AaxA porin very efficiently compared with other solutes, which seems to be attributable in part to its positively charged side chain.

Histidine and canavanine, which have lower pKa values for their side chains, have a neutral charge at the pH used for the liposome swelling assays and also had a much lower rate of uptake. Lysine, homoarginine, and citrulline, which have positively charged side chains under the tested conditions were taken up much more efficiently. It is worth noting that argininamide, an inhibitor of AaxB (Giles and Graham 2007), which has a higher net charge than arginine did not diffuse as efficiently through the AaxA porin as other positively charged molecules that were tested, most likely due to its larger size. Amino acids with negatively charged side chains were the worst of the solutes tested for diffusion through the AaxA porin. In general, uncharged polar and non-polar amino acids were not taken up as efficiently as the amino acids with positively charged side chains, although glycine was able to diffuse very efficiently through the porin, possibly because of its relatively small size. Isoleucine, cysteine, and proline were also taken up efficiently. Among polyamines tested, diffusion efficiency seemed size-dependant as was observed for the tested sugars.

C. pneumoniae AaxA has 2 cysteine residues located at position 69 and position 292 but these are not conserved in other Chlamydia species. Reducing conditions created using DTT had no significant affect on swelling rates in liposomes containing purified AaxA, suggesting the possibility that AaxA may be active in vivo under oxidizing conditions where chlamydial MOMP's conserved cysteine residues would form inactivating intramolecular disulfide bonds (Bavoil et al. 1984). Decarboxylase assays using whole *E. coli* cells under reducing conditions showed no significant change in

uptake for cells expressing AaxA while under the same conditions cells expressing MOMP showed a 38% enhancement of activity, suggesting a partial inactivation under oxidizing conditions that is not observed for AaxA.

Liposome swelling assays showed that arginine uptake through AaxA is highest at pH 6-8, dropping and leveling off at pH 9. The amino group of arginine has a pK value of 9, and the loss of positive charge at this pH is likely the cause of the reduction in uptake by AaxA at pH 9 and above. As the pH drops below 6, arginine uptake decreases markedly, approaching insignificance bellow pH 3. This relatively high optimal pH explains why AaxA had no significant impact on acid survival in *E. coli AdiAYC* mutants at pH 2.5 as reported previously (Smith and Graham 2008). This result suggests that AaxA may undergo a conformational change under acidic conditions as has been observed for *E. coli* OmpF and OmpC (Todt et al. 1992). Additional structural data and mutation experiments will be beneficial to determine the mechanics of AaxA's pH-sensitivity.

AaxA has been observed in this study to be a general porin with a preference for positively charged solutes. All tested solutes with a molecular weight less than that of sucrose showed efficient uptake with the exception of solutes with a net negative charge, which seem to be discriminated against by AaxA. It is worth noting that while aspartate and glutamate are not taken up at significant rates by AaxA, MOMP has been demonstrated to efficiently transport these amino acids (Jones et al. 2000). The largest

solute molecule tested that showed a significant rate of uptake was spermidine (MW 250). It was demonstrated in this study by whole cell decarboxylase assays using *E. coli* expressing AaxA and MOMP that both porins can enhance arginine uptake and while the question of whether they both do so under the same conditions in vivo has yet to be answered it does seem that the two porins may partially complement each other in their preferences for different amino acid substrates.

We hypothesized previously that the AaxABC arginine agmatine exchange system may have a role in virulence by consuming host arginine, the substrate for inducible nitric oxide synthase (iNOS), and thereby targeting the innate immune response (Smith and Graham 2008). While AaxA's role in such a system remains a possibility worthy of future investigation, it may also be important as a general porin involved in the uptake of a wide assortment of biomolecules required by Chlamydia for survival.

CHAPTER 5

Summary and future directions

5.1 SUMMARY

This study focuses on the discovery of two membrane proteins from *Chlamydia pneumoniae*, the inner membrane arginine-agmatine antiporter AaxC and the outer membrane general porin AaxA. In the first section it was determined that AaxC is localized to the cytoplasmic membrane when heterologously expressed in *E. coli* and that it is able to transport arginine into the cell. Arginine uptake by AaxC was most efficient between pH 5 and 6. Although *C. pneumoniae* is not able to synthesize its own arginine, it does have an operon regulated by the ArgR repressor that encodes a putative ABC-type arginine transporter. This suggests that AaxC should not be required for protein synthesis and must therefore serve another role.

Arginine uptake and degradation systems serve many different roles in bacteria and archaea ranging from ATP production to polyamine biosynthesis. However *C. pneumoniae* lacks the arginine deiminase gene required for producing ATP from arginine fermentation as well as the agmatine ureohydrolase required to convert agmatine to putrescine.

AaxB, the arginine decarboxylase from *C. pneumoniae* can decarboxylate arginine imported by AaxC to produce agmatine, but this reaction must serve a purpose other than polyamine biosynthesis. In *E. coli* a system coupling the arginine decarboxylase AdiA with the arginine-agmatine antiporter AdiC is one of several systems for amino acid uptake and decarboxylation used to allow these bacteria to survive the low pH environment of the stomach prior to colonizing the gut. In these systems amino acid decarboxylation uses up protons while creating an internal positive charge to repel protons and maintain an elevated pH inside the cell.

In spite of the fact that *C. pneumoniae* is not expected to encounter an extremely low pH environment during its life cycle it was found that coexpression of AaxB and AaxC in *E. coli adiAYC* mutants subjected to acid shock in the presence of arginine can rescue survival. This survival was comparable to wild type levels and was fully dependent on the presence of arginine in the acid shock medium. This result suggests that AaxC functions in the same way as the arginine-agmatine antiporter AdiC. The AaxA porin was unable to enhance survival due to its being inactive at pH 2.5.

To investigate AaxC in more detail the same *E. coli adiAYC* mutants expressing the *aax* genes were used to verify that agmatine was exported by AaxC using strong cation exchange HPLC to separate radiolabeled reaction products for analysis by liquid scintillation counting. Arginine-agmatine exchange by AaxBC was observed under the conditions used for acid shock in the previously mentioned complementation assays but

much greater conversion was observed at pH 5, the optimum pH for arginine uptake by AaxC alone. Whole cell decarboxylase assays verified that arginine-agmatine exchange by AaxBC was most efficient between pH 3 and 5, an apparent compromise between the higher pH optimum of AaxC and that of AaxB, which is most efficient at pH 3.4.

The second portion of this study focused on the *C. pneumoniae* porin AaxA. Although AaxA could not enhance acid survival by AaxBC, whole cell decarboxylase assays showed that it could significantly enhance arginine uptake and therefore decarboxylation in whole cells incubated at pH 3 and above. This enhanced uptake was most apparent from pH 3 to 6. Enhanced uptake at pH 7 and above was not detectable with this assay because AaxB is not active under these conditions.

AaxA was also able to enhance arginine uptake and decarboxylation by AdiA and AdiC in wild type *E. coli* as well as *E. coli* mutants defective for the native *E. coli* porins OmpC and OmpF. This result was not observed at pH 2.5 but was very clear at pH 4. Unlike *C. pneumoniae* MOMP, the most studied chlamydial porin, AaxA was fully functional under oxidizing conditions when MOMP is partially inactivated by the formation of disulfide bonds. This suggests the possibility that although AaxA and MOMP both facilitate arginine uptake, AaxA may be able to do so under conditions when MOMP cannot.

AaxA is a general porin and liposome swelling assays showed that in addition to arginine and agmatine, AaxA allows for the uptake of a wide variety of amino acids, polyamines, and sugars. Large molecules (> MW 260) as well as molecules with a net negative charge seem to be excluded while positively charged solute molecules, in general, are taken up most efficiently. It is noteworthy that with increasing pH, arginine is taken up very efficiently until pH 9 when it starts to lose its net positive charge. Above pH 9, the rate of arginine uptake drops and then levels off. Uptake of arginine is most efficient between pH 6 and 8 with a rapid drop bellow pH 6. Confirming results obtained in acid resistance and decarboxylase assays, AaxA is not active bellow pH 3.

The results obtained in this study show that AaxA and AaxC can act together with AaxB as a system for arginine-agmatine exchange. However, the broad specificity of AaxA suggests that it may have a role in chlamydial biology that goes beyond this one system. Together with MOMP, AaxA may play a major role in importing the various biomolecules that *C. pneumoniae* needs to survive during its parasitic life cycle.

The importance of arginine-agmatine exchange to *C. pneumoniae* has not yet been determined but there are two distinct possibilities. Because AaxB and AaxC have been shown to enhance *E. coli* survival under conditions of extreme pH, these proteins may play a role in maintaining pH homeostais in chlamydia. However, the environment inside the intracellular inclusion where *C. pneumoniae* grows is expected to have a neutral pH as fusion with acidifying lysosomes is believed to be blocked. Additionally,

C. pneumoniae should not encounter pH extremes not encountered by *C. trachomatis*, in which mutations have inactivated AaxB and with it the organism's system for arginine-agmatine exchange. The relaxed selection for the maintenance of AaxB in *C. trachomatis* but not in *C. pneumoniae* suggests that differences in tissue tropism may lead to different pressures affecting this system. *C. pneumoniae*, unlike *C. trachomatis*, is expected to encounter nitric oxide as part of the hosts innate immune system while infecting alveolar macrophages. Inducible nitric oxide synthase (iNOS) uses arginine as a substrate to produce nitric oxide and expression of iNOS is regulated by arginine abundance. If *C. pneumoniae* uses AaxB and AaxC to deplete host cell arginine in the same way as *Helicobacter pylori* with its arginine deiminase then this difference between these chlamydial species could be explained. Additionally agmatine may be converted by the host enzyme diamine oxidase to 4-guanidinobutyraldehyde, a potent inhibitor of iNOS. AaxABC may therefore be a virulence factor involved in countering the production of nitric oxide by the host.

5.2 FUTURE STUDIES

Although AaxC and AaxA have now been characterized in vitro and with the aid of a heterologous expression system their function in vivo has yet to be explored. It would be expected that the *aax* genes are co-transcribed to facilitate their cooperative action in arginine-agmatine exchange. Therefore, it must be verified that *aaxABC* is transcribed as a polycistronic mRNA in *C. pneumoniae* and it must be determined when these genes are

expressed. To this end, reverse transcriptase PCR analysis should be performed using mRNA extracted from cells harvested at different times post-infection. Polyclonal antibodies against AaxC and AaxA would be helpful in identifying these proteins in the cell extract from cells infected with *C. pneumoniae* using immunoblotting experiments. Together with RT PCR data these experiments would indicate the stages of the chlamydial life cycle when these proteins may be required.

Once the expression of the *aax* genes is determined, *C. pneumoniae* reticulate bodies or elementary bodies can be harvested under conditions where the Aax proteins can be expected to be active and subjected to the same whole cell decarboxylase assays used for *E. coli* expressing these genes. These assays would be able to show if these proteins are active in vivo and would be an important step in determining their significance to *C. pneumoniae*.

If *C. pneumoniae* uses the Aax proteins as a system for inhibiting host iNOS activity during infection, infection studies using macrophages capable of expressing iNOS would be essential to verify this role. Assays for the production of nitric oxide have been carried out using *C. pneumoniae* and it was determined that nitric oxide is able to inhibit *C. pneumoniae* infection (Carratelli et al., 2005). However these assays were carried out in the presence of arginine concentrations 10 times greater than concentrations found in the lung so it remains to be seen if *C. pneumoniae* expressing *aaxABC* is able to resist inhibition of growth by nitric oxide.

This dissertation highlights the two new chlamydial membrane proteins AaxC and AaxA and attempts to show how they may be important to the life cycle of the obligate intracellular parasite and human pathogen *C. pneumoniae*. Although their functions have been determined in vitro it will take in vivo studies to fully assess their role in the chlamydial life cycle.

Appendix: List of microorganisms and plasmids

Strain or Plasmid	Description and partial genotype	Source or reference
E. coli DEG0147	MG1655 *ΔAdiAYC::kan*	Smith and Graham, 2008
E. coli BW25113	*Δara*BAD_{AH33}	Datsenko and Wanner, 2000
E. coli JW0912-1	BW25113 *ΔompF*	Baba et al., 2006
E. coli JW2203-1	BW25113 *ΔompC*	Baba et al., 2006
E. coli DEG0156	BW25113 *ΔompFΔompC*	Smith and Graham, pending publication
pBAD/HisA	Expression vector, *bla araC*	Invitrogen
pDG339	*aaxB* in pBAD/HisA	Smith and Graham, 2008
pDG366	*aaxBC-HSV* in pBAD/HisA	Smith and Graham, 2008
pDG379	*aaxBC* in pBAD/HisA	Smith and Graham, 2008
pDG484	*aaxABC* in pBAD/HisA	Smith and Graham, 2008
pDG512	*aaxA-HSV* in pBAD/HisA	Smith and Graham, 2008
pDG552	*ompX* in pCOLADuet	Smith and Graham, 2008
pDG561	*lepA* in pCDFDuet-1	Smith and Graham, 2008
PDG569	*CpnMOMP* in pBAD/HisA	Smith and Graham, pending publication
PDG596	Truncated *aaxA* in PET43.1a	Smith and Graham, pending publication

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