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Arginine-Dependent Gene Regulation via the ArgR Repressor Is Species Specific in *Chlamydia*

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Some, but not all, *Chlamydia* spp. are predicted to encode a homolog of ArgR, a master regulatory molecule that modulates arginine biosynthesis and catabolism in bacteria in response to intracellular arginine levels. While genes for arginine biosynthesis are apparently missing in *Chlamydia*, a putative arginine transport system encoded by *glnP*, *glnQ*, and *artJ* is present. We found that recombinant *Chlamydia pneumoniae* ArgR functions as an arginine-dependent aporepressor that bound specifically to operator sequences upstream of the *glnPQ* operon. ArgR was able to repress transcription in a promoter-specific manner that was dependent on the concentration of the corepressor L-arginine. We were able to locate ArgR operators upstream of *glnPQ* in *C. pneumoniae* and *Chlamydophila caviae* but not *Chlamydia trachomatis*, which corresponded to the predicted presence or absence of ArgR in these chlamydial species. Our findings indicate that only some members of the family *Chlamydiaceae* have an arginine-responsive mechanism of gene regulation that is predicted to control arginine uptake from the host cell. This is the first study to directly demonstrate a species-specific mechanism of transcriptional regulation in *Chlamydia*.

The three species of *Chlamydia* that are human pathogens cause markedly different diseases in terms of site of infection and clinical presentation (24, 30). *Chlamydia pneumoniae* infects the respiratory tract, where it causes pneumonia, and it has also been linked to a role in atherosclerotic heart disease (1, 9). *Chlamydia trachomatis* causes two other distinct infections: involvement of the mucosal epithelium of the eye leads to the chronic blinding infection trachoma (19), while infection of the genital tract causes the most common form of bacterial sexually transmitted disease in the United States (2). A third species, *Chlamydia psittaci*, causes a systemic infection that begins with infection of the respiratory tract (3).

In the absence of genetics, a comparative bioinformatics analysis of the chlamydial genomes may yield clues to these differences in host tropism and pathogenesis. Of the approximately 1,000 genes in a representative chlamydial genome, about 800 are common to all chlamydial species and likely account for the conserved features of chlamydial structure and the unique intracellular developmental cycle. Superimposed upon this genetic backbone, *C. trachomatis* contains about 70 genes without homologs in *C. pneumoniae*, and *C. pneumoniae* has approximately 200 genes not present in *C. trachomatis* (8). At first glance, examining these species-specific genes for clues about their roles in tropism or pathogenesis has not been particularly illuminating as most of them are of unknown function. However, one gene that is present in *C. pneumoniae* but not *C. trachomatis* is predicted to encode a transcription factor with sequence similarity to the arginine regulator, ArgR, suggesting that the role of arginine-dependent gene regulation deserves closer scrutiny.

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ArgR is a regulatory protein that can function as both a transcriptional repressor and an activator. In *Escherichia coli*, ArgR negatively regulates the expression of the arginine biosynthetic genes (the *arg* regulon) in response to intracellular L-arginine levels (reviewed in reference 16). In some bacteria, ArgR can also function as a transcriptional activator of arginine catabolic genes (13, 28). In addition to regulating arginine metabolism, there is some evidence that ArgR also regulates genes encoding arginine transporters, which allow bacteria to import arginine from an extracellular source (17, 21). Whether as a repressor or activator, ArgR requires allosteric activation by L-arginine (7, 16) to form a hexamer (20, 38) that can bind to an 18-bp palindromic operator sequence called the ARG box (4, 14, 32, 36). ARG boxes are located in close proximity to ArgR-regulated promoters; in fact, for genes that are negatively regulated by ArgR, the ARG box frequently overlaps the promoter, allowing ArgR binding to block promoter occupancy by RNA polymerase through steric hindrance.

On the basis of the conserved sequence of ARG boxes in bacteria, Makarova et al. used a bioinformatics approach to predict ArgR binding sites upstream of *glnPQ* and *artJ* in *C. pneumoniae* (17). These predictions seemed biologically plausible as the three genes encode a potential arginine transport system. *glnP* and *glnQ* encode a predicted ABC transporter protein and ATPase, respectively, while *artJ* encodes a putative periplasmic arginine binding protein. A pair of well-conserved ARG boxes were predicted upstream of *C. pneumoniae glnPQ* in a tandem arrangement (Fig. 1). Two slightly less conserved ARG boxes were predicted for *C. pneumoniae artJ*, but they were in separate locations (Fig. 1). From this bioinformatics analysis alone, it was not possible to predict whether ArgR negatively or positively regulated these candidate target genes.

In this study, we have examined the function of *C. pneumoniae* ArgR and found it to be a transcriptional aporepressor that requires L-arginine for activity. Recombinant ArgR spe-

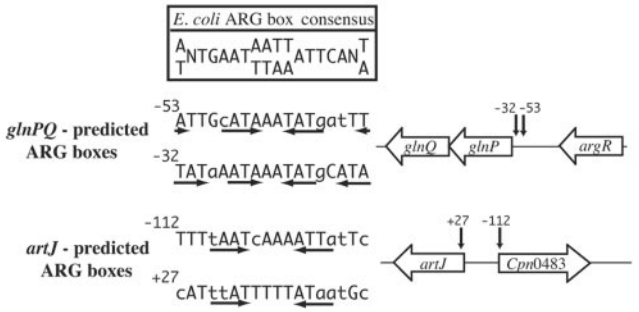

FIG. 1. Predicted ArgR operators upstream of *C. pneumoniae*. The 5′-to-3′ sequences of two predicted ArgR operators for *glnPQ* and another two for *artJ* are shown aligned with the *E. coli* ARG box consensus for comparison. Nucleotide differences from the *E. coli* consensus are shown in lowercase. Inverted repeat sequences are indicated with arrows below, and the numbers above the sequences indicate the positions relative to the translation start sites of *glnP* and *artJ*. The arrangement of each gene within the *C. pneumoniae* genome is shown on the right, with the location of each ARG box marked with a vertical arrow.

cifically bound both candidate ARG boxes upstream of *glnPQ* and repressed transcription. However, ArgR did not bind either ARG box predicted for *artJ*. *C. pneumoniae* ArgR was also able to bind an operator for *Chlamydophila caviae glnPQ*, but it did not bind upstream of *C. trachomatis glnPQ*. Our results suggest that a subset of chlamydial species have the necessary machinery for transcriptional regulation of an arginine transport system in response to intrachlamydial arginine levels.

MATERIALS AND METHODS

RT-PCR. *C. pneumoniae* CM1 RNA, obtained at 4, 12, 24, 48, and 70 h postinfection from infected HEp-2 cells, was a generous gift of Anatoly Slepenkin (UC Irvine) (27). RNA was also obtained from uninfected HEp-2 cells. To ensure that the RNA was free of genomic DNA contamination, each sample was treated with DNase I and incubated at 37°C for 30 min. After the DNase I was inactivated, the RNA samples were treated with DNase I for a second time with an on-column digestion using the RNeasy RNA cleanup kit (QIAGEN, Valencia, Calif.). cDNA was synthesized from 1 μg RNA using avian myeloblastosis virus (AMV) reverse transcriptase (RT) and specific 3′ primers designed according to the *C. pneumoniae* genome sequence (8) to anneal to sequences within the open reading frame regions of *argR*-T615 (5′-TAACAAAGCAGCGATCCAA), *artJ*-T613 (5′-GGTGCTATCAAAAGAACGGA), *glnP*-T611 (5′-TTCATTGGTTAAGGATGGTAAAA), and *glnQ*-T609 (5′-AATAGCCACACGTTGTTTTTGT). For each sample, parallel negative control reactions were performed in the absence of reverse transcriptase. Standard PCR assays with *Taq* polymerase were used to detect the presence or absence of cDNA using the following *C. pneumoniae* primer pairs: *glnP*-T610 (5′-GGATTGCCTGAAGTTCTCC) and T611, *glnQ*-T608 (5′-GGGGAGGCTCCAGCTCTA) and T609, *artJ*-T612 (5′-ATGTCCATTACTCCTTCGC) and T613, and *argR*-T771 (5′-CGTCTTGAAGGAGCGGC) and T772 (5′-ACAATAAGAGAGGCGTTATGGC), which yielded predicted DNA products of 231, 216, 213, and 218 bp, respectively. PCR was performed for 30 cycles with a 1-min annealing step at 55°C.

Cloning of *C. pneumoniae* ArgR. *C. pneumoniae argR* was cloned into the expression vector pRSET-C (Invitrogen, Carlsbad, Calif.) to produce plasmid pMT1227, which expressed full-length ArgR (except for the ATG start codon) with an N-terminal six-histidine tag. *argR* was amplified by PCR with *Tgo* DNA polymerase (Roche Diagnostics, Indianapolis, Ind.) using *C. pneumoniae* CWL029 genomic DNA and primers T257 (5′-AAAAAAAAAGTAACTATAGATGAGGCTTTAAA) and T258 (5′-GCGGTACCTTAATCCAAGAAAACTTGCAGTAAAT). The PCR product was digested with KpnI and cloned into pRSET-C between the KpnI and blunted BamHI sites. pMT1227 was sequenced to ensure that the coding region of ArgR matched the published nucleotide sequence (8).

Overexpression and purification of the ArgR protein. His_6-ArgR was overexpressed in *E. coli* BL21(DE3) cells (Stratagene, La Jolla, Calif.) freshly transformed with pMT1227. Cells (1.5 liters) were grown at 37°C to an optical density at 600 nm of 0.6 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 2.5 h, cells were collected by centrifugation, resuspended in 10 ml buffer B (20 mM Tris [pH 8.0], 500 mM NaCl) containing 6 M guanidine hydrochloride and 5 mM imidazole, and disrupted with a Branson Sonifier 450 (twice for 30 seconds). The denatured protein was separated from cell debris by centrifugation at 14,000 × *g* for 10 min at 4°C (Beckman JA-17 rotor), filtered, and then loaded onto a 1-ml HiTrap nickel column (Amersham Biosciences, Piscataway, N.J.) that had been equilibrated with 5 ml of buffer B containing 5 mM imidazole. The column was washed with 50 ml of buffer B containing 6 M guanidine hydrochloride and 75 mM imidazole at a flow rate of 1 ml/min on a Pharmacia fast protein liquid chromatography system, followed by 10 ml of buffer B containing 30 mM imidazole and 6 M urea. The His_6-ArgR was refolded on the column by removing the urea with a 30-ml gradient of buffer B containing 30 mM imidazole, starting with 6 M urea and ending with no urea. The column was washed with an additional 10 ml of buffer B containing 30 mM imidazole. His_6-ArgR was eluted with buffer N-500 (10 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10 mM 2-mercaptoethanol, 500 mM imidazole). One-milliliter fractions were collected, and fractions 2 and 3 were pooled and concentrated to <1 ml with an Amicon Centriplus YM-10 protein concentrator (Millipore, Bedford, Mass.) for 90 min at 3,000 × *g* (Beckman JA-17 rotor). The concentrated eluate was loaded onto a Pharmacia Superdex-75 gel filtration column (Amersham Biosciences) and eluted with 50 ml of buffer N (10 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10 mM 2-mercaptoethanol) at a flow rate of 0.5 ml/minute using a Pharmacia AKTA/fast protein liquid chromatography system. Fractions containing His_6-ArgR were pooled and concentrated with an Amicon Centriplus YM-10 protein concentrator for 90 min at 3,000 × *g* (Beckman JA-17 rotor) and then dialyzed against storage buffer (10 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 100 μM EDTA, 10 mM 2-mercaptoethanol, 100 mM NaCl, 30% glycerol) overnight and again for 4 h. His_6-ArgR concentration (~2 μM) was determined by the Bio-Rad protein assay, and aliquots were stored at −70°C. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by silver stain and Western blot analysis using an anti-RGS-His_6 monoclonal antibody. The final protein preparation was determined to be >95% pure by silver staining, with a concentration of 2.1 μM. ArgR concentrations were based on an ArgR monomer unless otherwise stated.

DNA templates for the EMSA. DNA fragments used as electrophoretic mobility shift assay (EMSA) probes were first cloned into the SmaI site of plasmid pGEM-7Zf(+) (Promega Biotech, Madison, Wis.). The following DNA fragments were amplified by PCR from *C. pneumoniae* CWL029 genomic DNA: a 156-bp *glnPQ* fragment containing the entire upstream intergenic region with tandem ArgR operators was amplified with primers T442 (5′-GCCGGAATTCTCCCATTGTTGATGGTTTC) and T478 (5′-GGTGTATCCGCATCCTCTTAA) to produce pMT1326; a 284-bp *artJ* fragment containing the entire upstream intergenic region with both predicted ArgR operators was amplified with primers T526 (5′-GCCGAATTCATTGGGCGGGCAATG) and T527 (5′-AAATTTGAAAAAGTTTTAACTTTGAATA) to produce pMT1317; a 199-bp *artJ* fragment containing the upstream intergenic region with predicted upstream ArgR operators was amplified with primers T526 (5′-GCCGAATTCATTGGGCGGGCAATG) and T670 (5′-CTATACGCTAAATTCTATCATGTGTTAA) to produce pMT1330; and a 230-bp *argR* fragment containing the entire upstream intergenic region was amplified with primers T662 (5′-TCCTGAGTTGCCGCTC) and T663 (5′-CTAATATCTGCTTATCCTCATTAACTATAG) to produce pMT1333. The following DNA fragments were amplified by PCR from *C. trachomatis* serovar D genomic DNA: a 358-bp *C. trachomatis glnP* fragment containing the entire upstream intergenic region was amplified with primers T664 (5′-CTTTGACTGGGTGCCTTTC) and T665 (5′-CACACACCAAAGAAATAGCTGTAAT), designed according to the *C. trachomatis* genome sequence (31), to produce pMT1335; a 221-bp restriction fragment containing the entire upstream region of *C. trachomatis artJ* was amplified with primers T666 (5′-CGTCAAACAAAAACTACAGACAA) and T667 (5′-CAACATATTCTTCCTTGTGAACTC) to produce pMT1334; and a 110-bp *C. trachomatis trpR* DNA fragment for use as a nonspecific competitor was amplified with primers T566 (5′-GAGGGGAGAATTCTAAGAAAAGA) and T567 (5′-GCCAGCCAGACTCCTCTT) to produce pMT1283. The following DNA fragment was amplified by PCR from *C. caviae* genomic DNA: a 120-bp restriction fragment containing the tandem ArgR operators upstream of *C. caviae glnPQ* was amplified with primers T677 (5′-CCTTAACAGCCAAAGATATAGAAAACTT) and T678 (5′-GCTGTAGCAACCACATGTTC), designed according to the *C. caviae* genome sequence (23), to produce pMT1337. Plasmids containing the EMSA restriction fragment templates were digested with XbaI and BamHI and dephosphorylated with calf intestinal alkaline phosphatase, and the EMSA fragments were gel purified from a 2% agarose gel.

Double-stranded oligonucleotide fragments were prepared by mixing equimolar amounts of complementary primers in annealing buffer (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA) at 94°C for 2 min and then allowing annealing to take place during cooling to 4°C over a 1-hour period. The specific oligonucleotide competitor containing the *C. pneumoniae glnPQ* tandem ArgR operators was constructed by annealing primers T690 (5-GATCCTAATTGCATAA ATATGATTTCATTATAAATAAATATGCATAAG) and T691 (5′-CTAGAC TTATGCATATTTATTTATAATGAAATCATATTTATGCAATTA). The nonspecific competitor containing the *C. trachomatis dnaK* CIRCE operator was constructed by annealing primers T224 (5′-AAACTAGCACTCTTTAGTTGC GAGCGCTAAAA) and T225 (5′-TTTTAGCGCTCGCAACTAAAGAGTGC TAGTTT). A *C. pneumoniae glnPQ* oligonucleotide probe containing only the downstream ArgR operator was constructed by annealing primers T744 (5′-GA TCCCTGCAAGTTTTCTTGGATTACATTATAAATAAATATGCATAAG) and T745 (5′-CTAGACTTATGCATATTTATTTATAATGTAATCCAAGAA AACTTGCAG); a *C. pneumoniae glnPQ* oligonucleotide probe containing only the upstream ArgR operator was constructed by annealing primers T742 (5′-G ATCCTAATTGCATAAATATGATTTCATAGAGGTCGGAGTGGATC ATT) and T743 (5′-CTAGAAATGATCCACTCCGACCTCTATGAAATCAT ATTTATGCAATTA).

EMSA. One-hundred nanograms of each DNA restriction fragment or double-stranded oligonucleotide probe was labeled with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase in a fill-in reaction. Free nucleotides were removed using a mini-Quick Spin DNA column (Roche Diagnostics), and the activity of the probe was quantified using a scintillation counter. EMSA reaction mixtures were assembled on ice and contained 20 mM Tris (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, 10 mM 2-mercaptoethanol, 7.5 mM L-arginine, 5% glycerol, and 0.62 nM α-^{32}P-labeled DNA probe in a final reaction volume of 12 μl. A range of concentrations of *C. pneumoniae* His_6-ArgR from 0 to 400 nM (monomer) were tested, with details in the legend of Fig. 3. Some reaction mixtures also included unlabeled competitor DNA fragments or oligonucleotides or anti-RGS-His_6 monoclonal antibody (QIAGEN) at a final concentration of 0.83 ng/ml. The binding reactions were performed at room temperature for 20 min, and then the reaction products were loaded onto a 6% polyacrylamide gel (prerun at 100 V for 1 h) containing 7.5 mM L-arginine. The gel was electrophoresed at 300 V in ArgR EMSA buffer (50 mM Tris [pH 8.8], 50 mM boric acid, 0.5 mM EDTA, 7.5 mM L-arginine). After electrophoresis, the gel was dried down and exposed to a phosphorimager screen. The screen was scanned using a Bio-Rad personal FX scanner, and the data were analyzed with Quantity One software (Bio-Rad, Hercules, Calif.). The dissociation constants for the binding reactions were calculated using KaleidaGraph software (Synergy Software, Reading, Pa.).

Construction of transcription plasmids. The promoter region of *C. pneumoniae glnPQ* (−104 to +12) was amplified by PCR from *C. pneumoniae* CWL029 genomic DNA with primers T442 (5′-GCCGGAATTCTCCCATTGT TGATGGTTTC) and T443 (5′-TTATGGATATTTATTTATAATGAAATCA TATTTATG). The G residues at −9 and −15, relative to the 5′ end of the translation start codon, were each changed to A residues to allow for transcription in the absence of GTP. This *glnPQ* promoter insert was cloned upstream of a promoterless G-less cassette transcription template in pMT1125 (39) to produce plasmid pMT1226. Transcription from this plasmid produced a 154-nucleotide transcript. The control plasmid, pMT1198, was constructed by cloning the *C. trachomatis* serovar D *omcB* promoter (−37 to +5) into pMT1125 (39), followed by a PacI digestion to remove 28 bp of the synthetic G-less cassette. Transcription of the control plasmid produced a 126-nucleotide transcript.

In vitro transcription. Transcription reactions were performed with heparin-agarose-purified *C. trachomatis* RNA polymerase as previously described (26). In some reaction mixtures, *C. pneumoniae* His_6-ArgR (over a range of concentrations from 40 to 240 nM monomer) and/or L-arginine (from 1 μM to 5 mM) was added, with more details given in the legends to Fig. 5 and 6. For the kinetic experiments, the reaction mixtures were scaled up to 70 μl, and 10-μl aliquots were removed at 2, 4, 6, 8, and 10 min after transcription had been initiated by the addition of RNA polymerase. The time course for the transcription experiments was normalized to a 1-minute lag in the appearance of radiolabeled transcripts after the reactions were started by the addition of RNA polymerase. Transcription rates were calculated by using KaleidaGraph software (Synergy Software).

RESULTS

Expression patterns of *C. pneumoniae glnP*, *glnQ*, *artJ*, and *argR*. We first determined whether the genes encoding the putative arginine transport system are expressed during the chlamydial developmental cycle. The expression patterns of the predicted arginine transport genes, *glnP*, *glnQ*, and *artJ*, as well as *argR*, the gene that encodes a putative arginine repressor, were measured by RT-PCR during the early, middle, and late stages of the *C. pneumoniae* developmental cycle. A transcript for each gene was detected at 4, 12, 24, 48, and 70 h postinfection (Fig. 2), indicating that *glnP*, *glnQ*, *artJ*, and *argR* are constitutively expressed during the *C. pneumoniae* developmental cycle under normal growth conditions. No PCR product was detected in the negative control reactions lacking RT, indicating that the template was cDNA and not genomic DNA.

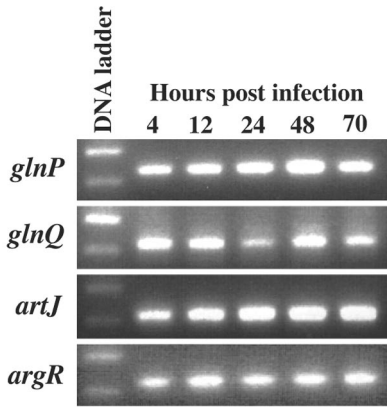

FIG. 2. RT-PCR analysis of the expression patterns of *C. pneumoniae glnP*, *glnQ*, *artJ*, and *argR* at 4, 12, 24, 48, and 70 h postinfection. The DNA ladder is shown for reference (upper band, 300 bp; lower band, 200 bp).

As *glnP* and *glnQ* are adjacent, with open reading frames that overlap by 11 bp, we examined their transcriptional organization to determine whether they are part of an operon. We repeated the RT-PCR assay with primers from the 5′ region of *glnP* and the 3′ region of *glnQ*. We were able to amplify the predicted 823-bp product (data not shown), indicating that *glnP* and *glnQ* are cotranscribed as a polycistronic mRNA.

ArgR binds to the upstream region of *C. pneumoniae glnPQ*. To study the function of chlamydial ArgR, we first examined its ability to bind DNA. We used an EMSA to determine whether purified recombinant *C. pneumoniae* ArgR can bind to the predicted ArgR operators located upstream of *glnPQ* (17). A 156-bp DNA restriction fragment containing the putative tandem ArgR operators was radiolabeled and used as the DNA probe. As ArgR in other bacteria is an aporepressor that is functional only in the presence of the corepressor L-arginine, we performed the binding reaction and the gel electrophoresis in the presence and absence of L-arginine. With L-arginine, His_6-ArgR bound to the *glnPQ* DNA fragment in a concentration-dependent manner, with complete binding of the probe by 400 nM ArgR (Fig. 3A, lanes 1 to 8). The gel shift complex consisted of at least two lower-mobility bands, which is consistent with the observation in *E. coli* that there is oligomerization of ArgR dimers at the operator site (32). This binding was not observed when L-arginine was absent (data not shown). The kinetics of binding were determined using EMSA reaction mixtures with a fixed DNA probe concentration (0.62 nM) over

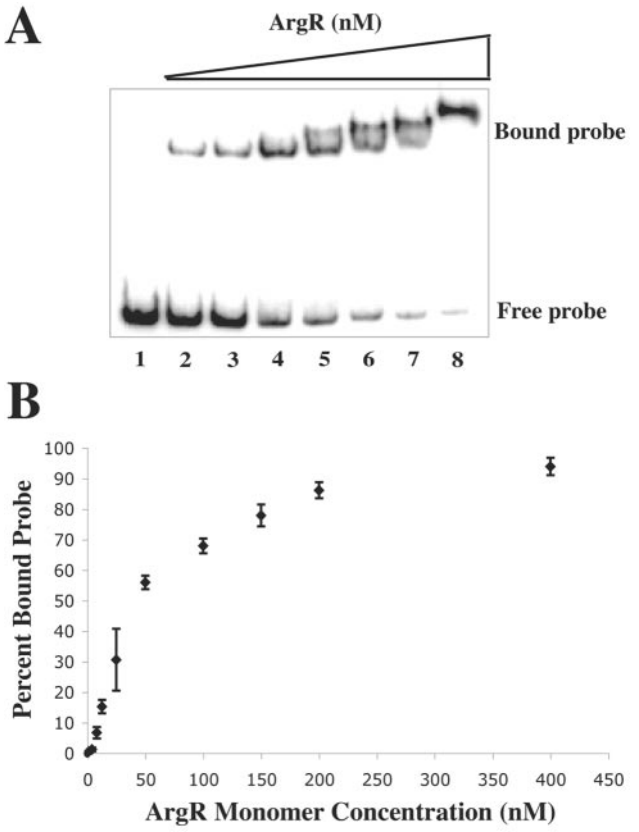

FIG. 3. EMSA with a *C. pneumoniae glnPQ* DNA fragment probe, containing tandem ARG boxes, and purified recombinant *C. pneumoniae* ArgR. The autoradiogram of the gel is shown with the positions of free and bound probes marked. (A) ArgR titration with final ArgR protein concentrations (monomer) of 0, 12.5, 25, 50, 100, 150, 200, and 400 nM in lanes 1 to 8, respectively. (B) Quantification of EMSA. EMSA reactions with 0.6 nM ^{32}P-labeled *glnPQ* DNA probe, 7.5 mM L-arginine, and various concentrations of recombinant ArgR were performed in triplicate and quantified by phosphorimager analysis. Error bars represent standard deviations from the means.

a range of ArgR concentrations (4, 8, 12.5, 25, 50, 100, 150, 200, and 400 nM) (Fig. 3B). Half depletion of the free DNA probe occurred at 49 nM ArgR monomer, corresponding to an apparent dissociation constant of 8.2 nM ArgR hexamer.

We performed control EMSA reactions to confirm that His_6-ArgR binding to *glnPQ* DNA was specific. The addition of anti-RGS-His_6 antibody to the binding reaction produced a supershift consisting of two slower-migrating species with loss of the original shifted band (Fig. 4A, lane 3), confirming that the gel shift was due to ArgR. In competition experiments, a molar excess of unlabeled *glnPQ* DNA fragment was able to compete for ArgR binding in a concentration-dependent manner, with complete loss of the gel shift at 64-fold excess competitor DNA (Fig. 4B, lanes 1 to 7). No competition was observed when a nonspecific DNA restriction fragment was used (Fig. 4B, lanes 8 to 12). These results demonstrate that His_6-ArgR binding to the *glnPQ* DNA fragment was specific.

We performed additional experiments to delineate the DNA sequence required for ArgR binding. A short, unlabeled double-stranded oligonucleotide containing only the putative tandem ARG boxes was able to compete with the labeled *glnPQ* DNA fragment for ArgR binding (Fig. 4C, lanes 1 to 6). In contrast, a nonspecific double-stranded oligonucleotide was unable to compete for binding (Fig. 4C, lane 7 to 10). There was no significant difference in ArgR binding affinity between the tandem *glnPQ* ARG boxes and either individual ARG box alone (data not shown), suggesting that a single ARG box was sufficient for *C. pneumoniae* ArgR binding under these in vitro binding conditions.

We were unable to detect specific binding of ArgR to sequences adjacent to *artJ* or *argR*, using EMSA with ArgR concentrations as high as 1 μM (data not shown). For *artJ*, we tested both of the ARG boxes that have been predicted by a bioinformatics analysis (17). These findings suggest that neither *artJ* nor *argR* contains binding sites for ArgR.

***C. pneumoniae glnPQ* is transcribed by σ^{66} RNA polymerase.** To study regulation by ArgR, we first defined the basal transcription of one of its target genes by identifying the promoter and determining the form of RNA polymerase involved in transcription. We chose *C. pneumoniae glnPQ* as we had located binding sites for ArgR upstream of this operon. By sequence inspection, we located promoter sequences upstream of *glnPQ* that were predicted to be recognized by the main chlamydial RNA polymerase, σ^{66} RNA polymerase. This candidate promoter contained −35 and −10 elements (TTGGAT and TTTCAT, respectively, spaced 17 bp apart) (Fig. 5A) with a 3/6 and a 4/6 respective match to the optimal promoter elements recognized by *C. trachomatis* σ^{66} RNA polymerase (25, 34). Further support for this predicted promoter was provided by the fact that it overlapped the tandem ARG boxes of *glnPQ* that had been shown to be binding sites for ArgR (Fig. 5A). As there is no available *C. pneumoniae* transcription assay, we tested this predicted *C. pneumoniae glnPQ* promoter with our *C. trachomatis* in vitro transcription assay (33) and found that it was transcribed by σ^{66} RNA polymerase (Fig. 5B, lane 1).

ArgR represses transcription of the *glnPQ* promoter. We next tested whether *C. pneumoniae* ArgR could modulate transcription of the *glnPQ* promoter. We tested a range of His_6-ArgR concentrations and performed the transcription assays with and without L-arginine. ArgR alone had no significant effect but in the presence of L-arginine, in vitro transcription of the *glnPQ* promoter was clearly repressed (Fig. 5B, compare lane 2 with lanes 3 to 7). This negative regulation by ArgR was concentration dependent, with 50% repression occurring at approximately 60 nM ArgR and complete repression occurring by 120 nM (Fig. 5C). As an internal negative control, we used the *C. trachomatis omcB* promoter in each transcription reaction. This promoter lacks an identifiable ARG box, and its transcription was not affected by ArgR (Fig. 5B).

To examine the role of L-arginine as a cofactor, we tested the effect of L-arginine concentrations on transcriptional repression by ArgR. In an in vitro transcription reaction with saturating amounts of ArgR, repression was dependent on the concentration of L-arginine (Fig. 6, lanes 1 to 5). At 1 μM L-arginine, corresponding to a 4:1 ratio of L-arginine to ArgR monomer, transcription was repressed by 25%, while complete repression was seen only at a ratio greater than 400:1, indicating that molar excess of the corepressor, L-arginine, is necessary for negative regulation by ArgR, as seen in other bacteria

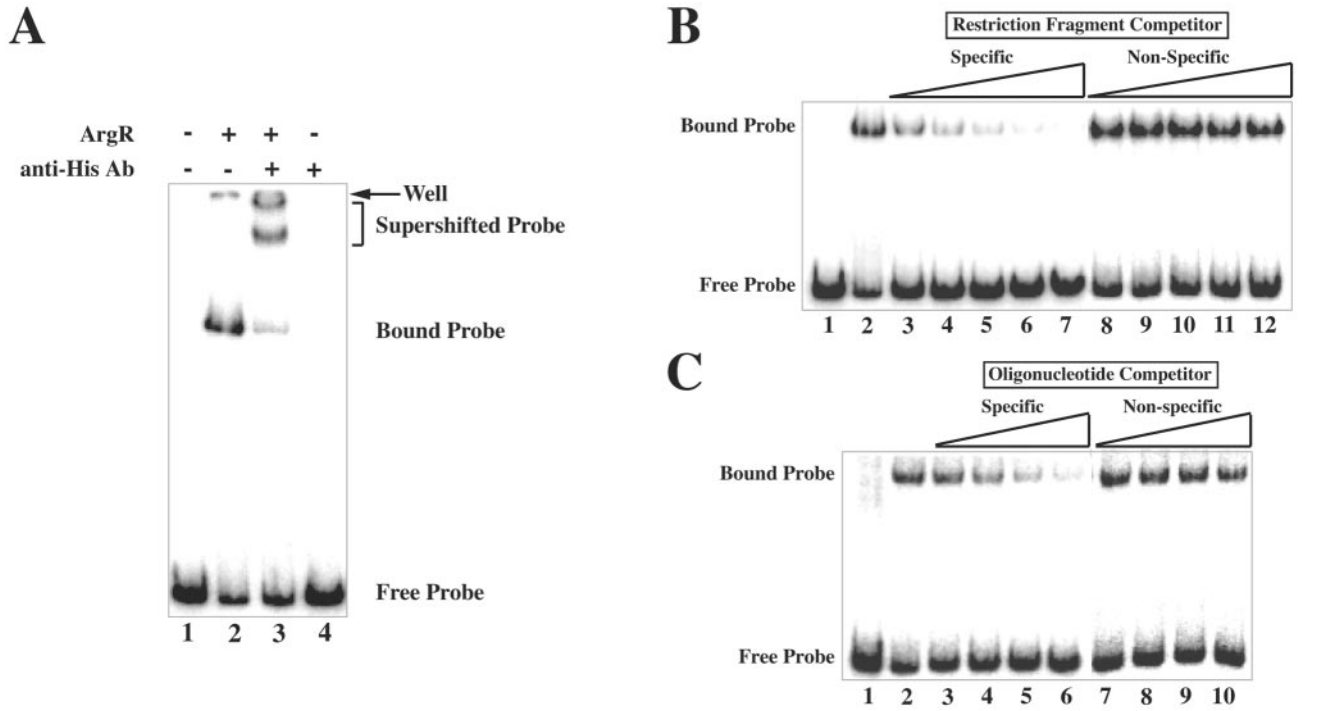

FIG. 4. EMSA specificity controls for ArgR binding to *C. pneumoniae glnPQ* DNA. (A) Antibody supershift EMSA. Lane 1, labeled probe alone; lane 2, addition of ArgR alone; lane 3, addition of ArgR and anti-RGS-His_6 monoclonal antibody (Ab); and lane 4, addition of anti-RGS-His_6 antibody alone. (B) Competition with DNA restriction fragments. Lane 1, labeled probe alone; lane 2, addition of ArgR alone; lanes 3 to 7, addition of unlabeled specific competitor containing tandem *glnPQ* ARG boxes (4, 8, 16, 32, and 64-fold excess compared to labeled probe); and lanes 8 to 12, addition of unlabeled nonspecific competitor (4-, 8-, 16-, 32-, and 64-fold excesses compared to labeled probe). (C) Competition with double-stranded oligonucleotides. Lane 1, labeled probe alone; lane 2, addition of ArgR alone; lanes 3 to 6, addition of unlabeled double-stranded oligonucleotide containing only the tandem *glnPQ* ARG boxes (8-, 16-, 32-, and 64-fold excesses compared to labeled probe); and lanes 7 to 10, addition of unlabeled nonspecific double-stranded oligonucleotide (128-, 256-, 512-, and 1,024-fold excesses compared to labeled probe). All reactions were performed with 0.6 nM ^{32}P-labeled *C. pneumoniae glnPQ* DNA fragment probe, 7.5 mM L-arginine, and 60 nM ArgR.

(32). We also tested other representative amino acids, such as L-alanine, L-serine, L-methionine, L-glutamine, L-tryptophan, and L-proline, but none of them functioned as a corepressor for ArgR (data not shown). Our results indicate that ArgR functions as an aporepressor that modulates *glnPQ* promoter activity in an L-arginine-dependent manner.

***C. pneumoniae* ArgR is a competitive inhibitor of σ^{66} RNA polymerase.** To further elucidate the molecular mechanism of *C. pneumoniae* ArgR-dependent repression, we measured the transcription kinetics of the *glnPQ* promoter alone and with medium and high concentrations of ArgR. Transcription of the *glnPQ* promoter in the absence of ArgR increased over time, reaching end point saturation by 8 min postinitiation (Fig. 7). Addition of 80 nM ArgR produced about a twofold decrease in transcription, with approximately 50% repression over all time points tested (Fig. 7). However, the rate of *glnPQ* transcription in the presence or absence of ArgR was about the same (0.44 $\pm$ 0.15 versus 0.46 $\pm$ 0.13, respectively). For comparison, complete repression of the *glnPQ* promoter by 160 nM ArgR is also shown (Fig. 7). The ability of ArgR to decrease end point transcription without affecting the rate of transcription indicates that ArgR acts as a transcriptional repressor by blocking RNA polymerase from binding to the promoter region.

***C. pneumoniae* ArgR binds to the upstream region of *glnPQ* in *C. caviae* but not *C. trachomatis*.** Of the five species of *Chlamydia* that have been completely sequenced to date (8, 22, 23, 31, 35), *C. caviae* is the only other species besides *C. pneumoniae* that contains an identifiable ArgR homolog. *C. caviae* is a pathogen of guinea pigs that was until recently known as *C. psittaci* GPIC strain, reflecting its phylogenetic relatedness to *C. psittaci*. *C. caviae* also contains the predicted target genes *glnP* and *glnQ* located downstream of *argR* in an arrangement that is similar to that of *C. pneumoniae*. By sequence inspection, we identified two candidate ARG boxes upstream of *C. caviae glnPQ* that closely resemble the tandem *C. pneumoniae glnPQ* operators in both sequence and spacing (Fig. 8A). Using EMSA, we demonstrated that *C. pneumoniae* ArgR was able to bind to the predicted *C. caviae glnPQ* operators in a concentration-dependent manner (Fig. 8B, lanes 1 to 8), with a binding affinity similar to that of the *C. pneumoniae* operators. These results suggest that ArgR may also negatively regulate transcription of *glnPQ* in *C. caviae*. Although *C. caviae* encodes a homolog of the predicted periplasmic arginine binding protein ArtJ, we were unable to demonstrate the presence of an ArgR binding site upstream of this gene (data not shown), providing further evidence that *artJ* is not regulated by ArgR in *Chlamydia*.

In light of the observation that *C. trachomatis* does not appear to encode ArgR (31), we were also interested in testing whether *C. pneumoniae* ArgR could recognize an operator

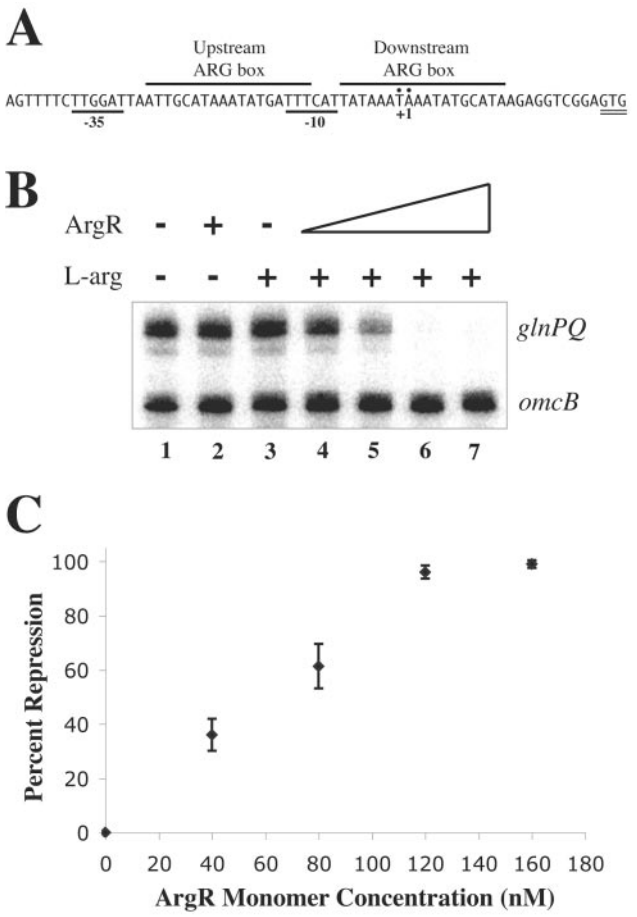

FIG. 5. Transcriptional regulation of *C. pneumoniae glnPQ*. (A) Sequence of the predicted *glnPQ* promoter. The −35 and −10 promoter elements are underlined. The in vitro transcription initiation sites are marked with a dot above the nucleotide, with an A residue designated as +1, and the translation start codon is underlined twice. The two ARG boxes are marked with bars above the sequence. (B) In vitro transcription with purified recombinant ArgR. The upper transcript is from the *C. pneumoniae glnPQ* promoter, and the lower transcript is from the *C. trachomatis omcB* promoter. Lane 1, no ArgR and no L-arginine; lane 2, 160 nM ArgR and no L-arginine; lane 3, no ArgR and 5 mM L-arginine; and lanes 4 to 7, increasing concentrations of ArgR (40, 80, 120, and 160 nM monomer) in the presence of 5 mM L-arginine. (C) Quantification of in vitro transcription. Transcription reactions for the *glnPQ* promoter in the presence of 5 mM L-arginine and various concentrations of purified recombinant ArgR were performed in triplicate and quantified by phosphorimager analysis. The error bars represent standard deviations from the means.

upstream of predicted arginine transport genes in *C. trachomatis*. In EMSA experiments, *C. pneumoniae* ArgR was unable to bind to the upstream intergenic regions of *C. trachomatis glnPQ* and *artJ* (data not shown). These results support the hypothesis that the predicted arginine transport genes *glnPQ* and *artJ* are not regulated by ArgR in *C. trachomatis*.

DISCUSSION

This study demonstrates that ArgR is a transcriptional aporepressor used by some, but not all, *Chlamydia* species to regulate gene expression in response to intrachlamydial arginine levels. The groundwork for this investigation was based on the wealth of information from comparative genomics, which allowed a chlamydial species-specific candidate regulator to be identified (8, 22, 31). A subsequent bioinformatics analysis predicted target genes with an associated DNA binding site for ArgR although it could not anticipate whether ArgR was a positive or negative regulator (17). Our experimental studies to test these predictions required identification of an ArgR-regulated promoter and a functional assay of promoter activity. We have found that *C. pneumoniae* ArgR can function as a transcriptional repressor in the presence of L-arginine and acts as a competitive inhibitor of RNA polymerase.

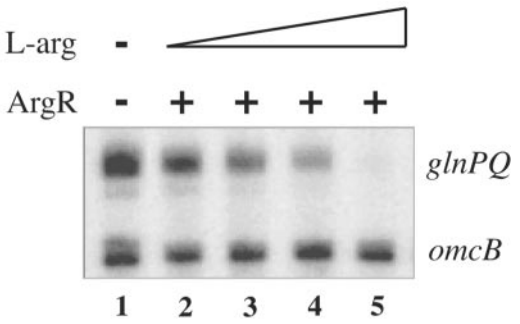

FIG. 6. Effect of L-arginine on the regulation of in vitro transcription of *C. pneumoniae glnPQ* by recombinant *C. pneumoniae* ArgR. The upper transcript is from the *C. pneumoniae glnPQ* promoter, and the lower transcript is from the *C. trachomatis omcB* promoter. Lane 1, no ArgR and no L-arginine (L-arg); and lanes 2 to 5, 240 nM ArgR and increasing concentrations of L-arginine (1, 50, 100, and 500 μM).

We were able to demonstrate that two of the three genes that together potentially encode an arginine transporter, and which were predicted to be target genes for ArgR in *C. pneumoniae* (17), are in fact regulated by this arginine-responsive

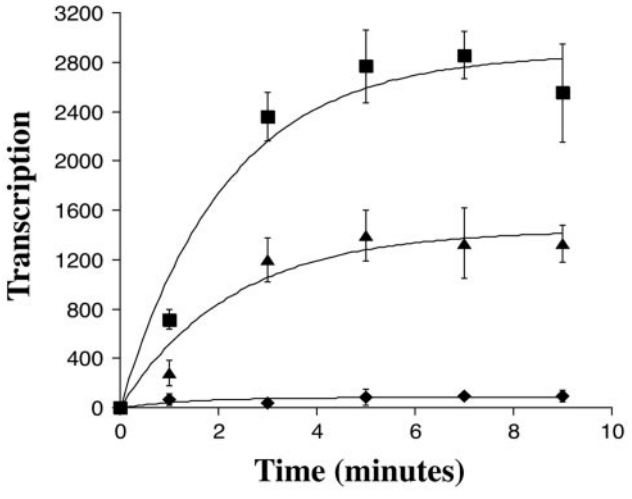

FIG. 7. Effect of purified recombinant *C. pneumoniae* ArgR on the rate of in vitro transcription of the *C. pneumoniae glnPQ* promoter. Transcription reactions were performed in the presence of no ArgR and 5 mM L-arginine (squares), 80 nM ArgR and 5 mM L-arginine (triangles), or 160 nM ArgR and 5 mM L-arginine (diamonds). Each reaction was performed in triplicate and quantified by phosphorimager analysis. Error bars represent standard deviations from the means.

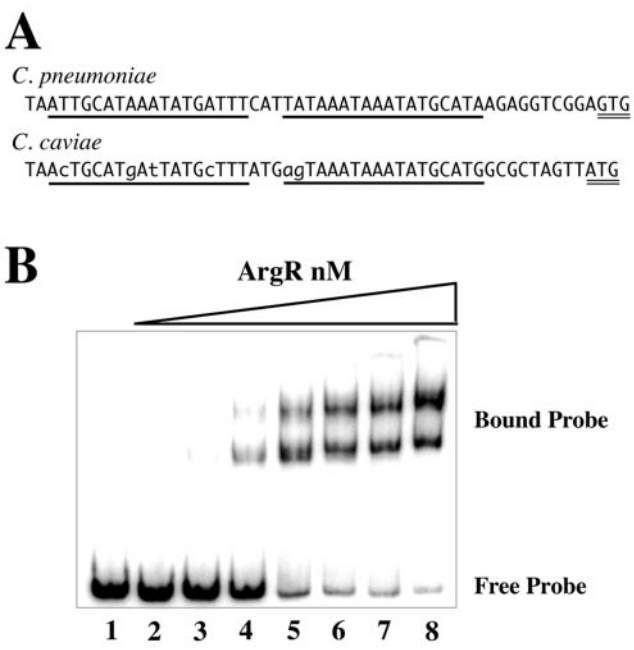

FIG. 8. Predicted ArgR operators upstream of *C. caviae glnPQ*. (A) Alignment of the sequences upstream of *C. pneumoniae* and *C. caviae glnPQ*. Predicted tandem ARG boxes are underlined, with nucleotide differences from the respective *C. pneumoniae* ARG box shown in lowercase. The translation start codon of *glnP* is underlined twice. (B) EMSA with *C. caviae glnPQ* DNA probe, containing tandem ARG boxes, and purified recombinant *C. pneumoniae* ArgR in the presence of 7.5 mM L-arginine. ArgR titration with final ArgR protein concentrations (monomer) of 0, 12.5, 25, 50, 100, 150, 200, and 400 nM in lanes 1 to 8, respectively.

transcription factor. We found that *glnP* and *glnQ*, which code for a predicted ABC transporter and ATPase, respectively, are part of an operon that is transcriptionally regulated by ArgR. However, we found no evidence that ArgR regulates the third gene, *artJ*. These findings were not too surprising as the evidence was stronger for *glnPQ*, which has two adjacent ARG boxes that resemble ArgR binding sites both in sequence and in location overlapping the *glnPQ* promoter. In particular, their tandem arrangement with a 3-bp spacing is typical of high-affinity ArgR binding sites and allows the centers of the ARG boxes to be 21 bp apart and thus aligned on the same face of the DNA. In contrast, the ArgR operators predicted for *artJ* were physically separated, with one site located within the coding region of *artJ* and the other located 119 bp upstream of the coding region. We were also unable to locate a functional promoter for *artJ* near either of the predicted ARG boxes. It remains formally possible that ArgR may bind to the predicted ARG boxes of *artJ* under conditions different from those tested in our in vitro assays. We also found that ArgR did not bind upstream of *argR*, supporting the prediction that *argR* expression is not autoregulated in *Chlamydia* (17) as it is in some bacteria (11, 12). Our results suggest that the sequence of the ARG box is conserved in *Chlamydia* but demonstrate the need for functional testing to confirm that predicted ARG boxes are indeed binding sites for ArgR.

The finding that ArgR is a regulator of genes that are likely to be involved in arginine transport provides further support for ArgR as a homeostatic regulator of arginine in bacteria. ArgR has been well studied as a transcriptional regulator of arginine biosynthesis (reviewed in reference 16), with complementary roles as a repressor of arginine biosynthetic genes and an activator of arginine catabolic genes (13, 28). ArgR has even been shown to function as a coactivator of the anaerobic activator ANR (15). The role of ArgR in regulating arginine transport has not been studied to the same extent. There is in vivo evidence that ArgR is involved in the positive regulation of arginine transport genes in *Pseudomonas aeruginosa* (21), and ARG boxes have been predicted upstream of genes encoding an arginine transport system in *E. coli* (17, 40, 41). Our cell-free studies provide direct evidence that ArgR is able to function as an arginine-dependent transcriptional repressor of genes encoding a predicted transporter.

The implication of this work is that some, but not all, chlamydial species utilize an ArgR-dependent mechanism to maintain intrachlamydial arginine levels by regulating uptake of arginine from the host cell. This mechanism appears to be conserved in two phylogenetically related species, *C. pneumoniae* and *C. caviae*, a pathogen of guinea pigs (6). We predict that another closely related species, *C. psittaci*, shares this arginine-responsive machinery, as it has a predicted ArgR homolog and ARG boxes can be identified upstream of *glnPQ* in its genome (23). In contrast, while *C. trachomatis* has genes that encode the same putative arginine transporter, it does not have an identifiable ArgR homolog and does not appear to regulate *glnPQ* through ArgR. It remains formally possible that ArgR is present in *C. trachomatis* but not well conserved and that an ArgR operator for *C. trachomatis glnPQ* could not be identified in our studies using the heterologous *C. pneumoniae* ArgR.

The simplest explanation for these species-specific differences is that *C. pneumoniae*, *C. caviae*, and *C. psittaci* require the ability to adjust to a range of arginine levels from the host cell and that *C. trachomatis* does not. One can speculate that arginine levels available to *C. trachomatis* from its host cell in the eye or genital tract are always in an acceptable range and never high enough to warrant a mechanism for limiting import of this amino acid. Alternatively, *C. trachomatis* may be able to tolerate high intrachlamydial arginine levels that *C. pneumoniae* and *C. caviae* actively prevent from accumulating. Studies examining the role of arginine levels during chlamydial growth in cell culture have been limited by host cell and chlamydial requirements for arginine (10, 37).

Parachlamydia UWE25, a chlamydia-like endosymbiont of free-living amoebae, also does not encode identifiable homologs of ArgR or the putative arginine transporter system components GlnP and GlnQ (M. Horn, personal communication). At this time, it is not possible to determine whether the ArgR-dependent regulatory mechanism was selectively lost during the evolution and divergence of *Chlamydiaceae* and *Parachlamydiaceae* or selectively acquired by the lineage that includes *C. pneumoniae* and *C. caviae*.

The acquisition of amino acids and other nutrients is a critical issue for an obligate intracellular parasite like *Chlamydia*, which has simplified its genome to such an extent that it is dependent on the host cell for growth and replication. As chlamydiae lack arginine biosynthetic genes and thus the abil-

ity to make their own arginine, they are dependent on arginine uptake from the host cell as the source of this essential amino acid. Regulation of the expression of transporter genes by ArgR provides a mechanism for maintaining homeostatic control of arginine levels by controlling arginine import. This is the first demonstration of an amino acid serving as a cofactor for a transcription factor in *Chlamydia*, but it is unlikely to be the only example. In fact, the regulation and autoregulation of intrachlamydial amino acid levels are being increasingly recognized as important factors in chlamydial pathogenesis. For instance, some chlamydial species also encode TrpR, a putative tryptophan aporepressor (42 and J. Akers and M. Tan, unpublished data) that has been shown to utilize tryptophan as a cofactor for regulation of the tryptophan biosynthetic genes in other bacteria (reviewed in reference 29). Tryptophan has been shown to play a pivotal role in chlamydial pathogenesis, and tryptophan limitation induces chlamydiae to enter a persistent growth state (reviewed in reference 18). As another example, strain variation in the *tyrP* tyrosine/tryptophan permease has been shown to correlate with *C. pneumoniae* tissue tropism (5). The finding that arginine-dependent regulation is present in only a subset of *Chlamydia* species suggests that the role of this essential amino acid in species-specific differences in tissue tropism and pathogenesis deserves a closer look.

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