A Single Point Mutation in CTP Synthetase of Chlamydia trachomatis Confers Resistance to Cyclopentenyl Cytosine*

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A Chlamydia trachomatis strain (L2/CPEC) resistant to the cytotoxic effects of cyclopentenyl cytosine (CPEC) was isolated by a stepwise selection procedure. This strain showed an approximate 350-fold increase in resistance to CPEC. Sequencing of the gene encoding CTP synthetase from this resistant strain revealed a single point mutation, resulting in a change of amino acid 149 from Asp to Glu. This appeared to be the only mutation in L2/CPEC, because no changes in CTP transport, CTP synthetase expression, or incorporation of CPEC into DNA or RNA could be detected. The mutation in the chlamydial CTP synthetase resulted in a loss of CTP feedback inhibition. This was demonstrated both in vivo using Escherichia coli cells carrying the cloned gene, and in vitro assay using partially purified preparations of CTP synthetase. As a result of the loss of feedback inhibition, E. coli cells carrying the CPEC-CTP synthetase showed a 22-fold increase in their CTP pools. However, examination of the CTP pools of L2/CPEC revealed no change in CTP levels when compared with wild type C. trachomatis.

Chlamydiae are obligate intracellular eubacterial parasites that infect a wide range of eukaryotic host cells. All members of the genus have evolved a unique biphasic life cycle, which consists of two distinct bacterial forms. The metabolically inert, infectious elementary body (EB) initiates the infection cycle, whereas the noninfectious, metabolically active reticulate body (RB) is the actively dividing intracellular form (1, 2). Growth of the RB takes place within the confines of a membrane-bound vacuole, the chlamydial inclusion that avoids fusion with host cell lysosomes (1).

Four species are currently recognized, Chlamydia trachomatis, Chlamydia pneumoniae, Chlamydia psittaci, and Chlamydia pecorum (1, 3, 4). C. trachomatis is one of the most prevalent sexually transmitted pathogens and is a leading cause of blindness in developing countries (5). Despite the clinical importance of C. trachomatis, relatively little is known about its biochemistry. This is largely due to the difficulty in conducting definitive studies on chlamydial metabolism. Conditions for cell free growth have not been established, host-free RBs show limited metabolic activity, and a genetic system has not been developed.

Our laboratory is currently interested in analyzing nucleotide acquisition and metabolism by chlamydiae in an attempt to understand how they obtain nucleotides for nucleic acid biosynthesis and other nucleotide-requiring metabolic processes. Previous analyses, using eukaryotic host cell lines with well defined mutations in nucleotide metabolism, have revealed that actively growing C. trachomatis RBs are impermeable to all deoxynucleoside triphosphates and lack or do not express the enzymes required for transport and/or metabolism of all purine and pyrimidine nucleobases or (deoxy)nucleosides (6, 7). Precursors for nucleic acid biosynthesis are acquired directly from the host cytoplasm in the form of the four ribonucleoside triphosphates, ATP, UTP, GTP, and CTP.

Although de novo synthesis pathways for purines and pyrimidines do not exist, C. trachomatis is auxotrophic for only three (ATP, UTP, and GTP) of the four ribonucleotides. C. trachomatis grows normally in a mutant Chinese hamster ovary (CHO) cell line (CR-2) that lacks CTP synthetase (7). CTP synthetase catalyses the amination of UTP to CTP (8); therefore, in the absence of exogenous cytidine CR-2 cells lack a CTP pool and growth rapidly ceases (16). Growth of C. trachomatis in this cell line suggested the ability of this bacterium to synthesize its own supply of CTP (7). This was subsequently verified by the cloning of a C. trachomatis gene encoding CTP synthetase (9).

Although C. trachomatis possesses a CTP synthetase enzyme, it is also capable of transporting CTP from host cells (7). The ability to obtain preformed CTP from the host cell raises the question of why the CTP synthetase enzyme is present in this bacterium. Chlamydiae draw on the host cell cytoplasm for a wide variety of macromolecules normally synthesized de novo by free living bacteria. This dependence on the host cell has been used as an explanation for the reduced size of the chlamydial genome and the limited biosynthetic capabilities of chlamydiae (10). Given the apparent loss of many biosynthetic enzymes from chlamydiae, the presence of CTP synthetase suggests that it may be an essential enzyme in the life cycle of C. trachomatis.

Our previous studies on nucleotide metabolism have been aided by the isolation and characterization of drug-resistant chlamydial mutants (11). The purpose of the present study was to isolate and characterize a C. trachomatis L2 mutant resistant to the cytotoxic effects of cyclopentenyl cytosine (CPEC) to gain a greater understanding of the role of CTP synthetase in the life cycle of this bacterium. CPEC is a carboxyclic analogue of cytidine in which the ribofuranose moiety is replaced by a cyclopentenyl ring (12). The mode of action of the triphosphate form of the drug (CPEC-TP) is through feedback inhibi-
tion of CTP synthetase, resulting in a marked reduction of CTP pools (12). CPEC is a pro-drug and must be raised intracellularly to its triphosphate form to become active.

We have isolated a mutant strain of C. trachomatis resistant to the effects of CPEC. We demonstrate that this chlamydial strain encodes a mutant CTP synthetase that has lost CTP feedback inhibition.

MATERIALS AND METHODS

Chemicals—[5,6-3H]Juridine triphosphate (40 Ci/mmol), [α-32P]dATP (3500 Ci/mmol), were obtained from New England Nuclear, DuPont Canada Inc. [2-3H]Thymidine (28 mCi/mmol) and [3-3H]Juridine (21 Ci/mmol) were from Moravek Biochemicals, Inc. (Brea, CA). [3H]CPEC was a gift from D. G. Johns, National Institutes of Health, Bethesda, Maryland. All other chemicals were of the highest purity available.

Bacterial Cells and Growth Conditions—C. trachomatis L2/434/Bu (L2) was obtained from R. Brunham, Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada. Chlamydial cells were propagated as described previously (6). 1 µg of cycloheximide/mL was present in the post-infection growth medium. Mock-infected host cell cultures were treated in the same fashion as infected cultures except that C. trachomatis was not added. Escherichia coli J F64 (F− thi-1 pyrE60 argE3 his4 proA2 thr-1 leu-6 cysG pyrG recA1 mit-1 yfi-5 5’ara-1 rpsL31 mraA3 lacY1 str−1 leu−7 lys−2 galK2 recA1) was obtained from H. Zalkin, Purdue University, Indiana (13, 14). DH5α (supE44 his3 recA1 endA1 gyrA96 thi-1 relA1) was obtained from B. Triggs-Raine, University of Manitoba, Winnipeg, Canada. Rich medium for E. coli was LB (15). Minimal medium was 60 mM K2HPO4, 33.1 mM KH2PO4, 7.6 mM (NH4)2SO4, 1.7 mM sodium citrate, 0.2 mg/ml MgSO4, 5 mg/ml glucose, 0.2 mg/ml thiamine (15).

Mammalian Cell Lines and Culture Conditions—Wild type mouse L929 cells were provided by K. Coombs, Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada. The cytidine requiring CTP synthetase-deficient CHO K1 (CR-2) cells (16) were obtained from M. Brown, Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada. The cytidine requiring CTP synthetase-deficient CHO K1 (CR-2) cells (16), the CPEC resistant CR-2 cells (17), and the revertant of Thy-49 (18) were provided by F. P. Holmes, Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada. The cytidine requiring CTP synthetase-deficient CHO K1 (CR-2) cells (16), the CPEC resistant CR-2 cells (17), and the revertant of Thy-49 (18) were provided by M. Brown, Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada. The cytidine requiring CTP synthetase-deficient CHO K1 (CR-2) cells (16), the CPEC resistant CR-2 cells (17), and the revertant of Thy-49 (18) were provided by M. Brown, Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada. L929 cells were provided by K. Coombs, Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada. The cytidine requiring CTP synthetase-deficient CHO K1 (CR-2) cells (16) were obtained from M. Brown, Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada.

RESULTS

CPEC Sensitivity of Wild Type and Mutant C. trachomatis—The effect of CPEC on the growth of C. trachomatis L2 was examined by exposing cells to increasing concentrations of CPEC. To differentiate between the effects of CPEC on host and parasite, C. trachomatis was grown in CR-2 host cells. This cell line lacks a functional CTP synthetase making these cells resistant to the effects of CPEC (data not shown). DNA synthesis activity, measured as incorporation of [3H]adenine into DNA, was used as a measure of chlamydial growth (6). The concentration of CPEC required to inhibit DNA synthesis activity by 50% for L2 in CR-2 cells was 0.15 µM (Fig. 1A),
The addition of 2 μM CPEC caused an 8-fold decrease in the CTP pool of L2 RBs, purified from L2-infected CR-2 host cells exposed to CPEC (effects of CPEC on wild type and mutant C. trachomatis nucleotide pools are considered in detail below). The reduction of the CTP pool indicates that the observed effect of CPEC toward C. trachomatis is identical to that seen in mammalian cells. A C. trachomatis strain (L2/CPEC) resistant to the effects of CPEC was sequentially selected in the presence of increasing concentrations of CPEC, as described under “Material and Methods.” The final concentration of CPEC used for the selection procedure was 5.0 μM. The concentration of CPEC required to inhibit DNA synthesis activity by 50% for L2/CPEC in CR-2 cells was 50 μM (Fig. 1B). To verify that the triphosphate form of CPEC was the agent active against C. trachomatis, both L2 and L2/CPEC were grown in UK – rat hepatoma host cells. UK – cells are deficient in uridine/cytidine kinase activity, and therefore do not phosphorylate CPEC. Neither L2 nor L2/CPEC are affected by CPEC when grown in UK – cells, verifying that only CPEC-TP is active in inhibiting chlamydial growth (Fig. 1, A and B).

Nucleotide Sequence of CTP Synthetase from L2/CPEC—Because CTP synthetase is the primary target of CPEC-TP (12), the nucleotide sequence of the CTP synthetase gene from L2/CPEC was determined. The ORF corresponding to the CTP synthetase gene of C. trachomatis L2 is the second of three overlapping ORFs (9). The CTP synthetase gene occurs at nucleotide 1090–2709 relative to a HindIII site upstream of ORF1. Three PCR products corresponding to various parts of the ORF were generated from genomic DNA isolated from L2/CPEC. These PCR products encompassed the entire CTP synthetase gene (bases 1074–2700) and two separate fragments encompassing bases 1309–2191 and 1835–2554. The PCR products were cycle sequenced and compared with the previously determined nucleotide sequence of the CTP synthetase gene from C. trachomatis L2 (9). The sequences were identical except for a single point mutation (T to G transition) at base 1536. This point mutation resulted in a change of amino acid 149 from Asp to Glu. The mutation was also confirmed by sequencing a genomic clone of the mutant CTP synthetase. This clone was obtained by colony hybridization of a pUC19 HindIII library prepared with genomic DNA isolated from L2/CPEC. The mutated region of the CTP synthetase enzyme identified is believed to be involved in formation of a CTP binding domain. Whelan et al. (17) isolated a series of 23 CHO cell lines resistant to arabinosylcytosine (araC) and 5-fluorouracil (5-FU). These same cell lines were subsequently shown to be cross-resistant to CPEC. Sequence analysis of the CTP synthetase gene from these cell lines revealed mutations at seven sites within the CTP synthetase ORF. Of these mutation sites, five were clustered within a stretch of 14 amino acids (residues 152–164 (17)). The two remaining sites were 37 and 69 amino acids N- and C-terminal to this region, respectively. The mutation identified in CPEC-resistant C. trachomatis fell within the same 14-amino acid region identified by Whelan et al. (17) (Fig. 2). However, the mutation in the chlamydial CTP synthetase enzyme was distinct from those reported by Whelan et al. (17) and has not previously been associated with CPEC (1). Whelan et al. (17) have proposed that these three regions of the CTP synthetase enzyme are associated with formation of a CTP binding domain. Wild type eukaryotic and prokaryotic CTP synthetase enzymes are feedback inhibited by CTP (26, 27). Mutations in the CTP binding domain could lead to a loss of CTP feedback inhibition, thereby preventing the normal cytotoxic effects of CTP analogues.

Phenotypic Characteristics of L2/CPEC—CPEC in mammalian cells has been associated with multiple mutational events (28–30); therefore, we compared pyrimidine metabolism in L2 and L2/CPEC to determine if other mutations had occurred which might contribute to CPEC resistance. Wild type C. trachomatis does not transport or anabolize cytosine or cytidine, therefore, mutations should be limited to alterations in CTP transport, changes in the expression or activity of CTP synthetase, or changes in the incorporation of CPEC-TP into DNA or RNA. Through a series of radiolabeling and immunochemical techniques these characteristics of L2 and L2/CPEC were compared.

C. trachomatis could potentially gain resistance to CPEC by eliminating or greatly reducing the transport of CTP (and therefore CPEC-TP) from the host cell and instead rely on endogenous production of CTP via its own CTP synthetase. To test this possibility, we measured the incorporation of [5-3H]cytidine into L2 and L2/CPEC DNA in chlamydia infected wild type mouse L929 cells. A significant reduction in the incorporation of [5-3H]cytidine into L2/CPEC DNA compared with wild type L2 would suggest a decrease in the transport of CTP by the mutant chlamydiae. Incorporation of radiolabeled adenine into parasite DNA was used as a control to verify that L2 and L2/CPEC were growing at approximately the same rate. The
results, shown in Table I, indicate that no decrease occurred in cytidine incorporation into L2/CPEC, suggesting that no major change in the transport of CTP from the host has occurred in the mutant organism.

C. trachomatis could also gain resistance to CPEC by eliminating or reducing the activity of its own CTP synthetase. Growth of L2 would then depend on a supply of CTP from host-derived CTP pools. Our selection procedure, using CR-2 host cells lacking a CTP pool, should have prevented this type of mutation from occurring. To eliminate this possibility, however, we examined the activity of the chlamydial enzyme in vivo to verify its activity. Enzyme activity was assayed by examining the DNA of C. trachomatis-infected CR-2 cells for \([3H] \text{deoxycytidine}\) following in situ labeling with \([5-3H] \text{Juridine}\). Uridine labeled in the 5 position labels specifically (d)CTP and not dTTP; therefore, any label detected in DNA originates only from CTP. Because CR-2 cells lack CTP synthetase activity any label incorporated as deoxycytidine in nucleic acids must be derived from activity of the chlamydial CTP synthetase. Initially, exogenous cytidine was not added to the medium; therefore, an unlabeled CTP pool was not present in the host. \([3H] \text{Adenine}\) incorporation was used as a standard to compare DNA synthesis activities of L2 and L2/CPEC. L2/CPEC incorporated more \([5-3H] \text{Juridine}\) label into DNA as deoxycytidine than L2 (Table I). In the presence of exogenous unlabeled cytidine (unlabeled CTP pool in host), the increased incorporation of label by L2/CPEC was more pronounced. Incorporation of exogenous uridine label into deoxycytidine of L2/CPEC DNA verifies that the CTP synthetase enzyme of L2/CPEC is still functional in vivo. The increase in incorporation by L2/CPEC, in comparison with L2, suggests increased production of CTP by CTP synthetase, consistent with the proposed loss of allosteric regulation of the enzyme. These results verify that the in vivo activity of the CTP synthetase of L2/CPEC has not been reduced or eliminated.

Overexpression of CTP synthetase, leading to increased levels of CTP production, could also reduce the cytotoxic effect of CPEC and could also account for the increase in the specific activity of labeled CTP pools, observed above. Whole cell proteins of purified L2 and L2/CPEC RBs were separated by SDS-PAGE and Western blotted to nitrocellulose membrane. The major outer membrane protein (Momp) of C. trachomatis was used as a standard on Coomassie Blue-stained gels to ensure equal amounts of protein were loaded. The CTP synthetase enzyme was immunodetected with rabbit polyclonal antiserum raised against purified L2 CTP synthetase. No change in the levels of CTP synthetase from L2 or L2/CPEC was apparent (Fig. 3), suggesting that a mutation leading to increased expression of CTP synthetase in L2/CPEC has not occurred.

CPEC toxicity could also potentially be due to incorporation of CPEC-TP or dCPEC-TP into RNA or DNA, respectively. Because the mode of action of CPEC in C. trachomatis has not been addressed, it is conceivable that incorporation of the nucleoside analogue into RNA or DNA could contribute to the cytotoxicity of the drug in this species. A mutation in the DNA or RNA polymerase enzymes could reduce or eliminate incorporation of (d)CPEC-TP and contribute to the development of resistance. Using \([3H] \text{CPEC}\) we examined the incorporation of label into nucleic acids. Table I illustrates that L2 incorporates very little (d)CPEC-TP into nucleic acids. Incorporation is unchanged in L2/CPEC. These results indicate that the incorporation of this analogue into nucleic acids is unlikely to be a significant source of toxicity and resistance to CPEC develops in C. trachomatis in the absence of any change in (d)CPEC-TP incorporation into nucleic acids.

**Allometric Regulation of Chlamydial CTP Synthetase**—Based on the previous analyses, the only detectable mutation in C. trachomatis associated with resistance to CPEC was a single point mutation in the structural gene encoding CTP synthetase. The occurrence of this mutation in a region of the protein believed to be a CTP binding domain (17), coupled with the apparent increase in vivo CTP production in L2/CPEC, suggested CPEC\(^b\) was due to a loss of CTP feedback inhibition of the chlamydial CTP synthetase. We therefore examined this characteristic of the wild type and mutant enzyme. The gene encoding the wild type enzyme has been previously cloned by functional complementation using a pyrG strain of E. coli (J F646) and expressed in E. coli (9). The mutant enzyme

![FIG. 2. Point mutation identified in L2/CPEC CTP synthetase. Hamster sequences show the mutations identified by Whelan et al. (17) in 15 independently isolated CHO cell lines showing multiple resistance to araC, 5-FU, and CPEC. Numbers in brackets indicate the number of strains identified with a given mutation.](http://www.jbc.org/)

| Cell line | Media supplement | \(^3H\)-Labeled precursor\(^a\) | Incorporation of precursor into nucleic acids (10\(^6\) dpm/10\(^6\) cells) |
|-----------|-----------------|-------------------------------|-----------------------------------------------|
|           |                 |                               | Mock-infected | L2       | L2/CPEC  |
| L29       | adenine         | ND                            | 116           | 176      |
| L29       | cytidine        | ND                            | 128           | 334      |
| In vivo conversion of UTP to CTP\(^c\) | | | | |
| CR-2      | adenine         | 12                            | 135           | 163      |
| CR-2      | uridine         | 5                             | 89            | 133      |
| CR-2      | cytidine        | 8                             | 151           | 189      |
| CR-2      | uridine         | 2                             | 24            | 56       |
| Incorporation of host-supplied CPEC-TP\(^d\) | | | | |
| L29       | cytidine        | 148                           | 217           | 204      |
| L29       | CPEC            | 6                             | 3             | 3        |

\(^a\) For utilization of host-supplied CTP and in vivo conversion of UTP to CTP, adenine incorporation is used as a growth control. For incorporation of host-supplied CPEC-TP, cytidine is used as a growth control (see text for details).

\(^b\) The ability of C. trachomatis L2 and L2/CPEC to utilize host-supplied CTP is determined by measuring \([5-3H] \text{cytidine}\) incorporation into DNA.

\(^c\) The ability of C. trachomatis L2 and L2/CPEC to convert UTP to CTP is determined by measuring \([5-3H] \text{uridine}\) incorporation into DNA.

\(^d\) The ability of C. trachomatis L2 and L2/CPEC to incorporate CPEC-TP is determined by measuring CPEC-TP incorporation into DNA and RNA.
FIG. 3. Immunodetection of CTP synthetase in L2 and L2/ CPEC EBs and RBs. Samples of purified EBs and RBs were heated at 95°C in the presence of SDS sample buffer prior to loading on the gel. Samples were blotted to nitrocellulose and CTP synthetase immunodetected with polyclonal antibodies raised against purified CTP synthetase. Eukaryotic CTP synthetase, which migrates with a greater mobility on SDS-PAGE gels than chlamydial CTP synthetase, is not detectable in preparations of purified EBs and RBs. Sizes are shown in kilodaltons. Cells were grown in the presence (+ CPEC) or the absence (− CPEC) of 2 μM CPEC. Lane 1, L2-EB; lane 2, L2-RB; lane 3, L2/ CPEC-EB (− CPEC); lane 4, L2/CPEC-RB (− CPEC); lane 5, L2/ CPEC-EB (+ CPEC); lane 6, L2/CPEC-RB (+ CPEC).

TABLE II
Chlamydial CTP synthetase activity as a function of CTP concentration

| CTP (μM) | L2 CTP5 activity | L2/CPEC CTP5 activity |
|----------|------------------|-----------------------|
| 0        | 49               | 25                    |
| 1        | 48               | 25                    |
| 10       | 42               | 32                    |
| 50       | 25               | 29                    |
| 100      | 7                | 25                    |
| 200      | ND               | 38                    |

was obtained following complete HindIII digestion of genomic DNA isolated from L2/CPEC. HindIII sites lie 178 and 11 base pairs upstream and downstream, respectively, of the CTP synthetase gene (9). A library of HindIII fragments was created in pUC19 and an E. coli JF646 clone potentially containing the mutant CTP synthetase enzyme was identified following colony hybridization using the wild type gene as a probe. Sequencing of a plasmid (pCP1) isolated from this clone, verified the cloning of the mutant enzyme. Additionally, pCP1 complemented the pyrG defect in E. coli JF646. Immunodetection of the enzyme in whole cell proteins from JF646 (pCP1) verified that the cloned mutant protein was expressed in E. coli (data not shown).

In vitro CTP synthetase assays were conducted with extracts prepared from E. coli JF646 containing either pCSWT1 (wild type L2 CTP synthetase) or pCP1. Enzyme activity was measured as a function of CTP concentration. Activity of the wild type L2 enzyme was inversely correlated with CTP concentration, with 50% inhibition occurring at approximately 50 μM CTP (Table I). In contrast, the CPEC CTP synthetase showed no measurable CTP feedback inhibition with CTP concentrations up to 200 μM. These findings verify that resistance to CPEC in C. trachomatis is associated with a loss of allosteric regulation of the chlamydial CTP synthetase.

Effect of the CPEC CTP Synthetase on E. coli Nucleotide Pools.—To determine the effect of the loss of CTP feedback inhibition on nucleotide pools in vivo, E. coli nucleotide pools were examined following overexpression of the wild type and CPEC chlamydial enzymes. pCSWT1, encoding the wild type chlamydial CTP synthetase, and pCP1, encoding CPEC CTP synthetase, were used to transform E. coli. Cells carrying pUC19 were used as a control. These experiments were conducted in two strains of E. coli. One, DH5α, is wild type for pyrimidine metabolism, and the other, JF646, is auxotrophic for pyrimidines (pyrE pyrG). Nucleotides were extracted from cells grown to late logarithmic phase in LB medium.

Overexpression of the wild type chlamydial CTP synthetase had relatively little effect on nucleotide pools of either strain (Table III). In contrast, expression of the CPEC CTP synthetase resulted in a marked increase in the CTP pool of both strains. The CTP pool of DH5α increased 19-fold. The UTP pool is maintained due to the presence of de novo synthesis of pyrimidines in this strain. The CTP pool of JF646 increased 2-fold, whereas the UTP pool decreased 16-fold. The smaller increase in the CTP pool in JF646 is due to the fact that UTP, the substrate for CTP synthetase by CTP synthetase, has become limiting. The dramatic reduction in the UTP pool results from depletion of pyrimidines from the growth medium (data not shown). In either case, results verify that the CTP synthetase enzyme isolated from L2/CPEC is no longer feedback inhibited by CTP.

CPEC CTP Synthetase Confers 5-Fluorouracil Resistance to E. coli.—The loss of CTP feedback inhibition of mammalian CTP synthetase is associated with multi-drug resistance to araC, 5-FU, and CPEC (17, 28). Therefore, we determined whether expression of the chlamydial CPEC CTP synthetase conferred resistance to these drugs in E. coli. We found that neither araC nor CPEC was cytotoxic to E. coli at concentrations up to 50 μg/ml. However, 5-FU was cytotoxic, requiring a concentration of 1 μg/ml to inhibit the growth rate of DH5α (pUC19) by 50% (IC50). The wild type chlamydial CTP synthetase did not confer a significant protective effect toward 5-FU (IC50 2 μg/ml). However, the IC50 value for DH5α (pCP1) increased to 25 μg/ml.

Nucleotide Pools in L2 and L2/CPEC—Given the effect of the CPEC CTP synthetase on E. coli nucleotide pools, we examined the nucleotide pools of L2 and L2/CPEC to determine if the CTP or UTP pools were increased or decreased, respectively. L2 and L2/CPEC RBs and L2/CPEC EBs were isolated from CR-2 host cells following 4- and 26-h exposures, respectively. L2 and L2/CPEC RBs were isolated from CR-2 host cells following 4- and 26-h exposures, respectively, to 2 μM CPEC (Table IV). As expected, CPEC caused an 8-fold reduction in the CTP pool of L2 RBs. In contrast, exposure to CPEC had little effect on the CTP pool of L2/CPEC RBs, demonstrating that the loss of allosteric control of CTP synthetase allows for the continued production and maintenance of a CTP pool in L2/CPEC. Surprisingly, unlike the results seen in E. coli expressing recombinant wild type and CPEC CTP synthetase, there were no significant changes in the size of the CTP/UTP pools in L2/CPEC RBs compared with L2 RBs. A similar result was obtained with EBs. Possible explanations to account for this phenomenon are presented under "Discussion."
acquire CTP either from its own CTP synthetase or from host pools. Therefore, the cytotoxic action of CPEC toward L2 in starved CR-2 cells does not indicate whether CTP synthetase is an essential enzyme for chlamydial growth.

In an attempt to determine whether the chlamydial CTP synthetase was essential, we examined the growth of L2 in Thy-49 and Thy-303 cell lines. These cells are resistant to the effects of CPEC due to a mutation in the mammalian CTP synthetase (17). However, because the enzyme is still functional, a CTP pool is maintained in these cells in the presence of CPEC. If CPEC inhibited the growth of L2 in these cell lines, it would indicate that L2 depended on its CTP synthetase to complete its life cycle, even in the presence of an exogenous source of CTP. However, we found that neither L2 nor L2/CPEC were able to grow in these cell lines, regardless of the presence or the absence of CPEC. The mutation in the CTP synthetase of Thy-49 and Thy-303 results in 3- and 6-fold increases, respectively, in the CTP pool of these cell lines (data not shown). L2 and L2/CPEC are able to grow in the Thy-49 revertant, Thy-5.1. Because the only apparent difference between these strains is the size of the CTP pool (Thy-5.1 reverts to wild type CTP pool and is sensitive to CPEC [data not shown]), an elevated host pool of CTP may adversely affect the growth of chlamydiae. We are currently investigating the effect of altered UTP/CTP host pools on chlamydial growth and will expand on these observations in a future publication.

Given the inability of Thy-49 and Thy-303 to support chlamydial growth we attempted an alternative means of assessing whether CTP synthetase is essential for the growth of L2. A CTP pool can be restored in CR-2 cells by the addition of exogenous cytidine. Under these conditions, growth of L2 in the presence of CPEC would suggest that cytidine had rescued L2 from the effects of CPEC by providing an alternate source of CTP. Although we found that cytidine was able to eliminate the effect of CPEC on L2, the addition of uridine to the medium was equally effective at restoring growth of L2 (data not shown). Based on these results we could not determine whether the presence of a host CTP pool rescued L2 or whether exogenous cytidine or uridine were competing with CPEC for transport into the mammalian cell or for phosphorylation at the level of the mammalian uridine/cytidine kinase. Given the difficulty in interpreting these results and the lack of a gene knockout system for chlamydiae, we cannot presently determine whether the CTP synthetase is essential for L2 survival.

**DISCUSSION**

The isolation of C. trachomatis mutants resistant to the effects of hydroxyurea (19), 6-thioguanine (31), and tri-methoprim and sulfisoxazole (32) have proven useful in understanding the nucleotide metabolism of this bacterial parasite. Here we report the isolation of a C. trachomatis strain resistant to the cytotoxic effects of CPEC. This is the first description of the mutations that are responsible for CPEC-resistant direct selection by CPEC. Mutations in CTP synthetase characterized by Whelan et al. (17) were induced by chemical mutagenesis or UV light followed by selection for resistance to araC or 5-FU and subsequently shown to confer cross-resistance to CPEC. Additionally, this is the first report of the characterization of a mutant chlamydial strain at the molecular level. Efforts to develop a genetic transfer system for chlamydiae have been hampered by the lack of selectable genetic markers. The characterization of a CPEC-resistant CTP synthetase in C. trachomatis may help to overcome this obstacle to chlamydiae research.

CPEC is a carboxycyclic analogue of cytidine that must be raised intracellularly to its triphosphate form to become active. The cytotoxicity of CPEC against C. trachomatis lies in the ability of this bacterium to transport CTP and other nucleotides from the host cell cytoplasm. CPEC, raised to its triphosphate form by the host cell, enters the chlamydial cell and exerts its cytotoxic effect. The known mode of action in eukaryotes is through feedback inhibition of CTP synthetase (12). The cytotoxic action of CPEC against C. trachomatis appears identical as a mutation was identified in the chlamydial CTP synthetase, which eliminated the allosteric regulation of the enzyme. This mutation allowed C. trachomatis L2/CPEC to maintain a CTP pool in the presence of normally cytotoxic concentrations of CPEC.

The loss of CTP feedback inhibition of the chlamydial enzyme was demonstrated with both an in vivo assay using E. coli cells carrying the cloned gene and an in vitro assay using partially purified preparations of CTP synthetase. The in vivo results are similar to results previously reported using mammalian cells in which mutations in CTP synthetase conferring resistance to nucleotide analogues resulted in elevated pools of CTP (28, 30). Additionally, the chlamydial CPEC-resistant CTP synthetase cloned in E. coli conveyed resistance to 5-FU, again identical to the multiple drug resistance that arises in mammalian cells expressing a CTP synthetase lacking allosteric regulation (17, 28). In vitro, we demonstrated that CTP concentrations up to 200 μM no longer inhibit the activity of the CTP synthetase derived from L2/CPEC. Together, these results demonstrated that the CPEC-resistant L2/CPEC was associated with a loss of CTP feedback inhibition of CTP synthetase.

Resistance to CPEC in C. trachomatis appears to be due solely to this mutation in CTP synthetase, because we could find no evidence of any other major alteration in nucleotide metabolism, which would influence the metabolism of CTP or its analogue, CPEC-TP. In contrast to mammalian cells, the means by which C. trachomatis can develop resistance to CPEC is limited due to the lack of cytosine/cytidine salvage pathways and its reliance on the host cell for preformed CTP/UTP (6, 7). Based on the limited nucleotide metabolizing capabilities of C. trachomatis, we felt mutations conferring CPEC-R in this bacterium would be associated with either alterations in CTP transport, CTP synthetase, and/or (d)CPEC-TP incorporation into nucleic acids. Of these possibilities the only change identified was the mutation in CTP synthetase rendering it resistant to CTP feedback inhibition.

Resistance to CPEC through a single mutational event is unlike the multiple resistance mechanisms that develop in mammalian cells resistant to CPEC. In these latter cells, resistance to CPEC has been linked to alterations in drug uptake (30), mutations in uridine/cytidine kinase (29), and mutations in CTP synthetase (17, 30). Multiple mutations in mammalian cells may correlate to the different levels of resistance to CPEC developed by mammalian cells versus C. trachomatis. Mamma-

**TABLE IV**

| Bacterial strain | CPEC added | Nucleotide pool | pmol/μg DNA |
|-----------------|------------|-----------------|-------------|
| L2 RB           | 1.4 20.3   | CTP UTP ATP GTP | 247.2 26    |
| L2 RB           | 0.1 20.7   | CTP UTP ATP GTP | 247.2 26    |
| L2/CPEC RB      | 0.1 20.7   | CTP UTP ATP GTP | 247.2 26    |
| L2/CPEC RB      | 0.3 20.7   | CTP UTP ATP GTP | 247.2 26    |
| L2 RB           | 0.1 20.7   | CTP UTP ATP GTP | 247.2 26    |
| L2 RB           | 0.1 20.7   | CTP UTP ATP GTP | 247.2 26    |
| L2/CPEC RB      | 0.1 20.7   | CTP UTP ATP GTP | 247.2 26    |
| L2/CPEC RB      | 0.1 20.7   | CTP UTP ATP GTP | 247.2 26    |
| L2 RB           | 0.1 20.7   | CTP UTP ATP GTP | 247.2 26    |
| L2 RB           | 0.1 20.7   | CTP UTP ATP GTP | 247.2 26    |
| L2/CPEC RB      | 0.1 20.7   | CTP UTP ATP GTP | 247.2 26    |

* CPEC (2 μM) added at 18–20 h post-infection L2 and L2/CPEC RBs were harvested approximately 24 h after the addition of CPEC. L2 and L2/CPEC EBs were harvested approximately 26 h after the addition of CPEC, at 44-48 h post-infection.
lavian cells show a 2000–12000-fold increase in resistance to CPEC (17, 30) in contrast to the 350-fold increase observed for C. trachomatis. Mutational events in mammalian cells leading to CPEC 

reduce both the levels of CPEC-TP accumulating in the cytoplasm and the sensitivity of CTP synthetase to CPEC-TP. In contrast, because C. trachomatis takes CPEC-TP preformed from the host cell, it has no control over the rate of phosphorylation of CPEC to CPEC-TP, and potential mutations may be limited to reducing the sensitivity of CTP synthetase to CPEC-TP. Although C. trachomatis could conceivably reduce CTP (and CPEC-TP) transport to reduce the accumulation of CPEC-TP, we could detect no decrease in CTP transport by L2/CPEC. We have not addressed the possibility as to whether continued selection at higher levels of CPEC would lead to mutations in CTP transport in L2/CPEC.

Expression of L2/CPEC CTP synthetase in E. coli DH5α or J646 resulted in an elevated CTP pool in these strains. Identical results have been observed in mammalian cells expressing CPEC 

CTP synthetase. However, L2/CPEC EBs or RBs did not show an elevated CTP pool. The mutant enzyme is functioning in C. trachomatis, because L2/CPEC grows normally in CR-2 lacking a CTP pool, and labeling with [5-3H]uridine indicated the in vivo formation of CTP in L2/CPEC. Several explanations are possible for the lack of an observed effect of the mutant enzyme on C. trachomatis nucleotide pools: 1) The CPEC 

enzyme may show reduced catalytic activity to compensate for the loss of allosteric regulation. A reduction in the rate of catalysis of UTP to CTP could allow continuous production of CTP, with relatively little effect on overall CTP pool size. 2) CTP transport could be reduced to compensate for the excess CTP produced by CTP synthetase in L2/CPEC. 3) Due to the extremely low levels of CTP normally seen in CR-2, the wild type CTP synthetase may rarely, if at all, be feedback inhibited by CTP, and hence no observable change occurs in the CTP pool of L2/CPEC. Loss of allosteric regulation in L2/CPEC may simply allow normal activity of the enzyme to continue in the presence of CPEC. 4) Excess CTP could be metabolized by L2/CPEC to maintain the size of the CTP pool.

Based on the results of in situ labeling experiments described above, 1) and 2) are unlikely. L2/CPEC-infected CR-2 cells appear to incorporate more [3H]deoxycytidine into DNA following [5-3H]uridine labeling than L2-infected CR-2 cells. If the CPEC 

enzyme was functioning below wild type levels, as outlined in the 1) above, less incorporation of label into L2/CPEC DNA would be expected. Similarly, L2/CPEC-infected L929 cells showed no decrease in incorporation of [3H]cytidine in comparison with L2-infected L929 cells, suggesting that no significant reduction in CTP transport has occurred. With respect to 3) and 4) above, our experimental data do not favor one explanation over the other. There appears to be an increase in the incorporation of [3H]deoxycytidine into L2/CPEC-infected CR-2 DNA following labeling with [5-3H]uridine, suggesting increased CTP production as a result of the loss of allosteric regulation of L2/CPEC CTP synthetase. However, the change is marginal, consistent with limited feedback inhibition occurring in the wild type chlamydial CTP synthetase. Any small increase in the chlamydial CTP pool may be compensated by increased metabolism or excretion back to the host cell.

Maintenance of CTP at wild type levels may be critical for chlamydiae because several lines of evidence suggest CTP may play an important role in the life cycle of C. trachomatis. First, as outlined in the introduction, the basis of our investigation into CTP metabolism originated with identification of a chlamydial CTP synthetase, rendering chlamydial cells prototropic for CTP, but auxotrophic for all other nucleotides. Second, the extremely small CTP pool in RBs and EBs is unusual in comparison with other prokaryotic and eukaryotic cells. The ratio of CTP:ATP in C. trachomatis L2 RBs is approximately 0.03 (Table IV) compared with 0.17 for E. coli (33). Third, in attempting to determine if the CTP synthetase of L2 was essential, we discovered that an elevated CTP host pool has a specific inhibitory effect on the growth of L2. Whether this is due to the ability of CTP to competitively inhibit the transport of other nucleotides, or whether host CTP levels act as a signal for chlamydial growth is currently unknown. Investigations into the role of host CTP levels on chlamydial growth are currently underway.

Growth of L2 in starved CR-2 cells demonstrates that the CTP synthetase of C. trachomatis can provide all of the CTP required for chlamydial growth. As a part of the present experimental analysis, we attempted to determine if the opposite was true. Can the life cycle of C. trachomatis be completed in the absence of an endogenous supply of CTP from CTP synthetase? However, we were unable to definitely answer this question, due to the lack of a genetic transfer system for chlamydiae and the inability of C. trachomatis to grow in mammalian cell lines carrying a CPEC 

CTP synthetase.

In analyzing the range of mutations that occurred in mammalian CTP synthetase as a result of drug selection, Whelan et al. (17) identified only a limited number of substitutions within a specific region of the gene. These authors argued that this apparent constraint on the distribution of mutations may relate to the necessity of retaining CTP synthetase function. Mutations were therefore limited to eliminating the sensitivity of the enzyme to the inhibitor, CPEC. This is in contrast to mutations identified in nonessential purine salvage pathways in mammalian cells, which are distributed throughout the target genes and can lead to inactivation of the pathway (34, 35). Based on these considerations, the identification of a mutation in the chlamydial CTP synthetase that eliminates sensitivity to CPEC yet maintains function may have some bearing on whether the enzyme is essential. As Whelan et al. (17) suggested for the mammalian CTP synthetase, there may be constraints on the functional alterations that can occur in the chlamydial enzyme. Repetition of our selection procedure, followed by identification of the locations of possible mutation sites may yield indirect evidence as to whether the CTP synthetase enzyme is essential in C. trachomatis.

In summary, the results presented indicate that C. trachomatis resistance to CPEC appears to be due to a single mutational event in the structural gene encoding CTP synthetase. No major changes in the transport of nucleotides, the expression of CTP synthetase, or the incorporation of (d)CPEC-TP into nucleic acids were observed.

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A Single Point Mutation in CTP Synthetase of Chlamydia trachomatis Confers Resistance to Cyclopentenyl Cytosine

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