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Narrow-Spectrum Inhibitors of *Campylobacter jejuni* Flagellar Expression and Growth

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***Campylobacter jejuni* is a major cause of food-borne illness due to its ability to reside within the gastrointestinal tracts of chickens. Multiple studies have identified the flagella of *C. jejuni* as a major determinant of chicken colonization. An inhibitor screen of approximately 147,000 small molecules was performed to identify compounds that are able to inhibit flagellar expression in a reporter strain of *C. jejuni*. Several compounds that modestly inhibited motility of wild-type *C. jejuni* in standard assays were identified, as were a number of small molecules that robustly inhibited *C. jejuni* growth, *in vitro*. Examination of similar bacterial screens found that many of these small molecules inhibited only the growth of *C. jejuni*. Follow-up assays demonstrated inhibition of other strains of *C. jejuni* and *Campylobacter coli* but no inhibition of the closely related *Helicobacter pylori*. The compounds were determined to be bacteriostatic and nontoxic to eukaryotic cells. Preliminary results from a day-of-hatch chick model of colonization suggest that at least one of the compounds demonstrates promise for reducing *Campylobacter* colonization loads *in vivo*, although further medicinal chemistry may be required to enhance bioavailability.**

Campylobacter jejuni is a leading cause of food-borne illness in the United States, with a projected 1.3 million infections annually (1). This prevalence of infection with *C. jejuni* is primarily due to its ability to reside, asymptomatically, within the gastrointestinal tracts of agriculturally relevant animals, especially chickens, where it can reach high bacterial loads ($\sim 10^9$ CFU/g of cecal contents) (2). During harvest, *C. jejuni* is released from the gastrointestinal tract, contaminating meat products. Human infection often occurs following consumption of either undercooked meat or food that contacted a contaminated surface. As such, much work has focused on identifying factors of *C. jejuni* that enable the organism to colonize the chicken gastrointestinal tract.

Genetic screens have been used to identify *C. jejuni* factors involved in colonization of the chicken. These include signature-tagged mutagenesis and transposon insertion sequencing (Tn-Seq) approaches to identify mutants with decreased cecal abundance; these studies demonstrated that flagella are required for full colonization of the chicken cecum (2, 3). Additionally, the regulatory hierarchy of flagellar biosynthesis has been characterized (4).

With widespread antibiotic resistance and concern about dysbiosis resulting from treatment with broad-spectrum antibiotics, new approaches that target pathogenic microbes are needed (5). *C. jejuni* has recently been determined to be a "serious threat" for developing antibiotic resistance by the Centers for Disease Control and Prevention (6). This report specifically notes an observed increase in ciprofloxacin resistance among *C. jejuni* isolates from 1997 to 2011, an increase from 13% to 25%, respectively. Two approaches to combat pathogens are (i) to identify chemical inhibitors of specific virulence factors and (ii) to identify narrow-spectrum compounds that selectively inhibit growth of a target pathogen (7–10). An example of the former is the high-throughput screen for inhibitors of ToxT-dependent regulation in *Vibrio cholerae*, which identified a small molecule, termed virstatin, that inhibited expression of both cholera toxin (*ctx*) and the toxin coregulated pilus (11). Virstatin inhibits ToxT dimerization, affecting the ability of the regulator to bind the *ctx* promoter (12). Additionally, virstatin protected infant mice from intestinal colonization by *V. cholerae* (11). An example of the latter is the mycobacterium-specific antimicrobial bedaquiline, which inhibits the F_1F_o-ATPase (10). While conserved across bacteria and mammals, variation in the mycobacterial F_1F_o-ATPase confers selectivity of the compound for these species. Bedaquiline, the first antimicrobial identified using a high-throughput screen of a commercially available library, was approved by the Food and Drug Administration (FDA) for treatment of human multidrug-resistant tuberculosis (MDR-TB) infection (10).

For a precise anti-*Campylobacter* strategy, we developed a screen to identify small-molecule inhibitors of flagellum expression in *C. jejuni*. We used a reporter strain with a gene encoding chloramphenicol acetyltransferase expression (*cat*) under the control of the *flgDE2* promoter. Given the nature of the regulatory hierarchy of flagellar biosynthesis in *C. jejuni*, any of several structural and regulatory gene products could be targets for inhibition with this strategy (4).

Following a screen of approximately 147,000 compounds against the *C. jejuni* reporter strain, we identified several potential flagellar inhibitors that ultimately exhibited modest effects on motility. Our approach, however, enabled simultaneous identification of compounds that specifically inhibited growth of our *C.*

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jejuni reporter strain but not of several other Gram-negative bacteria screened with the same library of compounds. Several lead compounds also inhibited growth of farm-isolated strains of *C. jejuni* and *Campylobacter coli* but did not inhibit growth of *Helicobacter pylori*. For *in vivo* efficacy, we determined that the *Campylobacter* growth inhibitors, which we term campynexins, are bacteriostatic and do not cause eukaryotic cell cytotoxicity. Finally, campynexins were administered to infected day-of-hatch chicks; one compound was effective, albeit inconsistently, toward *C. jejuni* in the gastrointestinal tract.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains used for subcloning were grown in LB medium at 37°C under aerobic conditions. *C. jejuni* strains and *C. coli* were grown at 37°C under microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) on either Mueller-Hinton (MH) agar containing 10% sheep's blood or in MH broth. *H. pylori* was grown on MH agar plates containing 10% sheep's blood under the same conditions as *C. jejuni*. In liquid culture, *H. pylori* was grown in 90% base medium (60% brucella broth and 40% calprotectin buffer [100 mM NaCl, 3 mM $CaCl_2$, 20 mM Tris, pH 7.5]) and 10% heat-inactivated fetal bovine serum (FBS). For *C. jejuni* experiments, the following antibiotics were used at the indicated concentrations: chloramphenicol (Cam), 7.5 μg/ml; kanamycin (Kan), 150 μg/ml; and trimethoprim (Tmp), 10 μg/ml.

Animal protocols used in this study were approved by the University of Michigan's University Committee on Use and Care of Animals.

Construction of reporter strain. A pUC19-based plasmid containing the *flgDE2* operon (DRH351) was mutagenized with a *mariner* transposon that contains a promoterless *cat* gene linked to a constitutive kanamycin resistance gene (DRH164) (4). Transposon insertions into *flgDE2* were identified by PCR and confirmed by sequencing. This plasmid was introduced into wild-type *C. jejuni* DRH212 (13) via electroporation, and transformants were selected for on MH blood plates containing kanamycin. Chromosomal integration was confirmed by PCR. To confirm that expression of *cat* was dependent on *flgDE2* expression, the reporter construct was also introduced into *C. jejuni* DRH212 Δ*flhA*, Δ*fliP*, and Δ*rpoN* mutant chromosomes and growth in chloramphenicol-containing media was examined (4). Briefly, each strain was used to inoculate MH medium containing 7.5 μg/ml chloramphenicol at an optical density at 600 nm (OD_{600}) of 0.025 in 96-well plates. Plates were incubated statically at 37°C under microaerobic conditions for 48 h before the cells were resuspended and the OD_{600} was recorded. Since several of these flagellar mutants exhibited decreased endpoint growth in medium lacking chloramphenicol, it was necessary to correct the results from chloramphenicol-containing medium in order to determine accurate statistical effect sizes (Z′ values). This was done by determining the extent of the growth defect for each mutant in medium lacking chloramphenicol and dividing mutant growth by wild-type growth (to determine % wild-type growth). The differences between wild-type growth (100%) and flagellar mutant growth (% wild-type growth as calculated above) were added to 1 and multiplied by those values observed for each mutant in chloramphenicol-containing medium.

Primary small-molecule screen. A primary screen was performed using the *C. jejuni flgDE2*::′*cat-kan* reporter strain (the prime denotes that the *cat* gene is promoterless). Approximately 147,000 compounds from the small-molecule collection at the University of Michigan Center for Chemical Genomics (UM-CCG) were tested. Assay plates were prepared, with 20 μl MH broth in each well of 384-well microtiter plates (Corning product no. 3680). Compounds were added in 0.2 μl of 2 mM dimethyl sulfoxide (DMSO) stock solutions using a Beckman Biomek FX liquid handler with a high-density replication (HDR) "pintool" (Beckman Coulter, Brea, CA). Columns containing either DMSO alone (the vehicle for all compounds) or ciprofloxacin were used as negative and positive controls, respectively. An equal volume (20 μl) of bacterial suspension containing *C. jejuni flgDE2*::′*cat-kan* at an OD_{600} of 0.05 with chloramphenicol at 15 μg/ml was added to all wells, resulting in the following final concentrations: compound, 10 μM; *C. jejuni flgDE2*::′*cat-kan*,0.025 (OD_{600}); and chloramphenicol, 7.5 μg/ml. Plates were incubated statically for 48 h at 37°C under microaerobic conditions before absorbances (OD_{600}s) were recorded.

Triage and confirmation. To identify inhibitors of flagellar biosynthesis, compounds that reduced the growth of *C. jejuni flgDE2*::′*cat-kan* by >40% in chloramphenicol-containing medium were selected for further confirmation. To eliminate leads that simply inhibited *C. jejuni* growth, compounds that inhibited by >40% were cherry-picked to triplicate wells in a set of 384-well plates by use of a TTP Labtech Mosquito X1 hit picking system (TTP Labtech, Inc., Cambridge, MA). Forty microliters of a suspension with an OD_{600} of 0.025 of *C. jejuni flgDE2*::′*cat-kan* without chloramphenicol was added to each well. Plates were grown exactly as described prior to recording the OD_{600}. Small molecules that did not inhibit *C. jejuni* growth by >50% in medium lacking chloramphenicol were considered further as potential flagellar inhibitors. Of these compounds, all were similarly picked in triplicate, and *C. jejuni flgDE2*::′*cat-kan* in medium containing chloramphenicol was added. Following growth in the presence of chloramphenicol, those compounds that inhibited reporter strain growth by >3 standard deviations in all three replicate wells were considered further.

To identify inhibitors of *C. jejuni* growth, compounds described above that inhibited *C. jejuni flgDE2*::′*cat-kan* growth in medium lacking chloramphenicol by >90% were examined in other bacterial screens within the UM-CCG database, MScreen, including those using the Gram-negative bacteria *E. coli* Δ*tolC*, *Shigella flexneri*, and *Vibrio cholerae* (14). Compounds that inhibited *C. jejuni* growth by >90% but did not inhibit other Gram-negative bacteria by >10% were considered further as possible *Campylobacter*-specific growth inhibitors.

Dose-response analysis. For both flagellar inhibitors and growth inhibitors, compounds from the small-molecule library were added to 384-well plates using the TTP Labtech Mosquito X1 system to achieve final concentrations between 1 and 100 μM. Forty microliters of 0.025 (OD_{600}) suspensions of either *C. jejuni flgDE2*::′*cat-kan* or wild-type *C. jejuni* in MH broth, with or without chloramphenicol, respectively, were added to each well and grown for 48 h at 37°C under microaerobic conditions. Similarly, for the growth inhibitor screen, a dose-response curve for *E. coli* EC2880 Δ*tolC* (a generous gift from Michael Hubband, Pfizer Scientific) was generated in parallel, under the same conditions, but the mutant was grown for 24 h (15).

For both screens, dose-response curve analyses were repeated at least twice, in a similar manner, using newly purchased compounds (ChemDiv, Inc., San Diego, CA). Briefly, compounds were resuspended in deuterated-DMSO (d_6-DMSO) to final concentrations between 10 and 20 mM and serially diluted (1:2 in MH broth) in 96-well plates to obtain 100 μl of medium containing compounds at concentrations between 3 and 200 μM. One hundred microliters of 0.05 (OD_{600}) suspensions of either *C. jejuni flgDE2*::′*cat-kan* or wild-type *C. jejuni* DRH212 in MH broth was added to each well, resulting in final compound concentrations between 1.5 and 100 μM at a final OD_{600} of 0.025 in 200 μl of MH broth. These plates were incubated without shaking for 48 h at 37°C under microaerobic conditions. For dose-response curves of growth inhibitors, *E. coli* EC2880 Δ*tolC* was treated similarly, in parallel, but was again grown for 24 h. Data were analyzed using SigmaPlot software, and either 50% effective concentrations (EC_{50}s), the effective molar concentrations of compound where 50% of bacterial growth was inhibited, or pEC_{50}s, the effective molar concentrations expressed as $-\log_{10}$, were calculated (Systat Software, Inc., San Jose, CA).

Motility assays. Compounds were added to a final concentration of 100 μM in 4 ml of MH motility agar (MH broth plus 0.4% agar), which was allowed to solidify in individual wells of a six-well tissue culture plate. Control wells were prepared similarly but contained only DMSO, the vehicle of all compounds. The center of each well was inoculated with an overnight culture of wild-type *C. jejuni*, except for a single DMSO control

well that was inoculated with a nonmotile *C. jejuni* Δ*rpoN* mutant. Cultures were grown for 24 h at 37°C under microaerobic conditions before wells were imaged and motility diameters were measured.

Dose-response analysis of additional strains. To examine for specificity of growth inhibition, dose-response curves for wild-type *C. jejuni* DRH212, *C. jejuni* MTVDSCj20 (16), *C. coli* MTVDSCc1 (M. Taveirne and V. J. DiRita, unpublished), and *E. coli* EC2880 Δ*tolC* were generated from 96-well plates, like those described above. For *H. pylori* J75 (17), the strain was grown under the same conditions but in medium containing brucella broth, calprotectin buffer, and FBS. After 24 h, OD_{600}s were recorded and analyzed using SigmaPlot.

MIC/MBC assays. Dose-response curves were generated for the wild type as described, but cultures were grown for 24 h at 37°C under microaerobic conditions. The concentration where endpoint growth was inhibited by approximately 90% (by OD_{600}) was identified, and cells were removed from these wells, serially diluted, and plated onto plates containing MH agar plus Tmp. Colonies were enumerated after growing microaerobically at 37°C for 48 h. Control wells contained DMSO or the inhibitory compounds chloramphenicol and kanamycin, which are bacteriostatic and bactericidal, respectively.

Cell viability assays. The effect of each compound on eukaryotic cell viability was determined using the CellTiter-Glo assay (Promega, Madison, WI). Briefly, 2-fold dilutions of each compound were made in Dulbecco's modified Eagle medium (reference no. 11965-092; Life Technologies, Carlsbad, CA) containing glucose and L-glutamine with 10% fetal bovine serum, 10 mM HEPES, and 1 mM sodium pyruvate (final concentrations). Fifty microliters of a 2×10^6/ml suspension (1×10^5) of Caco-2 cells was added to 50 μl of medium containing compound, resulting in final compound concentrations of 100, 50.0, 25.0, 12.5, 6.25. 3.12, and 1.56 μM. Additionally, Caco-2 cells were added to control wells containing either DMSO or 0.1% Triton X-100. Cultures were incubated for 24 h in 96-well Optilux plates (Becton Dickinson, Franklin Lakes, NJ) under standard tissue culture conditions. After incubation, 100 μl of CellTiter-Glo reagent was added to each well and ATP levels were determined according to the manufacturer's instructions.

Chicken colonization studies. To determine whether lead compounds can reduce *C. jejuni* loads within the chicken gastrointestinal tract, day-of-hatch chickens were inoculated by oral gavage with approximately 10^4 CFU (100 μl of a 10^5-CFU/ml suspension) of wild-type *C. jejuni* DRH212 in phosphate-buffered saline (PBS) containing compound. Briefly, a common inoculum was split into aliquots that received either compound CCG-84443, CCG-187741, CCG-187751, CCG-187769, or CCG-198215 to 100 μM concentrations. A positive-control group was infected with the same inoculum but did not receive compound, only an equivalent amount of DMSO. A negative-control group was mock infected with sterile PBS containing an equivalent amount of DMSO. Birds within each group were dosed further with their respective compound at days 3 and 6 postinoculation via oral gavage of 100 μl of sterile PBS containing 100 μM each compound. Control groups were dosed with sterile PBS containing only DMSO. At day 7 postinoculation, cecal contents were isolated and plated for viable *C. jejuni*.

RESULTS

An *flgDE2* reporter for screening compound libraries. Growth in chloramphenicol-containing medium was dependent on a chromosomally encoded *flgDE2*::′*cat-kan* reporter; this strain grew to an average OD_{600} of 0.347 ± 0.037, whereas the wild-type strain grew to an average OD_{600} of 0.039 ± 0.004. The average statistical effect size of this difference was calculated with a Z′ of 0.783 (18). To demonstrate that *flgDE2* reporter output was linked to flagellar expression, we moved the reporter into mutant strains (Δ*flhA*, Δ*fliP*, and Δ*rpoN* mutants). However, those strains exhibited a slight general growth defect even in medium lacking chloramphenicol. As this would magnify any differences in *flgDE2* expression observed in chloramphenicol-containing medium, the growth defect in medium without chloramphenicol was used to correct values obtained with chloramphenicol-containing medium. The corrected OD_{600} values for flagellar mutants expressing *flgDE2*::′*cat-kan* in medium containing chloramphenicol were as follows: Δ*flhA* mutant, 0.104 ± 0.011; Δ*fliP* mutant, 0.115 ± 0.011; and Δ*rpoN* mutant, 0.111 ± 0.018, representing >3-fold decreases in endpoint growth of the mutants (Fig. 1A). The corrected growth of each strain, while not significantly different from one another, was greater than that observed for wild-type *C. jejuni* and significantly less than the growth of the *flgDE2*::′*cat-kan* reporter strain ($P < 0.0001$). Compared to the *flgDE2*::′*cat-kan* reporter strain, these reductions produced Z′ values of 0.661 for the Δ*flhA* mutant, 0.656 for the Δ*fliP* mutant, and 0.561 for the Δ*rpoN* mutant.

Identification of putative flagellar inhibitors. After screening of the *flgDE2*::′*cat-kan* reporter strain against a library of 147,290 compounds, 3,141 compounds were found to inhibit growth of *C. jejuni flgDE2*::′*cat-kan* in medium containing chloramphenicol by >40% (Fig. 1B). Since a certain number of these hits likely represent nonspecific bacterial growth inhibitors, a counterscreen was performed by testing each compound in triplicate against *C. jejuni flgDE2*::′*cat-kan* in medium without chloramphenicol. This counterscreen identified 1,010 compounds that inhibited growth by <50%, indicating that these small molecules do not significantly inhibit bacterial growth and that the results observed in chloramphenicol are likely due to inhibition of *flgDE2* expression (Fig. 1B). Reproducibility of growth inhibition by these 1,010 compounds—and the likely inhibition of flagellar expression—was confirmed in triplicate using the *C. jejuni flgDE2*::′*cat-kan* strain in medium containing chloramphenicol. Of these 1,010 compounds, 40 inhibited *C. jejuni flgDE2*::′*cat-kan* growth in chloramphenicol-containing medium by more than 3 standard deviations from the uninhibited mean (Fig. 1B).

Dose-response analysis of flagellar inhibitors identified few reproducibly potent compounds. Of the 40 compounds that specifically inhibited *C. jejuni flgDE2*::′*cat-kan* growth in chloramphenicol-containing medium, four produced pEC_{50}s greater than 5.0, indicating adequate potency of *flgDE2* inhibition (Fig. 1B). These compounds—CCG-3485, CCG-4568, CCG-4586, and CCG-6448—are from a subset of UM-CCG's compound library (Chembridge 3028 and Chembridge 10000) and exhibited pEC_{50}s of 5.61, 5.67, 5.57, and 5.78, respectively (data not shown). Subsequent dose-response analysis of these inhibitors using fresh compound determined that they reproducibly inhibited growth of the reporter strain under the conditions used in the screen.

Flagellar inhibitors modestly reduce motility. After growth of wild-type *C. jejuni* in motility agar containing 100 μM CCG-3485, CCG-4568, CCG-4586, or CCG-6448, all compounds significantly reduced motility compared to an untreated control. Motility diameters were as follows: 2.84 ± 0.09 cm for the DMSO control, 2.08 ± 0.05 cm for CCG-3485 ($P < 0.0001$), 2.20 ± 0.11 cm for CCG-4568 ($P = 0.002$), 2.32 ± 0.05 cm for CCG-4586 ($P = 0.001$), and 2.18 ± 0.06 cm for CCG-6448 ($P < 0.001$) (Fig. 1C). While these represent reproducible reductions in motility, none of the compounds inhibited motility to the level of a Δ*rpoN* mutant (0.35 ± 0.05 cm, $P < 0.0001$).

Identification of campynexins, anti-*Campylobacter* compounds. While separating inhibitors of *flgDE2* expression from those that inhibited bacterial growth, we identified 1,853 com-

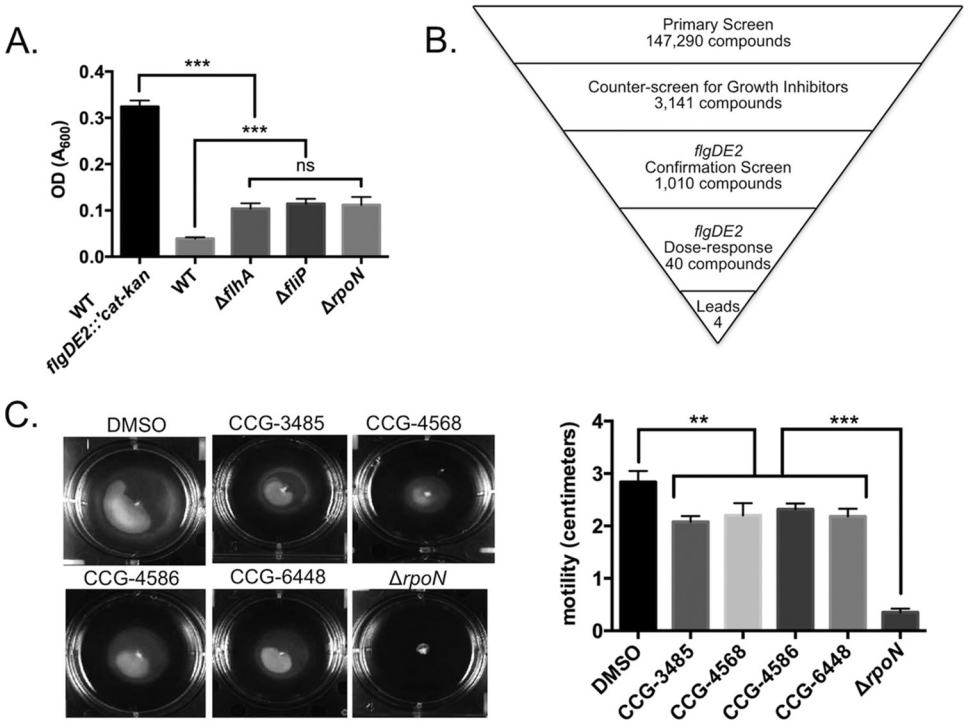

FIG 1 Identification of flagellar inhibitors by high-throughput screening of a small-molecule library. (A) Effect of flagellar mutations on *flgDE2* expression in a *C. jejuni flgDE2::'cat-kan* reporter strain; (B) schematic of triage process used to identify putative small-molecule inhibitors of *flgDE2* expression; (C) effect of compounds on wild-type *C. jejuni* motility in swim-agar medium. Statistical analyses were performed using Student's *t* test (**, $P < 0.001$, ***, $P < 0.0001$). ns, not significant.

pounds that inhibited *C. jejuni* growth by >90% in medium lacking chloramphenicol, indicating the presence of potent growth inhibitors (Fig. 2A). These were analyzed for general growth inhibition of Gram-negative bacteria by examining data from other screens performed at the UM-CCG using *E. coli* Δ*tolC*, *S. flexneri*, and *V. cholerae*. The *E. coli* Δ*tolC* mutant was of particular interest because its lack of the TolC efflux protein makes it hypersensitive to toxic compounds (19). From this analysis, 115 of the 1,853 *C. jejuni* inhibitors did not inhibit growth of the above-mentioned strains by >10% (Fig. 2A). Initial dose-response analysis determined that 57 of the 115 potential *C. jejuni*-specific growth inhibitors yielded pEC_{50}s greater than 4.0, with 31 of those compounds producing pEC_{50}s greater than 5.0 (Fig. 2A). Forty-four of these 57 compounds were available for purchase, and we performed dose-response analyses with these molecules. Following this analysis, the compounds selected for further study were CCG-84443, CCG-187741, CCG-187751, CCG-187769, and CCG-198215, which yielded average EC_{50}s of 8.18, 8.96, 11.9, 10.4, and 37.4 μM, respectively (Fig. 2B and C); we term these compounds campynexins A to E, respectively. Cultures containing *E. coli* EC2880 Δ*tolC* did not exhibit a dose response to any of the campynexins, and therefore EC_{50}s could not be calculated.

Campynexins exhibit activity toward *Campylobacter* but not *Helicobacter* bacteria. To rule out that growth inhibition by the campynexins was limited to our laboratory strain DRH212, and to examine how broadly the campynexins inhibited bacterial growth, dose-response assays against farm isolates *C. jejuni* MTVDSCj20 and *C. coli* MTVDCc1 were performed similarly to those described above. Compounds exhibited mean EC_{50}s for these strains that were similar to those observed for *C. jejuni* DRH212 (Table 1). In contrast, no response at any dose was observed with *H. pylori* strain J75, similar to the results using *E. coli* EC2880 Δ*tolC* (Table 1).

Inhibitors of *C. jejuni* growth are bacteriostatic. Prior to testing the compounds in infected chicks, we determined whether they act bacteriostatically or bactericidally. This was done for the five lead compounds, campynexins A to E, by identifying concentrations that inhibited endpoint growth by ≥90% and plating serial dilutions to determine the number of viable bacteria present. This level of inhibition occurred at concentrations of 50 μM for compounds CCG-187741 and CCG-187769 and at 100 μM for compounds CCG-84443, CCG-187751, and CCG-198215. Similarly, we also identified the lowest concentrations of chloramphenicol and kanamycin that were needed to inhibit growth by ≥90%. This level of inhibition occurred at concentrations of 2 μg/ml and 20 μg/ml for chloramphenicol and kanamycin, respectively. Following serial dilution, cultures treated with campnexins A to E had mean viable bacterial counts of 1.34×10^7, 9.65×10^6, 8.98×10^6, 2.73×10^7, and 3.38×10^6 CFU/ml, respectively (Fig. 3). These values were significantly different from those for control cultures treated with DMSO, with a mean of 1.42×10^9 CFU/ml ($P < 0.0001$), and those of the bactericidal control kanamycin, with a mean of 6.14×10^4 CFU/ml ($P < 0.05$). Viable cell counts from the bacteriostatic control, chloramphenicol, were not significantly different from those of the campynexins ($P > 0.05$), with a mean count of 1.41×10^7 CFU/ml.

Lead compounds do not affect eukaryotic cell viability. Human intestinal epithelial cells (Caco-2) were grown in the presence

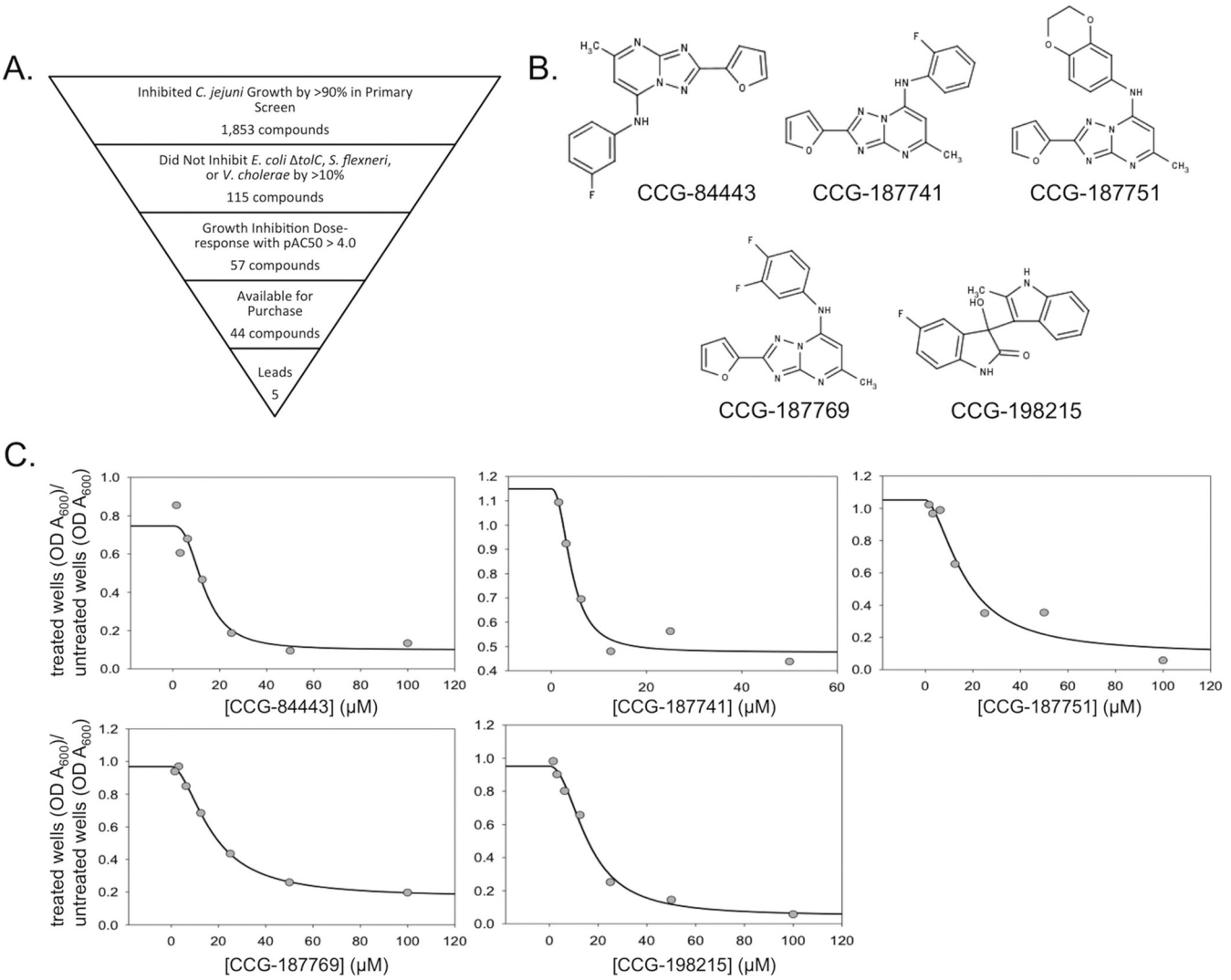

FIG 2 Identification of *C. jejuni*-specific growth inhibitors. (A) Triage process used to identify growth inhibitors from the high-throughput screen for flagellar inhibitors; (B) structures of selected lead compounds; (C) dose-response curves of selected lead compounds.

of increasing amounts of compound (1.56 μM to 100 μM) for 24 h under tissue culture conditions. Cell viability was examined by determining ATP levels using CellTiter-Glo reagent. Percent cell viability was determined by dividing luminescence units for each compound by those recorded from wells where Caco-2 cells were untreated. Cell viability for DMSO-treated Caco-2 cells was found to exhibit luminescence at a mean (± standard deviation) of 106.95% ± 53.03%, while nonviable, 0.1% Triton-treated control wells produced luminescence at a mean of 0.06% ± 0.03%. For the five lead compounds, mean percent cell viabilities of 120.82% ± 20.41%, 125.55% ± 24.71%, 161.46% ± 27.90%, 187.21% ± 25.74%, and 74.84% ± 37.54% were recorded at 100 μM concentrations of CCG-84443, CCG-187741, CCG-187751, CCG-187769, and CCG-198215, respectively. Based on these results, Caco-2 cells do not exhibit a cytotoxic dose-response to these compounds, so 50% cytotoxic concentrations (CC^{50}) could not be

TABLE 1 Campynexin effectiveness on different bacterial species

Strain	Compound EC_{50} (μM)[a]				
	CCG-84443	CCG-187741	CCG-187751	CCG-187769	CCG-198215
C. jejuni DRH212	12.93	9.83	16.14	2.95	51.78
C. jejuni MTVDSCj20	3.99	8.08	12.68	2.72	50.06
C. coli MTVDSCc1	7.55	16.8	15.4	3.82	37.54
E. coli EC2880 Δ*tolC*	>100	>100	>100	>100	>100
H. pylori J75	>100	>100	>100	>100	>100

[a] The EC_{50} was calculated from a dose-response curve that was generated from the average result for triplicate cultures at the given concentrations.

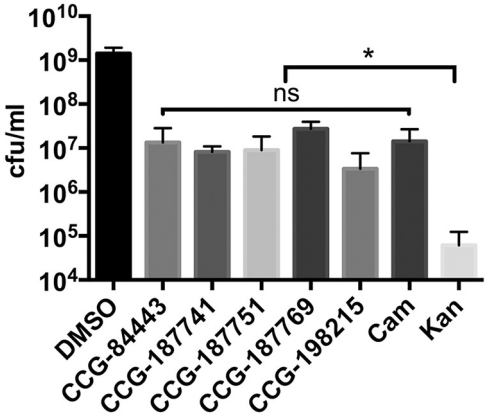

FIG 3 Determining the class of *C. jejuni* growth inhibitors (bacteriostatic versus bactericidal) by using selected compounds in an MIC/MBC assay. Statistical analysis was performed using Student's *t* test (*, $P < 0.05$).

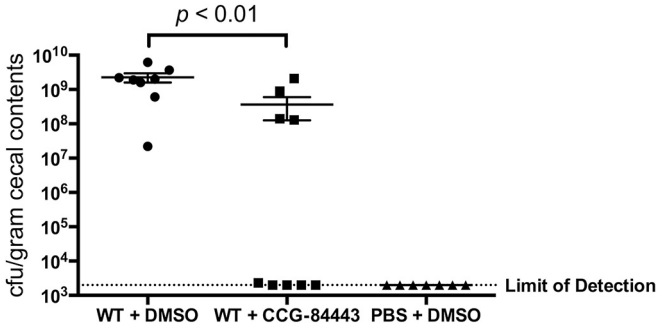

FIG 4 Colonization levels of *C. jeuni* in the chicken cecum following treatment with lead compound CCG-84443. Statistical analysis was performed using a Mann-Whitney test ($P < 0.01$).

calculated. Only cells treated with 0.1% Triton X-100 exhibited a significant decrease in cell viability.

Campynexin A reduces *C. jejuni* carriage in the chicken gastrointestinal tract. The ability of campynexins to reduce the load of *C. jejuni* was tested using day-of-hatch chickens, a standard colonization model in the natural host for *C. jejuni*. Following administration of the compounds at the time of inoculation and 3 and 6 days postinoculation, only campynexin A was capable of reducing *C. jejuni* loads, albeit inconsistently (Fig. 4). In more than half of the chickens (5 of 9) treated with campynexin A, the number of viable *C. jejuni* present was at or below the limit of detection (2×10^3 CFU/g cecal content) while approximately half of the chickens (4 of 9) exhibited full levels of cecal colonization ($\sim 10^8$ to 10^9 CFU/g cecal content). The mean cecal load of treated chickens was 3.63×10^8 CFU/g of cecal content, representing a nearly 1-log average decrease ($P < 0.01$) in colonization compared to the mean cecal load of chickens treated with only DMSO (2.28×10^9 CFU/g cecal content).

DISCUSSION

C. jejuni is a significant cause of food-borne infection in the United States, primarily due to its ability to reside within the gastrointestinal tracts of chickens. Infection of humans often occurs following ingestion of undercooked meat or by consuming uncooked food that has been cross-contaminated. As such, much emphasis has been placed on identifying and characterizing factors of *C. jejuni* with the goal of using those insights to inform the identification and/or development of strategies or compounds that limit *C. jejuni* loads in the food supply (2, 3). One such factor that has repeatedly been shown to be required for full colonization of the chicken gastrointestinal is the flagella of *C. jejuni*.

Other work has shown that a majority of the flagellar biosynthetic cascade terminates with expression of the flagellar hook-encoding operon, *flgDE2*, immediately upstream of flagellin production. We constructed an *flgDE2* reporter strain that links the expression of *flgDE2* to the expression of the gene that encodes *C. coli* chloramphenicol acetyltransferase, making growth in chloramphenicol-containing medium dependent on flagellar expression.

Following both the primary screen and the dose-response analysis of flagellar inhibitors, few compounds were found to reproducibly inhibit growth of the *flgDE2* reporter strain and many of those that were identified did not exhibit sufficient potency for us to pursue further. Instead, we chose to focus on those that were the most reproducible and potent, reasoning that those compounds were the most likely to provide us with detectable inhibition of flagellar motility. Unfortunately, at compound concentrations that almost completely inhibited growth of the reporter strain in the dose-response analysis, we observed very little inhibition of flagellar motility in a standard assay. This likely reflects that either (i) the assay is somewhat insensitive at detecting *flgDE2* expression, where even though we observe significant decreases in reporter strain growth, there is still productive *flgDE2* expression and flagellar biosynthesis, or (ii) the compounds are simply not amenable for use in this standard assay. We are currently pursuing both of these possibilities in the hope of optimizing the primary screen and/or motility assays for future identification of antiflagellar compounds.

In our primary screen for flagellar inhibitors, we identified ones that inhibited growth of the reporter strain, but these likely represented three different classes of molecules in terms of their effects: (i) those that inhibited *flgDE2* expression, which is the class we originally sought, (ii) those that inhibited the function of the chloramphenicol acetyltransferase enzyme, or (iii) those that inhibited growth of *C. jejuni* independently of *flgDE2* reporter inhibition. This made the screen attractive not only from the standpoint of identifying inhibitors of flagellar biosynthesis but also for identifying compounds capable of inhibiting growth of *C. jejuni in vitro*. While the ability to inhibit growth of *C. jejuni* is interesting by itself, ideally one would want to identify compounds that specifically inhibit *C. jejuni* growth while not affecting the viability or growth of other bacteria within the chicken gastrointestinal tract. As the facility in which we carried out the primary screen has performed multiple primary screens on other Gram-negative bacteria, we mined the data from these screens to eliminate compounds that broadly inhibit Gram-negative bacterial growth.

When many of these compounds were purchased and reexamined, many either did not inhibit to the level we would have expected or exhibited an inhibitory effect toward our bacterial toxicity control, *E. coli* EC2880 Δ*tolC*. This is likely due to the gradual degradation of the small molecules in the library; the initial growth inhibition may have been due to a degradation product rather than the original progenitor molecule. Nevertheless, several compounds were confirmed to inhibit the growth of *C. jejuni in*

vitro, and these, which we term campynexins, became the focus of additional study.

While a comparison of the results of our growth inhibitor screen to those using other Gram-negative bacteria indicated some measure of specificity, we also examined the campynexins for inhibition of farm-isolated strains of *C. jejuni* and *C. coli*, as well as the closely related epsilonproteobacterium *H. pylori* J75. Campynexins inhibited growth of members of the *Campylobacter* genus but not those of *Helicobacter*. This indicates differences in effectiveness within the order *Campylobacterales* but potentially uniform effectiveness within the *Campylobacter* genus. Such specificity would be attractive for infection control within an agricultural setting since one could potentially limit proliferation of all food-borne pathogens within the *Campylobacter* genus. We are currently expanding the above-described screen to determine whether this is a possibility by using *Campylobacter* strains from multiple sources in dose-response assays. Also of obvious further interest is the identification of targets of growth inhibition that make these compounds so specific to *Campylobacter* spp.

Initial tests to examine the feasibility of using these compounds to reduce *C. jejuni* colonization of the chicken gastrointestinal tract centered on determining whether compounds are bacteriostatic or bactericidal in nature and whether these compounds affect eukaryotic cell viability. The finding that these compounds are bacteriostatic indicates that they will likely need to be administered early and for an extended period of time to prevent outgrowth within the chicken gastrointestinal tract. Since we observed that eukaryotic cells tolerate high levels of these compounds *in vitro*, we attempted to reduce *C. jejuni* loads in the chicken by administering each compound individually at the time of inoculation and with doses at 3 and 6 days postinoculation. One of these compounds, campynexin A (CCG-84443), reduced *C. jejuni* loads on one occasion but was unable to significantly decrease *C. jejuni* carriage upon a subsequent attempt. The reasons for this are unclear and indicate that more information on the fates of these molecules *in vivo* is required. As such, pharmacokinetic analysis of these inhibitors is being carried out in order to determine their half-life within the chicken bloodstream and gastrointestinal tract. These data will inform the further development of these compounds for use in chickens. Additionally, studies are also under way to determine whether these compounds have any effect on other *Campylobacter* species in the chicken gastrointestinal tract, including *C. coli*. Long-term goals also include examining the efficacy of these compounds as feed or water additives in an agricultural setting.

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