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Characterization of the activity and expression of arginine decarboxylase in human and animal *Chlamydia* pathogens

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Abstract

Chlamydia pneumoniae encodes a functional arginine decarboxylase (ArgDC), AaxB, that activates upon self-cleavage and converts L-arginine to agmatine. In contrast, most *Chlamydia trachomatis* serovars carry a missense or nonsense mutation in *aaxB* abrogating activity. The G115R missense mutation was not predicted to impact AaxB functionality, making it unclear if AaxB variations in other *Chlamydia* species also result in enzyme inactivation. To address the impact of gene polymorphism on functionality, we investigated the activity and production of the *Chlamydia* AaxB variants. Since ArgDC plays a critical role in the *Escherichia coli* acid stress response, we studied the ability of these *Chlamydia* variants to complement an *E. coli* ArgDC mutant in an acid shock assay. Active AaxB was detected in four additional species: *C. caviae*, *C. pecorum*, *C. psittaci*, and *C. muridarum*. Of the *C. trachomatis* serovars, only E appears to encode active enzyme. To determine when functional enzyme is present during the chlamydial developmental cycle, we utilized an anti-AaxB antibody to detect both uncleaved and cleaved enzyme throughout infection. Uncleaved enzyme production peaked around 20 hours post-infection, with optimal cleavage around 44 hours. While the role ArgDC plays in *Chlamydia* survival or virulence is unclear, our data suggest a niche-specific function.

Keywords

Chlamydia evolution; arginine decarboxylase; inactivating mutation

Introduction

Infection with *Chlamydia*, a genus of Gram-negative obligate intracellular bacteria, may result in ocular, genital, or pneumonic disease, depending on route of entry and bacterial species/serovar. While the majority of *Chlamydia* species are zoonotic, infecting a wide range of mammalian and avian hosts, the *Chlamydia trachomatis* serovars are human-specific pathogens (Rohde *et al.*, 2010; Carlson *et al.*, 2005). All species undergo a unique biphasic developmental cycle transitioning between the extracellular, infectious elementary body (EB) and the intracellular, replicative form known as the reticulate body (RB) (AbdelRahman and Belland, 2005).

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Arginine decarboxylases (ArgDC), which catalyze the conversion of arginine into agmatine, are conserved in bacteria and play dual roles in acid resistance and the metabolism of polyamines such as putrescine (Lin *et al.*, 1995; Tabor and Tabor, 1984). In bacteria such as *Yersinia*, functional ArgDC is required to produce biofilms, making this enzyme essential for virulence (Patel *et al.*, 2006). Two ArgDC are encoded by *E. coli*: the acid-inducible *adiA* and a constitutive *speA* that functions in polyamine biosynthesis (Stim and Bennett, 1993). In *Chlamydia*, the only known ArgDC is encoded by *aaxB*, which resides in an operon between the putative porin *aaxA* and the characterized arginine-agmatine antiporter, *aaxC* (Giles and Graham, 2007) (Figure 1A). Although AaxB is functionally equivalent to *E. coli* AdiA, the enzyme itself is actually a member of the pyruvoyl-dependent ArgDC (PvlArgDC), and shares more similarities with ArgDC from organisms such as *Methanococcus jannaschii* (Graham *et al.*, 2002).

The AaxB proteins of *Chlamydia pneumoniae* and *C. trachomatis* serovars D and L2 were previously characterized (Giles and Graham, 2007; Giles *et al.*, 2009). All sequenced *C. pneumoniae* encode a 25 kDa proenzyme, which requires autocleavage between the conserved Thr⁵² Ser⁵³ residues to produce 16 kDa α and 9 kDa β subunits. The cleaved subunits are then free to assemble into the active ($\alpha\beta$)₃ complex.

In contrast, *C. trachomatis* serovars D and L2 have inactivated AaxB through one of two independent mutations (Giles *et al.*, 2009). The Gly¹¹⁵Arg substitution mutation in serovar D (also present and predicted to inactivate AaxB from B/D/G and F) disrupts the necessary auto-cleavage event; in serovar L2, a nonsense mutation midway through the gene results in early truncation. The Gly¹¹⁵Arg mutation present in strains of D was not predicted to result in enzyme inactivation based on sequence analysis alone, making it unclear if AaxB sequence variations seen in other *Chlamydia* alter AaxB activity.

To further our understanding of this enzyme and determine if inactivation of AaxB is restricted to the human-specific *C. trachomatis* serovars, we completed an activity panel using variant *Chlamydia* AaxB proteins in a surrogate *E. coli* acid shock assay. A pan-chlamydial anti-AaxB antibody was used to detect enzyme production and processing during the developmental cycle using a cell culture infection model. Collectively, our data indicate that non-*C. trachomatis* species (and a single *C. trachomatis* serovar: E) produce active AaxB.

Methods and Materials

Strains

Chlamydia strains used in this study include: *Chlamydia muridarum* strain Nigg, *Chlamydia trachomatis* serovar D strain UW-3/CX, *Chlamydia psittaci* strain 6BC, *Chlamydia caviae* strain SP6 (Binet *et al.*, 2010), and *Chlamydia trachomatis* serovar E strain UW-5/CX. *C. pecorum* strain E58 DNA was provided by Patrik Bavoil (University of Maryland). The previously unreported *aaxB* sequences for *C. caviae* SP6 and *C. trachomatis* E strain UW-5/CX were deposited in Genbank under accession numbers JX287368 and JX287367, respectively. *E. coli* strain MG1655 was used for the acid resistance complementation assays, while *E. coli* Rosetta-gami2 (DE3) (Novagen) was used for AaxB expression and purification.

Cloning of *aaxB*

A pBAD/HisA vector (modified during cloning to remove the histidine tag coding region) (Invitrogen) carrying *aaxB* from *C. pneumoniae* strain Kajaani 6 or *adiA* from *E. coli* strain MG1655 was provided by David Graham (Oak Ridge National Laboratory). Primers used to amplify the different *aaxB* variants are listed in Table 1. PCR-amplified products were

digested and ligated into the NcoI and HindIII sites on the pBAD/HisA vector (without the Histidine tag). Constructs were then electroporated into $\Delta adiA$ *E. coli* strain MG1655. The *aaxB* gene from *C. caviae* also was PCR-amplified (primers listed in Table 1) for cloning into a pET-19b expression vector (Invitrogen). PCR-amplified products were digested and ligated into the NdeI and BamHI sites on pET-19b, then electroporated into *E. coli* strain Rosetta-gami2 (DE3). All constructs were sequence verified at the Biomedical Instrumentation Center at the Uniformed Services University.

Deletion of *E. coli adiA*

The *adiA* gene was deleted from *E. coli* strain MG1655 using the lambda red method of linear recombination with the primers listed in Table 1 (Datsenko and Wanner, 2000). After PCR verification of the constructed $\Delta adiA::kan$ mutation, the allele was moved into a clean *E. coli* MG1655 background via PIL4 transduction (Miller, 1972). Transductants were selected on LB agar containing 100 $\mu\text{g mL}^{-1}$ kanamycin, verified by PCR, and checked for their acid resistance phenotype.

Acid resistance assay

The acid resistance assay of Castanie-Cornet *et al.* (Castanie-Cornet *et al.*, 1999), as modified by David Graham (Giles and Graham, 2007), was utilized. Acid resistance was expressed as the percentage of viable bacteria remaining after one hour acid shock compared to the number of viable bacteria determined immediately following acid shock.

Production of AaxB antibody

The highly conserved chlamydial AaxB peptide $^{137}\text{HAKMWLKKSLQHELDLRS}^{154}$ (part of the α subunit) was commercially synthesized by Pierce Custom Antibody production service and used to raise polyclonal rabbit antibodies using the standard 90-day protocol.

Purification of *C. caviae* AaxB

E. coli Rosetta-gami2 (DE3) was transformed with pET-19b carrying *aaxB* from *C. caviae* and grown in LB containing 100 $\mu\text{g mL}^{-1}$ ampicillin to an OD_{600} of 0.6. AaxB expression was induced with 1 mM IPTG for 23 hours at 20° C, and bacteria were collected by centrifugation. Bacteria were resuspended in equilibration buffer (50 mM monobasic sodium phosphate, 300 mM sodium chloride, and 10 mM imidazole, pH adjusted to 7.4) with 1x protease inhibitor (Roche) and 1x phosphatase inhibitors 2 and 3 (Sigma). Bacteria were lysed via sonication, centrifuged to remove debris, and the supernatant passed through a 0.45 μm filter (Millipore). HisPur™ cobalt resin (Thermo Scientific) was applied to the supernatant and the batch method of purification was carried out as per manufacturer's instructions. Purified protein samples were eluted (50 mM sodium phosphate, 300 mM sodium chloride, 500 mM imidazole, pH adjusted to 7.4), then applied to a 3K Amicon filter (Millipore) for concentration. Samples were resuspended in 50 mM Bis-Tris buffer (pH 6.0), and quantified by the Bio-Rad Protein Assay (Bio-Rad). Protein identity and purity was assessed using SDS-PAGE followed by Coomassie Brilliant Blue Staining or Western blotting with the anti-AaxB antibody (at a 1:250 dilution).

Detection of chlamydial AaxB in EBs and during infection

Chlamydia were grown in and harvested from mouse fibroblast L2 cells. EBs were titered using an infection forming unit assay (IFU) and stored at -80° C in Sucrose Phosphate Glutamic acid buffer (SPG) (7.5% w/v sucrose, 17 mM Na_2HPO_4 , 3 mM NaH_2PO_4 , 5 mM L-glutamic acid, pH 7.4) until use (Binet *et al.*, 2010). For time course experiments, L2 cells were infected at an MOI of 5 (10 hr samples), an MOI of 1 (20, 30, and 44 hr samples), or mock infected (Giles *et al.*, 2009). Samples were disrupted directly in Laemmli buffer and

run on 12% SDS-PAGE gels for Western blot analysis with either anti-AaxB antibodies or anti-Hsp60 antibodies (provided by Dan Rockey, Oregon State University; Yuan *et al.*, 1992). Detection of Hsp60 (60 kDa heat shock protein, GroEL) was used to demonstrate successful infection and equal loading of protein. To detect AaxB in EBs, bacteria were disrupted in Laemmli buffer and 1×10^7 IFU were used per SDS-PAGE gel lane.

Results

Alignment of *Chlamydia* AaxB

The AaxB sequences from the available *Chlamydia* genome projects were aligned to assess amino acid variability. All *Chlamydia* species have at least one sequenced strain available in the Genbank database except *C. suis*, a porcine pathogen. As many strains within the same species or serovar had identical protein sequences, duplicates were discarded and only unique AaxB sequences are shown in Figure 1B.

Despite differences in amino acid sequence, all AaxB variants carried the highly conserved Thr⁵² Ser⁵³ cleavage site. *C. trachomatis* serovars A/B/D/F and G carry a missense mutation, a glycine to arginine substitution (Gly¹¹⁵Arg) that was shown to abrogate cleavage of the protein and therefore activity in the serovar D variant (Giles *et al.*, 2009). In *C. trachomatis* serovar L2, an ochre codon at position 128 truncates the gene in mid-open reading frame. This truncated protein lacks activity (Giles *et al.*, 2009). Both inactivating mutations are present in high quality draft genomes of clinical isolates, suggesting that these mutations did not arise from laboratory adaptation. Neither *C. trachomatis* serovar E, nor any of the remaining *Chlamydia* species, carry either of the known mutations that have been shown to inactivate AaxB. However, there are variations in the amino acid sequence of these proteins compared to the amino acid sequence of the active *C. pneumoniae* AaxB. As the missense mutation in *C. trachomatis* serovars A/B/D/F and G was not indicative of protein inactivation, we measured the activity of the remaining variants.

Activity of *Chlamydia* AaxB

Previously, Giles and Graham demonstrated that expression of functional AaxB from *C. pneumoniae* can rescue an *E. coli* Δ *adiA* mutant from acid shock, demonstrating activity of the *Chlamydia* enzyme in a surrogate system (Giles and Graham, 2007). To test the remaining *Chlamydia* variants, an Δ *adiA* knockout of *E. coli* MG1655 was constructed and transformed with wild type *E. coli* *adiA* or *Chlamydia* *aaxB* genes cloned into a vector under control of an arabinose-inducible promoter. The different AaxB variants from *C. caviae*, *C. muridarum*, *C. trachomatis* serovar E, *C. psittaci*, and *C. pecorum* were tested in the acid resistance assay, with AaxB variants from *C. pneumoniae* and *C. trachomatis* serovar D serving as positive and negative controls, respectively (Figure 2A). All *Chlamydia* AaxB tested restored acid shock survival in the *E. coli* Δ *adiA* mutant, suggesting that *C. caviae*, *C. muridarum*, *C. trachomatis* serovar E, *C. psittaci*, and *C. pecorum* all encode active enzyme.

Protein expression and cleavage of the AaxB variants was measured via Western blotting with anti-AaxB antibody (Figure 2B). All constructs used in the acid shock experiments expressed uncleaved AaxB protein, and each active AaxB variant was capable of auto-cleavage as evidenced by detection of the α fragment (Figure 2B); i.e. the cleavage profile correlates with acid resistance. The deviation in protein size between the AaxB variants may be due to variation in molecular weight and isoelectric point; the predicted pI fluctuates within a range of approximately 0.3 pH and the predicted molecular weight fluctuates within a range of approximately 500 Da.

Time course expression of AaxB in *Chlamydia*

The timing of AaxB protein production and cleavage, and therefore activity, during infection is unknown. To determine when active enzyme is present during the chlamydial developmental cycle, the highly *Chlamydia*-conserved peptide $^{137}\text{HAKMWLKKSLQHELDLRS}^{154}$ was used to produce rabbit polyclonal antibodies. This antibody recognizes both the inactive, uncleaved proenzyme form of AaxB, as well as the activated α subunit, and therefore cleavage of this protein can be directly measured during infection.

L2 cells were infected with *C. caviae*, and the expression and cleavage of AaxB into active subunits over the course of infection was studied (Figure 3A). A unique band of ~20 kDa representing uncleaved proenzyme was initially detected at 20 hours post-infection, with very little cleaved protein (<20 kDa) appearing. Over the next 24 hours, this ratio slowly shifted, and by 44 hours post-infection, the majority of protein was in the cleaved, active state.

Interestingly, this pattern did not necessarily hold true across all the *Chlamydia* species (Figure 4A). In *C. muridarum*, while the majority of uncleaved protein also appeared at 20 hours post-infection, cleaved protein production likewise peaked at this time, then waned at subsequent time points. *C. psittaci* produced very little detectable cleaved protein.

Cleavage of AaxB also was assessed in EBs in comparison to samples from cells infected for 20 hours when full-length protein appears to be the predominant species (Supplementary Figure 1A). The cleaved form predominates in EBs, and very little, if any, detectable proenzyme remains. Despite equal loading of bacteria, AaxB was undetectable in *C. trachomatis* serovar D.

Discussion

Previously, a functional arginine decarboxylase enzyme, AaxB, was identified and characterized in *C. pneumoniae* (Giles and Graham, 2007). In this study, we demonstrate that several additional *Chlamydia* species, including *C. caviae*, *C. muridarum*, *C. psittaci*, and *C. pecorum*, encode functional AaxB. Although previous publications established that the majority of the *C. trachomatis* serovars encode nonfunctional AaxB due to one of two inactivating mutations (Graham *et al.*, 2009), we now show that the AaxB variant of *C. trachomatis* serovar E is capable of cleavage and activity.

AaxB undergoes maximal autocleavage during the mid to late *Chlamydia* developmental cycle, with slight variations on timing between the different species. At the extremes, optimal cleavage of *C. muridarum* AaxB occurs around 20 hours post-infection, with *C. caviae* AaxB cleaving around 44 hours. Although cellular conditions for autocleavage are not yet clear, timing of cleavage may be influenced by differences in amino acid composition between variants or post-translational modification.

We were unable to detect AaxB from *C. trachomatis* serovar D. Since this enzyme appears to be nonfunctional, production of AaxB would squander bacterial energy resources. While a transcriptome analysis by Belland *et al.* suggests that this gene is still transcribed, it is probable that expression of the inactive *aaxB* was maintained due to the necessity of transcribing the remaining operon genes (Belland *et al.*, 2003; Giles *et al.*, 2009). Due to the early truncation of the *C. trachomatis* serovar L2 AaxB, the anti-AaxB antibody, which was developed against a conserved peptide after the truncation, would not recognize this serovar if truncated protein is produced. However, previous data using an *E. coli* surrogate expression system indicates that this protein may not be produced (Giles *et al.*, 2009).

The total protein level of AaxB in *C. trachomatis* serovar E also appeared to be lower than the non-*C. trachomatis* variants (possibly indicating decreased expression levels), and the acid resistance phenotype of the serovar E AaxB producing strain was the weakest of the complementing strains. As the only *C. trachomatis* serovar expressing active AaxB, it is possible that the serovar E strains represent an intermediate phenotype between isolates that have maintained or lost enzyme functionality. Several studies suggest that there is no association between infections with *C. trachomatis* serovar E and presence or absence of clinical infection or specific symptoms, although this serovar is one of the most prevalent worldwide (Morré *et al.*, 2000; review, Byrne, 2010; van der Laar *et al.*, 1996). As the other genital serovars (D, F-K) occupy the same niche, it is unlikely that serovar E requires active AaxB when the other serovars have lost functionality. This, coupled with the low AaxB levels detected during *in vitro* infection, suggest that although *C. trachomatis* serovar E currently retains active AaxB, this serovar may be in the process of inactivating this enzyme.

While *C. pneumoniae* and many of the non-*C. trachomatis* serovars retain an active ArgDC, the function of this enzyme in *Chlamydia* remains obscure. Although ArgDCs in other bacteria play roles in acid resistance and/or polyamine metabolism, neither function appears relevant to *Chlamydia*. The *Chlamydia* inclusion remains at neutral pH throughout infection, so encounters with acidic environments are unlikely (Schramm *et al.*, 1996; Al-Younes *et al.*, 1999; Grieshaber *et al.*, 2002). Additionally, there are no known *Chlamydia* enzymes able to metabolize agmatine, such as the agmatine ureohydrolase, and therefore AaxB cannot contribute to polyamine synthesis. Finally, in certain cell lines, addition of exogenous agmatine alone may provide protection against cellular apoptosis (Arndt *et al.*, 2009), but investigation in our laboratory suggests that this is likely not a factor during *Chlamydia* infection (data not shown).

As Giles and colleagues have speculated previously, the most likely function for the arginine decarboxylase system during *Chlamydia* infection is depletion of host cell arginine reserves (Giles and Graham, 2007). L-arginine, a substrate for the nitric oxide synthase (iNOS) in macrophages and granulocytes, catalyzes the production of the reactive nitrogen intermediate nitric oxide (NO), which may play a role in decreasing bacterial infectivity and/or inhibiting the dissemination of *Chlamydia* to systemic sites (Xie *et al.*, 1992; Mayer *et al.*, 1993; Igietseme *et al.*, 1998). Removal of the substrate L-arginine (which would be degraded to agmatine and pumped back into the cytosol in counter-exchange for arginine by AaxC) could therefore promote *Chlamydia* survival and/or fitness, particularly in strains that are known to infect and replicate within these specialized host cells, such as *C. pneumoniae* and *C. psittaci* (Redecke *et al.*, 1998; Wyrick and Brownridge, 1978). The timing of cleavage, and presumably corresponding activity, of AaxB in these strains may correlate with optimal iNOS activation in infected macrophages, and ultimately allow *Chlamydia* to avoid the detrimental consequences of NO production prior to bacterial exit from the host cell. Alternatively, the presence of processed AaxB in EBs may indicate that EBs are 'pre-loaded' with functional AaxB that is used to protect against NO production during the immediate-early stage of infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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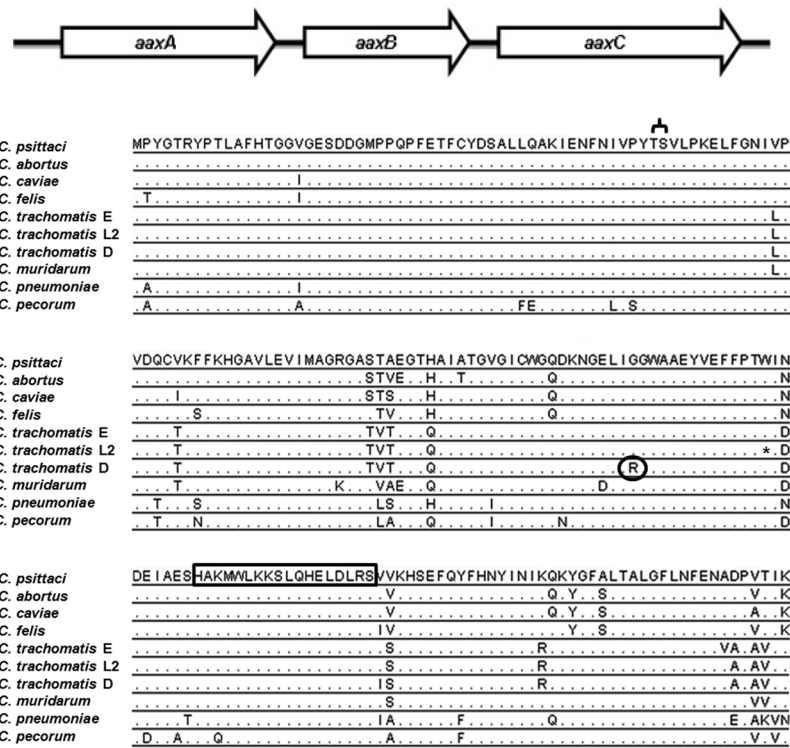


Figure 1. Gene organization and protein alignment

(a) *aaxB* is transcribed in an operon with *aaxA*, an outer membrane protein of unknown function, and *aaxC*, a characterized arginine-agmatine antiporter (Smith and Graham, 2008). (b) ClustalW alignment of different unique *Chlamydia* AaxB proteins, representative of all fully annotated strains and high quality draft genomes from clinical isolates found on the NCBI server as of June 23, 2012. The conserved Thr⁵²Ser⁵³ autocleavage site essential for activity is in brackets. * indicates the nonsense mutation in *C. trachomatis* serovar L2 that results in early truncation of the protein. A circle indicates the glycine to arginine mutation that abrogates cleavage and therefore activity; this mutation is conserved in all *C. trachomatis* A, B, D, and G lineages. Rabbit polyclonal antibody was raised against the highly conserved 18 amino acid peptide (outlined in the box) present in all AaxB sequences except the truncated *C. trachomatis* serovar L2.

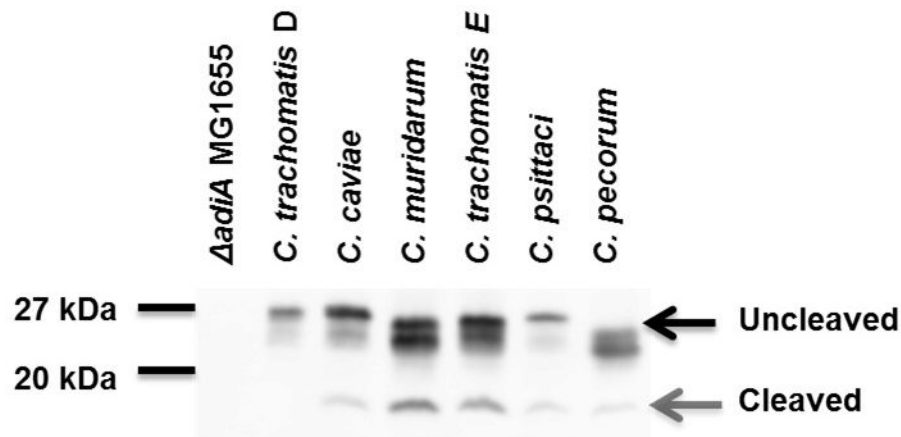
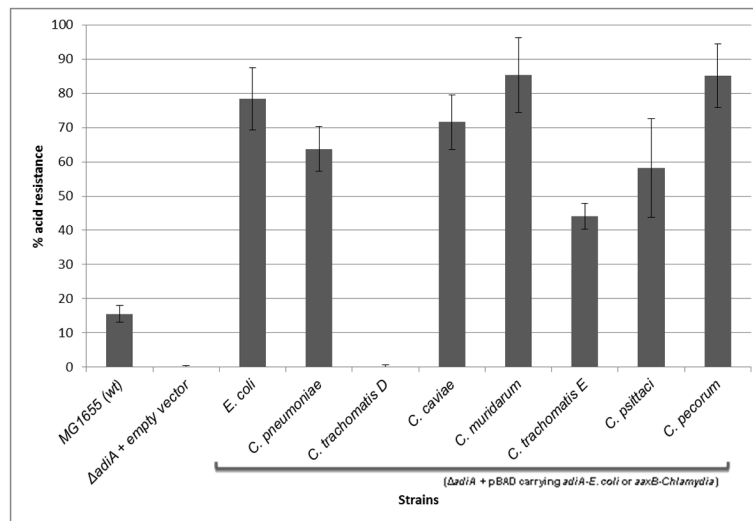


Figure 2. Activity analysis of AaxB proteins

(a) Strains were grown for 22 hours at 37° C in the presence of 0.2% arabinose. $\sim 3 \times 10^7$ cfu was exposed to acid shock buffer containing 0.4% arginine (pH 2.5) for one hour, and percent survival determined by viable plate counts. Strains were tested in three independent experiments performed in triplicate. Wild type MG1655 was significantly acid resistant compared to the Δ adiA *E. coli* empty vector control using a standard t-test with unequal variance ($p < 0.005$); all other strains, with the exception of *C. trachomatis* serovar D, were able to complement the Δ adiA *E. coli* mutant over wild type. The difference in acid resistance between the Δ adiA *E. coli* strain expressing AaxB from *C. trachomatis* serovar D and the empty vector control was not statistically significant ($p > 0.1$). (b) AaxB protein production and cleavage was measured using Western blotting with the anti-AaxB antibody. Molecular weight markers are shown to the left of the blot, and the proenzyme and α fragments (if present) are indicated with black and gray arrows, respectively.

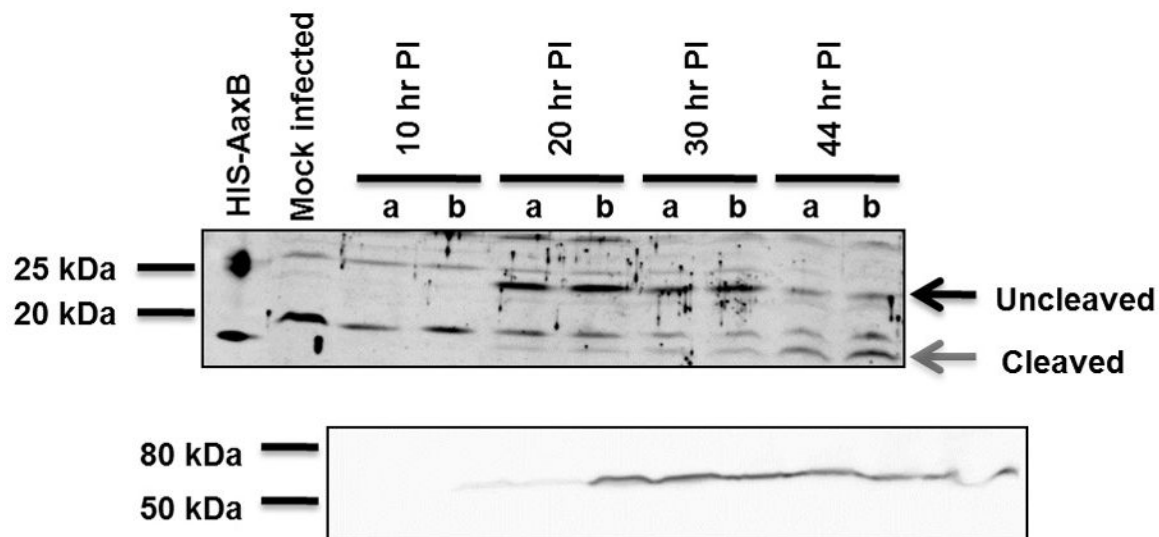


Figure 3. Time course of *C. caviae* AaxB production

L2 cells were infected at an MOI of 5 (10 hr samples), an MOI of 1 (20, 30, and 44 hr samples), or mock infected. Western blotting was performed with either anti-AaxB antibodies (a) or anti-Hsp60 antibodies as a control to demonstrate infection and for semi-quantitative comparison of chlamydial protein (b). Purified his-tagged AaxB is shown in the first lane. Two parallel-run infection samples are shown for each time point. Proenzyme and the α fragment are indicated by black and gray arrows to the right of the blot. Protein molecular weight marker positions are shown to the left of each blot.

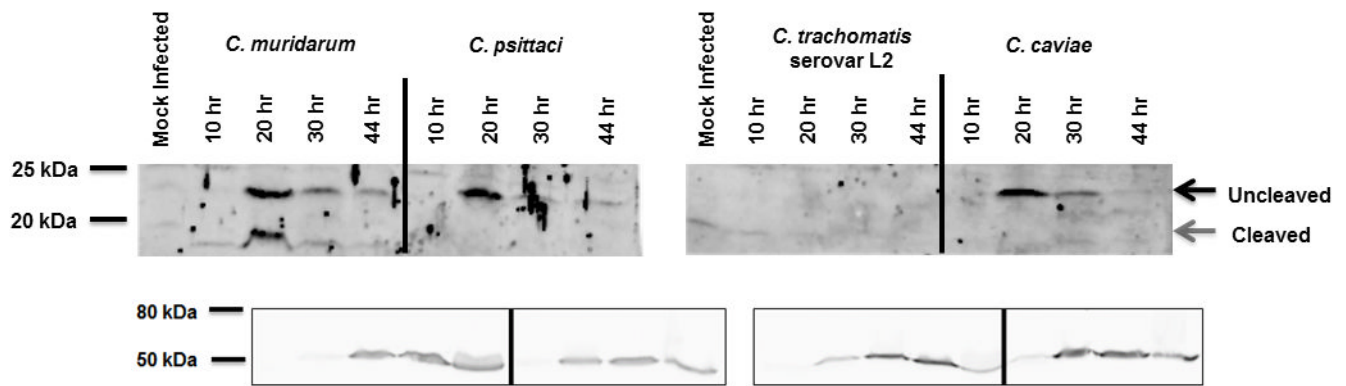


Figure 4. Time course of AaxB production by different *Chlamydia* spp

L2 cells were infected at an MOI of 10 (10 hr samples), an MOI of 1 (20, 30, and 44 hr samples), or mock infected. At the indicated times, samples were disrupted directly in Laemmli buffer and run on 12% SDS-PAGE gels. Protein was detected by Western blotting with either anti-AaxB antibodies (a) or anti-Hsp60 antibodies as a control to demonstrate infection and for semi-quantitative comparison of chlamydial protein (b). Full length AaxB is indicated by the black arrows and processed AaxB is indicated by the grey arrows. Protein molecular weight marker positions are shown to the left of each blot.