Role of RpoS in the Virulence of *Citrobacter rodentium*[▽]

Tao Dong,¹ Brian K. Coombes,² and Herb E. Schellhorn^{1*}

Department of Biology, Life Sciences Building, Rm. 433, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada,¹ and Department of Biochemistry and Biomedical Sciences, Health Sciences Centre, 4H17, McMaster University, 1200 Main St. West, Hamilton, Ontario L8N 3Z5, Canada²

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Citrobacter rodentium is a mouse enteropathogen that is closely related to *Escherichia coli* and causes severe colonic hyperplasia and bloody diarrhea. *C. rodentium* infection requires expression of genes of the locus of enterocyte effacement (LEE) pathogenicity island, which simulates infection by enteropathogenic *E. coli* and enterohemorrhagic *E. coli* in the human intestine, providing an effective model for studying enteropathogenesis. In this study we investigated the role of RpoS, the stationary phase sigma factor, in virulence in *C. rodentium*. Sequence analysis showed that the *rpoS* gene is highly conserved in *C. rodentium* and *E. coli*, exhibiting 92% identity. RpoS was critical for survival under heat shock conditions and during exposure to H₂O₂ and positively regulated the expression of catalase KatE (HPH). The development of the RDAR (red dry and rough) morphotype, an important virulence trait in *E. coli*, was also mediated by RpoS in *C. rodentium*. Unlike *E. coli*, *C. rodentium* grew well in the mouse colon, and the wild-type strain colonized significantly better than *rpoS* mutants. However, a mutation in *rpoS* conferred a competitive growth advantage over the wild type both in vitro in Luria-Bertani medium and in vivo in the mouse colon. Survival analysis showed that the virulence of an *rpoS* mutant was attenuated. The expression of genes on the LEE pathogenicity island, which are essential for colonization and virulence, was reduced in the *rpoS* mutant. In conclusion, RpoS is important for the stress response and is required for full virulence in *C. rodentium*.

Intestinal diseases caused by bacterial infection, such as the infection caused by *Escherichia coli* O157:H7, are major threats to public health (40). It is imperative to fully understand how the pathogens that cause these diseases are transmitted, propagate, and cause disease in the host. During transmission, pathogens likely have to survive many stresses, including environmental stresses (e.g., nutrient limitation) and host internal stresses (e.g., acidic exposure in the stomach and host defense), implying that stress response systems are important. One of the most important regulators in the stress response is RpoS, an alternative sigma factor of RNA polymerase that is present primarily in gamma-proteobacteria, including *E. coli* and *Salmonella* (11, 20).

RpoS is important for cell survival in stress conditions, such as oxidative stress and exposure to acid, in many pathogens, including *Salmonella* sp. (15), *Vibrio cholerae* (59), *Pseudomonas aeruginosa* (50), and *Yersinia enterocolitica* (22). However, RpoS has distinct roles in the pathogenesis of these organisms. RpoS is essential for virulence in *Salmonella* (15) and is important for the invasion of brain microvascular endothelial cells in *E. coli* K1 strains (55), but it is not required for virulence in *P. aeruginosa* (50) and *Y. enterocolitica* (22). The effects of RpoS on virulence may also differ within a species. For example, RpoS has been found to be either important (35) or not required (26, 59) for colonization of the mouse intestine by *V. cholerae*. This discrepancy is likely caused by differences in strain backgrounds or animal models. Although *rpoS* mutants of *E. coli* outcompete wild-type cells during competitive colo-

nization in the mouse large intestine (27), the role of RpoS in the enteropathogenesis of *E. coli* has not been clearly resolved yet due to the lack of an effective animal model. The human pathogens enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) do not cause severe disease in mice (36, 37, 58).

Citrobacter rodentium is a natural mouse pathogen that causes colonic hyperplasia and diarrhea (37). Similar to EPEC and EHEC strains in humans, *C. rodentium* utilizes attaching and effacing (A/E) lesions, induced by genes on the locus of enterocyte effacement (LEE) pathogenicity island, to colonize the large intestine of its host (37). The LEE island carries 41 open reading frames that are organized into five polycistronic operons encoding a type III secretion system and virulence factors essential for virulence (9). Expression of LEE genes is controlled by three LEE-encoded global regulators, Ler (the major regulator), Orf11 (GrlA; the Ler activator), and Orf10 (GrlR; the Ler repressor) (9).

Compared with the extensive information available regarding RpoS in *E. coli*, little is known about RpoS in *C. rodentium*. In this study, we examined the role of RpoS in the virulence of *C. rodentium*, which may help us understand the physiological function of stress response genes in human intestinal diseases caused by enteric bacteria.

MATERIALS AND METHODS

Media and growth conditions. Cultures were grown in Luria-Bertani (LB) broth or on LB plates with 1.5% agar. In the cell motility assay, 0.3% agar was used for LB plates. Samples isolated from the mouse colon were plated on brilliant green agar (BGA) (Oxoid, Nepean, Ontario, Canada), an indicator medium that differentiates *C. rodentium* from *E. coli* and other bacteria. Cell growth was monitored spectrophotometrically at 600 nm. When necessary, antibiotics were added at the following concentrations: ampicillin, 200 µg/ml; and chloramphenicol, 30 µg/ml. To visualize the RDAR (red, dry and rough) mor-

* Corresponding author. Mailing address: Department of Biology, Life Sciences Building, Rm. 433, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada. Phone: (905) 525-9140, ext. 27316. Fax: (905) 522-6066. E-mail: schell@mcmaster.ca.

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phototype, an indicator of production of extracellular components such as curli and cellulose (42, 43), cells were streaked on RDAR plates (non-salt-containing LB medium with 1.5% agar, 40 μ g/ml of Congo red dye, and 20 μ g/ml of Coomassie blue R-250) and incubated at 25°C for 48 h (2).

Construction of an *rpoS::cat* mutant of *C. rodentium*. A precise *rpoS* deletion mutant of *C. rodentium* was constructed using the Red recombination method (8). The *rpoS* sequence was retrieved from the genome sequencing project for *C. rodentium* (http://www.sanger.ac.uk/Projects/C_rodentium/). The *rpoS* gene was replaced by homologous recombination with the *cat* chloramphenicol resistance gene, which was amplified using the pKD3 plasmid (template) and primers FP1 (CCTCACAGAGACTGGTCTTTCTGATGGAACGGT GCGTGTAGGCTGGAGCTGCTTC) and RP1 (GCTTGTCTTTGTCAAGG GATCACGGGTAGGAGCCACCTTCATGAATATCCTCCTAG).

Native polyacrylamide gel electrophoresis (PAGE) for catalase activity. Cultures were grown in LB broth aerobically at 37°C to an optical density at 600 nm (OD_{600}) of 0.3 (exponential phase) and to an OD_{600} of 1.5 (stationary phase), harvested by centrifugation at 4,000 $\times g$ for 15 min at 4°C, and washed three times in potassium phosphate buffer (50 mM, pH 7.0). Cell extracts were prepared by sonication for 5 min at 4°C using a Heat Systems sonicator (Misonix, Inc.). Cell debris was removed by centrifugation for 15 min at 12,000 $\times g$ at 4°C. The protein concentration was determined by the Bradford assay using bovine serum albumin as a standard (3). Ten micrograms of each protein sample was loaded on a 10% native polyacrylamide gel and resolved by electrophoresis at 160 V for 50 min. The gel was then stained to determine catalase activity using horseradish peroxidase and diaminobenzidine (6, 46). Parallel gels were stained with Coomassie blue R-250 to verify equal protein loading.

Resistance to H_2O_2 and exposure to heat. Cell resistance to H_2O_2 and heat was tested as described previously (31). Stationary-phase LB medium cultures were washed with 0.9% NaCl and resuspended to obtain densities of 10^8 and 5,000 cells/ml for the H_2O_2 and heat exposure experiments, respectively. Viable cells were enumerated by serial dilution plating on LB media. Survival was quantified by determining the ratio of the number of viable cells after treatment to the starting number of cells.

In vitro competition. Equal volumes of overnight LB medium cultures (25 ml) of the wild type and *rpoS* mutants (chloramphenicol resistant) were mixed together, incubated at 37°C at 200 rpm, and sampled daily for 6 days. Numbers of CFU were determined by serial dilution plating on LB media and LB media with chloramphenicol. The competitive index was calculated as follows: (output mutant CFU/output wild-type CFU)/(input mutant CFU/input wild-type CFU).

In vivo colonization and competition. All experiments using mice were performed in accordance with Canadian Council on Animal Care guidelines. Female 6- to 8-week-old C3H/HeJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Groups of five mice were infected by oral gavage with 1×10^8 CFU of wild-type *C. rodentium*, an *rpoS* mutant, or a mixture (1:1) of the wild type and the *rpoS* mutant. Mice were sacrificed on day 6 postinfection. The colon of each animal was homogenized in 1 ml ice-cold phosphate-buffered saline, and the number of CFU was determined by serial dilution plating on selective BGA. Colonies were replica plated on BGA plates containing chloramphenicol for enumeration of *rpoS* mutants.

Survival analysis. To test whether RpoS is required for virulence, C3H/HeJ mice were inoculated with 1×10^8 CFU of the wild type or an *rpoS* mutant by oral gavage (9) and were euthanized when they had lost of 20% of their body weight.

Quantification of expression of the LEE genes by qPCR. Expression of the LEE genes was quantified by performing quantitative PCR (qPCR) using an Mx3000P qPCR system (Stratagene, La Jolla, CA). Cultures used for RNA isolation were prepared as described previously (10). Briefly, the wild type and *rpoS* mutants were inoculated into LB medium in triplicate, incubated aerobically at 37°C overnight, subcultured (1:50 dilution) in Dulbecco modified Eagle medium (DMEM), and grown in the presence of 5% CO_2 at 37°C to an OD_{600} of 0.7. RNA samples were extracted using acidic hot phenol, purified using NucleoSpin RNA II (Clontech, Palo Alto, CA), and reverse transcribed to obtain cDNA using the murine leukemia virus reverse transcriptase (NEB, Beverly, MA). A serial dilution of genomic DNA of *C. rodentium* was used as a standard for quantification. The *rrsA* gene, encoding the 16S RNA, was used as an endogenous control to normalize RNA quantity (12, 47).

LEE protein secretion assay. Protein secretion by *E. coli* and *C. rodentium* was examined as described previously (10). Cultures were grown in triplicate in DMEM in the presence of 5% CO_2 at 37°C to an OD_{600} of 0.7 and centrifuged twice at 12,000 $\times g$ for 10 min to completely remove cells from the supernatants. The supernatants were precipitated with 10% trichloroacetic acid, and the resultant protein pellets were dissolved in Laemmli sodium dodecyl sulfate loading

FIG. 1. Phylogeny of the *rpoS* genes of representative pathogens. The tree was constructed by the neighbor-joining method with bootstrap analysis (1,000 iterations). Scale bar = 5% sequence divergence. *E. coli* K-12 RpoD was included as an outgroup.

buffer (30). Proteins were then resolved by 10% sodium dodecyl sulfate-PAGE and stained with Coomassie blue.

Phylogenetic analysis and sequence alignment. The *rpoS* sequences were retrieved from the genome project for *C. rodentium* at the Sanger Institute and GenBank. Sequences were aligned using ClustalW (51). A phylogenetic tree was then generated by the neighbor-joining method (44) with bootstrap analysis (1,000 iterations).

RESULTS

RpoS is conserved in *C. rodentium* and *E. coli*. RpoS, an important stress response regulator, has been found in about 30 bacterial genera, primarily members of the class *Gamma-proteobacteria* (11, 19). It is particularly interesting that RpoS is present in many human pathogens. The *rpoS* gene is highly variable (17, 41). In different strains of *E. coli*, *rpoS* polymorphisms are common (1, 5, 17, 21). Thus, we first examined the evolutionary relationship of *rpoS* in *C. rodentium* and several representative pathogens. As expected, *C. rodentium* *rpoS* is very similar to *E. coli* *rpoS* and distinct from the *rpoS* genes of *P. aeruginosa* and *V. cholerae* (Fig. 1). The *rpoS* genes of *E. coli* O157:H7 strain EDL933 and *C. rodentium* exhibit 92% sequence identity. All but three variant sites contain synonymous mutations, and the RpoS protein sequences differ only in three amino acids (E19D, G21R, and D243R). To avoid the possibility that the observed differences between *C. rodentium* and *E. coli* are due to genome sequencing errors that have been reported previously for *E. coli* strains (7, 18), we sequenced the *rpoS* region of *C. rodentium* independently, and the results were consistent with the *C. rodentium* genome sequence.

RpoS is important for resistance to H_2O_2 and exposure to heat. Because of conservation of the *rpoS* gene in *E. coli* and *C. rodentium*, we expected RpoS in *C. rodentium* to play a role in stress response similar to its role in *E. coli*. To test this hypothesis, we examined the effect of the *rpoS* mutation on the survival of cells exposed to oxidative stress and heat (Fig. 2). Upon exposure to H_2O_2 , the viability of *rpoS* mutants was substan-

FIG. 2. Effect of RpoS on H₂O₂ resistance and thermotolerance. Stationary-phase cultures were washed with 0.9% NaCl and resuspended to obtain concentrations of 10⁸ and 5,000 cells/ml for H₂O₂ resistance and thermotolerance experiments, respectively. Cells were exposed to 15 mM H₂O₂ (A) or 55°C (B) and enumerated by plating on LB medium. Survival was expressed as a percentage determined by dividing the number of viable cells by the number of cells before treatment. WT, wild type; *rpoS*, *rpoS* mutant.

tially reduced compared with the viability of the wild type (Fig. 2). Under heat stress conditions, although the viability of both the wild type and *rpoS* mutants decreased, the number of CFU of the wild type was about 100-fold higher than the number of CFU of the *rpoS* mutants after 7 min of exposure. These results show that RpoS is required for both oxidative resistance and heat tolerance in *C. rodentium*.

Expression of catalase HPII (KatE) is highly RpoS dependent. In *E. coli*, there are two catalases, HPI encoded by *katG* and HPII encoded by *katE*, which protect cells from oxidative stress. Expression of *katE* is highly RpoS dependent (45). Since RpoS is critical for the oxidative response in *C. rodentium*, we examined the effect of RpoS on catalase production. Genes homologous to *E. coli katG* and *katE* were found in the *C. rodentium* genome using the BLAST algorithm (data not shown). Native PAGE analyses of the catalases showed that catalase HPII was induced in stationary phase (Fig. 3). HPII (KatE) was the major catalase expressed under the conditions investigated and was highly RpoS dependent.

Effect of RpoS on the RDAR morphotype, an important trait for virulence. Production of extracellular components, such as curli fimbriae and cellulose, is important for cell attachment in the pathogenesis of *E. coli* and *Salmonella*, and it is positively regulated by RpoS (42, 43, 53). The expression of curli and

FIG. 3. Expression of catalase is highly RpoS dependent. Cultures were collected in exponential phase (OD₆₀₀, 0.3) and in stationary phase (OD₆₀₀, 1.5) and washed three times in ice-cold phosphate buffer prior to sonication. Protein was quantified by the Bradford assay (3). Ten micrograms of each protein sample was loaded on the 10% native PAGE gel. Catalase activity staining was performed as described in Materials and Methods. WT, wild type; *rpoS*, *rpoS* mutant.

FIG. 4. Effect of RpoS on RDAR morphotype development. Cells were grown at 37°C in non-salt-containing LB medium in microtiter plates overnight and streaked on RDAR plates. The RDAR plates were incubated at room temperature for 48 h. Production of cellulose and curli was visualized by staining with Coomassie blue and Congo red in the RDAR plates. WT, wild type; *rpoS*, *rpoS* mutant.

cellulose can be visualized by growing cells at room temperature on RDAR plates, which results in a specific RDAR colony phenotype termed the RDAR morphotype. This morphotype is positively correlated with virulence (54). To test whether the RDAR morphotype is produced and/or controlled by RpoS in *C. rodentium*, the wild type and isogenic *rpoS* mutants were plated on RDAR plates and incubated at room temperature for 48 h. *E. coli* O157:H7 strain EDL933 and isogenic *rpoS* mutants of this strain were also included for comparison. Development of the RDAR morphotype was impaired in the *E. coli rpoS* mutants, consistent with previous findings (43) (Fig. 4). Wild-type *C. rodentium* exhibited a more pronounced RDAR morphotype than the *rpoS* mutants, indicating that the RDAR morphotype is positively regulated by RpoS.

Mutation in *rpoS* confers a growth advantage in vitro and in vivo. *rpoS* mutants of *E. coli* have a growth advantage in stationary phase, a phenotype designated growth advantage in stationary phase (GASP) (60, 61). To test whether *rpoS* mutants of *C. rodentium* also exhibit the GASP phenotype, we monitored changes in the cell population in a 1:1 mixture of overnight LB medium cultures of the wild type and *rpoS* mutants in vitro. The *rpoS* mutants dominated the coculture in about 24 h (Fig. 5). After 3 days of incubation, more than 90% of living cells in the mixture were *rpoS* mutants. During this time, the total number of CFU dropped dramatically from about 10⁸ cells/ml (day 0) to about 10⁴ cells/ml (day 6).

We then tested whether *rpoS* mutations confer a growth advantage in vivo in the mouse colon, a more complex environment than the in vitro environment. Mice were infected with a 1:1 mixture of the wild type and *rpoS* mutants. The results showed that the *rpoS* mutants outcompeted wild-type *C. rodentium* at day 6 (Fig. 6) and that the average competitive index was 5.9 (geometric mean), indicating that *rpoS* mutants have a growth advantage in vivo as well. Unlike the in vitro results, the number of CFU in the mixture remained similar (10⁸ to 10⁹ cells per colon) to the number of CFU in the initial inoculum. To test whether RpoS is important for colonization in the colon, mice were infected with either wild-type or *rpoS* mutant strains and colon samples were taken at day 6. The number of CFU per colon infected with wild-type cells was

FIG. 5. Competition between the wild type and *rpoS* mutants in vitro. Equal volumes of overnight cultures of the wild type and *rpoS* mutants were mixed and incubated aerobically at 37°C. The numbers of CFU were determined by serial dilution plating on LB medium (LB medium containing chloramphenicol for *rpoS* mutants). The competitive index (CI) was calculated as follows: (output *rpoS* mutant CFU/output wild-type CFU)/(input *rpoS* mutant CFU/input wild-type CFU). CFU represents the total count of bacteria.

significantly higher than number of CFU per colon infected with the *rpoS* mutants ($P < 0.05$) (Fig. 6). These results indicate that RpoS of *C. rodentium* may be beneficial for colonization but is likely a disadvantage for competition in the colon (see the Discussion). Colonic hyperplasia, a typical symptom of *C. rodentium* infection, was observed in mice infected with either the wild type or *rpoS* mutants, suggesting that the cause of *C. rodentium*-induced mortality was the same for both groups.

Because of the important role of RpoS in the stress response, it is also possible that the difference in colonization was

FIG. 6. Colonization and competitive index for the wild type and *rpoS* mutants of *C. rodentium*. C3H/HeJ mice were infected by oral gavage with 10^8 CFU of the wild type, *rpoS* mutants, or a 1:1 mixture of the wild type and *rpoS* mutants (CI data). Mice were sacrificed at day 6 after infection. The colon of each animal was homogenized in 1.0 ml of phosphate-buffered saline, and the number of CFU was determined by serial dilution plating on BGA. Each symbol indicates the data for one animal. WT, wild type; *rpoS*, *rpoS* mutant.

FIG. 7. Expression of the LEE genes in the wild type and *rpoS* mutants of *C. rodentium*. RNA samples were extracted from cultures grown in triplicate in DMEM to an OD₆₀₀ of 0.7 in the presence of 5% CO₂ at 37°C. Differential gene expression was determined by qPCR and is expressed as the ratio of the wild type to the *rpoS* mutant. WT, wild type; *rpoS*, *rpoS* mutant.

because wild-type cells survived better than *rpoS* mutant cells during passage through the gastrointestinal tract. However, this is not likely since the number of bacteria in mouse stools (CFU/g) after 5 h of infection was the same for the wild type and *rpoS* mutants (data not shown).

RpoS controls the expression of the LEE genes. The type III secretion system and virulence factors such as Tir and Eae on the LEE island are important for colonization through the formation of A/E lesions (9, 10). We therefore examined the role of RpoS in LEE expression. For *E. coli*, there are conflicting reports regarding the effect of RpoS on expression of the LEE genes, which may be due to differences in the strains and conditions tested. It has been shown that RpoS positively controls the expression of the LEE3 operon and of Tir (48). However, expression of the LEE genes has also been reported to be negatively regulated by RpoS (23, 28, 52).

Using LEE induction conditions (5% CO₂, 37°C, DMEM) (24), we examined the transcription of representative genes in each of the five LEE operons, including genes encoding three LEE regulators, Ler, GrlA, and GrlR, by qPCR. The levels of transcription of all genes tested were higher in the wild type than in the *rpoS* mutants (Fig. 7), although the RpoS effect seemed to be moderate (less than twofold difference). The similar expression ratios of these LEE genes may have been the result of the global regulatory effects of Ler, GrlA, and GrlR (9, 14, 34).

Many LEE genes encode either the type III secretion apparatus or secreted virulence effectors, and the secreted protein profile of wild-type *C. rodentium* has been characterized previously (9). We tested the effect of RpoS on LEE protein secretion. In *E. coli* O157:H7, the secretion of LEE factors is negatively controlled by RpoS (23). In this study, we included *E. coli* O157:H7 strain EDL933 and an *rpoS* knockout mutant of this strain in our assay as a control (Fig. 8). Consistent with previous reports, the secreted protein profile of *rpoS* mutants was enhanced in *E. coli*. However, in *C. rodentium*, there were more secreted proteins in wild-type cultures than in the *rpoS* mutant cultures, although the difference was moderate, consistent with the transcription results. The wild type and *rpoS* mutants of *C. rodentium* grew similarly in DMEM with generation times of ~76 min. Therefore, it is unlikely that the difference in LEE expression results from a difference in growth.

RpoS is required for full virulence. Many genes on the LEE island are essential for virulence (9). Some non-LEE-encoded virulence effectors also require the LEE type III secretion system for delivery to the host (57). In addition, the RDAR morphotype is associated with increased virulence in *E. coli*

FIG. 8. LEE protein secretion by wild-type *E. coli* O157:H7 strain EDL933 (A) and *C. rodentium* (B) and *rpoS* mutant derivatives. The positions of known secreted proteins are indicated. WT, wild type; *rpoS*, *rpoS* mutant.

(54). Because of the positive effect of RpoS on LEE expression and RDAR morphotype development, RpoS may play an important role in virulence in *C. rodentium*. To test this hypothesis, we performed a survival analysis using C3H/HeJ mice, a strain highly susceptible to *C. rodentium* infection. Although *rpoS* mutants were still lethal to C3H/HeJ mice, mice infected with *rpoS* mutants survived significantly longer than mice infected with the wild type ($P = 0.003$, Wilcoxon test), indicating that the virulence of *C. rodentium* is attenuated by *rpoS* mutation (Fig. 9).

DISCUSSION

Infection of mice by *C. rodentium* provides a robust model to examine enteropathogenesis and the interaction between the pathogen and its natural host under physiological conditions (9, 37, 58). Because *C. rodentium* utilizes the same A/E lesions as EPEC and EHEC strains, two classes of serious human pathogens, to initiate infection (37), results obtained with *C. rodentium* may be relevant to *E. coli* pathogenesis in humans. Here we used *C. rodentium* to examine the role of RpoS in virulence under physiological conditions. Our results show that both the sequence and the function of RpoS are conserved in *E. coli* and *C. rodentium*. RpoS positively regulated the expression of LEE genes and the development of the RDAR morphotype. Although *rpoS* mutants caused lethality in mice, there was a significant delay in both the onset of disease symptoms and lethality in infected mice. Thus, RpoS is important for full virulence of *C. rodentium*. This phenotype is similar to that caused by mutations in several LEE virulence genes (e.g., *rorfI*, *espF*, and *cesF*), which result in a 2- to 3-day increase in survival of infected mice (9).

RpoS controls a large regulon consisting of 10% of the genome in *E. coli* (29, 39, 56). We expect that the RpoS regulon in *C. rodentium* is a similar size, since *C. rodentium* and *E. coli* are closely related (37). Because *rpoS* mutations have pleiotropic physiological effects in *E. coli*, we cannot be sure that a single RpoS-controlled gene accounts for the observed virulence. It is likely that the attenuated virulence of *rpoS* mutants in vivo may be due to decreases not only in virulence factor expression but also in other RpoS-controlled functions. In this study, we found that the levels of expression of the LEE genes were higher in wild-type *C. rodentium* in vitro. Since the

FIG. 9. Survival analysis. C3H/HeJ mice were infected with 10^8 CFU of the wild type or *rpoS* mutants and euthanized when they had lost 20% of their body weight. Mice infected with *rpoS* mutants survived significantly longer than mice infected with the wild type ($P = 0.003$, Wilcoxon test). Data were obtained in two independent experiments. WT, wild type (14 mice); *rpoS*, *rpoS* mutant (11 mice).

LEE genes code for functions essential for virulence, decreased expression of the LEE genes in *rpoS* mutants may, at least in part, contribute to the longer survival of mice infected with *rpoS* mutants than of mice infected with wild-type *C. rodentium*. However, other non-LEE RpoS-regulated genes that have not been identified or tested yet may also contribute to virulence. Further studies are required to examine gene expression at the genomic scale to fully understand the role of RpoS during infection.

Flagella are essential for *Salmonella* pathogenesis in vivo (49), while *E. coli* mutants deficient in flagellum synthesis colonize the mouse intestine much better than the wild type (32). The level of expression of flagellum genes is higher in *E. coli* *rpoS* mutants (13, 39). We considered the possibility that flagella may be differentially expressed in the *C. rodentium* wild type and *rpoS* mutants as well, and this might have also contributed to the observed difference in colonization. However, this does not appear to be the case. Both the wild type and *rpoS* mutants of *C. rodentium* were nonmotile on soft agar plates (data not shown). The loss of motility in *C. rodentium* may contribute to the fact that it is better able to colonize the mouse colon than *E. coli*.

It seems paradoxical that, although RpoS was important for *C. rodentium* colonization, *rpoS* mutants had a substantial growth advantage over the wild type and predominated in cocolonization experiments (this study). Our results, however, are consistent with the results of a previous study in which the effect of RpoS on colonization was examined by using a non-pathogenic model with the *E. coli* BJ4 strain in mice (27). The finding that *rpoS* mutants outcompete the wild type in vivo is similar to the previously reported in vitro GASP growth phenotype of *E. coli* (60, 61). During in vitro growth in stationary phase, although RpoS is critical for long-term survival (31), *rpoS* mutants are dominant in mixed cultures with wild-type cells (61). The growth advantage of *rpoS* mutants may be explained by the sigma factor competition model (16, 17, 38). Sigma factors compete for a limited number of RNA core polymerase molecules, and mutations in *rpoS* may thus increase the number of molecules of RpoD-associated polymerase, the housekeeping sigma factor, thus increasing the transcription of many housekeeping genes that are important for nutrient scavenging in a nutrient-limiting environment. How-

ever, growth in the mouse colon is quite different from growth in vitro. We found that the size of the population of bacteria in the colon did not decrease drastically after infection (as it did in in vitro experiments) but rather remained constant, suggesting that nutrients in the colon are not limiting but are sufficient to support a stable population. In contrast, the viability of both the wild type and *rpoS* mutants rapidly decreased to 0.1% (for the wild type) or 0.001% (for *rpoS* mutants) during in vitro growth. How can *rpoS* mutants outcompete wild-type cells? There are at least two possible explanations. First, since RpoS is known to negatively regulate the expression of a large set of genes, including the genes involved in the tricarboxylic acid cycle (39), *rpoS* mutants may produce a factor, such as a secondary metabolite, that inhibits growth of the wild type. Alternatively, it is possible that *rpoS* mutants can utilize key limiting nutrients better than wild-type cells (25), perhaps through increased nutrient transport (25, 33).

In conclusion, this study showed that RpoS is important for full virulence in *C. rodentium* in its natural host. Because of the high levels of similarity of the sequence and function of RpoS in *C. rodentium* and *E. coli*, the use of a *C. rodentium*-mouse model is well suited to examination of the role of RpoS in enteropathogenesis in future studies. The effect of RpoS on LEE gene expression was found to be different in *C. rodentium* and *E. coli* O157:H7, suggesting that *C. rodentium*-specific factors are involved. Since whole-genome profiling has been successfully used to investigate carbon source utilization during *E. coli* colonization (4), it would be interesting to use microarrays to examine the expression of RpoS-regulated genes during *C. rodentium* infection in order to determine what kind of stresses bacteria may encounter during growth and pathogenesis or when they encounter the stresses, as well as to fully understand how RpoS functions in vivo.

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REFERENCES

- Atlung, T., H. V. Nielsen, and F. G. Hansen. 2002. Characterisation of the allelic variation in the *rpoS* gene in thirteen K12 and six other non-pathogenic *Escherichia coli* strains. *Mol. Genet. Genomics* **266**:873–881.
- Bokranz, W., X. Wang, H. Tschape, and U. Romling. 2005. Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. *J. Med. Microbiol.* **54**:1171–1182.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Chang, D. E., D. J. Smalley, D. L. Tucker, M. P. Leatham, W. E. Norris, S. J. Stevenson, A. B. Anderson, J. E. Grissom, D. C. Laux, P. S. Cohen, and T. Conway. 2004. Carbon nutrition of *Escherichia coli* in the mouse intestine. *Proc. Natl. Acad. Sci. USA* **101**:7427–7432.
- Chen, G., C. L. Patten, and H. E. Schellhorn. 2004. Positive selection for loss of RpoS function in *Escherichia coli*. *Mutat. Res.* **554**:193–203.
- Clare, D. A., M. N. Duong, D. Darr, F. Archibald, and I. Fridovich. 1984. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. *Anal. Biochem.* **140**:532–537.
- Coldewey, S. M., M. Hartmann, D. S. Schmidt, U. Engelking, S. N. Ukena, and F. Gunzer. 2007. Impact of the *rpoS* genotype for acid resistance patterns of pathogenic and probiotic *Escherichia coli*. *BMC Microbiol.* **7**:21.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
- Deng, W., J. L. Puente, S. Gruenheid, Y. Li, B. A. Vallance, A. Vazquez, J. Barba, J. A. Ibarra, P. O'Donnell, P. Metalnikov, K. Ashman, S. Lee, D. Goode, T. Pawson, and B. B. Finlay. 2004. Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proc. Natl. Acad. Sci. USA* **101**:3597–3602.
- Deng, W., B. A. Vallance, Y. Li, J. L. Puente, and B. B. Finlay. 2003. *Citrobacter rodentium* translocated intimin receptor (Tir) is an essential virulence factor needed for actin condensation, intestinal colonization and colonic hyperplasia in mice. *Mol. Microbiol.* **48**:95–115.
- Dong, T., C. Joyce, and H. E. Schellhorn. 2008. The role of RpoS in bacterial adaptation, p. 313–337. In W. M. El-Sharoud (ed.), *Bacterial physiology—a molecular approach*. Springer, Berlin, Germany.
- Dong, T., M. G. Kirchhof, and H. E. Schellhorn. 2008. RpoS regulation of gene expression during exponential growth of *Escherichia coli* K12. *Mol. Genet. Genomics* **279**:267–277.
- Dong, T., and H. E. Schellhorn. 9 October 2008. Control of RpoS in global gene expression of *Escherichia coli* in minimal media. *Mol. Genet. Genomics*. doi: 10.1007/s00438-008-0389-3.
- Elliot, S. J., V. Sperandio, J. A. Giron, S. Shin, J. L. Mellies, L. Wainwright, S. W. Hutcheson, T. K. McDaniel, and J. B. Kaper. 2000. The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **68**:6115–6126.
- Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney. 1992. The alternative sigma factor *katF* (*rpoS*) regulates *Salmonella* virulence. *Proc. Natl. Acad. Sci. USA* **89**:11978–11982.
- Farewell, A., K. Kvint, and T. Nystrom. 1998. Negative regulation by RpoS: a case of sigma factor competition. *Mol. Microbiol.* **29**:1039–1051.
- Ferenci, T. 2003. What is driving the acquisition of *mutS* and *rpoS* polymorphisms in *Escherichia coli*? *Trends Microbiol.* **11**:457–461.
- Hayashi, K., N. Morooka, Y. Yamamoto, K. Fujita, K. Isono, S. Choi, E. Ohtsubo, T. Baba, B. L. Wanner, H. Mori, and T. Horiuchi. 2006. Highly accurate genome sequences of *Escherichia coli* K-12 strains MG1655 and W3110. *Mol. Syst. Biol.* **2**:2006.
- Hengge-Aronis, R. 2000. The general stress response in *Escherichia coli*, p. 161–178. In G. Storz and R. Hengge-Aronis (ed.), *Bacterial stress response*. ASM Press, Washington, DC.
- Hengge-Aronis, R. 2002. Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev.* **66**:373–395.
- Herbelin, C. J., S. C. Chirillo, K. A. Melnick, and T. S. Whittam. 2000. Gene conservation and loss in the *mutS-rpoS* genomic region of pathogenic *Escherichia coli*. *J. Bacteriol.* **182**:5381–5390.
- Iriarte, M., I. Stainier, and G. R. Cornelis. 1995. The *rpoS* gene from *Yersinia enterocolitica* and its influence on expression of virulence factors. *Infect. Immun.* **63**:1840–1847.
- Iyoda, S., and H. Watanabe. 2005. ClpXP protease controls expression of the type III protein secretion system through regulation of RpoS and GrpL levels in enterohemorrhagic *Escherichia coli*. *J. Bacteriol.* **187**:4086–4094.
- Kenny, B., A. Abe, M. Stein, and B. B. Finlay. 1997. Enteropathogenic *Escherichia coli* protein secretion is induced in response to conditions similar to those in the gastrointestinal tract. *Infect. Immun.* **65**:2606–2612.
- King, T., A. Ishihama, A. Kori, and T. Ferenci. 2004. A regulatory trade-off as a source of strain variation in the species *Escherichia coli*. *J. Bacteriol.* **186**:5614–5620.
- Klose, K. E., and J. J. Mekalanos. 1998. Distinct roles of an alternative sigma factor during both free-swimming and colonizing phases of the *Vibrio cholerae* pathogenic cycle. *Mol. Microbiol.* **28**:501–520.
- Krogfelt, K. A., M. Hjulgaard, K. Sorensen, P. S. Cohen, and M. Givskov. 2000. *rpoS* gene function is a disadvantage for *Escherichia coli* BJ4 during competitive colonization of the mouse large intestine. *Infect. Immun.* **68**:2518–2524.
- Laaberki, M. H., N. Janabi, E. Oswald, and F. Repoila. 2006. Concert of regulators to switch on LEE expression in enterohemorrhagic *Escherichia coli* O157:H7: interplay between Ler, GrpA, HNS and RpoS. *Int. J. Med. Microbiol.* **296**:197–210.
- Lacour, S., and P. Landini. 2004. σ^S -dependent gene expression at the onset of stationary phase in *Escherichia coli*: function of σ^S -dependent genes and identification of their promoter sequences. *J. Bacteriol.* **186**:7186–7195.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Lange, R., and R. Hengge-Aronis. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol. Microbiol.* **5**:49–59.
- Leatham, M. P., S. J. Stevenson, E. J. Gauger, K. A. Krogfelt, J. J. Lins, T. L. Haddock, S. M. Autieri, T. Conway, and P. S. Cohen. 2005. Mouse intestine selects nonmotile *flhDC* mutants of *Escherichia coli* MG1655 with increased colonizing ability and better utilization of carbon sources. *Infect. Immun.* **73**:8039–8049.
- Maharjan, R. P., S. Seeto, and T. Ferenci. 2007. Divergence and redundancy of transport and metabolic rate-yield strategies in a single *Escherichia coli* population. *J. Bacteriol.* **189**:2350–2358.
- Mellies, J. L., A. M. Barron, and A. M. Carmona. 2007. Enteropathogenic and enterohemorrhagic *Escherichia coli* virulence gene regulation. *Infect. Immun.* **75**:4199–4210.

35. Merrell, D. S., A. D. Tischler, S. H. Lee, and A. Camilli. 2000. *Vibrio cholerae* requires *rpoS* for efficient intestinal colonization. *Infect. Immun.* **68**:6691–6696.
36. Mundy, R., F. Girard, A. J. FitzGerald, and G. Frankel. 2006. Comparison of colonization dynamics and pathology of mice infected with enteropathogenic *Escherichia coli*, enterohaemorrhagic *E. coli* and *Citrobacter rodentium*. *FEMS Microbiol. Lett.* **265**:126–132.
37. Mundy, R., T. T. MacDonald, G. Dougan, G. Frankel, and S. Wiles. 2005. *Citrobacter rodentium* of mice and man. *Cell. Microbiol.* **7**:1697–1706.
38. Nystrom, T. 2004. Growth versus maintenance: a trade-off dictated by RNA polymerase availability and sigma factor competition? *Mol. Microbiol.* **54**: 855–862.
39. Patten, C. L., M. G. Kirchhof, M. R. Schertzberg, R. A. Morton, and H. E. Schellhorn. 2004. Microarray analysis of RpoS-mediated gene expression in *Escherichia coli* K-12. *Mol. Genet. Genomics* **272**:580–591.
40. Rangel, J. M., P. H. Sparling, C. Crowe, P. M. Griffin, and D. L. Swerdlow. 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. *Emerg. Infect. Dis.* **11**:603–609.
41. Robbe-Saule, V., G. Algorta, I. Rouilhac, and F. Norel. 2003. Characterization of the RpoS status of clinical isolates of *Salmonella enterica*. *Appl. Environ. Microbiol.* **69**:4352–4358.
42. Romling, U. 2005. Characterization of the *rdar* morphotype, a multicellular behaviour in Enterobacteriaceae. *Cell. Mol. Life Sci.* **62**:1234–1246.
43. Romling, U., Z. Bian, M. Hammar, W. D. Sierralta, and S. Normark. 1998. Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation. *J. Bacteriol.* **180**:722–731.
44. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
45. Schellhorn, H. E., and H. M. Hassan. 1988. Transcriptional regulation of *katE* in *Escherichia coli* K-12. *J. Bacteriol.* **170**:4286–4292.
46. Schellhorn, H. E., and V. L. Stones. 1992. Regulation of *katF* (*rpoS*) and *katE* in *Escherichia coli* K-12 by weak acids. *J. Bacteriol.* **174**:4769–4776.
47. Shalel-Levanon, S., K. Y. San, and G. N. Bennett. 2005. Effect of ArcA and FNR on the expression of genes related to the oxygen regulation and the glycolysis pathway in *Escherichia coli* under microaerobic growth conditions. *Biotechnol. Bioeng.* **92**:147–159.
48. Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper. 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **96**:15196–15201.
49. Stecher, B., S. Hapfelmeier, C. Muller, M. Kremer, T. Stallmach, and W. D. Hardt. 2004. Flagella and chemotaxis are required for efficient induction of *Salmonella enterica* serovar Typhimurium colitis in streptomycin-pretreated mice. *Infect. Immun.* **72**:4138–4150.
50. Suh, S. J., L. Silo-Suh, D. E. Woods, D. J. Hassett, S. E. West, and D. E. Ohman. 1999. Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J. Bacteriol.* **181**:3890–3897.
51. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**:4876–4882.
52. Tomoyasu, T., A. Takaya, Y. Handa, K. Karata, and T. Yamamoto. 2005. ClpXP controls the expression of LEE genes in enterohaemorrhagic *Escherichia coli*. *FEMS Microbiol. Lett.* **253**:59–66.
53. Uhlich, G. A., P. H. Cooke, and E. B. Solomon. 2006. Analyses of the red-dry-rough phenotype of an *Escherichia coli* O157:H7 strain and its role in biofilm formation and resistance to antibacterial agents. *Appl. Environ. Microbiol.* **72**:2564–2572.
54. Uhlich, G. A., J. E. Keen, and R. O. Elder. 2002. Variations in the *csqD* promoter of *Escherichia coli* O157:H7 associated with increased virulence in mice and increased invasion of HEp-2 cells. *Infect. Immun.* **70**:395–399.
55. Wang, Y., and K. S. Kim. 2000. Effect of *rpoS* mutations on stress-resistance and invasion of brain microvascular endothelial cells in *Escherichia coli* K1. *FEMS Microbiol. Lett.* **182**:241–247.
56. Weber, H., T. Polen, J. Heuveling, V. F. Wendisch, and R. Hengge. 2005. Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S -dependent genes, promoters, and sigma factor selectivity. *J. Bacteriol.* **187**:1591–1603.
57. Wickham, M. E., C. Lupp, A. Vazquez, M. Mascarenhas, B. Coburn, B. K. Coombes, M. A. Karmali, J. L. Puente, W. Deng, and B. B. Finlay. 2007. *Citrobacter rodentium* virulence in mice associates with bacterial load and the type III effector NleE. *Microbes Infect.* **9**:400–407.
58. Wiles, S., W. P. Hanage, G. Frankel, and B. Robertson. 2006. Modelling infectious disease—time to think outside the box? *Nat. Rev. Microbiol.* **4**:307–312.
59. Yildiz, F. H., and G. K. Schoolnik. 1998. Role of *rpoS* in stress survival and virulence of *Vibrio cholerae*. *J. Bacteriol.* **180**:773–784.
60. Zambrano, M. M., and R. Kolter. 1996. GASping for life in stationary phase. *Cell* **86**:181–184.
61. Zambrano, M. M., D. A. Siegle, M. Almiron, A. Tormo, and R. Kolter. 1993. Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. *Science* **259**:1757–1760.