Characterization of the *In Vitro Chlamydia pecorum* Response to Gamma Interferon

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ABSTRACT *Chlamydia pecorum* is an important intracellular bacterium that causes a range of diseases in animals, including a native Australian marsupial, the koala. In humans and animals, a gamma interferon (IFN-γ)-mediated immune response is important for the control of intracellular bacteria. The present study tested the hypotheses that *C. pecorum* can escape IFN-γ-mediated depletion of host cell tryptophan pools. In doing so, we demonstrated that, unlike *Chlamydia trachomatis*, *C. pecorum* is completely resistant to IFN-γ in human epithelial cells. While the growth of *C. pecorum* was inhibited in tryptophan-deficient medium, it could be restored by the addition of kynurenine, anthranilic acid, and indole, metabolites that could be exploited by the gene products of the *C. pecorum* tryptophan biosynthesis operon. We also found that expression of *trp* genes was detectable only when *C. pecorum* was grown in tryptophan-free medium, with gene repression occurring in response to the addition of kynurenine, anthranilic acid, and indole. When grown in bovine kidney epithelial cells, bovine IFN-γ also failed to restrict the growth of *C. pecorum*, while *C. trachomatis* was inhibited, suggesting that *C. pecorum* could use the same mechanisms to evade the immune response *in vivo* in its natural host. Highlighting the different mechanisms triggered by IFN-γ, however, both species failed to grow in murine McCoy cells treated with murine IFN-γ. This work confirms previous hypotheses about the potential survival of *C. pecorum* after IFN-γ-mediated host cell tryptophan depletion and raises questions about the immune pathways used by the natural hosts of *C. pecorum* to control the widespread pathogen.

KEYWORDS *Chlamydia*, IFN-γ, tryptophan synthase, indoleamine 2,3-dioxygenase (IDO1), *in vitro* IFN-γ

Chlamydiae are significant obligate intracellular bacterial pathogens of humans and animals. An important characteristic of chlamydiae is their biphasic developmental cycle consisting of the extracellular infectious elementary body (EB) and the metabolically active reticulate body (1). At the beginning of infection, elementary bodies are taken up by the cell into a membranous vacuole. Within the vacuole, the elementary bodies transform into reticulate bodies, which then undergo replication. The reticulate bodies eventually transform back into infectious elementary bodies and are then released via host cell lysis to infect neighboring cells (2). Another well-characterized feature of the chlamydial cell cycle is the *in vitro* ability to convert to a viable but noninfectious aberrant form (3–5) as one phenotype of the chlamydial stress response (6). This stress response can be induced by different factors, including gamma interferon (IFN-γ), penicillin exposure, and chlamydia phage infection, as well as amino acid or iron deprivation (7–10). The first of these, IFN-γ, is an important cytokine secreted by T cells and is involved with the host defense against *Chlamydia* infection (11).

While IFN-γ induces many host cellular responses, including the activation of p47 GTPases in murine cell lines (12, 13), one of the most important responses in terms of

antichlamydial activity in human epithelial cells is the IFN-γ-mediated induction of the indoleamine 2,3-dioxygenase (IDO1) enzyme, which catalyzes L-tryptophan to L-kynurenine, resulting in inhibition of chlamydial growth and replication (14). In turn, one of the primary ways that chlamydial species appear to be able to resist IFN-γ exposure is via the presence and activity of the tryptophan biosynthesis operon in their genomes (15, 16). As in other bacteria, some chlamydial species have a complete or incomplete tryptophan biosynthesis operon regulated by an aporepressor, TrpR (17). When tryptophan is available, it enables TrpR to bind its cognate operator and repress the operon, while under tryptophan-limiting conditions, TrpR is unable to form the complex with the operator, resulting in tryptophan biosynthesis gene expression.

Genetic variation in the presence and completeness of the operon between chlamydial species, and indeed between strains of the same species, appears to be closely linked to the ability to survive IFN-γ-mediated tryptophan depletion (15, 16). *Chlamydia trachomatis* genital strains carry an incomplete tryptophan synthase gene (*trpBA*), which enables them to synthesize tryptophan only from indole (18, 19). In contrast, *Chlamydia pneumoniae* is highly sensitive to IFN-γ, as it does not have any tryptophan-biosynthetic genes in its genome (20–22). *Chlamydia caviae* contains an almost complete tryptophan operon (*trpRDFCAB*), *kynU*, and *prsA* (23) and, in HeLa cells, is completely resistant to IFN-γ, suggesting the presence of functional tryptophan biosynthesis enzymes that are capable of utilizing a range of metabolites, such as kynurenine, anthranilate, and indole, to synthesize tryptophan and restore growth under tryptophan-limiting conditions (24). While this situation appears to be consistent across most of the chlamydial species, there are exceptions: for instance, *Chlamydia muridarum* appears to resist the antichlamydial effects of IFN-γ in mouse cells via a different mechanism. IFN-γ priming induces p47 GTPases, not IDO1, in mouse epithelial cells (12). A large cytotoxin produced by *C. muridarum* was originally proposed to circumvent and escape the bactericidal effects of p47 GTPases (13); however, this has been refuted more recently following mutational studies (25). The genetic basis of *C. muridarum* IFN-γ resistance in murine cells thus remains unknown.

Chlamydia pecorum is recognized as an important pathogen that has been associated with diseases in livestock (cattle, sheep, goats, and pigs) and wild animals, including the koala (26–28). Clinical manifestations in livestock include polyarthritis, keratoconjunctivitis, encephalomyelitis, and abortion (29, 30). Chlamydial polyarthritis can appear rapidly and typically affects growing lambs from 4 to 8 months of age (28). Affected animals may develop debilitating arthritis that interferes with grazing and leads to weight loss and growth retardation (31). Beyond these reports, perhaps the most common outcome of *C. pecorum* infection is the absence of disease signs, with growing evidence that these subclinical infections may nevertheless impact the growth of affected animals, particularly dairy cattle (32, 33). *C. pecorum* is also a major driver of declines in the numbers of a native Australian marsupial, the koala (27). While much is still to be learned about the biology of the pathogen, recent breakthroughs in chlamydial genomics have revealed that the genome of the pathogen is largely conserved across different species and that if biological differences exist, they are influenced by only a limited number of single nucleotide polymorphisms (34, 35).

Unlike most of the other chlamydial species except *C. caviae*, *C. pecorum* carries an almost complete tryptophan biosynthesis operon containing the *trpRDFCAB* genes (35, 36). Notably, unlike in the other chlamydial species, this operon is not found within the chlamydial plasticity zone, a genetically variable region of chlamydial genomes normally found to contain virulence factors. In their place in this region, *C. pecorum* instead encodes several predicted cytotoxins (35), similar to those previously thought to be involved in *C. muridarum* IFN-γ resistance (25). Based on the presence of these genetic loci, we hypothesized that *C. pecorum* is resistant to IFN-γ exposure *in vitro*. Building on recent research that has begun to characterize the stress response of this important veterinary pathogen (37, 38), in the current study, we investigated the impact of IFN-γ on growth inhibition of *C. pecorum* in a range of different cell lines to explore the
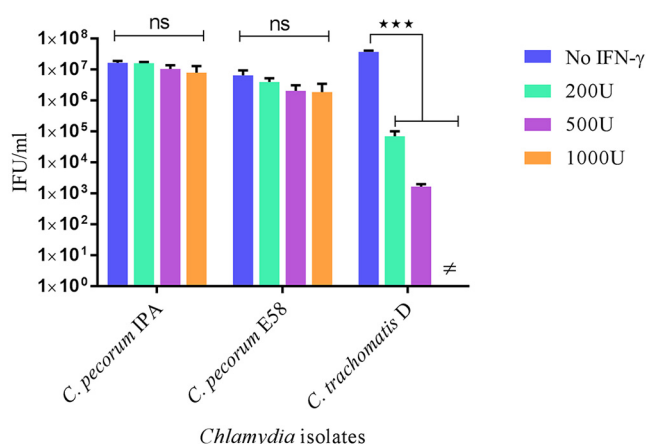
FIG 1 Viable infectious yields of *C. pecorum* IPA, *C. pecorum* E58, and *C. trachomatis* D strains after treatment with different concentrations of IFN-γ. Shown are the yields of *C. pecorum* IPA, *C. pecorum* E58, and *C. trachomatis* D generated after IFN-γ treatment of HEp-2 cell cultures. The HEp-2 cultures were pretreated with 0, 200, 500, and 1,000 U of IFN-γ/ml for 24 h and infected at an MOI of 0.5. The cultures were harvested at 36 hpi, and the numbers of infectious progeny were determined by measuring inclusion-forming units that formed infections in a new monolayer of host cells. ≠, no IFU were detected. The error bars indicate SD of the mean of three replicates ($n = 9$). Statistics were determined using unpaired two-tailed *t* tests. ***, $P < 0.001$ versus untreated controls; ns, not significant.

different mechanisms that *C. pecorum* may potentially use *in vivo* to avoid this important antichlamydial activity of the host.

RESULTS

Impact of IFN-γ treatment on the growth of *C. pecorum* in HEp-2 cells. Previous studies of other chlamydial species have investigated the chlamydial response to IFN-γ-mediated depletion of host cell tryptophan pools in various cell lines (7, 22, 39, 40). In order to understand how *C. pecorum* responds to this phenomenon, the growth characteristics of the *C. pecorum* strains (IPA and E58) were measured in human epithelial cells treated with IFN-γ. These characteristics were compared to those of *C. trachomatis* D grown under similar conditions. Previous studies also revealed that the most significant growth inhibition occurred when the host cells were pretreated with IFN-γ (13). Accordingly, HEp-2 cells were preexposed to various concentrations (0, 200, 500, and 1,000 U/ml) of IFN-γ for 24 h prior to infection. IFN-γ was replenished every 24 h until 36 h postinfection (hpi) to deplete intracellular tryptophan pools, as previously described (22).

Under these conditions, viability experiments to assess the production of infectious *C. pecorum* EBs revealed no significant effect on the growth of *C. pecorum* cultures treated with IFN-γ (Fig. 1), with minor reductions in detectable *C. pecorum* inclusion-forming units (IFU) observed at 1,000 U IFN-γ/ml. At similar and lower concentrations, IFN-γ treatment resulted in a significant decrease in the growth of *C. trachomatis* D in comparison to the control group supplied with normal Dulbecco's modified Eagle's medium (DMEM) ($P < 0.001$). In the presence of 500 U IFN-γ/ml, the number of *C. trachomatis* D IFU per milliliter was reduced from that in the control infection by approximately 50%, whereas treatment with 1,000 U of IFN-γ/ml resulted in no detectable remaining infectious *C. trachomatis* EBs (Fig. 1).

IDO1 expressed in IFN-γ-treated HEp2 cells infected by *Chlamydia*. IFN-γ treatment of human cells induces IDO1, leading to catalysis of L-tryptophan and subsequent reduction of chlamydial growth (41). In order to confirm activation of IDO1, we infected HEp-2 cells preexposed to 1,000 U IFN-γ/ml with both *C. pecorum* and *C. trachomatis* D isolates and measured the IFN-γ-induced IDO1 activation by (i) expression of the *IDO1* gene and (ii) Western blotting to monitor the presence of IDO1 in cultures. As shown in Fig. 2A, the level of IDO1 gene expression was significantly increased in *C. pecorum* IPA, *C. pecorum* E58, and *C. trachomatis* D cultures treated with IFN-γ compared to
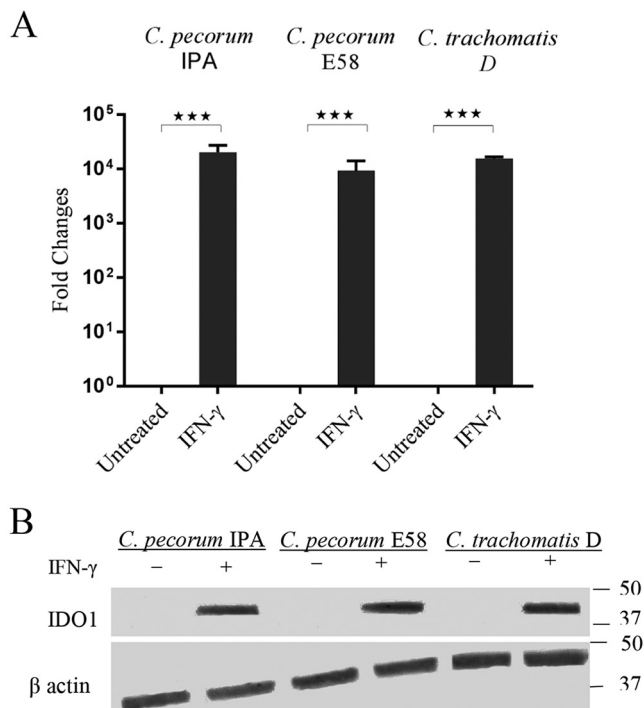
FIG 2 Presence of IDO1 protein in IFN-γ-treated *Chlamydia* cultures. (A) *IDO1* gene expression in HEp-2 cells stimulated with IFN-γ and infected with *C. pecorum* IPA, *C. pecorum* E58, or *C. trachomatis* D. Total RNA was collected from untreated (DMEM) and treated (1,000 U IFN-γ/ml) groups at 20 hpi. The results for *IDO1* were normalized against β-*actin*. The data are expressed as the fold increase relative to the untreated group. Statistics were determined using unpaired two-tailed *t* tests. ***, $P < 0.001$ versus untreated controls. (B) IDO1 protein expression of IFN-γ (1,000 U/ml)-treated HEp-2 cell lysate probed with anti-IDO1 monoclonal antibody. The sizes of relevant molecular mass markers in kDa are indicated on the right, and the Western blot band identities are indicated on the left.

untreated cultures, with peak IDO1 mRNA gene expression detected in HEp-2 cells treated with 1,000 U/ml ($P < 0.001$). To confirm translation of the IDO1 protein, we also detected IDO1 expression by Western blotting using anti-IDO1 rabbit monoclonal antibody (Fig. 2B). Consistent with the IDO1 gene expression detected, IDO1 protein was expressed when *Chlamydia*-infected cultures were exposed to IFN-γ. Taken together, these observations confirmed that *C. pecorum*-infected HEp-2 cells expressed IDO1 following IFN-γ exposure and that IDO1 expression was not inhibited by *C. pecorum* IPA, *C. pecorum* E58, or *C. trachomatis* D infection.

C. pecorum can utilize the intermediate substrates of tryptophan metabolism. The success of *C. pecorum* strains in avoiding inhibition of growth in IFN-γ-treated cultures led us to develop an alternate hypothesis that, as revealed in the genome sequences of the pathogen, *C. pecorum* may respond to tryptophan depletion through the activity of the encoded products of the tryptophan synthase operon. In the next experiments, we tested a hypothesis that *C. pecorum* can utilize kynurenine, anthranilic acid (AA), and indole to synthesize L-tryptophan through the activity of the intact tryptophan synthase operon. To do so, we infected HEp-2 cells with *C. pecorum* IPA, *C. pecorum* E58, and *C. trachomatis* D strains under the following conditions: (i) in DMEM, (ii) in tryptophan-free DMEM, (iii) in tryptophan-free DMEM supplemented with L-kynurenine, (iv) in tryptophan-free DMEM supplemented with anthranilic acid, and (v) in tryptophan-free medium supplemented with indole. Cultures grown in tryptophan-free medium and rescued by the addition of L-tryptophan were also prepared as controls.

A significant decrease in viable infectious yields for *C. pecorum* IPA and *C. pecorum* E58 was observed when they were grown in tryptophan-free medium compared to the growth of the isolates in DMEM ($P < 0.005$) (Fig. 3A and B). No infectious progeny were
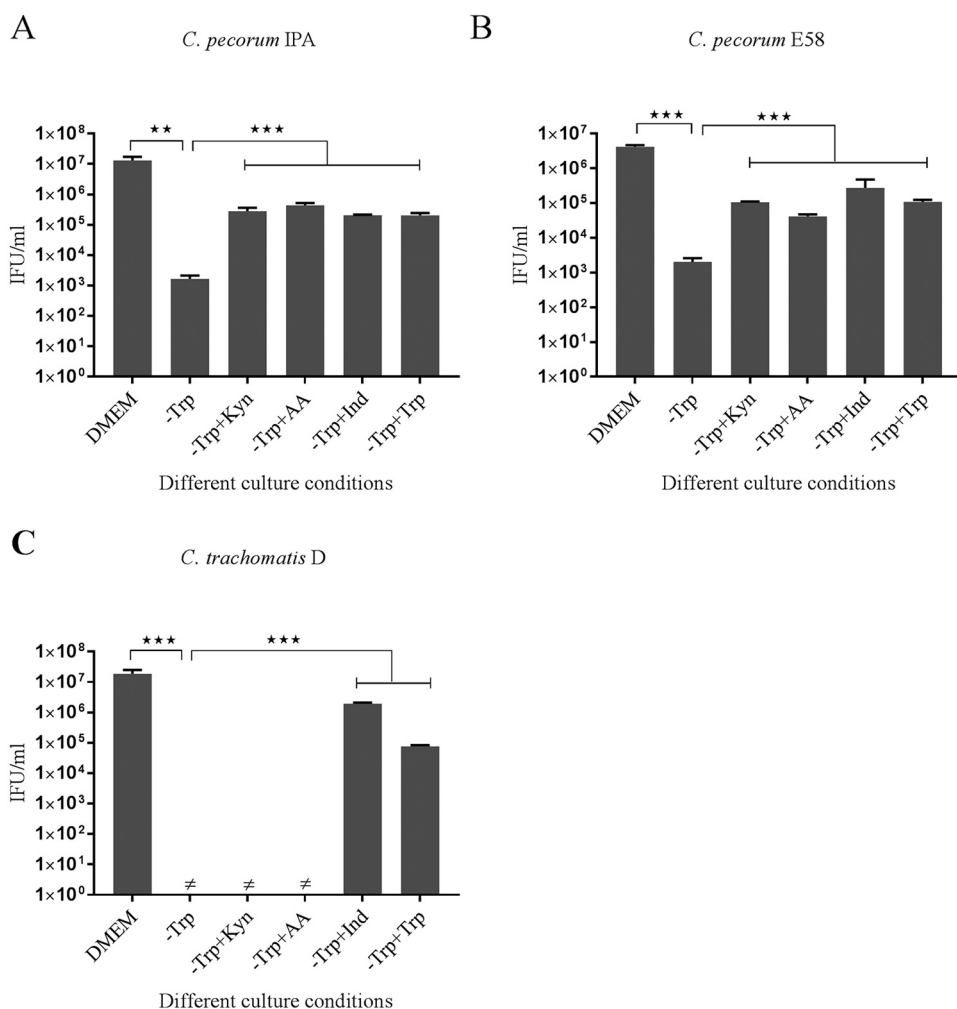
FIG 3 Viable infectious yield of *C. pecorum* IPA, *C. pecorum* E58, and *C. trachomatis* D after supplementation with alternative substrates of tryptophan biosynthesis. HEp-2 cells infected with *C. pecorum* IPA, *C. pecorum* E58, or *C. trachomatis* D were grown at an MOI of 0.5 in DMEM; tryptophan-free medium (−Trp); or tryptophan-free culture rescued by L-kynurenine (−Trp+Kyn), anthranilic acid (−Trp+AA), indole (−Trp+Ind), or tryptophan (−Trp+Trp). The cultures were harvested at 36 hpi, and the infectious progeny were determined by measuring inclusion-forming units that formed infections in a new monolayer of host cells. The error bars represent SD of the means of three replicates ($n = 9$). Statistics were determined using unpaired two-tailed t tests. **, $P < 0.005$; ***, $P < 0.001$ versus untreated controls.

observed for *C. trachomatis* D isolates grown in tryptophan-free medium (Fig. 3C). For the *C. pecorum* IPA isolate cultured in tryptophan-free medium, supplementation with alternative tryptophan substrates resulted in significantly increased yields of *C. pecorum* ($P < 0.001$). A similar trend was also observed for the *C. pecorum* E58 strains grown by the addition of kynurenine, anthranilic acid, and indole to the tryptophan-free medium ($P < 0.001$). In contrast, significant restoration of *C. trachomatis* D growth was achieved only when the isolates grown in tryptophan-free medium were supplemented with indole ($P < 0.001$). When the growth of all cultures in tryptophan-free medium was supplemented by exogenous tryptophan (fresh DMEM containing 16 mg/ml tryptophan according to the manufacturer's instructions), we were able to demonstrate rescue of all strains by observation of detectable IFU. For *C. pecorum*, this resulted in 10^3 to 10^5 IFU/ml recovered, while *C. trachomatis* experienced significant recovery of up to 10^4 IFU/ml ($P < 0.001$) (Fig. 3). Collectively, these results provide strong evidence that the capacity of *C. pecorum* to avoid the IFN-γ immune invasion *in vitro* is mainly due to the recycling abilities of different tryptophan precursors.
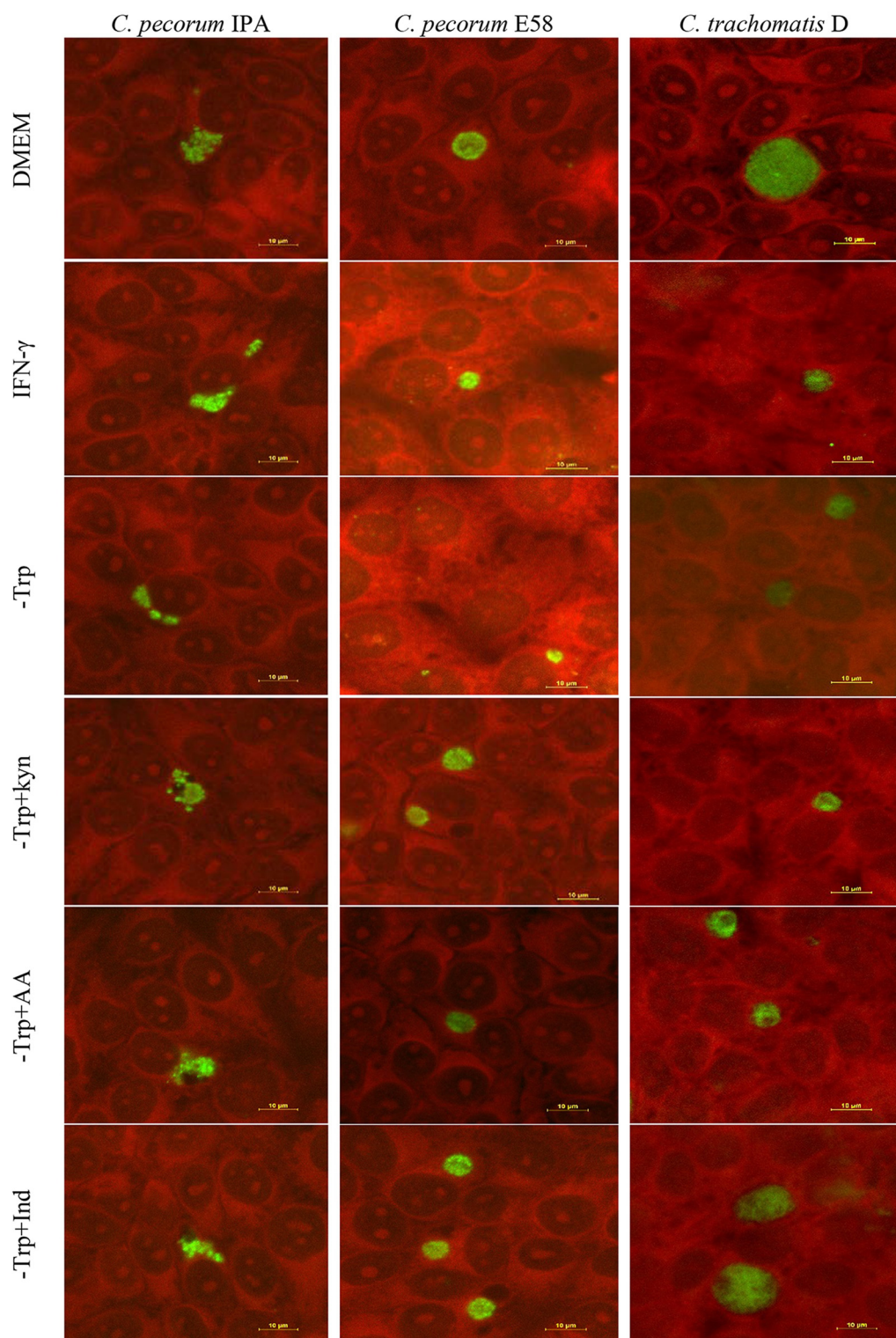
FIG 4 Representative confocal microscopic images of *C. pecorum* IPA, *C. pecorum* E58, and *C. trachomatis* D isolates grown in HEp-2 cells. The culture conditions are indicated on the left. The cultures were grown at an MOI of 0.5 in DMEM, IFN-γ-treated (1,000 U/ml) medium; tryptophan-free medium (−Trp); or tryptophan-free culture rescued by L-kynurenine (−Trp+Kyn), anthranilic acid (−Trp+AA), or indole (−Trp+Ind) and stained with *Chlamydia* cell LPS at 30 hpi. Distinct inclusions (green) were detected under each condition within the host cell (red). Scale bars, 10 μm.
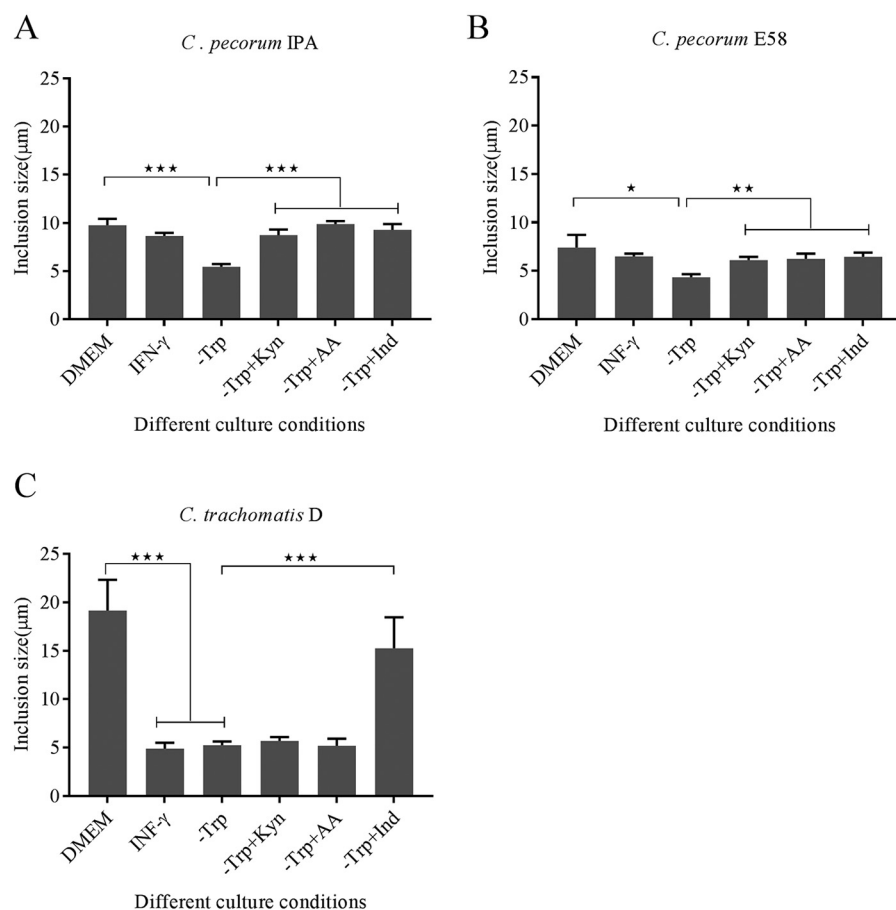
FIG 5 Inclusion size measurement in untreated DMEM; IFN-γ-treated (1,000 U/ml) medium; tryptophan-free medium (−Trp); or tryptophan-free culture rescued by L-kynurenine (−Trp+Kyn), anthranilic acid (−Trp+AA), or indole (−Trp+Ind) at 30 hpi. The inclusion sizes were measured under each condition from three independent coverslips. The error bars represent SD of the means of three replicates (n = 20). Statistics were determined using unpaired two-tailed t tests. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$ versus untreated controls.

The chlamydial stress response induced by IFN-γ and other factors is typically characterized by the presence of aberrant reticulate bodies and a smaller number of inclusions with reduced size *in vitro* (21). Therefore, cultures under the above-described conditions were examined by chlamydial immunofluorescence and confocal microscopy to examine chlamydial inclusion morphology at 30 hpi. *C. pecorum* IPA and *C. pecorum* E58 inclusion morphology was varied in shape, with the former most often irregularly crescent shaped and the latter round (Fig. 4). In addition, *C. trachomatis* inclusions were generally round (Fig. 4). With the exception of the tryptophan-free-medium cultures (mean inclusion diameter, 5.45 ± 0.22 μm for IPA and 4.36 ± 0.24 μm for E58), *C. pecorum* inclusion sizes remained relatively the same under all other conditions tested (Fig. 5A and B). In contrast, for *C. trachomatis* D, significantly smaller inclusions were observable under IFN-γ exposure (4.9 ± 0.49 μm), in tryptophan-free medium (5.26 ± 0.26 μm), and in tryptophan-free medium supplemented with kynurenine (5.69 ± 0.32 μm) and anthranilic acid (5.2 ± 0.58 μm) culture conditions than in the control cultures (19.17 ± 2.59 μm) ($P < 0.001$) (Fig. 5C). The inclusion size was restored only when the *C. trachomatis* cultures were supplemented with indole (14.58 ± 2.62 μm) (Fig. 5C).

Regulation of tryptophan gene expression in *C. pecorum*. The *trpR* gene, considered the tryptophan-dependent regulator of tryptophan metabolism, regulates the expression of the chlamydial tryptophan biosynthesis genes (17). To understand *trpR*
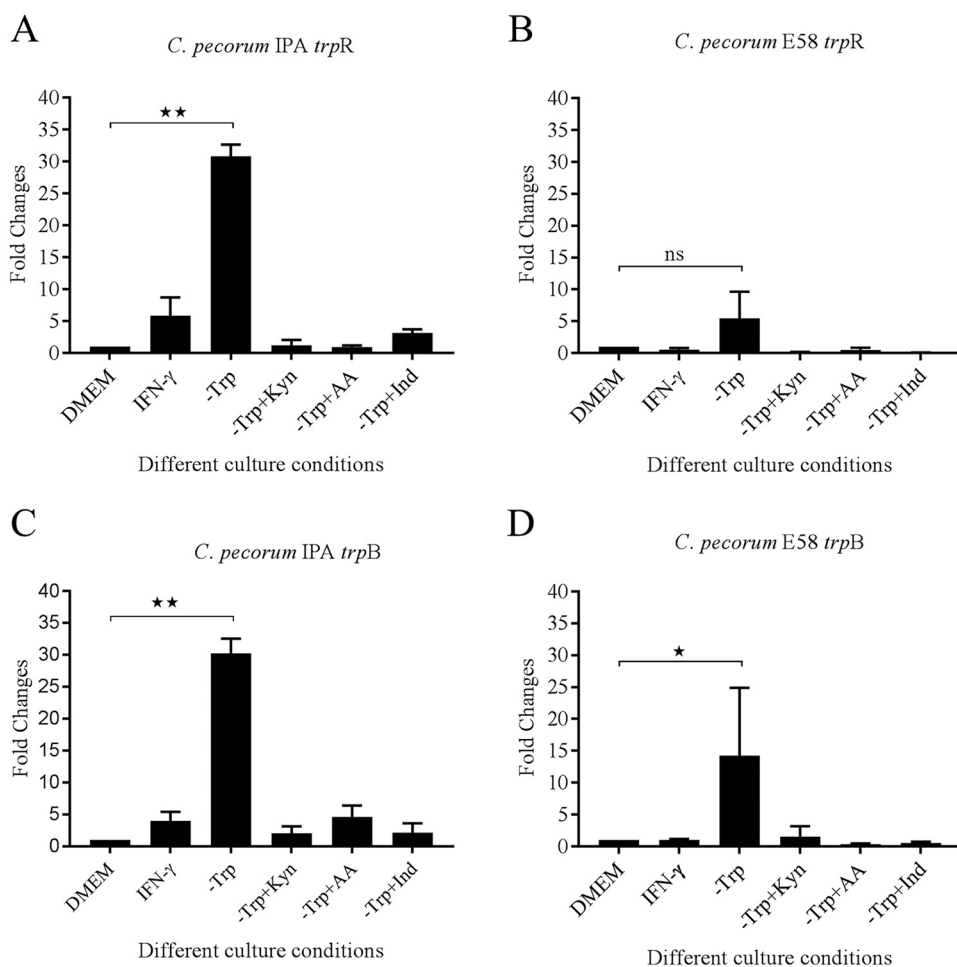
FIG 6 *trpR* and *trpB* gene expression in HEp-2 cells. Hep-2 cells were infected with *C. pecorum* IPA and *C. pecorum* E58 strains in DMEM (control group); DMEM with IFN-γ; tryptophan-deficient medium (−Trp); or tryptophan-free medium supplemented with L-kynurenine (−Trp+Kyn), anthranilic acid (−Trp+AA), or indole (−Trp+Ind). Total RNA was isolated from infected cultures at 20 hpi. The *trpR* and *trpB* results were then normalized with 16S rRNA transcripts. The results are expressed as fold changes relative to the untreated DMEM group. (A) *trpR* gene expression of *C. pecorum* IPA in Hep-2 cells ($n = 2$; means and SD). (B) *trpR* gene expression of *C. pecorum* E58 in Hep-2 cells ($n = 2$; means and SD). (C) *trpB* gene expression of *C. pecorum* IPA in Hep-2 cells ($n = 2$; means and SD). (D) *trpB* gene expression of *C. pecorum* E58 in Hep-2 cells ($n = 2$; means and SD). Statistics were determined using unpaired two-tailed *t* tests. *, $P < 0.05$; **, $P < 0.005$; ns, not significant versus untreated controls.

gene regulation in the *C. pecorum* response to tryptophan depletion, we investigated *trpR* gene expression in *C. pecorum*-infected HEp-2 cells upon exposure to IFN-γ or growth in tryptophan-free medium or following supplementation with tryptophan precursors by quantitative reverse transcription (qRT)-PCR. Compared to cultures grown in DMEM with tryptophan, we observed strong relative upregulation (>5-fold) of *trpR* gene expression for *C. pecorum* E58 cultured in tryptophan-free medium (Fig. 6B). This upregulation was even more significantly pronounced (>29-fold) for the *C. pecorum* IPA isolate grown in tryptophan-free medium ($P < 0.005$) (Fig. 6A). When we investigated *trpR* gene expression following addition of tryptophan precursors, such as L-kynurenine, anthranilic acid, and indole in the same cultures, *trpR* gene expression returned to baseline levels, similar to what was observed for the *C. pecorum* cultures grown in control DMEM. Similarly, when tryptophan was depleted via treatment with IFN-γ, the relative *trpR* transcript levels of *C. pecorum* IPA and E58 were similar to those of untreated DMEM cultures.

We also tested *trpB* gene expression under all the conditions tested for *C. pecorum* *trpR* expression to evaluate cotranscription of the genes within the operon (Fig. 6C and
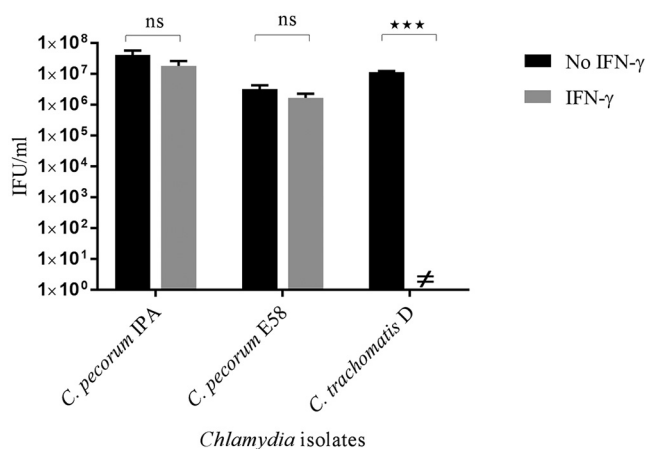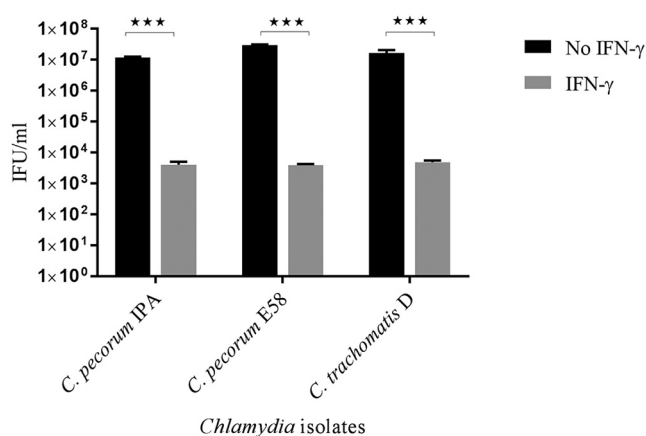
FIG 7 Effect of bovine IFN-γ on the growth of *C. pecorum* IPA, *C. pecorum* E58, and *C. trachomatis* D. BK cell monolayers pretreated with bovine IFN-γ (1,000 U/ml) were infected with the three strains at an MOI of 0.5. The cultures were harvested at 36 hpi, and the viable yield was determined by measuring inclusion-forming units that formed infections in a new monolayer of BK cells. ≠, no IFU were detected. The error bars represent SD of the means of three replicates ($n = 9$). Statistics were determined using unpaired two-tailed *t* tests. ***, $P < 0.001$; ns, not significant versus untreated controls.

D). In a pattern similar to that of *trpR*, and as expected, *trpB* gene expression was significantly upregulated in the *C. pecorum* cultures grown in tryptophan-free medium ($P < 0.05$). The relative expression of *trpB* was also observed to be similar to that of *trpR* for *C. pecorum* IPA and E58 when grown in tryptophan-free medium supplemented with tryptophan precursors.

C. pecorum strains grown in bovine epithelial cells are resistant to IFN-γ exposure. As humans are not the natural host of *C. pecorum*, we were interested to examine the response of *C. pecorum* strains E58 (bovine origin) and IPA (ovine origin) to bovine IFN-γ. Previous reports suggested that bovine cells expressed tryptophan-degrading enzyme, IDO1, after stimulation with bovine IFN-γ *in vitro* (42, 43). Therefore, in order to assess whether the bovine cells treated with IFN-γ inhibit the growth of *C. pecorum in vitro*, we pretreated bovine kidney epithelial (BK) cells with 1,000 U/ml bovine IFN-γ 24 h prior to infection with either *C. pecorum* E58, *C. pecorum* IPA, or *C. trachomatis* D. IFN-γ-treated cultures of *C. pecorum* IPA yielded 1.9×10^7 IFU/ml, while cultures with DMEM yielded 4.2×10^7 IFU/ml (Fig. 7). A similar pattern was observed for the *C. pecorum* E58 strain, demonstrating only a minor detectable loss of IFU when grown in IFN-γ-exposed cultures. In contrast, IFN-γ-treated cultures significantly inhibited *C. trachomatis* D strain growth (no detectable viable yield) compared to DMEM cultures ($P < 0.001$) (Fig. 7). Based on these results, we concluded that all the strains in this experiment could grow in BK cells and that IFN-γ stimulation did not inhibit *C. pecorum* growth.

C. pecorum strains are sensitive to IFN-γ in mouse cells. Unlike IDO1 induction by IFN-γ in human and bovine cells, mouse epithelial cells induce p47 GTPases when stimulated by mouse IFN-γ (13, 44). Therefore, in order to determine whether mouse IFN-γ had an effect on the growth of *C. pecorum* strains grown in McCoy cells, *C. pecorum* and *C. trachomatis* isolates were used to infect McCoy cell monolayers exposed to IFN-γ. Interestingly, *C. pecorum* IPA infection in mouse cells treated with IFN-γ resulted in a significant, approximately 4 $\log_{10}$-fold reduction in infectious progeny at 36 hpi ($P < 0.05$) (Fig. 8). Similarly, the growth of *C. pecorum* E58 in IFN-γ-treated cultures was reduced significantly, from 3.0×10^7 IFU/ml in the DMEM control group to 3.8×10^3 IFU/ml. Similar results were also observed in *C. trachomatis* D-infected cultures following IFN-γ treatment, with significant reductions in detectable yields of EBs observed in comparison to their untreated counterparts ($P < 0.001$).

FIG 8 Effect of murine IFN-γ on the growth of *C. pecorum* IPA, *C. pecorum* E58, and *C. trachomatis* D in mouse cells. McCoy cell monolayers pretreated with murine IFN-γ (1,000 U/ml) were infected with the three strains at an MOI of 0.5. The cultures were harvested at 36 hpi, and the viable yields were determined by measuring inclusion-forming units that formed infections in a new monolayer of McCoy cells. The error bars represent SD of the means of three replicates (*n* = 9). Statistics were determined using unpaired two-tailed *t* tests. ***, *P* < 0.001 versus untreated controls.

DISCUSSION

Tryptophan is an important amino acid essential for the normal development of *Chlamydia*. Chlamydial tryptophan synthesis or lack thereof is linked to tissue tropism (15, 17), species specificity (22), virulence (14, 45), and the chlamydial stress response (6). IFN-γ-induced IDO1-depleted tryptophan catabolism is a well-described method for tryptophan limitation in host cells *in vitro*. However, the response to IFN-γ is not universal among *Chlamydia* species and may be associated with different virulence characteristics. It has been shown that the variability in IFN-γ sensitivity is mainly due to the presence of different suites of genes in the tryptophan operon (45). *C. caviae* has an almost complete operon including all the genes for tryptophan biosynthesis except *trpE* (23). In contrast, *C. trachomatis* has only *trpB* and *trpA* genes in the tryptophan operon (15) and *C. pneumoniae* has no genes (46). *C. trachomatis* and *C. pneumoniae* have been well documented to produce a chlamydial stress response to IFN-γ exposure (7, 47). Surprisingly, no relevant studies have been conducted on the *C. pecorum*–IFN-γ relationship, although *C. pecorum* comparative genomics revealed that *C. pecorum* possesses an almost complete tryptophan operon, *trpRDCAB* (35, 36).

In this study, we noted that livestock *C. pecorum* isolates can escape the IFN-γ effect via tryptophan depletion in human epithelial cells. Both *C. pecorum* isolates showed very little sensitivity to IFN-γ, whereas human *C. trachomatis* strains showed complete loss of infectious progeny following confirmation of IDO1 expression in the cell culture. In human cells, IFN-γ induces IDO1, which catalyzes the intracellular tryptophan pools to kynurenine (7, 48). In the present study, we observed that in the absence of tryptophan, *C. pecorum* growth could be restored in human epithelial cells by supplementation with kynurenine, anthranilic acid, and indole. This rescue of *C. pecorum* in tryptophan-deficient medium is congruent with the presence of an almost complete tryptophan operon including the *trpRDFCAB*, *kynU*, and *prsA* genes. This phenotype is also consistent with that of the related chlamydial species *C. caviae*, which was previously demonstrated to be completely resistant to IFN-γ treatment in HeLa cells due to its capacity to reuse kynurenine from the host cell as a substrate to synthesize tryptophan (24) thanks to *trpRDFCAB*, *kynU*, and *prsA* in its tryptophan operon. Our results also revealed that, as expected, the expression of this operon in tryptophan-depleted medium was dependent on the presence of tryptophan following the addition of kynurenine, anthranilic acid, and indole to the cultures. It was previously demonstrated that repression of *trpR*, the transcriptional regulator of the operon, is dependent on the tryptophan concentration and that repression does not commence

until the ratio of tryptophan to TrpR approaches 100:1 (17). These results provide overwhelming evidence that adequate amounts of tryptophan can be synthesized by *C. pecorum* from these substrates and that this tryptophan can then bind to its cognate operator to repress transcription of the tryptophan operon.

While measuring the *C. pecorum* response to IFN-γ treatment in human cells provided an interesting comparison to *C. trachomatis*, the studies of *C. pecorum* resistance in other cell lines (i) highlighted differences in immune evasion strategies between *C. pecorum* and other veterinary chlamydiae (i.e., *C. muridarum*) and (ii) provided more information on the potential *in vivo* growth strategies utilized by the animal pathogen. Examination of whether *C. pecorum* can survive IFN-γ exposure in murine McCoy cells revealed that, like other chlamydial species except *C. muridarum* (12, 13, 22), *C. pecorum* growth was inhibited. We showed, however, that when grown in BK cells, *C. pecorum* isolates were resistant to bovine IFN-γ. In contrast, IFN-γ-treated BK cells inhibited *C. trachomatis* growth, possibly by an IDO1 depletion mechanism. Based on our previous data, we could then hypothesize that *C. pecorum* may avoid such an immune response due to the compensatory mechanisms available to the pathogen and courtesy of its nearly complete tryptophan biosynthesis operon. There is a reasonable amount of *in vivo* evidence to suggest that *C. pecorum* may indeed be able to do this in sheep and cattle. Subclinical *C. pecorum* gastrointestinal tract infections are common (28), with animals able to shed *C. pecorum* in fecal samples for extended periods (49). While evidence of aberrant chlamydial forms associated with *in vivo* infections are limited only to *Chlamydia suis* thus far (50), it is tempting to speculate that the IFN-γ resistance provided by the *C. pecorum* tryptophan biosynthesis operon may be an important virulence mechanism in the species to avoid mucosal responses in the gastrointestinal tracts of livestock hosts. In mouse models, experimental infections have revealed that downregulation of the gut immunity results in persistent subclinical infection with *C. muridarum* in the gastrointestinal tract with fecal shedding (51, 52). Studies of *Mycobacterium avium* subsp. *paratuberculosis* infection have also revealed that despite IDO1 gene and protein expression in infected gut tissues in a clinically infected sheep, the local adaptive immune response to the infection was inhibited, allowing the bacteria to survive and contributing to the chronicity of the infection (43). *C. pecorum* genetic experiments combined with *in vivo* infection studies will be required to verify this hypothesis. If true, however, it may have significance in influencing the future design of a *C. pecorum* vaccine, as vaccines for *C. pecorum* (53, 54) and other chlamydial species have, in part, focused on inducing pathogen-specific IFN-γ responses (55). The potential for IDO-independent effects of IFN-γ on chlamydial growth in bovine epithelial cells, as has been recently noted in both human (56) and murine (57) epithelial cells, as well as potential mechanisms that *C. pecorum* might use to evade them, requires additional investigation.

In summary, the results presented here demonstrate that *C. pecorum* produces complete resistance to IFN-γ-mediated tryptophan depletion due to its capacity to scavenge tryptophan from its metabolites from the host cell. Thus, the ability to synthesize tryptophan is likely to allow this intracellular pathogen to more readily adapt to the different environments within its natural host as a strategy to avoid host defenses. This study also provides further evidence that the relationship between IFN-γ and chlamydiae is host specific in nature. Further insights into the pathogenesis of *C. pecorum* will be needed for control strategies to be developed successfully for this enigmatic pathogen.

MATERIALS AND METHODS

Chlamydia isolates and culture conditions. The *Chlamydia* isolates used in this study included an ovine polyarthritis isolate, *C. pecorum* IPA (VR-629; American Type Culture Collection [ATCC]); a calf sporadic bovine encephalomyelitis isolate, *C. pecorum* E58 (kindly provided by Bernhard Kaltenboeck, Auburn University); and *C. trachomatis* D (VR-885; ATCC). All the isolates were routinely cultured in the HEp-2 cell line (CCL-23; ATCC) at 37°C and 5% CO_2 in DMEM growth medium (Gibco, Australia) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Life Technologies, Australia), 120 μg streptomycin ml^{-1} (Sigma-Aldrich, Australia), and 50 μg gentamicin ml^{-1} (Gibco, Australia). Bovine kidney (BK) cells (CCL-22; ATCC) and McCoy mouse fibroblasts (CRL-1696; ATCC) were routinely grown in

DMEM with 5% FCS, 50 μg gentamicin ml^{-1}, and100 μg streptomycin ml^{-1}. All experiments were conducted in 48-well plates (Sigma-Aldrich, Australia) at a multiplicity of infection (MOI) of 0.5 unless otherwise stated.

For IFN-γ experiments, 30,000 cells/well were seeded in a 48-well plate 24 h before infection with different concentrations of human IFN-γ (0, 200, 500, or 1,000 U/ml; Sigma-Aldrich, Australia), 1,000 U bovine IFN-γ/ml (Life Technologies, Australia), or 1,000 U murine IFN-γ/ml (Peprotech, USA) in HEp-2, BK, and McCoy cells, respectively. For the tryptophan-free experiment, 50,000 cells/well were seeded 24 h prior to infection in 48-well plates in 500 μl growth medium. Tryptophan precursors were added to the cultures at the following concentrations: 50 μM L-kynurenine (Sigma-Aldrich, Australia), 50 μM AA (Sigma-Aldrich, Australia), 50 μM indole (Sigma-Aldrich, Australia), and 16 μg/ml tryptophan (fresh DMEM [Gibco, Australia] according to the manufacturer's instructions). In all experiments, the indicated supplements were replenished at 24 hpi. Control cultures with no treatment were included in each experiment. The cultures were harvested at 36 hpi in sucrose-phosphate-glutamate (SPG) medium prior to storage at $-80°$C. The SPG medium consisted of 218 mM sucrose (Sigma-Aldrich, Australia), 3.76 mM KH$_2$PO$_4$ (Sigma-Aldrich, Australia), 7.1 mM K$_2$HPO$_4$ (Sigma-Aldrich, Australia), and 5 mM glutamic acid (Sigma-Aldrich, Australia), pH 7.4.

For Western blot analysis, 3×10^5 cells/well were seeded in a six-well plate (Sigma-Aldrich, Australia) in 3 ml growth medium 24 h prior to infection with IFN-γ. The cells were harvested at 36 hpi for subsequent use in Western blot analysis.

Determination of viable infectious yield. The viable infectious yield of *Chlamydia* was evaluated by calculating the number of IFU per milliliter in HEp-2, BK, and McCoy cells. Confluent host cells were infected with harvested cell lysate in serial dilutions in 96-well cell culture plates (Sigma-Aldrich, Australia). At 30 hpi, the host cell monolayer was washed with phosphate-buffered saline (PBS) and fixed with 100% methanol for 10 min. The fixed cells were permeabilized with Triton X-100 for 15 min and then blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, Australia) in PBS. Primary antibody (anti-*Chlamydia* HtrA) (10, 58) was incubated for 1 h at 1:500 dilution in 1% BSA, followed by three washes in 0.2% Tween 20 in PBS. Secondary antibody [goat anti-rabbit IgG(H+L)-Alexa Fluor 488; Invitrogen, Australia] was added at 1:600 dilution in 1% BSA for 1 h, followed by 4 washes in 0.2% Tween 20 in PBS. Triplicate samples at appropriate serial dilutions were counted in nine fields of view per dilution using fluorescence microscopy (Nikon Eclipse TiS fluorescence microscope). The viable infectious yield was determined as previously described (22). Graphical images were made and statistical analysis was conducted using GraphPad Prism (version 7).

Confocal microscopy. *Chlamydia*-infected HEp-2 cell monolayers cultured on 8-mm coverslips in a 48-well plate were used for confocal laser scanning microscopy. At 30 hpi, the coverslip cultures were fixed with 100% methanol for 10 min and washed with PBS. The coverslips were stained with fluorescein isothiocyanate (FITC)-labeled *Chlamydia* cell lipopolysaccharide (LPS) (Cell Labs, Australia) and mounted on slides using Prolong Gold antifade (Life Technologies, Australia). Confocal images were obtained using a Nikon Eclipse TiS fluorescence microscope with a 60$\times$ oil objective. Images were processed for presentation using the NIS software suite. Sizes of inclusions at 30 hpi were measured to determine the effect of IFN-γ and the addition of kynurenine, anthranilic acid, and indole to tryptophan-deficient medium using ImageJ.

Western blotting. Mock-infected cells and *Chlamydia*-infected cell cultures in 6-well plates were harvested at 36 hpi and washed with PBS. The cells were then lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, Australia) and protease inhibitor cocktail (Sigma-Aldrich, Australia) with sonication. Twenty microliters of cell lysates was diluted in 2$\times$ Laemmli sample buffer (Bio-Rad, Australia) supplemented with 5% beta-mercaptoethanol, incubated at 95°C for 5 min, and centrifuged at 10,000 $\times$ *g* for 2 min. Samples were loaded into 10% mini-Protean TGX precast gels (Bio-Rad, Australia) at 150 V for 1 h. Precision Plus protein dual-color standards (10 to 250 kDa; Bio-Rad, Australia) and HeLa cell lysate plus IFN-γ (Santa Cruz Biotechnology, USA) were used as positive controls. Proteins were separated by SDS-PAGE following transfer onto a nitrocellulose membrane. The blots were blocked with 1$\times$ PBS plus 5% skim milk for 1 h at room temperature and probed with an anti-IDO1 rabbit primary monoclonal antibody (Abcam, Australia) at 1:1,000 dilution or anti β-actin rabbit polyclonal antibody at 1:2,000 dilution overnight at 4°C. The blots were washed three times in a rocker for 5 min each time with 0.2% Tween 20 and 1$\times$ PBS. The blots were then probed with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Abcam, Australia) at 1:5,000 dilution for 1 h at room temperature. The nitrocellulose membranes were washed four times in a rocker for 5 min each time with 0.2% Tween 20 and 1$\times$ PBS. The blot was analyzed with an Odyssey CLx imaging system.

Reverse transcriptase qRT-PCR for *IDO1* and *trpR* genes. *C. pecorum*-infected cell cultures were harvested in RNAlater (Sigma-Aldrich, Australia) at 20 hpi. Total RNA was extracted from the harvested cells using the RNeasy minikit (Qiagen, Australia) according to the manufacturer's instructions. The purity and concentration of the RNA were determined using a Nanodrop spectrophotometer. Following digestion of residual DNA by treatment with DNase, cDNA synthesis from RNA was performed using a Roche reverse transcription kit (Roche, Australia) according to the manufacturer's instructions. For host cell *IDO1* gene expression, β-actin was used as a reference gene. The primers used for *IDO1* (59) and β-actin (60) are listed in Table 1. The 16S rRNA gene was used as a reference gene for the chlamydial *trpR* and *trpB* gene expression studies. The primers used for *trpR*, *trpB*, and 16S rRNA are also listed in Table 1. All reactions were carried out on a Rotor-GeneQ (Qiagen, Australia) real-time PCR machine in a 20-μl final volume, using a Quantitect SYBR green PCR kit (Qiagen, Australia). The PCR conditions were 95°C for 5 min and 35 cycles of 25 s at 95°C, 30 s at 58°C, and 35 s at 72°C. The results are presented as normalized values of $2^{-\Delta\Delta C_T}$ using the following equations (where C_T is threshold cycle): $\Delta C_T = (C_T$ of the IDO1 or *trpR*

TABLE 1 Primers used in this study

Gene target	Primer orientation	Sequence	Amplicon size (bp)	Reference
Human *IDO1*	Forward	5′-AGAAGTGGGCTTTGCTCTGC-3′	230	59
	Reverse	5′-TGGCAAGACCTTACGGACATCTC-3′		
Human *β-actin*	Forward	5′-CATGTACGTTGCTATCCAGGC-3′	250	60
	Reverse	5′-CTCCTTAATGTCACGCACGAT-3′		
trpR	Forward	5′-GATGGCTGGAATGCGTTTGT-3′	176	This study
	Reverse	5′-CTCACACCATATGCCTCAG-3′		
trpB	Forward	5′-AGACAGGAGCAGGACAAC-3′	198	This study
	Reverse	5′-GGAGAGCCTCATTTACGG-3′		
16S rRNA	Forward	5′-AGTCGAACGGAATAATGGCT-3′	204	62
	Reverse	5′-CCAACAAGCTGATATCCCAC-3′		

or *trpB* gene $- C_T$ of the *β-actin* or 16S rRNA gene) and $\Delta\Delta C_T = (\Delta C_T$ of the treated sample $- \Delta C_T$ of the calibrator sample) (61). To test for DNA contamination, we used eight randomly selected DNase-treated RNA samples from the above-mentioned set as templates for conventional PCR targeting a 204-bp fragment of the chlamydial 16S rRNA gene (62) and a 250-bp fragment of the human *β-actin* gene. Amplification was not achieved in any of the samples, confirming the digestion of residual DNA in our RNA samples.

Statistical analysis. Statistical significance was analyzed using an unpaired two-tailed *t* test in GraphPad Prism (version 7). A *P* value of <0.05 was considered significant, and results are presented as means and standard deviations (SD).

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