Salmonella enterica serovar Typhimurium utilizes the ClpPX and Lon proteases for optimal fitness in the ceca of chickens

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ABSTRACT *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a leading cause of salmonellosis. Poultry and poultry products are implicated in transmission of *Salmonella* to humans. In 2013, an outbreak of *S.* Typhimurium occurred that comprised 39 states within the United States and was associated with backyard flocks of chickens. Colonization of the avian host by *S.* Typhimurium requires numerous genetic factors encoded within the bacterium. Of particular interest are genetic factors induced by alternative sigma factors within *S.* Typhimurium since these genetic elements are important for adaptation to different environmental stresses. The heat shock response is a dedicated change in gene regulation within bacteria in response to several stresses, specifically growth at 42°C. Because chickens have a higher body temperature than other animals (42°C) the hypothesis was tested that components of the heat shock response are important for optimal fitness within the chicken. To this end, deletion of the heat shock proteases *clpPX* (BTNC0022) or *lon* (BTNC0021) was accomplished and the bacterial fitness *in vivo* was compared to the "wild-type" strain (NC1040) using a competition assay. One-day-old chicks were orally gavaged with an equal mixture of NC1040 and either BTNC0022 or BTNC0021. Quantification of viable bacteria over time by using plate counts indicated that deletion of either heat shock protease resulted in significantly reduced colonization of the chicken ceca compared to the wild-type strain. To satisfy the molecular Koch's postulates, *clpPX* and *lon* mutants were complemented *in trans* using a low-copy number plasmid for additional *in vivo* experiments. Complementation studies confirmed the importance of either heat shock protease to colonization of the chicken ceca. This report demonstrated that both ClpPX and Lon were important for optimal fitness within chickens. Moreover, these results suggested that components of the heat shock may be critical factors used by *S.* Typhimurium for colonization of poultry. The use of feed additives or other treatments that inactivate or inhibit ClpPX or Lon may reduce the bacterial burden of *S.* Typhimurium in poultry.

Key words: *Salmonella enterica*, poultry, heat shock, Clp, Lon

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INTRODUCTION

Infections caused by *Salmonella enterica* (*S. enterica*) account for the majority of bacterial food-borne illnesses [Center for Disease Control (CDC), 2015]. Although there are more than 2,500 serovars of *S. enterica*, serovar Typhimurium has been a leading cause of human salmonellosis in the United States. This serovar is particularly problematic because of its ability to colonize or infect multiple animal hosts. Poultry have been linked to the spread of Typhimurium to humans. In 2013, an outbreak occurred across 39 states that was associated with backyard flocks of chickens within the United States (http://www.cdc.gov/salmonella/typhimurium-live-poultry-04-13/). Typically, chickens colonized by Typhimurium are asymptomatic, which poses a potential source of infection to individuals that handle live poultry or poultry products.

The body temperature of poultry (41°C to 42°C) is unique and higher than the temperature of other animals. Temperature can have a profound influence on the gene regulation within pathogenic bacteria. For instance, recent work indicates that febrile temperatures cause a genetic reprogramming of the human-restricted *Salmonella* serovars (Elhadad et al., 2015). In addition, other bacterial pathogens, such as *Yersinia pestis*, *Borrelia burgdorferi*, and *Vibrio cholera*, regulate virulence factors in response to changing temperatures (Fukui et al., 1960; Parsot and Mekalanos, 1990; Stevenson et al., 1995; Stevenson et al., 1998). When bacteria are shifted to higher cultivation temperature, i.e., from 37 to 42°C, there is a dedicated change in gene expression,

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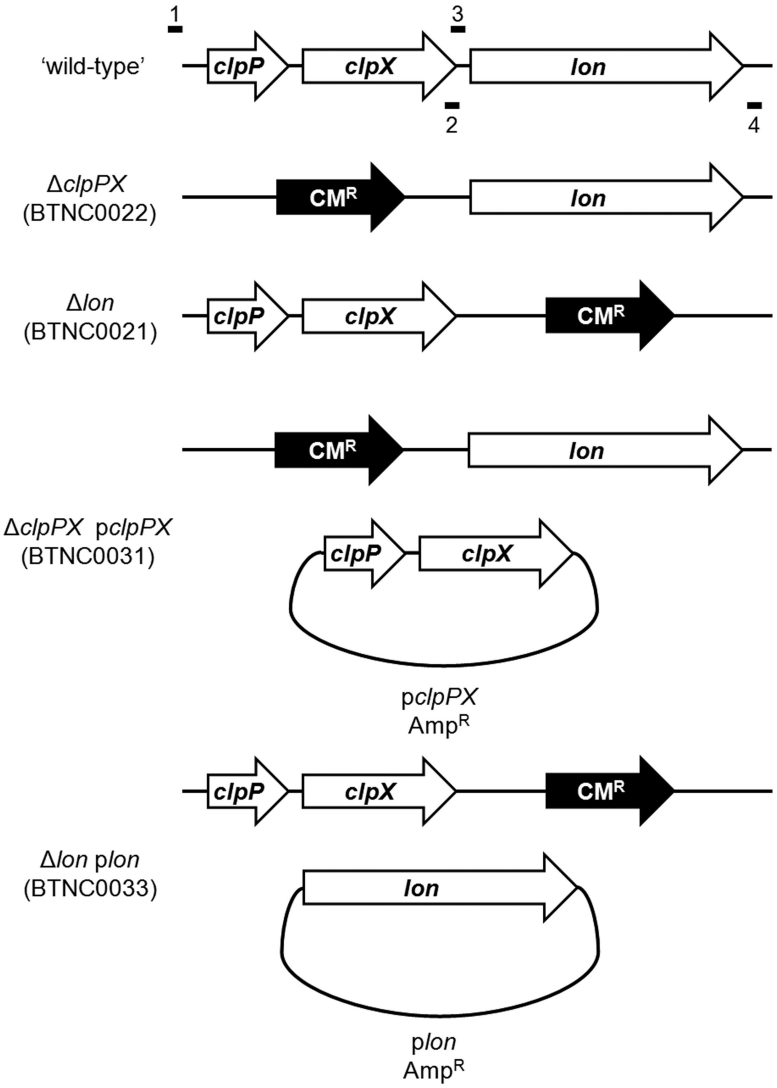

Figure 1. The genetic arrangement of the *clpP*, *clpX*, and *lon* genes and schematic of strains used in this study. In *S. enterica* serovar Typhimurium strain 14028s, these 3 genes are encoded in an apparent operon that is induced by σ^{32}. To generate deletion mutants, the open reading frames of *clpPX* or *lon* were replaced with the *cat* (chloramphenicol resistance gene) and confirmed by PCR and sequencing. For complementation studies, the wild-type copies of *clpPX* or *lon* were amplified by PCR and cloned into the low-copy plasmid pWKS30. Primers used for construction of p*clpPX* and p*lon* are shown by black bars and number. Primers 1 and 2 correspond to BamHIpClpPX fwd and HindIIIpClpPX rev (Table 2), respectively. Primers 3 and 4 correspond to EcoRIpLon fwd and HindIIIpLon rev (Table 2), respectively.

known as the heat shock response, reviewed in (Yura et al., 1993). In *S. enterica*, and other bacteria, this change in gene expression is initiated by the alternative sigma factor σ^{32} (encoded by the *rpoH* gene). When RNA polymerase is bound by σ^{32}, the holoenzyme recognizes a distinct promoter motif that is located upstream of genes whose protein products exhibit chaperone and/or protease function. Specifically, the genes encoding the major chaperones in the cell, *dnaK*, *dnaJ*, *grpE*, and *groES/EL*, contain a σ^{32}-dependent promoter and are induced during the heat shock response (Yura et al., 1993). In addition to the heat shock chaperones, heat shock proteases are induced by σ^{32}. The ATP-dependent proteases, Lon and Clp, execute a vast majority of the protein turnover in the cell. Not surprisingly, both Lon and Clp have been shown to be important for virulence in murine models of salmonellosis (Matsui et al., 2003; Takaya et al., 2003).

Mutations within the *lon* gene in *Salmonella* have been incorporated into serovars Typhimurium and Enteritidis in the development of vaccine strains for poultry (Nandre et al., 2011, 2012; Chaudhari and Lee, 2013; Matulova et al., 2013). Moreover, the approach of combining mutations in the *lon* gene with other mutations has been effective in the development of vaccine strains within the serovar Gallinarum, the causative agent of fowl typhoid (Matsuda et al., 2010; Chaudhari et al., 2011; Matsuda et al., 2011a,b; Jeon et al., 2013). Although the contribution of *lon* to fitness within poultry has been studied, there exists no published work regarding the role of Clp to *Salmonella* colonization of birds. This is somewhat surprising given the overlapping function of gene products and genetic arrangement of the *clpP*, *clpX*, and *lon* genes (Figure 1).

In this report, the contribution of the *clpPX* or *lon* genes to colonization of the cecum of chickens was

determined. In order to test the role of *clpPX* or *lon* to bacterial fitness *in vivo*, a competition assay was utilized (Freter et al., 1981). To accomplish this, one-day-old chicks from a commercial breed of egg layers were inoculated with a mixture of 2 strains of Typhimurium: 1) a "wild-type" Typhimurium isolate that is resistant to kanamycin and 2) a strain in which the chromosomal copy of either the *clpPX* or *lon* genes was deleted. The latter strains are resistant to chloramphenicol, which enabled quantification of viable wild-type and mutant bacteria within individual birds. Deletion of either *clpPX* (BTNC0022) or *lon* (BTNC0021) resulted in a decline in bacterial counts and a reduction in the number of culture-positive birds by d 10 post inoculation (**dpi**). By 21 dpi, the numbers of BTNC0022 or BTNC0021 cells in the cecal contents were below the limit of detection. Moreover, to satisfy the molecular Koch's postulates (Falkow, 1988), the contribution of *clpPX* or *lon* to cecal colonization of chickens was determined by providing the wild-type copy of *clpPX* or *lon in trans* using a low-copy number plasmid to strains BTNC0022 and BTNC0021, respectively. These *in vivo* competition experiments demonstrated that *clpPX* and *lon* were important for colonization of the chicken ceca. This study contributes to the understanding of how the heat-shock response of *Salmonella* promotes colonization of poultry.

MATERIALS AND METHODS

Reagents and Bacterial Strains

For molecular biology techniques, all PCR used Phusion® high-fidelity PCR master mix with HF buffer (2X) from New England Biolabs (NEB; Ipswich, MA). Ligation reactions were performed overnight at 14°C using T4 DNA ligase (NEB). Dimethyl sulfoxide (DMSO, Sigma-Aldrich; St. Louis, MO) was added to reactions to a final concentration between 1 and 3%, depending on the target DNA, and plasmid DNA was purified using a GenElute maxiprep kit (Sigma-Aldrich). PCR products were gel extracted using the QIAquick Gel extraction kit (Qiagen; Valencia, CA), and sequencing reactions were performed by Genewiz, Inc. (South Plainfield, NJ). Kanamycin sulfate and chloramphenicol were purchased from Sigma-Aldrich. Ampicillin, 3-morpholinopropane-1-sulfonic acid (**MOPS**), glucose, and rifampicin were purchased from Fisher Scientific (Pittsburgh, PA). Primers were ordered from Integrated DNA Technologies (IDT; Coralville, IA).

Strains and plasmids used throughout are listed in Table 1. American Type Culture Collection (**ATCC**) *S. enterica* serovar Typhimurium strain 14028s was the parental strain for all strains constructed. Strain 14028s was originally isolated from systemic organs of poultry by the Centers for Disease Control (Porwollik et al., 2014). Strains with spontaneous resistance to rifampicin were obtained as described previously (Troxell et al., 2015).

Primers used to construct and confirm plasmids are listed in Table 2. For the procedure and primers used to generate BTNC0022 and BTNC0021, see (Troxell et al., 2015). Briefly, the lambda Red method was used to create deletions of targeted genes (Datsenko and Wanner, 2000). The transducing phage P22 was used to transfer the mutation into the 14028s genetic background. Construction of p*clpPX* and p*lon* was performed as follows. DNA from 14028s was used as a template for PCR amplification of the *clpPX* or *lon* genes using the primers BamHIpClpPX fwd, HindIIIpClpPX rev, or EcoRI pLon fwd, HindIII pLon rev, respectively. PCR products were gel extracted, digested with BamHI-HindIII or EcoRI-HindIII, ligated into the BamHI-HindIII or EcoRI-HindIII digested pWKS30, and cloned into chemically competent *Escherichia coli* strain DH5α. Colony PCR was used to screen successful inserts in clones that were Amp^R using primers

Table 1. Bacterial strains and plasmids.

Strain	Genotype[1]	Source
Salmonella enterica serovar Typhimurium 14028s		ATCC[2]
Escherichia coli strain DH5α		Lab stock
NC1040	*fnr':ha* (Kan^R)	(Troxell et al., 2015)
BTNC0021	*lon::cat*	(Troxell et al., 2015)
BTNC0022	*clpPX::cat*	(Troxell et al., 2015)
BTNC0030	Rif^R BTNC0022	This study
BTNC0031	BTNC0022 with p*clpPX*	This study
BTNC0032	Rif^R BTNC0021	This study
BTNC0033	BTNC0021 with p*lon*	This study
Plasmids		
pWKS30	Amp^R, pSC101 origin	(Wang and Kushner, 1991)
p*clpPX*	*clpPX* cloned into the BamHI-HindIII sites of pWKS30	This study
p*lon*	*lon* cloned into the EcoRI-HindIII sites of pWKS30	This study

[1]Kan^R (kanamycin resistance), Rif^R (rifampicin resistance), Amp^R (ampicillin resistance).
[2]American Type Culture Collection.

Table 2. Primers used in this study.

Primer	Sequence[1]
BamHIpClpPX fwd	ATA TAT GGA TCC GGC GTA ATT TTT CGC GTT AAA
HindIIIpClpPX rev	ATA TAT AAG CTT TTT AAT TAT TCG CCA GAA GCC TGC G
EcoRI pLon fwd	ATA TAT GAA TTC TTA AAC ATT CAT ACA ATC AGT TAG CC
HindIII pLon rev	ATA TAT AAG CTT CTA TTT TGC GGT TAC AAC CTG C
pWKS30 fwd	TAG AAC TAG TGG ATC CCC CG
pWKS30 rev	AAG CGC GCA ATT AAC CCT CA

[1]Underlined sequences correspond to restriction enzyme sites.

pWKS30 fwd and pWKS30 rev. Strains BTNC0021 or BTNC0022 were electroporated with plasmid preparations from correct DH5α clones and the inserts were sequenced using pWKS30 fwd and rev. The complementing plasmids place expression of *clpPX* or *lon* under native control.

S. Typhimurium Challenge of One-Day-Old Chicks Using the Competition Assay

Animal experiments were performed as described previously (Troxell et al., 2015). Briefly, 30 one-day-old chicks of the egg laying line W-36 (Hy-Line North America West Des Moines, IA) were placed in a HEPA filtered 934-WP animal isolator (L. H. Leathers, Inc; Athens, GA). The isolator is equipped with wire mesh racks located above plastic trays for waste collection. Water and feed (Southern States All Grain Start-N-Grow; Richmond, VA) were provided ad libitum. The isolator temperature was controlled and maintained between 29 and 31°C for the duration of the experiment. The Institutional Animal Care and Use Committee (IACUC) approved the animal study protocol (protocol 15–065-A).

The competition assay (Freter et al., 1981) was used to measure fitness of bacterial mutants relative to the wild type. This assay is beneficial to host-pathogen studies for 2 reasons: 1) it reduces the number of animals needed for experiments and 2) it directly compares mutant to wild-type or complemented strain within individual animals. For complementation studies the mutant strain was competed against the complemented strain for the following reasons: 1) it tests reproducibility of the defective phenotype of the mutant strain, and 2) directly tests the role of fitness when providing the mutated gene(s) *in trans*. *S.* Typhimurium strains were cultivated overnight in standing conditions in Luria-Bertani broth (LB; per L: 10 g Tryptone, 5 g yeast extract, and 10 g NaCl) medium at 37°C. Cells were concentrated by centrifugation, washed with PBS, and the cell pellet was re-spuspended in PBS to a concentration of $\sim 1 \times 10^{10}$ CFU/mL (optical density at 600 nm ~ 10). To perform the competition assay, an equal volume of NC1040 (or complemented strain) was mixed with the mutant strain resulting in a cell suspension of $\sim 5 \times 10^9$ CFU/mL for each strain. Chicks were individually inoculated by oral gavage with 100 μL of the cell suspension ($\sim 5 \times 10^8$ CFU of each strain per bird; $\sim 10^9$ total CFU of *Salmonella*). The inoculum for each strain was confirmed by serial dilution and plating. For experiment #1, NC1040 and BTNC0022 were given to each bird at 5×10^8 CFU and 5.2×10^8 CFU, respectively. In addition, within experiment #1 using NC1040 and BTNC0021, each bird was given 5×10^8 CFU and 2×10^8 CFU, respectively. For experiment #2, each bird was given 3.2×10^8 CFU and 7×10^8 CFU of BTNC0030 and BTNC0031, respectively. For experiment #3, each bird was given 2×10^8 CFU and 2.9×10^8 CFU of BTNC0032 and BTNC0033, respectively.

At indicated dpi 5 birds from each treatment group were euthanized and the cecal contents were aseptically removed and placed in PBS with one mM $MgCl_2$. The weights of cecal content were recorded and samples were serially diluted and plated on XLT4 agar plates with 100 mM MOPS pH 7.4 without tergitol (BD Difco; Franklin Lakes, NJ). XLT4 agar plates contained kanamycin sulfate to select for strain NC1040 (65 μg/mL), chloramphenicol to select for strains BTNC0021, BTNC0022, and BTNC0030–0033 (25 μg/mL), rifampicin to select for strains BTNC0030 and BTNC0032 (75 μg/mL), and ampicillin to select for strains BTNC0031 and BTNC0033 (75 μg/mL). H_2S positive (black) and antibiotic resistant colonies were counted and the CFU counts were normalized to the weight of cecal contents.

Three animal experiments were conducted. In experiment #1, 2 isolators were used to compare the fitness of NC1040 with BTNC0022 (one isolator) and NC1040 with BTNC0021 (second isolator). During experiment #1, at 15 dpi, mutant bacteria were isolated from culture positive birds for use in complementation studies (experiments #2 and #3). This was accomplished by purification of chloramphenicol resistant bacteria from XLT4 plates, and PCR was used to confirm the lack of *clpPX* or *lon*. Isolated bacteria were transformed with the appropriate complementing plasmid for use in experiments #2 and #3. In addition, the reisolated mutant became resistant to rifampicin by cultivation of bacteria and plating $\sim 10^8$ CFU on chloramphenicol and rifampicin containing XLT4 plates. The chloramphenicol and rifampicin resistant isolates were designated BTNC0030 (for Δ*clpPX*) and BTNC0031 (for Δ*lon*). For experiment #2, one isolator was used to compare the fitness of BTNC0030 (Rif^R Δ*clpPX*) with

BTNC0031 (Δ*clpPX* p*clpPX*). For experiment #3, one isolator was used to compare fitness of BTNC0032 (Rif^R Δ*lon*) with BTNC0033 (Δ*lon* p*lon*).

Statistical Analysis

Figures and statistical analysis were accomplished using GraphPad Prism v4.0. Throughout the study, a one-way ANOVA with Bonferroni's correction for multiple comparisons was used to determine significance. CFU/g counts were $\log_{10}$ transformed. Because the mutant bacterial strains (either Δ*clpPX* or Δ*lon*) could not be quantified in all inoculated birds per time point a replacement approach was used to determine mean the $\log_{10}$ CFU/g. This method is acceptable when samples are <100% but >40% culture positive. The mean detection limit ($\log_{10}$ CFU/g 2.3±1.7) was substituted for zero to determine mean of samples for that population and time point (simple replacement approach).

RESULTS AND DISCUSSION

clpPX and lon are Important for Optimal Cecal Fitness Within Chicks

The heat shock genes *clpP*, *clpX*, and *lon* are encoded in an apparent operon (Figure 1). The promoters of each gene are activated by σ^{32}; however, the promoter of *clpP* appears to be the strongest of the 3 promoters (De la Cruz et al., 2015). Because these genes are induced during the heat shock response and since the body temperature of poultry is 41 to 42°C, it suggests that these genes would be important for survival *in vivo*. To test this, the open reading frames *clpPX* or *lon* genes were deleted and replaced with the *cat* gene, which encodes resistance to chloramphenicol. In experiment #1, 30 one-day-old female chicks of the W-36 line from Hy-Line International were given an oral dose of mixture of wild-type Typhimurium (NC1040) and mutant bacteria (BTNC0022 or BTNC0021). At 1, 3, 6, 10, 14/15, and 21 dpi, birds were euthanized and the bacterial burden of each strain was determined by plating of dilutions of cecal content on selective media.

Both the *clpPX* and *lon* genes appear important for optimal fitness within chickens. When chicks were infected with both NC1040 and BTNC0022, both strains were present in the cecal contents at ~10^8 CFU/g at 1 dpi (Figure 2A). Similar to previous results (Troxell et al., 2015), NC1040 was capable of colonizing the ceca of birds at >10^7 CFU/g for the duration of the study. However, the lack of *clpPX* (BTNC0022) resulted in a reduction of bacterial burden by 6 dpi (Figure 2A). By 10 dpi, only 2 birds were culture positive for BTNC0022 and the values were approaching the limit of detection ($\log_{10}$ CFU/g 2.3; Figure 2A). At 15 dpi, only one animal was positive and by 21 dpi all birds were negative for BTNC0022 (Figure 2A). These results suggested that *clpPX* was important for colonization of chicks. When one-day-old chicks were inoculated with NC1040 and BTNC0021, there was a pronounced difference in bacterial fitness between strains. Strain NC1040 was able to colonize the ceca at levels >10^7 CFU/g for the duration of the study. By 15 dpi, only 2 birds had BTNC0021 present in the ceca, which became below the limit of detection by 21 dpi (Figure 2B).

Providing clpPX and lon in trans Complements the Defect of Bacterial Fitness in Chicks

Data in Figure 2 suggest that *clpPX* and *lon* are important for optimal fitness in chicks. To confirm this and to satisfy the molecular Koch's postulates (Falkow, 1988), complemented strains were constructed as

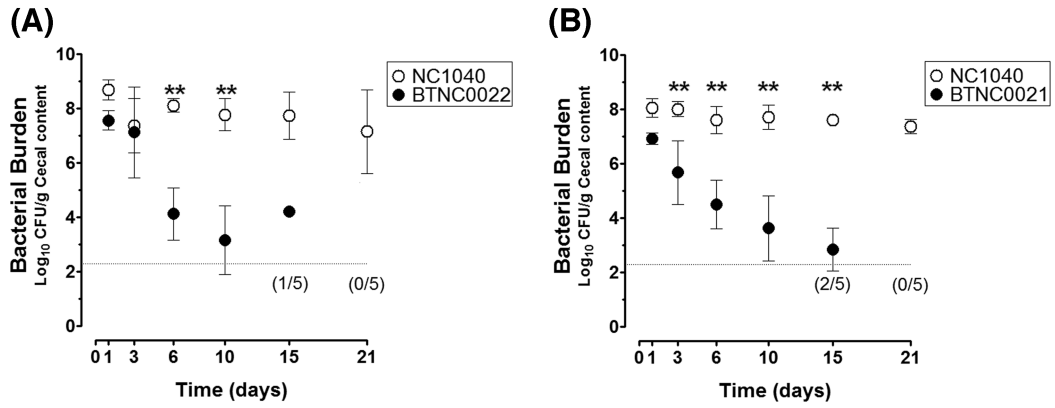

Figure 2. *S. enterica* serovar Typhimurium strain 14028s uses ClpPX and Lon for optimal fitness within chickens. Shown are the results from experiment #1. Open circles correspond to the wild-type strain (NC1040) and the filled circles correspond to mutant strains (BTN0022 or BTNC0021). **A.** Deletion of the *clpPX* genes resulted in reduced fitness within the ceca of chickens compared to NC1040. Each data point is the mean ± 1 standard deviation of the $\log_{10}$ CFU/g from 5 birds per time point. The dashed line on the y-axis shows the limit of detection for the assay. When not all samples were quantified by culture, the numbers in parentheses show the number of birds positive out of the number of birds sampled. **B.** Deletion of the *lon* gene resulted in reduced fitness within the ceca of chickens compared to NC1040. Data presented are as in **A.** and show the quantification of NC1040 and BTNC0021 within the ceca of birds over time. A one-way ANOVA was used to determine significance, ** $P < 0.01$.

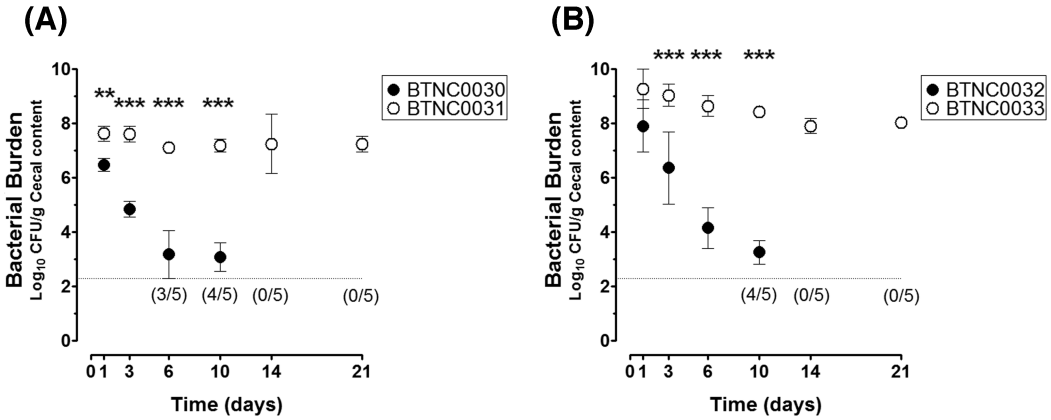

Figure 3. Complementation of mutant strains confirmed the importance of *clpPX* and *lon* for optimal fitness within chickens. Shown are the results from experiments #2 and #3. Open circles correspond to the complemented strain (either BTNC0031 or BTNC0033) and the filled circles correspond to mutant strains isolated from experiment #1 (BTN0030 or BTNC0032). **A.** Results of experiment #2 show that complementation of BTNC0030 with the *clpPX* genes resulted in enhanced fitness within the ceca of chickens compared to BTNC0030. Each data point is the mean ± 1 standard deviation of the $\log_{10}$ CFU/g from 5 birds per time point. The dashed line on the y-axis shows the limit of detection for the assay. When not all samples were quantified by culture, the numbers in parentheses show the number of birds positive out of the number of birds sampled. **B.** Results of experiment #3 show that complementation of BTNC0032 with the *lon* gene resulted in enhanced fitness within the ceca of chickens compared to BTNC0032. Data presented are as in **A.** and show the quantification of BTNC0032 and BTNC0033 within the ceca of birds over time. A one-way ANOVA was used to determine significance, ** $P < 0.01$, *** $P < 0.001$.

described in Materials and Methods. The *clpPX* genes were cloned into the low-copy plasmid pWKS30 (Wang and Kushner, 1991) and transformed into BTNC0022 generating a chloramphenicol (Δ*clpPX*) and ampicillin (p*clpPX*) resistant strain (BTNC0031). In addition, a spontaneous rifampicin resistant strain of BTNC0022 was obtained (BTNC0030). This was accomplished by reisolation of an isolate of BTNC0022 or BTNC0021 from the chicken at 15 dpi during experiment #1. Therefore, BTNC0030 is chloramphenicol and rifampicin resistant, whereas BTNC0031 is chloramphenicol and ampicillin resistant, and these strains were used in experiment #2. Similar to results from experiment #1, the lack of *clpPX* resulted in a decline of bacterial burden within the ceca (Figure 3A). By 14 dpi, BTN0030 was no longer detected in birds. This contrasts with the results obtained with BTNC0031. When *clpPX* was provided *in trans*, it was sufficient to dramatically restore colonization of the cecal contents of birds for the duration of the study (Figure 3A). In addition, since deletion of only the *lon* gene impairs fitness (Figure 2B) and providing *clpPX in trans* is sufficient to restore fitness of BTNC0022, these data indicate that disruption of the *clpPX* genes does not perturb expression of the downstream *lon* gene, as described previously (Yamamoto et al., 2001). For experiment #3, the lack of *lon* resulted in the inability to detect BTNC0032 after 14 dpi (Figure 3B). As with the complementation results in experiment #2, providing the *lon* gene *in trans* (strain BTNC0033) reverted the colonization defect to levels $>10^7$ CFU/g throughout the study (Figure 3B). Collectively, these *in vivo* studies show that both the ClpPX and Lon heat shock proteases are important for optimal fitness within the chicken ceca.

There are several key findings from this study. First, both *clpPX* and *lon* appear important for optimal fitness within chickens. Second, the importance of these genes appears temporal, i.e., the defective phenotype became more pronounced as chickens developed (d 6 and on). This may be due to the increase in body temperature from ∼40 to ∼42°C that occurs from day-of-hatch to 7 d post hatch during development (Troxell et al., 2015). It is expected that the heat shock response of *S. enteric* becomes increasingly important during this development. However, a caveat to this study is that no enrichment procedures were implemented to detect the *S.* Typhimurium strains used throughout. Therefore, the Δ*clpPX* and Δ*lon* strains may still be present within infected birds, but below the limit of detection. However, since the wild-type strain (NC1040) was ∼5 to 6 orders of magnitude above the limit detection for cecal samples, it indicates both *clpPX* and *lon* are important for optimal fitness. Finally, this study suggests that components of the heat shock response may be important for optimal function of *S. enterica in vivo*. Thus, targeting components of this pathway may be important for removal of bacteria from animals. Novel antimicrobials are already in development, which function through altering activity of the ClpP protease (Brotz-Oesterhelt et al., 2005; Gersch et al., 2015).

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