JOURNAL OF BACTERIOLOGY, Aug. 2007, p. 5839–5849 Vol. 189, No. 16
0021-9193/07/$08.00+0 doi:10.1128/JB.00083-07

Growth Rate Toxicity Phenotypes and Homeostatic Supercoil Control Differentiate *Escherichia coli* from *Salmonella enterica* Serovar Typhimurium[▽]†

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Received 16 January 2007/Accepted 19 March 2007

***Escherichia coli* and *Salmonella enterica* serovar Typhimurium share high degrees of DNA and amino acid identity for 65% of the homologous genes shared by the two genomes. Yet, there are different phenotypes for null mutants in several genes that contribute to DNA condensation and nucleoid formation. The mutant R436-S form of the GyrB protein has a temperature-sensitive phenotype in *Salmonella*, showing disruption of supercoiling near the terminus and replicon failure at 42°C. But this mutation in *E. coli* is lethal at the permissive temperature. A unifying hypothesis for why the same mutation in highly conserved homologous genes of different species leads to different physiologies focuses on homeotic supercoil control. During rapid growth in mid-log phase, *E. coli* generates 15% more negative supercoils in pBR322 DNA than *Salmonella*. Differences in compaction and torsional strain on chromosomal DNA explain a complex set of single-gene phenotypes and provide insight into how supercoiling may modulate epigenetic effects on chromosome structure and function and on prophage behavior in vivo.**

Dichotomous growth is an adaptation that allows certain bacteria to divide rapidly under nutrient-rich conditions. During dichotomous growth, wild-type (WT) *Escherichia coli* can divide every 20 min, even though it takes >50 min to replicate the genome. Cells can divide rapidly only as long as initiation of bidirectional DNA synthesis with the DnaA "initiator" complex at the *oriC* "replicator" (42) is well coordinated with the formation of nucleoids and the distribution of replicated copies of the chromosome to each daughter cell at the time of partition. Recent studies with live cells show that a highly organized traffic pattern deposits each replisome arm at opposing edges of growing nucleoids (2, 90). At high growth rates, the average cell has a complex chromosome structure, with nucleoids containing two or four partially replicated arcs spanning the *oriC* initiator region. To distribute nucleoids, the cell division machinery needs to find the cell midpoint, decatenate all cross-links between chromosomal replicas at the *dif* site (19, 49), and move the nucleoids into each new daughter cell.

The initiation frequency at *oriC* is critical for cell division. Hyperinitiation is toxic or lethal (77), with one problem being fork overrun. If a fork from a recent initiation cycle overtakes a previously initiated fork, large chromosome fragments with double-strand ends can be generated (4). We previously showed that the *Salmonella gyrB652* mutation causes "growth rate toxicity" that includes topological chaos near the *dif* site (68). Gyrase hypomorphs in *Salmonella* lose supercoiling near the terminus of replication, even though plasmids in the same cell maintain near-normal superhelical densities (68). Recent experiments suggest that a similar fate may occur in gyrase mutants of *E. coli* (29, 45).

The GyrB protein is highly conserved in *E. coli* and *Salmonella*. Of its 804 amino acids, 97% are identical in the two species, with most substitutions being in the C-terminal region of the protein (18 of 28 substitutions are beyond H567). To see if the *Salmonella* GyrB652 mutation would cause terminal chaos in *E. coli*, we designed an oligonucleotide to introduce a single base pair substitution (Fig. 1). The R436-S substitution was lethal in *E. coli*. Moreover, ectopic expression of the *Salmonella* WT GyrB or GyrB652 protein from a plasmid was toxic for *E. coli* whereas a multicopy plasmid containing the *E. coli* GyrB protein was tolerated in both *E. coli* and *Salmonella*. Using reporter plasmids, we found that the average supercoil density (σ) for mid-log cultures of WT *Salmonella* (-0.060) is 13% lower than that for *E. coli* (-0.069). Plasmids having 56 bp of alternating GC sequence proved that *E. coli* also has a significantly higher level of DNA torsional strain. These observations explain several mysterious phenotypic differences between the species, such as the growth rate toxicity of *mukA*- and *seqA*-null mutants and the behavior of Mu lysogens.

MATERIALS AND METHODS

Media. All cells were grown in LB broth (36). Antibiotics were added to the media at concentrations of 50 μg/ml for kanamycin (Kan) and ampicillin (Amp), 20 μg/ml for chloramphenicol, 15 μg/ml for tetracycline, 10 μg/ml for gentamicin, and 5 μg/ml for nalidixic acid (Nal).

Plasmids and strains. All bacterial strains and plasmids in this work are described in Tables 1 and 2. Plasmid pRW478 is derived from pBR322 by the introduction of a $(CG)_{28}$ repeat into the EcoRI site (43).

Chromosomal modification. Chromosome modifications were made with the λ RED recombination system (95). Synthetic oligonucleotides were from IDT (Coralville, IA), and the sequences are given in Table 3. PCR products used for recombineering were made using a 1:1 mixture of *Taq* polymerase (Sigma) and *Taq* extender additive (Stratagene).

Plasmid maintenance assay. The abilities of cells to maintain plasmids were measured by comparing serial dilutions of the culture after plating them on selective and nonselective media. LB medium containing 50 μg/ml Amp (and

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 30 March 2007.

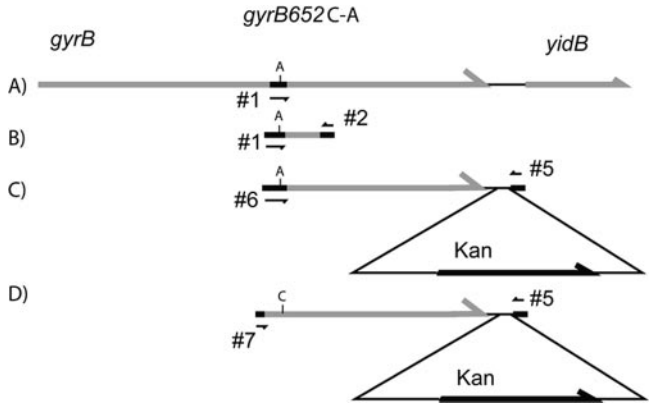

FIG. 1. Strategies for introducing the *gyrB652* C-to-A transversion mutation into the *E. coli* chromosome. Shown is a map of the *gyrB-yidB* region of *E. coli* and the *gyrB652* mutation (marked by "A"). (Strategy A) A synthetic, single-stranded, 70-mer oligonucleotide (oligonucleotide 1 [#1]) with the C-to-A transversion at position 36. (Strategy B) A PCR product made with oligonucleotides 1 and 2 to generate a 339-bp fragment with 35 bp of homology upstream and 303 bp downstream of the C/A substitution. (Strategy C) Oligonucleotides 5 and 6 make a 2,824-bp PCR fragment with the *gyrB652* mutation at bp 54, with 1,160 bp of further *gyrB* homology followed by a selectable Kan^r module and 133 bp of intergenic homology. (D) Control PCR. Oligonucleotides 5 and 7 make a 2,916-bp PCR product, with 1,306 bp of WT *gyrB* homology, with "C" marking the WT *gyrB* sequence, the Kan^r module, followed by 133 bp of downstream homology. Arrow tips with genes indicate transcription directions.

IPTG [isopropyl-β-D-thiogalactopyranoside] when indicated) was inoculated with fresh overnight culture by 1:100 dilution. For stationary-phase measurements, cultures were allowed to grow 8 h or more at 30°C. A portion of each culture was serially diluted 1:10 in 96-well microtiter plates. Finally, 3-μl aliquots of each dilution were spotted onto LB plates both with and without 50 μg/ml Amp and plates were incubated overnight in a 30°C incubator.

Supercoil density measurements. Plasmid supercoil density was determined by the band counting method using agarose gels containing chloroquine (75). DNA was prepared from log phase cells grown at 30°C. At indicated times, cells were collected and concentrated by centrifugation. Plasmid DNA was extracted with the Promega Wizard Plus midi prep DNA purification system and eluted into sterile water. DNA was further purified with phenol, phenol-chloroform, and chloroform extraction and concentrated with isobutanol. Ether was used to remove trace isobutanol. Purified plasmid DNA was separated in 25-cm 1.0% agarose gels containing chloroquine. For one-dimensional (1-D) gels, 1 to 2 μg of DNA was loaded into a gel containing 1.5 μM chloroquine and electrophoresis was carried out at room temperature at 2 V/cm for 46 h in 0.5× Tris-borate-EDTA (TBE), also containing 1.5 μM chloroquine. For 2-D gels, a topoisomer set from the first dimension was cut out, soaked in 0.5× TBE with 20 μM chloroquine for 4 hours, turned 90°, and recast in a new 25-cm 1.0% agarose gel containing 20 μM chloroquine. Electrophoresis in the second dimension was done at room temperature at 2 V/cm for 46 h in 0.5× TBE containing 20 μM chloroquine. After electrophoresis, the gels were soaked in deionized water for 30 min each for four changes and stained 60 min with ethidium bromide. DNA was visualized with UV light and captured on a Bio-Rad Zeta probe membrane using vacuum transfer. Southern blot analysis was done with probes made using Invitrogen's random-primed DNA labeling system with a pBR322 template. The membranes were exposed on a Molecular Dynamics phosphor screen and quantitated with Bio-Rad Gel Doc Software.

RESULTS

The GyrB652 protein is lethal in *E. coli*. The *Salmonella gyrB652* mutation (27) results from a single C_{1306}-to-A transversion mutation that substitutes serine for arginine at residue 436 (R436-S) (68). The 3-D model of the gyrase tetramer places this residue near a GyrB-GyrA interface (7, 47, 72). The GyrB652 form of gyrase has a lower in vitro catalytic efficiency (k_{cat}) than the WT enzyme. The temperature-sensitive (TS) growth defect is not caused by enzyme inactivation at 42°C (68) but is due to the inability of gyrase to keep up with high metabolic rates and multiple rounds of chromosomal DNA replication. The signature of this defect is topological chaos near the *dif* site, which is the terminus of replication (68). To learn whether this suboptimal form of gyrase would cause terminal chaos in *E. coli*, we tried to introduce at a permissive temperature (30°C) the GyrB R436-S substitution into strains of *E. coli* derived from W3110, NH3423, and NH3612.

Bacteriophage λ "recombineering" technology (95) exploits the regulated expression of λ recombination proteins Beta, Gam, and Exo to modify bacterial chromosomes efficiently using double-stranded or short single-stranded DNA substrates with only 30 bp of targeting homology (18). To introduce the *gyrB652* mutation, a synthetic 70-mer oligonucleotide (oligonucleotide 1) was made with 35 nucleotides of 5′-flanking homology followed by a single mismatch (the *gyrB652* C-to-A transversion mutation) and 34 nucleotides of 3′-flanking homology (Fig. 1) (see Table 3 for oligonucleotide 1 sequence). Because the primary *gyrB* sequences of this segment are identical in *E. coli* and *Salmonella*, we used λ recombineering to modify two *E. coli* strains (NH4323 and NH3612) and a WT *Salmonella* strain (NH3702). Expression of λ RED recombination proteins was induced for 15 min, oligonucleotide 1 was introduced into cells by electroporation, and 1-ml cultures were incubated overnight at 30°C in LB before plating them onto selective media.

The R436-S substitution was easy to introduce into the *Salmonella* strain NH3702 by using the λ recombineering technology. The phenotype includes TS growth at 42°C and resistance to 5 μg/ml of Nal (27). After 100-μl aliquots of the 1-ml NH3702 electroporation culture was spread onto Nal plates, an average of 125 Nal^r colonies was found on multiple plates. When 10 independent colonies from these plates were restreaked on LB agar at 30°C and 42°C, all 10 Nal^r strains failed to grow at 42°C. Sequence analysis of DNA PCR amplified from four of the *Salmonella* NH3702-derived Nal-resistant TS colonies confirmed that each had the *gyrB652* C-to-A transversion mutation. The efficiency of this chromosome modification reaction is similar to what others and we have observed for introducing single base pair changes into many different sites in both *E. coli* and *Salmonella* (13, 18).

However, introducing the R436-S substitution into *E. coli* was not easy. The same volume of the NH3423 or NH3612 strain electroporated with oligonucleotide 1 yielded an average of six Nal^r colonies. None of these colonies had a TS phenotype upon restreaking at 42°C. Low-level Nal resistance can be caused by mutations in several genes, including *gyrA*, *rpoB*, and *tolC*. The number of Nal^r colonies arising from the *E. coli* electroporation experiment was the same as that for the control experiment. When 100 μl of unmodified stationary-phase cultures of *E. coli* or *Salmonella* was spread on Nal plates, they gave an average of seven Nal^r colonies. Sequence analysis showed that none of the control strains of *E. coli* or *Salmonella* Nal^r colonies and none of the Nal^r *E. coli* NH3612 strains derived from plating electroporated cells carried the GyrB R436-S substitution.

The difficulty of establishing the *gyrB652* mutation in *E. coli*

TABLE 1. Strains used in this work

Strain	Name	Origin[a]	Genotype	Plasmid
NH0405	SL4213	*S.* Tm	*hsdL6 hsdSA29*(r_{LT}^- m_{LT}^+ r_s^- m_s^+) *metA22 metE551 ilv-452 trpB2 xyl-404 rpsL120 galE496* Fels2$^-$ *nml*	
NH0572	JTT1	*E. coli*	*pyrF gal25 nirA strA195*	
NH0742	LT2	*S.* Tm	*musA* Mu *cts62* pAp1	
NH0974	W3110	*E. coli*		
NH1013	N99	*E. coli*	*galE*	
NH1162	MC4100	*E. coli*	Mu *cts62* pAp1	
NH2002	LT2	*S.* Tm	WT	
NH2678	LT2	NH2002	*gyrB652 zib-6794*::Tn*10d*Tc	
NH3379	LT2	*S.* Tm	*seqA1*::(*swp*)::Tc	
NH3381	LT2	*S.* Tm	*mukB1*::(*swp*)::Tc	
NH3423	W3110	*E. coli*	λ*c*I857 (Δ*cro-bioA*) *thi nad*$^+$ Δ*lac169*	
NH3702	LT2	NH2002	WT	pSim5
NH3703	W3110	NH0974	*gyrB*::Kan	pSim5
NH3704	W3110	NH0974	*mutS*::Tn*10d*Tc	pSim5
NH3705	N99	NH1013	*galE*	pAG111
NH3706	W3110	NH0974	λ*c*I857 (Δ*cro-bioA*) *thi nad*$^+$ Δ*lac169*	pRC03
NH3707	W3110	NH0974	λ*c*I857 (Δ*cro-bioA*) *thi nad*$^+$ Δ*lac169*	pRC05
NH3708	N99	NH1013	*galE*	pTTQ18
NH3709	N99	NH1013	*galE*	pZT382
NH3710	SL4213	NH0405	See above	pAG111
NH3711	LT2	NH2002	WT	pRC03
NH3712	LT2	NH2002	WT	pRC05
NH3713	SL4213	NH0405	See above	pRW478
NH3715	SL4213	NH0405	See above	pBR322
NH3716	JTT1	NH0572	*pyrF gal25 nirA strA195*	pRW478
NH3718	N99	NH1013	*galE*	pBR322
NH3612	W3110	*E. coli*	WT	pSim5
CC4001	MM294	*E. coli*	F$^-$ *supE44 hsdR endoA1 pro thiA* Δ*seqA::tet*	
CC4208	MG1655	*E. coli*	Δ*mukB*::(*swp*) *kan* λ$^-$ F$^-$ *rph-1*	

[a] *S.* Tm, *S. enterica* serovar Typhimurium.

could be caused by intolerance of this variant protein. However, it could also be caused by a species-specific difference in *gyrB652* phenotype or by differences in λ recombineering efficiency between *E. coli* and *Salmonella*. To increase target homology, a second strategy (Fig. 1B) combined oligonucleotide 1 with a downstream 20-mer oligonucleotide (oligonucleotide 2) to generate a double-stranded, 339-bp PCR product. The *gyrB652* C-to-A transversion had 35 bp of upstream *gyrB* homology and 303 bp of downstream homology. Strains NH3702 and NH3612 were thermo-induced and electroporated with the PCR product of oligonucleotides 1 and 2. *E. coli* NH3612 produced fewer than 10 Nal^r colonies (the same as for the control plates), and none were TS. For the same experiment with *Salmonella*, 100-μl aliquots of the overnight electroporation culture yielded an average of more than 100 Nal^r TS colonies on each.

TABLE 2. Plasmids used in this work

Plasmid	Backbone	Insert	Source or reference
pSim5	pSC101	(ts *ori*) *imm*λ *c*I857-RED$^+$ *rex*::CAT	13
pTTQ18	pUC18		81
pZT382	pTTQ18	P_{tac} Tn*10 tnp* (G163-D)	Roth laboratory
pAG111	pTTQ18	*E. coli gyrB*	31
pRC03	pGem-T	*S. enterica* serovar Typhimurium *gyrB652*	68
pRC05	pGem-T	*S. enterica* serovar Typhimurium *gyrB*	68
pBR322			84
pRW478	pBR322	$(dC\text{-}dG)_{28}$	43

To eliminate the possibility that the GyrB652 substitution in *E. coli* does not exhibit low-level Nal resistance, we changed our genetic selection scheme. A new strain (NH3703) was made using λ recombineering to insert a Kan^r module in the intergenic space between *gyrB* and *yidB* (Fig. 1C). Using linkage to the Kan^r module, we could introduce mutant alleles by selection on Kan plates followed by screening of Kan^r recombinants for a linked TS phenotype and/or the presence of the C-to-A transversion in the *gyrB* gene. DNA from NH3703 was isolated and used as a template with two new PCR primers (oligonucleotides 5 and 6) to make a double-strand DNA product for electroporation. Oligonucleotide 6 had the *gyrB652* C-to-A transversion at position 54 of 74 nucleotides. The 2,808-bp PCR product includes 53 bp of upstream homology before the C-to-A transversion and 133 bp of homology beyond the Kan^r inset toward *yidB* (Fig. 1C and Table 3). *E. coli* NH3702 was electroporated with this PCR product, cells were incubated overnight, and 100-μl aliquots were spread onto Kan plates. An average of 20 Kan^r recombinants per plate spread with 100 μl of this electroporation culture was recovered. Each Kan^r colony was tested for Nal^r and TS phenotypes. None were found. The *gyrB* regions of 20 Kan^r strains were PCR amplified and sequenced. No C-to-A mutation was found among the Kan^r recombinants.

The failure to isolate an R436-S mutation linked to the Kan^r gene in the last experiment was surprising and unique in our recombineering experience. The result could mean that the

TABLE 3. Oligonucleotides

Oligonucleotide no.	Sequence[a]	Length (bp)
1	AAGGGGACTCCGCGGGCGGCTCTGCGAAGCAGGGGAGTAACCGCAAGAACCAGGCGA TTCTGCCGCTGAA	70
2	ACTTTGTACAGCGGCGGCTGAG	20
3	GCGAGCGTGCCTGATGCGCTACGCTTATCAGGCCTACGATGTAGGCTGGAGCTGCTTCG	59
4	TAGCTTCTTGCCGGATGCGGCGTGAACGCCTTACATATGAATATCCTCCTTAG	53
5	TGTAAAGCGTTACGTGTTGA	20
6	CCGAACTGTACCTGGTGGAAGGGGACTCCGCGGGCGGCTCTGCGAAGCAGGGGAGTA ACCGCAAGAACCAGGCG	74
7	CGCGCGTGAAATGACCCGCCGT	20

[a] In oligonucleotides 1 and 6, the underlined A indicates the C-to-A transversion that creates the *gyrB652* mutation. In oligonucleotides 3 and 4, the underlined sequences indicate the homology for amplifying the Kan cassette.

C-to-A mutation was lethal and that all Kan^r recombinants from the last experiment resulted from a mismatch repair that corrected a heteroduplex recombination intermediate to the WT sequence during recombination. Alternatively, the PCR product might have been degraded from the upstream end in vivo to eliminate the *gyrB652* segment, which would yield Kan^r recombinants that did not include the C-to-A transversion. To test these possibilities, the experiment was carried out with a *mutS*::Tn*10* strain (NH3704) that cannot perform mismatch repair (53). To determine how many Kan^r recombinants would be expected from this experiment, a WT *gyrB* sequence was made with oligonucleotides 5 and 7 (Table 3 and Fig. 1D). Both the mutant and the WT PCR products have >1,100 bp of *gyrB* homology upstream of the Kan module and 139 bp beyond the Kan module. Aliquots (100 μl) of the culture electroporated with the WT GyrB PCR (oligonucleotides 5 and 7) averaged 150 Kan^r colonies per plate. Not a single Kan^r colony was found on any plate using 100-μl aliquots of the *gyrB652* PCR product (oligonucleotides 5 and 6). This experiment confirms that the Kan^r colonies generated with the *gyrB652*-containing PCR product in the Mut$^+$ background (NH3702) arose after a MutS-dependent correction of a *gyrB652* heteroduplex to the WT *gyrB* sequence. The striking bias between WT and *gyrB652* PCR products is strong evidence that the GyrB R436-S substitution is lethal for *E. coli* under our experimental conditions.

WT *Salmonella gyrB* is toxic in *E. coli*. To study the *gyrB652* allele in *E. coli*, we introduced a plasmid with a copy of the *Salmonella gyrB652* gene. pRC03 is a pGEM-derived plasmid containing a copy of the *Salmonella gyrB652* gene cloned adjacent to a bacteriophage T7 promoter. This plasmid was used to express and purify the GyrB652 protein from *E. coli* BL21::DE3, a strain that harbors a Lac-inducible T7 RNA polymerase (68). However, in strains lacking the T7 RNA polymerase, the T7 promoter is slightly "leaky." pRC03 was introduced into *E. coli* NH3706 and maintained by continuous selection on Amp. But the strain grew more erratically than strains with the pGEM vector alone or pGEM vectors with other cloned inserts (not shown).

To test for a *gyrB652*-associated growth penalty, overnight cultures of NH3706 (with pRC03) were diluted 1:100 into triplicate LB-plus-Amp cultures and grown into stationary phase at 30°C. Aliquots (3 μl) of serially diluted samples were spotted onto both LB and LB-Amp plates (Fig. 2). There was a 4-order-of-magnitude difference between the numbers of CFU detected on LB and LB-Amp plates; pRC03 was lost from 99.99% of the stationary cells in culture. Control experiments with pGEM vector or pGEM vectors with other cloned genes showed that >99% of all cells retained these plasmids under the same experimental protocol. During growth of strains carrying plasmids with the Amp^r gene, Studier noted that Amp becomes degraded by the secreted penicillinase in early log phase, and during the latter stages of growth there is no selection for maintaining Amp-resistant plasmid (83). Thus, *Salmonella* GyrB652 protein is toxic and selectively eliminated from fast-growing cells, even when *E. coli* contains a WT chromosomal copy of *gyrB*. This explains our failure to isolate an *E. coli* strain bearing the R436-S substitution in the chromosome.

Since the mutant form of *gyrB* is not well tolerated by *E. coli*, we carried out the same experiment with strain NH3707, an *E. coli* strain that contains a pGEM plasmid with a cloned copy of the WT *Salmonella gyrB* gene. Surprisingly, after inoculation of cultures carrying pRC05 into fresh LB-Amp medium and growth to stationary phase, the plasmid was eliminated from 99.9% of the cells. Thus, even WT *Salmonella* GyrB is delete-

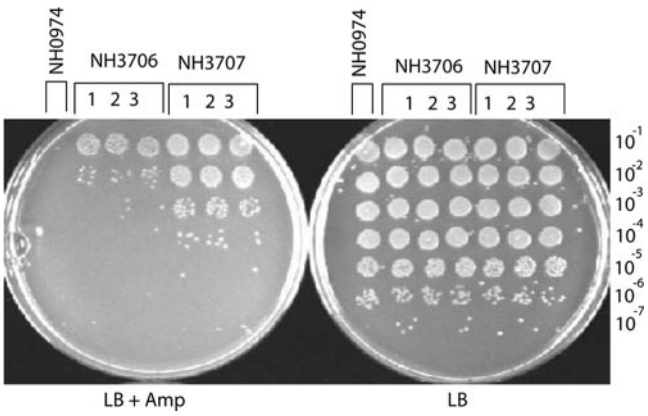

FIG. 2. Stabilities of plasmid pGem vectors carrying the *gyrB652* or WT *gyrB* gene of *S. enterica* serovar Typhimurium in *E. coli.* LB medium containing 50 μg/ml Amp was inoculated 1:100 in triplicate with overnight cultures of *E. coli* containing a pGem plasmid with the *Salmonella gyrB652* gene (NH3706) or a WT *Salmonella gyrB* gene (NH3707). Cultures were grown at 30°C to stationary phase in a shaking incubator. A portion of culture (labeled 1 to 3) was serially diluted into 96-well microtiter plates, and 3-μl aliquots were spotted onto LB plates with and without 50 μg/ml Amp. The dilution factor is shown on the right side. Pictures were taken after 24 h of incubation at 30°C.

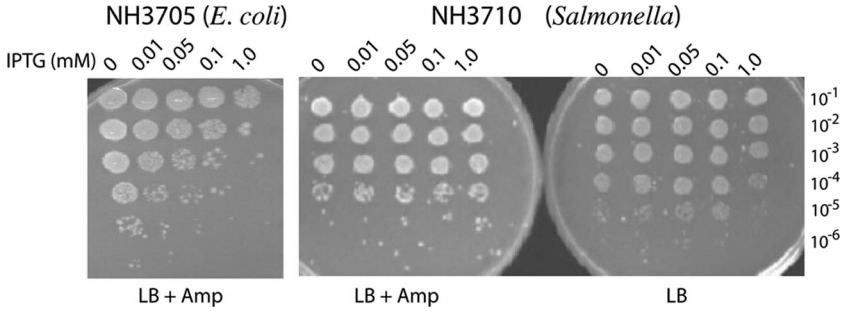

FIG. 3. Stability of plasmid pAG111, which expresses a WT *E. coli gyrB* gene, in *Salmonella* and *E. coli.* LB media containing 50 μg/ml Amp was inoculated with overnight cultures at 1:100 and grown at 30°C for 4 h in a shaking incubator with the indicated amounts of IPTG. A portion of each culture was serially diluted into 96-well microtiter plates, and 3 μl aliquots were spotted onto LB plates as indicated. The dilution factor is shown at the right. Pictures were taken after 24 h of incubation at 30°C.

rious in *E. coli*. To determine if the *E. coli gyrB* gene is toxic when cloned on a multicopy plasmid, pAG111 was introduced into both *E. coli* and *Salmonella*. In pAG111, the WT *E. coli gyrB* gene is cloned in the pTTQ-18 vector under Lac promoter control. Cells inoculated at a 1:100 dilution into fresh LB-Amp medium containing the indicated amounts of IPTG were grown to stationary phase at 30°C. Each culture was serially diluted, and 3-μl aliquots were spotted onto an LB-Amp plate (Fig. 3). In *E. coli*, there was no significant growth penalty when IPTG was absent. Over the range from 10 μM to 1 mM IPTG, a 1,000-fold plasmid destabilization was noted. However, IPTG induction of the *lacZ* gene in *E. coli* causes stochastic all-or-none high-level expression (66, 67, 76). GyrB protein expressed from the Lac promoter accumulates up to 40% of the total soluble cell protein in expressing cells (about 500-fold in excess of the normal level) (31). The *E. coli* GyrB protein was not toxic in *Salmonella*, which lacks the *lacY* permease, except when incubated with 1 mM IPTG. *Salmonella* may not give the all-or-none expression pattern observed in *E. coli* strains with the *lacY* gene, which actively concentrates the inducer in expressing cells. Nonetheless, *E. coli* is sensitive to large increases in its own GyrB protein and is extremely sensitive to the presence of low levels of the *Salmonella* GyrB protein (see below).

Species-specific supercoil set points. The phenotypic differences described above led us to ask whether a difference in mean supercoil density (σ) exists between these organisms. To compare values of σ, the linking difference of pBR322 was analyzed in strains with a WT complement of the four topoisomerases. *E. coli* JTT1 (NH0572) has been used in several important topological analyses (15, 52, 82). Experiments were carried out with *Salmonella* LT2 (NH0742) and SL4213 (NH0405), a strain with WT topoisomerases that carries a restriction mutation that makes it easy to move plasmids from *E. coli* directly to *Salmonella*. Actively growing cultures at mid-log phase (50 to 70 Klett units) were harvested, and plasmid DNA was purified. Topoisomers were separated in 2-D 1.0% agarose gels, and DNA was blotted to nylon membranes for quantitation. On 2-D gels, one can measure two quantities. First is the linking difference, which is obtained by assigning a number to each topoisomer relative to a relaxed DNA. The second parameter that can be scored is a supercoil-induced change in DNA structure, like the cooperative transition from right-handed to left-handed conformation in plasmids that have sequences that adopt the Z conformation (70). Remarkably, the σ of pBR322 in *Salmonella* was significantly lower than that in *E. coli* (Fig. 4). With DNA isolated from cells in mid-log phase, a three- to four-topoisomer difference existed between *E. coli* and *Salmonella*. By use of the band counting method in multiple experiments (75), the mean supercoil density (σ) values for plasmid DNA were found to be −0.069 for *E. coli* and 0.060 for *Salmonella*, a 13% difference. This difference means that *Salmonella* grows normally at a supercoil level that would be toxic for *E. coli* (16, 89). The *gyrB652* mutation of *Salmonella* lowers the linking number of pBR322 by approximately 1.5 topoisomers relative to the WT (68, 80), representing an additional 5% loss of supercoil density. This level is nonviable for *E. coli*. If the GyrB protein contributes to the control of mean supercoil density, this accounts for the dramatic effects of the GyrB652 protein in *E. coli*. Because gyrase is a heterotypic tetramer, GyrB652 expressed as only 10% of the total GyrB protein could alter nearly 20% of the gyrase

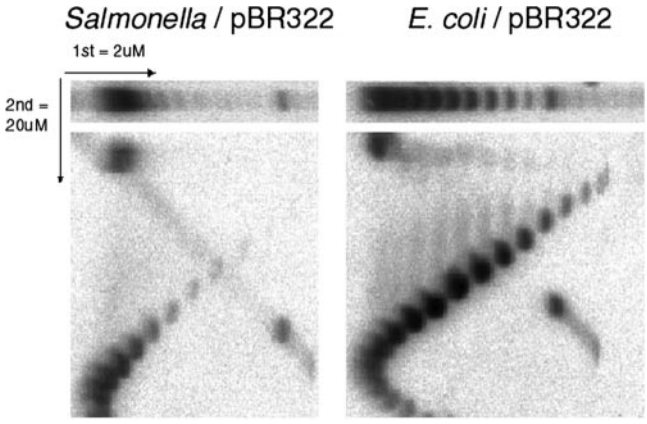

FIG. 4. *E. coli* and *Salmonella* supercoiling analysis of pBR322 on 2-D chloroquine gels. *Salmonella* (NH3715) and *E. coli* (NH3718) with WT topoisomerases were grown to mid-log phase (70 Klett units). Plasmid pBR322 DNA was purified, concentrated, and then separated in a 25-cm 1.0% agarose gel. Electrophoresis was for 46 h at 2.0 V/cm in 2 μM chloroquine for the first dimension and 46 h at 2.0 V/cm containing 20 μM chloroquine for the second dimension (70). The supercoil densities determined by the band counting method were −0.059 for *Salmonella* and −0.069 for *E. coli*.

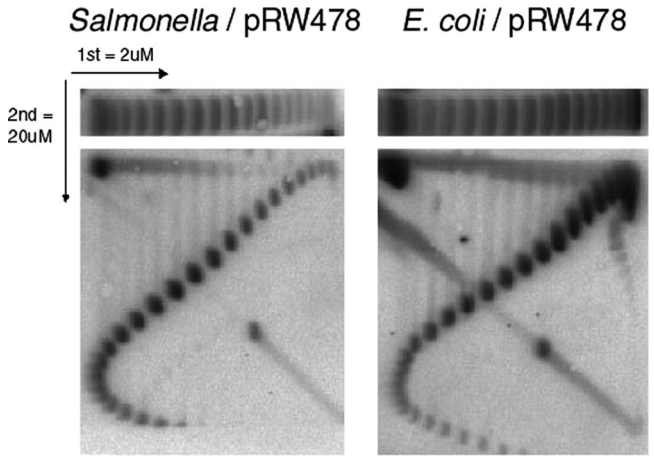

FIG. 5. *E. coli* and *Salmonella* supercoil comparison of a plasmid containing 56 bp of alternating GC repeats (pRW478) on a 2-D chloroquine gel. Strains of *Salmonella* (NH3713) and *E. coli* (NH3716) with WT topoisomerases and pRW478 were grown to mid-log phase (70 Klett units). Plasmid DNA was purified, and topoisomers were resolved in a 25-cm 1.0% agarose gel containing 2 μM chloroquine for 46 h at 2.0 V/cm in the first dimension and for 46 h in 20 μM chloroquine for the second dimension. More-negatively supercoiled topoisomers migrate to the upper-right region of this gel system. The pattern for *E. coli* shows a second distribution of highly supercoiled molecules with a break in the distribution (B/Z transition breaks in the downward sector of the arc in the right panel). The second very highly supercoiled distribution indicates the formation of Z-DNA in vivo (43).

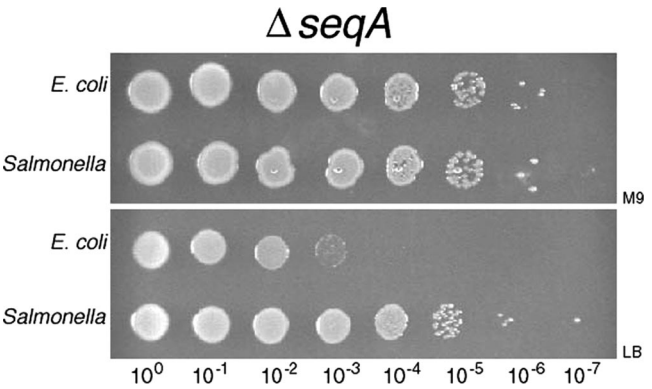

FIG. 6. Species-specific phenotypes for Δ*seqA* strains grown in rich medium. M9 medium containing 0.2% glucose, 1 μg/ml thiamine, and 40 μg/ml L-proline was inoculated with single colonies and grown overnight at 30°C in a shaking incubator. OD_{600} was measured, and cultures were diluted to an OD_{600} of 1.0. A portion of each culture was serially diluted (1:10) into 96-well microtiter plates, and 3-μl aliquots were spotted onto both LB plates and M9 plates with the above-mentioned additives. Pictures were taken after 24 h of incubation at 30°C.

molecules with a protomeric structure of $GyrA_2$-GyrB-GyrB652.

Bacteria partition supercoils into unconstrained and constrained conformations ($\Delta LK = S_U + S_C$) (see reference 43). Constrained supercoiling (S_C) is created by the unwound region of DNA bound to 3,000 molecules of transcribing RNA polymerase (26) and by the writhe associated with binding of abundant DNA binding proteins like HU, H-NS, and FIS (35, 46). Unconstrained supercoiling (S_U) causes torsion that stabilizes inter- and intramolecular triplex structures, left-handed Z-DNA, and cruciforms (35). In *E. coli*, S_U and S_C are distributed roughly 50:50 (5, 43, 71). To compare S_U in *E. coli* and *Salmonella*, the Z-DNA-forming plasmid pRW478 was introduced in both organisms. pRW478 has a 56-bp repeating (CG) insert at the EcoRI site of pBR322; when S_U is −0.025, 50% of the plasmid adopts a left-handed Z-DNA form in vitro (96). pRW478 was isolated from *E. coli* NH3716 and *Salmonella* NH3713 and analyzed on 2-D gels (Fig. 5). Relative to that of *Salmonella*, the *E. coli* pRW478 gel pattern showed a striking difference. Most of the plasmid was hypersupercoiled with bands migrating into the upper-right sector of the gel. This pattern indicates Z-DNA formation in vivo, because once the 56-bp GC segment flips to the left-handed conformation, gyrase can introduce about a dozen additional negative supercoils (43). One can also see the transition between topoisomers that had a left-handed conformation in the first dimension but were relaxed to the right-handed conformation by the higher chloroquine concentration in the second dimension (seen as a break in the continuous line in the upper-right part of Fig. 5, right). Most of the *E. coli* plasmid population (>70% of the total) is hypersupercoiled relative to the 2-D profile for pBR322 (Fig. 4 and 5). For *Salmonella*, some plasmids have adopted the left-handed conformation, but the fraction is substantially less than that for *E. coli* (<40% of the total). Therefore, the σ value and the mean torsional strain are both higher in *E. coli* than in *Salmonella*.

Species-specific phenotypes for SeqA and MukB. The differences in σ and S_U in *E. coli* and *Salmonella* predict different regulatory response thresholds in each organism. For example, a supercoil-sensitive promoter tuned to the *E. coli* S_U level would be inappropriately expressed in *Salmonella* unless a retuning mechanism alters the system. *E. coli* responded in a very negative way to the *gyrB652* mutation, but how do other chromosome dynamics proteins behave? Null mutation in two genes, *seqA* and *mukB*, showed substantially different phenotypes when these two organisms were compared.

The *seqA* gene encodes an A-methylation-sensitive DNA binding protein that recognizes the sequence GATC (51, 78, 88). SeqA binds preferentially to hemi-methylated GATC sites and sequesters the *oriC* region immediately after replication to block DnaA-dependent reinitiation of DNA synthesis for several minutes (6). *E. coli seqA* deletions were sensitive to growth in rich medium and failed to plate efficiently on rich LB plates at 30°C. A *Salmonella seqA* deletion plated efficiently on LB at 30°C (Fig. 6) and at temperatures up to 40°C (not shown). Cell cytometry also showed that *Salmonella seqA* mutants grown in LB at room temperature did not have the filamentation phenotype that is characteristic of *E. coli seqA* mutants (data not shown).

The second case is that of the bacterial "condensin" (37), a protein composed of MukB, MukE, and MukF (63, 64). *E. coli* deletions of *mukB*, *mukE*, or *mukF* exhibit the same phenotype, which is associated with growth rate toxicity, frequent loss of F plasmids, and segregation of DNA-less cells (62, 91). *E. coli mukB* deletions were nearly 100% viable in minimal medium, and they plated efficiently on LB medium as long as the temperature was 30°C or lower (74) (Fig. 7.) *Salmonella mukB* deletion mutants plated efficiently on minimal medium at 30°C

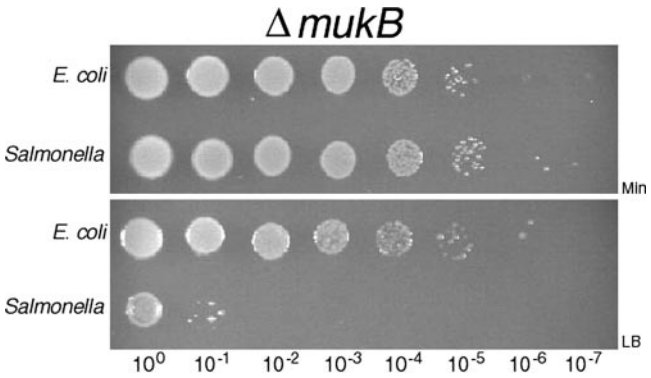

FIG. 7. Species-specific phenotypes for Δ*mukB* strains grown in rich medium. Minimal medium containing 0.2% glucose was inoculated with single colonies and grown 48 h at room temperature in a shaking incubator. OD_{600} was measured, and cultures were diluted to an OD_{600} of 1.0. A portion of each culture was serially diluted (1:10) into 96-well microtiter plates, and 3-μl aliquots were spotted onto both LB and minimal medium-glucose plates. Pictures were taken after 48 h of incubation at 30°C.

but did not form colonies on LB plates (Fig. 7). Growth in the 10^0 dilution spot (Fig. 7, bottom panel) is due to a low growth rate caused by high cell density and competition for nutrients, because spreading these cells across a whole plate leads to only a few colonies that appear to have suppressor mutations (data not shown). We interpret these results to indicate that WT *Salmonella* is more dependent on MukBEF for condensing and segregating chromosomes than *E. coli* (74).

DISCUSSION

All organisms store DNA in a supercoiled conformation to condense the material into a functionally useful form. Eukaryotic organisms organize supercoils on the surface of a highly conserved histone octamer and separate the chromosome from most of the protein synthetic activity by the nuclear membrane. Bacteria have no nuclear membrane to separate chromosomal DNA from protein translation. Rather, they exploit the ATP-dependent enzyme DNA gyrase to supercoil DNA, which compacts the molecule by using torsional stress (10, 59, 69, 89). A bacterial cell must compact the DNA over 1,000-fold while it is being simultaneously transcribed and replicated (38, 85). Compaction in both prokaryotes and eukaryotes is assisted by structural maintenance of chromosome proteins, which are ATP-binding, pincer-like proteins that are conserved from bacteria to humans (9).

The central importance of supercoiling in nucleoid formation requires that bacteria maintain DNA torsion levels within a narrow range (±15% of the normal value) (16, 89). Supercoil density is modulated by the activities of three topoisomerases, gyrase (56), Topo I (56), and Topo IV (97). These three enzymes are under strong selection to produce sufficient torsional strain to condense DNA, but not too much strain, which can trigger formation of alternative DNA conformations, such as Z-DNA, R loops, cruciforms, or intramolecular triplexes. These alternative DNA conformations stop replication forks, stall transcription complexes, and cause introduction of single- and double-strand chromosome breaks by several mechanisms (34).

Species-specific *gyrB* mutations. The A-to-C transversion that causes the R436-S substitution in GyrB is nonviable in an otherwise WT strain of *E. coli*. The strongest evidence of a severe *gyrB652* defect in *E. coli* comes from the electroporation of two PCR products into a *mutS* recombination-competent strain of *E. coli* (NH3704) (Fig. 1). The single C-to-A substitution in a >2-kb PCR product (Fig. 1C) caused at least a 1,000-fold reduction in the transduction efficiency of a Kan^r gene tightly linked to the *gyrB* locus. Moreover, the *Salmonella gyrB652* allele had a deleterious impact on *E. coli* as a merodiploid. Modest expression of the WT *Salmonella* GyrB was toxic in WT *E. coli*, and the GyrB652 mutation was >10 times more toxic. A pGEM plasmid bearing *Salmonella gyrB* cloned behind a T7 promoter was selectively eliminated from 99.99% of viable cells that did not contain T7 RNA polymerase (Fig. 2). Cells that spontaneously lost these plasmids rapidly overgrew cells with the plasmid. Although we do not know precisely how leaky the pRC03 GyrB652 expression was, it did not add enough protein to change the Western blot band intensity of WT *E. coli* GyrB (data not shown). Two- to threefold overexpression of *E. coli* gyrase subunits does not hinder *E. coli's* growth rate (44, 79), so *Salmonella* GyrB protein is exceptionally toxic in *E. coli*.

Species-specific supercoil set points. WT strains of *E. coli* growing exponentially in LB medium maintained significantly higher supercoiled densities than *Salmonella* (σ values of −0.069 versus −0.059, respectively [Fig. 4]). The analysis with plasmid pRW478 showed that *E. coli* also generated more torsional strain than *Salmonella*. In vitro, at S_U levels of −0.025, half of the pRW478 molecules adopt a left-handed conformation. Most of the plasmid molecules isolated from *E. coli* were hypersupercoiled, indicating that the mean in vivo S_U was >-0.025. The fraction of hypersupercoiled plasmid in *Salmonella* was less than half, indicating that the mean S_U was <-0.025 (Fig. 5). These facts suggest a supercoil explanation for phenotypic differences of *mukB*- and *seqA*-null mutations in otherwise WT strains of *E. coli* and *Salmonella* (38, 74).

Our results suggest that GyrB is part of the mechanism controlling σ. There are 28 species-specific substitutions (3.4%) among 804 amino acid residues of GyrB and 78 changes (8.9%) in 878 amino acids for GyrA. A significant fraction of the 15% difference in σ measured under identical growth conditions is likely caused by one or more of these substitutions. Internal metabolic flux changes the cellular phosphorylation state and ATP/ADP ratio, which modulates gyrase supercoiling activity (41, 44, 79, 92). But changes of metabolic flux are modest relative to the 15% difference shown in Fig. 4 and 5. Previous work indicated that *E. coli* had higher supercoiling levels than three other gram-negative organisms (43), so *E. coli* may have unusually high supercoiling among gram-negative organisms.

Species-specific phenotypes of chromosome dynamics. Supercoiling is critical during dichotomous growth for efficient formation of new nucleoids. Significant differences exist between *E. coli* and *Salmonella* in the phenotypes associated with several topologically sensitive genes other than gyrase, including *topA*, *seqA* (Fig. 6), *mukB* (Fig. 7), and *hns* (21, 61). Although there may be alternative explanations in each specific

case, the difference in WT supercoiling level between *E. coli* and *Salmonella* provides a unifying explanation for each of these observations.

First, *topA* amber mutations are lethal in WT strains of *E. coli* but are relatively healthy in *Salmonella enterica* serovar Typhimurium or *Shigella flexneri* (3, 15, 52). High torsional strain caused by elimination of *topA* from *E. coli* leads to formation of transcription-driven intermolecular triplexes or R loops (17, 40). R loops impede transcription and stall replication forks (35). Because *Salmonella* maintains a 15% lower level of σ, its supercoil level is already lower than that of *E. coli* strains carrying *gyrB* compensatory mutations that make introduction of *topA* mutations permissible (15, 43). Thus, R-loop formation would not cause the same type of problem in *Salmonella* that it does in *E. coli*.

Second, *seqA*-null mutants in *E. coli* exhibit growth rate toxicity in rich LB medium while *Salmonella* does not (Fig. 6). In *E. coli*, SeqA helps quell the lethal consequence of overinitiation by sequestering the hemi-methylated *oriC* region for many minutes after initiation, thus dampening DnaA-dependent replisome reassembly (8). Rapid reinitiation of replication at *oriC* leads to filamentation and cell death due replication fork failure (28, 77). One critical step in *oriC* initiation is the unwinding of A/T-rich sequences adjacent to DnaA binding sites at the origin (1, 24, 25). The 15% lower supercoil density that *Salmonella* attains during dichotomous replication may decrease *oriC* initiation to acceptable levels in rich media without the contribution of SeqA sequestration.

Third, an exception that proves the rule is the *mukB* mutation, which has a much more striking phenotype in *Salmonella* than in *E. coli* (Fig. 7). An *E. coli mukB* mutant plates with equal efficiency on minimal and LB medium at 30°C. Our *Salmonella mukB* mutant is viable on minimal medium but forms colonies on LB medium 6 orders of magnitude less efficiently than *E. coli*. In *E. coli*, supercoiling influences the phenotypes associated with *mukB* mutants. Sawitzke and Austin demonstrated that growth in the presence of low levels of the GyrB inhibitor novobiocin increased the reliance of *E. coli* on MukB, whereas increasing the median supercoil density by introducing a *topA* mutation made it possible for *E. coli mukB* mutants to plate on LB up to 42°C (38, 74). *Salmonella* fits this pattern. The lower level of σ correlates with growth of *E. coli* on novobiocin and a consequential heavier reliance on MukB for condensation and segregation in rich medium (Fig. 7).

The fourth example is the *hns* gene, which encodes a highly conserved gene for the abundant DNA binding protein H-NS. Like MukB, deletion of H-NS results in a more severe phenotype in *Salmonella* than it does in *E. coli*, where the growth rates and general physiologies of WT and *hns*-null mutations are nearly indistinguishable (21, 58). H-NS participates in chromosome condensation, regulation of gene expression, and the targeting of transposons (46). In *E. coli*, H-NS influences expression of about >200 genes directly or indirectly (39), while experiments with *Salmonella* indicate that expression levels of over 400 genes are altered in an *hns*-null mutant (61). Most of the genes in both organisms are derepressed when H-NS is eliminated. In addition to regulating genes, H-NS is one of the proteins that may organize loops in the bacterial chromosome (14). H-NS-induced looping in vitro has been demonstrated by atomic force microscopy (12) as well as by single-molecule manipulation using optical tweezers (11). When expressed, H-NS can cause a dramatic condensation of the nucleoid, which leads to inhibition of global transcription and an artificial stationary phase (55). The lower supercoiling level of *Salmonella* may contribute to the increased dependence of this organism on H-NS for maintaining nucleoid structure. Many of the genes that are bound by H-NS in *Salmonella* appear to be A/T rich and transferred horizontally from distantly related organisms (61).

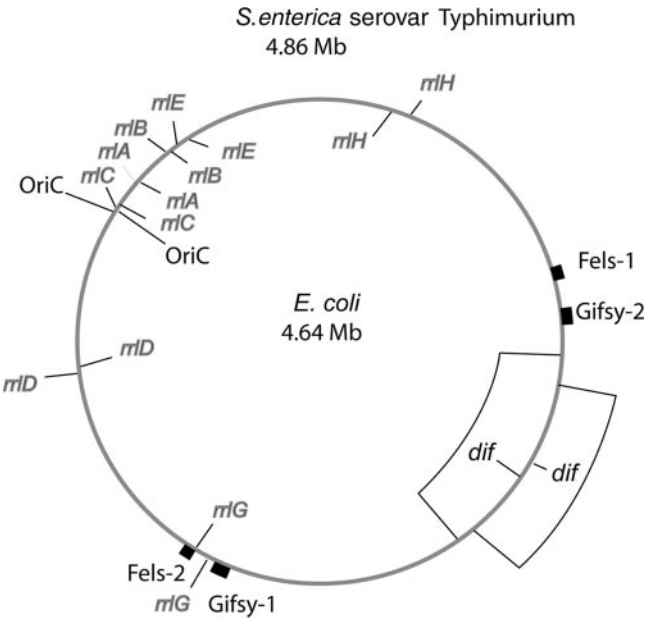

FIG. 8. Map of *E. coli* (inner circle) and *S. enterica* serovar Typhimurium (outer circle) showing the positions of the seven 23S rRNA operons (*rrlA* to *-H*) and four plaque-forming prophages in *Salmonella* (black arcs).

Why different supercoil densities in related enterics? Whereas the superhelical difference in *Salmonella* and *E. coli* provides a reasonable explanation for phenotypic differences in the *mukB*, *seqA*, *gyrB*, *topA*, and *hns* mutants, what selective force could lead to an altered state of mean superhelicity? We do not know the answer to this question. One strong selective pressure may come from prophages in the *Salmonella* genome (Fig. 8). *E. coli* K-12 harbors no prophage that can make an infectious virus in a lytic cycle, whereas *S. enterica* serovar Typhimurium contains four lysis-competent prophages; they are Gifsy-1, Gifsy-2, Fels-1, and Fels-2 (54). How long *Salmonella enterica* serovar Typhimurium has harbored four prophages is uncertain, but Gifsy-1 and Gifsy-2 contain genes that contribute to pathogenicity in infected mice (22). This could explain the selective pressure to preserve these elements.

Phage development changes in complex ways with the different growth conditions of the host. For lytic phages like T4, adsorption rate and burst size decrease at least an order of magnitude under poor growth conditions while eclipse time increases (30). For lysogenic phages Mu and λ, the lytic/lysogeny decision changes with physiology. Neither λ nor Mu produces significant single-cell bursts in stationary phase, and gyrase modulates the lytic/lysogeny outcome in both viruses. λ excision is reduced from some locations in the bacterial chro-

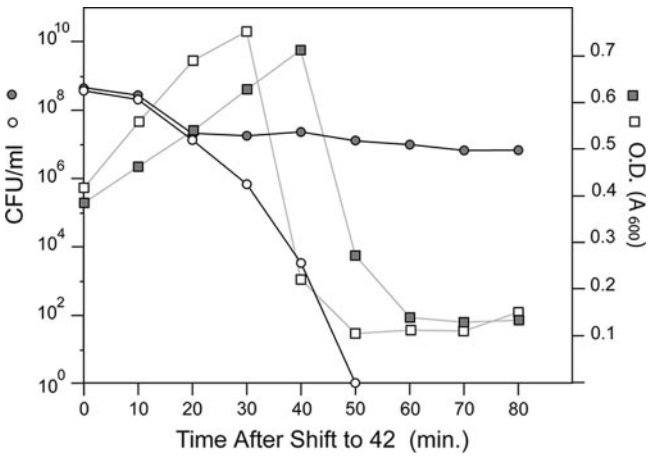

FIG. 9. Different Mu lytic profiles for *E. coli* and *Salmonella*. Mu lysogens of *E. coli* (NH1126) (open symbols) and *Salmonella* (NH742) (closed symbols) were diluted 1:100 in duplicate LB cultures and grown in a 30°C incubator to log phase (OD_{600} = 0.4). The cultures were shifted to a 42°C shaking water bath, and the OD_{600}s and numbers of CFU were measured at 10-min intervals. The *E. coli* cultures (open squares) began lysis at 30 min, and the *Salmonella* cultures (filled squares) began lysis at 40 min. The number of viable cells in *Salmonella* cultures (filled circles) dropped 50-fold, while for *E. coli* (open circles), viability dropped by 8 orders of magnitude.

mosome in *himB* (*gyrB*) mutants (57, 58). In *E. coli*, Mu burst size and plaque size are reduced in *gyrB* mutants (94) and the lysis/lysogeny fate is directly linked to negative supercoiling (33, 48).

To test the influence of host environment on the lytic/lysogenic behavior of Mu, plaque-forming MupAp1 monolysogens were isolated in *E. coli* (NH1126) and *Salmonella* (NH742), which also carries the *musA* allele that changes the surface polysaccharide structure to permit Mu adsorption (20). Mu *cts62* pAp1 carries the TS *cts62* repressor allele (87). At 30°C, the Mu *cts62* repressor binds to the operator and blocks p_E promoter activity, but a shift to 42°C induces conformation change in the C-terminal domain of the repressor, causing immediate release of DNA and initiation of the lytic transcription pathway (86). Duplicate overnight cultures were diluted 100-fold into fresh LB broth and incubated at 30°C to an optical density at 600 nm (OD_{600}) of 0.6, which corresponds to approximately 5×10^8 CFU/ml. Cultures were shifted to a 42°C shaking water bath, and the OD_{600} as well as the number of CFU that plated on LB agar at 30°C were sampled at 10-min intervals for the next 80 min. The OD_{600}s of *E. coli* cultures dropped 30 min after the temperature shift, while *Salmonella* cultures started lysis 10 min later (Fig. 9). Thus, the eclipse phase of phage development was longer in *Salmonella* than in *E. coli*. More dramatically, the numbers of cells surviving the shift to 42°C were different. In *E. coli*, the CFU number fell immediately after the shift and rapidly declined. By 50 min, the CFU number had dropped over 8 logs and no survivors were detectable. In *Salmonella*, the CFU number also dropped immediately after the temperature shift, but it leveled off at 20 min, and the viable cell count remained at about 10^7 CFU/ml for the next hour. Whereas both species are sensitive to the Mu lytic cycle, *Salmonella* survives thermo-induction 7 orders of magnitude better than *E. coli*.

The *E. coli-Salmonella* comparison. The supercoiling behavior of *Salmonella* differs from that of *E. coli* in at least two ways, either or both of which might contribute to the dominant-negative phenotype of expressing the *Salmonella* GyrB protein in *E. coli*. The first difference is that *E. coli* generates 13% more negative supercoils than *Salmonella* during rapid growth in rich medium (Fig. 4 and 5). Recent data from single-molecule experiments present new insight into the gyrase reaction mechanism. Nollmann et al. found that *E. coli* gyrase relaxes negative supercoils efficiently in the absence of ATP or other cofactors when DNA is held under strain from 0.5 to 1 pN (65). A model in which relaxation of negative supercoils occurs by a branched topoisomerase pathway in which supercoil relaxation is triggered by the energetic strain of negative supercoils has been proposed (32), i.e., the supercoil set point could be controlled by the level of DNA strain that causes gyrase to stop supercoiling and start relaxing negative supercoils. If this is correct, then a subset of the 28-amino-acid substitution that differentiates the *E. coli* and *Salmonella* GyrB proteins controls supercoil levels.

However, a second species difference is shown in Fig. S10 in the supplemental material. As *E. coli* enters stationary phase, the σ of pBR322 falls from −0.069 at the peak growth rate to below −0.055 as cells enter early stationary phase. This has been seen before in *E. coli* (43, 73). But in *Salmonella*, there is no loss of σ when cells enter or exit stationary phase. What controls the changes in supercoil levels in *E. coli* is not known, but the supercoiling recovery does not require new protein synthesis (73). One possibility is the GyrI gyrase inhibitory protein, which is under SOS regulatory control (60). Mechanisms notwithstanding, there is at least one system that modulates gyrase activity in stationary-phase *E. coli* that does not work in *Salmonella*.

The separation of *E. coli* and *Salmonella* from a common ancestor occurred about 200 million years before the present (50), which is about the time of separation of the lineages for mouse and human. Both organisms retain approximately 65% of their homologous genes, with 35% of the genome moving via horizontal transfer, but the genetic map is largely conserved. Maintaining four prophages that can all kill the cell may mean that *Salmonella's* fitness is improved by damping the lysis/lysogeny trigger. The four *Salmonella* prophages have expression and gene amplification effects that can be monitored using microarray technology under stress conditions (23). On the other hand, selection pressure in *E. coli* may have increased the supercoiling level to maximize its growth rate under dichotomous conditions (*E. coli* outgrows *Salmonella* in mixed culture). One added benefit might be that higher supercoiling levels lead to rapid curing once *E. coli* is lysogenized by a prophage. Recent work shows that Mu transposition hotspots in *Salmonella* are different from those in *E. coli* (D. Manna, S. Porwollik, M. McClelland, and N. P. Higgins, submitted for publication), and lower negative supercoiling could account for different locations of unusual DNA structure that act as transposition hotspots (93). If the supercoiling set point is established by the *gyrA* and *gyrB* genes, it would be very interesting to see how *Salmonella* fitness and dichotomous growth rates would change after swapping the normal *Salmonella* gyrase alleles with *E. coli gyrA* and *gyrB* sequences.

ACKNOWLEDGMENTS

We are grateful to Stuart Austin for carrying out flow cytometry on WT and *seqA* mutants of *Salmonella* and for providing *seqA* and *mukB* mutant strains of *E. coli*, to Don Court for providing the recombineering plasmid pSim5 prior to publication, and to members of the Higgins laboratory for critical comments on the manuscript.

This work was supported by grant GM33143 to N.P.H.

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