Tryptophan biosynthesis in chlamydiae

BY

HEIDI E. WOOD

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

PhD

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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DOCTOR OF PHILOSOPHY

ACKNOWLEDGEMENTS

To begin with, I would like to acknowledge Dr. Grant McClarty for his excellent supervision over the last three years. Without his support and guidance, none of the work in this thesis would have been possible. I consider myself extremely lucky to have been able to work in Grant's lab. I would also like to thank my committee members, Dr. Rosanna Peeling and Dr. Allan Becker, and Dr. Richard Morrison, the external examiner, for reviewing my thesis and offering helpful comments and suggestions.

I would also like to thank past and present members of the McClarty lab including Niki, Bonnie, John, Emma and Kate. In particular, I would like to acknowledge the contributions of Christine Roshick and Dr. Christine Fehlner-Gardiner, who were actively involved in the work presented in this thesis. I would also like to thank the members of the Health Canada lab – Liz and Zeena, who were there from the beginning and who were always willing to help me when I needed it, and Ken, a recent addition to the lab. As well, I would like to thank both Dr. Jody Berry and Dr. Ian Maclean for help and advice. I am also deeply grateful to Dr. Harlan Caldwell and members of the Rocky Mountain Laboratories, in particular John Carlson, for collaborating with us on our clinical studies.

Finally, I would like to thank my family - my parents and sisters, Heather and Linda, for their support and encouragement over the years.

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ABSTRACT

Comparative genomics indicates that vast differences in chlamydia *sp.* host range and disease characteristics can be traced back to subtle variations in gene content within a region of the chromosome, termed the plasticity zone. Genes required for tryptophan biosynthesis are located in the plasticity zone; however, the complement of genes encoded varies depending on the chlamydial species examined. *C. trachomatis* consists of multiple serovars that exhibit a clear dichotomy in organotropism. Ocular serovar A-C infections are largely restricted to the conjunctivae, whereas genital serovars D-L3 are primarily urogenital pathogens. Previous work demonstrated that differences in organotropism among laboratory reference strains or *C. trachomatis* could be consistently associated with mutations in the tryptophan synthase operon; specifically mutations producing a dysfunctional synthase were common to ocular serovars whereas genital serovars maintained a functional synthase. Here I have extended these findings to include a molecular characterization of the tryptophan synthase locus in numerous ocular and genital clinical isolates obtained from diverse geographical locations. My results indicate that the paradigm established with the reference serovars holds true for all clinical isolates examined. Further, my work indicates that *C. trachomatis* expresses a functional TrpR, and regulates the expression of these genes in response to changes in tryptophan availability. The tryptophan precursor, indole, repressed expression of the *trp* genes in serovar L2, which expresses a functional tryptophan synthase, but not in serovar A, which expresses a truncated and inactive TrpA. The fact that chlamydiae have retained the capacity to respond to tryptophan limitation suggests that these organisms are likely to encounter fluctuations in tryptophan levels *in vivo*, supporting the view that

IFNγ is critical in the host defense against *C. trachomatis*. Of the sequenced chlamydial genomes, *Chlamydia psittaci* GPIC contains the most complete tryptophan biosynthesis operon, encoding *trpRDCFBA*. Immediately downstream of the *trp* operon are genes encoding kynureninase and ribose phosphate pyrophosphokinase. Our results indicate that this complement of genes enables GPIC to recycle tryptophan and thus accounts for the IFN-γ phenotype displayed in indoleamine-2,3-dioxygenase-expressing host cells.

INTRODUCTION

Taxonomy

Chlamydiae are obligate intracellular gram-negative eubacteria (1). The genus *Chlamydia* consists of four species: *C. trachomatis*, *C. psittaci*, *C. pecorum*, and *C. pneumoniae*. *C. pecorum* infects ruminants and is not a human pathogen. *Chlamydia psittaci* strains are pathogens of birds and many other animal species, including humans. A strain of *C. psittaci* known as the agent of guinea pig inclusion conjunctivitis (GPIC) causes both ocular and genital infections in guinea pigs (2-4). The two medically important species of chlamydia for humans are *C. trachomatis* and *C. pneumoniae*. *C. pneumoniae* is a common cause of acute respiratory infections, including community-acquired pneumonia, with an estimated 40-60% of adult populations possessing antibodies to this organism. Seroepidemiological studies have demonstrated an association between *C. pneumoniae* and the development of chronic heart disease (5, 6). *C. trachomatis* has been divided into three biovars: trachoma, lymphogranuloma venereum (LGV), and mouse pneumonitis (MoPn). The trachoma biovar is further subdivided into 15 serovars, on the basis of serological responses to the major outer membrane protein (MOMP), designated A through K. The 15 different serovars display well-documented and unique tissue tropisms. Serovars A, B, Ba, and C preferentially infect conjunctival epithelial cells and are the causative agents of trachoma, an ocular infection which is the leading infectious cause of blindness worldwide (7). With the exception of serovar B variants, which have been associated with a very low incidence of urogenital disease (8-10), the trachoma serovars are rarely isolated from the genital tract

(11). Serovars D through K are responsible for localized genital tract infections. These serovars cause a variety of diseases including cervicitis, salpingitis, endometritis, urethritis, epididymitis and Reiter's syndrome (arthritis of the joints, urethritis, and conjunctivitis) (12). In women, repeated or persistent chlamydial infections can result in the development of adverse sequelae such as pelvic inflammatory disease (PID), ectopic pregnancy and tubal occlusion, which may lead to infertility (13). In fact, *C. trachomatis* is the leading cause of infertility in developed countries, including as Canada. Serovars D-K can, however, cause ocular infection when newborn infants acquire the organism during passage through the infected birth canal or when adults secondarily inoculate the eye with infected genital secretions. The LGV biovar consists of 4 serovars designated L1, L2, L2a, and L3. The LGV biovar strains are responsible for sexually transmitted infections but, unlike serovars D-K, they readily cause systemic infections and proliferate in monocytes within lymphatic tissues (14). In humans, progression from uncomplicated mucosal infections to serious sequelae such as blindness or tubal factor infertility has been linked epidemiologically to either reinfection or persistent infection. Re-exposure or persistent infection is thought to drive an immunopathological inflammatory response resulting in tissue fibrosis and scarring.

Developmental Cycle

Chlamydiae are obligate intracellular pathogens with a unique developmental cycle (1). They exist in two major morphological forms termed the elementary body (EB) and the reticulate body (RB). The EB is the extracellular, infectious form of the organism and is metabolically inactive. This spore-like structure possesses an osmotically resistant

outer membrane and a highly condensed chromosome. The EB is smaller (approximately 0.3 μm in diameter) than the RB (approximately 1.0 μm in diameter) and has a rigid cell wall conferred by extensive disulfide cross-linking of the cysteine-rich outer membrane proteins found in the cell envelope. The cysteine-rich proteins included: i) the major outer membrane protein (MOMP) which is 40 kDa and encoded by *ompA*; ii) the outer membrane cysteine-rich protein B or OmcB which is encoded by *omcB* and posttranscriptionally processed into two proteins of 60 kDa and iii) OmcA, a lipoprotein of 12-15 kDa encoded by *omcA*. Following infection of a eukaryotic host cell, the EB transforms into the larger, metabolically active RB. The RB is the noninfectious, intracellular form of the organism which replicates via binary fission. The transition from the EB to the RB involves structural changes in the outer membrane complex, including reduction of the disulfide cross-links between the outer membrane proteins and relaxation of the condensed nucleoid structure. The structural changes in the outer membrane complex increase its fluidity and allow for the transport of nutrients into the RB. The developmental cycle begins with the attachment of an EB to a susceptible host cell and entry of the EB into a host cell-derived phagocytic vesicle. Fusion of the host cell-derived vesicle and lysosomes is blocked, allowing the chlamydiae to grow within a protected environment. Once inside the host cell vesicle, the EB transforms into the metabolically active RB and begins RNA, DNA, and protein synthesis. The RBs divide by binary fission and grow within the expanding host cell vesicle, which is termed the chlamydial inclusion. After a period of growth and division which varies depending on the species and serovar, the RBs begin to reorganize to infectious EBs. The EBs are then released from the host cell and can continue on to infect a new round of cells (1).

Animal Models, Immunity and Pathology

Several lines of evidence support the current notion that chlamydial disease has an immunopathological component (13, 15). Chlamydial infection is characterized by inflammation, which exacerbated by reinfection or persistent infection, ultimately leads to tissue damage and scarring. Animal models have been especially helpful in defining the immunobiological features of *C. trachomatis* immunity and disease pathology (4, 16). As tools for the genetic manipulation of chlamydiae are not available, animal models have played a crucial role in deciphering the mechanisms of chlamydial pathogenesis and the host immune response to chlamydial infection. The mouse model has been particularly informative because of the availability of immune reagents for studies in mice. All three trachoma biovars can infect the mouse via a variety of routes of inoculation (17-19), although the MoPn biovar is most commonly used. MoPn was originally isolated from mouse lung tissue and is thought to be a natural pathogen of the mouse (20, 21). Hence, it offers an evolutionarily adapted pathogen for analyzing host-pathogen interactions during *C. trachomatis* infection. Experimental infections include ocular, respiratory and reproductive tract infections, inducing diseases such as conjunctivitis, pneumonitis, vaginitis and salpingitis (18, 22-24). The GPIC strain of *C. psittaci*, a natural pathogen of the guinea pig, has also been used to establish models for naturally occurring *C. trachomatis* infection and disease in humans. Even though GPIC is a member of the *C. psittaci*, it elicits ocular and genital infections paralleling the corresponding human diseases (4). MoPn and GPIC therefore represent evolutionarily adapted pathogens useful for analyzing host-pathogen interactions. However, these two animal models differ in terms of the immune responses required to clear chlamydial infection. The mouse

requires predominately CMI for resolution of genital tract infection and for resistance to reinfection, while the guinea pig requires both humoral immunity and CMI (4).

Chlamydial infection elicits both humoral and cellular immune responses in humans. Both arms appear necessary for the full expression of chlamydial immunity. The most important component of the humoral response appears to be mucosal IgA, directed against conformational epitopes on the chlamydial MOMP (25, 26). Recent studies with gene knock out mice have clearly demonstrated a major role for the cell-mediated immune (CMI) response (4, 13, 15, 16, 27-29). In particular, Th1 $CD4^+$ T cells have a predominant role for host defense against chlamydial infection. Morrison and colleagues observed that mice lacking either CD4 cells or MHC class II-bearing cells had longer infections with MoPn than did control mice. In fact, the class II-deficient mice were unable to resolve their infections. However, mice lacking β2 microglobulin, and thereby lacking CD8 cells, were able to resolve their infections in a more timely manner. These data indicate that $CD4^+$ T cells are essential for the resolution of MoPn genital infections (30, 31).

Cytokine knockout mice have also been widely used in chlamydial research, and the results have emphasized the importance of numerous cytokines, in particular the Th1 cytokine, IFN-γ (32, 33). The inhibitory effects of IFN-γ on chlamydiae replication have been demonstrated both *in vivo* (34, 35) and *in vitro* (36, 37). In addition, IFN-γ has been detected in individuals with *C. trachomatis* infection and strong IFN-γ responses have been directly correlated with healing trachoma (38). In cell culture models (reviewed in (39)), high levels of IFN-γ completely restrict chlamydial growth, while low levels induce the development of morphologically aberrant intracellular forms that are non-infectious.

These aberrant forms can persist for several weeks, with reactivation of viable infectious organism following the removal of IFN-γ (40). The persistent organisms display differential gene expression of key chlamydial antigens with continued synthesis of heat shock protein (HSP) 60, an immunopathological antigen and reduced synthesis of MOMP, a protective antigen (41, 42). A persistent infection is defined as a long-term association between chlamydiae and their host cell in which the organisms remain in a viable but culture-negative state. In cell culture, persistent chlamydial infections can be induced by a variety of conditions, including deprivation for essential amino acids, treatment with antibiotics, and exposure to cytokines, such as IFN-γ. These conditions prevent or delay RB division and maturation, or inhibit differentiation of RBs to infectious EBs. In all cases, the typical developmental cycle of chlamydiae is interrupted, resulting in morphologically aberrant forms which although viable, are noninfectious. However, infectious EBs can be recovered upon removal of the persistence-inducing stimulus (39). Because persistent infections are readily induced under suboptimal growth conditions, persistence may represent a survival strategy for chlamydiae by allowing them to persist within host cells in a viable state until optimal conditions are again present. In humans, persistent infections has been documented among women with *C. trachomatis* tubal infertility and individuals with scarring trachoma (13). The presence of unamplified chlamydial rRNA has been demonstrated in conjunctival swabs of patients with trachoma and in monkeys experimentally infected with *C. trachomatis*, even though infectious organism could not be recovered and antigen could not be detected. Because RNA molecules are highly labile, it is unlikely that they would be detectable in the absence of

metabolically active organisms, suggesting that chlamydiae may persist in ocular tissues in a viable, but culture-negative state (39).

Interferon-γ

A key mechanism for the control of chlamydial growth in human cells involves the production of interferon-γ (IFN-γ) by $CD4^+$ T cells and NK cells (13, 43-45). In human epithelial cells *in vitro*, IFN-γ induces the expression of indoleamine-2,3-dioxygenase (IDO), an enzyme which catalyzes the breakdown of L-tryptophan to L-kynurenine, resulting in the depletion of intracellular tryptophan (46, 47). The action of IDO effectively deprives the intracellular chlamydial RBs of tryptophan, inhibiting their growth and replication. Treatment of chlamydia-infected HeLa cells with IFN-γ results in the development of persistent infections characterized by the formation of aberrant but viable forms of the organism (42). However, different chlamydial species and serovars exhibit markedly different sensitivities to IFN-γ (48). *C. trachomatis* serovars A-C, which are responsible for ocular infections, are highly sensitive to the effects of IFN-γ. In contrast, many of the serovars responsible for genital tract infections, such as serovars D, E, and K, and serovars L1-L3, are much more resistant to IFN-γ. Interestingly, in human cells, strains of *C. psittaci*, including GPIC, are completely resistant to IFN-γ, in contrast to other chlamydial species and serovars (48). The varying sensitivities of the chlamydial species and serovars to IFN-γ is correlated, albeit not perfectly, with the complement of tryptophan biosynthesis genes that they possess.

The chlamydiae are not unique in their sensitivity to the depletion of tryptophan via the action of IDO. The relationship between IFN-γ, IDO, tryptophan limitation and persistent growth was first established for the intracellular parasite, *Toxoplasma gondii*. The growth of this eukaryotic intracellular protozoan is inhibited by exactly the same host mechanism used to inhibit the chlamydiae. In immunocompetent hosts, interleukin-12 (IL-12) produced by activated macrophages triggers cell-mediated immunity in early *T. gondii* infection by inducing the secretion of IFN-γ by NK cells. Many studies have demonstrated that IFN-γ production by NK cells is a major mechanism of innate defense against *T. gondii* infection. Furthermore, studies in mice indicate that IFN-γ gene knockout mice are incapable of controlling *T. gondii* growth, resulting in significantly shorter survival times compared to wild-type mice (49-51).

In contrast to human epithelial cells, IDO-induced tryptophan degradation is not the mechanism of IFN-γ-inhibition of chlamydial growth in mouse cells (52). In the murine system, IFN-γ induces an enzyme, inducible nitric oxide synthase (iNOS), which catalyzes the production of various anti-microbial reactive nitrogen intermediates, most notably nitric oxide (NO). However, studies in iNOS knockout mice indicate that NO generation is not essential for clearance of primary chlamydial genital infection (27, 53, 54). Thus, the exact mechanism by which IFN-γ inhibits chlamydial growth in mouse cells remains to be elucidated.

Tryptophan biosynthesis

Tryptophan is an essential amino acid required by all organisms and is generally synthesized by free-living prokaryotes, lower eukaryotes, and higher plants. The

biosynthesis of tryptophan *de novo* from chorismate requires the sequential action of a number of enzymes (Fig. 1a). In *E. coli*, these enzymes are encoded within a single operon, *trpEGDCFBA*, whose expression is regulated, in part, by the aporepressor encoded by *trpR*. The *trp* operon of *E. coli* has become one of the most intensely studied systems in biology, predominately due to the work of Charles Yanofsky and his coworkers. The seven conserved enzymatic domains required for tryptophan biosynthesis are encoded in six genetic regions in *E. coli*. The TrpD, TrpC, TrpF, TrpB, and TrpA proteins are each encoded by individual genes while TrpE and TrpG are fused in *E. coli* and are thus encoded together by a single gene (55). Yanofsky and co-workers have generated mutants of *E. coli* for each of the tryptophan biosynthesis genes. Such mutants have proven invaluable in the study of chlamydial tryptophan biosynthesis due to the lack of a genetic transformation system for chlamydiae. Thus, complementation of the *E. coli* mutants with the corresponding chlamydial homologue will allow the determination of the functional status of each encoded tryptophan biosynthesis protein in chlamydiae.

In *E. coli*, the component genes of the *trp* operon are organized into a single transcriptional unit. However, the structural gene for the *trp* repressor is unlinked from the *trp* operon. Tryptophan is the most biochemically expensive of the amino acid pathways, requiring the input of erythrose-4-phosphate, ATP, phosphoribosyl pyrophosphate (PRPP), two phosphoenolpyruvate molecules, L-glutamine, and L-serine. Thus, regulation of tryptophan biosynthesis in *E. coli* is quite sophisticated, being subject to the following multiple levels of control: (i) repression control via the Trp repressor, (ii) an attenuation mechanism mediated by a tryptophan-rich leader peptide (encoded by

trpL), and (iii) allosteric feedback inhibition of anthranilate synthase (TrpE) by tryptophan (56). Under conditions of tryptophan sufficiency, TrpR is complexed with tryptophan and represses transcription of the *trp* operon, as well as that of a number of additional genes including *aroH, aroL* (involved in chorismate biosynthesis), *mtr* (a tryptophan transporter), as well as *trpR* itself (57). When tryptophan levels decrease, the expression of these genes is induced due to the inability of the aporepressor to bind its cognate operator sequence in the absence of tryptophan. In combination with transcriptional attenuation, repression affords an exquisite regulation of expression of the tryptophan biosynthetic genes and allows the bacterium to respond quickly to changes in environmental conditions.

Chlamydial Genomics

The recent sequencing and subsequent annotation of seven chlamydial genomes including *C. trachomatis* serovar D, *C.* trachomatis serovar L2 (http://chlamydia-www.berkeley.edu:4231/), C. *pneumoniae* strain CWL029 (58), strain AR39 (59), strain J138 (60), *C. trachomatis* strain MoPn (59), and *C. psittaci* strain GPIC (61) has allowed for a comparative genomics approach to the analysis of potential virulence mechanisms in these organisms. In the absence of a genetic transfer system for chlamydiae, comparative genomic sequencing has provided unique insights into potential virulence genes in these organisms, as well as genes involved in host, organ and cellular tropism. The chlamydial genomes sequenced to date are highly conserved in gene content and order, not surprising considering that chlamydiae are isolated from genetic exchange with other bacteria owing to their obligate intracellular ecological niche (59). As an example, of the 1009 annotated

genes in the GPIC genome, 798 were conserved in *C. trachomatis* MoPn, *C. trachomatis* serovar D, and *C. pneumoniae*. These genes likely represent the 'minimal gene content' required for the developmental cycle and intracellular survival of the Chlamydiaceae. Genes in this group include those for DNA replication and structural components of the type III secretion system (61). One exception to this great deal of synteny between the genomes is a region of approximately 50 kb of the chromosome which has been termed the "plasticity zone" (62). This region of the genome, also known as the replication termination region (RTR), has undergone a greater degree of genetic reorganization than the rest of the chromosome (Fig. 2). Many of the unique genes are found in the plasticity zone, leading to the suggestion that differences in the plasticity zones of the various chlamydiae are likely to account for much of the phenotypic variation between chlamydial species, although subtle difference in expression of genes shared across the chlamydiae are also likely to play a role in this variation (59). A number of biotype-specific genes are encoded within the PZ of GPIC and include, in addition to an almost complete tryptophan biosynthesis operon, genes involved in nucleotide scavenging (*guaBA-add* cluster) and a toxin (tox) with homology to the large clostridial cytotoxins. Three orthologs of tox are clustered within the PZ of MoPn (59), while truncated ORFs matching portions of the N- and C-terminal regions of *tox* are found in *C. trachomatis* serovar D (63). It has been demonstrated that *C. trachomatis* strains that contain genes with homology to the large clostridial cytotoxins produced a cytopathic effect on HeLa cells that is virtually indistinguishable from the cytopathology caused by the toxins themselves (64). The PZ therefore appears to encode a number of virulence determinants, which undoubtedly account for much of the phenotypic variation between chlamydial

species and serovars. The plasticity zone also encodes enzymes required for the biosynthesis of tryptophan, with the exact complement of *trp* genes depending on the chlamydial species and serovar examined. *C. pneumoniae* and *C. trachomatis* mouse pneumonitis lack any tryptophan biosynthesis genes, while *C. trachomatis* encodes only a subset within the plasticity zone: *trpR*, encoding the tryptophan repressor, and *trpA* and *trpB*, encoding the α and β subunits of tryptophan synthase (65). Interestingly, *C. trachomatis* also encodes the gene for TrpF, which has been annotated in the *C. trachomatis* genome sequencing projects as a phosphoribosyl anthranilate isomerase homologue (59, 63); however, this gene is not encoded within the plasticity zone. Furthermore, *C. trachomatis* lacks the gene encoding the enzyme (TrpD) to provide the substrate for *trpF*, phosophribosyl anthranilate, as well as the gene encoding the enzyme (TrpC) to utilize the product of the TrpF reaction. Taken together, these observations suggest that *trpF* encodes an isomerase of a different specificity which does not play a role in tryptophan biosynthesis in *C. trachomatis.*

The genome of *C. psittaci* strain GPIC encodes genes for all reactions needed for tryptophan biosynthesis with the exception of the first, anthranilate synthase (*trpE/G*) (Read *et al.*, 2003; Xie *et al.*, 2002). Based on this *in silico* analysis, GPIC would be unable to utilize chorismate as a substrate for the biosynthesis of tryptophan and would require an alternative source of anthranilate (Fig. 1b). Two additional genes, *kynU* and *prsA*, encoding kynureninase and phosphoribosylpyrophosphate (PRPP) synthase, respectively, are located immediately downstream of the *trpDCFBA* genes in GPIC. These genes, along with *trpD* and *trpC*, are unique to GPIC among the chlamydial genomes sequenced to date. PRPP synthase catalyzes the formation of PRPP, a key

intermediate in tryptophan biosynthesis, while kynureninase catalyzes the conversion of kynurenine to anthranilate, which then combines with PRPP in a reaction catalyzed by TrpD to yield phosphoribosyl anthranilate (Fig. 1b). As kynurenine is a product of the IFN-γ-mediated degradation of tryptophan, it is possible that the presence of *kynU* and *prsA* in its genome may offer GPIC the opportunity to synthesize tryptophan when this essential amino acid becomes limited in the presence of IFN-γ (55, 61).

Tryptophan Synthase

Tryptophan synthase is a tetramer consisting of two α subunits (TrpA) and two β subunits (TrpB). The subunits form a bifunctional enzyme that catalyzes the final two reactions in the biosynthesis of tryptophan – namely, the cleavage of indole glycerol 3-phosphate (IGP) to indole by TrpA and the TrpB-mediated conversion of indole and serine to L-tryptophan (Fig. 1). Crystallographic studies of the tryptophan synthase $\alpha_2\beta_2$ complex from *E. coli* revealed that the four subunits are arranged in an extended αββα subunit with an overall length of ~150 Å. As shown in Fig. 3, the β_2 dimer is located at the center and the two α subunits are separated from each other at opposite ends of the complex. The active sites of the neighbouring α and β subunits are ~25 Å apart and are connected by a tunnel having a diameter and length sufficient to accommodate four molecules of indole. The tunnel permits the internal diffusion of indole between the heterologous active sites, preventing escape of indole into solution (66-68).

Comparative DNA sequencing of the plasticity zone of all 15 human *C. trachomatis* reference strains showed that 14 of 15 serovars encode homologues of *trpB* and *trpA* (63, 65). The chlamydial TrpB proteins (shown in Fig. 4) retained conserved

residues identified as essential for enzyme activity in other microorganisms (66, 67). In contrast, the chlamydial TrpA protein (Fig. 5) was shown to have several changes in its primary structure that differed considerably from its *trpA* orthologue in *E. coli.* Specifically, the *trpA* of ocular but not genital serovars had a common consensus triplet nucleotide deletion (nucleotides 408-410) that resulted in the loss of Phe-136 from the polypeptide (Fig. 6). Furthermore, a polymorphic mutational "hot spot" was identified in the *trpA* of ocular but not genital serovars. In ocular serovars, this region contained a single-nucleotide deletion that resulted in a frame-shift mutation and expression of a truncated nonfunctional TrpA polypeptide. Interestingly, the *trpA* of all genital serovars contained two point mutations at this same site giving rise to codon changes resulting in amino acid substitutions that differed among the serovars: YE in LGV serovars; CQ in D, E, and K serovars; and YQ in G, F, H, I, and J serovars. These amino acids are found in loop 6 of the *Salmonella* TrpA, a region identified in the tryptophan synthase crystal structure as being important for subunit-subunit interactions between TrpB and TrpA, metabolite channeling, and substrate binding (66, 67). Consistent with this conclusion was the finding by genetic complementation studies that the TrpBA of genital serovars was not capable of using IGP but could use exogenous indole for the synthesis of tryptophan (65). Further, these mutations resulted in measurable biological differences in TrpA function between ocular and genital serovars; the tryptophan synthase from ocular serovars was inactive whereas the synthase from genital serovars was active when indole was provided as a substrate (65). Since chlamydiae lack the ability to synthesize indole *de novo* and indole is not normally available as a metabolite in mammalian cells, it was proposed that *C. trachomatis* serovars that infect the genital tract may be able to use

indole produced by other microflora, either endogenous or as the result of infection, as a substrate to synthesize their own tryptophan for growth. In the same study, it was demonstrated that the infectivity of all human *C. trachomatis* serovars was inhibited when grown in tryptophan-deficient medium and, importantly, that the addition of exogenous indole to the growth medium rescued the infectivity of genital (D-K, L1-L3) but not ocular (A-C, Ba) serovars. This indole "rescuable" phenotype was unambiguously associated with a functional tryptophan synthase present in genital but not ocular serovars (65).

In addition, *trpBA* transcript was detected in infected HeLa cell cultures and the corresponding protein products were detected in purified EBs, when *C. trachomatis* was propagated in tryptophan-replete medium. These data indicated that a basal level of *trp* gene expression exists. However, in addition to *trpB* and *trpA*, *C. trachomatis* encodes a homologue of the tryptophan repressor, TrpR (59, 63), raising the possibility that expression of the *trp* genes may be regulated by transcriptional repression in a fashion similar to that observed in *E. coli* (69, 70). Given that during the *in vivo* infection process *C. trachomatis* is likely to encounter variations in tryptophan availability due to the effects of IFN-γ, regulation of *trp* gene expression in response to tryptophan levels may be advantageous for the intracellular survival of this organism.

The work in this thesis was undertaken to provide more information about tryptophan biosynthesis in chlamydiae. Specifically, I expanded upon earlier studies to include analyses of clinical ocular and genital isolate from diverse geographical locations. The paradigm established for laboratory reference strains – a functional tryptophan synthase in genital serovars versus a nonfunctional synthase in ocular serovars – was

confirmed in a larger, more heterogenous population of clinical isolates. The present studies were also undertaken to initiate studies on the molecular mechanisms regulating *trp* gene expression in *C. trachomatis* and *C. psittaci* GPIC. I characterized the regulation of tryptophan biosynthesis in chlamydiae by examining growth of chlamydiae and expression of the trp genes under various culture conditions and using various substrates for tryptophan biosynthesis. My results suggest that regulation of *trp* gene expression in response to tryptophan levels may be advantageous for the intracellular survival of this organism. The fact that chlamydiae have retained the capacity to respond to tryptophan limitation also suggests that these organisms are likely to encounter fluctuations in tryptophan levels *in vivo*, supporting the view that IFN-γ is critical in the host defense against *C. trachomatis*. In contrast, my results indicate that the complement of tryptophan biosynthesis genes encoded by GPIC enables this species of chlamydiae to recycle tryptophan and thus accounts for the IFN-γ-resistant phenotype displayed in indoleamine-2,3-dioxygenase-expressing host cells.

MATERIALS AND METHODS

1. Materials

Taq polymerase, the Thermoscript reverse transcriptase kit and Trizol were purchased from Invitrogen. Cell culture medium and fetal calf serum were purchased from either Gibco Life Technologies/Invitrogen or Signa Chemical Co. All chemicals were purchased from Sigma. The enhanced chemiluminescene kit was purchased from Amersham Pharmacia. IFN-γ was purchased from BD Pharmingen. Oligonucleotides were purchased from Invitrogen or synthesized by the DNA Core Facility, National Microbiology Laboratory, Health Canada.

2. Chlamydial strains and propagation

C. trachomatis serovars L2/LGV-434, A/Har-13, A/2497, E/IU823, B/genital isolate and *C. psittaci* strain GPIC were propagated in HeLa 229 cell monolayers as described previously (71). HeLa cell monolayers were inoculated with purified *C. trachomatis* EBs at a multiplicity of infection (MOI) of 3-5 inclusion forming units (IFU) per cell in 0.25 M sucrose-10mM sodium phosphate-5 mM L-glutamic acid (SPG) (pH 7.2). Infected HeLa cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine at 37°C in 5% CO_2. Where indicated, cycloheximide (1 μg/ml) was present in the post-infection medium. For growth under tryptophan-, tyrosine- or phenylalanine-free conditions, aromatic amino acid-free MEM

(UCSF Cell Culture Facility, San Francisco, CA) was supplemented with 10% FCS that had been previously dialyzed against Hank's Balanced Salt Solution (HBSS).

Strain abbreviation	Strain/isolate	Tissue tropism	Source
L2	*C. trachomatis* serovar L2	Genital	Laboratory strain
A	*C. trachomatis* serovar A	Ocular	Laboratory strain
2497	*C. trachomatis* serovar A	Ocular	Clinical isolate
IU823	*C. trachomatis* serovar E	Genital	Clinical isolate
B	*C. trachomatis* serovar B	Genital	Clinical isolate
GPIC	*C. psittaci* (guinea pig inclusion conjunctivitis)	Guinea pig	Laboratory strain

3. HeLa cell culture conditions

HeLa 229 cells were obtained from the American Type Culture Collection (ATCC) and were continuously maintained in our laboratory. HeLa cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine at 37°C in 5% CO_2.

4. Chlamydial proliferation assays

Monolayers of HeLa 229 cells in 6-well plates were inoculated with purified *C. trachomatis* or *C. psittaci* GPIC EBs at a multiplicity of infection (MOI) of 3-5 inclusion forming units (IFU) per cell. Where indicated, the cultures were supplemented with 5 ng mL^{-1} recombinant human IFN-γ (BD Biosciences), IFN-γ and 1 mg mL^{-1} tryptophan, or varying concentrations of tryptophan, indole, 5-fluoroindole, or 5-fluorotryptophan. HeLa cells were also pretreated for 24 h with 5 ng ml^{-1} IFN-γ and then infected with GPIC or serovar L2 in MEM-10 supplemented with 5 ng ml^{-1} IFN-γ. Where indicated, the medium was further supplemented with 50 μM indole, kynurenine, or anthranilate. The cultures were incubated for 48 to 72 h at 37°C, after which time the medium was

collected, the cells were lysed by sonication and aliquots of the combined HeLa cell lysates and culture medium were used to infect fresh HeLa cell monolayers. Recoverable IFUs were enumerated as previously described(71).

5. Determination of intracellular tryptophan levels

Uninfected HeLa cell monolayers were treated with 5 ng mL^{-1} IFN-γ or left untreated as a control. HeLa cell monolayers were infected with *C. trachomatis* serovar L2 at an MOI of 3-5 IFU $cell^{-1}$ in MEM-10 or MEM-10 supplemented with 5 ng mL^{-1} IFN-γ. Where indicated, infected cells were incubated with various concentrations of indole, tryptophan, 5-fluoroindole, or 5-fluorotryptophan. At the times indicated, the cell monolayer was extensively washed with ice-cold HBSS, 250 µl of 10% trichloroacetic acid (TCA) was added to each flask and the cells were scraped from the surface and incubated on ice for 30 min. The TCA precipitated material was pelleted by centrifugation and the tryptophan-containing supernatant was neutralized using 78.1:21.9 (v/v) freon-triN-octylamine (Tipples and McClarty, 1991). Tryptophan and 5-fluorotryptophan were separated and quantitated by high-performance liquid chromatography (HPLC) on a 12.5 cm Whatman µBondapak C_{18} column using 1 mM KH_2PO_4 (pH 4) in 10% methanol at 1 ml min^{-1} (Yong and Lau, 1979). Tryptophan and 5-fluorotryptophan were identified and quantified by comparing the absorbance and retention times to that of known standards. Data were analyzed with Beckman System Gold software.

6. RT-PCR analysis of *C. trachomatis* transcript expression

Total RNA was isolated from *C. trachomatis*-infected HeLa cells cultured in MEM supplemented with 10% FCS using the Trizol method (Invitrogen). HeLa cell monolayers were infected with *C. trachomatis* serovars at an MOI of 3-5 IFU $cell^{-1}$ in MEM-10 dialyzed FCS or MEM-10 FCS supplemented with 5 ng mL^{-1} IFN-γ for serovar L2 and E and 2 ng mL^{-1} IFN-γ for serovar A. Where indicated, the medium was further supplemented with indole or tryptophan at the specified concentration. HeLa cells were also infected with serovar L2 in MEM-10 dialyzed FCS lacking tyrosine or phenylalanine. In some cases, HeLa cells were treated with IFN-γ, but not infected with *C. trachomatis*. Following incubation for 24-36 h at 37°C, the culture medium was removed and the monolayer was rinsed with HBSS. Monolayers of HeLa 229 cells in T75 flasks were infected with *C. psittaci* strain GPIC at an MOI of 3-5 IFU $cell^{-1}$ in MEM-10 (+Trp), MEM-10 dialyzed FCS lacking tryptophan (-Trp) or lacking tryptophan but supplemented with 50 μM indole, kynurenine or anthranilate. HeLa cell monolayers were also pretreated for 24 h with 5 ng ml^{-1} IFN-γ and then infected with *C. psittaci* strain GPIC in MEM-10 supplemented with 5 ng ml^{-1} IFN-γ. The infected cells were incubated for 16 hours at 37°C. Total RNA was prepared from the cells using Trizol reagent according to the manufacturer's instructions (Invitrogen). One μg of total RNA was treated with DNase I (Invitrogen), reverse transcribed using random hexamer primers and Thermoscript reverse transcriptase (Invitrogen), and then treated with RNase H (Invitrogen). The nucleotide sequences of the primers used for conventional and quantitative PCR are listed in Tables 1 and 2. For conventional PCR, 1 μl of cDNA was

amplified in a 50 µl reaction mixture containing 0.2 µM primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1xTaq reaction buffer and 5 U Taq DNA polymerase (Invitrogen). The cycling program was 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C and 90 s at 72°C, with a final extension of 10 min at 72°C. Products were separated on a 1.5% TBE-agarose gel and visualized by ethidium bromide staining.

For quantitative PCR, amplifications were carried out according to manufacturer's instructions using the LightCycler and SYBR Green I as the fluorophore (Roche). Briefly, 2 µl cDNA was amplified in a 20 µl reaction mixture containing 0.5 µM primers, 5 mM $MgCl_2$, and 2 µl FastStart DNA Master SYBR Green I mix. The cycling program was: 95°C for 10 min followed by 40 cycles of 5 s at 95°C, 10 s at 60°C and 15 s at 72°C. Melting curves determined for all products indicated that primer dimers were not amplified in any of the reactions. Transcript levels were quantified using the LightCycler Data Analysis software from standard curves generated by amplification of known quantities of plasmid DNA containing the amplicon of interest. Results were normalized against the copy number of 16S rRNA transcripts in each cDNA preparation and are presented as transcript copy number per µg of total RNA.

7. Generation of plasmid standards for quantitative RT-PCR

Recombinant plasmids containing PCR products for each gene assayed were generated by amplifying 50 ng of *C. trachomatis* DNA template with each primer pair as described above. PCR products were purified from each reaction mixture using the Concert Rapid PCR Purification System (Invitrogen). The PCR products were then cloned into pCR2.1-

TOPO using the TOPO TA cloning kit from Invitrogen. Plasmids were purified using the Rapid Plasmid Purification kit (Qiagen) and quantitated by spectrophotometry. Serial dilutions of each purified plasmid were prepared and used as standards for quantitating chlamydial gene transcripts.

8. Western blot analyses

HeLa cells were preincubated in MEM-10 containing 5 ng mL^{-1} IFN-γ for 24 hr prior to infection for all experiments employing serovar L2. HeLa cell monolayers in T150 flasks (two per treatment group) were infected with *C. trachomatis* serovar L2 EBs at an MOI of 3-5 IFU $cell^{-1}$ and cultured in MEM-10, MEM-10 supplemented with 5 ng mL^{-1} IFN-γ or in tryptophan- free MEM-10 dialyzed FCS, with the addition of the specified concentrations of indole or tryptophan. Serovar L2-infected HeLa cells were also cultured in tyrosine- or phenylalanine-free MEM-10 supplemented with dialyzed FCS. HeLa cell monolayers were similarly infected with *C. trachomatis* serovar A, except that 2 ng mL^{-1} IFN-γ was used in the post-infection medium. For experiments with serovar A, HeLa cells were not pretreated with IFN-γ. Following incubation at 37°C, the cells were harvested by gentle scraping and were sonicated briefly. The sonicated cells were then centrifuged at 500 x g for 10 minutes to pellet host cell debris. The supernatant was then ultracentrifuged for 1 h at 16,000 x g on a 30% MD-76 cushion (Mallinckrodt). The pellet of semipurified EBs, RBs and aberrant forms was collected, mixed with an equal volume of 2 x Laemmli electrophoresis buffer, and heated at 95°C for 10 min. The proteins were resolved on a 12% acrylamide gel and electrophoretically transferred to a nitrocellulose membrane. TrpA and TrpB were detected using polyclonal antibodies

raised against the corresponding recombinant proteins, followed by enhanced chemiluminescence (Amersham). As positive controls, purified recombinant TrpA and TrpB were included on each gel. Duplicate blots were probed with monoclonal antibody to the chlamydial Hsp60 to approximate equal loading of all samples.

9. Sequencing of MOMP and tryptophan synthase from clinical samples

A 100 µl aliquot of each clinical sample or culture isolate was added to lysis buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.022% gelatin, 0.5% Nonidet P-40, 0.5% Tween-20) and digested with proteinase K at a final concentration of 100 µg/ml at 55°C for 2 h. The samples were then boiled for 10 min to inactivate the proteinase K. The primers used for PCR and sequencing are listed in Table 3. To determine the serovar of each clinical isolate, nested PCR was performed using primers designed to amplify the *ompA* gene. 5 µl DNA (approximately 50 ng) from each sample was amplified in a 100 µL reaction mixture containing 0.5 µM primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 x Taq reaction buffer and 1.25 U Taq DNA polymerase (Invitrogen). The first round of PCR used primers CT1 and CT5 to generate an 1142 bp product. The cycling program was 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 1.5 min at 72°C, with a final extension of 10 min at 72°C. For the second round of the nested PCR, 1 µl of the first round PCR product was amplified as described above using primers VD1 and VD4 to generate an 879 bp product. The cycling program for the second round of PCR was 5 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, with a final extension of 10 min at 72°C. The nested PCR product was purified using the QIAprep spin miniprep kit (Qiagen) and sequenced using primers VD1 and VD4.

For determination of the *trpA* and *trpB* gene sequences, PCR was performed on samples using the primers listed in Table 3. DNA (50 ng) was used in PCR reactions using primers that amplified the entire *trpRBA* operon (Trp.1 and Trp.2 for *trpRBA*). For sequencing of *trpB*, nested PCR was performed using primers 5'TrpB-L and 3'TrpB-L for the first round of PCR followed by 5'TrpB-M and 3'TrpB-M for the second round of PCR. The nested PCR product was sequenced using primer 5'TrpB-seq. For sequencing of the mutational "hotspot" around nucleotide 531 of *trpA*, nested PCR was performed using primers 5'TrpA-L and 3'TrpA-L for the first round of PCR followed by 5'TrpA-M and 3'TrpA-M for the second round of PCR. The nested PCR product was purified as described above and sequenced using primer 5'TrpA-seq. A second nested PCR was performed to sequence the region around nucleotide 118 of *trpA* using primers 5'TrpA-L#2 and 3'TrpA-L#2 for the first round of PCR followed by 5'TrpA-M#2 and 3'TrpA-M#2 for the second round of PCR. The cycling program was 5 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, with a final extension of 10 min at 72°C. The nested PCR product was purified and sequenced using primer 5'TrpA-#2-seq. All PCR products were sequenced by the DNA Core Facility (Health Canada, Winnipeg, Manitoba), using an ABI PRISM 377 DNA Sequencer.

10. Electron microscopy

HeLa cells were infected with *C. trachomatis* serovar L2 in MEM-10 dialyzed FCS lacking tryptophan but supplemented with 0.05 μg mL^{-1} indole, 50 μg mL^{-1} 5-fluoroindole, 0.5 μg mL^{-1} tryptophan or 10 μg mL^{-1} tryptophan. At 24-48 h post-

infection, infected HeLa cells were fixed and processed for electron microscopy as previously described (Hackstadt *et al.*, 1999).

11. *E. coli* culture media

LB broth, LB agar and SOC broth were prepared according to Sambrook et al, 1989.

12.* Expression cloning of *trp* genes, *kynU* and *prsA

C. psittaci strain GPIC, *C. trachomatis* serovar L2, and *E. coli trp* genes were amplified by PCR from purified chromosomal DNA as described previously (65). The PCR primer sequences are listed in Table 4, 5 and 6 and were designed to include unique restriction sites for cloning. For construction of plasmids to co-express *trpB* and *trpA* from *E. coli*, *C. trachomatis* serovar L2 and GPIC, the 5′-*trpB* and 3′-*trpA* primers were used in the PCR reaction. The PCR products were gel-purified, restricted with *Kpn*I and *Pst*I (for GPIC), *Bam*HI and *Kpn*I (for *E. coli*), and *Kpn*I and *Sal*I (for serovar L2) and ligated to expression vector pQE80-L (Qiagen) cut with the corresponding restriction enzymes. For construction of plasmids expressing *trpD-A, trpD-kynU, kynU* or *prsA*, the PCR products were restricted with *KpnI* and *SalI* and ligated to pQE80-L as described above. Constructs were transformed into DH5α for screening, purified by miniprep, and then used to transform *E. coli* strains for complementation assays.

13. Complementation assays

The cells from stationary phase cultures of *E. coli* trp transformants were harvested by centrifugation and washed three times with sterile phosphate-buffered saline. The cell

suspensions were then streaked onto minimal agar (1 x M9 salts, 0.2% glucose, 0.2% casamino acids, 2 mM $MgSO_4$, 0.2 mM L-serine, 100 μg/ml ampicillin, and 50 μg/ml each thiamine, cysteine, and uracil) containing 100 μM indole, 50 μg/ml L-tryptophan, 4 μg/ml anthraniliate, 4 μg/ml kynurenine, 4 μg ml^{-1} chorismate, or without additional supplements. The plates were incubated for 48 h at 37°C and then photographed.

RESULTS

A. Regulation of tryptophan synthase gene expression in *C. trachomatis*

1. Effect of IFN-γ on HeLa cells

It was previously shown that growth of all human *C. trachomatis* serovars is inhibited in tryptophan-deficient medium (65). During the course of an *in vivo* human infection, *C. trachomatis* is likely to encounter tryptophan limitation due to the effects of host-produced IFN-γ (13, 19, 39, 44). Since our aim was to examine *trp* gene regulation under physiologically relevant conditions of tryptophan limitation, initial experiments were carried out to confirm the effects of IFN-γ on IDO expression and tryptophan levels in HeLa cells, as well its effects on *C. trachomatis* growth. HeLa cells were used for all cell culture experiments as this is the prototypical cell line used to culture chlamydiae. Polarized epithelial cells have also been used to establish a persistent infection model with results analogous to those obtained with nonpolarized HeLa cells. RT-PCR analyses were carried out to confirm that IDO was induced under the experimental conditions. Total RNA was prepared from HeLa cells treated for 24 h with IFN-γ, as well as from control cells without exposure to IFN-γ. Following reverse transcription, the cDNA was amplified with primers specific for IDO and for a housekeeping gene (rig/S15) encoding a small ribosomal subunit (72). PCR products of the expected size were obtained from both IFN-γ-treated and -untreated HeLa cells using the rig/S15 primers (Fig. 7a). In contrast, IDO products were only detectable in HeLa cells treated with IFN-γ and not in the control cells.

The tryptophan pool in HeLa cells treated with IFN-γ was measured over time to determine the kinetics of tryptophan depletion by IDO. As shown in Fig. 7b, treatment of uninfected HeLa cells with IFN-γ for at least 10 h was required in order to detect a significant decrease in intracellular tryptophan levels. Treatment of HeLa cells for 24 h resulted in a nearly complete degradation of intracellular tryptophan compared to untreated cells. Similarly, HeLa cells, whether pretreated with IFN-γ or left untreated, then infected with *C. trachomatis* serovar L2 and incubated with IFN-γ for 24 h were almost completely depleted of intracellular tryptophan (Table 7). As shown in Fig. 7c, the growth of serovar L2 in HeLa cells is decreased by approximately 50% when IFN-γ is added at the time of infection (T=0). However, a much more significant decrease in the growth of serovar L2 (approximately 3 logs) is observed if HeLa cells are pretreated with IFN-γ for 24 h prior to infection (T=-24). The growth of ocular serovar A, in contrast, is largely inhibited even if IFN-γ is added at the time of infection.

2. Regulation of *trp* gene expression in reference *C. trachomatis* serovars

To determine if tryptophan can regulate the expression of the *trp* genes real-time quantitative RT-PCR analyses were carried out. Two different culture conditions were used to attain tryptophan limitation. IFN-γ (5 ng ml^{-1}) pretreated HeLa cells were infected with *C. trachomatis* serovar L2 and incubated in the presence of IFN-γ or serovar L2-infected HeLa cells were cultured in tryptophan-free MEM-10. Total RNA was prepared 24 h post-infection and used for RT-PCR. As shown in Fig. 8a, *C. trachomatis* serovar L2 grown under tryptophan-replete conditions (+trp) contained very few copies of

trpBA mRNA. In contrast, those grown under tryptophan-deficient conditions (IFN-γ and –trp) expressed significantly higher levels of *trpBA* transcripts. Addition of excess tryptophan (1 mg ml^{-1}) to IFN-γ treated cultures (IFN-γ +trp) resulted in the repression of *trpBA* expression. In *E. coli*, *trpR* expression is auto-regulated (69, 70). This was also the case for *C. trachomatis* serovar L2(Fig. 8b). Thus, expression of *trpR* mirrored that of *trpBA*, with induction of expression under tryptophan-deficient conditions (IFN-γ and –trp) and repression in the presence of excess trp (IFN-γ +trp). The differences observed in *trpBA* mRNA levels were reflected in the protein expression levels of both subunits of tryptophan synthase (Fig. 8c). Thus, *C. trachomatis* serovar L2 propagated in HeLa cells cultured in tryptophan replete MEM-10 expressed a basal level of TrpB and TrpA, detectable in Western blots by specific antisera (Fig. 8c, lane 1). Growth of serovar L2 in IFN-γ (5 ng ml^{-1}) pretreated Hela cells (lane 2) resulted in an increase in both TrpB and TrpA. Similar expression patterns for both *trpR* and *trpBA* were observed for *C. trachomatis* genital serovar I (Fig. 9).

C. trachomatis ocular serovar A encodes a *trpR* homologue as well as a full length *trpB*, but unlike serovar L2, has a mutated *trpA* (65, 73). These mutations result in the production of a non-functional tryptophan synthase (65). To determine whether expression of the *trp* genes was also regulated by tryptophan availability in serovar A, similar transcriptional analyses were carried out. Transcripts for *trpR* and *trpBA* were undetectable in the control cells grown in tryptophan-replete media (MEM-10). However, when serovar A was grown under tryptophan-deficient conditions (IFN-γ or -trp), significantly higher levels of the *trpBA* and *trpR* transcripts were observed (Fig. 10a and b). As expected, when serovar A was grown in the presence of IFN-γ and excess

tryptophan (IFN-γ + trp), expression of the *trpBA* and *trpR* transcripts was repressed. The Western blots for serovar A showed the same pattern of expression for the TrpA and TrpB proteins as the RT-PCR results for the *trpBA* transcripts (Fig. 10c). Thus, when serovar A was grown in tryptophan-replete media, TrpA and TrpB could not be detected by Western blot (lane 1). However, when serovar A was grown in the presence of IFN-γ (lane 2), the expression of TrpA and TrpB was induced. Upon addition of excess tryptophan to IFN-γ treated cultures expression was reduced (lane 4).

Transcript levels for a number of other genes, which are part of the TrpR regulon in *E. coli* (74, 75), including homologues of phosphoribosylanthranilate isomerase, encoded by *trpF*, the chorismate biosynthesis genes *aroH* and *aroL,* the *Mtr* tryptophan transporters, *tyrP.1* and *tyrP.2* were examined under tryptophan limiting conditions in serovar L2-infected HeLa cells. mRNA levels were not affected for any of these genes by tryptophan limitation (Figs. 11, 12 and 13). Thus, in *C. trachomatis*, TrpR appears to only regulate its own expression and that of the genes encoding the tryptophan synthase subunits (*trpBA*).

To determine whether induction of *trp* gene expression was specific to tryptophan limitation, I examined the expression of the *trp* genes in *C. trachomatis* serovar L2 cultured in the absence of two other aromatic amino acids, tyrosine and phenylalanine. The growth of serovar L2 in the absence of tyrosine or phenylalanine is severely decreased, analogous to growth in the absence of tryptophan, but can be rescued by supplementation of the media with the missing amino acid (Fig. 14c). As shown in Fig. 14b, *trpBA* was not induced when serovar L2 was grown in the absence of either of these two amino acids. Similarly, *trpR* was not induced in the absence of tyrosine or

phenylalanine (Fig. 15). I also examined the expression of TrpB and TrpA, by Western blot analysis, in serovar L2 grown in the absence of tyrosine or phenylalanine. Consistent with the RT-PCR results, I found that TrpB and TrpA were not induced in the absence of either of these two amino acids (lanes 3 and 4, Fig. 14b). In contrast, induction of TrpB and TrpA was observed when serovar L2 was grown in the absence of tryptophan (lane 2).

3. Regulation of chlamydial growth and *trp* gene expression by indole

It was previously shown that in the absence of tryptophan growth of genital, but not ocular, serovars of *C. trachomatis* can be rescued from tryptophan starvation by supplementation of the culture medium with the tryptophan precursor indole (65). An induction of *trpBA* transcript and protein was observed with serovar L2- (Fig. 8) and A- (Fig. 10) infected HeLa cells cultured in the presence of IFN-γ. For serovar L2, which encodes a functional tryptophan synthase, supplementation of the IFN-γ containing medium with indole (Fig. 8, lane 3) resulted in repression of both TrpB and TrpA. In keeping with the fact that the serovar A tryptophan synthase is inactive, addition of indole to IFN-γ containing medium does not result in repression of *trpBA* expression. (Fig. 10).

To determine if there is a threshold concentration of indole required to meet the tryptophan biosynthesis needs of serovar L2, infected HeLa cells were cultured in various concentrations of indole. Culture in the presence of 1 μM indole or less resulted in a dramatic reduction in recoverable IFUs (Fig. 16a). Furthermore, consistent with a tryptophan starved phenotype, this condition was accompanied by the development of aberrant inclusions in the HeLa cells, as shown by electron microscopy (Fig. 17a),

induction of *trpBA* and *trpR* transcripts (Fig. 18a and b) and TrpA and B protein levels (Fig. 18c, lane 3). Culture of serovar L2-infected HeLa cells with 1 or 10 μg/ml indole resulted in optimal EB production, similar to levels seen in the presence of non-limiting amounts of tryptophan (Fig. 16), and normal inclusions as assessed by electron microscopy. Furthermore, under these conditions, *trpBA* and *trpR* expression was repressed and TrpA and TrpB protein levels were decreased (Fig. 18c, lane 4), returning to levels similar to that observed in tryptophan-replete medium (Fig. 18c, lane 1). Together, these data suggest that *trp* gene induction aids in *C. trachomatis* serovar L2 survival when indole is available for conversion to tryptophan.

4. Sequence analysis of *trp* genes from *C. trachomatis* clinical isolates

Sequence analysis of the *trpRBA* operon genes of the 14 *C. trachomatis* reference serovars showed that these serovars possessed a mutational hot spot at nucleotide position 531 of the *trpA* gene; the exception was serovar B, in which the entire t*rpRBA* operon was deleted from the chromosome (65). It was imperative to extend the finding obtained with *C. trachomatis* reference serovars to more recently isolated naturally occurring strains because of the potential importance these mutations might have in the pathogenesis of infections and as novel epidemiological markers. Therefore, the trpRBA region was sequenced from a total of 94 trachoma isolates and 214 genital isolates obtained from distinct geographical areas within Africa, China, Canada and the USA. A summary of the clinical isolates by disease, *ompA*, and *trpA* genotype is shown in Tables 8 and 9 and schematically in Figure 19. The results showed that there was an absolute

correlation between ocular and genital isolates and the presence of specific mutations in the *trpA* genes. Remarkably, every ocular isolate displayed mutations that resulted in an interrupted *trpA* or *trpB* open reading frame or a complete loss of both genes due to large deletions (Table 8 and Figure 19). As striking, no such mutations were found in *trpA* for any of the genital isolates (Table 9 and Figure 19). Six different types of inactivating mutations were identified among the ocular isolates.

The vast majority (65 of 66) of the Gambian isolates had the same mutation (single-nucleotide deletion at position 531) preciously described for trachoma reference serovar A, Ba, and C. The bulk of the Tanzanian isolates, and a single Gambian isolate, were found to have a 2-bp (TG) insertion at nucleotide 118 in *trpA*. Two Tanzanian isolates had a 22-bp deletion (nucleotides 11-32) in *trpB*, and one had a single-base deletion (nucleotide 470) in *trpA*. Finally, one Tanzanian isolate that *ompA* typed as serovar B and one isolate from China, *ompA* type serovar C, had the entire *trp* region deleted, much like the mutation in reference serovar B (65, 73), although the limits of the deleted regions were different. Of particular interest, we observed a three-base deletion (nucleotides 408-410) in *trpA* for all ocular isolates that had *trp* genes, a finding previously reported for reference ocular strains (65). This deletion was not present in the *trpA* of any genital strain and occurred irrespective of the geographical location or *ompA* genotype of the ocular isolate. Thus, the *trpA* genotypic marker might prove useful for epidemiological typing of ocular and genital strains.

Conversely, the *trpA* genes of all genital serovars encoded intact open reading frames giving rise to a functional tryptophan synthase, regardless of *ompA* genotype or

the geographic location of the isolate (Table 9 and Figure 19). Furthermore, each isolate typed as one of the three distinct *trpA* genotypes that were previously found with the reference serovars based on changes surrounding the mutational hot spot at nucleotide 531 (65). These changes resulted in a region of polymorphism in TrpA which are referred to as Group 1 (YQ), Group 2 (CQ), and Group 3 (YE); the Group 3 genotype was found exclusively in LGV isolates. In contrast, Group 1 and 2 polymorphisms occurred independently of *ompA* (D-K) genotype, and there was a predominance of Group 1 over Group 2 sequences (136 out of 170) among genital isolates.

One particular ocular clinical isolate from Tanzania was chosen for further analysis. This ocular isolate, referred to as 2497, is a serovar A isolate, as determined by typing of its *ompA* gene, encoding MOMP. As shown in Fig. 20, this clinical A isolate displayed similar growth kinetics as compared to the reference serovar A seed stock as determined by measurement of recoverable IFUs at various times during the developmental cycle. However, the clinical isolate displayed an increased resistance to the growth inhibitory effects of IFN-γ compared to the seed stock serovar A (Fig. 21), although the growth of both strains was completely inhibited at 5 ng mL^{-1} IFN-γ.

5. trpRBA gene expression in clinical ocular and genital *C. trachomatis* isolates

To determine if the *trp* genes were expressed and regulated by tryptophan availability in the clinical isolates during a HeLa cell infection, representative ocular (A-2497) and genital (E-IU823) serovars were chosen for more in-depth analysis. For real-

time quantitative RT-PCR analysis, total RNA was isolated from midcycle (36-hour)-infected HeLa cell cultures grown in the presence of complete DMEM-10, complete DMEM-10 plus IFN-γ, complete DMEM-10 plus IFN-γ and indole, and complete DMEM-10 plus IFN-γ and excess tryptophan. As shown in Figure 22, *trpR* and *trpBA* expression is detectable in HeLa cells infected with either the ocular or the genital isolate. In general, the level of expression is lower in cells infected with the ocular isolate, a result that could be caused by Rho-dependent termination due to the premature stop codon in *trpA* of this isolate. In both the ocular and the genital isolates, *trpR* and *trpBA* expression are dramatically upregulated by the presence of IFN-γ, suggesting that there is induction of expression under tryptophan-limiting conditions. Addition of excess tryptophan to IFN-γ-treated cultures repressed the expression of *trpR* and *trpBA* in both serovars. In contrast, indole reversed the IFN-γ-induced increase in *trp* gene expression in the genital but not the ocular isolate. This results suggests that, unlike the genital isolate, the ocular isolate is incapable of converting indole into tryptophan.

Polyclonal antibodies to TrpA and TrpB were used for Western blot analysis to assess whether the encoded messenger RNA was translated into protein products (Figure 23). For the genital serovar E strain, protein bands of the same electrophoretic mobility as purified recombinant TrpA and TrpB were detected in semipurified chlamydiae prepared from IFN-γ-treated cultures, which corresponds well with the increased level of transcripts detected under these culture conditions. The amounts of immunoreactive TrpA and TrpB are substantially reduced when indole is added and are below the level of detection in control cultures or cultures treated with excess tryptophan. Different results

were observed for the ocular serovar A isolate in that no TrpA protein band was detected under any of the conditions tested. This is not surprising since the two-base insertion mutation in *trpA* in this isolate occurs at bases 118-119. This frame shift mutation gives rise to a new stop codon (nucleotides 136-138) with a resulting truncated protein product of 4.7 kDa, a size not detectable under the experimental conditions. An anti-TrpB immunoreactive band, of the correct mobility, was detected in IFN-γ-treated cultures and IFN-γ-treated cultures plus indole but not in control or IFN-γ-treated cultures plus excess tryptophan. These results correlate well with the RT-PCR data and suggest that, even in the ocular serovars in which tryptophan synthase is inactive, *trp* gene expression is regulated by tryptophan availability.

6. Effect of various culture conditions on the yield of infectious chlamydial EBs

To determine if indole could rescue the clinical serovar A and E isolates from the effects of IFN-γ *in vivo*, the level of recoverable IFUs was calculated from infected HeLa cell cultures treated with IFN-γ. As shown in Figure 24, both serovars were sensitive to IFN-γ, and the inhibition was reversed by the addition of excess exogenous tryptophan. Addition of indole to the culture medium reversed the growth inhibitory effect of IFN-γ on the genital serovar E but not the ocular serovar A. These findings are in keeping with the expression data and suggest that the genital, but not the ocular, isolate produces a functional tryptophan synthase.

7. Characterization of *C. trachomatis* genital serovar B

An interesting group of clinical isolates among the samples tested were the genital samples *ompA* sequenced that typed as serovar B. Although serovar B is most commonly associated with ocular trachoma (7), it has also been isolated from patients with urogenital disease (8-10). Since these genital Bs represent an unusual example of a crossover serovar, they were characterized in more detail. The *trpA* and *trpB* genes from these clinical isolates were sequenced. In contrast to what was found with the ocular B serovars, all of the genital B isolates showed intact tryptophan synthase genes similar to those found with all the classical genital serovars (D-K). As with the other genital isolates, both the Group 1 and 2 genotypes were found in the *trpA* hot-spot region.

To determine if these isolates expressed TrpA and TrpB and whether their expression was regulated, Western blot analyses were carried out on semipurified chlamydiae particles prepared from IFN-γ-treated and untreated infected HeLa cells. The results shown in Figure 25 indicate that immunoreactive material with the same electrophoretic mobility as full-length recombinant TrpA and TrpB was detected. The expression of both TrpA and TrpB was upregulated by IFN-γ, and the induction was repressed by the addition of excess tryptophan or indole. Finally, just as had been seen with the genital serovar E, genital serovar B growth, as assessed by quantitation of recoverable IFUs, was inhibited by IFN-γ, and the inhibition was reversed by the addition of exogenous tryptophan or indole (Figure 25).

8. Effect of host competition for tryptophan on *trp* gene expression

It was previously demonstrated that host cell competition for limiting amino acids restricts the growth of *C. psittaci* (76) and *C. trachomatis* (77) in mouse cells, resulting in the development of persistently-infected, aberrant organism-containing cells. The effect of host competition for tryptophan on the growth of serovar L2 and the expression of the *trp* genes was examined by culturing serovar L2-infected HeLa cells in the presence of various concentrations of tryptophan and in tryptophan-deficient medium with or without cycloheximide. As shown in Fig. 16a, the growth of serovar L2 is significantly reduced when cultured in medium containing less than 5 mg mL^{-1} tryptophan. Furthermore, growth under tryptophan-limiting conditions results in the development of aberrant organisms (Figure 17), an increase in *trpBA* transcript (Fig. 18a) and TrpB and TrpA proteins (Fig. 18c, lane 2). Culture of serovar L2-infected HeLa cells in tryptophan-deficient medium supplemented with cycloheximide, an inhibitor of host cell protein synthesis, resulted in a greater than 3 $\log_{10}$ rescue in growth (Fig 16b), normal inclusion morphology (data not shown) and reduced expression of *trpBA* (Fig. 16c). The size of the intracellular tryptophan pool was also measured under several of these conditions (Table 7). As expected, the size of the tryptophan pool in cells cultured in tryptophan-replete (10 $\mu g\ mL^{-1}$) medium is dramatically larger than the pool size in cells cultured in tryptophan-deficient media. Interestingly, despite the fact that there is a substantial difference in serovar L2 growth and *trpBA* expression, the size of the tryptophan pool is similar in cells cultured in medium containing a low concentration of tryptophan (1 $\mu g\ mL^{-1}$) and

tryptophan-deficient medium supplemented with cycloheximide. In total, these data indicate that it is not necessarily the absolute size of the host cell tryptophan pool that determines whether serovar L2 can grow, but whether this tryptophan pool is available to chlamydiae in the absence of host competition.

9. Effect of 5-fluoroindole and 5-fluorotryptophan on *trp* gene expression

To further characterize the regulation of *trp* gene expression, the effect of 5-fluoroindole and 5-fluorotryptophan on *trp* gene expression and growth of serovar L2 was examined. 5-fluoroindole is a prodrug which is converted by tryptophan synthase into 5-fluorotryptophan (78). This toxic tryptophan analog is incorporated into cellular proteins and, depending on the protein, can result in loss of function and subsequent inhibition of cell growth (79-81). HeLa cells were infected with serovar L2 and cultured in tryptophan-free medium supplemented with 100 μM 5-fluoroindole, 10 μg/ml 5-fluorotryptophan, or 10 μM indole and 100 μM 5-fluoroindole. I found that expression of *trpBA* was repressed in the presence of either of these two compounds, suggesting that 5-fluoroindole is converted, by tryptophan synthase, into 5-fluorotryptophan, which is then capable of binding to TrpR and repressing transcription of the *trp* operon (Fig. 26a). To examine the effect of 5-fluoroindole on the growth of serovar L2, HeLa cells were infected in the presence of tryptophan and increasing concentrations of 5-fluoroindole. Only a small decrease in the growth of serovar L2 was observed in the presence of tryptophan and increasing concentrations of 5-fluoroindole (Fig. 26b). We reasoned that the lack of inhibition could result from the fact that 5-fluoroindole requires tryptophan

synthase to be converted into 5-fluorotryptophan and that tryptophan synthase expression is repressed when tryptophan is available. Therefore, the growth of serovar L2 was examined in the presence of indole and increasing concentrations of 5-fluoroindole. Under these conditions, a concentration dependent decrease in the growth of serovar L2 was observed (Fig. 26b). This indicates that 5-fluoroindole is converted by tryptophan synthase into 5-fluorotryptophan, which is then incorporated into chlamydial proteins, with subsequent inhibition of growth. Consistent with this, culture of serovar L2 in the presence of 5-fluoroindole resulted in the development of aberrant inclusions in the HeLa cells, as shown by electron microscopy (Fig. 17b). In addition, expression of *trpBA* was repressed in the presence of 10 μM indole and 100 μM 5-fluoroindole (Fig. 26a), indicating that 5-fluoroindole is converted to 5-fluorotryptophan, which can then repress *trp* gene expression and inhibit the growth of serovar L2. As shown in Table 6, tryptophan or 5-fluorotryptophan could not be detected in chlamydia-infected HeLa cells cultured in the presence of 5-fluoroindole or indole. This is likely due to the fact that the chlamydial intracellular pools of these tryptophan metabolites are below the limit of detection of the HPLC assay. In contrast, an intracellular pool of 5-fluorotryptophan was readily detected when HeLa cells were cultured in the presence of the analogue.

The ability of indole and tryptophan to reverse the inhibition of growth of serovar L2 by 5-fluorotryptophan was also examined. As shown in Fig. 27, the growth of serovar L2 is reduced by approximately 3 $\log_{10}$ in the presence of 5-fluorotryptophan compared to growth in the presence of tryptophan or indole. 5-fluorotryptophan-induced L2 growth inhibition could not be reversed by indole, likely because 5-fluorotryptophan repressed

trp gene expression, thus preventing the conversion of indole into tryptophan. 5-fluorotryptophan-induced growth inhibition was, however, reversed by addition of tryptophan in a dose dependent manner.

B. Tryptophan recycling is responsible for the interferon-γ resistance of *Chlamydia psittaci* GPIC in indoleamine-2,3-dioxygenase-expressing host cells

1. Organization and expression of the tryptophan biosynthesis genes of *C. psittaci* GPIC

For simplicity, I will refer to the *trpDCFBA*, *kynU* and *prsA* genes as the tryptophan biosynthesis genes of GPIC (Fig. 28a and b). Characteristics of the proteins encoded by the tryptophan biosynthesis genes present in the genome of GPIC are shown in Table 10. Of particular interest is the TrpA homologue because it was previously shown that the *C. trachomatis* TrpA protein is incapable of converting IGP to indole (65). The GPIC TrpA protein contains 257 amino acids, giving a calculated molecular mass of 28.2 kDa. A comparison of the TrpA protein sequences of *E. coli*, *C. trachomatis* serovar L2 and *C. psittaci* GPIC (Fig. 29) indicates that amino acids essential for subunit-to-subunit interactions (Gly^{51}, Pro^{53}, Asp^{56}, Pro^{57}, Pro^{62}, Tyr^{102}, Asn^{104}, Val^{126}, Pro^{132}) and for catalytic activity (Glu^{49} and Asp^{60}) (82-84) are conserved between the *E. coli* and chlamydial TrpA proteins. However, amino acids which form the active site pocket and/or have been identified by mutagenesis as essential for TrpA activity in *E. coli* (Ser^{178}, Gly^{181}, Thr^{183}, Gly^{184}, Gly^{211}, Gly^{213}, Gly^{234}, Ser^{235}) (66-68) (85-89) are conserved in the GPIC TrpA but not in the TrpA of serovar L2 (65). Based on this alignment, we

predicted that the TrpA of GPIC, unlike serovar L2 (65), should be capable of utilizing IGP as a substrate for the biosynthesis of indole.

The organizational structure of the tryptophan biosynthesis genes suggests that they may be encoded in an operon. To determine if *C. psittaci* strain GPIC expresses the tryptophan biosynthesis genes encoded by its genome, and to determine which genes are coexpressed, RT-PCR was performed with primers designed to overlap the junction between each pair of genes starting at *trpR* and ending at *prsA* (Fig. 28b). As shown in Fig. 28c, PCR products for all of the overlapping primer sets were detected when GPIC was cultured in medium lacking tryptophan (-Trp medium). Thus, we found that all of the genes involved in tryptophan biosynthesis encoded by GPIC are co-transcribed starting at *trpR* and ending at *prsA*. As a comparison, the organization of the tryptophan biosynthesis genes in *C. trachomatis* serovar L2 was examined to determine if *trpR* is coexpressed along with the *trpBA* operon by designing primers to overlap *trpR* and *trpB*. In contrast to GPIC, *trpR* is not coexpressed with *trpBA*, as indicated by the inability to detect a PCR product with primers specific for the *trpR-trpB* junction (Fig. 28d).

2. Regulation of *trp* gene expression

To determine whether expression of the tryptophan biosynthesis operon is regulated by tryptophan levels, real-time quantitative RT-PCR analyses were performed. Two different culture conditions were used to attain tryptophan limitation. HeLa cells pretreated with 5 ng mL^{-1} IFN-γ for 24 h were infected with *C. psittaci* strain GPIC and incubated in the presence of IFN-γ or GPIC-infected HeLa cells were cultured in tryptophan-free MEM-10. Total RNA was prepared 16 h post-infection and used for RT-

PCR. As shown in Fig. 30a-e , relatively low levels of transcripts for *trpAB, prsA-kynU, trpD, trpC, and trpF* were detected when GPIC was grown in tryptophan-replete medium (+Trp). In contrast, when GPIC was grown in tryptophan-deficient medium (–Trp), significantly higher levels of transcripts for the tryptophan biosynthesis genes were detected, indicating induction of the *trp* operon. However, when the potential tryptophan precursors, indole, anthranilate or kynurenine, were present in the media in the absence of tryptophan, *trp* gene expression was repressed, with the transcript levels comparable to the levels observed in tryptophan-replete medium. This suggests that GPIC is capable of using these three substrates for the biosynthesis of tryptophan, which then negatively regulates the expression of the tryptophan biosynthesis operon via TrpR. In contrast to *C. trachomatis* serovar L2 where *trpBA* expression is upregulated (90), we found that expression of the GPIC tryptophan biosynthesis genes was not induced in the presence of IFN-γ.

3. Genetic Complementation Studies

To determine whether the tryptophan biosynthesis genes encoded by *C. psittaci* strain GPIC are functional, a heterologous complementation system was utilized. The *trp* genes from *C. psittaci* strain GPIC and *C. trachomatis* serovar L2 were cloned into an *E. coli* expression vector and transformed into *E. coli* mutants lacking various components of the tryptophan biosynthesis pathway (Table 11). The ability of the *E. coli* mutants expressing the chlamydial *trp* genes to grow on minimal medium was then assessed (Fig. 31a-c). The *E. coli* mutant KS463 expresses a non-functional TrpA but expresses active

TrpB. As expected, all KS463 transformants were able to grow on minimal medium supplemented with tryptophan (Fig. 31a). KS463 cells transformed with either the expression vector alone or constructs expressing serovar L2, GPIC, or *E. coli* TrpA were also capable of growth on minimal medium supplemented with indole. These data are consistent with published observations indicating that *E. coli* TrpB can utilize indole in the presence or absence of functional TrpA (65, 91). However, in the absence of indole, KS463 cells transformed with either the expression vector alone or constructs expressing serovar L2 or GPIC TrpA were unable to grow on M9 medium. These results suggested that the serovar L2 and GPIC TrpA proteins could not efficiently utilize the indole glycerol-3 phosphate (IGP) produced by KS463, either due to a loss of catalytic activity for this substrate or due to an inability of the chlamydial TrpA to interact with the *E. coli* TrpB.

To distinguish between these two possibilities, the *E. coli trpB* transposon mutant BW7622, which does not express either *trpB* or *trpA*, was transformed with constructs co-expressing the serovar L2 or GPIC *trpA* and *trpB*. The BW7622 transformants expressing TrpA and TrpB from either *E. coli* or GPIC were capable of growth on M9 medium alone, indicating that tryptophan synthase from GPIC is capable of utilizing IGP produced by BW7622 as a substrate for tryptophan biosynthesis (Fig 31a). This is in contrast to the tryptophan synthase of *C. trachomatis* serovar L2 which is unable to utilize IGP as a substrate as previously reported (65). When the BW7622 transformants were grown on minimal medium supplemented with indole, the constructs expressing TrpA and TrpB from serovar L2, GPIC and *E. coli* all exhibited growth. This indicates that the

TrpA and TrpB proteins of GPIC are indeed functional enzymes capable of utilizing IGP or indole as a substrate for the biosynthesis of tryptophan.

In contrast to the *E. coli* TrpB which can function alone, the *C. trachomatis* TrpB requires the presence of full-length TrpA in order to utilize indole as a substrate for the biosynthesis of tryptophan (65). To determine whether the GPIC TrpB also requires full-length TrpA for activity, *E. coli* deletion mutant CY15077, which contains a deletion of the entire *trp* operon from *trpE* to *trpA*, was transformed with constructs expressing either *trpB* alone or *trpA* and *trpB*. None of the CY15077 transformants expressing TrpB were capable of growth on M9 medium alone as expected based on the absence of a functional TrpA, although the growth of all transformants was rescued by the presence of tryptophan in the media (Fig. 31a). However, CY15077 transformed with the *E. coli* or GPIC *trpB* construct was capable of growth on M9 medium supplemented with indole. In contrast, CY15077 transformed with the *trpB* construct from serovar L2 failed to grow on indole. These results indicate that the TrpB of GPIC, like *E. coli*, does not require TrpA for activity (91, 92). When *E. coli* CY15077 Δ*trpE-A* was transformed with a construct expressing both *trpA* and *trpB*, none of the transformants were capable of growth on M9 medium alone, as the genes required for conversion of chorismate to IGP (*trpE/G*, *trpD*, *trpF* and *trpC*) are missing in this strain (Fig. 31a). However, the growth of CY15077 transformed with constructs expressing TrpA and TrpB from *E. coli*, serovar L2 or GPIC was rescued by supplementation of the M9 medium with indole, as expected.

To examine the function of the *trpD*, *trpF* and *trpC* genes of GPIC, *E. coli* CY15077 Δ*trpE-A* was transformed with a construct expressing *trpD-A* from GPIC and growth in the presence of anthranilate was examined. TrpD, TrpF and TrpC are required

for the conversion of anthranilate to IGP, which is then converted by TrpBA into tryptophan (Fig. 31a). The transformant expressing *trpD-trpA* was capable of growth on M9 medium supplemented with anthranilate (Fig. 17b), indicating that the protein products of these genes can indeed use anthranilate as a substrate for tryptophan biosynthesis and that GPIC encodes homologues of TrpD, TrpF and TrpC that are functional.

To examine the function of the PRPP synthase of GPIC, which catalyzes the formation of PRPP, a key intermediate in tryptophan biosynthesis (Fig. 28a), the *E. coli* transposon mutant H0965, which does not express PRPP synthase (93), was transformed with a construct expressing *prsA* from GPIC. The *prsA* gene product is required for growth of the transformant on LB agar. In the absence of this gene, *E.* coli strain H0965 cannot grow on LB agar unless it has been supplemented with NAD (93). As expected, the transformant expressing the PRPP synthase from GPIC was capable of growth on LB agar in the absence of NAD, while the vector control was only capable of growth on LB agar supplemented with NAD (Fig. 31b). This indicates that the *prsA* gene product of GPIC is functional.

The function of the kynureninase of GPIC, encoded by *kynU*, was also examined. The *E. coli* strain T3D contains a missense mutation in *trpE* resulting in a nonfunctional TrpE gene product. Therefore, this strain cannot grow on M9 media containing chorismate, but requires an alternative source of anthranilate for growth. The T3D strain was transformed with a construct expressing the *kynU* gene of GPIC and growth in the presence of anthranilate and kynurenine was examined (Fig. 31c). As expected, both the transformant and vector control were able to grow on M9 medium supplemented with

tryptophan or anthranilate. However, only the *kynU* transformant was capable of growth on kynurenine, indicating that the GPIC *kynU* gene product is indeed functional and capable of converting kynurenine to anthranilate which is subsequently converted to tryptophan.

Finally, to examine the function of the tryptophan biosynthesis operon of GPIC, *E. coli* CY15077 Δ*trpE-A* was transformed with a construct expressing *trpD*, *trpC*, *trpF*, *trpB*, *trpA* and *kynU* from GPIC and growth in the presence of anthranilate and kynurenine was examined (Fig. 17c). The *kynU* gene product is required for growth on kynurenine but not anthranilate. As expected, the transformant expressing these six genes was able to grow on M9 medium supplemented with tryptophan or anthranilate, while the vector control was only capable of growth on M9 medium supplemented with tryptophan. The transformant expressing *trpD-kynU*, but not the vector control, was also capable of growth on M9 medium supplemented with kynurenine, indicating that the *kynU* gene product can convert kynurenine to anthranilate, which is then converted by the protein products encoded by *trpD-trpA* into tryptophan.

4. Growth of *C. psittaci* strain GPIC using alternative substrates for tryptophan biosynthesis

It is clear from the genetic complementation studies that *E. coli* transformants expressing the GPIC tryptophan biosynthesis genes can utilize kynurenine as a substrate for growth. To determine whether the tryptophan biosynthesis operon of *C. psittaci* strain GPIC is functional *in vivo*, HeLa cells were infected in tryptophan-deficient media or tryptophan-deficient media supplemented with indole, anthranilate, or kynurenine. A

large decrease in growth, as determined by a decrease in recoverable IFUs of approximately 4 $\log_{10}$, was observed when GPIC was grown in tryptophan-deficient media compared to control cells infected in tryptophan-replete media (Fig. 32). The growth of GPIC in the absence of tryptophan was rescued by supplementation of the media with indole, anthranilate or kynurenine, indicating that GPIC can utilize these substrates for tryptophan biosynthesis. The growth of *C. trachomatis* serovar L2 was also significantly decreased when cultured in tryptophan-deficient medium. However, in contrast to GPIC, the growth of serovar L2 was only rescued by the addition of indole to the media, indicating that serovar L2 cannot utilize anthranilate or kynurenine as precursors for tryptophan biosynthesis.

HeLa cell infections were also carried out in the presence of IFN-γ to deplete the cells of intracellular tryptophan, and chlamydial growth was assessed after supplementation of the media with the potential tryptophan precursors. As shown in Fig. 33, no decrease in the growth of GPIC was observed in the presence of IFN-γ, as reported previously (48). This likely reflects the ability of GPIC to scavenge tryptophan degradation products, such as kynurenine and anthranilate, from the host cell as substrates for growth. As a control, *C. trachomatis* serovar L2 was grown under the same set of conditions as GPIC. The large decrease in recoverable IFUs of over 4 $\log_{10}$ observed when serovar L2 was grown in the presence of IFN-γ did not change after supplementation of the media with anthranilate or kynurenine. However, the growth of serovar L2 was rescued by the addition of tryptophan or indole to the media, as expected.

DISCUSSION

A. Regulation of tryptophan synthase gene expression in *C. trachomatis*

Transcriptional repression and attenuation are common means of regulating gene expression in prokaryotes (94). Such mechanisms allow the bacteria to respond rapidly to changes in environmental conditions and make possible the conservation of energy when metabolites are in abundant supply. To date, only three putative transcriptional regulators have been characterized in chlamydiae – Fur, a repressor that inhibits the transcription of genes involved in iron uptake in response to an increased availability of iron (95); HrcA, a transcriptional repressor of heat shock gene expression (96); and TrpR. It has been speculated that the relatively small number of transcriptional regulators identified in the chlamydial genome may be related to the somewhat stable environment in which this intracellular parasite exists compared to extracellular pathogens, such as *E. coli* (95). However, bacterial pathogens, such as chlamydiae, must still be able to sense and adapt to minute changes in their environment, such as changes in nutrient availability, by coordinately altering the expression of essential genes. In the present work, I have observed transcriptional regulation of the expression of *trpR, trpB* and *trpA* genes of *C. trachomatis* in response to changes in tryptophan availability. Thus, transcription was repressed when infected cells were cultured in tryptophan-replete medium or in the presence of indole, as long as the encoded tryptophan synthase was functional. When tryptophan or indole concentrations were limiting, *trpR* and *trpBA* expression was induced. IFN-γ treatment of HeLa cells resulted in the induction of IDO expression and essentially complete degradation of intracellular tryptophan by 24 h post-treatment. Under

these conditions of IFN-γ-induced tryptophan starvation, expression of the *trpBA* and *trpR* genes was induced and the changes in the transcripts levels were reflected in the amount of the corresponding TrpB and TrpA protein. Addition of super-physiological concentrations of tryptophan or indole repressed *trp* gene expression. These data indicate that transcriptional regulation is a significant determining factor in the level of tryptophan synthase subunit expression in *C. trachomatis.*

The fact that human genital serovars of *C. trachomatis* have retained a functional tryptophan synthase, the expression of which responds to tryptophan limitation, suggests that these organisms are likely to encounter fluctuations in tryptophan levels *in vivo.* These findings therefore support the view that, as demonstrated *in vitro*, the *in vivo* inhibitory effect of IFN-γ on chlamydiae is via IDO-induced tryptophan degradation (19, 43-45).

The depletion of tryptophan in HeLa cells by the action of IDO is not immediate - a significant decrease in tryptophan levels was not detected until 10 h after treatment with IFN-γ. The difference in sensitivity of serovar L2 and serovar A to IFN-γ is likely related to differences in the developmental cycle of these serovars. The more rapid growth of serovar L2 compared to serovar A may allow it to establish a successful infection before tryptophan levels become limiting in HeLa cells treated with IFN-γ. These results could explain why significant inhibition of serovar L2 growth, but not serovar A growth, requires pretreatment of HeLa cells with IFN-γ prior to infection. Furthermore, it has been demonstrated that *C. trachomatis* serovar L2 secretes a protease which degrades host cell proteins (97). Specific protein degradation was first detected 17 h post-infection, when chlamydial protein synthesis approached its maximum (97). As a result

of the action of this protease, a pool of amino acids would become available to chlamydiae. Together, these observations provide a reasonable explanation for the effects of IFN-γ on chlamydia-infected HeLa cells. For rapidly growing serovars like L2, IFN-γ addition at the time of infection results in only a small decrease in recoverable IFUs because the infection can be established before host pools of tryptophan become limiting, as a result of IDO activity. Once established, the chlamydial-specific protease is secreted and the resultant degradation of cellular proteins "feeds" the chlamydiae with needed tryptophan, so growth and development continue essentially unabated. In contrast, with IFN-γ pretreatment the host cell, the tryptophan pool is depleted prior to chlamydial infection. As a result, even for rapid growing serovars like L2, chlamydial development is restricted and the protease, which appears later in development (97), is likely not synthesized and/or secreted; therefore, no tryptophan is made available and growth is drastically reduced.

Like *C. trachomatis* serovar L2, serovar A induces expression of *trpBA* and *trpR* under conditions of tryptophan limitation, such as in the presence of IFN-γ. However, the *trpA* gene of serovar A contains a frameshift mutation resulting in a truncated product. The truncated TrpA cannot activate TrpB and therefore serovar A cannot utilize indole as a substrate for the synthesis of tryptophan (65). In keeping with this, the RT-PCR and Western blot analyses indicate that indole is incapable of down-regulating *trpBA* expression in serovar A. In contrast to indole, tryptophan down-regulated *trp* gene expression. That expression of the *trp* genes in serovar A was regulated by tryptophan was somewhat unexpected, given the lack of a functional gene product from *trpA*. *C. trachomatis* has a streamlined genome and has lost biosynthetic capabilities for many

precursors of metabolism (63, 98). Given this propensity for genetic reduction, it is curious that a regulatory system for the production of a non-functional enzyme has been maintained. In the absence of function, there is undoubtedly selective pressure for the loss of the *trp* genes from the genome. In support of this, serovar B, another ocular serovar, contains a deletion encompassing the entire *trp* region (65, 73). In addition, total deletions of the *trp* genes have been observed in several ocular serovar clinical isolates (99).

My results demonstrate that host competition for available tryptophan plays a key role in influencing *trp* gene expression and growth of chlamydiae. In the presence of low levels of tryptophan, chlamydiae cannot successfully compete with the host cell for this essential amino acid unless cycloheximide, an inhibitor of eukaryotic protein synthesis, is present. Under tryptophan-limiting conditions, the growth of serovar L2 was significantly decreased and *trp* gene expression was induced. However, upon addition of cycloheximide to tryptophan-deficient medium, the growth of serovar L2 was rescued and expression of the *trp* operon was repressed, despite the fact that only a small intracellular tryptophan pool was available from the host cell. This suggests that the limited pool of tryptophan available is sufficient to support the growth of chlamydiae. The amount of intracellular tryptophan in HeLa cells cultured under tryptophan-limiting conditions (1 μg ml^{-1}) in the absence of cycloheximide is not significantly different from the amount available in HeLa cells cultured in tryptophan-deficient medium in the presence of cycloheximide (295 vs. 228 pmoles/10^7 cells). This indicates that it is not necessarily the absolute size of the tryptophan pool that determines whether expression of the *trp* genes

is repressed and whether serovar L2 can grow, but whether there is competition for the tryptophan pool from the host cell.

In other Gram negative bacteria such as *E. coli*, expression of the tryptophan biosynthetic genes is regulated by both transcriptional repression (mediated by TrpR-tryptophan complexes) and by transcriptional attenuation (dependent upon the availability of charged $tRNA^{Trp}$) (69, 70, 100). The presence of a tryptophan repressor homologue whose expression is also regulated by tryptophan suggests that repression is utilized in the regulation of the *C. trachomatis trp* genes. Identification of putative repressor binding sites upstream of *trpB* and *trpR* will further characterize the role of transcriptional repression in the regulation of *trp* gene expression in chlamydiae. To provide further evidence of regulation of the *C. trachomatis trp* operon by TrpR, we have used primer extension studies to map the repressor binding sites, which are indeed located upstream of the *trpBA* operon and upstream of *trpR*. However, the results presented here do not rule out a role for transcriptional attenuation as an additional regulatory mechanism. This question could be addressed by examination of *trp* gene expression in a *trpR* mutant. In this regard, the results presented here demonstrate that 5-fluoroindole is recognized by the chlamydial tryptophan synthase and converted into 5-fluorotryptophan, a compound which is inhibitory to the growth of chlamydiae. Therefore, 5-fluorotryptophan and/or 5-flouroindole will be useful tools for selecting mutants of chlamydiae with mutations in genes involved in tryptophan metabolism. Such mutants will allow for further characterization of *trp* gene regulation in chlamydiae. The availability of reagents for mutant selection is invaluable since, at the present time, no system for gene transformation exists for chlamydiae.

In *E. coli*, TrpR not only represses the tryptophan biosynthetic genes, but also other genes involved in aromatic amino acid biosynthesis, including *aroG* and *aroH*, as well as a tryptophan transporter encoded by *mtr*. The data presented in this thesis indicate that the complement of genes regulated by tryptophan levels in *C. trachomatis* appears to be limited to *trpBA* and *trpR*. It is not surprising that *aroG* and *aroH* expression is not regulated in chlamydiae, since they lack homologues of *trpE, G, D,* and *C* (63). In the absence of these genes, there is no biosynthetic link between chorismate and tryptophan. It is surprising, however, that the expression of the chlamydial Mtr homologues, *tyrP.1* and *tyrP.2,* (58, 59, 63), was not affected by tryptophan availability. It appears that the primary response of *C. trachomatis* serovar L2 to tryptophan limitation is the upregulation of biosynthetic capability rather than increased tryptophan transport, and is a significant difference between *C. trachomatis* and *E. coli*. Notably, expression of the *trpF* gene, which has been annotated in the *C. trachomatis* genome sequencing projects as a phosphoribosyl anthranilate isomerase homologue (59, 63), was not affected by tryptophan levels. In *C. trachomatis*, the *trpF* homologue is not encoded in an operon with the tryptophan synthase genes. Furthermore, *C. trachomatis* lacks the gene encoding the enzyme (TrpD) to provide the substrate for *trpF*, phosophribosyl anthranilate, as well as the gene encoding the enzyme (TrpC) to utilize the product of the TrpF reaction. Taken together, these observations strongly suggest that *trpF* encodes an isomerase of a different specificity which does not play a role in *trp* biosynthesis in *C. trachomatis.* This is further supported by the fact that *C. trachomatis* MoPn has a *trpF* homologue but lacks *trpRBA* (59). In addition, a sequence comparison of TrpF from *E. coli* with the corresponding chlamydial TrpF sequences indicates that critical residues required for

catalytic activity have been mutated to nonconserved amino acids in the *C. trachomatis* TrpF homologue (55). This strongly suggests that TrpF does not function as a phosphoribosyl anthranilae isomerase in *C. trachomatis*.

In addition to the induction of *trp* gene expression, tryptophan limitation results in the generation of aberrant organisms in HeLa cells (39, 43). The induction of *trp* gene expression was the direct result of tryptophan depletion and not an indirect consequence of the aberrant phenotype. This conclusion is supported by the observation that similar aberrant organisms could be induced by starving the cells for two other aromatic amino acids, tyrosine and phenylalanine, with no effect on *trp* gene expression. Furthermore, 5-fluorotryptophan induced the formation of aberrant organisms and at the same time repressed *trp* gene expression. The development of aberrant chlamydial forms can also be induced with antibiotics (43, 101, 102), and glucose deprivation (77); however, no induction of *trp* gene expression was observed under either of these conditions.

As an obligate intracellular bacterium, *C. trachomatis* develops in a specialized and relatively homeostatic environment with respect to temperature, pH, and osmolarity. In addition, there is a relative abundance of substrates of metabolism as well as preformed mediators, which decrease the requirements for biosynthesis by this intracellular bacterium. However, infection with *C. trachomatis* is known to alter the composition of the intracellular environment; in particular, tryptophan is depleted as a result of IFN-γ-induced IDO production. Results from previous work demonstrated that serovars of *C. trachomatis* infecting the genital tract have maintained the ability to synthesize tryptophan (65). In this thesis, I have furthered this work by demonstrations that *C. trachomatis* can regulate expression of the tryptophan biosynthesis genes in response to

changes in tryptophan availability. The ability to synthesize tryptophan and to regulate the expression of the necessary enzymes clearly has implications for the survival of *C. trachomatis*, and likely contributes to the ability of this pathogen to persist in the presence of a rigorous host immune response. The fact that chlamydiae have retained the capacity to respond to tryptophan limitation suggests that these organisms likely encounter fluctuations in tryptophan levels *in vivo*, supporting the view that IFN-γ is critical in the host defense against *C. trachomatis*. Finally, it may be possible to use the *trp* genes for the construction of an inducible expression plasmid, which may be useful as a vector for developing a gene transformation system for chlamydiae.

B. Sequence Analysis of *trp* Genes from *C. trachomatis* Clinical Isolates

Comparative DNA sequencing of the PZ of all 15 human *C. trachomatis* reference strains showed that 14 of 15 serovars encode homologues of *trpB* and *trpA* (63, 65). The chlamydial TrpB proteins retained conserved residues identified as essential for enzyme activity in other microorganisms (66, 67). In contrast, the chlamydial TrpA protein was shown to have several changes in its primary structure that differed considerably from its *trpA* orthologue in *E. coli*. Specifically, the *trpA* of ocular but not genital serovars had a common consensus triplet nucleotide deletion (nucleotides 408-410) that resulted in the loss of Phe-136 from the polypeptide. Furthermore, a polymorphic mutational "hot spot" was identified in the *trpA* of ocular but not genital serovars. In ocular serovars, this region contained a single-nucleotide deletion that resulted in a frame-shift mutation and expression of a truncated nonfunctional TrpA polypeptide. Interestingly, the *trpA* of all

genital serovars contained two point mutations at this same site giving rise to codon changes resulting in amino acid substitutions that differed among the serovars: YE in LGV serovars; CQ in D, E, and K serovars; and YQ in G, F, H, I, and J serovars (Figure 7). These amino acids are found in loop 6 of the *Salmonella* TrpA, a region identified in the tryptophan synthase crystal structure as being important for subunit-subunit interactions between TrpB and TrpA, metabolite channeling, and substrate binding (66, 67). Consistent with this conclusion was the finding by genetic complementation studies that the TrpBA of genital serovars was not capable of using IGP but could use exogeneous indole for the synthesis of tryptophan (65).

The findings obtained with *C. trachomatis* reference serovars were extended to include more recently isolated naturally occurring strains because of the potential importance these mutations might have in the pathogenesis of infection and as novel epidemiological markers. These original findings were made with reference serovars that had been passaged many times *in vitro*; consequently, recent clinical specimens that represented diverse epidemiological and geographical populations were analyzed analogous to the reference serovars. The trpRBA region was sequenced from a total of 94 trachoma isolates and 214 genital isolates obtained from distinct geographical areas within Africa, China, Canada, and the U.S.A. As with the reference serovars, there was an absolute correlation between ocular and genital isolates and the presence of specific mutations in the *trpA* genes. Every ocular isolate displayed mutations that result in an interrupted *trpA* or *trpB* open reading frame or a complete loss of both genes due to large deletions. Six different types of inactivating mutations were identified among the ocular isolates (Table 8). Sequencing of the *trpA* region from ocular clinical isolates uncovered

four new inactivating mutations in addition to the complete *trp* region deletion and *trpA* frame-shift mutation (nucleotide 531) originally described in the ocular reference strains (65, 73). Interestingly, the chlamydial isolate(s) infecting a particular geographic region appear to be clonal with regard to the *trp* locus. For example, 65 out of 66 isolates from the Gambia, regardless of *ompA* serotype, had a frame-shift mutation at base 531 of *trpA*, the same mutation originally described with reference serovars A, Ba, and C (65, 73). Similarly, all isolates from Tanzania had the same inactivating mutation, a two-base insertion at nucleotide 118 of *trpA*. These results suggest that the chlamydial strains infecting these populations are clonal and that the *trp* inactivating mutation predates *ompA* variation. The clonality of these populations may reflect the limited opportunities for interchange of the strains that occur between the remote trachoma-endemic villages where the samples were collected. Determination of the *trp* genotype may prove useful for molecular epidemiological studies aimed at identifying chlamydial reservoirs and evaluating transmission patterns.

Interestingly, the clinical ocular serovar A isolate displayed an increased resistance to IFN-γ compared to the reference serovar A, even though both strains displayed similar growth kinetics as determined by the measurement of recoverable IFUs as various times during the developmental cycle. As mentioned earlier, the difference in the sensitivity of laboratory reference strains of serovar L2 and serovar A to IFN-γ is likely related to well-documented differences in the developmental cycle of these serovars. However, this cannot account for the increased resistance of the clinical 2497 strain compared to the reference serovar A, as both strains display the 72 hour developmental cycle expected. Complete genomic sequencing of the clinical 2497 strain

and the reference serovar A will shed light on potential differences between these two strains. In addition, a comparison of changes in global gene expression patterns between these two strains using a chlamydial genome microarray may provide added insight into the differences between these two serovar A strains in terms of their differences in IFN-γ sensitivity.

In contrast to the ocular serovars, no mutations were observed in the *trpA* of any of the genital isolates (Table 9). Furthermore, each isolate typed as one of three distinct *trpA* genotypes that were previously characterized with the reference serovars based on changes surrounding the mutational hot spot at nucleotide 531. These changes resulted in a region of polymorphism in TrpA which were referred to as Group 1 (YQ), Group 2 (CQ), and Group 3 (YE); the Group 3 genotype was found exclusively in LGV isolates. In contrast, Group 1 and 2 polymorphisms occurred independently of *ompA* (D-K) genotype, with a predominance of Group 1 over Group 2 sequences among genital isolates. The fact that no other amino acid changes were found suggests that the spectrum of mutations allowed may be limited, presumably by the need for a functional enzyme. Given that these amino acids lie in TrpA loop 6, it is quite possible that the mutations have an effect on enzyme catalytic activity or efficiency. These findings suggest that genital strains have evolved to utilize indole for the synthesis of tryptophan as an immunoavoidance strategy, thereby explaining the retention of the *trpR*, *trpB* and *trpA* genes. Clearly, there is strong selective pressure for genital but not ocular serovars to retain the ability to synthesize tryptophan. Because *C. trachomatis* lacks the other genes of the tryptophan operon needed for de *novo* indole biosynthesis (*trpE*, *G*, *D*, *C*), and because under normal physiological conditions indole is not present in mammalian cells,

genital strains probably acquire indole from their environment. The most likely source of exogenous indole is the other microorganisms known to colonize the female genital tract. While the predominant organism present in the normal vagina, lactobacillus, does not produce indole, many other organisms present in the vaginal microflora, including *Peptostreptococcus* species, *Fusobacterium* species, *Bacteroides* species, and aerobic gram-negative rods such as *E. coli*, do produce indole (103-105). Interestingly, many of these microbes, especially the anaerobes, are present in much higher concentrations in women with bacterial vaginosis, whereas lactobacilli are reduced or absent in these women (106-109). It is known that coinfection with the organisms that cause bacterial vaginosis and chlamydiae is common. Moreover, these coinfections significantly increase the risk of complications such as pelvic inflammatory disease (110-112). Thus, it is possible that genital strains evolved the capability to synthesize tryptophan from indole produced by other vaginal microbes and that this host-microbe interplay is essential in the ability of these strains to avoid IFN-γ-mediated host defense mechanisms and establish persistent infection, particularly in the female host. The studies presented here indicate that even low concentrations of indole (0.1-1.0 μM) allow recovery of EBs in the presence of IFN-γ. This highly efficient escape mechanism from the inhibitory effect of IFN-γ could result in the development of infectious EBs that would maintain the pathogen's ability to persist in the presence of an active immune response and to be transmitted from person to person.

Perhaps the strongest evidence of the paradigm established with laboratory reference serovars – namely, that ocular serovars possess a dysfunctional tryptophan synthase, whereas genital serovars possess a functional tryptophan synthase – is the

finding that the rare genital isolates of serovar B, a serovariant usually associated with ocular disease (11, 113), had intact *trp* genes that encoded a functional TrpBA complex, a characteristic associated exclusively with genital serovars. From an epidemiological perspective, this makes the *trp* locus useful for differentiating ocular and genital infections with serovar B isolates.

While the genital serovars have maintained a functional tryptophan synthase, the trachoma serovariants have evolved an equally powerful negative selection to mutate the synthase. Like chlamydial genital infection, chlamydial ocular infections also persist, and persistence has been suggested as a mechanism that leads to the late damaging inflammatory and scarring stages of disease (11, 114, 115). Genital serovars can infect the conjunctivae of infants during their passage through an infected birth canal. However, these infections produce a self-limiting neonatal conjunctivitis that does not progress to more chronic eye disease characteristic of trachoma (11, 113). This suggests that genital strains are highly susceptible to the inhibitory effects of IFN-γ in the environment of the eye, perhaps because the eye represent a more sterile habitat lacking other microflora that could provide a source of exogenous indole. In the late stages of trachoma, there are reports that coinfection might contribute to disease transmission (11).

As mentioned, many organisms present in the vaginal microflora produce indole (103-105). However, in contrast to the flora of the genital tract, the ocular microflora are more limited in diversity (114, 115), and of those associated with trachoma, only *H. influenza* is an established producer of indole (116). The conjunctival epithelium is an immunocompetent tissue in which infection induces IFN-γ, and analysis of both systemic and local cytokine profiles among individuals differing in trachoma disease status

implicate an IL-12-driven Th1-type of immunity in resistance to the progression of chronic scarring inflammatory disease (38, 117). However, there may be alternate sites of infection in the eye other than the conjunctival epithelium, and the trachoma serovars may have adapted a strategy of interacting with these different cellular environments that allows for both productive and persistent infection. If the secondary infection targets exist in immunoprivileged areas of the eye that are sites in which chlamydiae establish persistent growth, it would be an advantage for these serovars to have a dysfunctional (non-indole rescuable) synthase. Clearly, there would be little need to maintain a functional synthase if (a) there were not environmental sources of exogenous indole (in the conjunctivae) and (b) secondary infection sites were devoid of lymphocytes and IFN-γ, as is the situation in immunoprivileged tissues of the eye (118, 119). In fact, being "rescuable" in a persistent immunocompromised environment could be a disadvantaged, since it would compromise the ability of the pathogen to sustain persistent infection. Indirect support for the possibility that the ocular serovars might reside in an immunoprivileged environment is the presence of a limited number of serovariants (A, B, Ba and C) associated with trachoma, as compared with the numerous (D, E, F, G, H, I, J, K, L1, L2, L3) genital serovariants. This suggests that the ocular serovars have been exposed to less immune selection than the genital serovars.

C. Tryptophan recycling in *C. psittaci* GPIC

In the absence of a genetic transfer system for chlamydiae, comparative genomic sequencing has provided unique insights into potential virulence genes in these

organisms, as well as genes involved in host, organ and cellular tropism (61, 63). Currently, six chlamydial genomes have been sequenced and published allowing the identification of core genes conserved among all genomes (59, 61, 63). Of 1009 annotated genes in the *C. psittaci* GPIC genome, 798 were conserved in *C. pneumoniae*, *C. trachomatis* serovar D, and *C. trachomatis* MoPn (61). These core genes most likely represent those involved in the developmental cycle and intracellular survival of all *Chlamydiaceae*. The GPIC genome contains 68 genes that lack orthologs in any other completed chlamydial genomes. These biotype-specific genes presumably encode functions necessary for survival and virulence of GPIC in the guinea pig (61). A comparison of the chlamydial genomes sequenced to date indicates that the gene order and content are remarkably conserved with the exception of one region of the genome which has been termed the plasticity zone or replication termination region. A number of biotype-specific genes are encoded within the PZ of GPIC and include, in addition to an almost complete tryptophan biosynthesis operon, genes involved in nucleotide scavenging (*guaBA-add* cluster) and a toxin (*tox*) with homology to the large clostridial cytotoxins. Three orthologs of *tox* are clustered within the PZ of MoPn (59), while truncated ORFs matching portions of the N- and C-terminal regions of *tox* are found in *C. trachomatis* serovar D (63). It has been demonstrated that *C. trachomatis* strains that contain genes with homology to the large clostridial cytotoxins produced a cytopathic effect on HeLa cells that is virtually indistinguishable from the cytopathology caused by the toxins themselves (64). The PZ therefore appears to encode a number of virulence determinants, which undoubtedly account for much of the phenotypic variation between chlamydial species and serovars.

Unlike other chlamydiae, *C. psittaci* strain GPIC encodes all of the genes required for *de novo* tryptophan biosynthesis, except for *trpE/G*. The data presented here indicate that the tryptophan biosynthesis genes encoded by GPIC are expressed when this organism is cultured under tryptophan limiting conditions, and that these genes are co-transcribed as a single transcript starting at *trpR* and ending at *prsA*. To show definitively that the eight genes are co-transcribed, we are currently mapping the transcriptional start site of the operon. In contrast, the *trpR* of *C. trachomatis* serovar L2 is not coexpressed with *trpBA*, a notable difference between these two chlamydial species. Since the open reading frames (ORFs) of almost all of the genes involved in tryptophan biosynthesis overlap in GPIC (the exceptions are *trpR* and *trpD*, and *trpA* and *kynU*), it is not surprising that these genes together constitute a single operon. To date, the *C. psittaci* GPIC genome is the only bacterial genome that has this collection of genes clustered together in a single operon. Not only are the *kynU* and *prsA* genes not components of the classical tryptophan biosynthesis operon, but the gene for kynureninase is present in only a few prokaryotes (55). The genetic complementation studies reported here indicate that *trpD*, *trpC*, *trpF*, *trpB*, *trpA*, *prsA* and *kynU* of GPIC are functional. This cluster of genes provides a means for GPIC to evade tryptophan limitation resulting from IDO expression.

In cultured human epithelial cells, IFN-γ induces the expression of IDO, the activity of which results in the degradation of intracellular tryptophan to kynurenine (46, 47). The genital serovars of *C. trachomatis* have maintained functional tryptophan synthase (*trpBA*) genes, as well as the ability to regulate the expression of these genes via TrpR, but lack the additional tryptophan biosynthesis genes encoded by GPIC (90, 99). It

has been demonstrated that indole can rescue the growth of genital serovars exposed to IFN-γ. However, since indole is not produced by mammalian cells and because *C. trachomatis* does not encode the enzymes required for *de novo* indole biosynthesis, it has been speculated that microflora of the female genital tract may provide a source of indole which could rescue the growth of genital serovars in the presence of IFN-γ (99). Consequently, indole produced by genital microflora would provide selective pressure on the genital serovars to maintain functional tryptophan synthase genes.

In contrast to human epithelial cells, IDO-induced tryptophan degradation is not the mechanism of IFN-γ-inhibition of chlamydial growth in mouse cells (52). Interestingly, the MoPn strain of *C. trachomatis* does not encode any tryptophan biosynthesis genes within the PZ, suggesting that the absence of tryptophan degradation in mouse cells exposed to IFN-γ has effectively eliminated any selective pressure on MoPn to maintain these genes. It has been demonstrated that antibody is not required for resolution of genital MoPn infection in the mouse (31, 120). Rather, $CD4^+$ T cells capable of secreting IFN-γ are the primary mediators of immunity in the mouse infection model (27, 30). In the murine system, IFN-γ induces an enzyme, inducible nitric oxide synthase (iNOS), which catalyzes the production of various anti-microbial reactive nitrogen intermediates, most notably nitric oxide (NO). However, studies in iNOS knockout mice indicate that NO generation is not essential for clearance of primary chlamydial genital infection (27, 53, 54). Thus, the exact mechanism by which IFN-γ inhibits chlamydial growth in mouse cells remains to be elucidated.

In contrast to the mouse model for genital tract infection, numerous studies have demonstrated that antibody plays an important role in the resolution of GPIC infection in

the guinea pig (121-124). The *in vitro* data presented here are consistent with these observations in that they suggest that GPIC encodes the genes required to circumvent cell mediated IFN-γ-induced tryptophan degradation. The data presented here indicate that in GPIC-infected IDO-expressing cells, kynurenine serves as the main substrate for the biosynthesis of tryptophan. In fact, sufficient kynurenine is recycled to tryptophan to repress *trp* gene expression. These results provide a reasonable explanation for the complete resistance of GPIC to the growth inhibitory effects of IFN-γ in IDO-expressing cells. At the present time, it is unknown if guinea pig cells express IDO in response to IFN-γ treatment and clearly, the effect of IFN-γ on the growth of GPIC in guinea pig cells needs to be examined. However, reagents required for such *in vivo* or *in vitro* studies, including knock-out guinea pigs and recombinant guinea pig IFN-γ, are not commercially available.

A comparison of the TrpA protein sequences from *C. trachomatis* serovar L2, *C. psittaci* strain GPIC, and *E. coli* indicates that amino acids essential for subunit-to-subunit interactions (Gly-51, Pro-53, Asp-56, Pro-57, Pro-62, Tyr-102, Asn-104, Val-126, Pro-132) and for catalytic activity (Glu-49 and Asp-60) are conserved between the *E. coli* and chlamydial TrpA proteins. However, amino acids that form the active site pocket and/or have been identified by mutagenesis as essential for TrpA activity in *E. coli* (Ser-178, Gly-181, Thr-183, Gly-184, Gly-211, Gly-213, Gly-234, Ser-235) (66, 67, 85-89, 125) are conserved in the GPIC TrpA but not in the TrpA of serovar L2 (65). Specifically, amino acids in the active site pocket identified as key residues involved in the binding of IGP in the *Salmonella* TrpBA crystal structure (66, 85, 89) are conserved in the GPIC TrpA but not in the TrpA of serovar L2. Based on this alignment, it is not

surprising that the GPIC TrpA was capable of utilizing IGP as a substrate for the biosynthesis of indole, like the *E. coli* TrpA. In contrast, the TrpA of *C. trachomatis* serovar L2 was incapable of using IGP as a substrate for the biosynthesis of indole (65). The genetic complementation studies also demonstrated that the GPIC TrpB, like the *E. coli* TrpB, does not require the presence of TrpA in order to utilize indole as a substrate for the biosynthesis of tryptophan. This is in contrast to the serovar L2 TrpB, which requires the presence of full-length TrpA for activity (65). While the final goal is to synthesize tryptophan, it is obvious that each organism has compiled a complement of genes needed to utilize the metabolic precursors available from the environment.

Consistent with the presence of a tryptophan repressor in the *C. psittaci* strain GPIC genome, I observed regulation of the expression of the *trp* operon of *C. psittaci* strain GPIC in response to changes in tryptophan availability. When *C. psittaci* strain GPIC was cultured in the absence of tryptophan, expression of the *trp* genes was induced. However, the addition of indole, anthranilate or kynurenine to the medium repressed *trp* gene expression, indicating that sufficient tryptophan was synthesized by GPIC in the presence of these substrates to bind to the aporepressor, TrpR, and repress expression of the *trp* operon. In the presence of IFN-γ, *trp* gene expression was also repressed, again suggesting that sufficient tryptophan was produced by the sequential activity of the tryptophan biosynthesis genes, *kynU* and *prsA* to bind to TrpR and repress the *trp* operon. In contrast, expression of *trpR*, *trpB* and *trpA* transcripts remains induced in *C. trachomatis* serovar L2-infected HeLa cells treated with IFN-γ (90).

Like all of the genes involved in tryptophan biosynthesis, mRNA for the phosphoribosyl anthranilate isomerase orthologue, encoded by *trpF*, was induced by

tryptophan limitation, indicating that expression of this gene is regulated by TrpR in GPIC. This is in contrast to the *trpF* gene of *C. trachomatis* which is not induced upon tryptophan limitation. Because the *trpF* gene of *C. trachomatis* is not encoded within the *trpBA* operon in the PZ and is not induced by tryptophan limitation, it is likely that *trpF* encodes an isomerase of a different specificity which does not play a role in tryptophan biosynthesis in *C. trachomatis.* In contrast, the *trpF* gene is encoded within the tryptophan biosynthesis operon in GPIC (61). Thus, *C. psittaci* strain GPIC has retained *trpF* as part of the tryptophan biosynthesis pathway.

The data presented in this thesis have demonstrated that the complete resistance of GPIC to the growth-inhibitory effects of IFN-γ *in vitro* is the result of the ability of this chlamydial species to recycle kynurenine from the host cell for use as a substrate for tryptophan biosynthesis. Thus, GPIC is capable of circumventing the growth-inhibitory effects of IFN-γ in IDO-expressing epithelial cells by intercepting an early intermediate of tryptophan catabolism and recycling it back to tryptophan. The fact that GPIC has retained the genes required for the biosynthesis of tryptophan from kynurenine suggests that this organism is indeed exposed to tryptophan limitation via the action of IDO in the guinea pig, and that these genes are required for survival of GPIC in its natural host. The ability of GPIC to synthesize tryptophan within an IFN-γ-rich environment would therefore be an important virulence determinant and a parasitic strategy for evading host defense.

In total, the results presented here indicate that the ability to regulate *trp* gene expression in response to tryptophan availability is advantageous for the intracellular survival of chlamydiae. As the such, the tryptophan biosynthesis genes represent niche-

specific virulence determinants. In contrast to the ocular serovars, the genital serovars of *C. trachomatis* have retained a functional tryptophan synthase, allowing them to utilize indole provided by the microflora of the female genital tract as a precursor for growth. *C. psittaci* strain GPIC has retained an almost complete tryptophan biosynthesis operon (and has recruited two additional genes, *prsA* and *kynU*, not found in the *trp* operon of any other organism sequenced to date), allowing this species to recycle tryptophan from its IFN-γ-induced degradation products. In contrast, *C. trachomatis* MoPn has lost all *trp* genes within the plasticity zone, a finding in keeping with the fact the mouse cells do not induce IDO in response to exposure to IFN-γ and thus do not degrade tryptophan. These results strongly suggest that the tryptophan biosynthesis operon encoded by the various chlamydial species and serovars has evolved to reflect the environment in which each organism resides and the tryptophan precursors available for growth.

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Table1. Primers used for RT-PCR and genes analyzed in *C. trachomatis*

***C. trachomatis* genes analyzed**	**Gene description**	**Primers used for RT-PCR**	
		Name of primer	**Sequence**
16S rRNA	Ribosomal subunit	5′ 16S rRNA	5′ GGAGAAAAGGGAATTTCACG 3′
		3′ 16S rRNA	5′ TCCACATCAAGTATGCATCG 3′
trpBA	Tryptophan synthase α and β subunits	5′ TrpBA	5′ GCATTGGAGTCTTCACATGC 3′
		3′ TrpBA	5′ ACACCTCCTTGAATCAGAGC 3′
trpR	Tryptophan repressor	5′ TrpR	5′ AATCAAGAGGAGTCTGGCT 3′
		3′ TrpR	5′ GAGGATCTGATCCTTTAAG 3′

Table 2. Primers used for RT-PCR and genes analyzed in *C. psittaci* strain GPIC

***C. psittaci* genes analyzed**	**Gene description**	**Primers used for RT-PCR**	
		Name of primer	**Sequence**
16S rRNA	Ribosomal subunit	5′ 16S rRNA	5′ ACGAAAGTCTGACGAAGCGA 3′
		3′ 16S rRNA	5′ CTTCGCCACTGGTGTTCTTC 3′
trpR	Tryptophan repressor	5′ TrpR	5′ TGTCCAAGCGTTACTGCGGA 3′
		3′ TrpR	5′ GGTTTCCATATTTTCTAT 3′
trpDC	Anthranilate phosphoribosyl transferase/ Indoleglycerol phosphate synthase	5′ TrpDC	5′ GCAATGCTGCAACTTTAGAA 3′
		3′ TrpDC	5′ GCAAGATCTGCTGGACTATC 3′
trpF	Phosphoribosyl anthranilate isomerase	5′ TrpF	5′ TGCCGAGCCTGTAGGCGTGT 3′
		3′ TrpF	5′ CGGTAGCAACGTCAACTCCA 3′
trpBA	Tryptophan synthase α and β subunits	5′ TrpBA	5′ ACATAGCTCCTACTCTTCCA 3′
		3′ TrpBA	5′ GGACCATCAGCAACGGGATC 3′
kynU	Kynureninase	5′ KynU	5′ CACGTGCAGGTGAGGATTTA 3′
		3′ KynU	5′ CGCAAGAAGAGGCATTAGAG 3′
prsA	PRPP synthase	5′ PrsA	5′ GATGTTCGAGGTCGTGATGT 3′
		3′ PrsA	5′ ATCACAGCCAGTCCAGTATC 3′
trpRD	Tryptophan repressor/ Anthranilate phosphoribosyl transferase	5′ TrpRD	5′ TGAGTATCGCACAAATTACAAGAGG 3′
		3′ TrpRD	5′ GCTGCTATTTGATGCGGATCAGC 3′
trpCF	Indoleglycerol phosphate synthase/ Phosphoribosyl anthranilate isomerase	5′ TrpCF	5′ GCCCTTTGTAGCTCTAAAGATC 3′
		3′ TrpCF	5′ ACACGCCTACAGGCTCGGCA 3′
trpFB	Phosphoribosyl anthranilate isomerase/ Tryptophan synthase β subunit	5′ TrpFB	5′ TGGAGTTGACGTTGCTACCG 3′
		3′ TrpFB	5′ GTGGTCCATGTATTGCTTTGGC 3′
trpA-kynU	Tryptophan synthase α subunit/ Kynureninase	5′ TrpA/KynU	5′ GCTGATGGCATTGTTGTAGGTT 3′
		3′ TrpA/KynU	5′ TCTGACCAGCGTTGCAGTAG 3′
kynU-prsA	Kynureninase/PRPP synthase	5′ KynU/PrsA	5′ GAGTAACGGCAACACCTCTT 3′
		3′ KynU/PrsA	5′ ACATCACGACCTCGAACATC 3′

Table 3. Primers used for sequencing of *C. trachomatis* clinical isolates

***C. trachomatis* genes analyzed**	**Gene description**	**Primers used for RT-PCR**	
		Name of primer	**Sequence**
ompA	Major outer membrane protein	CT1	5′ GCCGCTTTGAGTTCTGCTTCCTC 3′
		CT5	5′ ATTTACGTGCAGCTCTCTCAT 3′
		VD1	5′ TGACTTTGTTTTCGACCGTGTTTT 3′
		VD4	5′ TTTTCTAGATTTCATCTTGTTCAAYTG 3′
trpBA	Tryptophan synthase α and β subunits	Trp.1	5′ GCGACATTACTGAAGACG 3′
		Trp.2	5′ GCAAGACTATCAAGAGTG 3′
		5′ TrpB-L	5′ AGCTGGCGCTTATCTACT 3′
		3′ TrpB-L	5′ ACACTGACCAAGAGCATT 3′
		5′ TrpB-M	5′ ATAGAAGTAAGAGCGTC 3′
		3′ TrpB-M	5′ CTCCCCGCATAGTTTTTC 3′
		5′ TrpA-L	5′ ATGAGTAAATTAACCCAA 3′
		3′ TrpA-L	5′ TCCATTGTTGTCTGATGA 3′
		5′ TrpA-M	5′ GCTACTACAATCCGCTTC 3′
		3′ TrpA-M	5′ TTCTATCTACAATTGGAA 3′
		5′ TrpA-L#2	5′ CTGATTGCATGTATCGGA 3′
		3′ TrpA-L#2	5′ TCCAATCCTACAGCTAAA 3′
		5′ TrpA-M#2	5′ GAAGCGGATTGTAGTAGC 3′
		3′ TrpA-M#2	5′ TATACATTAGCCACCGAT 3′

Table 4. Primers used for cloning of *C. trachomatis* serovar L2 genes for complementation studies

***C. trachomatis* genes analyzed**	**Gene description**	**Primers used for Cloning**	
		Name of primer	**Sequence**
trpA	Tryptophan synthase α subunit	5′ CtTrpA	5′ CCCC**GGTACC**ATGATGAAATTAACC 3′
		3′ CtTrpA	5′ CCCC**GTCGAC**TTATCCAGGAATAAAC 3′
trpB	Tryptophan synthase β subunit	5′ CtTrpB	5′ CCCC**GGTACC**ATGTTCAAACATAAAC 3′
		3′ CtTrpB	5′ CCCC**GTCGAC**TTACTCATAAATTCC 3′

Table 5. Primers used for cloning of *E. coli trpA* and *trpB* genes for complementation studies

***E. coli* genes analyzed**	**Gene description**	**Primers used for Cloning**	
		Name of primer	**Sequence**
trpA	Tryptophan synthase α subunit	5′ EcTrpA	5′ AGC*GGATCC*GAACGCTACGAATCT 3′
		3′ EcTrpA	5′ GG**GGTACC**TAAGCGAAACGGTAAA 3′
trpB	Tryptophan synthase β subunit	5′ EcTrpB	5′ CGC*GGATCC*ACAACATTACTTAACC 3′
		3′ EcTrpB	5′ TCGTAG**GGTACC**ATCAGATTTCCC 3′

Bold lettering indicates *SalI* sites for cloning
Underlined lettering indicates *PstI* sites for cloning
Italicized lettering indicates *BamHI* sites for cloning
Bold and underlined lettering indicates *KpnI* sites for cloning

Table 6. Primers used for cloning of *C. psittaci* strain GPIC genes for complementation studies

***C. psittaci* genes analyzed**	**Gene description**	**Primers used for Cloning**	
		Name of primer	**Sequence**
trpA	Tryptophan synthase α subunit	5′ TrpA	5′ GC**GGTACC**AATAGAATTGAAACAGC 3′
		3′ TrpA	5′ AACTGCAGTTACTGACGGGGATCT 3′
trpB	Tryptophan synthase β subunit	5′ TrpB	5′ GC**GGTACC**AAACATCCTTATCCAT 3′
		3′ TrpB	5′ GGCTGCAGCTATTCATTGTTGCCA 3′
trpD-A	Anthranilate phosphoribosyl transferase/ Indoleglycerol phosphate synthase/ Phosphoribosyl anthranilate isomerase/ Tryptophan synthase α and β subunits	5′ TrpD-A	5′ CCCC**GGTACC**ATGCTACAGACCTA 3′
		3′ TrpD-A	5′ CCCC**GTCGAC**TTACTGACGGGGAT 3′
prsA	PRPP synthase	5′ PrsA	5′ CCCC**GGTACC**ATGAATAAACAACC 3′
		3′ PrsA	5′ CCCC**GTCGAC**TCAGAAAGATAATG 3′
kynU	Kynureininase	5′ KynU	5′ CCCC**GGTACC**ATGAATGAAATTTTAAA ACA 3′
		3′ KynU	5′ CCCC**GTCGAC**GATTTGATCTCTAACACTTC 3′
trpD-kynU	Anthranilate phosphoribosyl transferase/ Indoleglycerol phosphate synthase/ Phosphoribosyl anthranilate isomerase/ Tryptophan synthase α and β subunits/ Kynureininase	5′ TrpD-KynU	5′ CCCC**GGTACC**ATGCTACAGACCTATTTGCA 3′
		3′ TrpD-KynU	5′ CCCC**GTCGAC**GATTTGATCTCTAACACTTC 3′

Bold lettering indicates *SalI* sites for cloning
Underlined lettering indicates *PstI* sites for cloning
Bold and underlined lettering indicates *KpnI* sites for cloning

Table 7. Tryptophan and 5-fluorotryptophan pool sizes in *C. trachomatis* serovar L2-infected HeLa cells cultured under various conditions.

Culture condition	Tryptophan or 5 fluorotryptophan pool size (pmoles/10^7 cells)
10 μg ml^{-1} tryptophan	6895
1 μg ml^{-1} tryptophan	295
IFNγ no pre-treatment (T=0)	200
IFNγ pre-treatment (T=-24)	233
-tryptophan – cycloheximide	n.d.*
-tryptophan + cycloheximide	228
100 μM indole	n.d.
100 μM 5-fluoroindole	n.d.
1 μg ml^{-1} 5-fluorotryptophan	324
10 μg ml^{-1} 5-fluorotryptophan	6754
10 μM indole + 100 μM 5-fluoroindole	n.d.

* n.d. = below the level of detection of the HPLC assay (<75 pmole/10^7 cells)
All analyses were made in triplicate with results varying by less than 10%

Table 8. Sequence polymorphism in *trpBA* from clinical ocular isolates

		Tryptophan synthase inactivating mutation				
***ompA* type**	**Number of isolates**	Single deletion *trpA* hot spot ta-caa (nucleotide 531)	Single deletion *trpA* tc-gga (nucleotide 470)	2-bp addition *trpA* gtg*t*gtc (nucleotide 118)	22-bp deletion *trpB* (nucleotides 11-32)	Complete *trp* deleted region
A	48	25	-	23	-	-
B	32	28	1	-	2	1
Ba	13	13	-	-	-	-
C	1	-	-	-	-	1
Total	94	66	1	23	2	2

All ocular isolates that have a trp region have the specific *trpA* codon deletion (nucleotides 408-410). None of the ocular isolates tested were indole rescuable.

Table 9. Sequence polymorphism in *trpA* from clinical genital isolates

		***trpA* genotype sequence per serovar**		
***ompA* type**	**Number of isolates**	tatcaa (1)[A] (B, D-K)	tgtcaa (2) (B, D-K)	tatgaa (3) (L1-L3)
D	47	38	9	0
E	39	38	1	0
F	14	13	1	0
G	7	3	4	0
H	10	8	2	0
I	20	17	3	0
J	8	7	1	0
K	10	8	2	0
B	9	4	5	0
L1	15	0	0	15
L2	35	0	0	35
Total	214	136	28	50

All of the genital isolates tested were indole rescuable. [A]Genotypic groups within the *trpA* mutation hot-spot region: Group 1 (encodeing YQ), Group 2 (encoding CQ), and Group 3 (encoding YE).

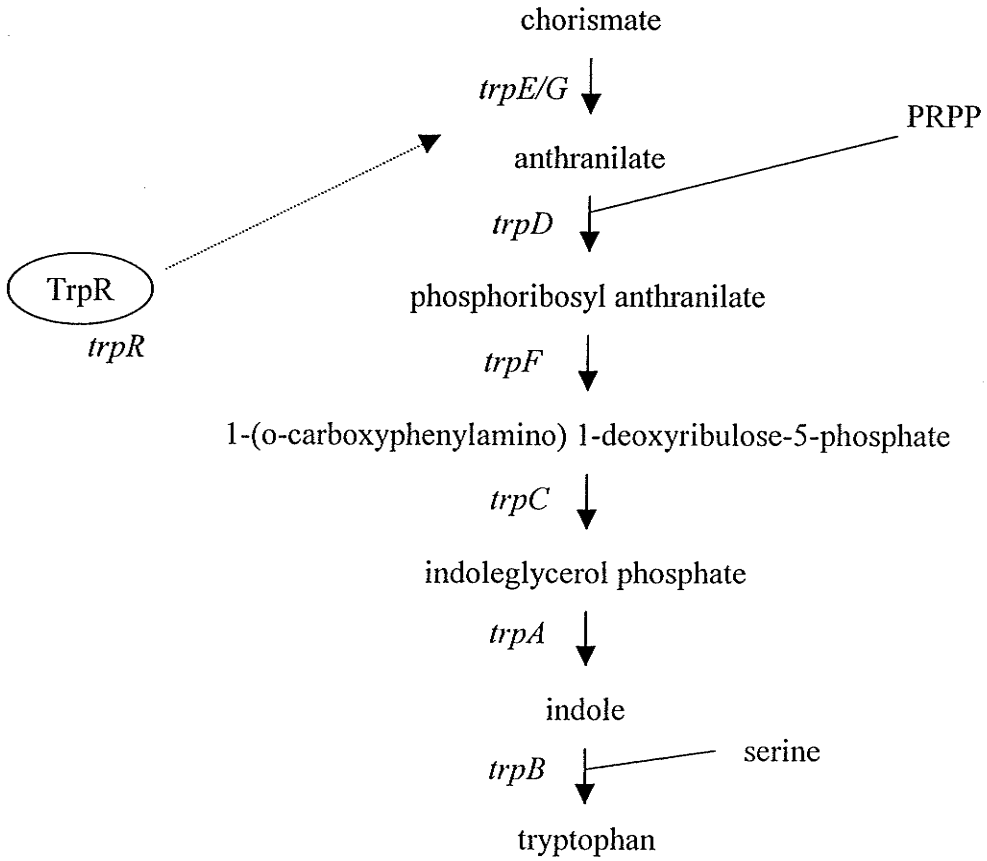
Table 10. Characteristics of the tryptophan biosynthesis genes of *C. psittaci* strain GPIC

Gene	Protein encoded	MW (kDa)	Non-chlamydial organism with highest homology	Accession number	% identity
trpR	Tryptophan repressor	11.6	*Vibrio cholera*	gi 15640720	37%
trpD	Anthranilate phosphoribosyl transferase	36.8	*Arabidopsis thaliana*	gi 15238711	45%
trpC	Indoleglycerol phosphate synthase	35.7	*Streptomyces coelicor*	gi 21220520	41%
trpF	Phosphoribosyl anthranilate isomerase	22.5	*Arabidopsis thaliana*	gi 5031261	35%
trpB	Tryptophan synthase β-subunit	42.2	*Aquifex aeolicus*	gi 15606106	59%
trpA	Tryptophan synthase α- subunit	28.2	*Xylella fastidosa*	gi 15837977	40%
kynU	Kynureninase	48.2	*Xanthomonas campestris*	gi 21231007	42%
prsA	PRPP synthase	32.9	*Microbulbifer degradans*	gi 23029846	43%

Table 11. Bacterial strains and plasmids used in this study

Bacterial Strain	Nonfunctional gene(s)
E. coli	
KS463	*trpA*
BW7622	*trpA*, *trpB*
CY15077	*trpA-trpE*
H0965	*prsA*
T3D	*trpE*
Plasmid	
pQE-80L expressing GPIC *trpA*	
pQE-80L expressing GPIC *trpB*	
pQE-80L expressing GPIC *trpBA*	
pQE-80L expressing GPIC *trpD-A*	
pQE-80L expressing GPIC *prsA*	
pQE-80L expressing GPIC *kynU*	
pQE-80L expressing GPIC *trpD-kynU*	
pQE-80L expressing serovar L2 *trpA*	
pQE-80L expressing serovar L2 *trpB*	
pQE-80L expressing serovar L2 *trpBA*	

Fig. 1. Reaction scheme for the biosynthesis of tryptophan. a) Tryptophan biosynthesis pathway of *E. coli*. b) Tryptophan biosynthesis pathway of *C. psittaci* strain GPIC as deduced from the genome sequence.


chorismate
trpE/G
anthranilate
PRPP
trpD
TrpR
trpR
phosphoribosyl anthranilate
trpF
1-(o-carboxyphenylamino) 1-deoxyribulose-5-phosphate
trpC
indoleglycerol phosphate
trpA
indole
trpB
serine
tryptophan

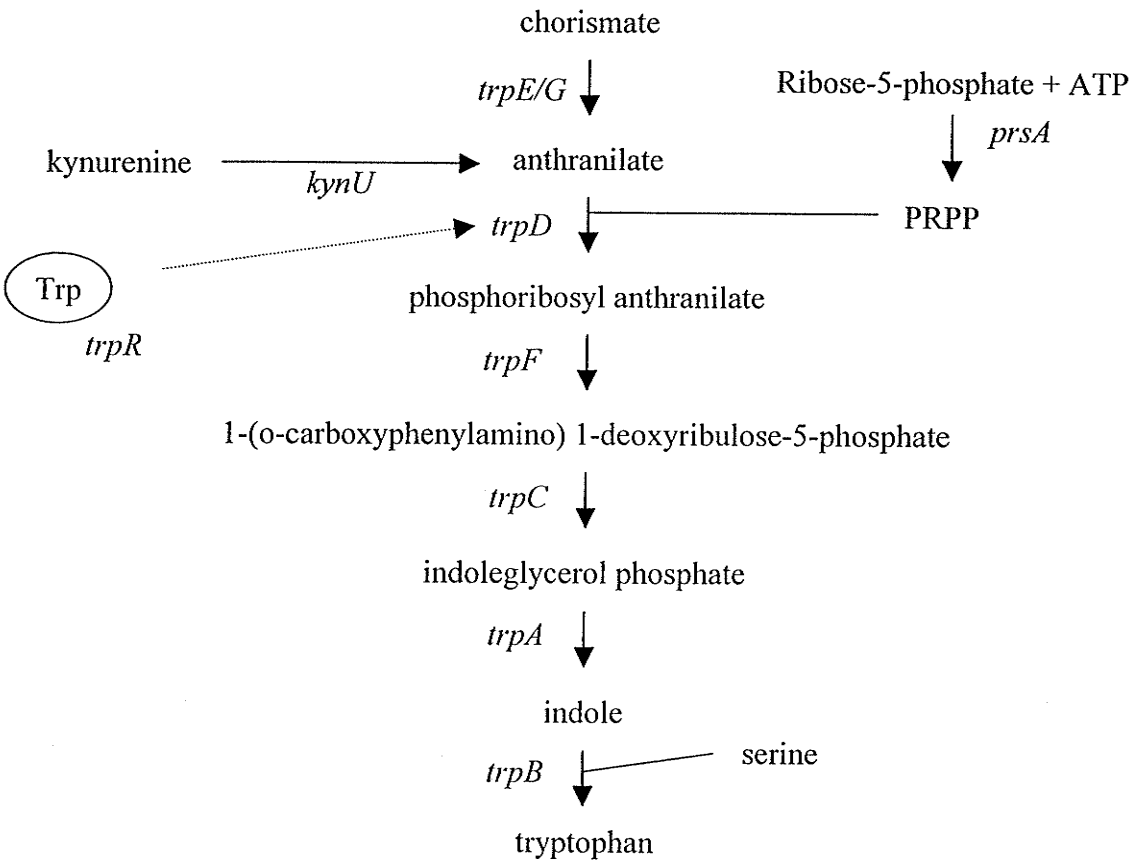

chorismate
trpE/G
Ribose-5-phosphate + ATP
prsA
kynurenine
kynU
anthranilate
trpD
PRPP
Trp
trpR
phosphoribosyl anthranilate
trpF
1-(o-carboxyphenylamino) 1-deoxyribulose-5-phosphate
trpC
indoleglycerol phosphate
trpA
indole
trpB
serine
tryptophan

Fig. 2. Schematic comparing chlamydial plasticity zones of *C. pneumoniae* AR39, *C. psittaci* strain GPIC, *C. trachomatis* MoPn, and *C. trachomatis* serovar D. Genes are colored according to role category assignments: tryptophan biosynthesis, yellow; purine interconversion (*guaBA-add*), green; toxin with homology to the large clostridial toxins, red; phospholipase D-endonuclease (PLD) family, blue; other function, white. Figure reproduced from reference {Read, 2000 #209}.

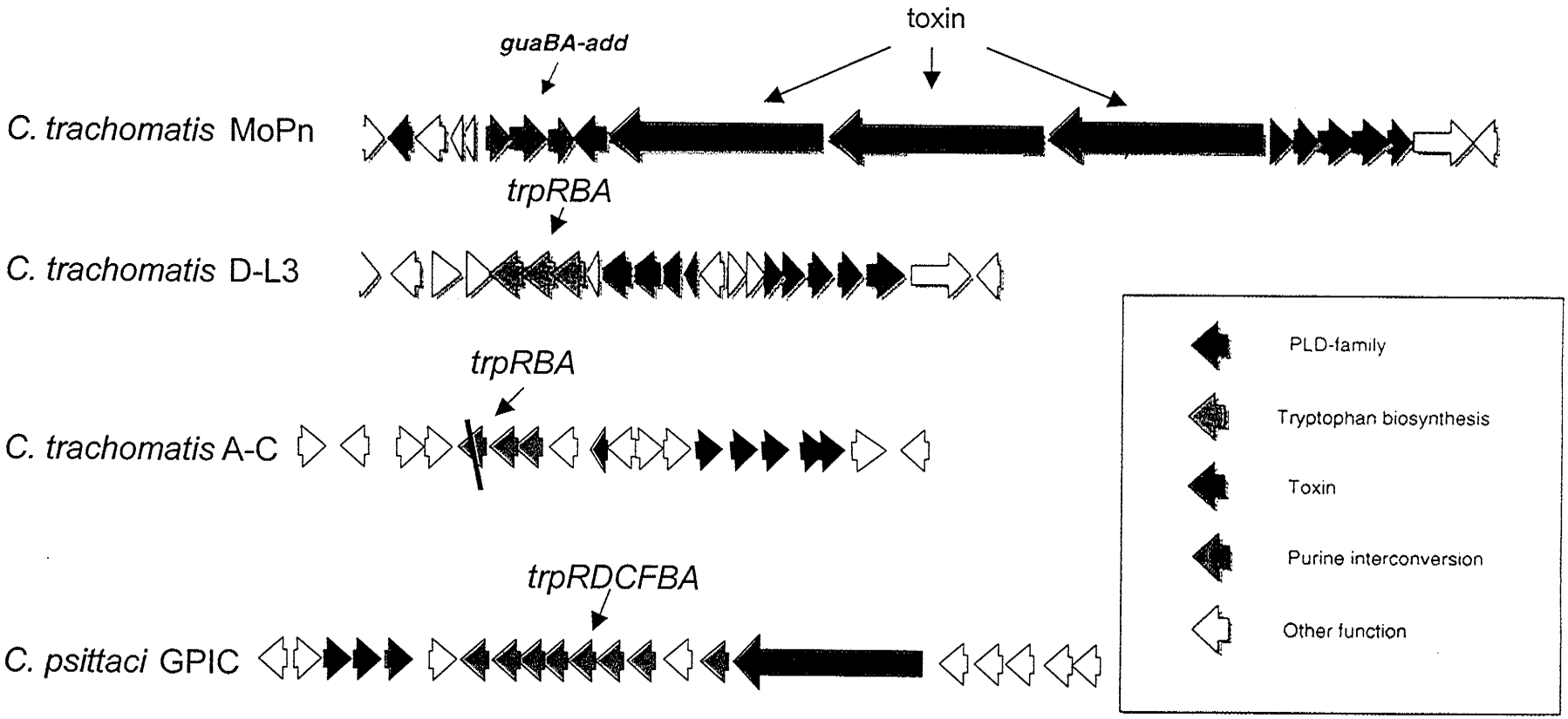

toxin
guaBA-add
C. trachomatis MoPn
trpRBA
C. trachomatis D-L3
trpRBA
C. trachomatis A-C
trpRDCFBA
C. psittaci GPIC
PLD-family
Tryptophan biosynthesis
Toxin
Purine interconversion
Other function

Fig. 3. Crystal structure of the *E. coli* tryptophan synthase.

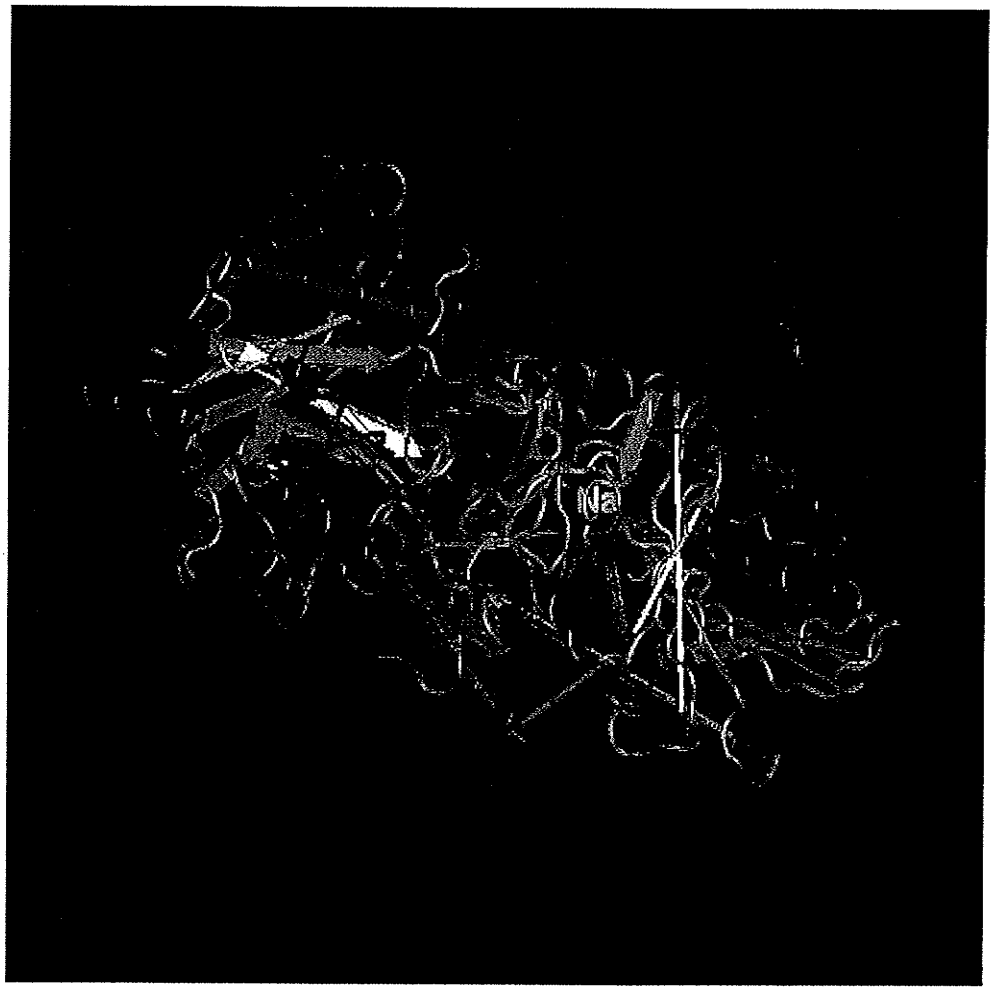

Na

Fig. 4. Comparison of the *C. trachomatis* serovar D (CHLTR) and *C. psittaci* GPIC (CHLCV) TrpB protein sequences with those from *E. coli* (ECOLI), *Salmonella typhimurium* (SALTY), *Zea mays* (MAIZE), Synechococcus sp. strain WH8102 (SYNPX), Bacillus subtilis (BACSU), and Methanosarcina acetivorans (METAC). Critical conserved residues identified as necessary for TrpB activity including His-86, Lys-87, Glu-109, Arg-148, Leu-188, Cys-230, Asp-305, Phe-306, and Glu-350 are in bold.

```
ECOLI     ----------------------------------------------TTLLNPYFGEFGGM 14
SALTY     ----------------------------------------------TTLLNPYFGEFGGM 14
MAIZE     PGPPPPAPEGRRRRGRGRNAAGQAVAAEASPAAVEMGNGAAAPGLQRPDAMGRFGRFGGK 60
SYNPX     ------------------MTSTLPNASTPDPASLQPS--------VRPGAHGRFGRFGGQ 34
BACSU     ----------------------------------------MYPYPNEIGRYGDFGGK 17
METAC     -------------------------------------MTETKVSEFQLKGKYGKYGGQ 21
CHLCV     ----------------------------------------------------MKHPYPFGGQ 10
CHLTR     ----------------------------------------------------MFKHKHPFGGA 11
                                                                  :**

ECOLI     YVPQILMPALRQLEEAFVSAQKDPEFQAQFNDLLKNYAGRPTALTKCQNITAGTN----- 69
SALTY     YVPQILMPALNQLEEAFVSAQKDPEFQAQFADLLKNYAGRPTALTKCQNITAGTR----- 69
MAIZE     YVPETLMHALTELESAFHALATDDEFQKELDGILKDYVGRESPLYFAERLTEHYKRADGT 120
SYNPX     YVPETLMPALAELEQAAAQAWNDPAFTAELNQLLKNYVGRATPLYEAERLTAHYRRADG- 93
BACSU     FVPETLMQPLDEIQTAFKQIKDDPAFREEYYKLLKDYSGRPTALTYADRVTEYLG----- 72
METAC     YVPEVLMPALEELDVGYEKYKNDPESLAELDHYLRDFAGRETPLYFARNLSKKYG----- 76
CHLCV     FMPEILMAPVQDLSNSWKSLQNHSDFKNELDSVLKNYAGRPTPLTEIKNFAKAIH----- 65
CHLTR     FLPEELLAPIQNLKAEWEILKTQQSFLSELDCILKNYAGRQTPLTEVKNFARAID----- 66
          ::*: *: .: ::.        .     :    *::: ** :.*    ..:

ECOLI     -TTLYLKREDLLHGGAHKTNQVLGQALLAKRMGKTEIIAETGAGQHGVASALASALLGLK 128
SALTY     -TTLYLKREDLLHGGAHKTNQVLGQALLAKRMGKSEIIAETGAGQHGVASALASALLGLK 128
MAIZE     GPLIYLKREDLNHTGAHKINNAVAQALLAKRLGKQRIIAETGAGQHGVATATVCRRFGLQ 180
SYNPX     GPRIWLKREDLNHTGAHKINNALGQALLALRMGKKRIIAETGAGQHGVATATVCARFGLE 153
BACSU     GAKIYLKREDLNHTGSHKINNALGQALLAKKMGKTKIIAETGAGQHGVAAATVAAKFGFS 132
METAC     -TKVYLKREDLVHGGAHKLNNALGQALLAKFMGKTRLIAETGAGQHGTATAMVGATLGFE 135
CHLCV     GPRIFLKREDLLHTGAHKINNALGQCLLAKHLGKTRIIAETGAGQHGVATATACGCLGLE 125
CHLTR     GPRVFLKREDLLHTGAHKLNNALGQCLLAKYLGKTRVVAETGAGQHGVATATACAYLGLD 126
           . ::****** * *:** *:.:.*.***  :** .::*********.*:* .   :*:.

ECOLI     CRIYMGAKDVERQSPNVFRMRLMGAEVIPVHSGSATLKDACNEALRDWSGSYETAHYMLG 188
SALTY     CRIYMGAKDVERQSPNVFRMRLMGAEVIPVHSGSATLKDACNEALRDWSGSYETAHYMLG 188
MAIZE     CIIYMGAQDMERQALNVFRMRLLGAEVRAVHSGTATLKDATSEAIRDWVTNVETTHYILG 240
SYNPX     CVIYMGAEDMRRQALNVFRMRLLGATVQPVTAGTATLKDATSEAIRDWVTNVESTHYILG 213
BACSU     CTVFMGEEDVARQSLNVFRMKLLGAEVVPVTSGNGTLKDATNEAIRYWVQHCEDHFYMIG 192
METAC     TIVYMGAKDIKRQQMNAYRMELMGTEVRAVETGSKTLKDAINEAMRDWVTNIENTHYLIG 195
CHLCV     CVIYMGAKDIERQKPNVDKMNLLGAEVISVNQGAQTLKDAVNEALRDWSKNYESTHYCLG 185
CHLTR     CVVYMGAKDVERQKPNVEKMRFLGAEVVSVTKGSCGLKDAVNQALQDWATTHSFTHYCLG 186
            ::** :*: **  *. :*.::*: * .*  *   **** .:*:: *    .  .* :*

ECOLI     TAAGPHPYPTIVREFQRMIGEETKAQILEREGRLPDAVIACVGGGSNAIGMFADFINETN 248
SALTY     TAAGPHPYPTIVREFQRMIGEETKAQILDKEGRLPDAVIACVGGGSNAIGMFADFINDTS 248
MAIZE     SVAGPHPYPMMVREFHKVIGKETRRQAMDKWGGKPDVLVACVGGGSNAMGLFHEFVEDQD 300
SYNPX     SVAGPHPYPMLVRDFHAVIGQEARQQCREAFGRLPDVLMACVGGGSNAMGLFHPFVECTN 273
BACSU     SVVGPHPYPQVVREFQKMIGEEAKDQLKRIEGTMPDKVVACVGGGSNAMGMFQAFLN-ED 251
METAC     SVVGPHPYPMIVRDFQSVIGKEVKEQAMEKEGRLPDSIIACAGGGSNAMGTFHPFIEDRE 255
CHLCV     SALGPSPYPEMVRNFQSVISLEVKSQLQEIG-FSPDLLIACVGGGSNAIGFFHHFIPDTR 244
CHLTR     SALGPLPYPDIVRFFQSVISAEVKEQIHAVAGRDPDILIACIGGGSNAIGFFHHFIPNPK 246
          :. ** *** :** *: :*. *.: *         ** ::** ******:* *  *:

ECOLI     VGLIGVEPGGHGIET----GEHGAPLKHGRVGIYFGMKAPMMQTEDGQIEESYSISAGLD 304
SALTY     VGLIGVEPGGHGIET----GEHGAPLKHGRVGIYFGMKAPMMQTADGQIEESYSISAGLD 304
MAIZE     VRLVGLEAAGHGVDT----DKHAATLTKGQVGVLHGSMSYLLQDDDGQVIEPHSISAGLD 356
SYNPX     VRLIGVEAAGDGVAT----GRHAATITEGRAGVLHGAMSLLLQDSDGQVQEAHSISAGLD 329
BACSU     VELIGAEAAGKGIDT----PLHAATISKGTVGVIHGSLTYLIQDEFGQIIEPYSISAGLD 307
METAC     VKLIAVEAGGKGLKCTEKAALHSASLCIGEEGILHGARTKILQDKNGQILESESVSAGLD 315
CHLCV     VKLVGVEAGGLGIES----GHHAARFATGRPGVLHGFYSYLLQDNEGQVLPTHSISAGLD 300
CHLTR     VQLIGVEGGGLGISS----GKHAARFATGRPGVFHGFYSYLLQDDDGQVLQTHSISAGLD 302
          * *:. * .* *:       *.* :  *  *: .*  : ::*   **:  . *:*****

ECOLI     FPSVGPQHAYLNSTGRADYVSITDDEALEAFKTLCLHEGIIPALESSHALAHALKMMREN 364
SALTY     FPSVGPQHAYLNSIGRADYVSITDDEALEAFKTLCRHEGIIPALESSHALAHALKMMREQ 364
MAIZE     YPGVGPEHSFLKDIGRAEYDSVTDQEALDAFKRVSRLEGIIPALETSHALAYLEKLCPTL 416
SYNPX     YPGVGPEHSYLREIGRAEYGAVTDQQALGALRLVSELEGIIPALETAHAFAWLEQLCPTL 389
BACSU     YPGIGPEHAYLHKSGRVTYDSITDEEAVDALKLLSEKEGILPAIESAHALAKAFKLAKGM 367
METAC     YSGVGPELAYLSESGRVTARYVTDDEALDAFNELSRLEGIIPALESSHALAYLKKAAE-S 374
CHLCV     YPAVGPDHAVLYESGRASYTVATDKAALEAFFLLSRLEGIIPALESSHALAELINIAPTL 360
CHLTR     YPSVGPDHAEMHESGRAFYTLATDEEALRAFFLLTRNEGIIPALESSHALAHLVSIAPSL 362
          :..:**: : : . **.     **. *: *:  :   ***:**:*::**:*   .
```

```
ECOLI       PDKEQLLVVNLSGRGDKDIFTVHDILKARGEI-- 396
SALTY       PEKEQLLVVNLSGRGDKDIFTVHDILKARGEI-- 396
MAIZE       AD-GVRVVVNCSGRGDKDVHTASKYLDV------ 443
SYNPX       PD-GSEVVINCSGRGDKDVNTVAEKLGDQL---- 418
BACSU       DR-GQLILVCLSGRGDKDVNTLMNVLEEEVKAHV 400
METAC       GELGEFVVVNLSGRGDKDLETVLSLRRGV----- 403
CHLCV       PK-ESVVVVNLSGRGDKDLEQITNLIKAGNNE-- 391
CHLTR       PK-EQIVIVNLSGRGDKDLPQIIRRNR-GIYE-- 392
                  :::  *******:
```

Fig. 5. Comparison of the *C. trachomatis* serovar D (CHLTR) and *C. psittaci* GPIC (CHLCV) TrpA protein sequences with those from *E. coli* (ECOLI), *Salmonella typhimurium* (SALTY), *Zea mays* (MAIZE), Synechococcus sp. strain WH8102 (SYNPX), Bacillus subtilis (BACSU), and Methanosarcina acetivorans (METAC). Conserved catalytic amino acids Glu-49 and Asp-60 are in bold and underlined. Critical residues in the active site that have been shown to be necessary for TrpA activity in *E. coli* are in bold. Amino acids in loop 6 are overlined.

```
ECOL6     ------------------------------------------------------------
SALTY     ------------------------------------------------------------
METAC     ------------------------------------------------------------
MAIZE     MAFAPKTSSSSSLSSALQAAQSPPLLLRRMSSTATPRRRYDAAVVVTTTTTARAAAAAVT 60
SYNP2     ------------------------------------------------------------
BACSU     ------------------------------------------------------------
CHLCV     ------------------------------------------------------------
CHLTR     ------------------------------------------------------------

ECOL6     -------------------------MERYESLFAQLKERKEGAFVPFVTLGDPGIEQSLK 35
SALTY     -------------------------MERYENLFAQLNDRREGAFVPFVTLGDPGIEQSLK 35
METAC     --------------MATELRATEHGRQKISEKFDELKQKKEGALIGYVMAGDPSAEATFG 46
MAIZE     VPAAPPQAGRRRRCHQSKRRHPQRRSRPVSDTMAALMAKGKTAFIPYITAGDPDLATTAE 120
SYNP2     -------------------------MTSVSERFRSLKQAGQCALIPFITAGDPDLETTEQ 35
BACSU     -------------------------------MFKLDLQPSEKLFIPFITAGDPVPEVSIE 29
CHLCV     -------------------------MNRIETAFKNTK-----PFIGYLTGGDGGFDYSVA 30
CHLTR     -------------------------MSKLTQVFKQTK-----LCIGYLTAGDGGTSYTIE 30
                                          :           : ::  **     :

ECOL6     IIDTLIEAGADALELGIPFSDPLADGPTIQNATLRAFAAGVTPAQCFEMLALIRQKHPTI 95
SALTY     IIDTLIDAGADALELGVPFSDPLADGPTIQNANLRAFAAGVTPAQCFEMLALIREKHPTI 95
METAC     IVKALVNGGADIIELGFPFSDPVADGPTIQAAGQRALAAGMDIEHYFE---LVRGLGVEV 103
MAIZE     ALRLLDGCGADVIELGVPCSDPYIDGPIIQASVARALASGTTMDAVLEMLREVTP-ELSC 179
SYNP2     ALKILDAAGADFIELGVPYSDPLADGPTIQAAATRALSRGVTLEQVLAIVQRVHG-QLTA 94
BACSU     LAKSLQKAGATALELGVAYSDPLADGPVIQRASKRALDQGMNIVKAIELGGEMKKNGVNI 89
CHLCV     CAHALIRGGVDILEIGFPFSDPVADGPIIQKAHTRALEEKTDSTTILEIAKALRET-SNI 89
CHLTR     AAKALIQGGVDILELGFPFSDPVADNPEIQVSHDRALAENLTSETLLEIVEGIRAFNQEV 90
              *   *.  :*:*.. ***  *.* ** :  **:         :     :

ECOL6     PIGLLMYANLVFNKGIDEFYAECEKVGVDSVLVADVPVEESA----PFRQAALRHNVAPI 151
SALTY     PIGLLMYANLVFNNGIDAFYARCEQVGVDSVLVADVPVEESA----PFRQAALRHNIAPI 151
METAC     PLVCMTYYNPVFRYGVDKFVEHAADAGISGLIIPDIPVEEAA----DLKSSCEKYGLDLI 159
MAIZE     PVVLLSYYKPIMSR----SLAEMKEAGVHGLIVPDLPYVAAH----SLWSEAKNNNLELV 231
SYNP2     PIILFTYYNPIFYRGIDAFMAQVAAAGVKGLVIPDLPLEESQ----MVLDAATSHGLDLI 150
BACSU     PIILFTYYNPVLQLNKEYFFALLRENHIDGLLVPDLPLEESN----SLQEECKSHEVTYI 145
CHLCV     PLVLFSYYNPLLQKGPQ-YLHQLKAAGFDAVLIVDLPIPQHANESEPFFQALIEAKLFPI 148
CHLTR     PLILYSYYNPLLQRDLD-YLRRLKDAGINGVCVIDLPAPLSHGEKSPFFEDLLAVGLDPI 149
          *:    * : ::          . .: : *:*         . .     :  :

ECOL6     FICPPNADDDLLRQIASYGRGYTYLLSRAGVTGAENRAALPLNHLVAKLKEYNAAPPLQG 211
SALTY     FICPPNADDDLLRQVASYGRGYTYLLSRSGVTGAENRGALPLHHLIEKLKEYHAAPALQG 211
METAC     FLVAPTTTDARIRKILERGSGFIYLVSRLGVTGARADVSGSTKELLSRVKT--DIPKAVG 217
MAIZE     LLTTPAIPEDRMKEITKASEGFVYLVSVNGVTGPRANVNPRVESLIQEVKKVTNKPVAVG 291
SYNP2     LLVAPTSPTERIEAIAKASQGFIYLVSVTGVTGARTSVASRVGELLPKLRQVTDKPIGVG 210
BACSU     SLVAPTS-ESRLKTIIEQAEGFVYCVSSLGVTGVRNEFNSSVYPFIRTVKNLSTVPVAVG 204
CHLCV     VLATPSTREERLLQIRKLAKGFLYYVSQKGTTGIRSKLSDDFSTQIARLRCYFQIPIVAG 208
CHLTR     LLISAGTTPERMSLIQEYARGFLYYIPCQATRDSEVGIKEEFR----KVREHFDLPIVDR 205
           : ..      :  : . . *: * :.  .. . .             ::     *

ECOL6     FGISAPDQVKAAIDAGAAGAISGSAIVKIIEQ------HINEPEKMLAALKAFVQPMKAA 265
SALTY     FGISSPEQVSAAVRAGAAGAISGSAIVKIIEK------NLASPKQMLAELRSFVSAMKAA 265
METAC     FGISTGKQAAEVRKAGADAVIVGSVFVRIIEE------GNGVNEKLEALARELKSGILGA 271
MAIZE     FGISKPEHVKQIAQWGADGVIIGSAMVRQLG-------EAASPKQGLRRLEEYARGMKNA 344
SYNP2     FGVSDPAQARQLKEWGADGVIVGSAVVKRL--------ATGTPAEGLAAVKEFCESLKEA 262
BACSU     FGISNREQVIKMNEI-SDGVVVGSALVRKIEELKDRLISAETRNQALQEFEDYAMAFSGL 263
CHLCV     FGIANRASAAAALKH-ADGIVVGSAFVEKLEK-----------KISPEELTTFAQSIDPR 256
CHLTR     RDICDKKEAAHVLNY-SDGFIVKTAFVHQTTM-----------DSSVETLTALAQTVIPG 253
           .:.    .       : . :  :..*.                              .

ECOL6     TRS- 268
SALTY     SRA- 268
METAC     N--- 272
MAIZE     LP-- 346
SYNP2     IA-- 264
BACSU     YSLK 267
CHLCV     Q--- 257
CHLTR     ----
```

Fig. 6. a) Alignment of the *trpA* gene sequences from the 14 human *C. trachomatis* reference serovars. A ClustalW alignment of the nucleotide regions (in bold) containing sequence polymorphisms is illustrated. As compared with genital serovars, ocular serovars have a three-base (nucleotides 408-410) deletion that results in the loss of a phenylalanine. The various serovars have been grouped, in accordance with their nucleotide mutational "hotspot" sequence. The ocular serovars have a single base-deletion (nucleotide 528) resulting in a non-functional truncated TrpA protein. Genital serovar specific missense mutations (nucleotides 530 and 532) that result in amino acid changes in loop6 of TrpA are shown below the nucleotide sequence; b) Alignment of the TrpA protein sequences resulting in the tryptophan synthase inactivating mutations (in bold and underlined) identified in clinical ocular serovars and the missense mutations identified in the clinical genital serovars.

A

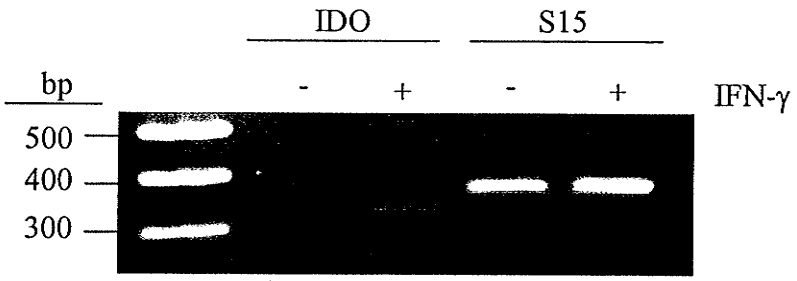
```
TRPA_D          ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATGTCAAGCTACG 540
TRPA_K          ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATGTCAAGCTACG 540
TRPA_E          ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATGTCAAGCTACG 540
TRPA_H          ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATATCAAGCTACG 540
TRPA_J          ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATATCAAGCTACG 540
TRPA_I          ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATATCAAGCTACG 540
TRPA_G          ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATATCAAGCTACG 540
TRPA_F          ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATATCAAGCTACG 540
TRPA_L1         ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATATGAAGCTACG 540
TRPA_L2         ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATATGAAGCTACG 540
TRPA_L3         ATGTCTTTAATACAAGAATACGCAAGAGGCTTTCTGTATTATATCCCATATGAAGCTACG 540
TRPA_A          ATGTCTTTAATACAAGAACACGCAAGAGGCCTTCTGTATTATATCCCATA-CAAGCTACG 536
TRPA_C          ATGTCTTTAATACAAGAACACGCAAGAGGCCTTCTGTATTATATCCCATA-CAAGCTACG 536
TRPA_Ba         ATGTCTTTAATACAAGAACACGCAAGAGGCCTTCTGTATTATATCCCATA-CAAGCTACG 536
                ****************** ********** ******************   ********
```

B

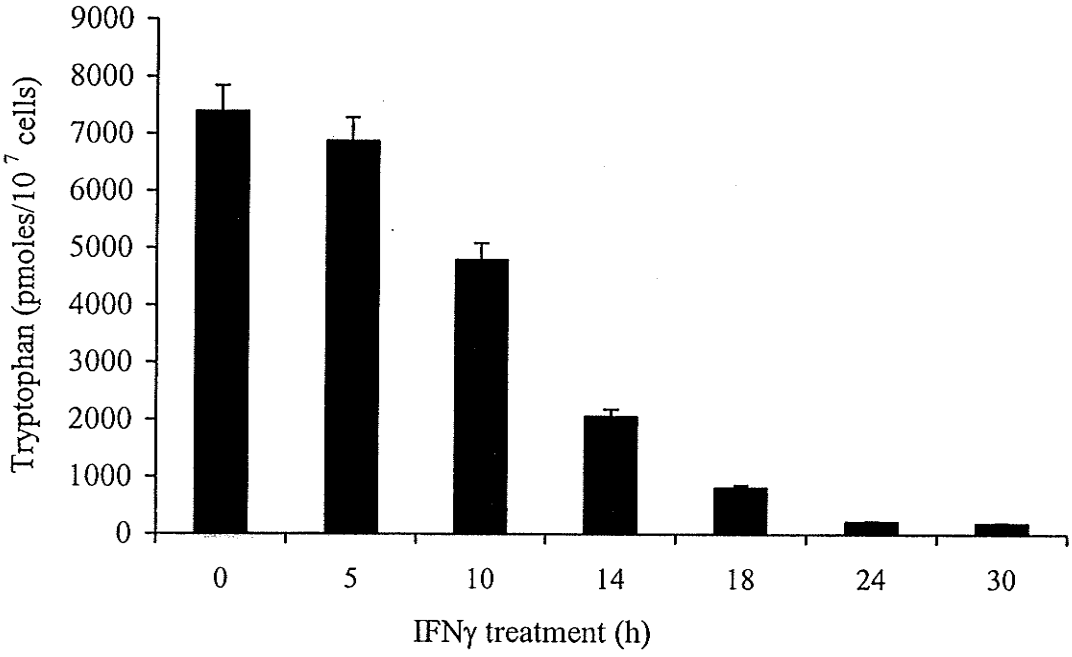
```
TRPA_D          PERMSLIQEYARGFLYYIPCQATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_K          PERMSLIQEYARGFLYYIPCQATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_E          PERMSLIQEYARGFLYYIPCQATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_H          PERMSLIQEYARGFLYYIPYQATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_J          PERMSLIQEYARGFLYYIPYQATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_I          PERMSLIQEYARGFLYYIPYQATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_G          PERMSLIQEYARGFLYYIPYQATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_F          PERMSLIQEYARGFLYYIPYQATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_L1         PERMSLIQEYARGFLYYIPYEATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_L2         PERMSLIQEYARGFLYYIPYEATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_L3         PERMSLIQEYARGFLYYIPYEATRDSEVGIKEEFRKVREHFDLPIVDRRDICD 210
TRPA_A          PERMSLIQEHARGLLYYIPY--******************************** 176
TRPA_Ba         PERMSLIQEHARGLLYYIPY--                                  176
TRPA_C          PERMSLIQEHARGLLYYIPY--                                  176
                *********:***:*****
```

Fig. 7. Effect of IFN-γ on IDO expression and tryptophan levels in HeLa cells. a) RT-PCR analysis of IDO transcript expression. HeLa cells were treated with 5 ng mL^{-1} IFN-γ (+) or left untreated as a control (-). cDNA was amplified with primers specific for *ido* and for *rig/S15*, a housekeeping gene. b) Analysis of tryptophan levels in HeLa cells. Uninfected HeLa cells were treated with IFN-γ or left untreated as a control. At the time indicated, total intracellular tryptophan levels were determined as described in Materials and Methods. c) Growth of *C. trachomatis* serovar L2 and A in IFN-γ-treated HeLa cells. HeLa cells were infected with serovar L2 or serovar A and cultured with IFN-γ or HeLa cells were pre-treated with IFN-γ for 24 hr and then infected and cultured in IFNγ-containing medium. Recoverable IFUs were determined as described in Materials and Methods. Data are presented as the mean IFU (log_{10}) of triplicate determinations from three separate experiments.

A


IDO
S15
bp
-
+
-
+
IFN-γ
500
400
300

B

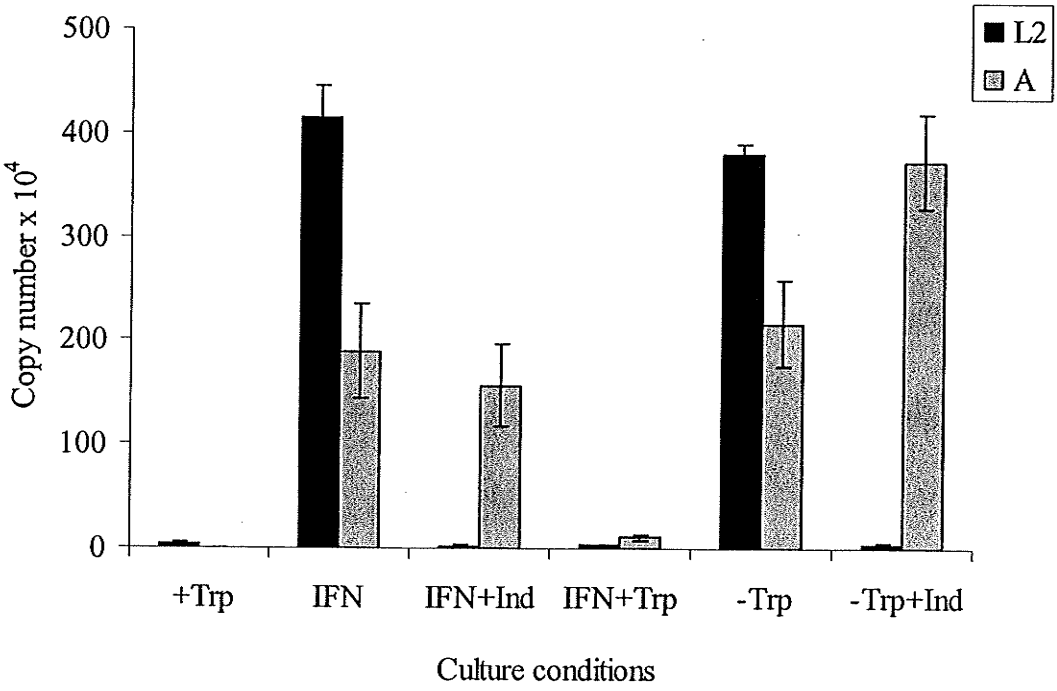

Tryptophan (pmoles/10 7 cells)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
14
18
24
30
IFNγ treatment (h)

C

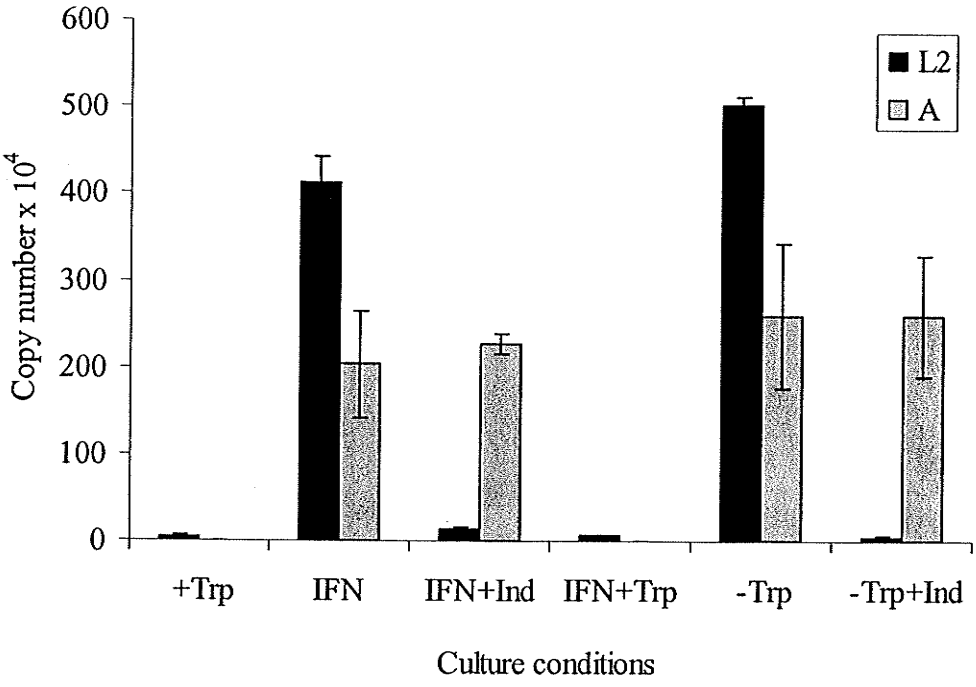

Recoverable IFUs/ml
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
L2
A
Serovar
MEM
MEM + IFN T=0
MEM + IFN T=-24

Fig. 8. Analysis of *trp* gene expression in *C. trachomatis* serovar L2 and A. HeLa cells were infected with *C. trachomatis* serovars L2 or A EBs in MEM-10 (+Trp); MEM-10 containing IFN-γ (IFN); MEM-10 containing IFN-γ supplemented with 10 μg mL^{-1} indole (IFN+Ind); MEM-10 containing IFN-γ supplemented with 1 mg mL^{-1} tryptophan (IFN+Trp); MEM-10 dialyzed FCS lacking tryptophan (-Trp) or lacking tryptophan but supplemented with 10 μg mL^{-1} indole (-Trp+Ind). Results for *trpBA* and *trpR* were normalized against the copy number of 16S rRNA transcripts in each cDNA preparation. a) *trpBA*. b) *trpR*. c) Expression of TrpA and TrpB in *C. trachomatis* serovars L2 and A. Protein extracts were prepared from infected cells and analyzed as described in Materials and Methods.

A


500
400
300
200
100
0
Copy number x 10^4
L2
A
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

B


600
500
400
300
200
100
0
Copy number x 10^4
L2
A
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

C

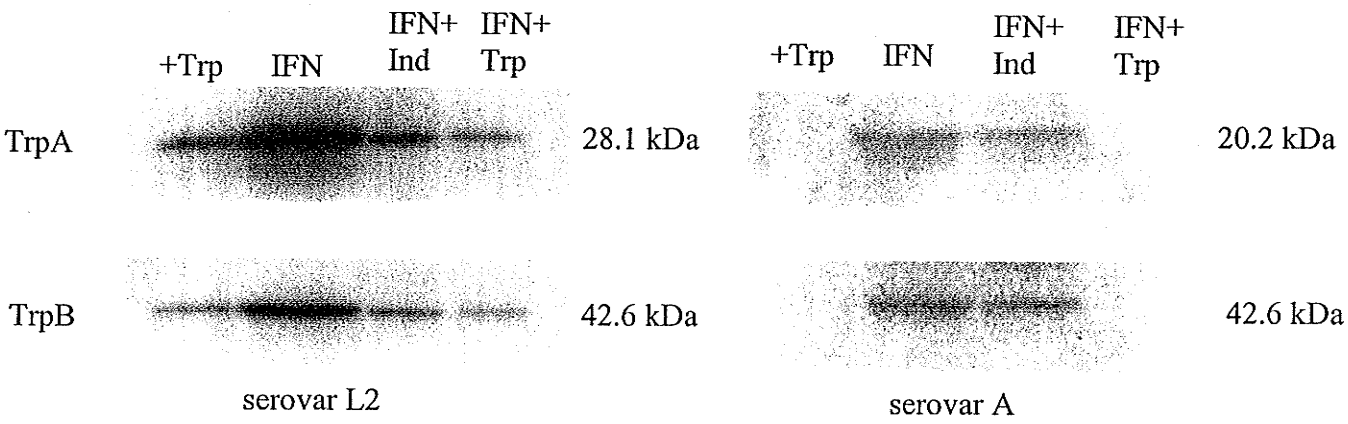
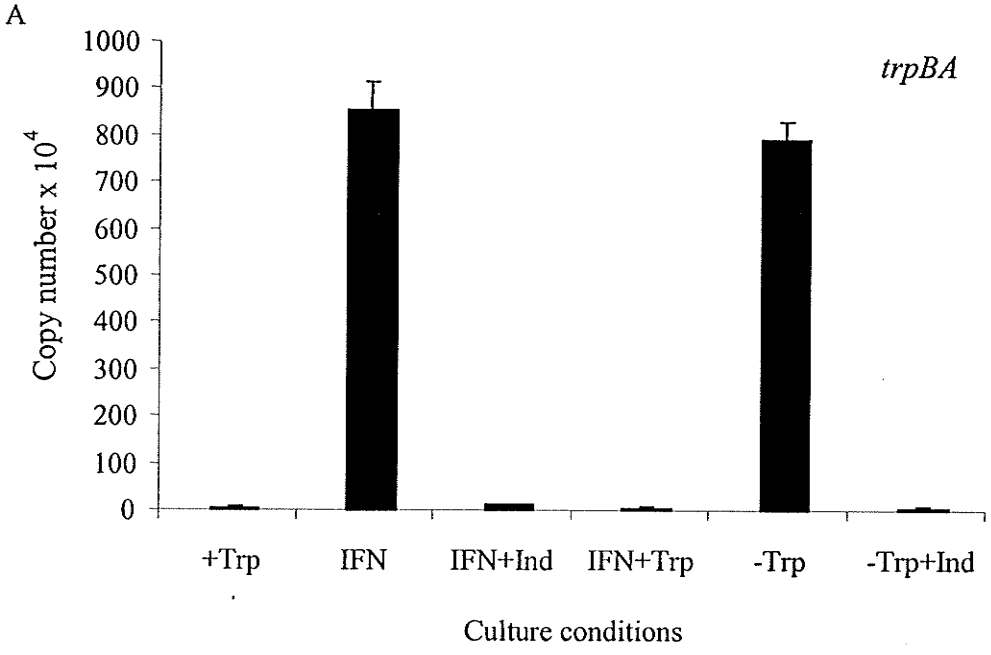

+Trp
IFN
IFN+ Ind
IFN+ Trp
TrpA
28.1 kDa
TrpB
42.6 kDa
serovar L2
+Trp
IFN
IFN+ Ind
IFN+ Trp
20.2 kDa
42.6 kDa
serovar A

Fig. 9. Analysis of *trpBA* and *trpR* gene expression in *C. trachomatis* serovar I. HeLa cells were infected with *C. trachomatis* serovars I EBs in MEM-10 (+Trp); MEM-10 containing IFN-γ (IFN); MEM-10 containing IFN-γ supplemented with 10 μg mL^{-1} indole (IFN+Ind); MEM-10 containing IFN-γ supplemented with 1 mg mL^{-1} tryptophan (IFN+Trp); MEM-10 dialyzed FCS lacking tryptophan (-Trp) or lacking tryptophan but supplemented with 10 μg mL^{-1} indole (-Trp+Ind). Transcript levels were quantitated as described in the legend to Fig. 8. a) *trpBA*; b) *trpR*.

A


trpBA
Copy number x 10^4
1000
900
800
700
600
500
400
300
200
100
0
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

B

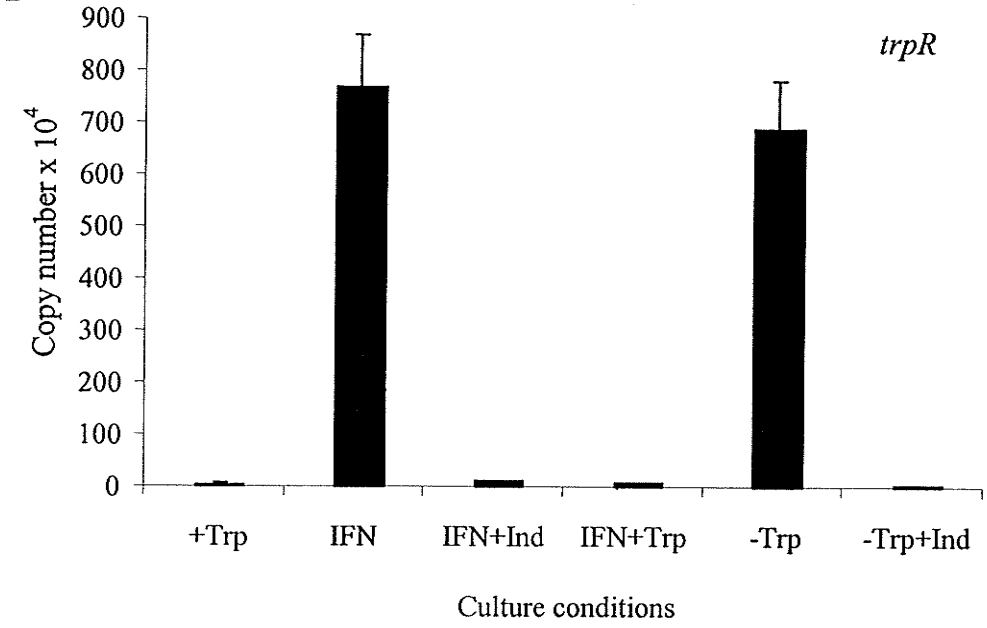
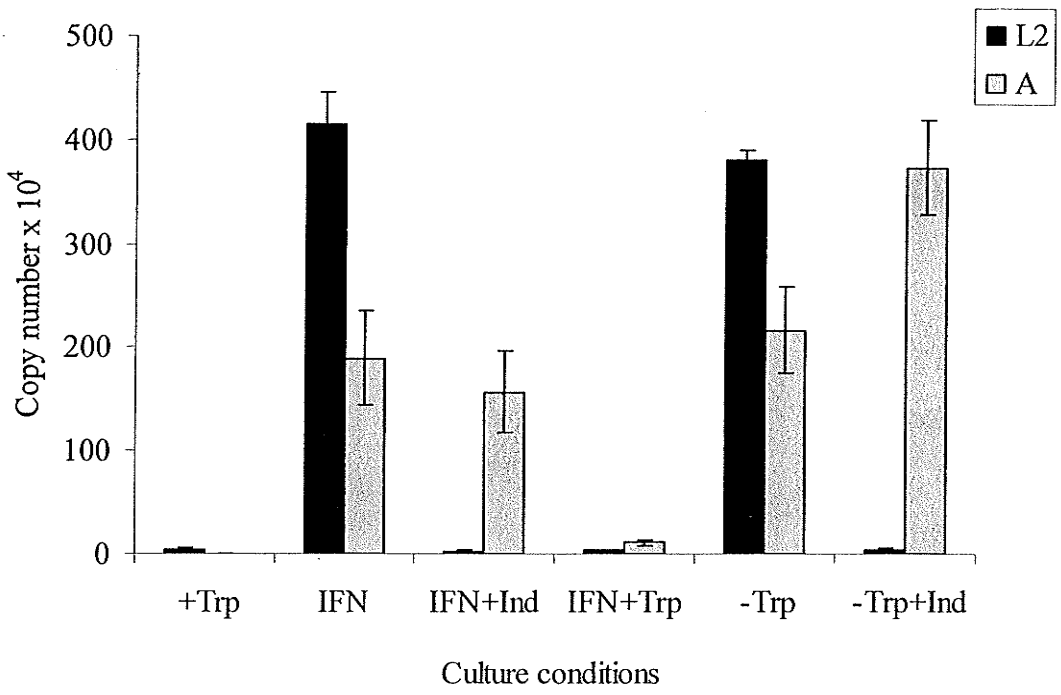
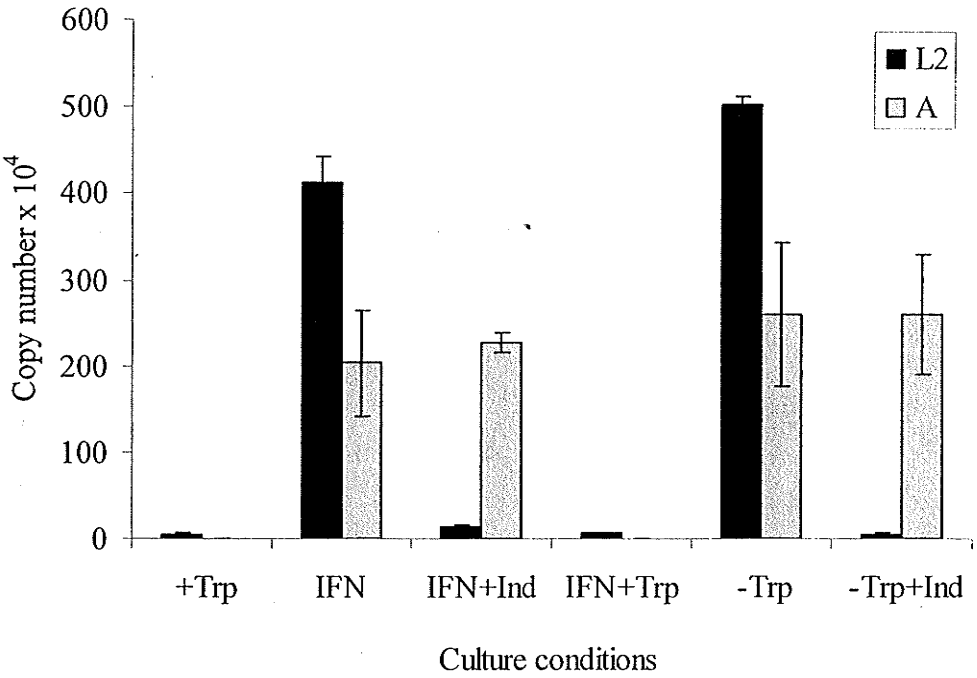

trpR
Copy number x 10^4
900
800
700
600
500
400
300
200
100
0
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

Fig. 10. Analysis of *trp* gene expression in *C. trachomatis* serovar L2 and A. HeLa cells were infected with *C. trachomatis* serovars L2 or A EBs in MEM-10 (+Trp); MEM-10 containing IFN-γ (IFN); MEM-10 containing IFN-γ supplemented with 10 μg mL^{-1} indole (IFN+Ind); MEM-10 containing IFN-γ supplemented with 1 mg mL^{-1} tryptophan (IFN+Trp); MEM-10 dialyzed FCS lacking tryptophan (-Trp) or lacking tryptophan but supplemented with 10 μg mL^{-1} indole (-Trp+Ind). Results for *trpBA* and *trpR* were normalized against the copy number of 16S rRNA transcripts in each cDNA preparation. a) *trpBA*. b) *trpR*. c) Expression of TrpA and TrpB in *C. trachomatis* serovars L2 and A. Protein extracts were prepared from infected cells and analyzed as described in Materials and Methods.

A


500
400
300
200
100
0
Copy number x 10^4
L2
A
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

B


600
500
400
300
200
100
0
Copy number x 10^4
L2
A
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

C

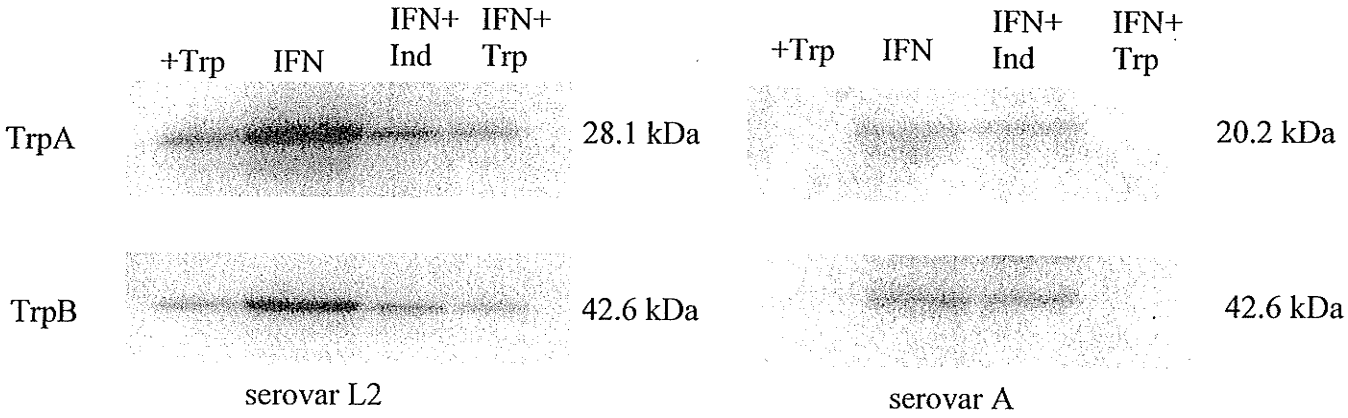
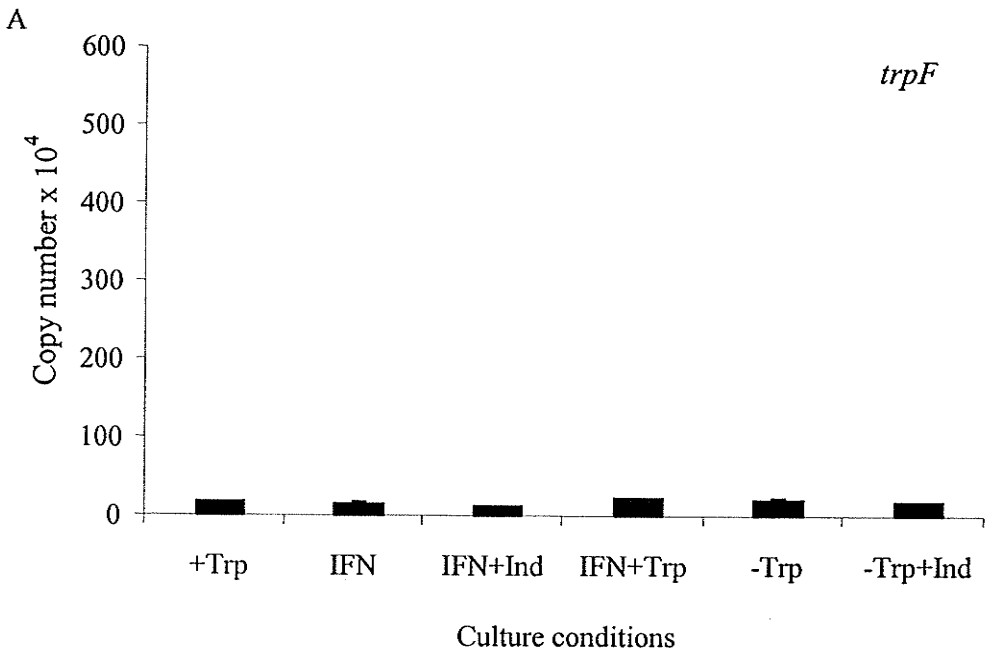
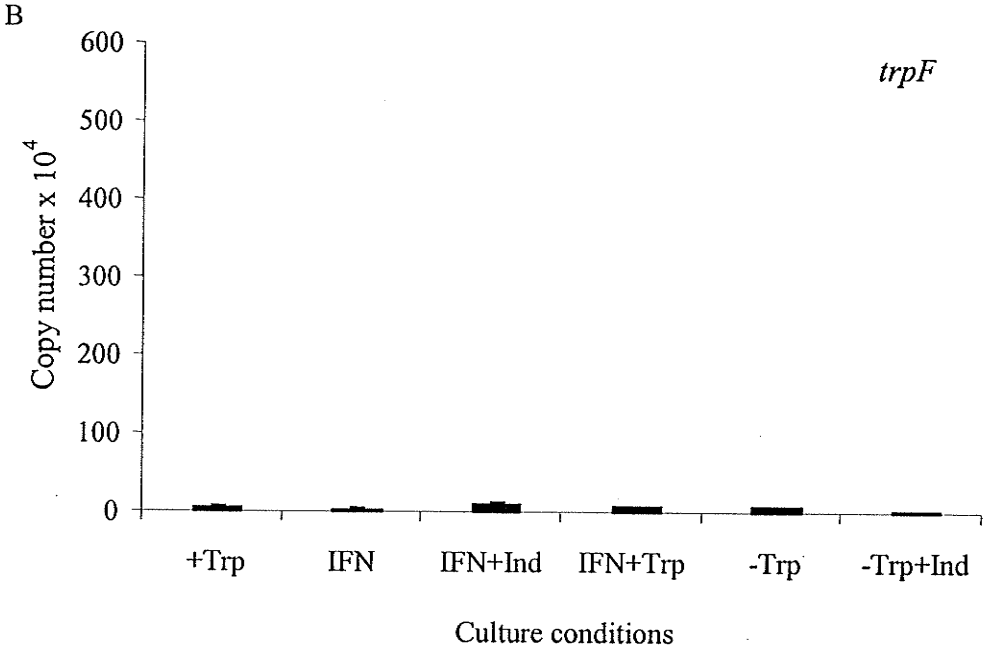

+Trp
IFN
IFN+ Ind
IFN+ Trp
TrpA
28.1 kDa
TrpB
42.6 kDa
serovar L2
+Trp
IFN
IFN+ Ind
IFN+ Trp
20.2 kDa
42.6 kDa
serovar A

Fig. 11. Analysis of *trpF* gene expression in *C. trachomatis* serovars L2, A and I. HeLa cells were infected with *C. trachomatis* serovars L2, A, or I EBs in MEM-10 (+Trp); MEM-10 containing IFN-γ (IFN); MEM-10 containing IFN-γ supplemented with 10 μg mL^{-1} indole (IFN+Ind); MEM-10 containing IFN-γ supplemented with 1 mg mL^{-1} tryptophan (IFN+Trp); MEM-10 dialyzed FCS lacking tryptophan (-Trp) or lacking tryptophan but supplemented with 10 μg mL^{-1} indole (-Trp+Ind). Transcript levels were quantitated as described in the legend to Fig. 8. a) serovar L2; b) serovar A; c) serovar I.


A
600
500
400
300
200
100
0
Copy number x 10^4
trpF
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

B


trpF
600
500
400
300
200
100
0
Copy number x 10^4
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

C

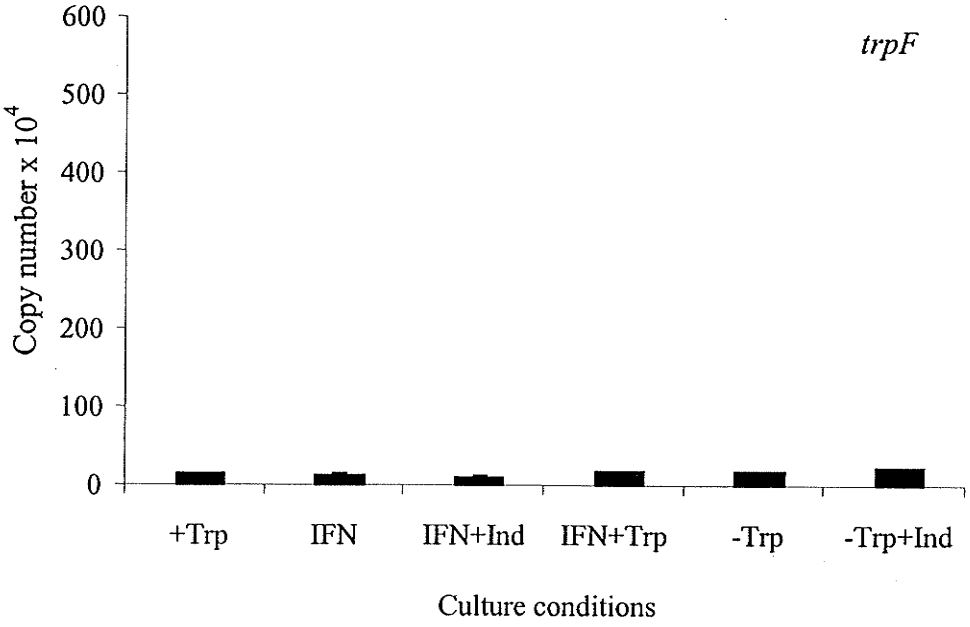
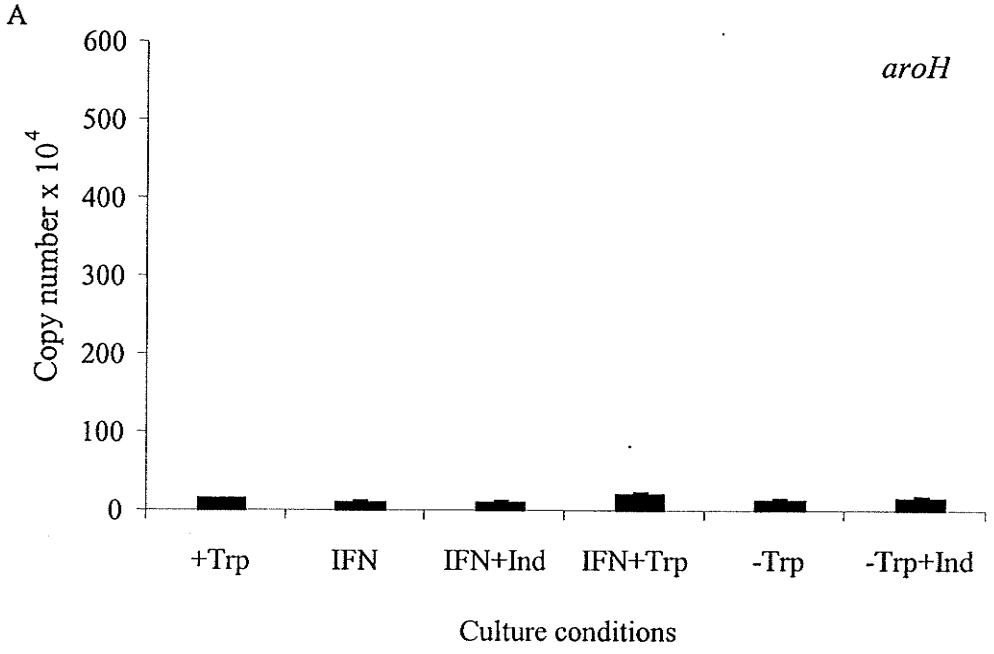

trpF
600
500
400
300
200
100
0
Copy number x 10^4
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

Fig. 12. Analysis of *aroH* and *aroL* gene expression in *C. trachomatis* serovar L2. HeLa cells were infected with *C. trachomatis* serovar L2 EBs in MEM-10 (+Trp); MEM-10 containing IFN-γ (IFN); MEM-10 containing IFN-γ supplemented with 10 μg mL^{-1} indole (IFN+Ind); MEM-10 containing IFN-γ supplemented with 1 mg mL^{-1} tryptophan (IFN+Trp); MEM-10 dialyzed FCS lacking tryptophan (-Trp) or lacking tryptophan but supplemented with 10 μg mL^{-1} indole (-Trp+Ind). Transcript levels were quantitated as described in the legend to Fig. 8. a) *aroH*; b) *aroL*.

A


600
500
400
300
200
100
0
Copy number x 10^4
aroH
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

B

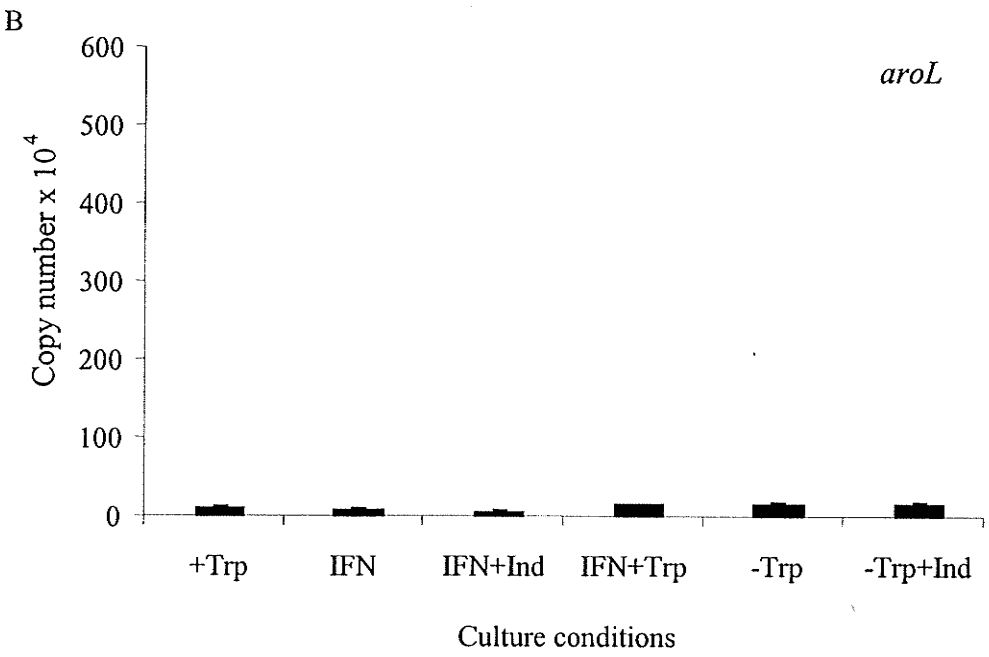
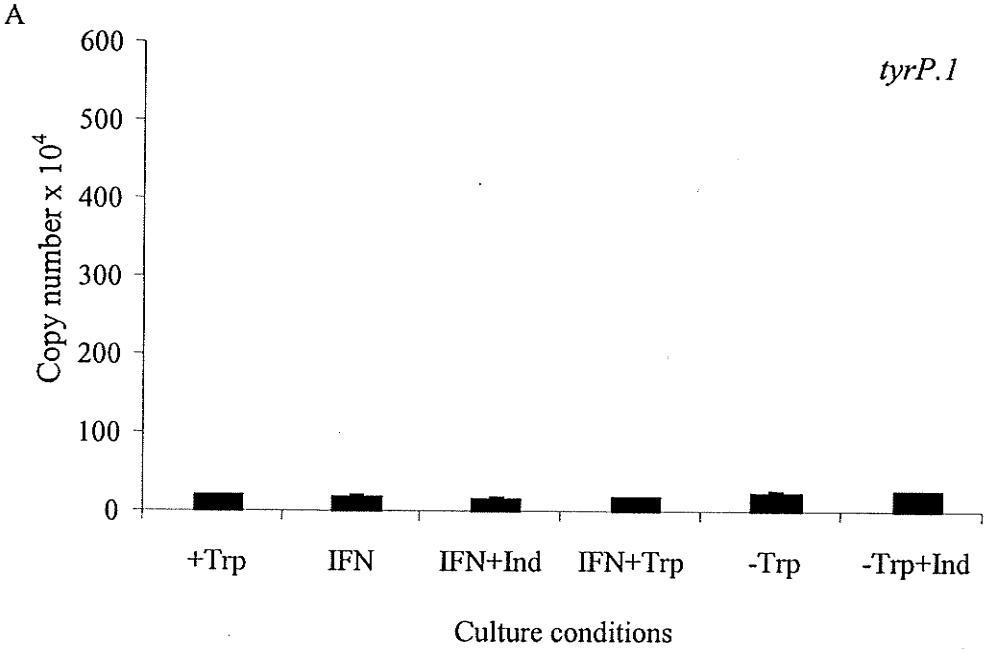

aroL
Copy number x 10^4
600
500
400
300
200
100
0
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

Fig. 13. Analysis of *tyrP.1* and *tyrP.2* gene expression in *C. trachomatis* serovar L2. HeLa cells were infected with *C. trachomatis* serovar L2 EBs in MEM-10 (+Trp); MEM-10 containing IFN-γ (IFN); MEM-10 containing IFN-γ supplemented with 10 μg mL^{-1} indole (IFN+Ind); MEM-10 containing IFN-γ supplemented with 1 mg mL^{-1} tryptophan (IFN+Trp); MEM-10 dialyzed FCS lacking tryptophan (-Trp) or lacking tryptophan but supplemented with 10 μg mL^{-1} indole (-Trp+Ind). Transcript levels were quantitated as described in the legend to Fig. 8. a) *tyrP.1*; b) *tyrP.2*.

A


tyrP.1
Copy number x 10^4
600
500
400
300
200
100
0
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

B

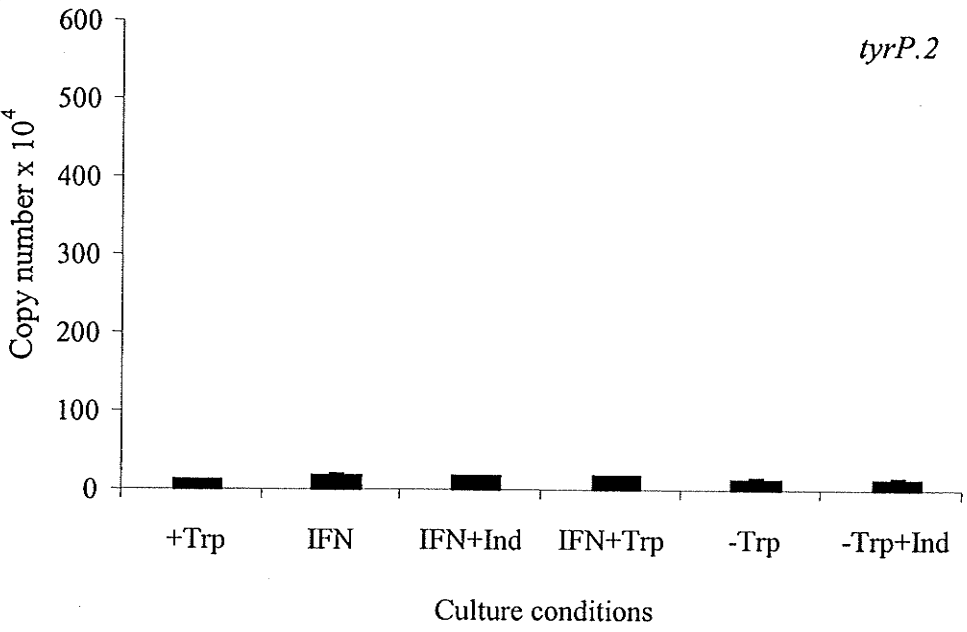
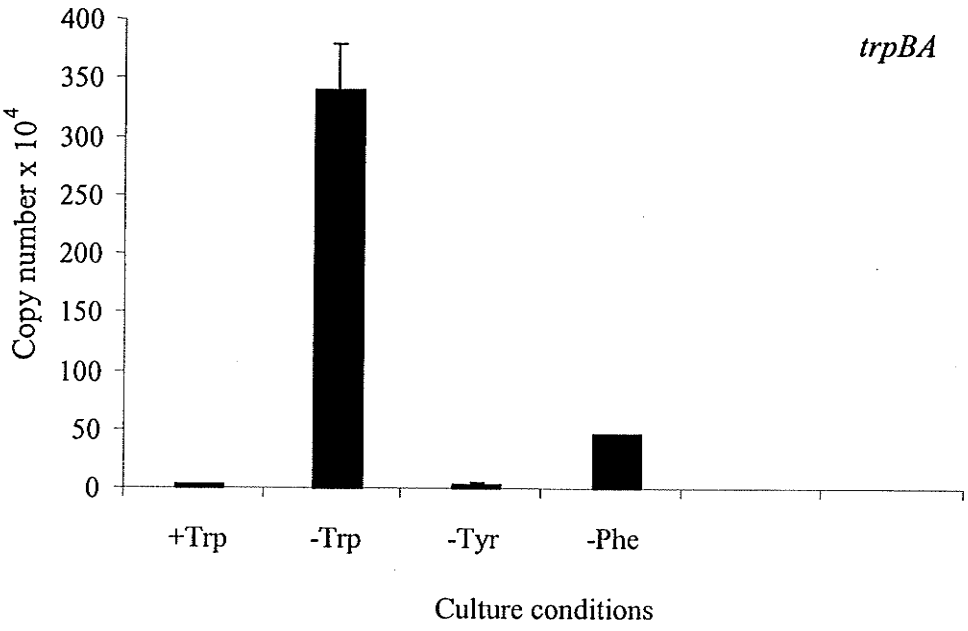
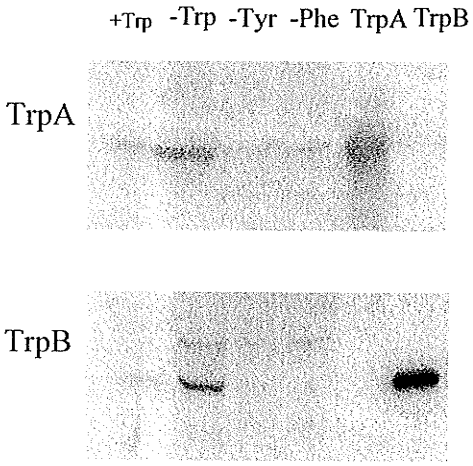

600
500
400
300
200
100
0
Copy number x 10^4
tyrP.2
+Trp
IFN
IFN+Ind
IFN+Trp
-Trp
-Trp+Ind
Culture conditions

Fig. 14. Effect of tyrosine or phenylalanine limitation on *trp* gene expression and growth of *C. trachomatis* serovar L2. HeLa cells were infected with *C. trachomatis* serovar L2 EBs at an MOI of 3-5 IFU $cell^{-1}$ in MEM-10 dialyzed FCS lacking tryptophan (W), tyrosine (Y) or phenylalanine (F). Where indicated, cultures were supplemented with the missing amino acid. A) Quantitative RT-PCR analysis of *trpBA* gene expression. Transcript levels were quantitated as described in the legend to Fig. 8. B) Expression of TrpA and TrpB. At 48 h post-infection, the infected HeLa cells were washed with HBSS and detached by gentle scraping. TrpA and TrpB were analyzed as described in the legend to Fig. 8. C) Growth of *C. trachomatis* serovar L2. After 48 h, infected cells and culture supernatants were collected and used to infect a fresh HeLa cell monolayer for enumeration of recoverable IFU. Data are presented as the mean IFU (log_{10}) of triplicate determinations from three separate experiments. The S.E. of any determination never exceeded $0.5log_{10}$.

A


trpBA
Copy number x 10^4
400
350
300
250
200
150
100
50
0
+Trp
-Trp
-Tyr
-Phe
Culture conditions

B



C

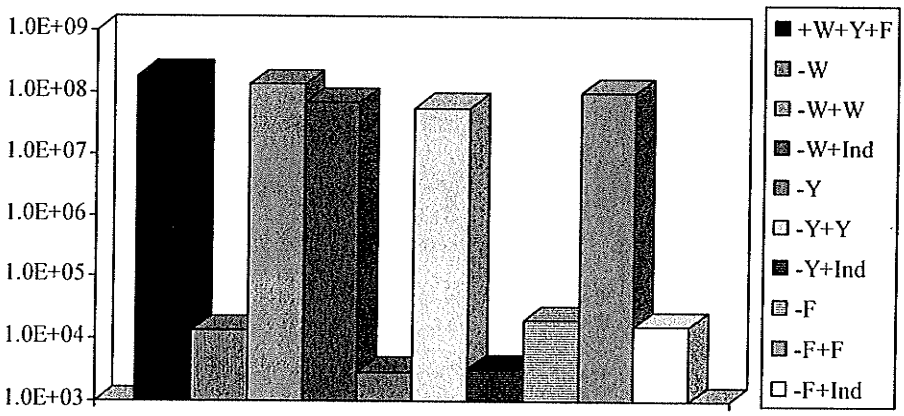

1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
+W+Y+F
-W
-W+W
-W+Ind
-Y
-Y+Y
-Y+Ind
-F
-F+F
-F+Ind

Fig. 15. Effect of tyrosine or phenylalanine limitation on *trpR* gene expression. HeLa cells were infected with *C. trachomatis* serovar L2 EBs at an MOI of 3-5 IFU $cell^{-1}$ in MEM-10 dialyzed FCS lacking tryptophan (W), tyrosine (Y) or phenylalanine (F). Where indicated, cultures were supplemented with the missing amino acid. Transcript levels were quantitated as described in the legend to Fig. 8.

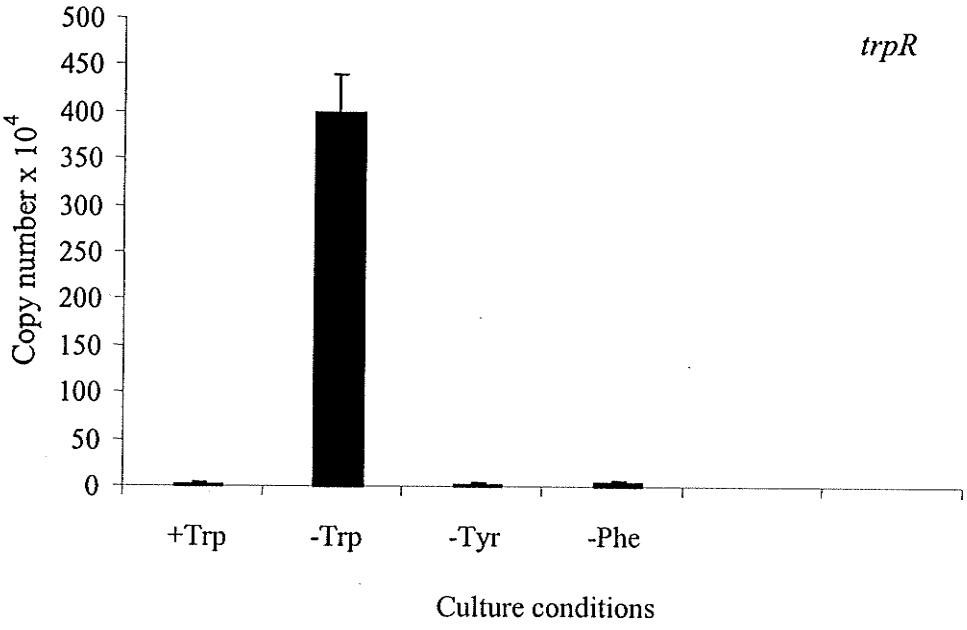
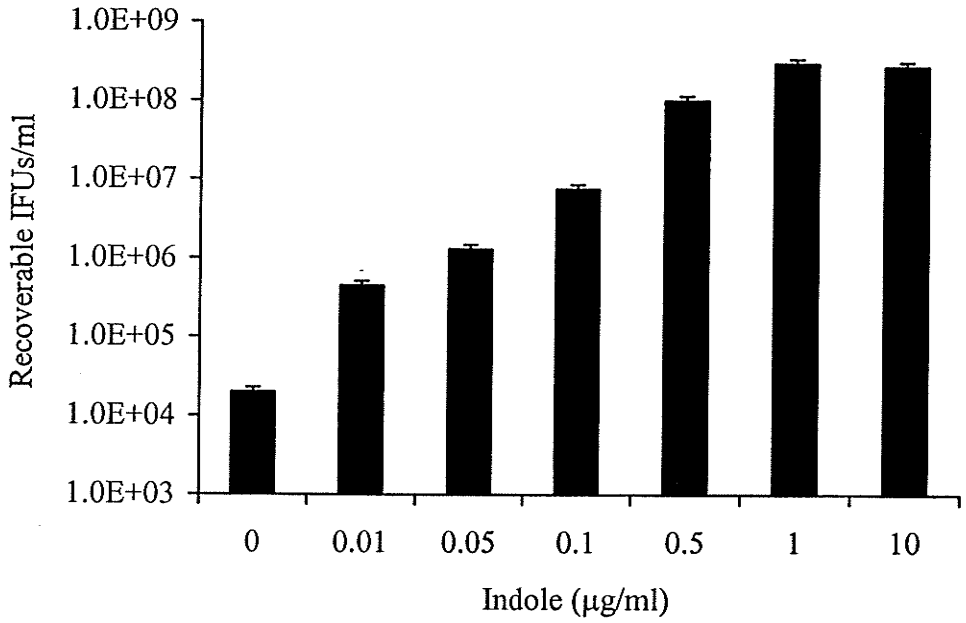
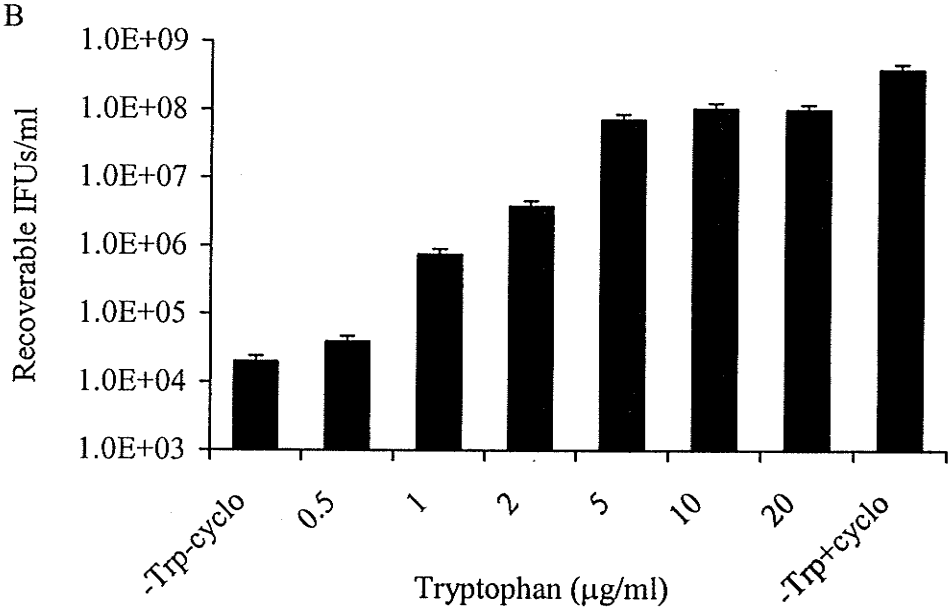

trpR
Copy number x 10^4
500
450
400
350
300
250
200
150
100
50
0
+Trp
-Trp
-Tyr
-Phe
Culture conditions

Fig. 16. Effect of tryptophan and indole on the growth of *C. trachomatis* serovar L2. HeLa cells were infected with *C. trachomatis* serovar L2 EBs in MEM-10 dialyzed FCS supplemented with the specified concentrations of a) indole or b) tryptophan, or MEM-10 dialyzed FCS lacking tryptophan and cycloheximide (-Trp-cyclo), or lacking tryptophan but supplemented with 1 $\mu g\ mL^{-1}$ cycloheximide (-Trp+cyclo). Recoverable IFUs were determined as described in Materials and Methods. c) Effect of host competition for tryptophan on *trpBA* gene expression. Transcript levels were quantitated as described in the legend to Fig. 8.

A


1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
Recoverable IFUs/ml
0
0.01
0.05
0.1
0.5
1
10
Indole (µg/ml)

B


1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
Recoverable IFUs/ml
-Trp-cyclo
0.5
1
2
5
10
20
-Trp+cyclo
Tryptophan (µg/ml)

C

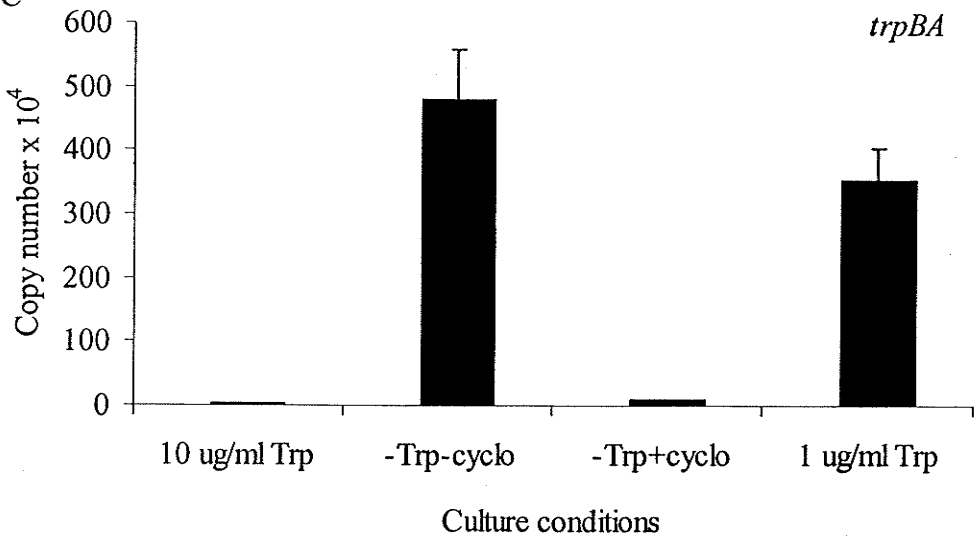

trpBA
600
500
400
300
200
100
0
Copy number x 10^4
10 ug/ml Trp
-Trp-cyclo
-Trp+cyclo
1 ug/ml Trp
Culture conditions

Fig. 17. Transmission electron micrographs of *C. trachomatis* serovar L2. HeLa cells were infected with *C. trachomatis* serovar L2 EBs in MEM-10 dialyzed FCS lacking tryptophan but supplemented with a) 0.05 μg mL^{-1} indole, b) 0.5 μg mL^{-1} tryptophan, c) 50 μg mL^{-1} 5-fluoroindole or d) 10 μg mL^{-1} tryptophan as a control . Infected cells were processed for transmission electron microscopy 24 h (D) or 48 h post-infection (A-C) as the aberrant chlamydiae require a longer incubation period to develop.

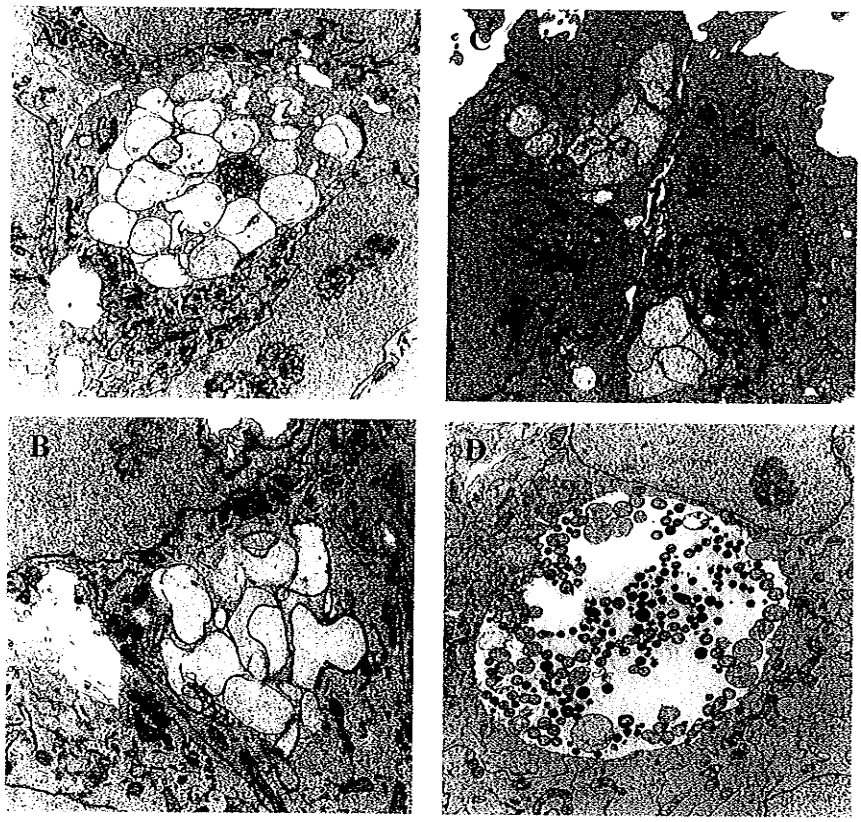
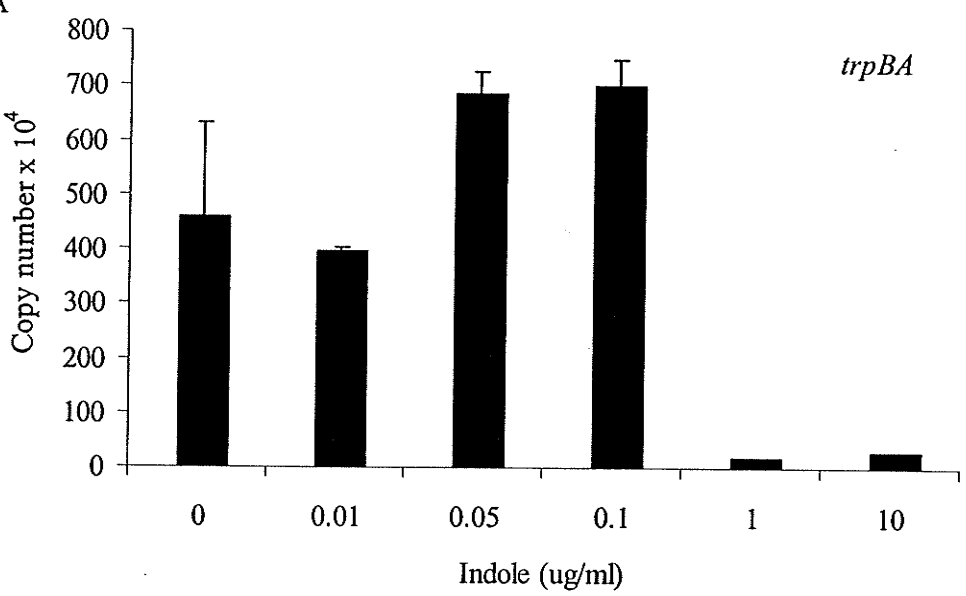
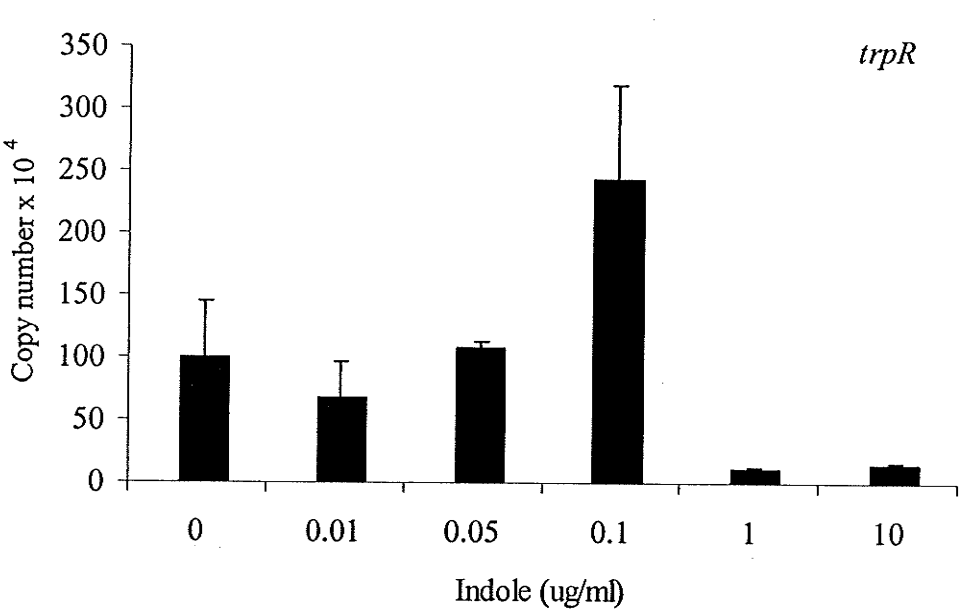

A
C
B
D

Fig. 18. *C. trachomatis* serovar L2 *trp* gene expression in the presence of indole. HeLa cells were infected with *C. trachomatis* serovar L2 EBs in MEM-10 dialyzed FCS lacking tryptophan but supplemented with the indicated concentrations of indole. Transcript levels were quantitated as described in the legend to Fig. 8. a) *trpBA*. b) *trpR*. c) Expression of TrpA and TrpB. HeLa cells were infected with *C. trachomatis* serovar L2 EBs MEM-10 dialyzed FCS lacking tryptophan but supplemented with the indicated concentrations of tryptophan or indole. TrpA and TrpB were analyzed as described in the legend to Fig. 8.

A


trpBA
Copy number x 10^4
800
700
600
500
400
300
200
100
0
0
0.01
0.05
0.1
1
10
Indole (ug/ml)

B


trpR
Copy number x 10^4
350
300
250
200
150
100
50
0
0
0.01
0.05
0.1
1
10
Indole (ug/ml)

C

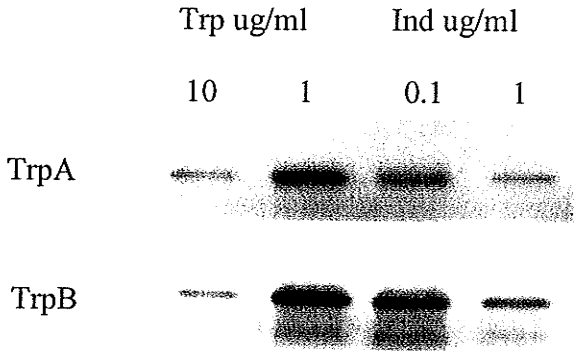
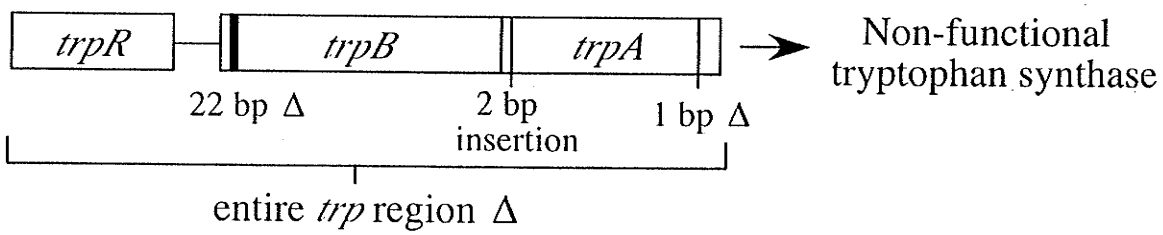


Fig. 19. a) Alignment of partial sequences from the *trpA* gene from the 14 human *C. trachomatis* reference serovars. A ClustalW alignment of the nucleotide regions containing sequence polymorphisms is illustrated. As compared with genital serovars, ocular serovars have a three-base (nucleotides 408-410) deletion that results in the loss of a phenylalanine. The various serovars have been grouped, in accordance with their nucleotide mutational "hotspot" sequence. The ocular serovars have a single base-deletion (nucleotide 528) resulting in a non-functional truncated TrpA protein. Genital serovar specific missense mutations (nucleotides 530 and 532) that result in amino acid changes in loop6 of TrpA are shown below the nucleotide sequence. b) Schematic summary of the tryptophan synthase inactivating mutations identified in clinical ocular serovars and the missense mutations identified in the clinical genital serovars.

Laboratory Reference Serovars

```
A_A   ...CC---TTTTGAAGAT 417   ...GAACACGCAAGAGGCCTTCTGTATTATATCCCATA-CAAGCTACGAGAGATTCTGAAGTAG 552
A_Ba  ...CC---TTTTGAAGAT 417   ...GAACACGCAAGAGGCCTTCTGTATTATATCCCATA-CAAGCTACGAGAGATTCTGAAGTAG 552
A_C   ...CC---TTTTGAAGAT 417   ...GAACACGCAAGAGGCCTTCTGTATTATATCCCATA-CAAGCTACGAGAGATTCTGAAGTAG 552
        ΔF                                                    deletion                    stop

A_G   ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATATCAAGCTACGAGAGATTCTGAAGTAG 556
A_F   ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATATCAAGCTACGAGAGATTCTGAAGTAG 556
A_I   ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATATCAAGCTACGAGAGATTCTGAAGTAG 556
A_H   ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATATCAAGCTACGAGAGATTCTGAAGTAG 556
A_J   ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATATCAAGCTACGAGAGATTCTGAAGTAG 556
        F                                                     Y  Q  Group 1

A_D   ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATGTCAAGCTACGAGAGATTCTGAAGTAG 556
A_K   ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATGTCAAGCTACGAGAGATTCTGAAGTAG 556
A_E   ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATGTCAAGCTACGAGAGATTCTGAAGTAG 556
        F                                                     C  Q  Group 2

A_L1  ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATATGAAGCTACGAGAGATTCTGAAGTAG 556
A_L2  ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATATGAAGCTACGAGAGATTCTGAAGTAG 556
A_L3  ...CCATTTTTTGAAGAT 420   ...GAATACGCAAGAGGCTTTCTGTATTATATCCCATATGAAGCTACGAGAGATTCTGAAGTAG 556
        F                                                     Y  E  Group 3
```

Clinical Ocular Serovars (A-C, Ba)



Clincal genital Serovars (B, D-K, LGV)

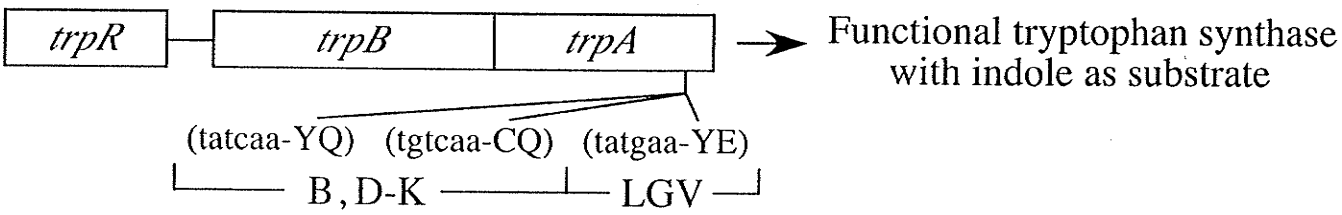


Fig. 20. Growth curves for *C. trachomatis* serovar A clinical (2497) isolate and reference serovar A. HeLa cells were infected with *C. trachomatis* serovar A clinical or reference strains in MEM-10 with or without 1 μg mL^{-1} cycloheximide. Recoverable IFUs were determined as described in Materials and Methods.

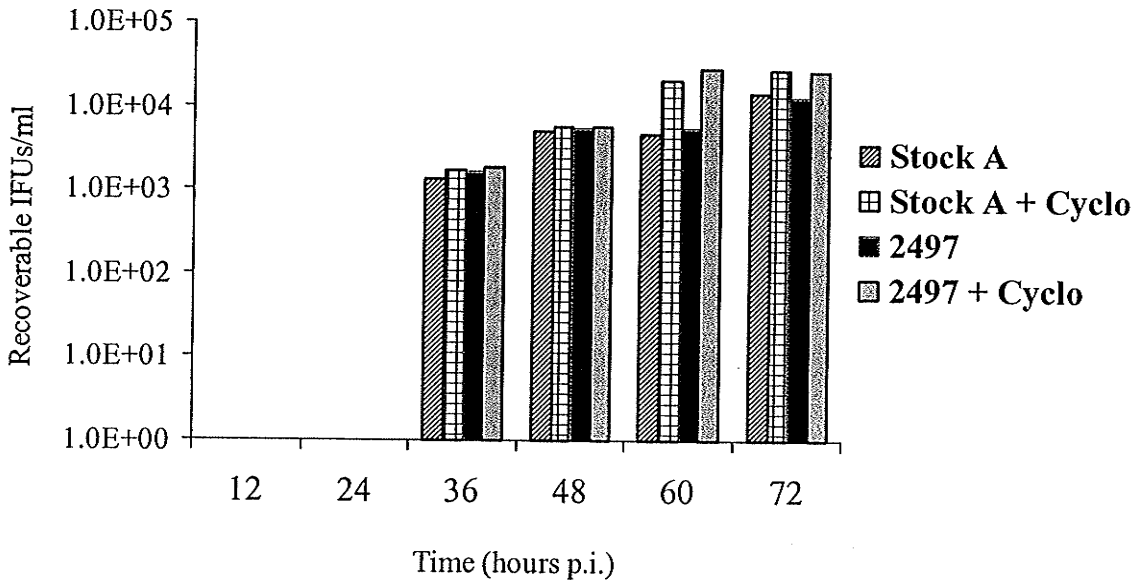

1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Recoverable IFUs/ml
12
24
36
48
60
72
Time (hours p.i.)
Stock A
Stock A + Cyclo
2497
2497 + Cyclo

Fig. 21. Effect of IFN-γ on the growth of *C. trachomatis* ocular clinical A (2497) and reference serovar A. HeLa cells were infected with the serovar A strains in MEM-10 supplemented with increasing concentrations of IFN-γ. Recoverable IFUs were determined as described in Materials and Methods.

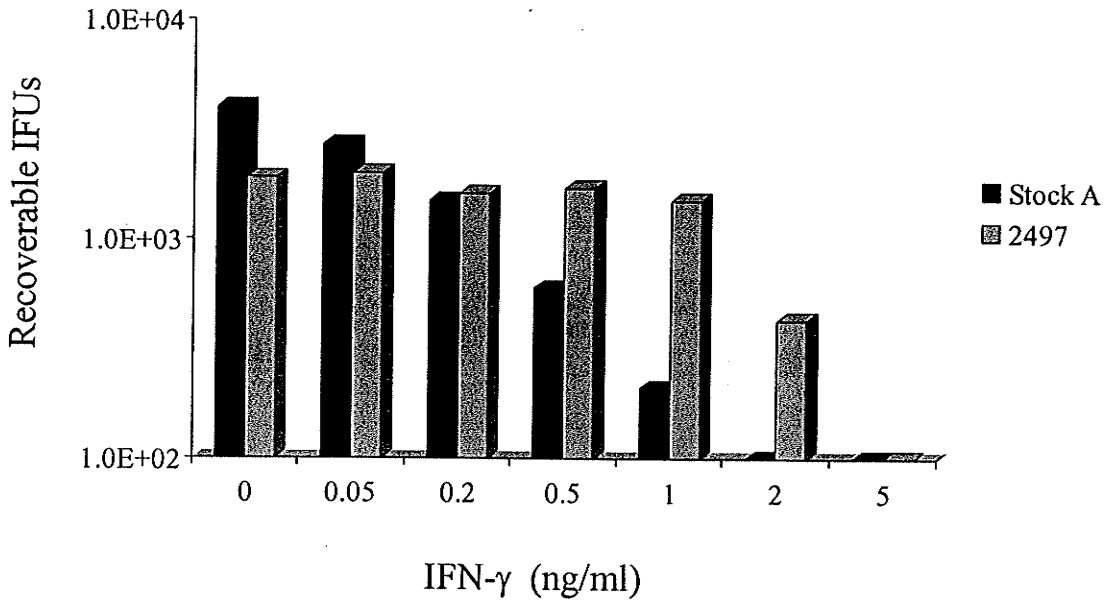
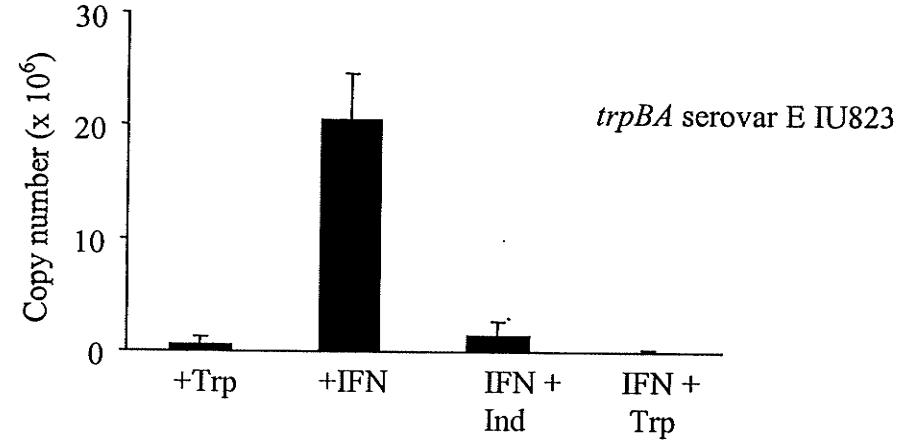
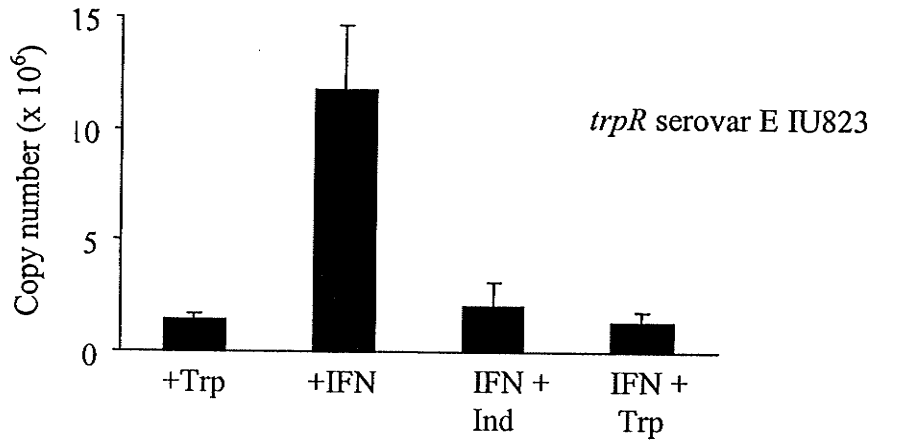

1.0E+04
1.0E+03
1.0E+02
Recoverable IFUs
0
0.05
0.2
0.5
1
2
5
IFN-γ (ng/ml)
Stock A
2497

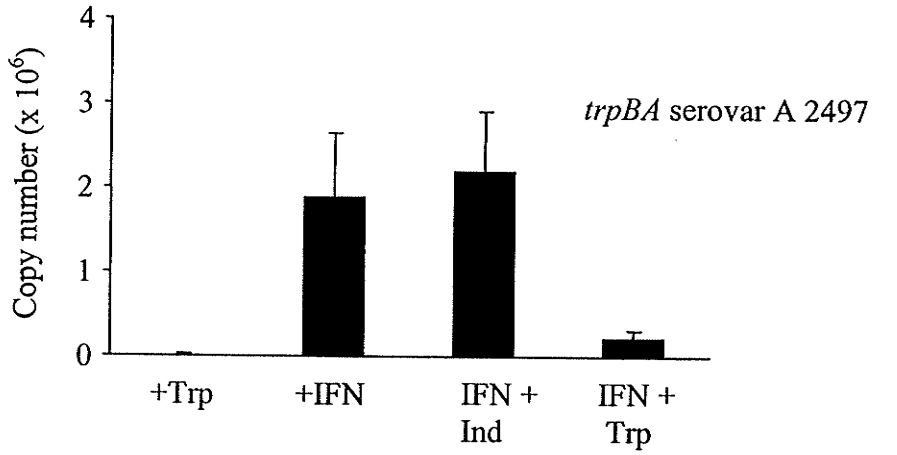
Fig. 22. RT-PCR quantitation of *trpBA* and *trpR* mRNA isolated from HeLa cell monolayers infected with *C. trachomatis* ocular (serovar A 2497) and genital (serovars E IU823) clinical isolates cultured in the presence of IFN-γ. Culture and chlamydiae infection conditions were as described in the legend to Figure 8. Infected HeLa cells were cultured in the presence of complete DMEM-10 (+Trp), complete DMEM-10 plus 5 ng/ml IFN-γ (+IFN), complete DMEM plus 5ng/ml IFN-γ and 100 μM indole (IFN+Ind), and complete DMEM-10 plus 5 ng/ml IFN-γ and supplemented with 1 g L^{-1} tryptophan (IFN+Trp). RNA was isolated 36 h after infection. The RNA was reverse-transcribed using random hexamer primers, and the cDNA was used for quantitative PCR amplifications with primers specific for *trpBA* or *trpR*. Quantitative PCR reactions were carried out using the LightCycler and SYBR Green I as the fluorophore. See Materials and Methods for details.



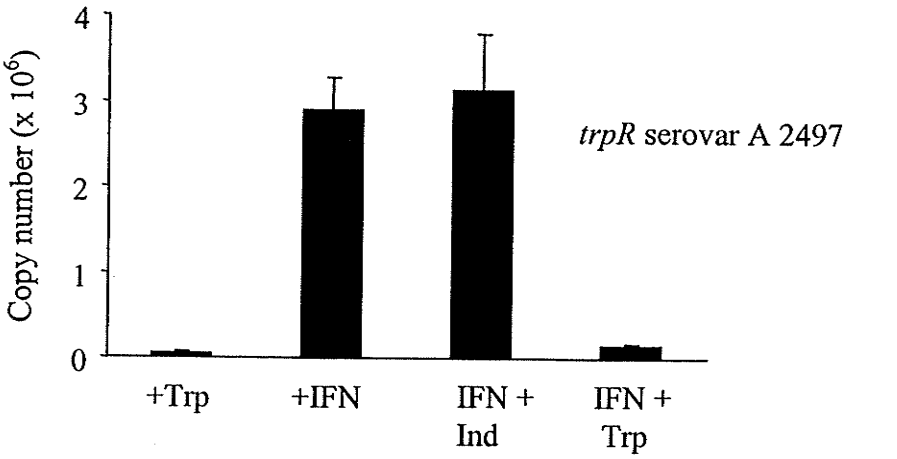
trpBA serovar E IU823



trpR serovar E IU823



trpBA serovar A 2497



trpR serovar A 2497

Fig. 23. Western blot analysis of TrpA and TrpB expression in HeLa cell monolayers infected with *C. trachomatis* ocular clinical isolate serovar A 2497 or genital clinical isolate serovar E IU823 cultured in the presence of IFN-γ. Infected HeLa cells were cultured in the presence of complete DMEM-10 (+Trp), complete DMEM-10 plus 5 ng/ml IFN-γ (+IFN), complete DMEM plus 5ng/ml IFN-γ and 100 μM indole (IFN+Ind), and complete DMEM-10 plus 5 ng/ml IFN-γ and supplemented with 1 g L^{-1} tryptophan (IFN+Trp). Infected cells were harvested 48 h after infection, sonicated and chlamydial particles were semi-purified over a 30% MD-76 cushion. The resulting pellet was lysed in Laemmli sample buffer, and proteins were separated by SDS-PAGE (12%) and then transferred to nitrocellulose. Proteins were detected using polyclonal antibodies raised against recombinant L2 TrpB or TrpA. The respective recombinant proteins (Recomb) were included as positive controls.

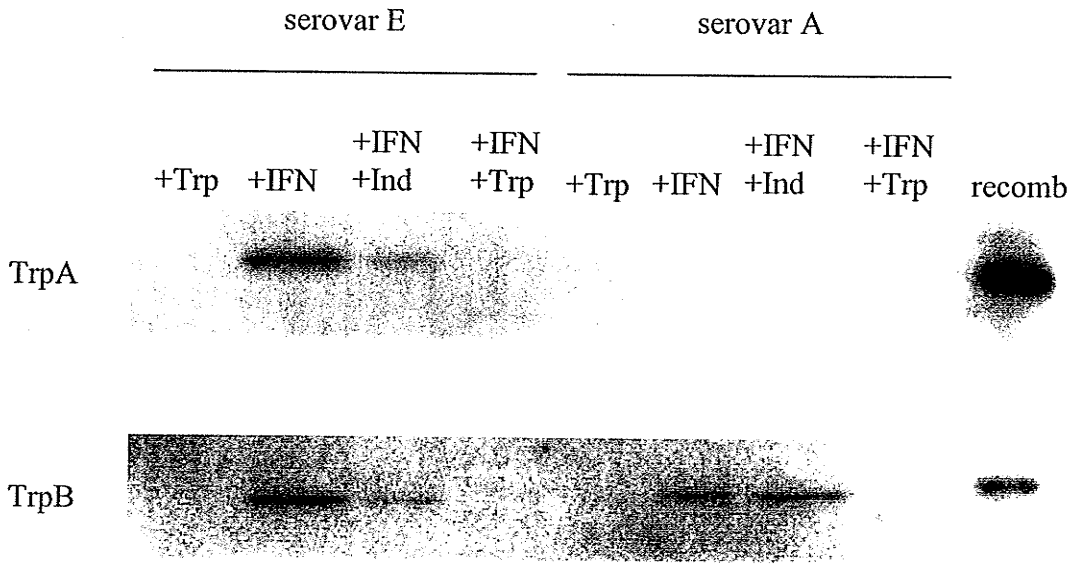

serovar E
serovar A
+Trp
+IFN
+IFN +Ind
+IFN +Trp
+Trp
+IFN
+IFN +Ind
+IFN +Trp
recomb
TrpA
TrpB

Fig. 24. Effect of indole or tryptophan on the growth of *C. trachomatis* ocular (serovar A 2497) and genital (serovars E IU823) clinical isolates cultured in the presence of IFN-γ. Culture and chlamydiae infection conditions were as described in the legend to Figure 8. Infected HeLa cells were cultured in the presence of complete DMEM-10 (+Trp), complete DMEM-10 plus 5 ng/ml IFN-γ (+IFN), complete DMEM plus 5ng/ml IFN-γ and 100 μM indole (IFN+Ind), and complete DMEM-10 plus 5 ng/ml IFN-γ and supplemented with 1 g L^{-1} tryptophan (IFN+Trp). After 72 h, cultures were harvested and recoverable IFUs were enumerated. Data are presented as IFUs (log_{10}) and represent the means ± SD of triplicate determinations.

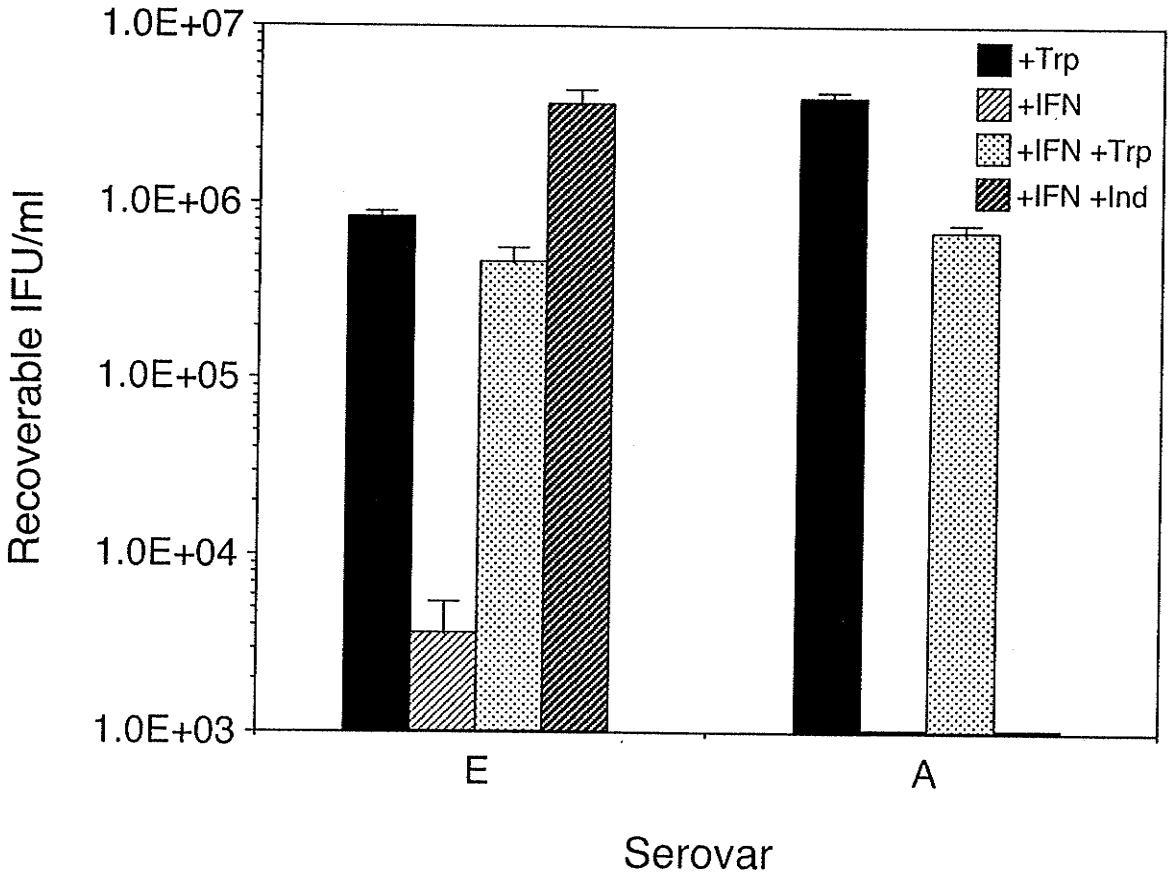
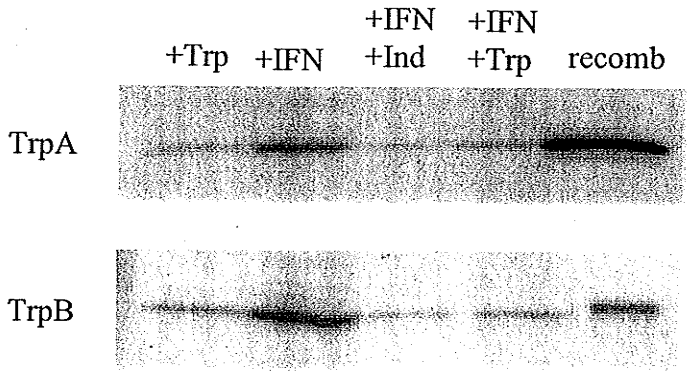

1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
Recoverable IFU/ml
+Trp
+IFN
+IFN +Trp
+IFN +Ind
E
A
Serovar

Fig. 25. a) Western blot analysis of TrpA and TrpB expression in HeLa cell monolayers infected with a *C. trachomatis* clinical B isolate. Cell culture conditions, harvesting, chlamydial protein sample preparation and Western blot procedure were as described in the legend to Figure 17. b) Effect of indole or tryptophan on the growth of *C. trachomatis* genital serovar B clinical isolate cultured in the presence of IFN-γ. Cell culture conditions, harvesting at 72 h post infection and enumeration of recoverable infectious chlamydial EBs were as described in the legend to Fig. 8.

A


+Trp
+IFN
+IFN +Ind
+IFN +Trp
recomb
TrpA
TrpB

B

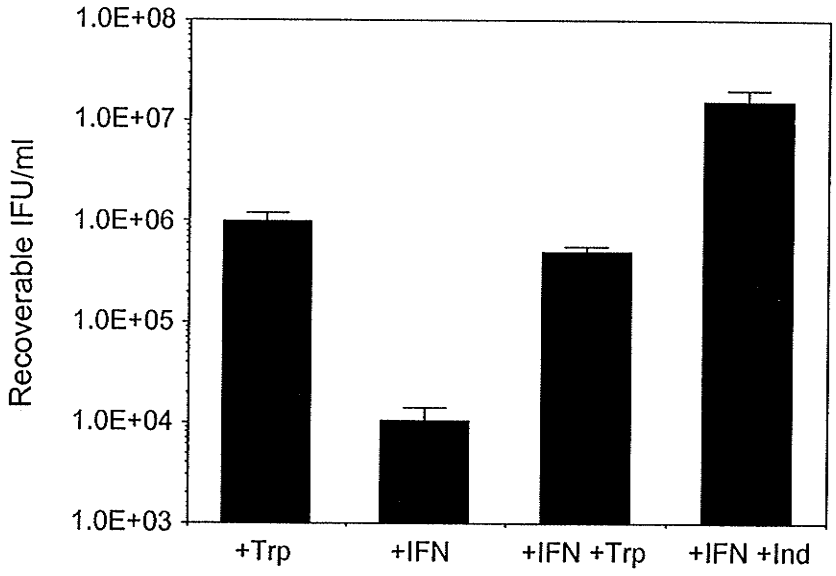
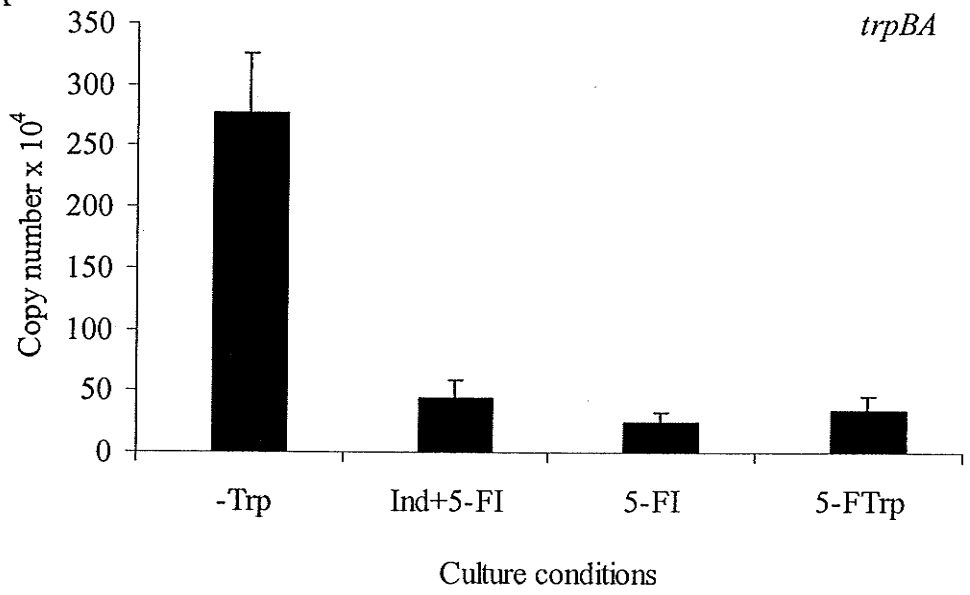

Recoverable IFU/ml
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
+Trp
+IFN
+IFN +Trp
+IFN +Ind

Fig. 26. Effect of 5-fluoroindole and 5-fluorotryptophan on *trpBA* gene expression and growth of *C. trachomatis* serovar L2. a) Quantitative RT-PCR analysis of *trp* gene expression. HeLa cells were infected with *C. trachomatis* serovar L2 EBs in MEM-10 (+Trp); MEM-10 dialyzed FCS containing 1 μg mL^{-1} indole and 10 μg mL^{-1} 5-fluoroindole (Ind+5-FI); or MEM-10 dialyzed FCS containing 10 μg mL^{-1} 5-fluoroindole (5-FI) or 10 μg mL^{-1} 5-fluorotryptophan (5-FTrp). Transcript levels were quantitated as described in the legend to Fig. 8. b) Growth of serovar L2 in the presence of 5-fluoroindole. HeLa cells were infected in MEM containing 10 μg mL^{-1} tryptophan and increasing concentrations of 5-fluoroindole or 1 μg mL^{-1} indole and increasing concentrations of 5-fluoroindole. Recoverable IFUs were determined as described in Materials and Methods.

A


trpBA
Copy number x 10^4
350
300
250
200
150
100
50
0
-Trp
Ind+5-FI
5-FI
5-FTrp
Culture conditions

B

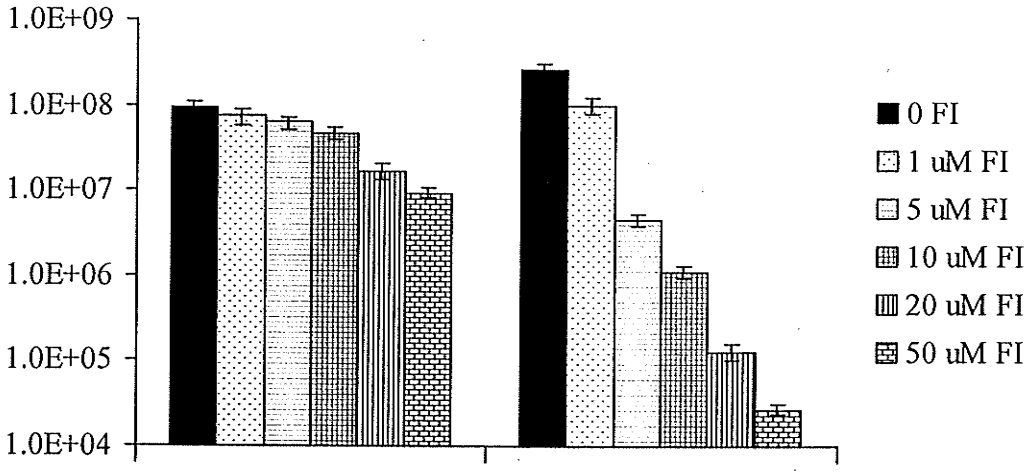

1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
0 FI
1 uM FI
5 uM FI
10 uM FI
20 uM FI
50 uM FI

Fig. 27. Effect of indole and tryptophan on the growth of *C. trachomatis* serovar L2 cultured in the presence of 5-fluorotryptophan. HeLa cells were infected with EBs in MEM-10 dialyzed FCS supplemented with 10 μg mL^{-1} tryptophan (+Trp) or 1 μg mL^{-1} indole (Ind). HeLa cells were also infected in the presence of 1 μg ml^{-1} 5-fluorotryptophan (5-FTrp) alone or further supplemented with 1 μg mL^{-1} indole, 10 μg mL^{-1} indole, 10 μg mL^{-1} tryptophan or 100 μg mL^{-1} tryptophan. Recoverable IFUs were determined as described in Materials and Methods.

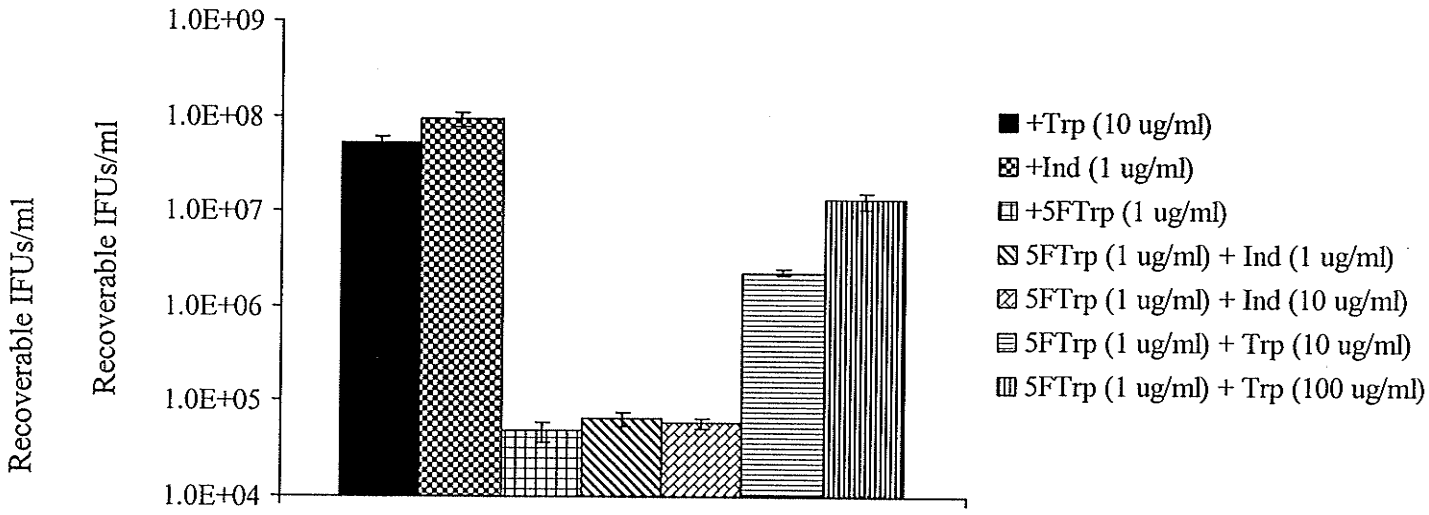
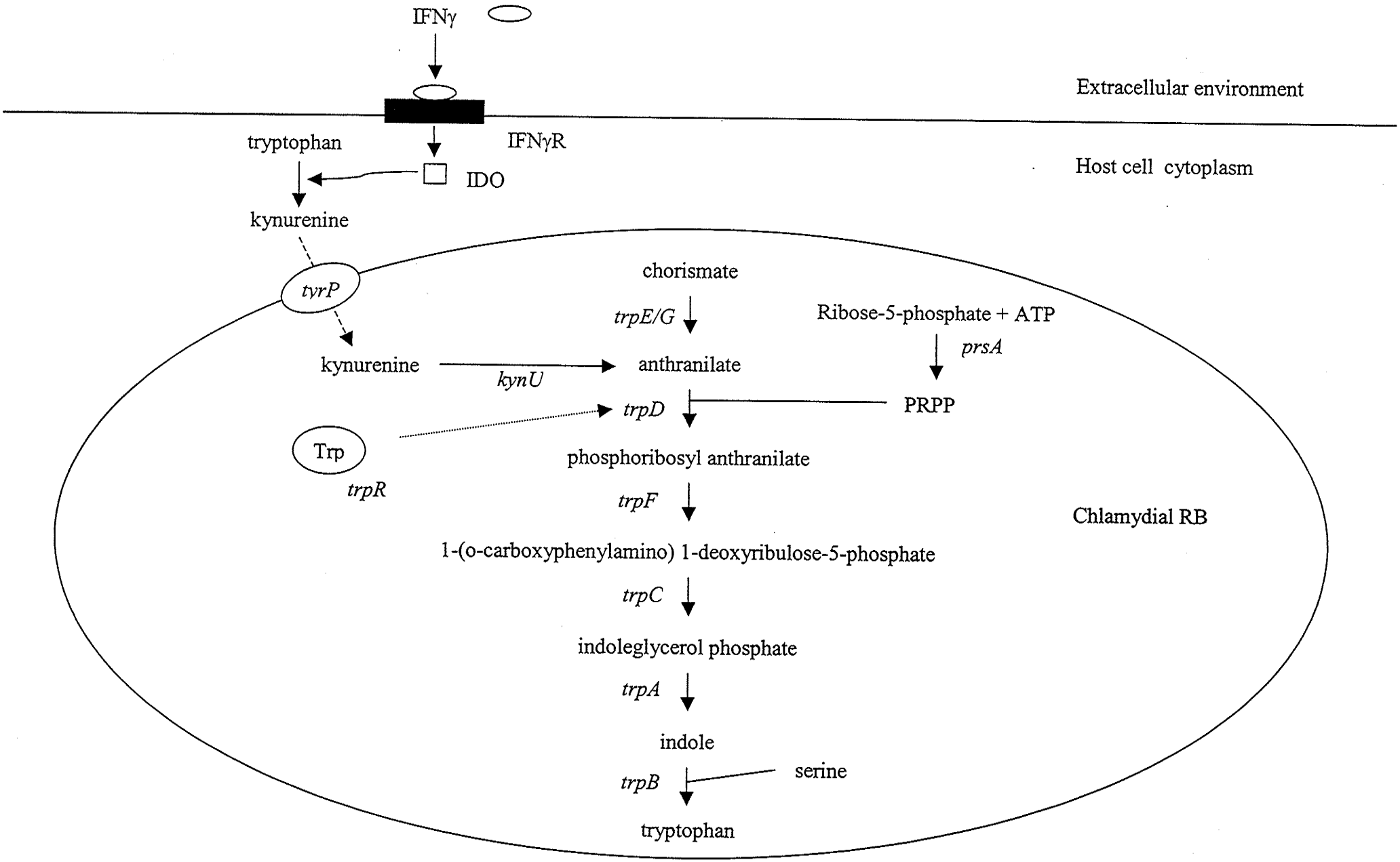
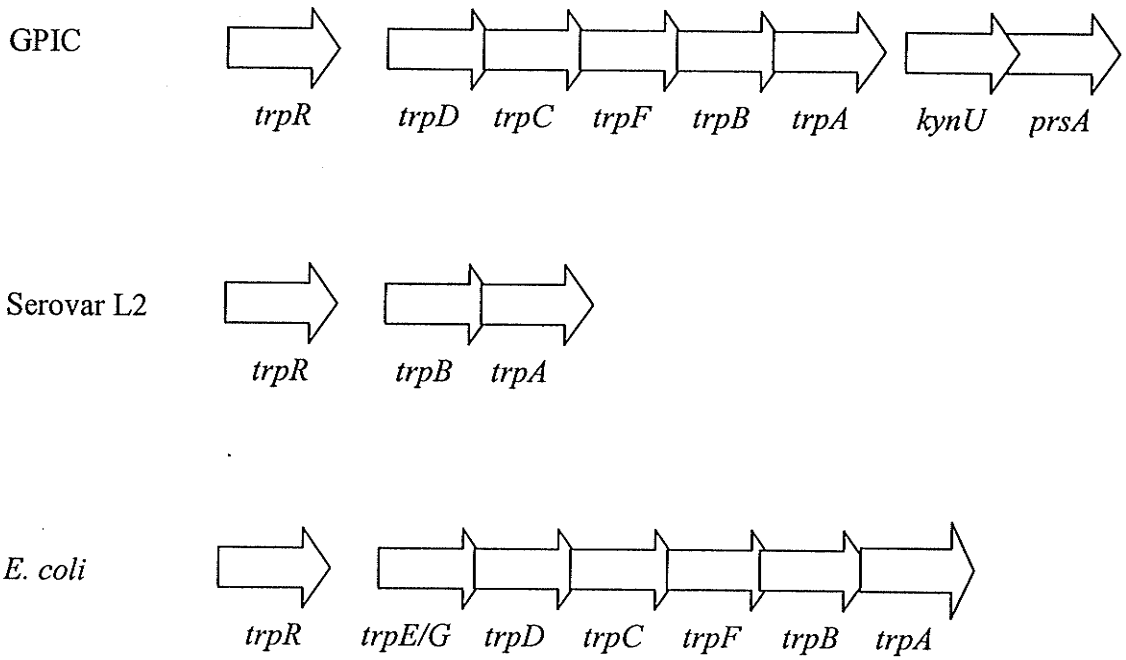
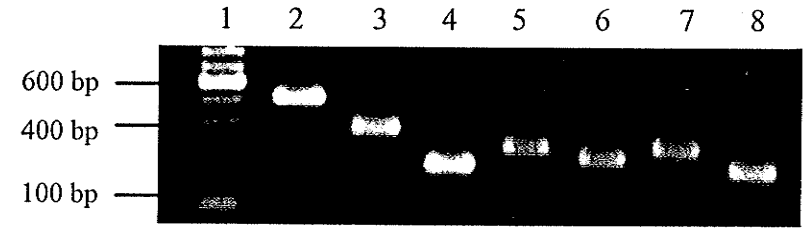

Recoverable IFUs/ml
Recoverable IFUs/ml
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
+Trp (10 ug/ml)
+Ind (1 ug/ml)
+5FTrp (1 ug/ml)
5FTrp (1 ug/ml) + Ind (1 ug/ml)
5FTrp (1 ug/ml) + Ind (10 ug/ml)
5FTrp (1 ug/ml) + Trp (10 ug/ml)
5FTrp (1 ug/ml) + Trp (100 ug/ml)

Fig. 28. Tryptophan biosynthesis in *C. psittaci* strain GPIC. a) Reaction scheme for the biosynthesis of tryptophan. *C. psittaci* strain GPIC encodes all genes with the exception of *trpE* and *trpG*, boxed reaction. b) Schematic diagram depicting gene order in the tryptophan biosynthesis operon of GPIC, *C. trachomatis* serovar L2, and *E. coli*. The *trpR* and *trpE-A* genes are not contiguous in *E. coli*. c) RT-PCR analysis of *trp* transcript expression. HeLa cells were infected with *C. psittaci* GPIC EBs at an MOI of 3-5 IFU $cell^{-1}$ in MEM-10 dialyzed FCS lacking tryptophan (-Trp). RNA was isolated 20 h post-infection and reverse transcribed using random hexamer primers. The cDNA was amplified with primers designed to overlap the junction between each pair of genes sequentially along the entire operon starting at *trpR* and ending at *prsA*. Lanes are as follows: 1) 100 bp ladder; 2) *trpR-D*; 3) *trpD-C*; 4) *trpC-F*; 5) *trpF-B*; 6) *trpB-A*; 7) *trpA-kynU*; 8) *kynU-prsA*. d) RT-PCR analysis of *trp* operon organization. HeLa cells were infected as described above in MEM-10 dialyzed FCS lacking tryptophan (-Trp). RNA was isolated and cDNA was amplified with primers overlapping the junction between *trpR* and *trpB* in serovar L2 or *trpR* and *trpD* in GPIC. Lanes are as follows: 1) 100 bp ladder; 2) –RT control; 3) serovar L2 cDNA; 4) serovar L2 DNA; 5) –RT control; 6) GPIC cDNA; 7) GPIC DNA.


IFNγ
Extracellular environment
IFNγR
tryptophan
IDO
Host cell cytoplasm
kynurenine
tyrP
chorismate
trpE/G
Ribose-5-phosphate + ATP
prsA
kynurenine
kynU
anthranilate
trpD
PRPP
Trp
trpR
phosphoribosyl anthranilate
trpF
Chlamydial RB
1-(o-carboxyphenylamino) 1-deoxyribulose-5-phosphate
trpC
indoleglycerol phosphate
trpA
indole
trpB
serine
tryptophan


GPIC
trpR
trpD
trpC
trpF
trpB
trpA
kynU
prsA
Serovar L2
trpR
trpB
trpA
E. coli
trpR
trpE/G
trpD
trpC
trpF
trpB
trpA



d

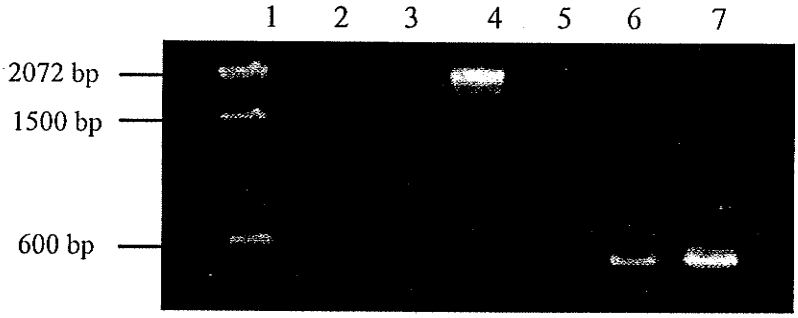
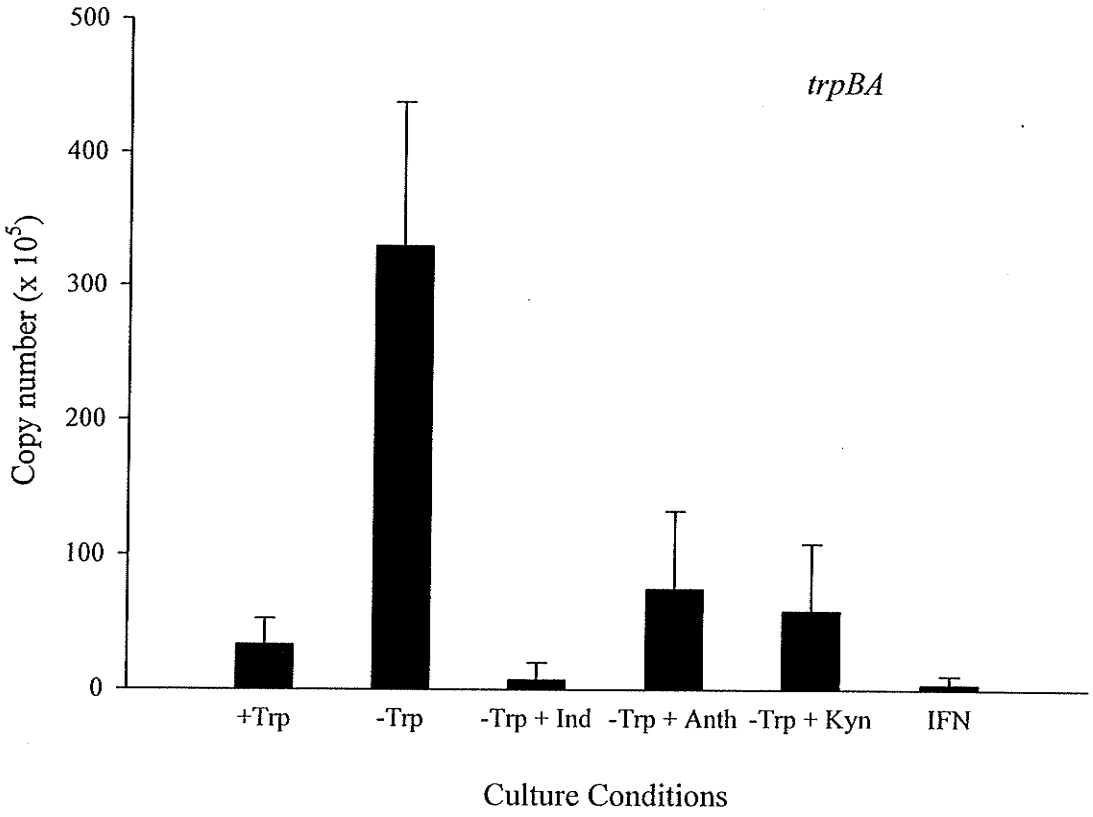


Fig. 29. Comparison of the *C. psittaci* GPIC, *C. trachomatis* serovar L2, and *E. coli* TrpA amino acid sequences. Critical conserved residues identified as necessary for subunit-to-subunit interaction with TrpB are in bold. Critical residues in the active/substrate binding site are in bold italics, while residues critical for catalytic activity are in bold underlined. See "Results" for details.

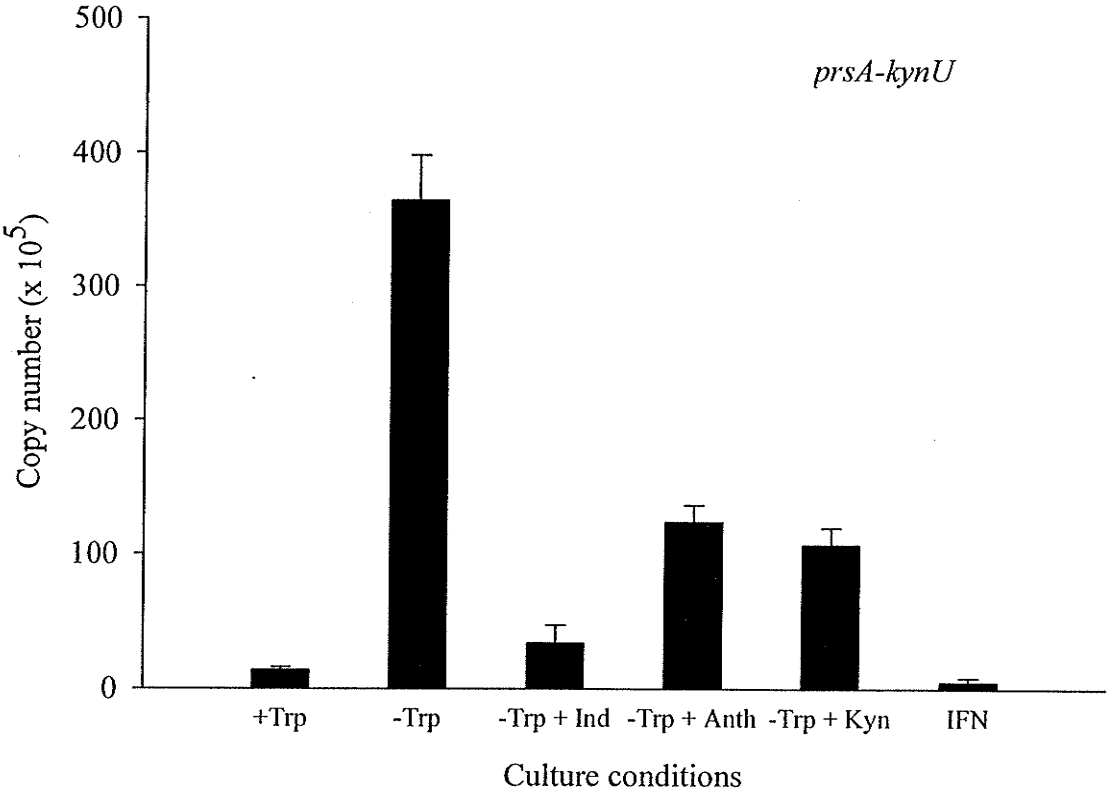
```
TRPA_GPIC       MNRIETAFKNTKP-----FIGYLTGGDGGFDYSVACAHALIRGGVDILEIGFPFSDPVAD 55
TRPA_L2         MSKLTQVFKQTKP-----CIGYLTAGDGGTSYTIEAAKALIQGGVDILELGFPFSDPVAD 55
TRPA_E.coli     MERYESLFAQLKERKEGAFVPFVTLGDPGIEQSLKIIDTLIEAGADALELGIPFSDPLAD 60
                *.:    * : *       : ::* ** * . ::   .:**..*.* **:*:*****:**

TRPA_GPIC       GPIIQKAHTRALEEKTDSTTILEIAKALRET-SNIPLVLFSYYNPLLQKGP-QYLHQLKA 113
TRPA_L2         NPEIQVSHDRALAENLTSETLLEIVEGIRAFNQEVPLILYSYYNPLLQRDL-DYLRRLKD 114
TRPA_E.coli     GPTIQNATLRAFAAGVTPAQCFEMLALIRQKHPTIPIGLLMYANLVFNKGIDEFYAQCEK 120
                .* ** :  **:     .   :*:   :*     :*: *  * * ::::.  ::  : :

TRPA_GPIC       AGFDAVLIVDLPIPQHANESEPFFQALIEAKLFPIVLATPSTREERLLQIRKLAKGFLYY 173
TRPA_L2         AGINGVCVIDLPAPLSHGEKSPFFEDLLAVGLDPILLISAGTTPERMSLIQEYARGFLYY 174
TRPA_E.coli     VGVDSVLVADVPV----EESAPFRQAALRHNVAPIFICPPNADDDLLRQIASYGRGYTYL 176
                .*.:.* : *:*      *. ** :  :   : **.: ...:  : :  * . .:*: *

TRPA_GPIC       VSQKGTTGIRSKLSDDFSTQIARLRCYFQIPIVAGFGIANRASAAAALKH-ADGIVVGSA 232
TRPA_L2         IPYEATRDSEVGIKEEFR----KVREHFDLPIVDRRDICDKKEAAHVLNY-SDGFIVKTA 229
TRPA_E.coli     LSRAGVTGAENRAALPLNHLVAKLKEYNAAPPLQGFGISAPDQVKAAIDAGAAGAISGSA 236
                :.  . . . .      :     ::: :   * :   .*.   ..  .:.  : * :  :*

TRPA_GPIC       FVEKLEKKIS-----PEELTTFAQSIDPRQ-- 257
TRPA_L2         FVHQTTMDSS-----VETLTALAQTVIPG--- 253
TRPA_E.coli     IVKIIEQHINEPEKMLAALKVFVQPMKAATRS 268
                :*.    . .        *..:.*.: .
```

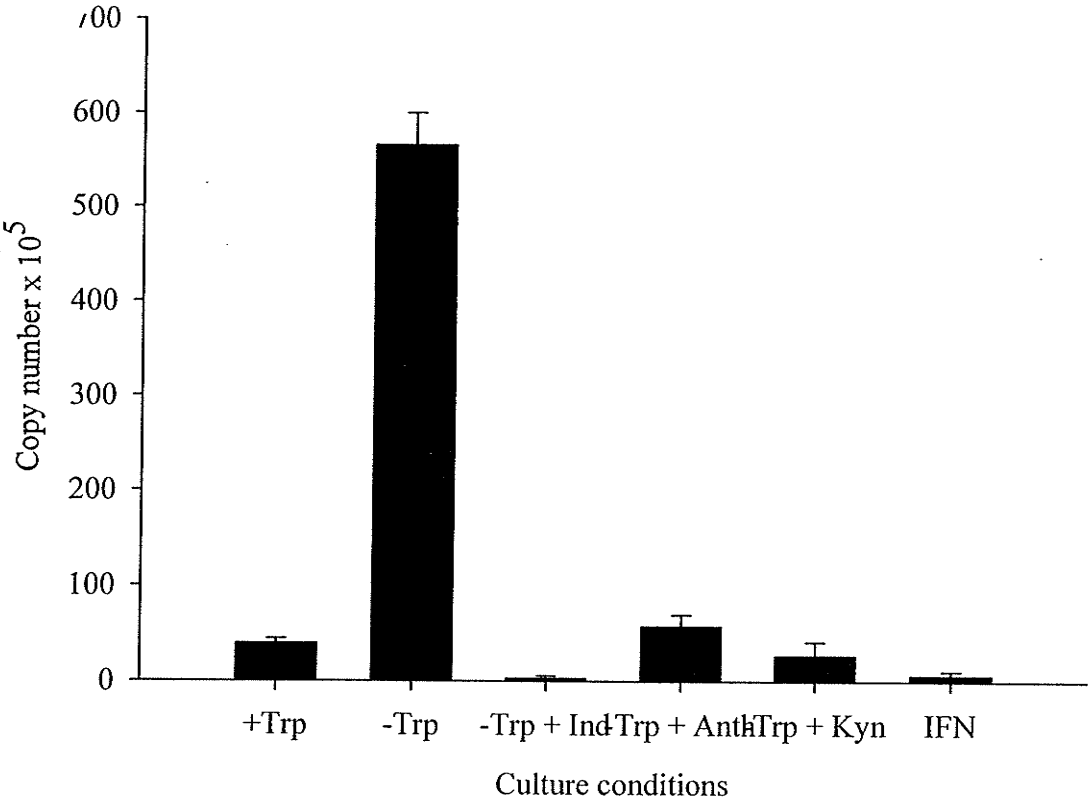
Fig. 30. Quantitative RT-PCR analysis of *trp* gene expression in *C. psittaci* strain GPIC. HeLa cells were infected with *C. psittaci* GPIC EBs at an MOI of 3-5 IFU $cell^{-1}$ in MEM-10 (+Trp); MEM-10 supplemented with 5 ng ml^{-1} IFN-γ; MEM-10 dialyzed FCS lacking tryptophan (-Trp) or lacking tryptophan but supplemented indole, anthranilate, or kynurenine. RNA was isolated 20 h post-infection and reverse transcribed using random hexamer primers. Results for each gene were normalized against the copy number of 16S rRNA transcripts in each cDNA preparation. cDNA was amplified with primers overlapping the junction between a) *trpA* and *trpB*; b) *prsA* and *kynU*; c) *trpD*; d) *trpC*; and e) *trpF*.

A

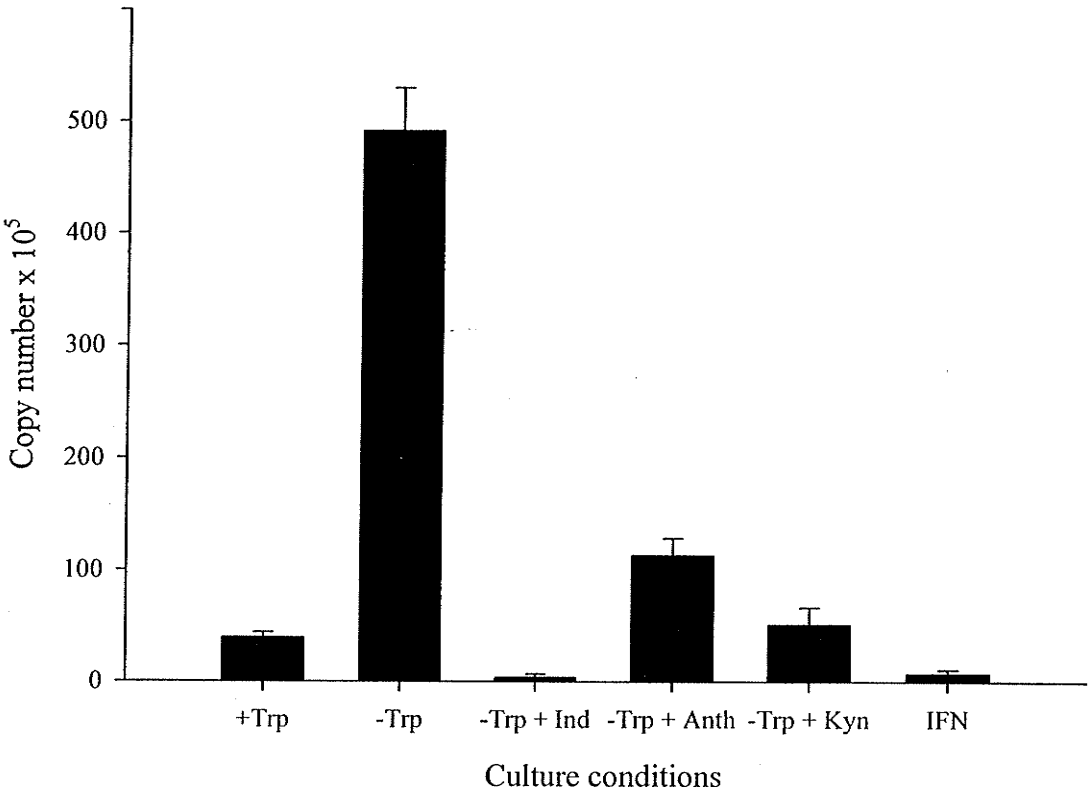

trpBA
Copy number (x 10^5)
500
400
300
200
100
0
+Trp
-Trp
-Trp + Ind
-Trp + Anth
-Trp + Kyn
IFN
Culture Conditions

B


prsA-kynU
Copy number (x 10^5)
500
400
300
200
100
0
+Trp
-Trp
-Trp + Ind
-Trp + Anth
-Trp + Kyn
IFN
Culture conditions


700
600
500
400
300
200
100
0
Copy number x 10^5
+Trp
-Trp
-Trp + Ind
-Trp + Anth
-Trp + Kyn
IFN
Culture conditions

D


Copy number x 10^5
500
400
300
200
100
0
+Trp
-Trp
-Trp + Ind
-Trp + Anth
-Trp + Kyn
IFN
Culture conditions

E

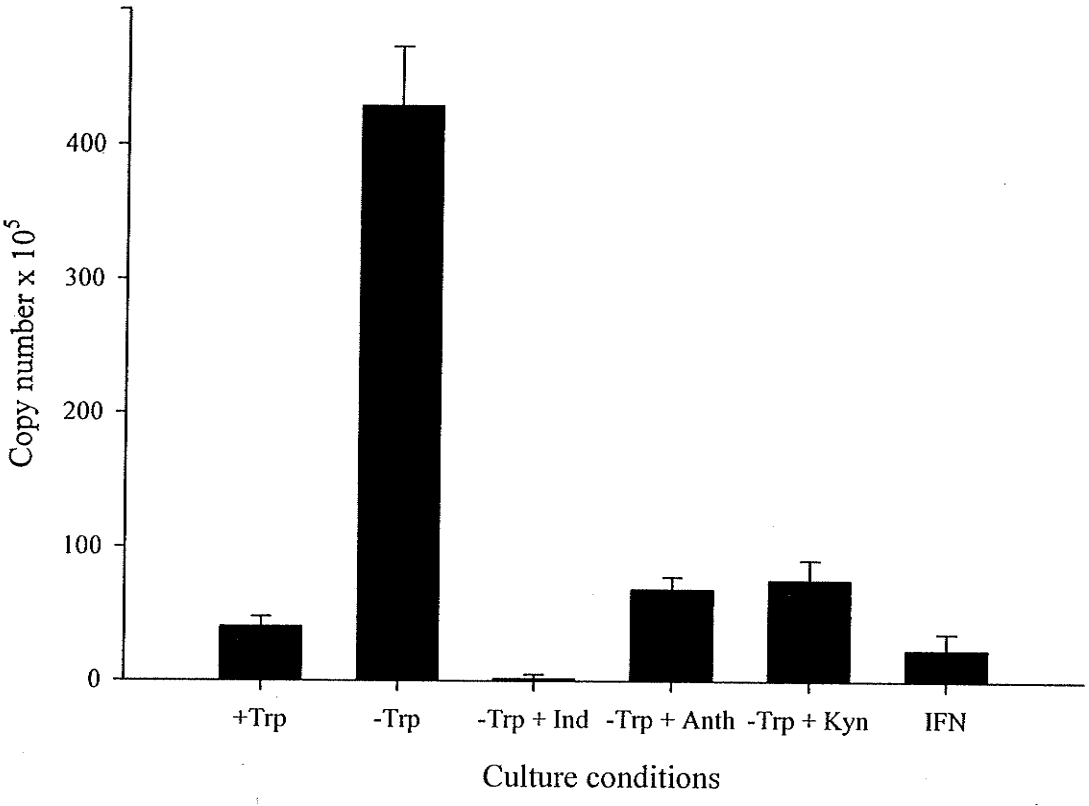
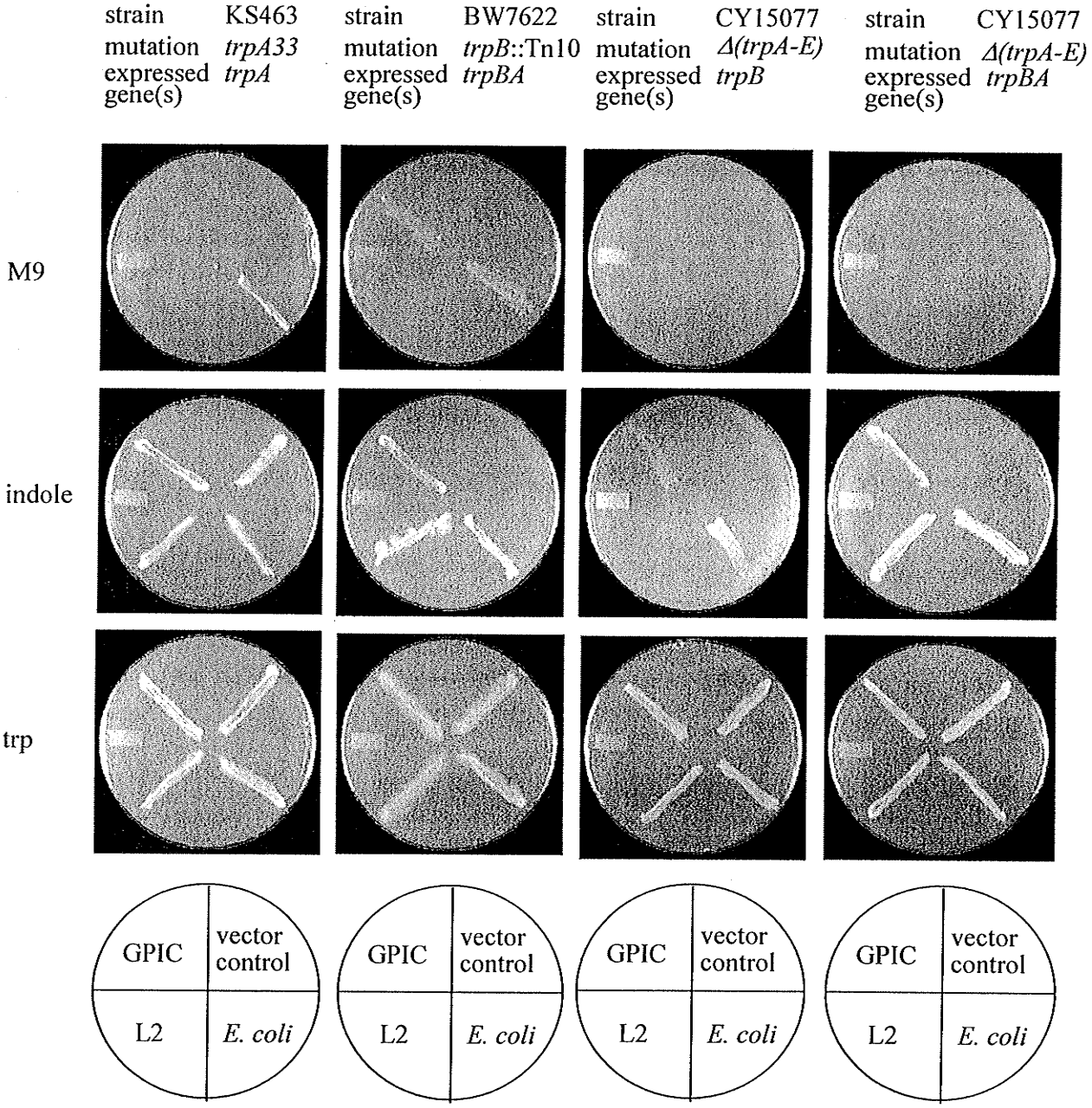
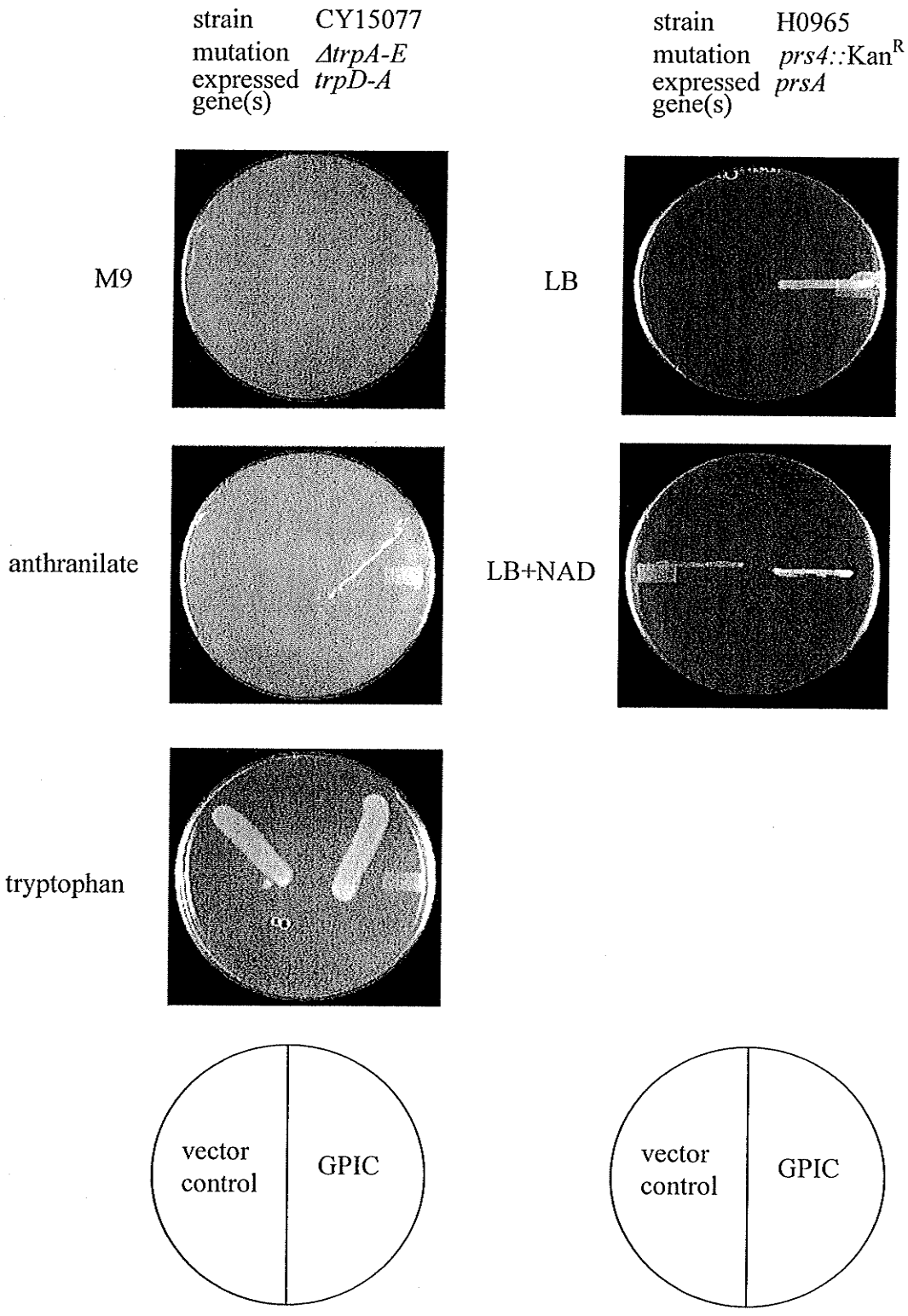

Copy number x 10^5
400
300
200
100
0
+Trp
-Trp
-Trp + Ind
-Trp + Anth
-Trp + Kyn
IFN
Culture conditions

Fig. 31. Analysis of *C. psittaci* strain GPIC and *C. trachomatis* serovar L2 Trp protein function by genetic complementation. The *trp* genes from *C. psittaci* GPIC and *C. trachomatis* serovar L2 were cloned into the *E. coli* expression vector pQE-80L either individually or together (*trpBA*, *trpD-A or trpD-kynU*). *E. coli trpA* and *trpB* were also cloned for use as controls. Constructs were transformed into *E. coli* mutants a) KS463 (*trpA33)*, BW7622 (*trpB*::Tn10), and CY15077 (Δ*trpE-A*), b) CY15077 (Δ*trpE-A*) and H0965 (*prs-4*::Kan^R) or c) CY15077 (Δ*trpE-A*) and T3D (*trpE61*), and growth of the transformants was assessed on M9 minimal agar containing 100 μM indole, 4 ug ml^{-1} anthranilate, 50 ug ml^{-1} tryptophan, 50 ug ml^{-1} kynurenine or no additional supplements.


strain KS463
mutation *trpA33*
expressed gene(s) *trpA*
strain BW7622
mutation *trpB*::Tn10
expressed gene(s) *trpBA*
strain CY15077
mutation *Δ(trpA-E)*
expressed gene(s) *trpB*
strain CY15077
mutation *Δ(trpA-E)*
expressed gene(s) *trpBA*
M9
indole
trp
GPIC
vector control
L2
E. coli


strain CY15077
mutation ΔtrpA-E
expressed gene(s) trpD-A
strain H0965
mutation prs4::Kan^R
expressed gene(s) prsA
M9
LB
anthranilate
LB+NAD
tryptophan
vector control
GPIC
vector control
GPIC

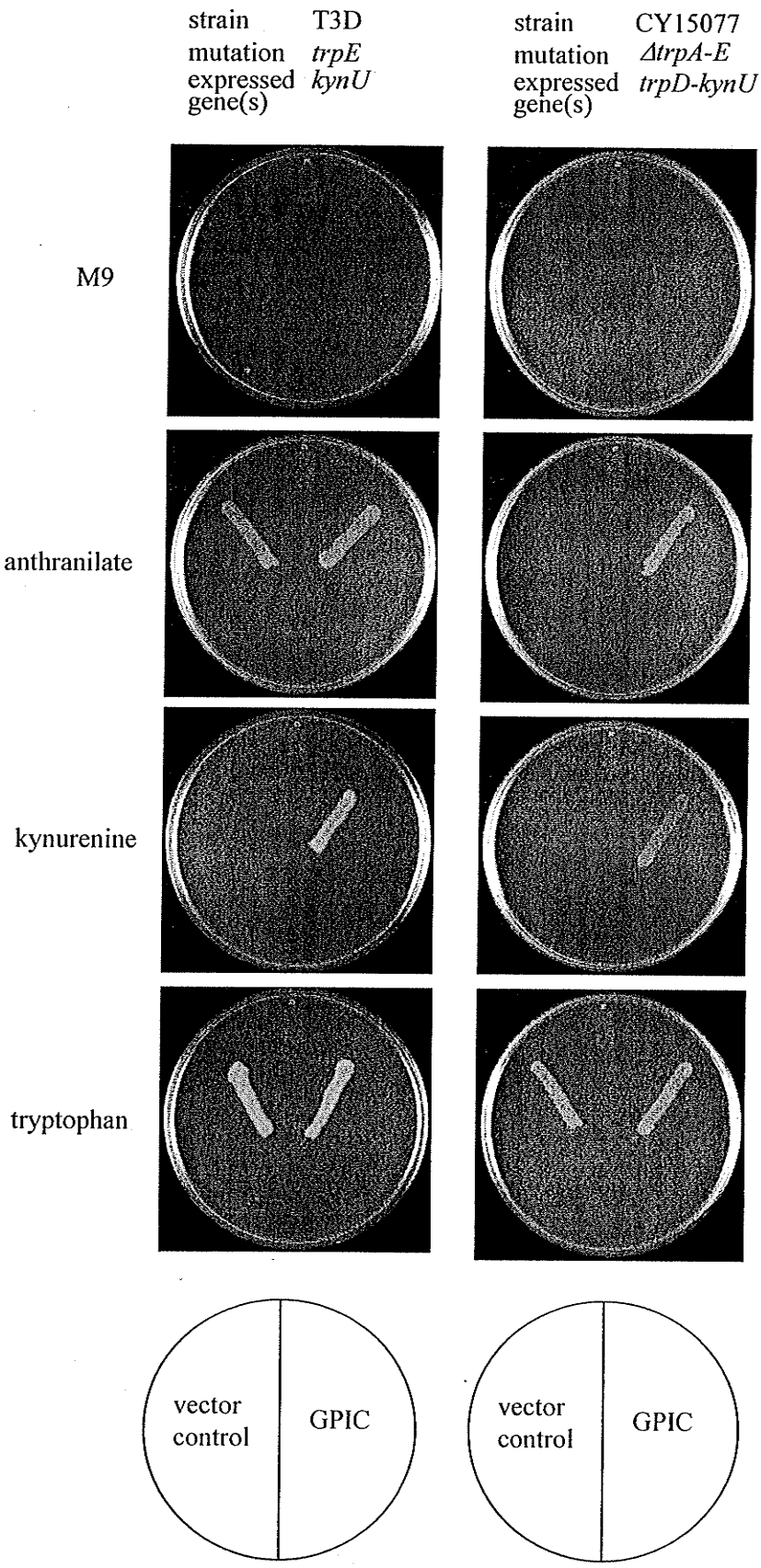

strain T3D
mutation *trpE*
expressed gene(s) *kynU*
strain CY15077
mutation *ΔtrpA-E*
expressed gene(s) *trpD-kynU*
M9
anthranilate
kynurenine
tryptophan
vector control
GPIC
vector control
GPIC

Fig. 32. Effect of alternative substrates for tryptophan biosynthesis on the growth of *C. psittaci* strain GPIC and *C. trachomatis* serovar L2. HeLa cells were infected with *C. psittaci* GPIC or *C. trachomatis* serovar L2 EBs at an MOI of 3-5 IFU $cell^{-1}$ in MEM-10 (+Trp); MEM-10 dialyzed FCS lacking tryptophan (-Trp) or lacking tryptophan but supplemented indole, anthranilate, or kynurenine. After 48 h, infected cells and culture supernatants were collected and used to infect a second HeLa cell monolayer for enumeration of recoverable IFU. Data are presented as the mean IFU(log_{10}) of triplicate determinations from three separate experiments. The S.E. of any determination never exceeded $0.5log_{10}$.

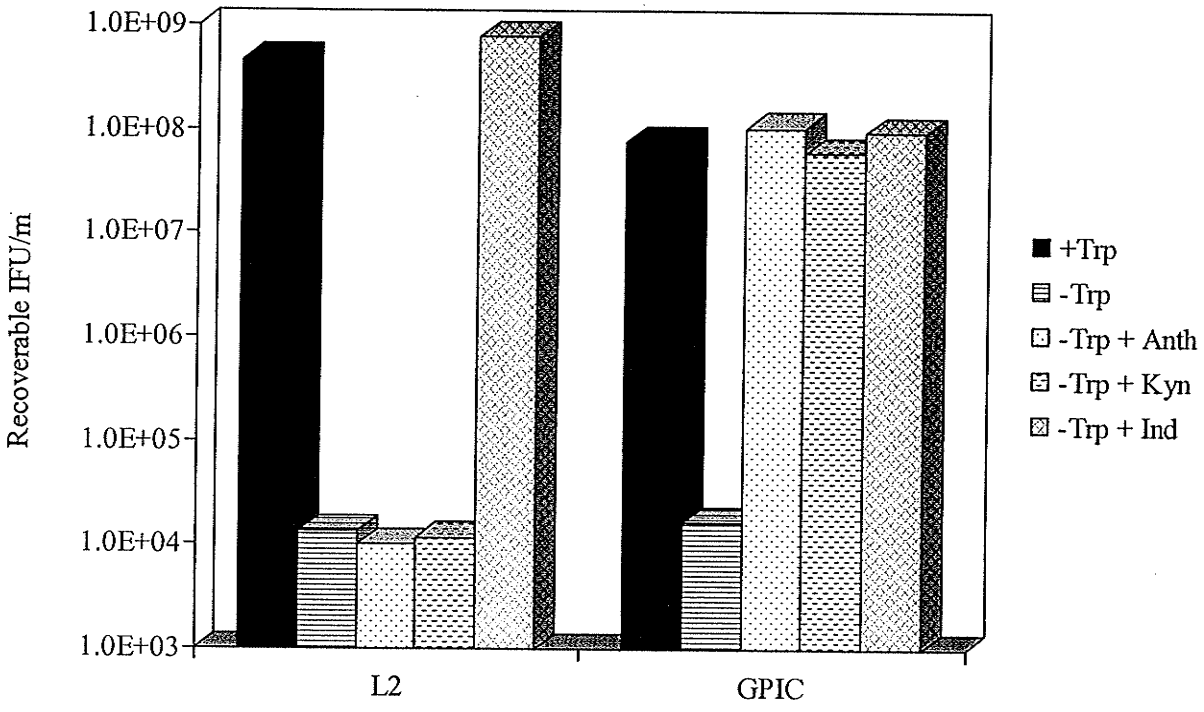
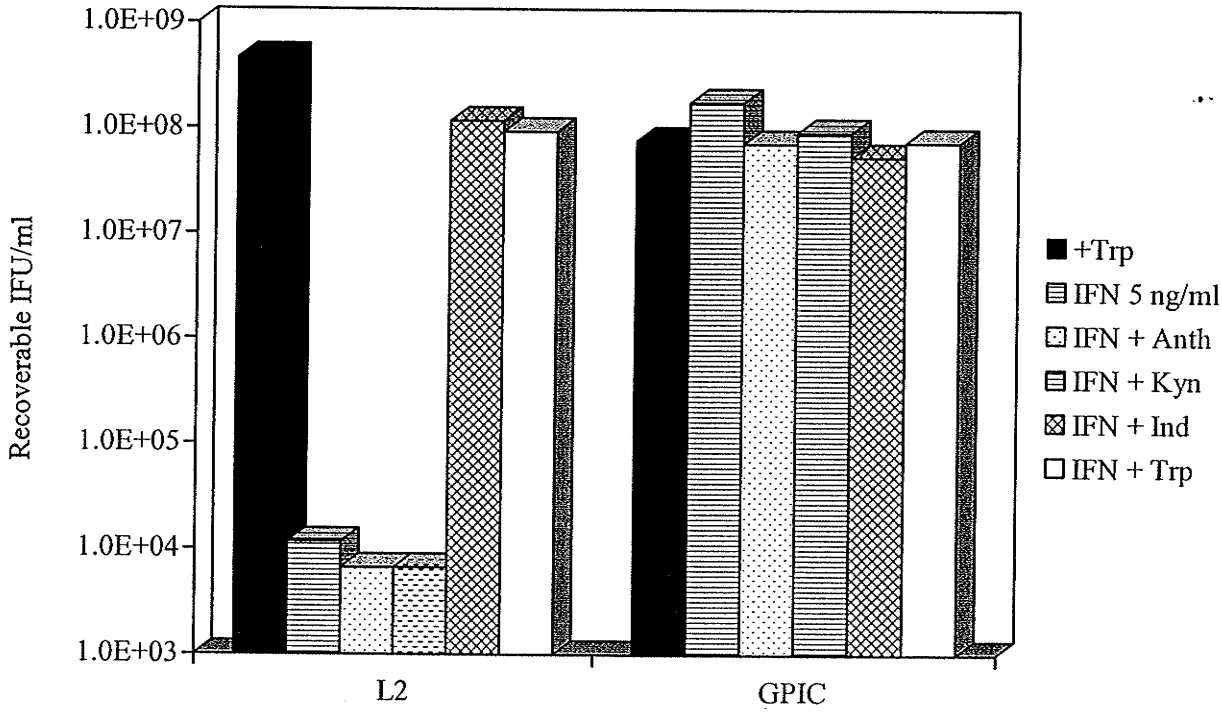

1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
Recoverable IFU/m
L2
GPIC
+Trp
-Trp
-Trp + Anth
-Trp + Kyn
-Trp + Ind

Fig. 33. Effect of alternative substrates for tryptophan biosynthesis on the growth of *C. psittac* strain GPIC and *C. trachomatis* serovar L2 in the presence of IFN-γ. HeLa cells were pretreated with 5 ng mL^{-1} for 24 h and infected with *C. psittaci* GPIC or *C. trachomatis* serovar L2 EBs at an MOI of 3-5 IFU $cell^{-1}$ in MEM-10 containing 5 ng mL^{-1} IFN. Where indicated, the medium was further supplemented with indole, anthranilate, or kynurenine. After 48 h, infected cells and culture supernatants were collected and used to infect a second HeLa cell monolayer for enumeration of recoverable IFU. Data are presented as the mean IFU(log_{10}) of triplicate determinations from three separate experiments. The S.E. of any determination never exceeded $0.5log_{10}$.


1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
Recoverable IFU/ml
L2
GPIC
+Trp
IFN 5 ng/ml
IFN + Anth
IFN + Kyn
IFN + Ind
IFN + Trp

APPENDIX

Abbreviations

CMI	cell-mediated immunity
dNTPs	deoxynucleotide triphosphates
EB	elementary body
ECL	enhanced chemiluminescence
FCS	fetal calf serum
GPIC	*C. psittaci* strain GPIC
HPLC	high performance liquid chromatography
hr	hour
IDO	indoleamine-2,3-dioxygenase
IFN-γ	interferon-γ
IFU	inclusion forming unit
IgG	immunoglobulin G
IGP	indole glycerol 3-phosphate
IL-12	interleukin-12
iNOS	inducible nitric oxide synthase
LGV	lymphogranuloma venereum
MEM	minimal essential media
MOI	multiplicity of infection
MOMP	major outer membrane protein
MoPn	*C. trachomatis* mouse pneumonitis
NAD	nicotinamide adenine dinucleotide

NK cells	natural killer cells
p.i.	post-infection
PID	pelvic inflammatory disease
PRPP	phosphoribosyl pyrophosphate
PZ	plasticity zone
RB	reticulate body
RT	reverse transcription
RTR	replication termination region
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TNF-α	tumor necrosis factor-α

Amino acid Codes

A	alanine
C	cysteine
D	aspartate
E	glutamate
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine