The Effects of Modeled Microgravity on Growth Kinetics, Antibiotic Susceptibility, Cold Growth, and the Virulence Potential of a *Yersinia pestis ymoA*-Deficient Mutant and Its Isogenic Parental Strain

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Abstract

Previously, we reported that there was no enhancement in the virulence potential (as measured by cell culture infections) of the bacterial pathogen *Yersinia pestis* (YP) following modeled microgravity/clinorotation growth. We have now further characterized the effects of clinorotation (CR) on YP growth kinetics, antibiotic sensitivity, cold growth, and YP's virulence potential in a murine model of infection. Surprisingly, none of the aforementioned phenotypes were altered. To better understand why CR did not enhance YP's virulence potential as it did for other bacterial pathogens, a YP *ΔymoA* isogenic mutant in the KIM/D27 background strain that is unable to produce the histone-like YmoA protein and influences DNA topography was used in both cell culture and murine models of infection. YmoA represses type three secretion system (T3SS) virulence gene expression in the yersiniae. Similar to our CR-grown parental YP strain data, the CR-grown *ΔymoA* mutant induced reduced HeLa cell cytotoxicity with concomitantly decreased *Yersinia* outer protein E (YopE) and low calcium response V (LcrV) antigen production and secretion. Important, however, were our findings that, although no significant differences were observed in survival of mice infected intraperitoneally with either normal gravity (NG)- or CR-grown parental YP, the *ΔymoA* mutant induced significantly more mortality in infected mice than did the parental strain following CR growth. Taken together, our data demonstrate that CR did enhance the virulence potential of the YP *ΔymoA* mutant in a murine infection model (relative to the CR-grown parental strain), despite inducing less HeLa cell rounding in our cell culture infection assay due to reduced T3SS activity. Therefore, CR, which induces a unique type of bacterial stress, might be enhancing YP's virulence potential *in vivo* through a T3SS-independent mechanism when the histone-like YmoA protein is absent. Key Words: Type three secretion system (T3SS)—Low-shear modeled microgravity—High-aspect ratio vessel—Cell culture infection models—Mouse infection model. Astrobiology 13, 821–832.

1. Introduction

PROLONGED MANNED SPACE EXPLORATION has raised health concerns for astronauts as they relate to the virulent nature of infectious agents to which they are exposed in a microgravity (MG) environment (Nickerson *et al.*, 2004; Rosenzweig *et al.*, 2010). As a result, several experiments have been conducted to explore bacterial virulence following actual spaceflights as well as under conditions of ground-based modeled microgravity (MMG) (Nickerson *et al.*, 2004; Rosenzweig *et al.*, 2010 and references therein).

In addition to a spaceflight study that examined the impact of MG on biofilm production by the opportunistic pathogen *Pseudomonas aeruginosa* (Klaus and Howard, 2006), two additional spaceflight experiments have been carried out that evaluated the virulence potential of space-flown *Salmonella enterica* serovar Typhimurium. Following growth on board STS-115, *S. Typhimurium* exhibited 167 differentially expressed genes when compared to normal gravity (NG)-grown bacteria, and a hypervirulent phenotype following MG exposure (Wilson *et al.*, 2007). Furthermore, when *S. Typhimurium* was flown to space a second time on board

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STS-123, the hypervirulent phenotype of the pathogen was not noted under environmental/nutritional changes (Wilson *et al.*, 2008).

Due to the infrequent nature of shuttle missions, ground-based MMG systems were developed by NASA (Nickerson *et al.*, 2004). The use of such alternative approaches has enabled astrobiologists to overcome both the infrequency and the cost-prohibitive limitations associated with spaceflight experiments. Although no system can emulate true space MG, these ground-based alternatives provide meaningful data that eventually can be correlated with, and preparative for, actual space mission studies. The most commonly utilized vessels that produce low-shear MMG are clinostats, rotating wall vessels (RWVs), random positioning machines, and magnetic levitation instruments. Clinostats have the longest track record of producing MMG; however, slow clinorotation (CR) becomes problematic and can actually promote hypergravity conditions (Hoson *et al.*, 1997). The random positioning machines and magnetic levitation instruments are more expensive and complex. The former uses a computer to randomly assign speed and orientation to sample rotation in efforts to negate unilateral gravity, while the latter employs a spatially varied magnetic field that suspends the cells by negating the force of gravity (Beuls *et al.*, 2009).

The most commonly employed CR instruments are RWVs. The high-aspect ratio vessel (HARV), a type of RWV, was designed by former NASA scientists Charles D. Anderson and Ray Schwartz for the purpose of cultivating fastidious eukaryotic cells in a three-dimensional culture system (www.synthecon.com/company/our-journey.html). HARVs (Synthecon Inc., Houston, TX) have since been used to cultivate bacteria and other microorganisms under CR, generating a MMG environment (Rosenzweig *et al.*, 2010 and references therein). The HARV can easily be positioned to achieve CR or NG conditions. When the axis of rotation is perpendicular to the force of gravity, CR is achieved by low-shear fluid forces offsetting the weight of the cells, which results in their being suspended in a state of terminal velocity. Conversely, when the axis of rotation is parallel to the gravitational vector, cells can move down a linear path that results in NG and sedimentation (Fig. S1; Supplementary Data are available online at www.liebertonline.com/ast). In fact, the vast majority of CR-based studies of bacteria have employed the HARV (Rosenzweig *et al.*, 2010).

Mirroring spaceflight studies, CR-grown *S. Typhimurium* (in the HARV) was also hypervirulent in both cell culture and murine models of infection (Nickerson *et al.*, 2000, 2003; Wilson *et al.*, 2002a), and differential gene expression profiles following CR growth closely resembled those following spaceflight both qualitatively and quantitatively (Wilson *et al.*, 2002b, 2007; Chopra *et al.*, 2006; Rosenzweig *et al.*, 2010). Unexpectedly, the CR/MG-induced hypervirulence of *S. Typhimurium* was not due to increased expression of two independent type three secretion system (T3SS)-associated genes required for invasion and survival within eukaryotic host cells, respectively (Wilson *et al.*, 2002b). The fact that the hypervirulent *S. Typhimurium* demonstrated repressed T3SS gene expression strongly suggests that CR and MG were enhancing the *S. Typhimurium* virulence potential in an unknown, T3SS-independent manner. The observed *S. Typhimurium* hypervirulence could have been due to enhanced

biofilm formation that occurs following both CR and spaceflight (Wilson *et al.*, 2007) or due to increased expression of *virK*, since *VirK* contributes to remodeling of the bacterial outer membrane in response to the host environment (Chopra *et al.*, 2006).

In addition to *S. Typhimurium*, enterotoxigenic and enteropathogenic *E. coli* (Chopra *et al.*, 2006), adherent-invasive *E. coli* (Allen *et al.*, 2008), and *P. aeruginosa* (Crabbé *et al.*, 2008, 2011) have also been characterized following CR growth. Interestingly, all the aforementioned pathogens responded to CR growth by similarly exhibiting enhanced virulence potentials. While *P. aeruginosa* produced enhanced biofilm (Crabbé *et al.*, 2008), CR promoted the upregulation of the heat-labile (LT-1) enterotoxin gene in enterotoxigenic *E. coli* (Chopra *et al.*, 2006) and resulted in adherent-invasive *E. coli* becoming hyper-adherent vis-à-vis a gastrointestinal epithelial cell line (Allen *et al.*, 2008). Interestingly, CR appears not to affect all bacterial pathogens in the same manner. More specifically, when the Gram-positive *Staphylococcus aureus* was exposed to a CR environment, it displayed a reduced virulence potential (Castro *et al.*, 2011). Similarly, we demonstrated that CR was unable to enhance the virulence potential of the pigmentation (*pgm*-) locus minus strain KIM/D27 of *Yersinia pestis* (YP), a Gram-negative, facultative anaerobic, bacterial human pathogen (Lawal *et al.*, 2010).

Yersinia pestis, the etiological agent of bubonic, septicemic, and pneumonic plague, is an Enterobacteriaceae family member that, like the salmonellae, also possesses a T3SS, encoded by its 70 kb virulence plasmid (pCD1). This formidable pathogen has caused over 200 million deaths stemming from the three major human plague pandemics that it has caused (Inglesby *et al.*, 2000). Effector *Yersinia* outer membrane proteins (Yops) are injected into the host cell cytoplasm via the T3SS injectisome and exert multiple anti-host effects, including the prevention of actin polymerization, the disruption of cell signaling, and apoptosis (Cornelis, 2002; Viboud and Bliska, 2005; Pujol and Bliska, 2005). Following CR, we previously reported that YP KIM/D27 strain was reduced in its capacity to proliferate vis-à-vis cultured macrophage-like cells and was greatly diminished in its ability to induce HeLa cell rounding and cytotoxicity due to decreased T3SS factor expression/production and secretion (Lawal *et al.*, 2010).

In this study, we further characterized the phenotype of CR-grown parental YP KIM/D27 strain, evaluated its virulence potential in a murine infection model, and employed a *YmoA* deletion mutant (with a de-repressed Yop regulon) to evaluate whether CR could similarly repress expression of Yop-encoding genes. The chromosomally encoded *ymoA* gene expresses a small histone-like protein, YmoA, homologous to the *Escherichia coli* Hha protein, which influences DNA super-coiling and gene expression (Balsalobre *et al.*, 1996). In YP, YmoA represses the transcription and expression of T3SS genes at room temperature, but at higher temperatures, particularly at 37°C, it is quickly degraded by ClpXP and Lon proteases, enabling the full expression of the virulence-associated T3SS genes (Jackson *et al.*, 2004). We chose to characterize this mutant strain to determine whether CR could reduce expression of the de-repressed Yop regulon, as compared to the isogenic parental YP KIM/D27 strain, in efforts of gaining insight into how CR modulates the YP virulence potential.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

All studies were conducted with the exempt (not a select agent as designated by the Centers for Disease Control and Prevention, Atlanta, GA) and attenuated strain *Y. pestis* KIM5/D27, derived from strain KIM5-3001 by elimination of the pigmentation locus *pgm* (Lindler *et al.*, 1990) (Table 1). The isogenic *ΔymoA* (Jackson *et al.*, 2004) mutant was derived by using the lambda red recombinase system (Datsenko and Wanner, 2000), while the *ΔyopB* mutant (Rosenzweig *et al.*, 2005, 2007) was generated by using a previously published allelic replacement method (Day *et al.*, 2003). Various ribonuclease mutant strains, for example, *Arnb* (ribonuclease II), *Arnr* (ribonuclease R), and *Apnp* (polynucleotide phosphorylase) were previously characterized in a bacterial cell culture infection assay (Rosenzweig *et al.*, 2005), while the *ΔcspA* (cold shock protein A) mutant strain was not studied earlier but developed by Dr. Greg Plano, University of Miami Health System, Miami, Florida, who used the aforementioned lambda red recombinase system (Table 1). Bacterial strains were cultivated by using brain heart infusion or heart infusion broth medium (Difco/Becton Dickinson, Franklin Lakes, NJ) at 28°C or 37°C with constant agitation or were grown on tryptic soy agar (TSA) plates (Difco/Becton Dickinson).

2.2. HARV cultures

Our previously published HARV culturing methods were employed (Lawal *et al.*, 2010). In short, for NG growth conditions, the HARV (Synthecon Inc.) was positioned such that the axis of rotation was parallel to the gravitational vector. For cultivation under CR, the HARV was positioned such that the axis of rotation was perpendicular to the gravitational vector (Fig. S1). The TSA plates were also used for solid phase cultivation. For growth kinetic studies, absorbance of bacterial cultures was monitored over a 10 h period at 600_{nm} by a UV-vis spectrophotometer (Thermo Fischer Scientific Inc., Pittsburgh, PA). Viable colony counts (colony-forming units, cfu) were also determined at selected time points on TSA plates.

2.3. Antibiotic susceptibility

Previously published disk diffusion methods were employed (Bauer *et al.*, 1959, 1966). In short, disks impregnated

with common antibiotics [streptomycin (10 μg), kanamycin (30 μg), chloramphenicol (30 μg), penicillin (10 IU), and tetracycline (30 μg)] (Sigma-Aldrich, St. Louis, MO) were aseptically placed (using forceps) at equidistant locations (relative to one another) on a TSA plate previously inoculated with a saturated/stationary-phase culture of the parental YP strain grown under either NG or CR conditions. Following 16 h of incubation, zones of inhibition were measured (in mm) from three independent experiments, and averages were graphed with error bars displayed.

2.4. Cold growth assay

Our previously published cold growth assay (Rosenzweig *et al.*, 2005, 2007) was used to evaluate growth capabilities of various YP mutant strains, including *ΔymoA* at 4°C. The bacterial strains grown at 28°C for 2 days served as a positive control for growth. In short, saturated cultures of the parental YP strain, as well as the *ΔymoA*, *Δpnp*, *Arnr*, *Arnb*, and *ΔcspA* mutants were streaked onto TSA medium. Growth was observed after 10 or 16 days at 4°C. Additionally, saturated cultures grown under CR or NG were serially diluted, spot-plated on TSA + 5% sheep's blood plates (in duplicates), and incubated at 4°C for 16 days. Colonies were assessed for differences in morphology and numbers.

2.5. HeLa cell cytotoxicity assay

To measure the effect of simulated microgravity on the virulence potential of parental YP and its *ΔymoA* mutant strain, our previously published HeLa cell cytotoxicity assay was employed (Rosenzweig *et al.*, 2005, 2007; Lawal *et al.*, 2010). Briefly, six-well plates were seeded with 1.0×10^5 HeLa cells and infected with the parental YP or its *ΔymoA* mutant strain (grown under NG and CR conditions) and a *ΔyopB* mutant (negative control) at a multiplicity of infection (MOI) of ~30 for 2–2.5 h at 37°C with 5% (v/v) of gaseous CO₂. The severity of HeLa cell rounding was then quantified with a scoring system (1 = no rounding; 2 = some rounding; 3 = intermediate rounding; 4 = severe rounding) by evaluating ~150–200 HeLa cells.

2.6. Yop expression and secretion

Our previously published Yop secretion assay (Rosenzweig *et al.*, 2005, 2007; Lawal *et al.*, 2010) was employed to evaluate the effects of CR on YopE and low calcium response V (LcrV) production and secretion. In short, saturated NG and CR cultures were used to inoculate pre-warmed (37°C) heart infusion broth medium (5 mL) that contained 2.5 mM Ca²⁺ to an OD_{620nm} of 0.2. Following 1 h of growth at 37°C with agitation, the T3SS was either induced with the addition of 5.0 mM ethylene glycol tetraacetic acid (EGTA) or left uninduced (*i.e.*, no EGTA was added to the cultures). At various time points, 1 mL samples were collected in pre-chilled microcentrifuge tubes. Supernatants and whole cells were fractionated in a refrigerated microcentrifuge (12 min at 13,000g). Secreted proteins were captured by removing 500 μL of supernatant followed by precipitation with 55 μL of 100% (v/v) trichloroacetic acid (TCA) overnight at 4°C.

The TCA-precipitated proteins were then pelleted by centrifugation for 10 min at 13,000g. Whole cell lysates and

TABLE 1. STRAIN LIST FOR THIS STUDY

Strain	Reference
KIM5-3001	Lindler <i>et al.</i> , 1990
KIM5/D27sm (WT)	Jackson <i>et al.</i> , 2004; Rosenzweig <i>et al.</i> , 2005, 2007
KIM5/D27sm <i>ΔymoA</i>	Jackson <i>et al.</i> , 2004
KIM5/D27sm <i>ΔyopB</i>	Rosenzweig <i>et al.</i> , 2005, 2007
KIM5/D27sm <i>Δpnp</i>	Rosenzweig <i>et al.</i> , 2005
KIM5/D27sm <i>Arnr</i>	Rosenzweig <i>et al.</i> , 2005
KIM5/D27sm <i>Arnb</i>	Rosenzweig <i>et al.</i> , 2005
KIM5/D27sm <i>ΔcspA</i>	This study

secreted proteins were dissolved in the same volume of the sample buffer, and equal amounts of proteins were then loaded onto sodium dodecyl sulfate 4–12% polyacrylamide gels (SDS-PAGE) (Invitrogen Corp. Carlsbad, CA) and separated electrophoretically. Proteins were transferred to nitrocellulose membranes, which were blocked with 10% (w/v) nonfat milk for 1 h at room temperature, and then probed for 1 h at room temperature with rabbit polyclonal anti-YopE or anti-LcrV antigen antibodies at 1:5000 dilutions. Following several washes with Tris-buffered saline (TBS) + 0.05% (v/v) Tween 20 (1X TBST), the membranes were treated with goat-anti-rabbit secondary antibodies conjugated with horseradish peroxidase at a dilution of 1:2000. The protein bands were visualized by using enhanced chemiluminescence (Amersham/GE Healthcare, Piscataway, NJ).

2.7. Murine infections

For murine infections, 5- to 6-week-old, female Swiss Webster mice (Charles River Laboratories, Wilmington, MA) were intraperitoneally (i.p.) infected with CR- or NG-grown parental or CR-grown $\Delta ymoA$ mutant. Infectious doses of either 1.0×10^7 cells/100 μ L or 1.0×10^8 cells/100 μ L of all strains were used to challenge 5 or 10 animals per group (Sha *et al.*, 2008; Liu *et al.*, 2010). Mice were subsequently examined for morbidity and mortality over a 10-day period, and survival curves were generated.

2.8. Statistical analysis

For growth kinetics and antibiotic susceptibility, the Student *t* test was employed (Microsoft Excel). The *p* values of less than 0.5 were considered significantly different. For murine infection studies, Kaplan Meier's survival estimates (GraphPad Software, Inc. La Jolla, CA) were used with *p* values of ≤ 0.05 considered significant.

3. Results

3.1. YP growth under CR

Considering previous observations that *S. Typhimurium* sensed and responded to changes in culture conditions when grown in low-fluid-shear environment of microgravity (Nickerson *et al.*, 2003), we hypothesized that CR may induce some alterations in the growth kinetics of YP. To test this, YP parental strain and its $\Delta ymoA$ mutant were grown as liquid cultures under CR and NG conditions at 28°C for 10 h; absorbance was measured at 620_{nm} every hour. As shown in Fig. S2A, both parental and the $\Delta ymoA$ mutant strain proliferated similarly whether grown under CR or NG, and no statistically significant differences were observed in their growth rates. Additionally, colony-forming units were also determined at selected time points for NG-grown cultures for comparative purposes. Paralleling growth curve data from liquid cultures, the number of viable NG-grown parental and NG-grown $\Delta ymoA$ mutant colony-forming units produced a very similar growth curve (Fig. S2B), validating our liquid culture-based growth curve data (Fig. S2A). Our results in liquid culture, therefore, show that YP growth was neither negatively impacted nor enhanced by CR and that the histone-like YmoA regulator

of gene expression was not required for growth under low-shear forces associated with CR.

3.2. Antibiotic resistance of parental YP strain remains unaltered in the CR environment

The fact that CR increased antimicrobial resistance (to penicillin and chloramphenicol) in *E. coli* (Lynch *et al.*, 2006) prompted us to investigate whether CR could similarly alter sensitivity of YP to these antibiotics as well as to kanamycin, tetracycline, and streptomycin. The latter two are used to treat patients with plague (Rosenzweig *et al.*, 2011). To our surprise, no significant differences between CR- and NG-cultivated YP susceptibility patterns were observed when evaluating each of the above-mentioned five antimicrobial-induced zones of inhibition (Fig. S3). Since the YP KIM/D27 strain we employed harbored a chromosomally encoded streptomycin resistance gene (Jackson *et al.*, 2004; Rosenzweig *et al.*, 2005, 2007), we did not observe a zone of inhibition surrounding the streptomycin disk (Fig. S3). Coupled with the observation that the histone-like YmoA protein (a regulator of gene expression) was not required for CR growth, the fact that our KIM/D27 strain remained streptomycin-resistant following CR strongly suggests that YP's genomic topography did not dramatically change during CR (at least not in the chromosomal region proximal to the streptomycin gene).

3.3. CR does not influence cold growth phenotype

The yersiniae are psychrotrophic bacteria capable of growing at refrigerated temperatures (Rosenzweig *et al.*, 2005, 2007; Goverde *et al.*, 1998; Neuhaus *et al.*, 2000). Therefore, we sought to evaluate whether YmoA was required for cold growth as well as whether CR could impact YP's cold-growth capability. Interestingly, the $\Delta ymoA$ mutant displayed cold-growth sensitivity as evident by significantly smaller colonies following growth at 4°C for 10 days when compared to colonies formed by the parental bacteria grown under the same conditions. However, no growth defect was noted in the $\Delta ymoA$ mutant at 28°C when grown for 2 days (data not shown).

This finding prompted us to evaluate additional YP mutant strains, including reevaluating the $\Delta ymoA$ mutant following 16 days of growth at 4°C, an endpoint that we have commonly used in the past (Rosenzweig *et al.*, 2005, 2007). To our surprise, the $\Delta ymoA$ mutant appeared to no longer demonstrate cold sensitivity following 16 days of growth at 4°C, and its growth was indistinguishable from that of the isogenic parental strain (Fig. 1A). Previously, we observed that the 3'-5' exoribonuclease polynucleotide phosphorylase (PNPase, encoded by the *pnp* gene) was required for cold-growth in YP and *Y. pseudotuberculosis* (Rosenzweig *et al.*, 2005, 2007; Henry *et al.*, 2012), so we included a YP Δpnp mutant in our study as a negative control. Not surprisingly, and in agreement with our previously published data (Rosenzweig *et al.*, 2005, 2007; Henry *et al.*, 2012), the Δpnp mutant was unable to grow at 4°C (Fig. 1A); however, at 28°C, like all other tested strains, the Δpnp mutant did not suffer any compromised growth (Fig. 1B).

We also evaluated the cold growth of other ribonuclease-deficient mutants devoid of ribonuclease II (*Arnb*) and ribonuclease R (*Arnr*). Unlike PNPase, RNase II and RNase R

FIG. 1. Cold growth evaluation of the $\Delta ymoA$ mutant and additional YP mutant strains at 16 days. Evaluation of 16 days of cold growth at 4°C (A) of parental YP, $\Delta ymoA$, Δpnp , Δnr , Δrnb , and $\Delta cspA$ strains or 2 days of growth at 28°C (B) of the same strains. Only the Δpnp mutant demonstrated cold-growth sensitivity following 16 days of growth at 4°C. Color images available online at www.liebertonline.com/ast

were dispensable for YP's cold growth (Fig. 1A). Similarly, the $\Delta cspA$ mutant, unable to produce the chief cold shock protein, CspA, also retained its cold growth capability (Fig. 1A), demonstrating that of all our mutants tested, only PNPase and YmoA appeared necessary for cold growth, although the latter was required for only early cold growth and became less important during prolonged cold growth periods (data not shown and Fig. 1). Finally, we tested whether CR could influence the cold growth capability of the parental and $\Delta ymoA$ strains following 16 days of incubation at 4°C. Neither the parental nor the $\Delta ymoA$ strains exhibited altered cold growth phenotypes following CR relative to their NG-grown counterparts (data not shown). Seemingly, CR did not influence two distinct YP stress responses (*i.e.*, chemical/antimicrobial and cold stress responses).

3.4. CR influences YP-induced HeLa cell cytotoxicity

To investigate whether CR modulates T3SS function, HeLa cells were infected with the parental YP or its $\Delta ymoA$ mutant strain at an MOI of ~ 30 . As a negative control, we also infected HeLa cells with a $\Delta yopB$ mutant, which is un-

able to inject Yops into targeted host cells. HeLa cell cytotoxicity was measured 2 h following infection (Fig. 2). As was observed before (Rosenzweig *et al.*, 2005, 2007; Lawal *et al.*, 2010), the parental YP strain induced severe rounding of HeLa cells, whereas the $\Delta yopB$ mutant strain, unable to translocate effector Yops into the host cell cytoplasm, did not induce much rounding (Fig. 2). Interestingly, the $\Delta ymoA$ mutant induced very severe HeLa cell rounding that appeared to be more intense than that of the isogenic parental strain (Fig. 2). This was likely due to the $\Delta ymoA$ mutant's de-repressed *yop* regulon (Jackson *et al.*, 2004). Interestingly, following CR, there was an apparent reduction in the degree of $\Delta ymoA$ -induced HeLa cell rounding relative to its NG-grown gravity counterpart (Fig. 3A). As we reported earlier (Lawal *et al.*, 2010), CR reduced the amount of parental YP strain-induced HeLa cell rounding as well (Fig. 3A). The Yop translocation-incompetent $\Delta yopB$ mutant induced little rounding, as was expected, and one well was left uninfected for evaluating the phenotype of the HeLa cells (Fig. 3A). In an attempt to quantify the degree of rounding induced by each of the strains tested, between 150 and 200 cells were scored individually and assigned a number ranging from 1

FIG. 2. HeLa cell cytotoxicity assay. Cultured HeLa cells were infected with the parental YP, $\Delta ymoA$, and $\Delta yopB$ (negative control) mutant strains at MOIs of ~ 30 , and rounding/cytotoxicity was imaged 2 h post-infection.

FIG. 3. HeLa cell cytotoxicity assay employing NG- and CR-grown YP strains. **(A)** Cultured HeLa cells were infected with NG- and CR-grown parental YP, $\Delta ymoA$ mutant, and the NG-grown $\Delta yopB$ mutant (negative control) at MOIs of ~ 30 , and rounding/cytotoxicity was imaged 2 h post infection. **(B)** Approximately 200 cells in multiple fields from each panel were scored 1, 2, 3, or 4 depending on their severity of rounding, where 1 represents unaffected HeLa cells and 4 represents severely rounded HeLa cells. Numbers 2 and 3 represent intermediate rounding. The percentage of rounding for each of the four categories is shown graphically, and a pictorial scale is provided below demonstrating how a cell in each of the four categories appears.

to 4 (where 1 represented no rounding, 4 indicated severe rounding, and 2–3 represented degrees of increasing rounding). Representative images corresponding to each of the four levels of rounding illustrated the variations (Fig. 3B pictorial legend). CR-grown parental YP strain induced the most severe rounding (4) in $\sim 37\%$ of HeLa cells compared to $\sim 62\%$ of the NG-grown, parental strain-infected HeLa cells (Fig. 3B). Similarly, the most severe HeLa cell rounding induced by CR-grown $\Delta ymoA$ mutant was observed in $\sim 52\%$

of scored cells compared to $\sim 73\%$ of NG-grown $\Delta ymoA$ mutant-infected cells (Fig. 3B).

The reduction in $\Delta ymoA$ -induced HeLa cell rounding following CR exposure occurred despite the $\Delta ymoA$ mutant inducing more severe rounding (4) than did its isogenic parental YP strain, 74% versus 62% scored HeLa cells respectively, under NG conditions (Fig. 3B). As was expected for the translocation-incompetent, negative control $\Delta yopB$ mutant, only 18% of scored HeLa cells were severely rounded

FIG. 4. YopE production and secretion by NG- and CR-grown parental YP and its $\Delta ymoA$ mutant strain. Immunoblot analysis of cell pellet and secreted YopE proteins induced in bacteria in the presence (+) and absence (-) of 5.0 mM EGTA (a calcium chelator) is shown. Pellet and supernatant fractions of bacteria were blotted and probed with rabbit polyclonal anti-YopE antibodies. Color images available online at www.liebertonline.com/ast

(4), while the majority of HeLa cells appeared unaffected, with 38% and 40% being scored a 1 or 2, respectively (Fig. 3B). Taken together, CR reduced the $\Delta ymoA$ -induced HeLa cell rounding to a number comparable to that of CR-grown parental YP strain, indicating that at least in a cell culture infection model, CR could reduce the virulence potential of an otherwise hypervirulent, *yop* regulon-de-repressed mutant.

3.5. CR modulates Yop secretion and production in the $\Delta ymoA$ mutant

Since a reduction in T3SS function following CR could have accounted for the observed decrease in HeLa cell rounding (Fig. 3), we sought to evaluate YopE and LcrV expression and secretion levels in NG- and CR-grown YP strains. T3SSs were either induced with the addition of a calcium chelator while growing at 37°C or left uninduced (with no chelator added). Immediately following T3SS induction (0 min time point), there was a significant decrease in the amount of YopE produced (pellet fraction) and secreted in the CR-grown parental YP strain (Fig. 4, lanes 5 and 6) relative to its NG-grown counterpart (lanes 7 and 8), consistent with our earlier findings (Lawal *et al.*, 2010). The

aforementioned dramatic disparity between CR- and NG-grown parental strain YopE production and secretion profiles was maintained 20 min following T3SS induction (lanes 13–16).

In agreement with the HeLa cell rounding data, the CR-grown $\Delta ymoA$ mutant showed a decrease in YopE production and secretion at the 0 and 20 min time points similar to the CR-grown parental YP strain (lanes 1 and 2 and 9 and 10 relative to lanes 5 and 6 and 13 and 14, respectively) when compared to the NG-grown $\Delta ymoA$ mutant (lanes 3 and 4 and 11 and 12). Still, the impressive degree of reduction in CR-grown $\Delta ymoA$ mutant strain's YopE secretion at the 0 min time point relative to its NG-grown counterpart was surprising considering its de-repressed T3SS. However, the CR-grown $\Delta ymoA$ YopE secreted fractions were slightly greater than that of the simulated microgravity-grown parental strain at both the 0 and 20 min time points (compare lanes 1 and 2 to 5 and 6 and lanes 9 and 10 to 13 and 14), showing that although CR reduced secreted levels of YopE, *yopE* transcript levels were likely greater to begin with due to $\Delta ymoA$'s de-repressed Yop regulon phenotype.

When we evaluated the 0 min time point for LcrV secretion profiles in CR-grown parental YP and $\Delta ymoA$ mutant strains (Fig. 5), we observed reduced secreted LcrV relative

FIG. 5. LcrV secretion by NG- and CR-grown parental YP and its $\Delta ymoA$ mutant strain. Immunoblot analysis of secreted LcrV protein induced in bacteria in the presence (+) of 5.0 mM EGTA (a calcium chelator) is shown. Supernatant fractions of bacteria were blotted and probed with rabbit polyclonal anti-LcrV antibodies.

FIG. 6. Murine infections with NG- and/or CR-grown parental YP strain and CR-grown $\Delta ymoA$ mutant strain. (A) Female Swiss Webster mice ($n=5$ /group) were i.p. challenged with 1×10^8 cfu of NG- and CR-grown parental YP KIM/D27. Mouse survival curves are graphically represented, and a Kaplan-Meier survival estimate revealed no significant difference in survival curves of mice infected with NG- and CR-grown bacteria ($p=0.429$). (B) Female Swiss Webster mice ($n=5-6$ /group) were i.p. challenged with 1.0×10^7 cfu of CR-grown parental YP and its $\Delta ymoA$ mutant strain. Mouse survival curves are graphically represented, and a Kaplan-Meier survival estimate revealed a significant difference in survival curves of mice infected with CR-grown $\Delta ymoA$ vs. CR-grown parental YP ($p=0.008$).

to their respective NG-grown counterparts (Fig. 5 compare lane 2 to 1 and lane 4 to 3). Furthermore, more secreted LcrV was observed in the CR-grown $\Delta ymoA$ mutant than in the CR-grown parental YP strain (compare lane 4 to 2), in agreement with YopE secretion profiles (Fig. 4). Taken together, CR profoundly reduced the amounts of YopM (Lawal *et al.*, 2010), YopE (Fig. 4), and LcrV (Fig. 5) production and/or secretion in the parental YP strain. Furthermore, CR was able to overcome the de-repressed Yop regulon phenotype of the $\Delta ymoA$ mutant strain. The observed reduction in T3SS-associated gene expression and secretion likely accounted for the observed reduction in induced HeLa cell rounding.

3.6. Murine infection studies

To extend our cell culture infection studies to a biologically relevant animal model, mice ($n=5$) were i.p. challenged with either the parental or the $\Delta ymoA$ mutant strain cultivated under CR or NG conditions. Following a 1×10^8 cfu i.p. challenge, both CR- and NG-grown parental bacteria induced 40–50% mortality by day 2 post-infection (p.i.); and by day 6 p.i., both CR- and NG-grown parental YP strain induced 100% mouse lethality (Fig. 6A). Although the mean time to death was somewhat delayed when the parental YP strain was grown under CR, ultimately there was no statistically significant difference between the observed survival rates of both groups of infected mice, which is in strong agreement with our previously published cell culture infection data (Lawal *et al.*, 2010).

To further compare the effects of CR on the parental YP and $\Delta ymoA$ mutant strains' virulence potentials in mice, a separate murine infection was carried out by using a lower i.p. challenge dose of 1×10^7 cfu. Mice infected with CR-grown parental YP experienced 100% survival up to 3 days p.i. However, by day 4 p.i., 20% of CR-grown parental-infected mice had succumbed to infection followed by a steady decline in survivors until only 20% of infected mice

had survived by day 6 p.i. (Fig. 6B). By contrast, mice i.p. challenged with the CR-grown $\Delta ymoA$ mutant experienced only 40% survival on day 2 p.i. compared to 100% survival of mice infected with CR-grown parental bacteria (Fig. 6B). Furthermore, by day 4 p.i., all CR-grown $\Delta ymoA$ mutant-infected mice had succumbed to infection compared to 80% mouse survival in the CR-grown parental-infected group (Fig. 6B). The difference between the survival curves of the two groups of infected mice was statistically significant ($p=0.008$). These findings demonstrate that despite compromised T3SS function, CR was unable to reduce the virulence potential of the $\Delta ymoA$ mutant to levels observed in CR-grown parental YP in mice.

4. Discussion

The spaceflight environment is characterized by weightlessness in confined enclosures in which life support is dependent on recycled/reclaimed air and water; this unusual condition alters the immune response of flight members and facilitates the transmission of infectious diseases aboard (Chopra *et al.*, 2006). Due to the infrequent nature of spaceflight experiments, ground-based alternatives that generate MMG have been developed and extensively employed (Nickerson *et al.*, 2004; Rosenzweig *et al.*, 2010). The HARV is one such instrument capable of generating CR and has been the most widely used in bacterial virulence studies (Rosenzweig *et al.*, 2010 and references therein). In fact, CR-grown salmonellae and *P. aeruginosa* were found to respond by increasing biofilm production (Klaus and Howard, 2006; Wilson *et al.*, 2007). YP has been shown to produce biofilm under various environmental conditions, including the flea intestine, soil, and exposed surfaces (Kutyrev *et al.*, 2009; Zhou and Yang, 2011) and can inhibit biofilm formation through repression of the diguanylate cyclase gene (*hmsT*) expression (Sun *et al.*, 2012). The YP strains used in this study were unable to produce biofilm on account of their lacking

the *pgm* locus, which contains iron acquisition genes (*e.g.*, *hms*) (Lillard *et al.*, 1997) as well as genes involved in biofilm production (Forman *et al.*, 2006). However, *Y. pseudotuberculosis* could be employed in future studies to determine whether CR influences yersinia biofilm production as well. In that same vein, CR-grown *Candida albicans* (pathogenic yeast) produced greater amounts of biofilm, which were detected on the HARV siliconized gas exchange membranes (Searless *et al.*, 2011).

In the past, we reported that unlike the salmonellae, which developed a hyper-virulent phenotype following CR and spaceflight (Nickerson *et al.*, 2000, 2003; Chopra *et al.*, 2006; Wilson *et al.*, 2007, 2008), YP's virulence potential in cell culture infection assays was not enhanced following CR (Lawal *et al.*, 2010). Further, we demonstrated that CR diminished YP's T3SS function and speculated that a sub-optimally functioning T3SS was likely responsible for the observed decrease in HeLa cell cytotoxicity (Lawal *et al.*, 2010). Interestingly, the salmonellae hypervirulence phenotype observed following spaceflight (Wilson *et al.*, 2007) was masked when spaceflight salmonellae were nutritionally starved (Wilson *et al.*, 2008), suggesting that, when facing multiple stressors (in this example low-shear force associated with MG and nutritional stress), bacteria might respond to one stress at the exclusion of another. This realization motivated us to evaluate growth curves, stress responses (to antibiotic and cold temperatures), and virulence potentials of NG- and CR-grown parental YP and its *ΔymoA* mutant strain. The *ΔymoA* mutant was selected for this study due to its de-repressed Yop regulon phenotype in which it displays de-regulated Yop secretion and expression (Jackson *et al.*, 2004). Since we earlier observed CR compromising YP's T3SS (Lawal *et al.*, 2010), we wanted to determine whether CR could similarly compromise the de-repressed T3SS of the *ΔymoA* mutant.

As it turned out, growth kinetics of neither the parental strain nor the *ΔymoA* mutant were altered during CR, in agreement with earlier *E. coli* studies (Lynch *et al.*, 2006). However, unlike in *E. coli* and *Salmonella* (Wilson *et al.*, 2002a; Lynch *et al.*, 2004), YP did not experience increased resistance to chemical stress (in the form of antimicrobials). Evaluating bacterial resistance to common antimicrobial agents following CR exposure is a concern for spaceflight programs and their ability to manage infectious diseases during extended spaceflight missions. Similarly, CR did not influence the cold-growth potential of the psychrotrophic YP parental strain or the *ΔymoA* mutant following 16 days of cold growth.

In addition to *S. Typhimurium*, different strains of *E. coli* (Chopra *et al.*, 2006; Allen *et al.*, 2008) and *P. aeruginosa* (Crabbé *et al.*, 2008, 2011) also exhibited a hypervirulence phenotype or an enhanced virulence potential following CR. Our previous CR-related YP studies were the first published exception to the hypervirulence trend of CR-exposed Gram-negative pathogens (Lawal *et al.*, 2010). More recently, *Staphylococcus aureus* exhibited downregulated expression of *hly* following CR (Castro *et al.*, 2011) in a manner consistent with what was formerly observed in *P. aeruginosa* and *S. Typhimurium* (McLean *et al.*, 2001; Crabbé *et al.*, 2011). However, unlike the salmonellae (Nickerson *et al.*, 2004; Rosenzweig *et al.*, 2010), which exhibited a hypervirulence phenotype, the *S. aureus* response to CR resembled that of the yersinia (Lawal *et al.*, 2010), resulting in diminished virulence potential (Castro *et al.*, 2011). The fact that YP and *S. aureus*

have responded to CR differently than expected forced us to consider the reality that not all pathogens respond to stresses similarly. This should not come as that much of a surprise since pathogens, regardless of how closely related they are, have adopted very diverse pathogenic strategies as well as unique stress responses.

In this study, cell culture infection assays revealed no enhanced virulence potential of CR-grown YP parental and *ΔymoA* mutant strains. Further, CR compromised the T3SS activity in both of the aforementioned, despite the *ΔymoA* mutant having a deregulated T3SS (Jackson *et al.*, 2004). Interestingly, YopE and LcrV production and secretion were reduced in both strains following CR. LcrV, the backbone of the T3SS translocator complex, is required to establish contact with the target host cell to form a channel in the host-cell membrane through which other Yop effector proteins are translocated into the host cytosol (Holmström *et al.*, 2001). Therefore, a deletion or repression of LcrV secretion and/or expression results in outright reduced virulence, which could have also accounted for the reduced HeLa cell cytotoxicity induced by CR.

In a murine model of infection, CR did not enhance the virulence potential of the parental YP strain, which is consistent with our earlier cell culture infection data (Lawal *et al.*, 2010). Surprisingly, the virulence potential of the CR-grown *ΔymoA* mutant was significantly different and greater than that of the CR-grown parental strain in our murine model of infection. This suggests that despite a compromised T3SS following CR growth and a reduction in induced HeLa cell rounding, the *ΔymoA* mutant (by an unknown mechanism) was significantly more virulent than CR-grown parental YP strain *in vivo*. This underscores the importance of employing an animal infection model (when possible) in addition to cell culture infection assays to best gauge a pathogen's virulence potential. Perhaps CR prompted the upregulation of a particular low-shear stress responsive gene that was already more accessible due to the deletion of the *YmoA*-encoding gene. Such a scenario could have resulted in overexpression of a virulence-associated gene accounting for the CR-grown *ΔymoA* mutant strain's enhanced virulence potential in mice. A global transcriptome profile may reveal such candidate genes and could be the basis for a future study to help further expand our understanding of the yersinia CR response.

5. Conclusions and Implications

During a human-bacterial pathogen interaction, microbes may experience low-shear stress/forces during their systemic spread while exposed to various bodily fluids (*e.g.*, blood). Consequently, numerous studies of CR-exposed/grown bacterial pathogens have been undertaken to better understand this complex scenario. Furthermore, we can envision the various low-shear forces experienced by systemic bacteria that help shape the coevolution of both host and pathogen (as both battle for supremacy). Of course, bacterial pathogens that cause fulminant infections would be less susceptible to the effects of low-shear forces in human bodily fluids due to their shorter infection time courses. However, such a view could help us better evaluate which pathogens might become hypervirulent when present in certain bodily fluids that produce possible low-shear forces.

With regard to the possibility of future, long-term space travel, astrobiologists have become increasingly concerned with alterations in the virulence potentials of MG-exposed opportunistic bacteria that double as normal flora within and on astronauts. Consequently, a comprehensive reference list of bacterial pathogens' responses to CR and, when possible, bona fide MG (associated with spaceflight) should be established. Such a list could easily be referenced during space travel and help guide spaceflight clinical responses to bacterial infections when medicine is limiting and immediate returns to Earth are impossible.

In this study, we furthered our earlier findings that demonstrated no significant alternation in the virulence potential of CR-grown YP (Lawal *et al.*, 2010) by evaluating the effects of CR on YP growth kinetics, antibiotic susceptibility, and cold growth. Further, we characterized the effects of CR on a YP *ΔymoA* mutant strain with a de-repressed T3SS in both cell culture and murine models of infection. Contrary to what had been observed earlier in *Salmonella* spp. and *E. coli* (Lynch *et al.*, 2006; Wilson *et al.*, 2007), CR-grown YP did not exhibit increased resistance to antibiotic/antimicrobial challenge, nor was an effect on cold growth observed. Interestingly, and for reasons unclear at present, CR significantly enhanced the virulence potential of the *ΔymoA* mutant strain in a murine infection model when compared to the CR-grown parental YP, despite reducing its T3SS effectors' production and secretion. This strongly suggests that CR is enhancing the *ΔymoA* mutant strain's virulence potential in a T3SS-independent manner and warrants further investigation.

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Abbreviations

cfu, colony-forming units; CR, clinorotation; EGTA, ethylene glycol tetraacetic acid; HARV, high-aspect ratio vessel; i.p., intraperitoneally; LcrV, low calcium response V; MG, microgravity; MMG, modeled microgravity; MOI, multiplicity of infection; NG, normal gravity; p.i., post-infection; RWVs, rotating wall vessels; T3SS, type three secretion system; TCA, trichloroacetic acid; TSA, tryptic soy agar; Yops, *Yersinia* outer membrane proteins; YopE, *Yersinia* outer protein E; YP, *Yersinia pestis*.

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