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Competitive Inhibition of Amino Acid Uptake Suppresses Chlamydial Growth: Involvement of the Chlamydial Amino Acid Transporter BrnQ▽†

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Chlamydiaceae **are obligate intracellular bacterial pathogens that strictly depend on host metabolites, such as nucleotides, lipids, and amino acids. Depletion of amino acids in cell culture media results in abnormal chlamydial development in vitro. Surprisingly, enrichment of certain amino acids also retards chlamydial growth. Our experiments revealed that the antichlamydial effects are largely independent of changes in the host cell transcriptome or proteome and in the major signal transduction pathway modulated by amino acids, the mTOR (mammalian target of rapamycin) pathway. Furthermore, the chlamydial growth inhibition induced by leucine, isoleucine, methionine, or phenylalanine was completely reversed by concomitant addition of valine. In contrast, the growth inhibition induced by serine, glycine, or threonine was not reversed by valine addition. Functional characterization of the only predicted chlamydial transporter for branched-chain amino acids, BrnQ, revealed that it can be blocked by leucine, isoleucine, methionine, or phenylalanine but not by serine, glycine, or threonine. This chlamydial transporter is the only known BrnQ homolog possessing specificity for methionine, suggesting a unique strategy for methionine uptake among gram-negative bacteria. The antichlamydial effects of leucine, isoleucine, methionine, and phenylalanine could be explained as competitive inhibition of the BrnQ transporter and subsequent valine starvation.**

Members of the order *Chlamydiales* are gram-negative, obligately intracellular bacterial pathogens capable of infecting a wide variety of hosts. In humans, most chlamydial infections are caused by *Chlamydia trachomatis* and *Chlamydophila* (formerly *Chlamydia*) *pneumoniae*. *C. trachomatis*, a species consisting of 18 different serovars, is the most common agent of sexually transmitted diseases and is responsible for over 90 million new infections every year worldwide (41). The ocular serovars A to C are responsible for trachoma, a major cause of blindness in developing countries (39). Infections with the urogenital serovars D to K can lead to pelvic inflammatory disease, ectopic pregnancy, and infertility (35). The L1 to L3 serovars not only infect genital epithelial cells but also monocytes and macrophages, leading to a systemic infection of the inguinal lymph nodes called lymphogranuloma venereum (28). Acute *C. pneumoniae* infections cause pulmonary diseases such as pharyngitis, bronchitis, and atypical pneumonia (14, 24, 31), whereas chronic infections are linked to cerebrovascular diseases (9), atherosclerosis (23, 33), and reactive arthritis (44).

Chlamydiales have a unique biphasic developmental cycle that alternates between the extracellular, infectious, metabolically inactive elementary bodies (EBs) and the intracellular, replicating reticulate bodies (RBs) (1). After infection of the host cell, chlamydiae reside inside a vacuole termed an inclusion, which escapes lysosomal degradation. The EBs transform into RBs, which multiply inside the growing inclusion, until they redifferentiate into EBs. After completion of a developmental cycle (48 to 72 h, depending on the chlamydial strain), both EBs and RBs are released, and EBs infect neighboring cells. A conventional infection may deviate toward a persistent infection, in which RBs transform into a distinct form called "aberrant bodies" (ABs). ABs are metabolically active but fail to proliferate and transform back into EBs and therefore cannot be cultivated. Persistent infections can be induced in vitro by gamma interferon, antibiotics, or iron or nutrient deprivation (30). Upon restoration of favorable growth conditions, ABs differentiate back into RBs, and the developmental cycle is completed.

Genome analysis has revealed that many metabolic pathways are incomplete in members of the *Chlamydiaceae* (20, 36). Therefore, these pathogens rely on a variety of metabolic precursors from the host cell, including amino acids. Removal of amino acids from the cell culture medium leads to reduced intracellular amino acid concentrations and subsequently to nutrient starvation and inhibition of the growth of the intracellular pathogens (15). Recent observations in our laboratory suggest that elevated concentrations of certain amino acids result in even stronger inhibition of chlamydial growth than amino acid depletion (4). Addition of leucine (Leu), isoleucine (Ile), methionine (Met), or phenylalanine (Phe) to the medium of infected cell cultures led to the formation of undersized inclusions containing ABs. Remarkably, the production of in-

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fectious EBs was completely blocked under these conditions. Other amino acids, such as threonine (Thr), tyrosine (Tyr), or serine (Ser), were also able to inhibit chlamydial growth, but they were less effective. These inhibitory effects are not specific to *C. trachomatis*, as similar effects were observed upon treatment of *C. pneumoniae*. The most potent amino acids against both species are Leu, Ile, and Phe, whereas Met has stronger activity against *C. trachomatis* than against *C. pneumoniae* (5).

Here, we assessed the molecular basis underlying the inhibitory effects of the amino acids Leu, Ile, Met, and Phe on *C. trachomatis* serovar L2 growth. We found that these four amino acids are antagonists of valine (Val) and that the antagonism probably leads to a Val shortage inside the bacteria, remarkably without depriving the host cell of this essential amino acid. In addition, we functionally characterized the chlamydial BrnQ homolog, a branched-chain amino acid (BCAA) transporter. Our results show that this transporter is responsible for bacterial Val uptake and that it is competitively inhibited by Leu, Ile, Met, and Phe. We concluded that the BrnQ transporter is the molecular target of the chlamydial growth inhibition elicited by excess amino acids.

MATERIALS AND METHODS

Growth media, chemicals, and antibodies. RPMI medium and Hanks' balanced salt solution were purchased from Invitrogen GmbH (Karlsruhe, Germany), and fetal bovine serum (FBS) was purchased from Biochrom KG (Berlin, Germany). Cell growth medium (CGM) consisted of RPMI medium supplemented with 2 mM glutamine, 25 mM HEPES, 10% (vol/vol) FBS, and 10 μg/ml gentamicin, and infection medium (IM) consisted of RPMI medium supplemented with 2 mM glutamine, 25 mM HEPES, and 5% FBS. IM containing elevated concentrations of amino acids was prepared as described elsewhere (4).

Cycloheximide, sodium hydroxide, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), trichloroacetic acid (TCA), L-amino acids, D-lactate, U0126, Trizol, epidermal growth factor, and glutaraldehyde were provided by Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), or Fluka (Buchs, Germany). Rapamycin was obtained from LC Laboratories (Woburn, MA), and [U-^{14}C]valine was purchased from Hartmann Analytic GmbH (Braunschweig, Germany). Scintillation fluid (Zinsser Aquasafe 300 plus) was purchased from Zinsser Analytic GmbH (Frankfurt, Germany), and cellulose nitrate filters with a pore size of 0.45 μm were obtained from Millipore (Eschborn, Germany). A BCA protein assay kit and an Imagen kit for chlamydial detection were purchased from Pearce (Rockford, IL) and Dako (Hamburg, Germany), respectively. The antibodies used in this study were rb-α-phospho-p70-S6K (catalog no. 9205; Cell Signaling), m-α-tubulin (catalog no. T-9026; Sigma-Aldrich), m-α-Hsp60 (catalog no. 804-071-R100; Alexis Biochemicals), and horseradish peroxidase-conjugated secondary antibodies donkey-α-rb and sheep-α-m (catalog no. NA934 and NA931, respectively; Amersham Biosciences).

Microarray experiments. HeLa cells were grown to 80% confluence in six-well plates and incubated in IM containing 10 mM Leu for 24 h at 37°C in the presence of 5% CO_2. As a control, HeLa cells were incubated in IM without supplements. RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) and was purified using an RNeasy RNA purification kit (Qiagen). The quality was verified both with an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE) and electrophoretically with an Agilent Bioanalyzer (Santa Clara, CA). Cy3- and Cy5-labeled cRNA was produced using an Agilent low-RNA-input fluorescent linear amplification kit. Labeled specimens were hybridized on Agilent whole-human-genome oligonucleotide microarrays for 17 h. The microarrays were washed according to the manufacturer's instructions and were scanned with an Agilent microarray scanner. The mean expression ratios for two technical replicates with reverse labeling were calculated for each gene, and the replicates were considered one experiment. Experiments were performed in duplicate.

Two-dimensional gel electrophoresis. HeLa cells were grown to 80% confluence in 75-cm^2 cell culture flasks and incubated in IM containing 10 mM Leu at 37°C in the presence of 5% CO_2 for 24 h. As a control, HeLa cells were incubated in IM without supplements. After incubation, the cells were washed once with 30 mM Tris-HCl (pH 7.3) containing 1 mM NaF and 1 mM $NaVo_3$ and detached using a cell scraper. Samples containing 60 μg total protein were prepared and separated on 15% polyacrylamide gels (30 by 40 cm) as previously described (19). Protein spots were visualized by silver staining. Biologically independent quadruplicate experiments were performed, and only spots showing the same regulation tendency in three of four experiments were considered significantly regulated.

Growth and purification of chlamydial stocks. *C. trachomatis* LGV serovar L2 (ATCC VR-902B) was routinely propagated in HeLa cells (ATCC CCL-2). Stock organisms were prepared as previously described (6, 7).

Chlamydial infections, amino acid treatment, and progeny infectivity titration. HeLa cells were seeded onto six-well plates in CGM and incubated overnight in the presence of 5% CO_2 at 37°C to allow adherence. *C. trachomatis* was suspended in IM, and cells were infected at a multiplicity of infection (MOI) of 0.5. Infected monolayers were rinsed once after 2 h in the presence of 5% CO_2 at 35°C, amino acid-supplemented IM was added, and incubation was continued under the same conditions. Infected cells were incubated in IM as a control. Infected cells were detached at 44 h postinfection (p.i.) using sterile glass beads, lysed by vortexing, and titrated onto fresh HeLa cells as previously described (4). Infectivity titers were determined, and the progeny infectivity was expressed as the percentage of inclusion-forming units compared to the untreated control.

Confocal immunofluorescence microscopy. HeLa cells seeded onto coverslips were infected as described above. Amino acid treatment was started 2 h p.i. Cells were washed with phosphate-buffered saline (PBS) at 44 h p.i., fixed with methanol, and subsequently stained with an Imagen *Chlamydia* detection kit. Coverslips were mounted on glass slides with Mowiol, and the preparations were examined with a Leica TCS-SP laser scanning confocal microscope with a krypton/argon laser. Images were processed using Adobe Photoshop 6.0.

Transmission electron microscopy. Infected cells were washed with PBS and fixed in 2.5% glutaraldehyde, and then they were postfixed with 1% osmium tetroxide, contrasted with uranyl acetate and tannic acid, dehydrated, and embedded in Polybed (Polysciences, Warrington, PA). After polymerization, 60-nm sections were cut and contrasted with lead citrate. Specimens were analyzed with a Leo 906E transmission electron microscope (Oberkochen, Germany) using a Morada digital camera (SIS, Münster, Germany). Images were processed using Adobe Photoshop 6.0.

High-performance liquid chromatography analysis of intracellular amino acids. HeLa cells were seeded onto six-well plates in CGM and incubated overnight in the presence of 5% CO_2 at 37°C to allow adherence. The cells were then rinsed once and incubated under the same conditions in IM containing 10 mM of the indicated amino acids. The medium was removed after 24 h, and the cells were rapidly chilled on ice and washed four times with 2 ml of ice-cold PBS. Free amino acids were extracted by incubating each monolayer in 5% TCA for 2 h at 4°C without detaching the cells from the cell culture dish, as described previously (15). The amino acid-containing TCA was carefully removed and centrifuged for 10 min at 20,000 × *g* and 4°C. Amino acids in the supernatant were identified by ion-exchange chromatography using a Biochrom 30 amino acid analyzer (Biochrom Ltd., Cambridge, United Kingdom) with a lithium hydroxide/lithium acetate system and a postcolumn ninhydrin reaction. All buffers and reagents were obtained ready to use from Laborservice Onken (Gründau/Breitenborn, Germany). The remaining intact cell monolayers were lysed in 0.1 M NaOH for protein determination with the BCA protein assay kit. Intracellular Val concentrations were normalized to the protein contents and expressed as percentages of the untreated control.

Quantitative RT-PCR. HeLa cells were infected with *C. trachomatis* at an MOI of 1 and incubated in IM for 2, 8, 16, 24, or 44 h. RNA was isolated with Trizol (Invitrogen) used according to the manufacturer's instructions, and quantitative real-time PCR was performed with the Qiagen SYBR green reverse transcription (RT)-PCR system using an ABI Prism 7000 sequence detection system. The primers used were CT554-RT-F (5′-CCT CGT TGG GAG ACA AAC AT-3′), CT554-RT-R (5′-AAG GCT TGT TGC TTC GAA AA-3′), 16S-RT-F (5′-GGA GAA AAG GGA ATT TCA CG-3′), and 16S-RT-R (5′-TCC ACA TCA AGT ATG CAT CG-3′) (BioTez, Berlin, Germany). Expression was normalized to chlamydial 16S rRNA, and mRNA levels relative to those at 24 h p.i. were calculated as previously described (32).

Bacterial strains, cloning, and functional complementation. *Escherichia coli* TOP10 cells (Invitrogen) were used for cloning and isolation of genomic DNA. The Ile auxotrophic *E. coli* K-12 derivative strain B7634 that is deficient for transport of BCAA (*ileA hrbBCD brnQ*) was kindly provided by Y. Anraku, Science University of Tokyo, Tokyo, Japan (42). All strains were grown in Luria-Bertani medium supplemented with 100 μg/ml ampicillin for selection and propagation of transformed clones. Isolation of genomic DNA and plasmids, PCR, restriction digestion, and electrotransformation were carried out as described by Sambrook et al. (34). Restriction enzymes were purchased from New

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England Biolabs (Ipswich, MA), and primers were purchased from BioTez. The *E. coli brnQ* gene and its homolog in *C. trachomatis* serovar L2, CT554, were both cloned into the low-copy-number plasmid pACYC177 (EMBL accession number X06402), because BCAA transporters were shown to be toxic at high expression levels (37). The natural *E. coli* promoter region of *brnQ* (promoter 191) was inserted upstream of *brnQ* and CT554 for comparable and constitutive expression. The primers used for gene isolation and cloning were Promo191-F (5′-ATG GAT CCT ACT GGT TGG TGA TTT CTT ATC-3′), Promo191-R (5′-ATC CCG GGT ACT GCC TGT GGA TGT GGT-3′), EcoQ-F (5′-ATC CCG GGA TGA CCC ATC AAT TAA GAT CG-3′), EcoQ-R (5′-ATC TCG AGT TAG TGA GCG CTG GAG GTC-3′), CT554-F (5′-ATC CCG GGA TGC ATA AGA AAA CAC AAT CAC-3′), and CT554-R (5′-TAC TCG AGT TAA ACG GAC AGC TTA TAG AG-3′). Plasmids were verified by DNA sequencing (SMB, Berlin, Germany). Functional expression of the cloned transporters was verified using *E. coli* strain B7634 cultured on M9 minimal medium agar plates supplemented with 50 μg/ml ampicillin and 5 μg/ml Ile. Transformants expressing the cloned transporters grew at this limiting Ile concentration, in contrast to B7634 carrying the empty vector.

Valine transport assays. The B7634 *E. coli* strain harboring pACYC177 derivatives was grown in 50 ml Luria-Bertani medium supplemented with 100 μg/ml ampicillin to an optical density at 600 nm (OD_{600}) of 0.5, harvested by centrifugation (10 min, 3,000 × *g*, 4°C), resuspended in 10 ml of ice-cold assay buffer (50 mM potassium phosphate [pH 6.5], 5 mM $MgSO_4$, 10 mM D-lactate), and centrifuged again. The pellet was then resuspended in fresh assay buffer to an OD_{600} of 5, and [^{14}C]valine uptake assays were performed at 30°C. For these assays, bacteria were diluted in prewarmed assay buffer to obtain an OD_{600} of 1.7 and preincubated for 5 min. Then [^{14}C]valine was added to produce the final concentrations indicated, and 180-μl aliquots were removed after 1 min, filtered through a 0.45-μm cellulose nitrate membrane, and immediately washed with 5 ml of ice-cold assay buffer. Filters were transferred to scintillation tubes containing 4.5 ml of scintillation fluid, and the radioactivity was measured with a Packard Tri-Carb 2900TR liquid scintillation analyzer. The initial uptake rates in the presence of 1 to 80 μM Val were used to determine the V_{max} and K_m values of Val transport using a Hanes-Wilkinson transformation and linear regression.

Statistical analysis. Experiments were conducted in triplicate unless otherwise stated. The data were expressed as means ± standard errors. Differences were considered significant if the *P* value was ≤0.01, as calculated using the Student *t* test.

RESULTS

The host cell is not affected by high amino acid concentrations. We tested whether high concentrations of amino acids resulted in stress or dramatic changes to the host cells. The host cell viability was not affected by high concentrations of antichlamydial amino acids, as measured using the WST-1 assay (see Fig. S1 in the supplemental material). Microarray analysis also revealed that there were no changes in the host transcriptome. None of the >41,000 human genes represented on this microarray were regulated more than 1.8-fold differently after the addition of 10 mM Leu. Analysis on two-dimensional gels also revealed no differences in the proteomes of Leu-treated and control HeLa cells (data not shown).

The mTOR signal transduction pathway is not involved in chlamydial growth arrest. BCAAs, particularly Leu, are known to activate the host signal transduction pathway of the mTOR (mammalian target of rapamycin) kinase, to upregulate host protein synthesis, and to inhibit autophagy (22). We detected mTOR pathway activation during chlamydial infection (see Fig. S2 in the supplemental material). The mTOR pathway-specific inhibitor rapamycin was employed to examine the influence of this pathway on amino acid-induced chlamydial growth inhibition. Rapamycin treatment changed neither the outcome of the productive infection nor the rate of chlamydial growth in the presence of excess Leu, Ile, Met, or Phe (Fig. 1).

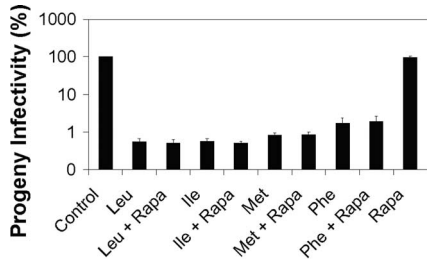

FIG. 1. The mTOR kinase is not involved in chlamydial growth arrest. *C. trachomatis*-infected HeLa cells were treated with 5 mM Leu, Ile, Met, or Phe from 19 to 44 h p.i., with or without treatment with 100 nM rapamycin (Rapa) from 30 min before amino acid treatment until 44 h p.i. Cells were lysed 44 h p.i. and titrated onto fresh cells, and inclusions were counted 24 h later. Progeny infectivity is expressed as a percentage of the untreated control. Means standard errors of the means from triplicate experiments are shown. mTOR kinase inhibition did not alter chlamydial progeny infectivity in the absence or presence of Leu, Ile, Met, or Phe.

Inhibition of host cell protein synthesis partially restores chlamydial growth. We investigated the influence of host cell protein synthesis during amino acid-induced chlamydial growth inhibition. Blocking protein synthesis in infected, amino acid-treated cells with cycloheximide resulted in inclusions that were larger than the inclusions observed without cycloheximide treatment (Fig. 2). Infected cell cultures treated with cycloheximide together with Leu, Ile, or Met contained inclusions that were roughly one-half the diameter of the inclusions in infected cells grown without additives. Unexpectedly, infectious EBs could not be recovered from these samples. Transmission electron micrographs revealed that these inclusions contained more chlamydiae than the inclusions in amino acid-treated specimens without cycloheximide but that the chlamydiae had a characteristic aberrant morphology. The bacteria were larger, and no EBs were detected. In brief, blocking host protein synthesis in infected cells treated with Leu, Ile, or Met led to resumption of inclusion growth and chlamydial proliferation but not to completion of the developmental cycle and production of infectious progeny. In Phe-treated cell cultures, cycloheximide treatment resulted in inclusions that were similar sizes but reduced the infectivity of progeny (54.6% ± 3.2% of the untreated control). Electron micrographs showed inclusions with few ABs and a high number of EBs (Fig. 2). These experiments showed that cycloheximide treatment led to partial reconstitution of chlamydial growth and development in infected, amino acid-treated cells. This rescue effect was more effective against the growth suppression caused by Phe than against the growth suppression caused by Leu, Ile, or Met.

Valine supplementation reverses chlamydial growth inhibition by leucine, isoleucine, methionine, or phenylalanine. The inability of cycloheximide to efficiently overcome the adverse effects of Leu, Ile, Met, or Phe indicates that a mechanism independent of the host cell is responsible for the inhibition of chlamydial growth. One attractive hypothesis is that the added amino acid may compete with one or more amino acids that are essential for chlamydial development and are transported by the same amino acid transporter. To test this hypothesis, cultures exposed to Leu, Ile, Met, or Phe were concomitantly supplemented with other single amino acids essential for *C. trachomatis* serovar L2 growth (2, 21). The growth arrest induced by elevated levels of Leu, Ile, Met, or Phe was com-

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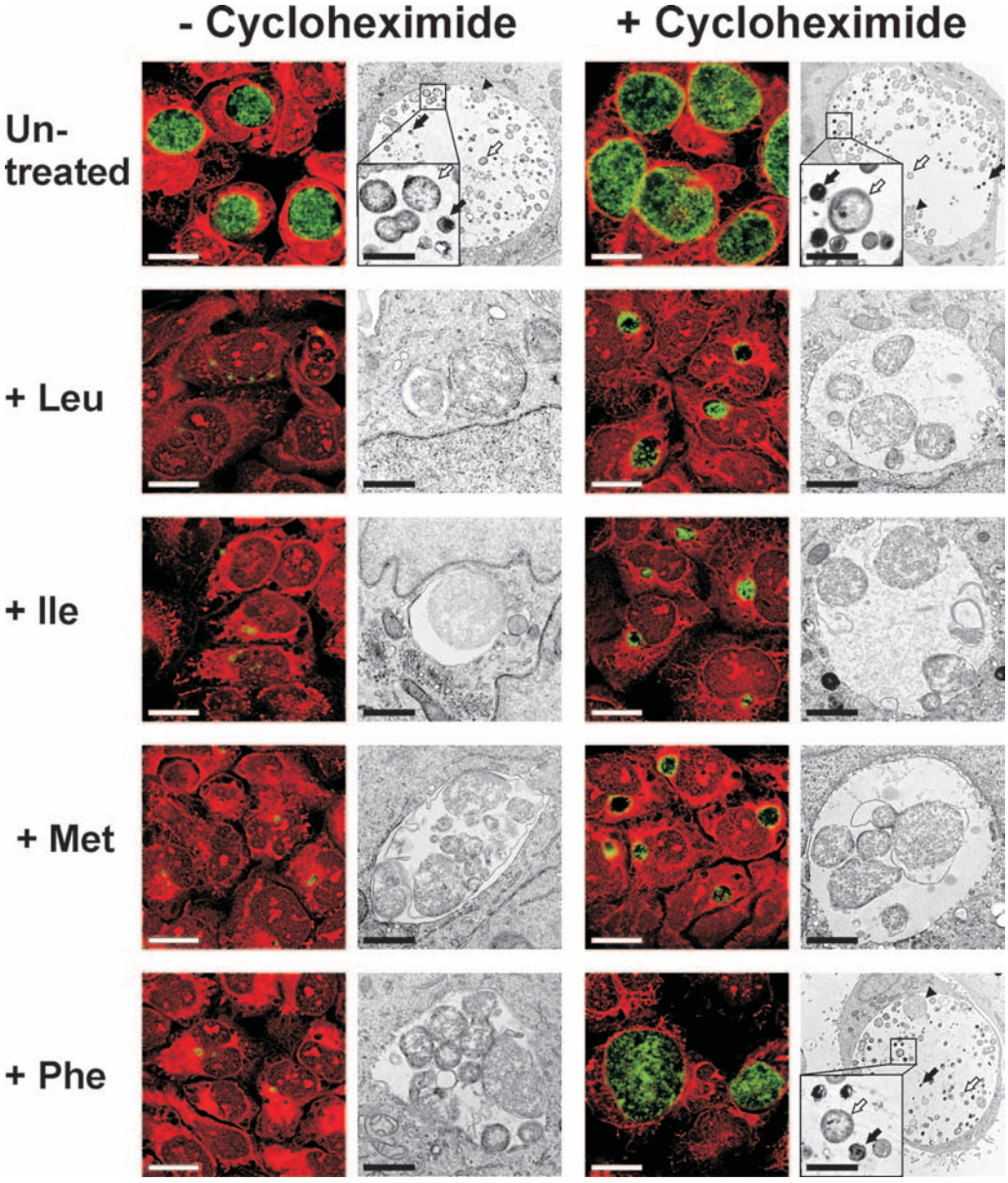

FIG. 2. Inhibition of host cell protein synthesis partially restores chlamydial growth. *C. trachomatis*-infected HeLa cells were treated with 10 mM Leu, Ile, Met, or Phe from 2 h p.i. until the end of the infection cycle, with or without 5 μg/ml cycloheximide. Infected cells were fixed 44 h p.i. and prepared for confocal immunofluorescence or transmission electron microscopy analysis. In the fluorescence micrographs, chlamydial inclusions were stained green, and cells were counterstained red with Evans blue. White bars = 20 μm. White arrows indicate RBs, black arrows indicate EBs, and black arrowheads indicate ABs. For better comparison, sections containing typical EBs and RBs were enlarged fourfold. Black bars = 2 μm. Chlamydial inclusion growth was suppressed by Leu, Ile, Met, or Phe and was partially restored by simultaneous addition of cycloheximide. Phe-induced growth suppression was more efficiently reversed than the antichlamydial effects induced by Leu, Ile, or Met.

pletely reversed by simultaneous administration of Val (Fig. 3). Val supplementation fully restored both primary inclusion development and the production of infectious progeny. This ability to overcome amino acid-induced growth suppression was specific for Val, as other single amino acids did not have this effect. Even addition of a mixture containing elevated concentrations of all other essential amino acids except Val (Leu, Ile, Phe, Met, glutamine [Gln], histidine [His], and tryptophan [Trp]) did not restore chlamydial growth (Fig. 3). These results indicate that antagonism between amino acids is the principal mechanism underlying the inhibitory effects of elevated amino acid concentrations. Furthermore, Leu, Ile, Met, and Phe are all antagonists of the essential amino acid Val.

Valine supplementation does not reverse the chlamydial growth suppression caused by glycine, serine, or threonine. In our previous work, we found that addition of Gly, Ser, or Thr substantially impaired the development of infectious progeny but that the effects were not as strong as those of the hydrophobic amino acids discussed above (5). Here we investigated whether concomitant addition of Val could restore chlamydial

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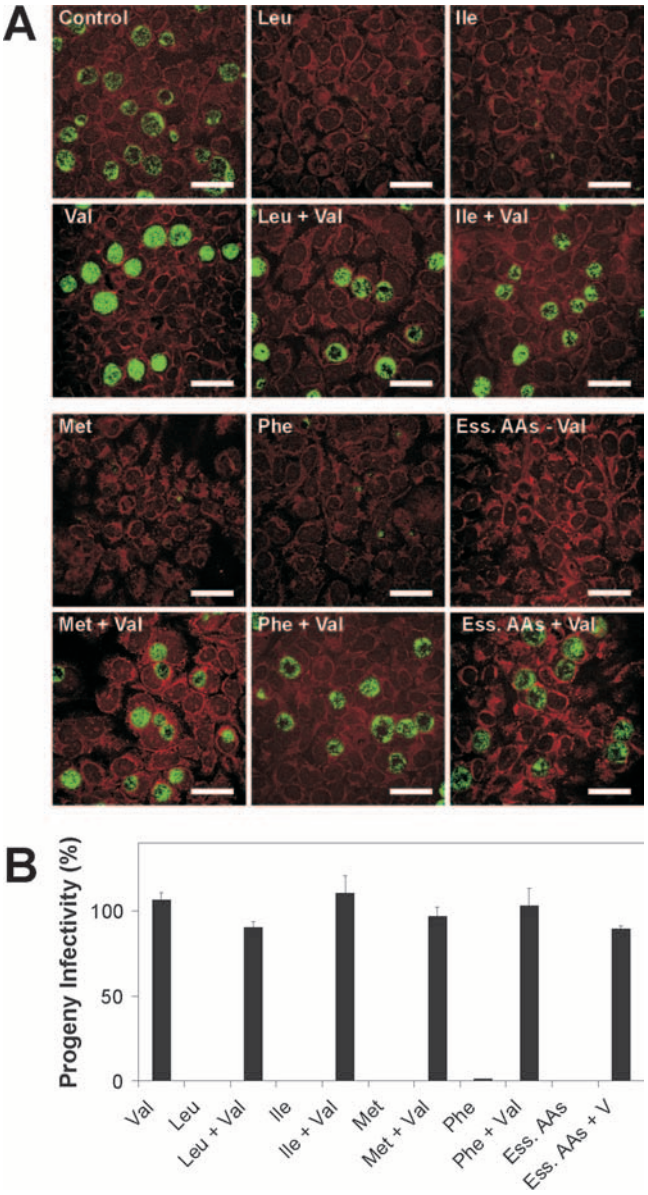

FIG. 3. Valine addition reverses the abrogation of chlamydial growth induced by leucine, isoleucine, methionine, or phenylalanine. (A) HeLa cells were infected using an MOI of 0.5 and were treated with the indicated amino acids at a concentration of 5 mM from 2 h p.i. until the end of the infection cycle. The essential amino acid mixture (Ess. AAs) contained 0.5 mM Trp, 5 mM Leu, 5 mM Ile, 5 mM Met, 5 mM Phe, 5 mM Gln, and 5 mM His. Infected cells were fixed at 44 h p.i. and immunostained for chlamydial inclusions (green). Cells were counterstained red. Bars = 40 μm. (B) HeLa cells were infected and treated as described above for panel A. Cells were lysed at 44 h p.i. and titrated onto fresh cells, and inclusions were counted 24 h later. Progeny infectivity is expressed as a percentage of the untreated control. Means and standard errors of the means for triplicate experiments are shown. Chlamydial inclusion growth and progeny infectivity were restored upon addition of Val to infected cells treated with Leu, Ile, Met, or Phe.

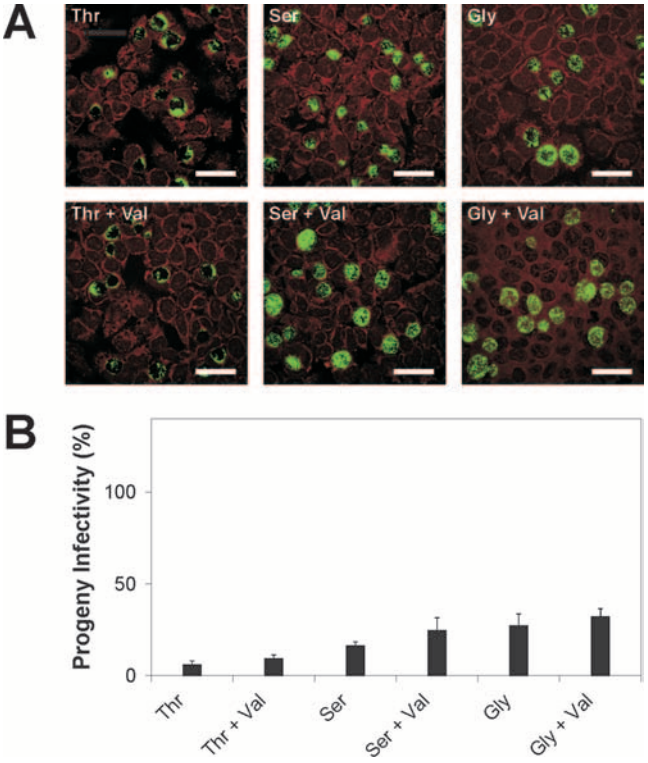

FIG. 4. Valine supplementation does not inhibit chlamydial growth suppression induced by glycine, serine, or threonine. (A) HeLa cells were infected using an MOI of 0.5 and treated with the indicated amino acids at a concentration of 5 mM from 2 h p.i. until the end of the infection cycle. Cells were fixed at 44 h p.i. and immunostained for chlamydial inclusions (green). Cells were counterstained red. Bars = 40 μm. (B) HeLa cells were infected and treated as described above for panel A. Cells were lysed at 44 h p.i. and titrated onto fresh cells, and inclusions were counted 24 h later. Progeny infectivity is expressed as a percentage of the untreated control value. Means and standard errors of the means for triplicate experiments are shown. Val could not significantly restore chlamydial growth when it was added to cells treated with Thr, Ser, or Gly, as Val treatment increased neither the inclusion size nor the progeny infectivity.

development. Val did not reverse the antichlamydial effects of Gly, Ser, or Thr, since cell cultures treated with these amino acids with and without additional Val contained inclusions that were similar sizes (Fig. 4A). The yield of infectious chlamydial progeny in cultures treated with Gly, Ser, or Thr in the presence of Val was no different from the yield in the absence of Val (Fig. 4B). In contrast to valine's counteracting effect on Leu, Ile, Met, and Phe, no rescue was observed for the growth-inhibitory effects of Gly, Ser, or Thr, indicating that these hydrophilic amino acids do not act as Val antagonists.

The cytoplasmic valine concentration is not disturbed by the addition of other amino acids. Our results indicate that the growth-inhibitory effects of Leu, Ile, Met, or Phe on *Chlamydia* most likely result from competition with Val for a common membrane transporter, depriving *Chlamydia* of this essential amino acid. To localize this antagonism to a cellular compartment, we compared the intracellular Val concentrations in amino acid-treated and untreated cell cultures. Untreated HeLa cells had an average intracellular Val concentration of 10.0 ± 3.3 pmol per mg cellular protein. The intracellular Val concentration was not reduced upon treatment with Leu, Ile, Met, or Phe as potential competitors or with the noncompetitive control amino acid Thr (Fig. 5). To examine whether the antichlamydial amino acids were transported into the host cell,

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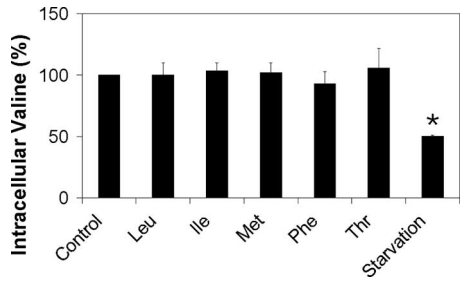

FIG. 5. Valine concentrations in the host cytoplasm are not disturbed by addition of other amino acids. HeLa cells were cultured for 24 h in medium supplemented with the amino acid indicated at a concentration of 10 mM or for 2 h with Hanks' balanced salt solution for total amino acid starvation. After thorough washing, free intracellular amino acids were extracted from the monolayers using 5% TCA and analyzed by high-performance liquid chromatography. The remaining intact monolayers were dissolved in 0.1 M NaOH for subsequent protein determination. Intracellular Val concentrations were normalized to the total protein and were expressed as a percentage of the untreated control plus the standard error of the mean. Intracellular Val levels were not reduced upon treatment with other amino acids, indicating that cellular Val transport is not impaired by addition of Leu, Ile, Met, Phe, or Thr.

their intracellular concentrations were also assessed. The intracellular concentrations increased with increases in the extracellular concentrations (data not shown). These findings suggest that competitive inhibition of Val transport by either Leu, Ile, Met, or Phe does not occur at the host cell membrane.

The chlamydial homolog of *brnQ*, CT554, is expressed throughout chlamydial infection. Since the host cell membrane was ruled out as the site of competition, we speculated that amino acid antagonism might occur at the inclusion membrane, the chlamydial membrane, or both. We searched the chlamydial genome (http://stdgen.northwestern.edu/stdgen/bacteria/chlamy/index.html) for genes encoding transporters for hydrophobic amino acids, which could serve as potential molecular sites of Val competition. The only candidate found was the CT554 gene, predicted to encode a BCAA transport system of the BrnQ (or LIV-II) family. This protein has amino acid sequence similarities of 47% to the BrnQ transporter of *E. coli* and up to 53% to the BrnQ transporters of other gram-negative bacteria (e.g., *Clostridium tetani* E88). These permeases are located in the inner bacterial membrane and rely on the proton motive force to transport Leu, Ile, and Val into the bacterium (37, 40). All sequenced members of the *Chlamydiaceae* and *Parachlamydiaceae* possess homologs of this gene, implying that it has an essential role. We examined the expression of the chlamydial *brnQ* homolog throughout the developmental cycle in *C. trachomatis*. Quantitative real-time RT-PCR of chlamydial RNA isolated at various time points revealed that this gene was expressed throughout the developmental cycle of *C. trachomatis* and that the expression was strongest during the early phase of infection (Fig. 6).

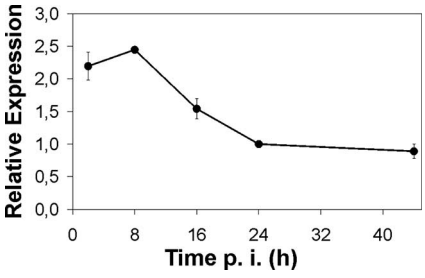

FIG. 6. CT554 is expressed throughout the entire chlamydial developmental cycle. HeLa cells were infected using an MOI of 1, and total RNA was prepared at 2, 8, 16, 24, and 44 h p.i. using Trizol for analysis by quantitative real-time RT-PCR. The expression of CT554 was normalized to chlamydial 16S rRNA, and the average mRNA levels relative to the level at 24 h p.i. are shown; the error bars indicate standard errors. Experiments were done in duplicate.

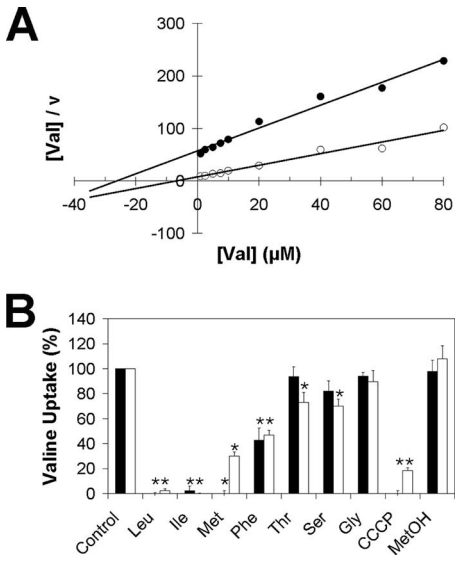

FIG. 7. Characterization of the chlamydial BrnQ homolog. (A) Hanes blot of Val transport. *E. coli* and *C. trachomatis* BrnQ homologs were expressed in *E. coli* B7634, and uptake of ^{14}C-labeled Val was measured using substrate concentrations of 1 to 80 μM. K_m and V_{max} values were calculated directly from regression lines. The regression lines intercept the horizontal axis at $-K_m$ and have slopes of $1/V_{max}$. The results for one of three experiments with similar outcomes are shown. ●, *C. trachomatis* BrnQ; ○, *E. coli* BrnQ. [Val], Val concentration; v, Val transport rate in μmol · min^{-1} · mg of total protein^{-1}. (B) [^{14}C]valine uptake was measured in the presence of 5 mM competitive amino acid, 60 μM CCCP, or 0.1% methanol. The results for Val uptake were normalized to the total protein and expressed as percentages of the transported Val in the absence of inhibitors (untreated control). The uptake rates of the untreated controls were 247 and 848 nmol · min^{-1} · mg of total protein^{-1} for the *C. trachomatis* and *E. coli* homologs, respectively. Asterisks indicate preparations in which Val transport was significantly suppressed compared to controls. Filled bars, *C. trachomatis* BrnQ; open bars, *E. coli* BrnQ. The bars indicate means, and the error bars indicate standard errors. The competition profiles show that there was complete inhibition of the chlamydial transporter by Leu, Ile, Met, and Phe. Met inhibited the *E. coli* transporter to a lesser extent.

Characterization and competitive inhibition of the chlamydial BrnQ transporter. In order to functionally characterize the putative chlamydial BrnQ transporter, the CT554 gene was heterologously expressed in an *E. coli* strain deficient for BCAA transport, and the uptake of [^{14}C]valine was monitored. *C. trachomatis* BrnQ facilitated transport of Val into *E. coli* with a V_{max} of 428 nmol · min^{-1} · mg total protein^{-1} under standard conditions, compared to 1,046 nmol · min^{-1} · mg total protein^{-1} for the *E. coli* transporter. Both transporters were inhibited in the presence of the ionophore CCCP, confirming

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their dependence on the proton gradient at the inner bacterial membrane. The K_m value for Val transport by the chlamydial BrnQ was 26.6 μM, compared to the 4.3 μM observed for Val transport by *E. coli* BrnQ (Fig. 7A).

To verify our hypothesis that this transporter could be involved in the amino acid-induced growth inhibition, we examined which amino acids could competitively inhibit Val transport. We tested not only the hydrophobic amino acids Leu, Ile, Met, and Phe but also Gly, Ser, and Thr. Only Leu, Ile, Met, and Phe, which also cause valine-reversible growth inhibition in *Chlamydia*, completely blocked the chlamydial BrnQ transporter. In contrast, Met inhibited Val uptake by the *E. coli* BrnQ transporter much less. Gly, Ser, and Thr had little impact on Val transport by both transporters (Fig. 7B). Taken together, our results support the hypothesis that competitive inhibition of Val uptake by the chlamydial BrnQ transporter is a major mechanism underlying the antichlamydial effects of hydrophobic amino acids.

DISCUSSION

The amino acid requirements of different chlamydial strains have been extensively studied. Omission of individual amino acids from cell culture media has been used by various groups to identify amino acids essential for survival of this intracellular pathogen. The results of these studies are partially inconsistent, probably due to the complex experimental system consisting of infected cells and the use of different chlamydial isolates (2, 3, 17, 21). Genome sequencing of *C. trachomatis* and *C. pneumoniae* has revealed the absence of complete functional pathways for amino acid synthesis (20, 36). More recently, Harper and colleagues were able to show that *C. trachomatis* grown in the presence of amino acid concentrations equivalent to those in human serum produced more ABs and fewer infectious EBs than *Chlamydia* grown in standard cell growth medium. Accordingly, they suggested that serum amino acid concentrations could contribute to the induction of persistence in vivo (15).

Amino acid competition in chlamydial infections was first described by Coles and Pearce (10) in the context of amino acid deprivation. It was not clear, however, whether bacterial or host cell pathways were responsible for suppression of chlamydial growth. We previously reported that not only reduced but also elevated concentrations of certain amino acids in the growth medium impaired chlamydial growth. Excess Leu, Ile, Met, or Phe strongly suppressed chlamydial growth and led to the production of ABs (4, 5).

As amino acid availability is known to affect mammalian cells (16, 22, 38), we first investigated the influence of altering amino acid concentrations on the viability, transcriptome, and proteome of the host cell. These experiments detected no significant changes in the host cell during culture with elevated amino acid levels. However, this global assessment may have missed subtle changes in the regulation of single factors at the mRNA or protein levels; hence, more focused approaches were employed.

BCAAs, especially Leu, are known to regulate protein synthesis and autophagy in mammalian cells via the mTOR kinase pathway (38). The influence of this kinase was investigated more closely since autophagy plays a role in many intracellular bacterial and viral infections (18, 26) and mTOR is involved in the macrophage response to mycobacteria (43). mTOR was found to be activated predominantly at early time points during chlamydial infection (see Fig. S2 in the supplemental material). However, specific inhibition of mTOR (see Fig. S3 in the supplemental material) revealed that chlamydial growth was completely independent of this kinase both under normal growth conditions and in the presence of high concentrations of Leu, Ile, Met, or Phe. This finding is consistent with the findings of Lundemose et al. (27), who reported that chlamydial growth was inhibited by high concentrations (25 μM) of rapamycin, whereas lower concentrations (2.5 μM) blocked mTOR but could not impair chlamydial development. The authors attributed this effect to the inhibition of a chlamydial peptidyl-prolyl *cis*/*trans* isomerase.

We show here that the inhibition of host cell protein synthesis led to restoration of chlamydial inclusion growth in the presence of elevated concentrations of Leu, Ile, Met, or Phe. Nonetheless, transmission electron micrographs revealed that inclusions inside cells treated with Leu, Ile, or Met contained fewer chlamydiae than inclusions inside control cells and that the bacteria had an aberrant morphology. Inhibition of host protein synthesis leads to increases in the levels of all intracellular amino acids (15). Elevated Val concentrations might support chlamydial growth to a certain extent but not be sufficient to complete the developmental cycle. Indeed, *Chlamydia* grown in the presence of cycloheximide and high Leu, Ile, or Met concentrations strongly resembles *Chlamydia* grown under general amino acid starvation conditions, as reported by Harper et al. (15).

The fact that a concomitant increase in Val concentrations leads to reversal of the growth inhibition induced by Leu, Ile, Met, or Phe indicates that competition of these amino acids with Val might be the key mechanism underlying the antichlamydial effects. Here we showed that each of these four hydrophobic amino acids competed with Val for a shared membrane transporter, subsequently leading to inhibition of Val uptake. These findings extend our previous work (5), where the influence of excess amino acids on chlamydial development was studied alongside other established modulators of autophagy. Here we provide a molecular explanation for the previously observed effects.

Elevated levels of Gly, Ser, and Thr also strongly inhibit chlamydial growth. However, addition of Val to infected cell cultures treated with these amino acids did not reverse their antichlamydial effects. However, since Gly, Ser, and Thr belong to the group of hydrophilic amino acids, it is not surprising that the restraining effects of these amino acids are independent of Val deprivation. Gly, Ser, and Thr could influence *Chlamydia* via a variety of other mechanisms, including competition with other amino acids.

We investigated the role of the host cell during amino acid-induced chlamydial growth suppression. Intracellular Val concentrations were determined in cells treated with elevated concentrations of competitive amino acids. Excess Leu, Ile, Met, Phe, and Thr entered the host cell (data not shown) but did not lead to reduced cytoplasmic Val concentrations. Thus, deprivation of the host cell cytoplasmic Val pool, either by competitive inhibition of Val membrane transporters or by inhibition

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of BCAA metabolism, can be excluded as a cause of chlamydial growth suppression.

Most gram-negative bacteria possess at least two transport systems for BCAA uptake, a high-affinity ABC transporter and a low-affinity BCAA permease (8, 42). Only homologs of the latter have been identified in chlamydial genomes. The activity of this single transporter, encoded by the *brnQ* gene, appears to be indispensable for the uptake of these essential amino acids in *Chlamydiales*. In *C. trachomatis* serovar L2, the BrnQ transporter is predicted to be a 45-kDa protein encoded by the CT554 gene. We showed here that CT554 is expressed during the entire chlamydial developmental cycle. Expression is highest in the early phase, when intensive protein synthesis requires a sufficient supply of amino acids. Heterologous expression and functional analysis of this previously uncharacterized protein revealed a number of interesting results. The CT554 gene encodes a secondary transporter, dependent on the proton motive force at the inner membrane of gram-negative bacteria. We also determined the V_{max} and K_m values for the BrnQ transporters of both *E. coli* and *C. trachomatis*. The V_{max} of the chlamydial transporter was less than one-half the V_{max} of the *E. coli* homolog. Since expression of both genes was regulated by the same promoter, the mRNA levels as well as the protein levels were considered to be similar, although a possible bias cannot be fully excluded for the V_{max} values obtained. However, since K_m values and the extent of competitive inhibition are independent of expression levels, this limitation does not affect the following results. We determined a K_m of 4.3 μM for the *E. coli* BrnQ transporter, which is close to the K_m for Val (~2 μM) reported by Guardiola et al. (12, 13). The K_m for the *C. trachomatis* homolog was approximately six times higher than that for the *E. coli* homolog. This low affinity reflects the fact that *Chlamydia* resides in a special niche that provides comparatively high concentrations of hydrophobic amino acids.

In order to examine whether the chlamydial BrnQ protein is susceptible to competitive inhibition by Leu, Ile, Met, or Phe but not by Gly, Ser, or Thr, competition by these amino acids was assessed. The chlamydial BrnQ transporter was inhibited by only those amino acids that also induced valine-reversible growth suppression, making it a highly plausible candidate for the molecular site of this competition. It was previously shown that Ile uptake by BrnQ from *Lactobacillus delbrückii* is competitively inhibited by Leu and Val (37). A similar effect was described for the BrnQ transporter of *Corynebacterium glutamicum* VAL1 (25). In contrast to these transporters, the Ile transport activity of the chlamydial homolog seems not to be inhibited by Val, since excess Val does not suppress chlamydial growth in cell culture (5). These differences may be explained by the lower affinity of the chlamydial BrnQ for Val than for its competitors. The substantial sequence differences between *C. trachomatis* BrnQ and its homologs in *C. glutamicum* and *L. delbrückii* (25 and 23% amino acid sequence identity, respectively) also support the hypothesis that the protein functions in these organisms are different.

We also showed that chlamydial BrnQ has an affinity for Met, unlike the *E. coli* homolog. An affinity for Met has not been described yet for any BCAA transporter encoded by *brnQ* homologs. The only other permease known to possess a transport activity for Met besides BCAA is BcaP from *Lactococcus lactis*, as recently described by den Hengst et al. (11). Surprisingly, even though the chlamydial BrnQ protein exhibits between 59% (*C. pneumoniae* CWL029) and 86% (*Chlamydia muridarum*) sequence identity to homologs from other *Chlamydiales* and up to 30% sequence identity to homologs from other bacteria (*Clostridium* sp.), it shows no significant homology to BcaP from *L. lactis*.

Most gram-negative bacteria possess a methionine-specific ABC transporter encoded by *metD* (29). Interestingly, a homolog of this ABC transporter gene was not identified in the *C. trachomatis* genome. This supports the idea that *C. trachomatis* possesses a unique uptake strategy for Met which involves employing the BrnQ permease to transport this essential amino acid. In contrast, *C. pneumoniae* appears to possess an ABC transporter for Met, as Cpn0278, Cpn0279, and Cpn0280 encode proteins that are highly homologous to such a system in *E. coli*. The presence of two independent Met transport systems might explain the fact that this organism is less susceptible to elevated Met levels than *C. trachomatis* (5).

The present study identified the only chlamydial BCAA transporter as the probable molecular site for inhibition of chlamydial growth by excess Leu, Ile, Met, or Phe. Furthermore, functional characterization of the chlamydial BrnQ transporter revealed a high specificity for Met, a feature not described yet for other BrnQ transporters. This specificity and the absence of a Met-specific ABC transporter suggest a strategy for Met uptake unique among gram-negative bacteria. In addition, the culture technique used allows specific induction of starvation-mediated persistence in cell culture models without affecting the host cell, thus providing a new tool for *Chlamydia* research.

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