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HOST RESPONSE AND INFLAMMATION

Distinct Roles of Chromosome- versus Plasmid-Encoded Genital Tract Virulence Factors in Promoting *Chlamydia muridarum* Colonization in the Gastrointestinal Tract

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ABSTRACT The genital pathogen *Chlamydia* is known to colonize the gastrointestinal tract. Orally delivered *Chlamydia muridarum* can reach the colon and maintain a long-lasting colonization there. However, *C. muridarum* with mutations in chromosomal genes *tc0237* and *tc0668* (designated a chromosomal mutant) or deficient in plasmid-encoded pGP3 (designated a plasmid mutant) is unable to do so. We now report that the chromosomal mutant is still able to reach the colon while the plasmid mutant fails to do so following an oral delivery, suggesting that lack of colon colonization by different mutants may involve distinct mechanisms. Consistently, a direct intracolonic delivery selectively restored the ability of the plasmid mutant, but not the chromosomal mutant, to colonize the colon. The chromosomal mutant was rescued only in the colon of mice deficient in gamma interferon (IFN-γ). Thus, the chromosomal mutant's deficiency in colonizing colonic mucosal tissue is likely due to its increased susceptibility to IFN-γ-mediated immunity. Furthermore, IFN-γ deficiency was sufficient for rescuing colon colonization of an orally delivered chromosomal mutant but not plasmid mutant while mice deficient in gastric acid production rescued the plasmid mutant but not the chromosomal mutant. Both mutants are attenuated in inducing genital tract pathology. Thus, we propose that chlamydial chromosomal-gene-encoded genital tract virulence factors may be essential for *Chlamydia* to maintain long-lasting colonization in the colon while the plasmid may enable *Chlamydia* to reach the colon by promoting evasion of gastric barriers.

KEYWORDS *Chlamydia muridarum*, IFN-γ susceptibility, chromosomal genes *tc0237* and *tc0668*, intestinal colonization, plasmid gene *pgp3*

Although *Chlamydia trachomatis* is transmitted among humans mainly via genital tract contacts (1), it is also frequently detected in the gastrointestinal (GI) tract (2–5). However, the medical significance of GI tract *C. trachomatis* remains unclear. It has been proposed that the GI tract *Chlamydia* may serve as a reservoir for autoinoculating the genital tract to promote chlamydial pathogenicity in the genital tract (6, 7). Although there is no direct experimental model-based evidence or clinical investigation for supporting this hypothesis, it is obvious that human sexual behavior may be sufficient for spreading *C. trachomatis* between the human genital and GI tracts. Thus, addressing how *C. trachomatis* colonizes the human GI tract may provide information for understanding chlamydial pathogenicity in the genital tract and for developing strategies for controlling GI *C. trachomatis*. Achieving these goals may require large-scale clinical investigations. We have been using a mouse model to investigate how *Chlamydia muridarum* passes through the mouse gut, which should provide essential mechanistic information for guiding clinical studies.

We chose the *C. muridarum* model because it has been used for investigating the mechanisms of *C. trachomatis* pathogenesis in the genital tract (8–16) and because the

Citation Koprivsek JJ, Zhang T, Tian Q, He Y, Xu H, Xu Z, Zhong G. 2019. Distinct roles of chromosome- versus plasmid-encoded genital tract virulence factors in promoting *Chlamydia muridarum* colonization in the gastrointestinal tract. Infect Immun 87:e00265-19. https://doi.org/10.1128/IAI.00265-19.

Editor Andreas J. Bäumler, University of California, Davis

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Received 8 April 2019
Returned for modification 13 May 2019
Accepted 30 May 2019
Accepted manuscript posted online 3 June 2019
Published 23 July 2019

genital *C. muridarum* organisms can readily spread into the GI tract (17). In addition, *C. muridarum* either spreads from the genital tract or, when orally delivered, can reach the colon to establish long-lasting colonization (6, 17–21). The spread of *C. muridarum* from the genital to GI tract still occurred in mice prevented from coprophagy (17, 19), suggesting that the spreading was likely independent of the oral-fecal route. Instead, the spreading might be mediated via a hematogenous route since intravenously inoculated *C. muridarum* was able to establish long-lasting colonization in the GI tract (22). Interestingly, once in the GI tract, *C. muridarum* failed to auto-inoculate the genital tract (21). Although GI *C. muridarum* cannot auto-inoculate the genital tract, it may still be able to promote chlamydial pathogenicity in the upper genital tract via an indirect mechanism (23). The hypothesis is that GI *Chlamydia* may induce profibrotic lymphocytes that can be subsequently recruited to oviduct tissue previously infected with *Chlamydia* to convert transient tubal tissue repairing into pathogenic fibrosis. Thus, investigating how *Chlamydia* interacts with the GI tract to induce profibrotic responses may promote our understanding of chlamydial pathogenicity in the upper genital tract. In contrast, when naive mice were first exposed to *C. muridarum* in the GI tract, the mice were immunized against subsequent challenge infections in the genital tract (24) or airway (25). These observations suggest that chlamydial colonization in the GI tract may be explored as oral vaccination. Revealing how *Chlamydia* colonizes the GI tract may promote such efforts.

We have previously demonstrated that *C. muridarum* clones carrying mutations in the chromosomal gene *tc0237* and/or *tc0668* or plasmid gene *pgp3* are significantly attenuated in inducing hydrosalpinx in the mouse upper genital tract (26–30). Thus, the *C. muridarum* chromosomal gene-encoded TC0237/TC0668 and plasmid-encoded pGP3 have been defined as genital tract virulence factors (23, 31). These same *C. muridarum* mutant clones are defective in colonizing the mouse GI tract (32–34), indicating that the genital tract virulence factors are also chlamydial colonization factors in the GI tract. Indeed, a *C. muridarum* clone carrying loss-of-function mutations in both chromosomal genes *tc0237* and *tc0668* (designated chromosomal mutant G28.51.1 [32]) and a *C. muridarum* clone deficient in the plasmid-encoded pGP3 (designated plasmid mutant CMpGP3S for comparison purposes [34]) can no longer colonize mouse GI tracts following oral delivery, although both the chromosomal mutant G28.51.1 and the plasmid mutant CMpGP3S can still cause a significant ascending infection following intravaginal inoculation. Thus, these genital tract virulence factors are more important for chlamydial growth in mouse GI tracts than genital tracts. We recently found that orally delivered plasmid mutant CMpGP3S was rapidly cleared by gastric acid in the stomach and unable to reach the colon to establish stable colonization (35). In the current study, we found that the chromosomal mutant G28.51.1 was still able to reach the colon although the plasmid mutant CMpGP3S failed to do so in the same experiments, suggesting that lack of GI tract colonization by different mutants may involve distinct mechanisms. Indeed, a direct intracolonic delivery selectively restored the plasmid mutant but not the chromosomal mutant to colonize the colon. The latter was rescued only in the colon tissues of mice deficient in gamma interferon (IFN-γ). Thus, we have identified the chromosomal mutant G28.51.1 as a colon colonization-deficient mutant that is susceptible to IFN-γ-mediated immunity. More importantly, comparing the orally delivered chromosomal and plasmid mutants for colonizing the GI tracts of mice deficient in IFN-γ or gastric acid production has led us to propose that chlamydial chromosomal-gene-encoded genital tract virulence factors may be essential for *Chlamydia* to maintain long-lasting colonization in the colon while the plasmid may enable *Chlamydia* to reach the colon.

RESULTS

***C. muridarum* with mutations in two chromosomal genes is still able to reach the colon following an oral inoculation.** We previously reported that a series of *C. muridarum* mutants with mutations in either chromosomal genes or plasmid genes were both attenuated in inducing hydrosalpinx in the mouse upper genital tract (26,

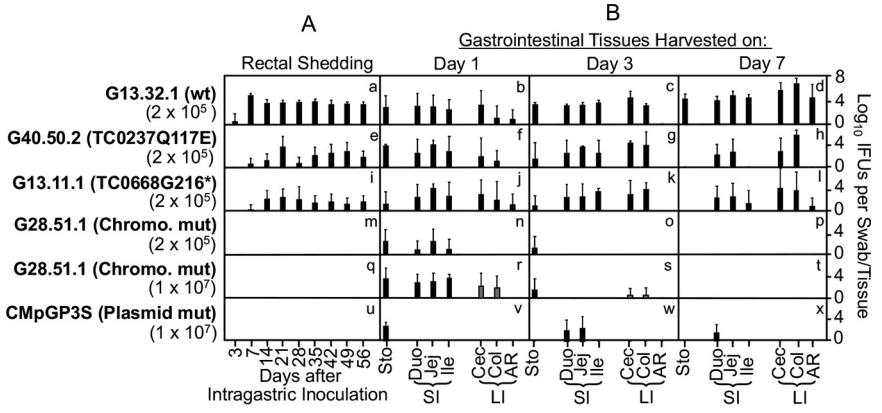

FIG 1 Comparing *C. muridarum* organisms with or without mutations in chromosomal genes *tc0668* and/or *tc0237* or plasmid gene *pgp3* for colonizing the gastrointestinal tract following oral/intragastric inoculation. (A) The wild-type (wt) clone G13.32.1 or *C. muridarum* with a substitution mutation in chromosomal gene *tc0237* (G40.50.2 [TC0237/Q117E]), a premature stop codon in chromosomal gene *tc0668* (G13.11.1 [TC0668/G216*]), a *tc0668* premature stop codon plus a *tc0237* substitution mutation (G28.51.1 [TC0668/G216* TC0237/Q117E]), designated a chromosomal mutant, or a premature stop codon in plasmid gene *pgp3* (CMpGP3S, designated a plasmid mutant) was orally/intragastrically inoculated into female C57BL/6J mice at the indicated doses (IFUs) ($n = 5$ mice per group). On days 3 and 7 and weekly thereafter, after inoculation as shown along the *x* axis, rectal swabs were taken for monitoring live chlamydial organism shedding. Note that both the chromosomal mutant G28.51.1 and the plasmid mutant CMpGP3S failed to colonize the gut. $P < 0.05$ (for comparison of the area under curve between panels a and m, q, or u; Wilcoxon test). (B) Parallel groups of mice ($n = 5$) were sacrificed on the indicated days after intragastric inoculation for monitoring live chlamydial organisms in different segments of the gastrointestinal tract including the stomach (Sto), small intestine (SI) tissues of the duodenum (Duo), jejunum (Jej), and ileum (Ile) and large intestine (LI) tissues of the cecum (Cec), colon (Col), and anorectum (AR) as indicated. Note that the chromosomal mutant G28.51.1 reached the colon following intragastric inoculation (panels r and s) while the plasmid gene mutant CMpGP3S failed to do so (panels v to x). $P < 0.05$ (for a comparison of the numbers of IFUs recovered from cecum or colon in G28.51.1- and CMpGP3S-infected mice, as highlighted in panels r and v; Wilcoxon test).

27) and defective in colonizing the gastrointestinal (GI) tract (32–34). In the current study, we further determined where in the GI tract the colonization deficiency took place for both the chromosomal mutant G28.51.1 and the plasmid mutant CMpGP3S. G28.51.1 contains both a substitution mutation in chromosomal gene *tc0237*, resulting in mutated protein TC0237/Q117E, and a premature stop codon in chromosomal gene *tc0668*, resulting in truncated and unstable protein TC0668/G216* (27), while CMpGP3S harbors a premature stop codon in the *pgp3* gene on the plasmid, resulting in a lack of pGP3 protein (29, 36). As shown in Fig. 1, following oral/intragastric inoculation, although the wild-type *C. muridarum* organisms (clone G13.32.1) stably colonized the GI tract, both the chromosomal mutant G28.51.1 and plasmid mutant CMpGP3S failed to do so. No live organisms could be recovered from rectal swabs of mice inoculated with these mutant organisms. The clones that carry either the substitution mutation in *tc0237* (G40.50.2 [TC0237/Q117E]) or a premature stop codon in *tc0668* (G13.11.1 [TC0668/G216*]) alone were still able to productively colonize the GI tract although each at a reduced level, suggesting that these two chromosomal genes may play a redundant role in promoting chlamydial colonization in the GI tract. We then further monitored the distribution of live chlamydial organisms in different segments of the GI tract. We found that, like the wild-type clone G13.32.1, both single chromosomal mutant clones G40.50.2 and G13.11.1 productively colonized all GI tract tissues although at reduced levels. However, G28.51.1 with mutations in both chromosomal genes *tc0237* and *tc0668* was quickly cleared from the GI tissues by day 7 after oral inoculation. It is worth pointing out that G28.51.1 did initially reach the colon. In contrast, the plasmid mutant CMpGP3S never reached the colon at any time point following oral inoculation, which is consistent with our recent finding that CMpGP3S is extremely susceptible to the clearance by gastric acids (35). These observations together suggest that plasmid genes may mainly promote chlamydial resistance to

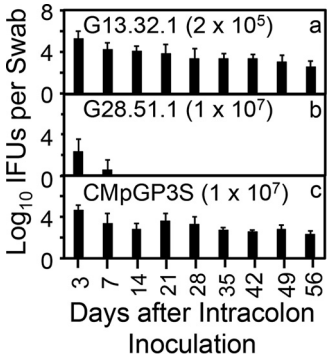

FIG 2 Comparison of *C. muridarum* with or without mutations in chromosomal genes *tc0668* and *tc0237* or plasmid gene *pgp3* for the ability to colonize the mouse gastrointestinal tract following an intracolonic inoculation. *C. muridarum* without (G13.32.1) or with mutations in chromosomal genes *tc0668* and *tc0237* (G28.51.1) or plasmid gene *pgp3* (CMpGP3S) was used to infect groups of C57BL/6J mice ($n = 5$) via intracolon inoculation. On days 3 and 7 and weekly thereafter, after the intracolon inoculation, rectal swabs were taken for monitoring live chlamydial organism shedding. Note that intracolonic inoculation fully rescued the plasmid gene mutant CMpGP3S but not the chromosomal gene mutant G28.51.1. $P < 0.05$ (for the area under curve for panel a versus panel b but not for panel a versus panel c; Wilcoxon test).

gastric barriers while chromosomal genes may be necessary for promoting chlamydial survival in the colon.

The chromosomal mutant is rapidly cleared from the colon while the plasmid mutant is able to continuously colonize the colon following an intracolonic inoculation. To test whether the chromosomal mutant G28.51.1 and plasmid mutant CMpGP3S differ in their abilities to colonize the colon, we directly inoculated them into mouse colon and compared their recoveries in rectal swabs (Fig. 2). As we have previously shown (35), intracolonic inoculation with CMpGP3S successfully rescued its colonization defect in the GI tract. High levels of live organisms were recovered from the rectal swabs of the inoculated mice throughout the experiment. However, the chromosomal mutant G28.51.1 was rapidly cleared following the intracolonic inoculation. When we monitored the tissue distribution of live chlamydial organisms (Fig. 3), we found that the wild-type chlamydial organisms continuously colonized tissues of the large intestine, with spreading to the entire GI tract. Although the chromosomal mutant G28.51.1 was initially recovered from cecum/colon tissue, it was rapidly cleared

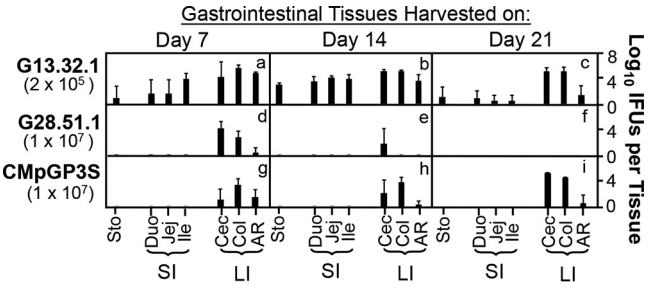

FIG 3 Comparison of *C. muridarum* with or without mutations in chromosomal genes *tc0668* and *tc0237* or plasmid gene *pgp3* for survival in mouse gastrointestinal tissues following an intracolonic inoculation. Groups of C57BL/6J mice intracolonically infected with *C. muridarum* as described in the legend of Fig. 2 were sacrificed on days 7 ($n = 5$), 14 ($n = 5$), and 21 ($n = 3$ to 5) after the intracolonic inoculation for monitoring live chlamydial organisms in different segments of the gastrointestinal tract including stomach (Sto), small intestine (SI) tissues of the duodenum (Duo), jejunum (Jej), and ileum (Ile) and large intestine (LI) tissues of the cecum (Cec), colon (Col), and anorectum (AR) as indicated. Note that the chromosomal gene mutant G28.51.1 rapidly reduced its survival in the mouse colon by day 14 and completely cleared by day 21 while the plasmid gene mutant CMpGP3S was able to productively colonize the colon following an intracolonic inoculation. Thus, we designated G28.51.1 a colon colonization-deficient mutant. $P < 0.05$ (for results from colons of G28.51.1- versus CMpGP3S-infected mice in panel e versus panel h; Wilcoxon test).

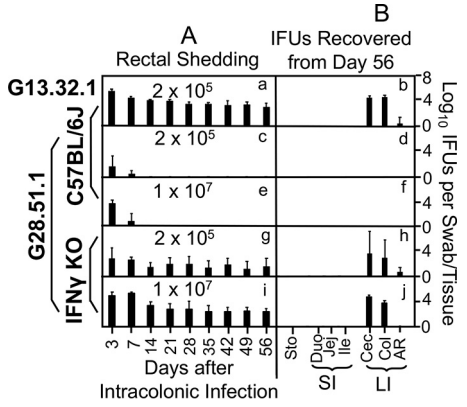

FIG 4 Rescuing chromosomal mutant G28.51.1 to colonize colon in IFN-γ-deficient mice. (A) Groups ($n = 5$) of C57BL/6J mice without or with deficiency in IFN-γ (KO) were intracolonically infected with *C. muridarum* without (G13.32.1) or with mutations in chromosomal genes *tc0668* and *tc0237* (G28.51.1) at the indicated doses. On days 3 and 7 and weekly thereafter, after the intracolon inoculation, rectal swabs were taken for monitoring live chlamydial organism shedding. Note that the ability of the chromosomal gene mutant G28.51.1 to colonize the gastrointestinal tract was fully rescued in mice deficient in IFN-γ. $P < 0.01$ (for the area under curve in panel a versus panel c or e, for panel c versus panel g, or panel e versus panel i; Wilcoxon test). (B) All mice were sacrificed on day 56 after intracolon infection for monitoring live chlamydial organisms in different segments of gastrointestinal tract including the stomach (Sto), small intestine (SI) tissues of the duodenum (Duo), jejunum (Jej), and ileum (Ile) and large intestine (LI) tissues of the cecum (Cec), colon (Col), and anorectum (AR), as indicated. Note that IFN-γ deficiency rescued the colon colonization mutant G28.51.1, allowing it to fully establish productive and long-lasting colonization in the colon.

at late time points. However, the plasmid mutant CMpGP3S continued to colonize the cecum/colon tissues. These observations have demonstrated that the plasmid gene *pgp3* is not required while the two chromosomal genes *tc0237* and *tc0668* are necessary for promoting chlamydial colonization in the colon.

The chromosomal mutant is rescued to colonize the colon of IFN-γ-deficient mice. The above-described experiments have demonstrated distinct differences between the chromosomal and plasmid mutants in their abilities to reach and colonize the colon. Since the chromosomal mutant G28.51.1 is not able to maintain colonization of the colon, we tested whether IFN-γ is responsible for restricting G28.51.1 colonization in the colon (Fig. 4). This is because IFN-γ is the most powerful host defense effector molecule for inhibiting *Chlamydia* identified so far (10). We found that the growth of G28.51.1 in the colon of mice deficient in IFN-γ was significantly rescued regardless of whether G28.51.1 was intracolonically inoculated at 2×10^5 or 1×10^7 inclusion-forming units (IFUs)/mouse. The colonization pattern of the chromosomal mutant G28.51.1 in IFN-γ-deficient mice mimicked that of wild-type *C. muridarum* in wild-type mice, suggesting that IFN-γ is a key host factor for controlling the colonization of G28.51.1 in the colon. Thus, we designated the chromosomal mutant G28.51.1 an IFN-γ-susceptible clone.

IFN-γ deficiency rescues an orally delivered chromosomal mutant while gastrin deficiency rescues the plasmid mutant but not vice versa. Since the growth of intracolonically inoculated G28.51.1 in the colon was significantly rescued in mice deficient in IFN-γ (Fig. 4) and since orally inoculated G28.51.1 could transiently reach the colon (Fig. 1), we next tested whether IFN-γ deficiency can also rescue orally inoculated G28.51.1 to colonize the GI tract. As shown in Fig. 5, live organisms were indeed recovered from the rectal swabs of IFN-γ-deficient mice orally inoculated with G28.51.1 throughout the experiment, indicating that the orally inoculated G28.51.1 organisms were able to resist the mouse defense mechanisms in the stomach and small intestine to reach the colon of IFN-γ-deficient mice. We did note a delay in live-organism shedding in these mice (the first positive detection was on day 21), which may reflect the time required for the orally inoculated G28.51.1 organisms to overcome the barriers in the stomach and small intestine. Indeed, as we have shown previously

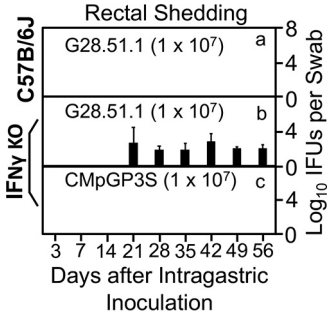

FIG 5 IFN-γ deficiency is sufficient for rescuing orally delivered chromosomal mutant G28.51.1 to colonize the gastrointestinal tract. C57BL/6J mice ($n = 5$) without or with a deficiency in IFN-γ (KO) were intragastrically infected with *C. muridarum* with mutations in chromosomal genes *tc0668* and *tc0237* (G28.51.1) or a mutation in plasmid gene *pgp3* (CMpGP3S) as indicated. On days 3 and 7 and weekly thereafter, after the intragastric inoculation, rectal swabs were taken for monitoring live chlamydial organism shedding. Note that the ability of the chromosomal gene mutant G28.51.1 but not the plasmid mutant CMpGP3S to establish long-lasting colonization in the gastrointestinal tract was successfully rescued in mice deficient in IFN-γ. $P < 0.05$ (for the area under curve in panel a versus panel b or panel b versus panel c; Wilcoxon test).

(35), IFN-γ-deficient mice still maintain robust acidic barrier function in the stomach since the gastric acid-susceptible plasmid mutant CMpGP3S was unable to overcome these barriers. No live organisms were ever recovered from the IFN-γ-deficient mice orally inoculated with CMpGP3S. In contrast, in gastrin-deficient mice that are defective in gastric acid secretion without significant alterations in other host defense mechanisms (37), the plasmid mutant CMpGP3S was rescued, as shown previously (35), while the chromosomal mutant G28.51.1 was not, as shown in Fig. 6. Thus, the above observations together indicate a clear separation of function between the plasmid-encoded and chromosomal-gene-encoded chlamydial genital tract virulence factors or gut colonization factors.

DISCUSSION

Chlamydial organisms have been detected in GI tracts of both human and animal hosts (2–6, 17–19, 22, 38–45). *C. muridarum* readily spreads from the genital tract to the GI tract and persists in the colon for long periods of time (6, 17, 21, 22, 44). Although GI *Chlamydia* is nonpathogenic to the GI tract (21, 24), it may impact chlamydial pathogenicity in the genital tract by either serving as a reservoir for repeatedly infecting the genital tract (6, 7, 20) or inducing profibrotic responses to exacerbate pathology in the upper genital tract (23). On the other hand, the chlamydial colonization in the GI

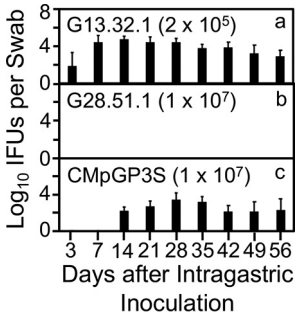

FIG 6 Comparing *C. muridarum* with or without mutations in chromosomal genes *tc0668* and *tc0237* or plasmid gene *pgp3* for colonizing the gastrointestinal tracts of gastrin-deficient mice. *C. muridarum* without (G13.32.1) or with mutations in chromosomal genes *tc0668* and *tc0237* (G28.51.1) or plasmid gene *pgp3* (CMpGP3S) was intragastrically inoculated into gastrin-deficient mice ($n = 5$ per group). On days 3 and 7 and weekly thereafter, after inoculation rectal swabs were taken for monitoring live chlamydial organism shedding. Note that gastrin deficiency rescued the plasmid mutant CMpGP3S but not the chromosomal mutant G28.51.1, allowing it to colonize the gut. $P < 0.01$ (for the area under curve in panel a versus that in panel b; Wilcoxon test).

tract may be explored as oral vaccination. When naive mice were first exposed to *C. muridarum* in the GI tract, the mice were immunized against subsequent challenge infections in the genital tract (24) or airway (25). Thus, revealing the molecular and cellular basis of chlamydial interactions with the GI tract may both promote our understanding of chlamydial pathogenic mechanisms in the genital tract and provide information for developing chlamydial vaccines. Thus, in the current study, we have compared the roles of chlamydial plasmid- and chromosome-encoded virulence factors (identified during chlamydial infection in the genital tract) in chlamydial colonization of the GI tract. We have presented evidence for supporting the hypothesis that chlamydial chromosomal-gene-encoded genital tract virulence factors may be essential for *Chlamydia* to maintain long-lasting colonization in the colon while plasmid-encoded factors may help *Chlamydia* reach the colon. First, although the wild-type *C. muridarum* is able to colonize the mouse colon for long periods of time following oral delivery, chlamydial organisms deficient in either chromosome- or plasmid-encoded genital tract virulence factors are no longer able to do so. Second, an orally delivered chromosomal but not plasmid mutant can reach the colon. Third, direct intracolonic inoculation can restore the plasmid but not the chromosomal mutant to colonize the colon. Fourth, the chromosomal mutant can be rescued to colonize the colon of IFN-γ-deficient mice. Finally, following an oral delivery, the ability of the chromosomal but not plasmid mutant to colonize the colon was rescued in IFN-γ-deficient mice while that of the plasmid but not the chromosomal mutant was rescued in gastrin-deficient mice.

Careful comparison of the plasmid mutant and chromosomal mutant has revealed that the plasmid mutant is more defective in ascending infection following intravaginal inoculation (26–30). The chromosomal mutant maintained an ascending infection as robust as that of the wild-type organisms. The difference between the plasmid and chromosomal mutants in their abilities to maintain an ascending infection in the genital tract reflects the difference in their abilities to promote chlamydial evasion of the gastric acid barrier. The plasmid mutant CMpGP3S is more defective in maintaining an ascending infection because the plasmid-encoded pGP3 is essential for *Chlamydia* to overcome acidic barriers in both the stomach and vagina (35). It is likely that *Chlamydia* may have acquired the plasmid for facilitating its own trafficking to the colon during its adaptation to the GI tract. The ability to evade the gastric acid barrier may enable chlamydial organisms to overcome the lactic acid barrier in the vagina when *Chlamydia* is introduced to the genital tract. This hypothesis is consistent with the finding that the plasmid mutant CMpGP3S is ~100-fold more defective than the wild-type *C. muridarum* in surviving the stomach while only ~8-fold more defective in surviving the vagina (35).

It is worth noting that chromosomal mutant G28.51.1 (with loss-of-function mutations in both *tc0237* and *tc0668*) maintained as robust an ascending infection in the genital tract as wild-type *C. muridarum* (27, 32), which has prevented us from determining its susceptibility to IFN-γ. Only in the colon did G28.51.1 become deficient in colonizing mucosal tissue, which has allowed us to discover that the two chromosomal-gene-encoded proteins TC0237 and TC0668 may promote chlamydial colonization of mucosal tissues by targeting IFN-γ-mediated immunity. Clearly, *C. muridarum* interaction with mouse colon is a more productive platform for revealing the secrets of chlamydial biology. However, it remains unclear why the chromosomal mutant G28.51.1 can colonize the genital tract as robustly as its isogenic wild-type control but becomes highly susceptible to IFN-γ inhibition in the colon. Obviously, the colon environment is different from the genital tract environment although both are mucosal tissues. One possibility is that colonic IFN-γ can more efficiently access *Chlamydia*-infected cells than genital tract IFN-γ does, which is testable by comparing the spatial relationships between IFN-γ-producing and *Chlamydia*-infected cells in the colon and genital tract. Nevertheless, the finding from chlamydial colonization in the colon has allowed us to propose that *C. muridarum* may have acquired the chromosomal genes *tc0237* and *tc0668* for maintaining long-lasting colonization in the colon during its adaptation to the gastrointestinal tract. It makes sense for *Chlamydia* to use chromosomal genes but not plasmid genes to evade IFN-γ, the most powerful host immune

effector for inhibiting chlamydial growth. The next question is how exactly TC0237/TC0668 can redundantly promote chlamydial resistance to IFN-γ in the colon.

Both TC0237 and TC0668 are conserved chlamydial proteins with no known function. TC0237 has a total of 159 amino acids (aa) and contains a domain of unknown function 720 (DUF720) motif (46). DUF720 is also found in the neighboring hypothetical proteins TC0236 (172 aa) and TC0235 (170 aa). These proteins are paralogous to each other and share ~90% amino acid sequence identity with their *C. trachomatis* serovar D counterparts CT849, CT848, and CT847. The TC0237 mutation was previously shown to result in enhanced attachment to cultured cells (26, 27), suggesting that the three proteins may participate in initial chlamydial interactions with host cells. The TC0237 mutation in G28.51.1 organisms may make the organisms less efficient in invading new colonic epithelial cells in the presence of IFN-γ. TC0668 has a total of 408 aa, sharing ~90% amino acid identity with its homologue CT389 from *C. trachomatis* serovar D. CT389 is predicted to associate with the chlamydial outer membrane complex (47). It contains a series of transmembrane domains in the N terminus and a domain of unknown function 1207 (DUF1207) motif in the remaining region. TC0668 might inherit the DUF1207 motif from a distant chlamydial progenitor (27). An I-TASSER search reveals TC0668 homology with various eukaryotic integrins (27). Because these integrins are involved in diverse cellular activities, it is difficult to predict the function of TC0668. Like its homologue CT389 from *C. trachomatis*, TC0668 may also be an outer membrane protein. Although the TC0668 mutations resulted in loss of the TC0668 protein that did not alter chlamydial attachment efficiency in cultured cells, the TC0668 mutation in G28.51.1 may make chlamydial organisms more susceptible to colonic IFN-γ inhibition. Apparently, further investigations are necessary for defining the exact functions of TC0237 and TC0668. Nevertheless, since the wild-type control G13.32.1 is isogenic to G28.51.1 (26, 27), we can conclude that these two chromosomal-gene-encoded proteins can redundantly maintain chlamydial long-lasting colonization in the colon by promoting evasion of colonic IFN-γ. It is possible that the TC0237/TC0668-deficient *C. muridarum* organisms may become susceptible to an interferon (IFN)-inducible guanylate binding protein (GBP)-mediated cell-autonomous host mechanism while wild-type *C. muridarum* organisms are known to resist this IFN-γ mechanism (48).

Although G28.51.1 is highly attenuated in inducing hydrosalpinx in the genital tract, it still maintains a robust ascending infection to the oviduct (32). This observation suggests that an ascending infection alone may not be sufficient for inducing the long-lasting hydrosalpinx although it may be necessary (16). The chlamydial colonization in the gut mucosal tissue seems to corelate with its pathogenicity in the upper genital tract. The question is how the colonic *Chlamydia* may contribute to chlamydial pathogenicity in the upper genital tract. It has been hypothesized that the chlamydial long-term colonization in the GI tract or colon may serve as a reservoir for auto-inoculating the genital tract (6, 44). We have recently found that *C. muridarum* failed to auto-inoculate the genital tract after colonization in the GI tract of the same mouse for 70 days (21). Thus, it is likely that the colonic chlamydial organisms may promote chlamydial pathogenicity in the genital tract via an indirect mechanism.

There is a big caveat in applying the knowledge learned from the murine system to humans due to the difference between *C. muridarum* interactions with mice and *C. trachomatis* interactions with humans. Most animal chlamydial species, including *C. muridarum*, may have adapted to the GI tracts of their corresponding host species. However, *C. trachomatis* has been forced to adapt to the human genital tract due to the fact that sexual transmission between modern humans is more efficient than oral-fecal route transmission. Nevertheless, the genomes of *C. muridarum* and *C. trachomatis* are still largely conserved (46, 49). Specifically, both TC0237 and TC0668 share >90% amino acid identity with their *C. trachomatis* counterparts. *C. trachomatis* has been frequently detected in the human GI tract (50). Furthermore, like *C. muridarum* (18, 44), *C. trachomatis* has not been associated with any significant GI tract pathologies (51). Thus, further revealing the specific mechanisms by which DUF720- and/or DUF1207-containing homologues promote chlamydial resistance to IFN-γ during chlamydial

colonization in the colon may produce useful information for combating highly prevalent urogenital *C. trachomatis* disease in humans.

MATERIALS AND METHODS

***Chlamydia muridarum* organism growth.** *C. muridarum* strain Nigg3 (GenBank accession no. CP009760.1) and its derived mutants were all propagated and purified using HeLa cells (human cervical carcinoma epithelial cells; ATCC CCL2) as described previously (17, 52). The *C. muridarum* mutant clones were generated using multiple *in vitro* passages as described previously (26, 27). The following three isogenic passaging clones were used in the current study: G13.32.1 (retaining the wild-type Nigg3 genome sequence), G40.50.2 (a substitution mutation in gene *tc0237* resulting in a Q[glutamine] 117E[glutamic acid] mutation in TC0237 protein, TC0237/Q117E), G13.11.1 (a frameshift mutation in *tc0668* resulting in a premature stop codon at the 216th codon [for glycine], TC0668/G216*), and G28.51.1 (carrying both the premature stop codon in *tc0668* and the substitution mutation in *tc0237* resulting in TC0668/G216* TC0237/Q117E). Since clone G28.51.1 with the double mutation displayed a consistent striking phenotype in both mouse genital and GI tracts (27, 32), we designated this mutant a chromosomal mutant in the current study. In addition, a plasmid-free clone, CMUT3G5 (GenBank accession number CP006974.1), was initially derived from Nigg3 (28) and used for transformation with the plasmid pCM-GFP carrying a premature stop codon in the *pgp3* gene to create CMpGP3S (designated a plasmid mutant in the current study for comparison purposes), as described previously (29, 36). All *C. muridarum* organisms were purified as elementary bodies (EBs) and stored in aliquots at −80°C until use. The stocks used for the current study were resequenced after purification, and their genomes and plasmid sequences were confirmed to be identical to those described previously (27, 29, 36).

Mouse infection. The mouse experiments were carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* endorsed by the National Institutes of Health (53). The protocol was approved by the Committee on the Ethics of Laboratory Animal Experiments of the University of Texas Health Science Center at San Antonio.

Purified *C. muridarum* EBs were used to inoculate 5- to 7-week-old mice (Jackson Laboratories, Inc., Bar Harbor, ME) orally/intragastrically, intracolonically, or intravaginally with different amounts of inclusion-forming units (IFUs), as indicated in individual experiments and as described previously (16, 17, 29, 34). The wild-type C57BL/6J (stock number 000664) and IFN-γ knockout (KO) (B6.129S7-*Ifng*^{tm1Ts}/J; stock number 002287) mice were from Jackson Laboratory (Bar Harbor, ME) while breeding pairs of gastrin-deficient mice were from the University of Michigan (37).

For oral delivery or intragastric inoculation, live *C. muridarum* EBs suspended in 200 μl of SPG buffer (containing sucrose, phosphate, and glutamic acid) were delivered to each mouse using a straight, ball-tipped needle (N-PK 020; Braintree Scientific, Inc., Braintree, MA). For intracolonic infections, EBs were diluted in 50 μl of SPG buffer that contained the desired number of IFUs as indicated in individual experiments and delivered to the colon using a straight ball-tipped needle designed for mouse oral gavage (N-PK 020; Braintree Scientific, Inc., Braintree, MA). After the inoculations, mice were monitored for rectal live organism shedding or sacrificed for titrating live organisms in corresponding organs/tissues.

Titrating live chlamydial organisms from mouse swabs and tissues. For monitoring live organism shedding from genital and GI tracts, rectal swabs were taken on day 3 postinoculation and weekly thereafter. Each swab was soaked in 0.5 ml of SPG buffer and vortexed with glass beads to release infectious EBs for quantitation. For titrating live chlamydial organisms recovered from mouse tissues, various organs/tissues were harvested on designated days after inoculation as specified in individual experiments. Each organ or tissue segment was transferred to a tube containing 0.5 ml (for each segment of the genital tract tissue) or 2 ml (for each remaining tissues/organ) of SPG buffer. Each GI tract was divided into the following segments: stomach; small intestine tissues of duodenum, jejunum, and ileum; large intestine tissues of the cecum, colon, and anorectum. The organs and tissue segments were homogenized in cold SPG buffer. The live *C. muridarum* organisms released into swab suspensions or tissue supernatants were titrated on HeLa cells in duplicate as described previously (16, 17, 21, 22, 54, 55). The total number of IFUs/swab or tissue was converted into $\log_{10}$ values for calculating the mean and standard deviation across mice of the same group at each time point. The detection limits of the above titration method are 10 IFUs per swab and 40 IFUs per tissue sample. This is because we always used 50 μl of the total volume (500 μl for swab and 2,000 μl for tissue), starting with neat tissue homogenates without dilution and then following with serial dilutions to titrate the numbers of IFUs of a given sample. We counted the entire titration culture well for chlamydial inclusions when the inclusion density was low, allowing the detection of a single IFU per 50-μl sample.

Immunofluorescence assay. An immunofluorescence assay was used for titrating live organisms as described previously (52, 56). Briefly, HeLa cells grown on coverslips were fixed with paraformaldehyde (Sigma) and permeabilized with saponin (Sigma). After being washed and blocked, the cell samples were subjected to a combination of antibody and chemical staining. Hoechst (blue; Sigma) was used to visualize nuclear DNA. A rabbit anti-chlamydial antibody (raised by immunization with *C. muridarum* EBs [data not shown]) plus a goat anti-rabbit IgG conjugated with Cy2 (green) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used to visualize chlamydial inclusions. The cell samples after immunolabeling were used for counting inclusions under an Olympus AX-70 fluorescence microscope equipped with multiple filter sets (Olympus, Melville, NY).

Statistics analyses. The numbers of live organisms expressed as IFU counts were compared between groups using a Wilcoxon rank sum test. Rates of samples positive for IFUs between groups were evaluated using Fisher's exact probability test (http://vassarstats.net/tab2x2.html).

ACKNOWLEDGMENT

This work was supported in part by grants (G.Z.) from the U.S. National Institutes of Health (grants R01AI121989 and R01AI047997).

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