Consequences of producing DNA gyrase from a synthetic gyrBA operon in Salmonella enterica serovar Typhimurium

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Abstract

DNA gyrase is an essential type II topoisomerase that is composed of two subunits, GyrA and GyrB and has an A₂B₂ structure. Although both subunits are required in equal proportions to form DNA gyrase, the gyrA and gyrB genes that encode them in Salmonella (and in many other bacteria) are at widely separated locations on the chromosome, are under separate transcriptional control and are present in different copy numbers in rapidly growing bacteria (gyrA is near the terminus of chromosome replication while gyrB is near the origin). We generated a synthetic gyrBA operon at the oriC-proximal location of gyrB to test the significance of the gyrase gene position for Salmonella physiology. Producing gyrase from an operon did not alter growth kinetics, cell morphology, competitive fitness index, or sensitivity to some gyrase-inhibiting antibiotics. However, the operon strain had altered DNA supercoiling set points, its SPI-2 virulence genes were expressed at a reduced level and its survival was reduced in macrophage. The gyrB gene could not be deleted from its oriC-proximal location, even in a gyrB merodiploid strain. We discuss the physiological significance of the different gyrA and gyrB gene arrangements found naturally in Salmonella and other bacteria.
**Introduction**

DNA gyrase is an essential type II topoisomerase that introduces negative supercoils into DNA through an ATP-dependent mechanism (Gellert *et al.*, 1976a; Higgins *et al.*, 1978; Nöllmann *et al.*, 2007); it can also relax negatively supercoiled DNA via an ATP-independent mechanism (Gellert *et al.*, 1977; Higgins *et al.*, 1978; Williams and Maxwell, 1999). The enzyme is composed of two copies of two subunits, GyrA and GyrB, giving it an $A_2B_2$ structure (Bates and Maxwell, 2005). Topoisomerase activity is required to eliminate the over-wound (positively supercoiled) and under-wound (negatively supercoiled) zones of the DNA template that are generated by transcription and DNA replication (Liu and Wang, 1987; Stracy *et al.*, 2019). DNA gyrase relaxes the positively supercoiled DNA by introducing negative supercoils in an ATP-dependent manner (Ashley *et al.*, 2017). Transcription and the associated disturbance to local DNA topology contribute to the architecture of the bacterial nucleoid by influencing the probability of DNA-DNA contacts between parts of the genome that border long transcription units that are heavily transcribed (Le and Laub, 2016). The changes to local DNA supercoiling that are caused by transcription and DNA replication also affect the activities of some transcription promoters (Ahmed *et al.*, 2016; 2017; Chong *et al.*, 2014; Dorman, 2019; Higgins, 2014; Rahmouni and Wells 1992; Rani and Nagaraja, 2019; Wu *et al.*, 1988; Tobe *et al.*, 1995). A large subset of promoters is sensitive to alterations in DNA supercoiling and to ensure appropriate gene expression, topoisomerases play an important role in maintaining supercoiling set points within a range that is tolerable by the cell (Cheung *et al.*, 2003; Dorman and Dorman, 2016; Peter *et al.*, 2004; Sutormin *et al.*, 2019). The promoters of gyrA and gyrB, the genes that encode the A and B subunits, respectively, of gyrase, are stimulated by DNA relaxation (Menzel and Gellert, 1983; 1987; Straney *et al.*, 1994; Unnirahman and Nagaraja, 1999). This is part of a mechanism that maintains DNA supercoiling homeostasis, keeping average DNA supercoiling values within the tolerable range (DiNardo *et al.*, 1982; Dorman *et al.*, 1989; Pruss *et al.*, 1982; Raji *et al.*, 1985; Richardson *et al.*, 1988; Steck *et al.*, 1984). As a corollary to this, the transcription of topA, the gene encoding DNA topoisomerase I (Topo I), is
stimulated by negative supercoiling (Ahmed et al., 2016; Tse-Dinh and Beran, 1988). Topo I relaxes negatively supercoiled DNA using an ATP-independent type I mechanism (Dekker et al., 2002).

Several studies have shown that gene position on the chromosome is physiologically significant in bacteria (Bogue et al., 2020; Bryant et al., 2014; Gerganova et al., 2015; Scholz et al., 2019). In Salmonella enterica serovar Typhimurium, the gyrA and gyrB genes are widely separated on the genetic map of the circular chromosome: the gyrB gene is located close to the origin of chromosome replication, oriC, while gyrA is located near to the terminus region, Ter (McClelland et al., 2001). This arrangement closely resembles that seen in the model organism, Escherichia coli (Berlyn, 1998; Blattner et al., 1997). It has been proposed that the order of genes along each replichore in the bidirectionally replicated circular chromosome of E. coli correlates with the peak levels of expression of genes as a culture passes through each of the major stages of its growth cycle in batch culture (Sobetzko et al., 2012). DNA supercoiling plays an important role in the initiation of chromosome replication, so locating gyrB close to oriC is consistent with the gene location hypothesis. Bacteria emerging from lag phase and entering a period of rapid growth in exponential phase, experience a build up of negative DNA supercoiling and this stimulates the transcription of genes whose products support rapid growth (Colgan et al., 2018; Conter et al., 1997). Rapidly growing bacteria undergo multiple rounds of initiation of chromosome replication, so genes close to oriC are present in more copies per cell than those close to the terminus (Cooper and Helmstetter, 1968). GyrA and GyrB are required in equal amounts to form active DNA gyrase molecules, so the physical separation of gyrA from gyrB on the chromosome, and their organisation as independent transcription units, seem counterintuitive.

The genetically separated pattern of gyrA and gyrB gene location seen in S. Typhimurium and E. coli is not found universally among bacteria: many possess a gyrBA operon, although none appears to have a gyrAB operon. Examples of bacteria with a gyrBA setup include, inter alia, Listeria monocytogenes (Glaser et al., 2001), Mycobacterium tuberculosis (Cole et al., 1998) and Staphylococcus aureus (Baba et al., 2008). The operon
arrangement appears to offer a number of advantages over the individual transcription unit model. Co-expression allows *gyrA* and *gyrB* to share the same promoter and the same transcription regulatory signals. Production of GyrA and GyrB from a common, bicistronic mRNA is likely to facilitate the establishment of equal amounts of each protein. The co-production of GyrA and GyrB might also be expected to enhance the efficiency of gyrase enzyme assembly. It should be noted that the *gyrA* and *gyrB* genes are only seen to be widely separated from one another on the unfolded, circular genetic map of *S. Typhimurium*: the genes may be brought into closer proximity in the folded chromosome within the nucleoid. Furthermore, in the tiny universe of the bacterium's interior, the problem of gyrase assembly from GyrA and GyrB subunits produced from spatially separated mRNA molecules may be an insignificant one (Moffitt et al., 2016). We investigated this issue by building a derivative of *S. Typhimurium* with a *gyrBA* operon and comparing its physiology with that of the wild type.
Results

Constructing a derivative of S. Typhimurium with a synthetic gyrBA operon

A kanamycin resistance cassette, kan, was inserted adjacent to the gyrA gene in S. Typhimurium strain SL1344 to serve as a selectable marker (Experimental procedures). This gyrA-kan combination was amplified by PCR, leaving behind the transcription control signals of gyrA, and inserted immediately downstream of the gyrB gene, creating a gyrBA operon with an adjacent kan gene that was bordered by directly-repeated copies of the FRT sequence; the kan gene was then deleted by FLP-mediated site-specific recombination at the frt sites. A kan gene cassette, flanked by directly repeated frt sites, was used to replace the gyrA gene at the native gyrA location in the gyrBA-operon-containing strain; this kan cassette was then excised by FLP-mediated recombination. This process produced a derivative of SL1344 that had a gyrBA operon at the chromosomal position that is normally occupied by just gyrB and had no gyrA gene at the chromosomal site where this gene normally resides (Fig. 1). The whole genome sequence of this new strain was determined to ensure that no genetic changes, other than the desired ones, were present; none were detected.

An attempt was also made to build a derivative of SL1344 with a gyrAB operon at the native location of the gyrA gene using a similar strategy to that used in constructing the synthetic gyrBA operon. Although the desired gyrAB operon was produced, it proved to be impossible to delete the second copy of gyrB from its native location. This suggested that the presence of the gyrB gene at its native oriC-proximal location is essential, even when a second copy of gyrB is present at another chromosomal position.

The growth characteristics of the gyrBA operon strain

The growth kinetics of the wild type and the strain with the gyrBA operon were compared in batch liquid culture. Lysogeny broth (LB) cultures had identical growth curves when measured by plating and colony counting or by optical density measurements (Fig. S1A, S1B). Growth was also assessed in a minimal medium in an experiment that included low magnesium stress, an important environmental challenge that S. Typhimurium encounters in the
macrophage vacuole during infection (Colgan et al., 2018). The wild type and the gyrBA operon strains were grown in minimal medium N with either 10 µM (low magnesium) or 10 mM (high magnesium) MgCl₂. Once again, the two strains had identical growth kinetics (Fig. S1C).

**Morphology of the strain with the gyrBA operon**

The identical growth characteristics of the strain with the gyrBA operon and the wild type, both in LB and in minimal medium, showed that producing DNA gyrase from an operon made no difference to the growth cycle and suggested that the cell cycle was unlikely to be altered either. Interference with the timing of major events in the cell cycle (initiation, replication fork passage and termination) can lead to delays in cell division, resulting in filamentation of the bacterial cell (Martin et al., 2020; Sharma and Hill, 1995). When we compared the morphologies of mid-exponential-phase cultures of the wild type and the gyrBA operon strain by light microscopy, no differences in the shapes of the cells or the frequency of cell filamentation were detected (Fig. S2). Taken together with the growth kinetic data, these findings showed that the operonic arrangement of gyrA and gyrB was well tolerated by S. Typhimurium.

**Sensitivity to gyrase-inhibiting antibiotics**

The minimum inhibitory concentrations of gyrase-inhibiting antibiotics were compared for wild type SL1344 and SL1344 gyrBA (Fig. 2). Four drugs were tested: coumermycin and novobiocin are coumarins that target the GyrB subunit of DNA gyrase (Lewis et al., 1996) while nalidixic acid and ciprofloxacin are quinolones that target GyrA (Drlica and Zhao, 1997). The two strains were equally sensitive to the quinolones, but the SL1344 gyrBA strain was more resistant than SL1344 to novobiocin while SL1344 was more resistant than SL1344 gyrBA to coumermycin (Fig. 2). The reasons for the differential sensitivity patterns of the strains to the two classes of antibiotics, and for the opposing patterns of resistance to the two coumarins were not determined. However, the results indicated that producing the subunits of gyrase from a gyrBA operon resulted in coumarin MIC data that were not
equivalent to those measured for the strain producing the subunits from physically separate genes.

Motility and competitive fitness measurements

The gyrBA operon strain was compared with the wild type to assess relative motility on agar plates and competitive fitness in liquid co-culture. The operonic strain showed a small, but statistically significant, reduction in motility compared to the wild type (Fig. 3A). The reasons for this were not determined and may reflect changes at one or more levels in the production and operation of the complex motility machinery of the bacterium. In contrast, the two strains were equally competitive when growing in LB (Fig. 3B). To perform the competition, the two strains were each marked genetically by insertion on a chloramphenicol resistance (cat) cassette that is located in the pseudogene SL1483. This cat insertion has a neutral effect on fitness and serves simply to allow the competing bacteria to be distinguished by selection on chloramphenicol-containing agar (Lacharme-Lora et al., 2019). The competitions were performed between a cat-marked wild type and the unmarked gyrBA operon strain and separately between a cat-marked gyrBA operon strain and the unmarked wild type (Fig. 3B). No differences in the competitive indices of the two strains were detected in either version of the competition.

Transcription of the separate and the operonic gyr genes

The output of mRNA from the gyrA and gyrB genes was measured by quantitative PCR in wild type SL1344 and in SL1344 gyrBA, using the transcript of the hemX gene as a reference. (Expression of the hemX gene does not change under the growth conditions used here [Kröger et al., 2013]). Gyrase gene transcription in both strains was found to vary with growth cycle stage, with mRNA outputs being highest in early exponential phase (2-h time point) and lowest in stationary phase (Fig. 4). In the wild type, the gyrA gene (located near Ter, the terminus of chromosome replication) was expressed to a significantly higher level than gyrB (located close to oriC) at 2 h. This was interpreted as a reflection of the need to compensate for the effect of
increased gyrB gene dosage relative to that of gyrA in rapidly growing cells. As the culture approached stationary phase, the levels of gyrA and gyrB transcripts equalised, in line with the convergence of oriC-proximal and Ter-proximal gene dosages (Fig. 4). The formation of the gyrBA operon eliminated the difference in gyrB and gyrA mRNA levels because each became part of the same bicistronic transcript and has adopted the expression profile of gyrB (Fig. 4).

227 DNA supercoiling in the strain with a gyrBA operon

228 The distributions of the topoisomers of the pUC18 reporter plasmid isolated from the wild type and the gyrBA operon strain were compared by electrophoresis in a chloroquine-agarose gel (Fig. 5). The cultures were grown in LB medium (Fig. 5A; S3A) or in minimal medium N with high or low concentrations of MgCl2 (Fig. 5B; S3B). In LB, the reporter plasmid was more relaxed in the gyrBA strain than in the wild type (Fig. 5A, S3B). Low-magnesium growth was used to mimic one of the stresses experienced by Salmonella in the macrophage vacuole. In the high MgCl2 control, the wild type and the gyrBA operon strain differed in their reporter plasmid distributions: DNA from the gyrBA strain was more negatively supercoiled than that from the wild type and showed a linking number difference (ΔLk) of -3 (Fig. 5B, S3B). At the low MgCl2 concentration, the topoisomer distributions were more relaxed in both strains than in the high MgCl2 controls (ΔLk = +3). The reporter plasmid from the gyrBA operon strain was also more negatively supercoiled than that from the wild type, with the peak in its topoisomer distribution being approximately 2 linking numbers below that of the wild type (Fig. 5B, S3B). DNA relaxation occurs in Salmonella cells as they adjust to the macrophage vacuole and this forms part of the activation mechanism for the genes in the SPI-2 pathogenicity island (Cameron and Dorman, 2012; O Cróinín et al., 2006; Quinn et al., 2014). The products of these virulence genes protect the bacterium by inhibiting fusion of the vacuole with lysosomes (Garvis et al., 2001). We therefore monitored SPI-2 gene transcription in our two strains.
SPI-1 and SPI-2 gene expression in the gyrBA operon strain

The SPI-1 and SPI-2 pathogenicity islands encode distinct type 3 secretion systems and effector proteins that are used to invade epithelial cells (SPI-1) or to survive in the intracellular vacuole (SPI-2) (Figueira and Holden, 2012; Hensel, 2000; van der Heijden and Finlay, 2012). Transcription of SPI-1 genes was monitored using a gfp+ reporter fusion under the control of the prgH promoter, P_{prgH}, while a gfp+ fusion to the ssaG promoter (P_{ssaG}) was used to monitor SPI-2 gene transcription. Wild type and gyrBA operon strains harbouring these fusions were grown in LB medium (Fig. 6A, 6B) or in minimal medium N, supplemented with high or low concentrations of MgCl₂. (Fig. 6C-F). The cultures were grown with aeration at 37°C in 96-well plates and green fluorescence was measured throughout the growth cycle. The results obtained showed that in LB medium and in minimal medium N, SPI-1 transcription was indistinguishable between the wild type and the gyrBA operon strains (Fig. 6A, 6C, 6E). SPI-2 transcription was equivalent in both strains growing in LB (Fig. 6B) or in minimal medium with high MgCl₂ (Fig. 6D). However, SPI-2 transcription occurred at reduced levels in the gyrBA strain in the later stages of the growth cycle under low magnesium conditions (Fig. 6F). These findings showed that when the subunits of gyrase are produced from an operon, rather than from separate gyrA and gyrB genes in their native chromosome locations, the normal expression profile of the SPI-2 virulence gene cluster is disrupted, but that this is conditional on growth in a low magnesium medium.

Impact of the gyrase operon on cell infection by Salmonella

The abilities of the wild type and the gyrBA operon strains to invade and to replicate in cultured mammalian cells were compared. Bacteria, grown to mid-exponential phase to promote SPI-1 gene expression, were used to infect RAW264.7 macrophage. When intracellular bacteria were then enumerated post-invasion, fewer of the gyrBA operon strain cells were detected than wild type cells at and after the 16-h time point (Fig.7). This reduction in bacterial survival correlated with the diminished SPI-2 expression detected in the gyrBA operon strain in low Mg²⁺, a macrophage relevant condition.
Discussion

The genes in *Salmonella* that encode DNA gyrase, *gyrA* and *gyrB*, are located at the opposite ends of the left replichore of the chromosome (Fig. 1). In contrast, many other bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Mycobacterium tuberculosis*, possess a *gyrBA* operon (Baba et al., 2008; Cole et al., 1998; Glaser et al., 2001). As a first step in assessing the significance of the stand-alone gyrase gene arrangement versus the operon model, we constructed a derivative of *S. Typhimurium* with a *gyrBA* operon at the chromosomal location that is normally occupied by *gyrB* alone, while removing the individual *gyrA* gene from its native position in the genome. This strain, with the *gyrA* and *gyrB* genes transcribed from a common promoter (*P_{gyrB}* and located close to the origin of chromosomal replication, had normal growth characteristics and cell morphology. The deletion of the (now redundant) *gyrA* gene copy from its native position near the terminus of chromosome replication was tolerated well. In contrast, it proved to be impossible to delete the *gyrB* gene from its native location in a strain that had a *gyrAB* operon at the chromosomal position that is normally occupied by *gyrA* alone. These findings show that there are limits to the rearrangements of *gyrA* and *gyrB* genes that *Salmonella* will tolerate.

Although the production of DNA gyrase from an operon was well tolerated by *S. Typhimurium*, the operon strain differed from the wild type in a number of phenotypic characteristics. Subtle differences in sensitivities to coumarin antibiotics, but not quinolones, distinguished the operon strain from the wild type (Fig. 2). The reasons for the different responses to coumarins were not determined and may have involved indirect effects of operonic gyrase production on processes involved in drug uptake. A modest decrease in competitive fitness in the operon strain hinted at impacts on physiology. In light of the fact that *S. Typhimurium* is a facultative intracellular pathogen, it was interesting to note a difference in the expression of the horizontally acquired SPI-2 pathogenicity island. The *gyrBA* operon strain expressed SPI-2 less well (Fig. 6) and survived less well than the wild type in the macrophage (Fig. 7), whose *Salmonella*-containing vacuole is a stressful, low
magnesium environment where SPI-2 plays a key protecti
and Holden, 2012; Hensel, 2000; van der Heijden and Finlay, 2012). The
 gyrBA operon strain maintained its DNA in a less relaxed state in a low-
magnesium environment (Fig. 5). Since DNA relaxation contributes to full
expression of SPI-2 genes (Cameron and Dorman, 2012; Quinn et al., 2014)
and DNA in S. Typhimurium becomes relaxed when the bacterium is in the
macrophage (O Cróinín et al., 2006), this may explain the poorer transcription
of ssaG that was seen in low magnesium growth (Fig. 6).

We have shown experimentally that there is no absolute barrier to the
organisation of the gyrA and gyrB genes as a gyrBA operon in Salmonella.
Furthermore, there are many examples of naturally occurring gyrBA operons
among bacterial species. Why is this arrangement not found universally?
Sharing a common promoter, common transcriptional regulatory features and
a common chromosomal location would appear to offer the advantages of
coordinated gene expression (Price et al., 2005) and physical co-production of
protein products that will need to combine with a fixed stoichiometry to form
an active product (Dandekar et al., 1998; Pal and Hurst, 2004; Swain, 2004).
Indeed, the coupling of transcription and translation in prokaryotes may aid
the production of operon-encoded proteins that are required in stoichiometric
amounts (Li et al., 2014; Rocha, 2008). Often, but not invariably, operons are
composed of genes that contribute to a common pathway (de Daruvar et al.,
2002; Lawrence and Roth, 1996; Price et al., 2006; Rogozin et al., 2002) and
that is true of the gyrBA operon. Colocation of genes within an operon
facilitates their collective translocation via horizontal gene transfer, allowing
them to replace lost or mutated copies in the recipient cell (Lawrence and
Roth, 1996). According to this "selfish operon" hypothesis, this creates a
selective pressure for the maintenance of an operon structure. However,
since loss of either gyrA or gyrB is lethal, the gyrase operon may be one of
the exceptions to the selfish operon rule, because gyrase-deficient recipients
cannot exist.

We conducted a survey of gyrase gene locations in bacteria to assess
the frequency of the stand-alone arrangement seen in Salmonella and the
 gyrBA operon arrangement seen in other species (Experimental procedures).
We were unable to find any examples of bacteria that naturally possess a gyrAB operon. The results of the survey are shown in Table 1, where bacteria are grouped according to their gyrase gene arrangement, using oriC as a reference point. Fig. 8 shows a phylogenetic tree summarising the occurrence of different gyrase gene arrangements among bacteria from the four groups listed in Table 1.

Inversions of DNA between the left and the right replichores were seen frequently and these followed no obvious patterns. This is in agreement with the previous finding that, while distance to the origin is highly conserved, inversions of genes around the Ter region of a chromosome are frequent and well tolerated between *E. coli* and *Salmonella* (Alokam et al., 2002). Various relative arrangements of gyrA and gyrB were observed and subdivided into four groups: Group 1 had gyrB and gyrA positioned separately, with gyrB near oriC; Group 2 had gyrB and gyrA positioned separately, with the position of gyrB being variable; Group 3 had gyrB and gyrA arranged as a gyrBA operon in the immediate vicinity of oriC; Group 4 had gyrB and gyrA arranged as a gyrBA operon at a distance from oriC. The arrangements of gyrA and gyrB genes were categorised into the four Groups not only according to the relative positions of gyrA and gyrB but also according to the degree of conservation of the genetic environment of gyrB.

Table 1 suggests that all members of the class gamma-proteobacteria (phylum Proteobacteria), including *E. coli* and *Salmonella*, some alpha-proteobacteria, beta-proteobacteria and epsilon-proteobacteria are in Group 1. Group 2 contains some alpha-, beta- and delta-proteobacteria, members of the family Streptococcaceae (order Lactobacillales, phylum Firmicutes); members of the class Flavobacteriia; multiple members of the phylum Bacteroidetes; Acidobacteria and *Deinococcus radiodurans*. Group 3 was composed of members of the phylum Actinobacteria, classes Clostridia and Bacilli (phylum Firmicutes), family Enterococcaceae and family Lactobacillaceae (order Lactobacillales, phylum Firmicutes) order Fusobacteria (phylum Fusobacteria) and phylum Terenicutes. Finally, Group 4 consisted of members of the phylum Chlamydiae. There is perhaps more variation within Group 4, but this was not detected using the method.
Mycoplasma is an anomaly of Group 3, since not all its species clearly belong to this group. Some Mycoplasma possess the expected conserved genes 5' to gyrB, but not in its immediate vicinity. However, the orientation of genes 5' to gyrB remains favourable for the initiation of its transcription, therefore, Mycoplasma is placed in Group 3. It was clear from the analysis that members of the same taxonomic rank do not necessarily have to belong to the same Group, especially in diverse phyla. For example, both Group 2 and Group 3 arrangements are present within the Firmicutes. Moreover, both arrangements are present within the order Lactobacillales alone. Some less diverse phyla such as Fusobacteria and Chlamydiae belong to only one Group. No variation was found within families.

It was difficult to conclude if given taxons were enriched in particular groups in Table 1, so a phylogenetic tree was plotted that included all of the bacteria in the table (Fig. 8). The tree was constructed using the phylogenetic tree generator phyloT, based on NCBI taxonomy (Letunic & Bork, 2019) and the positions of the branches were manually reviewed with the aid of the NCBI taxonomy browser. It is apparent that one Group can be present in multiple unrelated phyla and that one phylum can contain members of several Groups, illustrating a high level of diversity of gyrA and gyrB chromosomal arrangements. However, certain patterns are discernable. Bacteria from Group 1 are exclusively located in phylum Proteobacteria. All the members of phylum Bacteroidetes that were investigated belong to Group 2, but other phyla can also contain some members of Group 2. The Group 3 arrangement occurs with high frequency in the superphylum Terrabacteria (Firmicutes, Tenericutes, Actinobacteria, Deinococcus), although this arrangement can be encountered elsewhere too. Finally, all of the tree members of Group 4 shown belong to the phylum Chlamydiae. No other phylum was found to contain bacteria of Group 4, but the existence of the Group 4 arrangement outside of the Chlamydiae cannot be ruled out. It is also difficult to draw clear parallels between the lifestyle of an organism and the Group to which it belongs, since bacteria of various lifestyles can be members of the same Group. The analysis presented here is indicative rather than exhaustive: it is possible that further sampling will broaden the existing Groups and reveal further details.
We found no examples of a naturally occurring gyrAB operon in bacteria. Nevertheless, we attempted to construct one in S. Typhimurium. Although gyrB could be inserted successfully downstream of gyrA, attempts to delete the gyrB copy from its native locus failed repeatedly. The strong impediment to deleting gyrB from its original locus suggests the particular importance of the position of this gene. What are the constraints on removing gyrB from its natural location on the S. Typhimurium chromosome and why is gyrA free from such constraints? The gyrB and gyrA genes respond differently to treatment with DNA gyrase inhibitors (Neumann & Quiñones, 1997). In particular, the expression of both gyrA and gyrB can be induced by coumarins (inhibitors of the GyrA subunit), while only gyrA is induced by quinolones (inhibitors of the GyrB subunit). This is despite DNA relaxation occurring in response to both classes of gyrase-inhibiting drugs. This suggests that gyrB differs from gyrA in its sensitivity to DNA relaxation. Another characteristic that is different between these genes is the degree of sequence conservation in the regulatory regions. FIS regulation is important for gyrA and gyrB of in both E. coli and Salmonella. FIS regulatory binding sites are located upstream from the ORFs of both genes (Schneider et al., 1999; Keane & Dorman, 2003). However, the 5' region of gyrA is significantly diverged between these bacteria, while the 5' region of gyrB is, in contrast, highly conserved (Keane & Dorman, 2003). Various mutual arrangements of gyrA and gyrB were found among bacteria (Table 1), but the relative position of gyrB seems to be more conserved than that of gyrA.

When the immediate genetic environment of both genes in bacteria listed in Table 1 was studied, one distinct pattern was found – homologues of dnaA (encoding chromosomal replication initiation protein DnaA), dnaN (encoding the beta subunit of DNA polymerase III) and recF (encoding the DNA repair protein RecF) or at least one of these three genes, are found directly upstream of gyrB gene in all bacteria in which gyrB is located in the immediate vicinity of oriC, such as most bacteria of Groups 1 and 3 (Table 1). Transcription from these co-oriented neighbouring genes provides a strong input of DNA relaxation (Sobetzko, 2016) that stimulates transcription of supercoiling-sensitive PgyrB (Menzel & Gellert, 1987). This is true of most
bacteria where gyrB is in the immediate vicinity of oriC and gyrA is located either about 20% of the chromosome further away or is a part of a gyrBA operon. Bacteria with a gyrBA operon that is not in the immediate vicinity of oriC (such as Chlamydia psittaci) and bacteria with the two gyr genes separated by about 20% of the chromosome, such as Myxococcus xanthus (together with some Bacteroidetes) that satisfy the gene positional parameters characteristic of Group 1, possess gyrB with a non-conserved genetic neighbourhood. Bacteria of Groups 2 and 4 do not have a conserved genetic environment around gyrB. The conservation of the genetic neighbourhood 5' to gyrB seems to be more important than the subjective proximity to oriC. Therefore, genetic neighbourhood conservation was used as a parameter to decide the groupings in Table 1. The frequent association of gyrB with the dnaA, dnaN and recF genes; the higher conservation of gyrB's position in comparison to gyrA; the transcriptional response of gyrB to quinolones and our inability to delete gyrB from the gyrAB + gyrB merodiploid Salmonella strain – all indicate that conservation of the physical location of gyrB, but not gyrA, is essential in many bacteria. These findings reveal important information about chromosome composition in natural bacteria and can help guide attempts at synthetic chromosome design.
Experimental procedures

Bacterial strains and culture conditions

The bacterial strains used in this study were derivatives of S. Typhimurium strain SL1344 and their details are listed in Table 1. Bacteriophage P22 HT 105/1 int-201 was used for generalized transduction during strain construction (Schmieger, 1972). Phage lysates were filter-sterilized and stored at 4°C in the dark. Bacterial strains were stored as 35% glycerol stocks at -80°C and freshly streaked on agar plates for each biological replicate. Four ml LB broth was inoculated with a single colony and grown for 18 h. This overnight culture was sub-cultured into fresh 25 ml LB broth normalizing to an OD$_{600}$ of 0.003, unless otherwise stated, and grown to the required growth phase. The standard growth conditions for all bacterial strains were 37°C, 200 rpm, unless otherwise stated. For culturing in minimal medium, overnight cultures were prepared as described above. 1 ml of overnight culture was washed three times with minimal medium N of the required MgCl$_2$ concentration to remove nutrients, sub-cultured into minimal medium of the corresponding MgCl$_2$ concentration in a total volume of 25 ml and grown for 24 h to pre-condition the bacteria. The pre-conditioned culture was sub-cultured into 25 ml of fresh minimal medium N adjusted to an OD$_{600}$ of 0.03 and grown for a further 24 h to obtain a culture in the stationary phase of growth.

To measure growth characteristics of a bacterial culture, an overnight culture was adjusted to an OD$_{600}$ of 0.003 in 25 ml of fresh LB broth and grown at the standard conditions for 24 h in the appropriate liquid medium. The optical density of the culture at OD$_{600}$ was measured at 1-h intervals for the first 3 hours and then every 30 min until 8 hours; the last reading was taken at 24 h. Measurements were taken using a Thermo Scientific BioMate 3S spectrophotometer with liquid cultures in plastic cuvettes. To measure the growth characteristics of a bacterial culture in minimal medium with altered Mg$^{2+}$ concentration, an overnight bacterial culture was washed in minimal medium with an appropriate concentration of MgCl$_2$ and pre-conditioned for 24 h. The pre-conditioned culture was adjusted to an OD$_{600}$ of 0.03 in 25 ml of fresh medium in two flasks and the OD$_{600}$ was
measured every hour beginning from 2 h post time zero until 8 h. Separate
cultures were set up similarly to measure OD_{600} every hour from 8 h until 15 h.
In this way, the number of times each flask was opened and sampled was
minimized to yield reliable and reproducible measurements.

The growth characteristics of bacterial cultures in LB broth were also
measured by viable counts. The culture was grown in the same way as for
spectrophotometry, and an aliquot was taken at 2 h, 4 h, 6 h, 8 h and 24 h,
serially diluted and spread on LB agar plates to give between 30 and 300
colonies after overnight incubation at 37°C. The bacterial colony counts were
expressed as colony forming units per millilitre (cfu ml$^{-1}$).

**Bacterial motility assays**

Assays were carried out precisely as described to achieve agreement
between biological replicates. 0.3% LB agar was melted in a 100 ml bottle in a
Tyndall steamer for 50 min, allowed to cool in a 55°C water bath for 20 min,
six plates were poured and left to dry near a Bunsen flame for 25 min. 1 µl of
bacterial overnight culture was pipetted under the agar surface with two
inocula per plate. Plates were placed in a 37°C incubator without stacking to
ensure equal oxygen access. After 5 h, the diameters of the resulting swarm
zones were measured and expressed as the ratio of the WT zone to that of
the mutant.

**Competitive fitness assays**

Flasks of broth were inoculated with the pair of competing bacterial strains in
a 1:1 ratio. Derivatives of each competitor were constructed that carried a
chloramphenicol acetyl transferase (cat) gene cassette within the
transcriptionally silent pseudogene SL1483. This cat insertion is known to be
neutral in its effects on bacterial fitness (Lacharme-Lora et al., 2019) and
allows the marked strain to be distinguished from its unmarked competitor.
Competitions were run in which wild type SL1344 was the marked strain or in
which SL1344 gyrBA was the marked strain. Strains to be competed were
pre-conditioned in separate 25 ml cultures for 24 h without antibiotics. Then
$10^5$ cells of each strain were mixed in 25 ml of fresh LB broth and grown as a
mixed culture for another 24 h. The number of colony forming units was determined by plating the mixture on chloramphenicol-containing plates and on plates with no antibiotic at T=0 h and T=24 h. Taking the wild type SL1344 Vs SL1344 gyrBA competition as an example, SL1344 was competed against SL1344 gyrBA SL1483::cat and, as a control, SL1344_SL1483::cat was competed against SL1344 gyrBA. The competitive fitness index (f.i.) was calculated according to the formula:

$$f.i. = \frac{\ln (Nc(24)/Nc(0))}{\ln ((Nwt(24)/Nwt(0))},$$

where Nc(0) and Nc(24) are the initial and final counts of a competitor and Nwt(0) and Nwt(24) are initial and final counts of the WT. Competitor is a strain other than the WT; f.i. <1 means that the competitor is less fit than the WT; f.i. >1 indicates the opposite.

Construction of a gyrBA operon strain and an attempt to construct a gyrAB operon strain

A derivative of S. Typhimurium with an artificial gyrBA operon was constructed by Lambda-Red homologous recombination (Datsenko & Wanner, 2000). Briefly, a kan cassette was amplified from plasmid pKD4 with primers Kan ins gyrA F and Kan ins gyrA R, (Table S1) using Phusion high-fidelity DNA polymerase. The amplicon, with overhangs homologous to a region immediately downstream of gyrA, was transformed into the WT strain harbouring plasmid pKD46, then grown in the presence of arabinose to activate the Lambda Red system in order to tag gyrA with the kan cassette. The gyrA::kan construct, including 20 nucleotides upstream from the gyrA translational start codon, was amplified using primers gyrB.int.gyrA::kan_Pf and gyrB.int.gyrA::kan_Prev (Table S1). The amplicon had overhangs that were homologous to sequences immediately downstream of gyrB. This allowed translation of the GyrA protein from the bicistronic gyrBA mRNA because several sequences closely matching to a consensus ribosome binding site (5'-AGGAGG-3') were located in this 20 bp region. The gyrA::kan amplicon was inserted by Lambda Red-mediated recombination immediately downstream of the gyrB protein-coding region to construct the gyrBA operon. The original gyrA gene was deleted by an in-frame insertion of a kan cassette.
The kan resistance cassettes were subsequently eliminated via FLP-mediated site-specific recombination (Cherepanov & Wackernagel, 1995). The resulting gyrBA strain had the genes that encode both subunits of DNA gyrase arranged as a bicistronic operon under the control of a common promoter, \( P_{\text{gyrB}} \) (Table 2). Although no gyrAB operon has been reported to occur naturally in bacteria, the construction of an artificial gyrAB operon in Salmonella was attempted in parallel with the gyrBA construction, using an identical strategy. The gyrB gene was tagged with a kan cassette that was amplified using primers Kan ins gyrB F and Kan ins gyrB R (Table S1). The gyrB::kan construct was amplified using primers gyrA.int.gyrB::Kan_Pf and gyrA.int.gyrB::Kan_Prev (Table S1) and inserted immediately downstream of gyrA to construct a gyrAB operon. To enable translation of the GyrB protein from the bicistronic gyrAB mRNA, 20 nucleotides upstream from the gyrB translation start codon were included in the insertion, providing gyrA with the best match to a consensus ribosome binding site found there: 5'-ACGAGG-3'.

The resulting strain had a gyrAB operon at the gyrA locus and was merodiploid for gyrB (Table 2). Repeated attempts to delete gyrB from its original locus were unsuccessful. This indicated that the original position of gyrB is important and that it cannot be deleted even when gyrB is expressed from a synthetic operon located elsewhere on the chromosome. No further work was performed with the gyrAB operon strain.

**DNA isolation for whole genome sequencing**

To obtain high-quality chromosomal DNA for whole genome sequencing, a basic phenol-chloroform method was used (Sambrook & Russell, 2006). 2 ml of an overnight culture were centrifuged at 16000 x g for 1 min to harvest cells and the cell pellet was resuspended in 400 µl of TE buffer pH 8 (100 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 (BDH, Poole, England)). 1% SDS and 2 mg/ml protease K were added and incubated for 2 h at 37°C to complete lysis. DNA was isolated by the addition of 1 volume of phenol pH 8.0 : chloroform : isoamyl alcohol (25 : 24 : 1) (AppliChem, Darmstadt, Germany), thorough mixing and centrifugation at 16000 x g for 15 min at 4°C in phase-
lock tube. The upper aqueous layer containing DNA was collected and the phenol: chloroform extraction was repeated two more times. To remove contaminants and to precipitate DNA, sodium acetate pH 5.2 at 0.3 M and isopropanol at 60% of the final volume were added and kept for 1 h at -20°C. DNA was pelleted by centrifugation at 16000 x g for 15 min at 4°C. The DNA pellet was washed with 70% ethanol, dried at 37°C until translucent and resuspended in 100 µl TE pH 8.0. The sample was electrophoresed on agarose gel to check for degradation and the DNA concentration was determined as follows: to remove RNA contamination from DNA samples, 100 mg/ml RNase A was added and incubated for 30 min at 37°C. Phenol-chloroform extraction was performed as above. To precipitate DNA, 0.5 M of ammonium acetate (Merck, Darmstadt, Germany) and a half volume of isopropanol were added and incubated for 2 h at -20°C. DNA was pelleted by centrifugation at 16000 x g for 15 min at 4°C. The DNA pellet was washed twice with 70% ethanol, dried at 37°C until translucent and resuspended in 50 µl water. The sample was run on an agarose gel to check for degradation.

The concentration of DNA extracted was determined by measuring absorbance at 260 nm on a DeNovix DS-11 spectrophotometer (Wilmington, Delaware, USA). The shape of the absorbance curve was ensured to have a clear peak at 260 nm. The purity of samples was assessed by the ratio of $A_{260}/A_{280}$ – a measure of protein and phenol contamination and $A_{260}/A_{230}$ – a measure of contaminants such as EDTA, where both should be as close as possible to 2. Only high-quality samples were chosen for further work.

**Whole genome sequencing**

Whole genome sequencing was performed on final versions of the constructed strains to ensure that no compensatory mutations were introduced into their genomes. The sequencing was performed by MicrobesNG (Birmingham, UK) using Illumina next generation sequencing technology. The output reads were assembled using Velvet (Zerbino, 2010) and aligned to the reference SL1344 sequence NC_016810.1 Breseq software (Deatherage & Barrick, 2014). The data are available through the Sequence Read Archive (SRA) with accession number PRJNA682874.
RNA extraction, DNase treatment and RT-qPCR.

RNA for measuring gene expression by qPCR was isolated using an acidic phenol-chloroform method. An overnight culture was subcultured into 25 ml of fresh LB broth normalising to an OD600 of 0.003. The bacterial culture was grown to the required timepoint and mixed with 40% volume of 5% acidic phenol (pH 4.3) in ethanol and placed on ice for at least 30 min to stop transcription. The cells were harvested by centrifugation at 3220 x g for 10 min at 4°C and resuspended in 700 µl of TE buffer pH 8 containing 0.5 mg/ml lysozyme. 1% SDS and 0.1 mg/ml proteinase K were added and incubated for 20 min at 40°C to complete lysis. 1/10 volume of 3 M sodium acetate was added to precipitate RNA, 1 volume of 1:1 solution of acidic phenol and chloroform was added, mixed well on a vortex mixer and centrifuged at 16000 x g for 15 min at 4°C to extract RNA into aqueous phase. To precipitate RNA the aqueous layer was harvested, mixed with 1 volume of isopropanol and incubated at -20°C for 1 hour. RNA was harvested by centrifugation at 16000 x g for 15 min at 4°C. The RNA pellet was washed with 70% ethanol and dried at 37°C until translucent. The total RNA was dissolved in 50 µl DEPC-treated water and its concentration was determined using DeNovix DS-11 spectrophotometer.

For DNase-treatment, RNA was diluted to 20 µg in 80 µl, denatured at 65°C for 5 min and kept on ice. 1x DNase I buffer including MgCl2 and 10 U DNase I (ThermoFisher Scientific, Waltham, US) were added and incubated for 45 min at 37°C. 100 µl of 1:1 acidic phenol : chloroform was added to DNase I digestion samples, mixed and transferred to a phase-lock tube. RNA was extracted by centrifugation at 16000 x g for 12 min at 15°C. The upper aqueous layer was harvested and RNA was precipitated by adding 2.5 volumes of 30:1 ethanol : 3 M sodium acetate pH 6.5 for 2 h or overnight at -20°C. RNA was harvested by centrifugation at 16000 x g for 30 min at 4°C. The RNA pellet was washed with 70% ethanol and dried at 37°C until translucent. The total RNA was dissolved in 30 µl DEPC-treated water and its concentration was determined as in 2.5.5. RNA was checked for DNA...
contamination by the end point PCR and for integrity on a HT gel (Mansour & Pestov, 2013).

400 nm of the total extracted and DNase I treated RNA was converted to cDNA using GoScriptTM Reverse Transcription System kit (Promega) according to manufacturer’s guidelines. Then, 5.33 ng of cDNA in 20 µl reaction was used as a template for Real Time quantitative PCR (RT-qPCR) using 1x FastStart Universal SYBR Green Master (ROX) (Roche, Mannheim, Germany) and gene-specific pair of primers (0.3 µM each). For each pair of primers, a standard curve was generated using 10-fold serially diluted gDNA. PCR and fluorescence detection were carried out in StepOne Real Time PCR system (Applied Biosystems). Analysis was performed in the accompanying software. The cycling conditions were as follows: 10 min at 95°C; 40 cycles of 15 sec at 95°C and 1 min at 60°C.

Minimum inhibitory concentration (MIC) of antibiotics determination

MIC90 of antibiotics (a minimal concentration at which 90% of bacterial growth is inhibited) was found by serially diluting antibiotics and spectrophotometrically testing the ability of different dilutions to inhibit bacterial growth. On a 96-well plate, all wells (excluding column 12) were filled with 60 µl of sterile LB broth. 1 ml solutions of antibiotics to be tested were prepared at the highest desired concentration in LB. 300 µl of the prepared antibiotics were added to the wells of column 12 and homogenised by pipetting up and down 5 times with a multichannel pipette. 240 µl was transferred to the next wells in column 11, homogenisation was repeated and serial 1:1.25 dilutions were sequentially continued until column 3. The final 240 µl from column 3 were discarded. All the wells were inoculated with bacterial cultures adjusted to an OD\textsubscript{600} of 0.003 except column 1. In this way column 1 contained negative controls (no bacteria and no antibiotic), column 2 contained positive controls (no antibiotics) and columns 3-12 contained serially diluted antibiotics inoculated with the identical number of bacteria. The plate was covered, sealed between plastic sheets and incubated for 18 h at the standard growth conditions. The plate was read by measuring OD\textsubscript{600} values on a plate reader (Multiscan EX, Thermo Electronics).
SPI-1 and SPI-2 reporter assays

Salmonella pathogenicity island (SPI) activity was accessed by measuring the expression of gfp+ reporter gene fusions to promoters of prgH and ssaG to look at SPI-1 and SPI-2 expression, respectively. The gfp+ reporter fusions were transduced into each strain by P22 generalized transduction and selected with chloramphenicol. 100 µl of overnight culture of the gfp+ reporter-carrying strain was diluted 1:100 in LB broth. Black 96 plate with transparent flat bottom was filled with 100 µl of the diluted culture in six technical replicates, negative controls were included. The plate was sealed with parafilm and incubated at 300 rpm, 37°C for 24 h in the Synergy H1 microplate reader (Biotek, Vermont, USA). Bacterial growth was measured at 600 nm and GFP fluorescence was read using 485.5 nm excitation frequency at 528 nm emission frequency, measurements were taken every 20 min. For measurements in the minimal medium, the culture was adjusted to OD600 of 0.03 in the medium of the required MgCl2 concentration and measurements proceeded as above.

Global supercoiling determination

Global DNA supercoiling was assayed in bacterial strains transformed with a reporter plasmid pUC18. An overnight culture of pUC18-containing strain was adjusted to an OD600 of 0.003 and grown to the late stationary growth stage (24 h) in 25 ml LB broth or in 25 ml of minimal medium N of the required MgCl2 concentration pre-conditioned as above. Fourteen OD600 units (6 OD600 units for minimal medium) were harvested and pUC18 was isolated with the aid of QIAprep Spin miniprep kit (QIAGEN, Hilden, Germany) according to manufacturer’s guidelines.

To observe the range of DNA supercoiling states characteristic of a strain at a given growth stage, extracted pUC18 samples were resolved on 0.8% agarose gel supplemented with the DNA intercalating agent chloroquine. 2 L of 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA pH 8.0) and 1 ml of 25 mg/ml chloroquine were made. 0.8% agarose solution was made from 300 µl TBE and melted in a Tyndall steamer. When
the gel cooled down, it was supplemented with 2.5 µg/ml chloroquine. The 27 cm long gel was poured, left to polymerise for 2 h and covered with 1.7 litres of the running buffer containing 1x TBE and chloroquine at 2.5 µg/ml. 1 µg or 500 ng of the plasmid samples in 15 µl volumes were mixed with 5x loading dye (80% glycerol, 0.01% bromophenol blue) and loaded on a gel. The gel was electrophoresed for 16 h at 100 V. The gel was washed in distilled water for 24 h changing water a few times, stained in 1 µg/ml ethidium bromide for 1 h rocking in the dark. The stain was poured off and the gel was washed in distilled water for further 1 h. The plasmid topoisomers were visualised under UV on the ImageQuant LAS 4000 imager. ImageJ software was used to outline plasmid topoisomer distribution profiles.

Determining the patterns of gyrA and gyrB locations in bacterial chromosomes

The location of oriC in each organism examined was determined using the DoriC 10.0 database (tubic.org/doric) (Luo and Gao, 2019) and the coordinates of their gyrA and gyrB genes were obtained using the Ensembl bacteria browser (bacteria.ensembl.org). Distance in base pairs between the oriC and the gene was calculated and converted into the percentage of the total chromosome size. An attempt was made to cover bacterial taxonomy as broadly as possible, encompassing members of the major bacterial phyla, well studied, and clinically important organisms in the analysis (Table 1). The table is neither complete nor does it claim to include all the existing possibilities of gyrA and gyrB arrangements in bacterial chromosomes, but instead, exemplifies the arrangement possibilities mentioned in this work. Closely related species and those belonging to the less diverse phyla were found to share the chromosomal positions of gyrA and gyrB frequently. Thus, one representative of a taxonomic rank was often deemed sufficient for the purpose of inclusion in the table. Lower classification ranks were analysed within more diverse and studied phyla.

Mammalian cell culture conditions

RAW264.7 murine macrophages were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM), (Sigma, catalogue number D6429) supplemented
with 10% fetal bovine serum (FBS) in a humidified 37°C, 5% CO₂ tissue-
culture incubator grown in 75 cm³ tissue-culture flasks. When approximately
80% confluent growth was achieved, cells were split to a fresh flask. Cells
within the 9-16 passage number range were used for infections. All media and
PBS used for cell culture were pre-warmed to 37°C. To split cells, old DMEM
was removed and the monolayer was rinsed with 10 ml of sterile PBS. Ten ml
of fresh DMEM was pipetted into the flask and the monolayer was scraped
gently with a cell scraper to dislodge the cells. Scraped cells were centrifuged
at 450 x g for 5 min in an Eppendorf 5810R centrifuge and the cell pellet was
resuspended in 5 ml DMEM+FBS. One ml of the cell suspension was added
to 14 ml of fresh DMEM+FBS in a 75 cm³ flask, gently rocked to mix and
incubated at 37°C, 5% CO₂. To seed cells for infection, cells were treated as
for splitting. After resuspension in 5 ml DMEM+FBS, viable cells were counted
on a haemocytometer using trypan blue exclusion dye. A 24-well tissue
culture plate was filled with 500 µl DMEM+FBS. 1.5×10⁵ cells were added to
each well, gently rocked to mix and incubated at 37°C, 5% CO₂ for 24 h.

Macrophage viability assay in SPI-1 inducing conditions

Overnight bacterial cultures were subcultured 1:33 in 10 ml of fresh LB broth
in 125 ml conical flask and grown for 3.5 h to maximize SPI-1 expression
(Steele-Mortimer et al., 1999). 500 µl of the culture was centrifuged at 16000 x
g for 1 min and resuspended in 500 µl of HBSS-/-/. Monolayers were washed
twice with 500 µl of HBSS+/+ and infected with bacteria at MOI of 5 in three
technical replicates for each timepoint and strain. The plate was centrifuged at
200 x g for 10 min to synchronize infections and incubated for 30 min at 37°C,
5% CO₂. In the meantime, the infection medium was plated for enumeration
on LB agar plates – T=0 h. Gentamycin protection assay was used to
determine bacterial counts inside macrophages. To kill all extracellular
bacteria, the monolayers were washed once with HBSS+/+ and high
gentamycin (100 µg/ml) treatment diluted in DMEM+FBS was added to the
wells. The plate was incubated at 37°C, 5% CO₂ for 1 h. At 1 h post infection
the monolayers were washed three times with HBSS+/+, macrophages were
lysed by adding 1 ml of ice-cold water, pipetting up and down ten times with
scraping and intracellular bacteria were plated for enumeration. The
monolayers which were intended for other timepoints, were washed once with
HBSS+/+, low gentamycin (10 µg/ml) treatment in DMEM+FBS was added
and the plate was incubated at 37°C, 5% CO2. The low gentamycin
concentration is to ensure that any extracellular bacteria are killed, but at the
same time to avoid gentamycin permeabilizing plasma membrane of a
macrophage (Kaneko et al., 2016). At later timepoints monolayers were
washed three times with HBSS+/+, macrophages were lysed by adding 1 ml
ice-cold water, pipetting up and down ten times with scraping and intracellular
bacteria were plated for enumeration.

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Conflicts of interest
The authors declare that they have no conflicts of interest.
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### Table 1. Relative positions of gyrB and gyrA across bacterial species

| Organism | gyrB to oriC distance in bp, Left or Right replicore | gyrB to oriC distance as % total chromosome | gyrA to oriC distance in bp, Left or Right replicore* | gyrA to oriC distance as % total chromosome |
|----------|------------------------------------------------------|---------------------------------------------|------------------------------------------------------|---------------------------------------------|
| **Group 1** | | | | |
| *Escherichia coli* | 45409, Right | 0.98% | 1585679, Right | 34.20% |
| *Salmonella enterica* serovar Typhimurium | 42033, Left | 0.86% | 1730509, Left | 35.48% |
| *Salmonella enterica* serovar Gallinarum | 37634, Right | 0.81% | 1382662, Left | 29.68% |
| *Shigella flexneri* | 57368, Left | 1.25% | 1577518, Left | 34.24% |
| *Yersinia pestis* | 37176, Left | 0.79% | 1073028, Right | 22.82% |
| *Vibrio cholerae* | 10386, Right | 0.35% | 1329436, Right | 44.90% |
| *Pseudomonas aeruginosa* | 2248, Right | 0.04% | 2708909, Right | 43.26% |
| *Xanthomonas axonopodis* (all above are Proteobacteria, class Gammaproteobacteria) | 3161, Right | 0.06% | 1876940, Right | 36.27% |
| *Azospirillum* sp. (Proteobacteria (α), order Rhodospirillales) | 179561, Left | 5.42% | 1595259, Right | 48.17% |
| *Caulobacter crescentus* (Proteobacteria (α), order Caulobacteriales) | 166112, Right | 4.14% | 1744542, Right | 43.43% |
| *Azoarcus* sp. (Proteobacteria (β), order Rhodocyclales) | 32299, Right | 0.61% | 1182414, Left | 22.49% |
| *Burkholderia cepacia* (Proteobacteria (β), Burkholderiales) | 160154, Right | 4.62% | 859834, Left | 24.82% |
| *Campylobacter jejuni* (Proteobacteria, class Epsilonproteobacteria) | 635, Right | 0.04% | 653170, Left | 40.12% |
| **Group 2** | | | | |
| *Myxococcus xanthus* (Proteobacteria (Δ), order Myxococcales) | 310304, Right | 3.40% | 872133, Right | 9.54% |
| *Bacteroides thetaiotaomicron* (Bacteroidetes, family Bacteroidaceae) | 246107, Right | 3.93% | 2199265, Left | 35.10% |
| *Bacteroides fragilis* (Bacteroidetes, family Bacteroidaceae) | 155636, Right | 2.99% | 2420574, Left | 46.50% |
| *Rickettsia prowazekii* (Proteobacteria (α), order Rickettsiales) | 382412, Left | 34.40% | 250129, Right | 22.50% |
| *Neisseria gonorrhoeae* | 412632, Left | 19.16% | 616174, Right | 28.61% |
(Proteobacteria (β), order Neisseriales)

| Species                        | Left % | Right % |
|--------------------------------|--------|---------|
| *Streptococcus pneumoniae*     | 37.25% | 45.05%  |
| *(Firmicutes, family Streptococcaceae)* |        |         |
| *Streptococcus pyogenes*       | 27.53% | 47.40%  |
| *(Firmicutes, family Streptococcaceae)* |        |         |
| *Flavobacterium columnare*     | 14.19% | 39.22%  |
| *(Bacteroidetes, class Flavobacteria)* |        |         |
| *Prevotella intermedia*        | 12.64% | 32.89%  |
| *(Bacteroidetes, family Prevotellaceae)* |        |         |
| *Sphingobacterium sp.*         | 47.03% | 35.06%  |
| *(Bacteroidetes, class Sphingobacteriia)* |        |         |
| *Porphyromonas gingivalis*     | 23.59% | 37.26%  |
| *(Bacteroidetes, family Porphyromonadaceae)* |        |         |
| *Deinococcus radiodurans*      | 34.45% | 26.88%  |
| *(Deinococcus-Thermus, class Deinococi)* |        |         |
| *Acidobacterium capsulatum*    | 16.62% | 0.82%   |
| *(Acidobacteria, class Acidobacteria)* |        |         |

**Group 3**

| Species                        | Left % | Right % | Downstream of gyrB |
|--------------------------------|--------|---------|---------------------|
| *Geobacter sulfurreducens*     | 0.05%  |         |                     |
| *(Proteobacteria (Δ), order Desulfuromonadales)* |        |         |
| *Pelobacter carbinolicus*      | 0.06%  |         |                     |
| *(Proteobacteria (Δ), order Desulfuromonadales)* |        |         |
| *Streptomyces coelicolor*      | 0.05%  |         |                     |
| *(Actinobacteria, class Actinobacteria)* |        |         |
| *Mycobacterium tuberculosis*   | 0.06%  |         |                     |
| *(Actinobacteria, class Actinobacteria)* |        |         |
| *Micrococcus luteus*           | 0.11%  |         |                     |
| *(Actinobacteria, family Micrococcaceae)* |        |         |
| *Clostridium tetani*           | 0.09%  |         |                     |
| *(Firmicutes, class Clostridia)* |        |         |
| *Lactobacillus brevis*         | 0.11%  |         |                     |
| *(Firmicutes, family Lactobacillaceae)* |        |         |
| *Enterococcus faecalis*        | 0.20%  |         |                     |
| *(Firmicutes, family Enterococcaceae)* |        |         |
| *Listeria monocytogenes*       | 0.13%  |         |                     |
| *(Firmicutes, order Bacillales)* |        |         |
| *Bacillus subtilis*            | 0.06%  |         |                     |
| *(Firmicutes, order Bacillales)* |        |         |
| *Spirochaeta thermophila*      | 0.38%  |         |                     |
| *(Spirochaetes, class Spirochaetia)* |        |         |
| *Fusobacterium nucleatum*      | 0.15%  |         |                     |
| *(Fusobacteria, class Fusobacteria)* |        |         |
| *Borrelia burgdorferi*         | 0.14%  |         |                     |
| *(Spirochaetes, class Spirochaetia)* |        |         |
| *Mycoplasma haemofelis*        | 2.94%  |         |                     |
| *(Tenericutes, class Mollicutes)* |        |         |
### Group 4

|                         | Accession | Location | Percent | Relative Location |
|-------------------------|-----------|----------|---------|-------------------|
| *Chlamydia psittaci*    | 573957, Left | 48.97%   | downstream of gyrB |
| (Chlamydiae, class Chlamydiia) |           |          |         |                   |
| *Chlamydia trachomatis* | 504740, Left | 48.42%   | downstream of gyrB |
| (Chlamydiae, class Chlamydiia) |           |          |         |                   |
| *Waddlia chondrophila*  | 1044601, Right | 49.36%   | downstream of gyrB |
| (Chlamydiae, class Chlamydiia) |          |          |         |                   |

*Where gyrB and gyrA form an operon, gyrA is universally located downstream of gyrB*
### Table 2. Bacterial strains

| Strain name | Genotype/Description | Source/reference |
|-------------|----------------------|------------------|
| SL1344      | rpsL hisG            | Hoiseth and Stocker, 1981 |
| SL1344 gyrA::kan | Kanamycin resistance cassette inserted downstream of the gyrA protein-coding region | This work |
| SL1344 gyrB::kan | Kanamycin resistance cassette inserted downstream of the gyrB protein-coding region | This work |
| SL1344 gyrBA | gyrBA operon under the control of the gyrB promoter, P<sub>gyrB</sub> | This work |
| SL1344 gyrAB<sub>_gyrB</sub> | gyrAB operon under the control of the gyrA promoter, P<sub>gyrA</sub>; merodiploid for gyrB | This work |
| SL1344 prgH::gfp<sup>+</sup> | prgH-gfp<sup>+</sup> [LVA]/R::cat fusion of a gfp<sup>+</sup> gene encoding a destabilised version of GFP to the SPI-1 promoter, P<sub>prgH</sub> | Ibarra et al., 2010 |
| SL1344 gyrBA prgH::gfp<sup>+</sup> | Fusion of a gfp<sup>+</sup> derivative encoding a destabilised version of GFP to the SPI-1 promoter, P<sub>prgH</sub> in the gyrBA background | This work |
| SL1344 ssaG::gfp<sup>+</sup> | ssaG-gfp<sup>+</sup> [LVA]/R::cat fusion of a gfp<sup>+</sup> derivative encoding a destabilised version of GFP to the SPI-2 promoter, P<sub>ssaG</sub> | Ibarra et al., 2010 |
| SL1344 gyrBA ssaG::gfp<sup>+</sup> | Fusion of a gfp<sup>+</sup> derivative encoding a destabilised version of GFP to the SPI-2 promoter, P<sub>ssaG</sub> in the gyrBA background | This work |
| SL1344 SL1483::cat | Insertion of a chloramphenicol resistance cassette into the pseudogene SL1483 | This work |
| SL1344 gyrBA SL1483::cat | Insertion of a chloramphenicol resistance cassette into the pseudogene SL1483 in the gyrBA background | This work |
Table 3. Plasmids used in this study

| Plasmid name | Description | Reference |
|--------------|-------------|-----------|
| pKD3         | Amp<sup>R</sup> (Carb<sup>R</sup>), Cm<sup>R</sup> | (Datsenko & Wanner, 2000) |
| pKD4         | Amp<sup>K</sup> (Carb<sup>K</sup>), Kan<sup>R</sup> | (Datsenko & Wanner, 2000) |
| pKD46        | Amp<sup>K</sup> (Carb<sup>K</sup>), λ Red genes γ, β, exo under the control of an arabinose inducible promoter | (Datsenko & Wanner, 2000) |
| pCP20        | Amp<sup>K</sup> (Carb<sup>K</sup>), Cm<sup>K</sup>, FLP recombinase expressing, temperature sensitive replicon | (Cherepanov & Wackernagel, 1995) |
| pUC18        | Amp<sup>K</sup> (Carb<sup>K</sup>) | (Yanisch-Perron et al., 1985) |

Abbreviations: Amp<sup>R</sup>, ampicillin (carbenicillin) resistance; Cm<sup>R</sup>, chloramphenicol resistance; Kan<sup>R</sup>, kanamycin resistance.
**Figures and legends**

**Fig. 1.** Construction of a derivative of *S. Typhimurium* strain SL1344 with a *gyrBA* operon.

Chromosomal maps of the WT SL1344 and SL1344 *gyrBA* strains. Positions of *oriC*, *dif* and macromdomains are shown. Promoter, protein coding region (open reading frame, ORF) and the terminator of the genes of interest are shown and colour coded. The *gyrA* ORF is green and the *gyrB* promoter and ORF are red. Not to scale.
**Fig. 2.** Minimum inhibitory concentrations of DNA gyrase-inhibiting antibiotics in the wild type SL1344 and SL1344 gyrBA strains. Cells were grown in a 96-well plate with 1:1.25 serially diluted antibiotics in LB broth for 18 h at 37°C and aeration. Cell density was measured by OD\textsubscript{600}. A. Percentage survival of the WT and SL1344 gyrBA in 65.54-160 µg/ml novobiocin. MIC\textsubscript{90} of the WT = 128 µg/ml, MIC\textsubscript{90} of SL1344 gyrBA = 160 µg/ml. B. Percentage survival of the WT and the gyrBA in 2.62-8 µg/ml coumermycin, MIC\textsubscript{90} = 6.4 µg/ml. C. Percentage survival of the WT and the gyrBA in 1.31 – 3.2 µg/ml nalidixic acid, MIC\textsubscript{90} = 2.56 µg/ml. D. Percentage survival of the WT and the gyrBA in 0.0084 – 0.0205 µg/ml ciprofloxacin, MIC\textsubscript{90} = 0.0164 µg/ml. Error bars represent the standard deviation of at least three biological replicates. Significance was found by unpaired Student’s t-test, where * = P<0.05 and ** = P<0.01.
Fig. 3. Motility and competitive fitness of strain SL1344 gyrBA.

A. Diameters of swimming motility were measured after 5 h incubation at 37°C on soft 0.3% LB agar. The gyrBA strain is slightly, but statistically significantly, less motile than the WT. Values below 1 indicate that the strain is less motile than the WT.

B) Fitness of the gyrBA strain was compared to the WT SL1344 in LB broth grown for 24 h with aeration at 37°C. Fitness index (f.i.) = 1 means that the competed strains were equally fit, f.i. < 1 indicates that the competitor strain is less fit than the WT, f.i. > 1 indicates that the competitor is more fit than the WT. The gyrBA and the WT were equally fit. Significance was determined by one sample T-test, where P<0.05.
Fig. 4. Expression of the gyrA and gyrB genes in wild type SL1344 (WT) and SL1344 gyrBA during growth in liquid culture. Cells were grown in LB broth at 37°C with aeration and samples were taken at 2 h, 3.5 h, 5 h and 7 h representing the lag, exponential, exponential-stationary transition and early stationary phases of growth, respectively. Transcription of gyrA and gyrB was measured and is reported relative to that of the hemX reference gene. Three biological replicates were used. Statistical significance was found by unpaired Student’s T-test, where P<0.05.
Fig. 5. Reporter plasmid DNA supercoiling in SL1344 gyrBA.
The pUC18 reporter plasmid was extracted from the WT and the SL1344 gyrBA strains at the stationary phase of growth and electrophoresed on a 0.8% agarose gel containing 2.5 µg/ml chloroquine. The arrow shows the direction of migration, with the more supercoiled plasmid topoisomers at the right of the gel. A. Global DNA supercoiling pattern of the WT and the gyrBA strain when grown in LB. B. Global DNA supercoiling pattern of the WT and the gyrBA strain when grown in minimal medium N with high (10 mM) MgCl₂ or low (10 µM) MgCl₂. Sample lanes are supplemented with densitometry profiles that were generated with ImageJ. The analysis is representative of four biological replicates.
A.

![Graph A]

B.

![Graph B]

C.

![Graph C]
D. 

![Graph showing fluorescence/OD600 over time for WT and gyrBA strains.]

E. 

![Graph showing fluorescence/OD600 over time for WT and gyrBA strains.]

F. 

![Graph showing fluorescence/OD600 over time for WT and gyrBA strains.]

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Fig. 6. Expression of genes in the SPI-1 and SPI-2 pathogenicity islands in wild type SL1344 (WT) and SL1344 gyrBA. Expression of gfp+ reporter gene fusions was measured in the wild type and SL1344 gyrBA strains every 20 min over a 24-h. period. A. SPI-1 expression in the gyrBA strain was identical to that in the WT in LB. B. SPI-2 expression in the gyrBA strain was identical to that of the WT in LB. C. SPI-1 expression in both the WT and the gyrBA strain. D. SPI-2 expression in minimal medium N with high MgCl₂ concentration (10 mM) was repressed in both the WT and the gyrBA strains. E. SPI-1 expression in minimal medium N with a low MgCl₂ concentration (10 μM) was repressed in both the WT and the gyrBA strains. F. SPI-2 expression in minimal medium N with low MgCl₂ concentration was lower in the gyrBA strain than in the WT at the stationary phase of growth. All plots are the results of at least three biological replicates; error bars represent the standard deviation. Statistical significance was found by Student’s unpaired T-test, where P<0.05.
Fig. 7. SPI-1-mediated entry and survival of the WT and SL1344 gyrBA strain in cultured RAW264.7 macrophage cells. Cells were infected with SPI-1-induced bacteria, grown to mod-exponential phase to promote SPI-1-mediated invasion. Survival and replication were measured by enumerating colony forming units (CFUs) at 3 h, 8 h, 16 h and 20 h post-infection. Fold replication represents the number of CFUs recovered at a particular time point divided by the CFU number at 1 h. Mean and individual replicates are shown. Significance at 16 h was found by unpaired Student’s T-test, where P<0.05.
**Fig. 8.** Phylogenetic tree of bacteria that belong to different groups based on their *gyrA* and *gyrB* arrangement. The phylogenetic tree was built in phyloT, a phylogenetic tree generator based on NCBI taxonomy (Letunic & Bork, 2019). Each of the four groups (see Table 1) of *gyrA* and *gyrB* arrangements is indicated by colour. Group 1, blue: *gyrA* and *gyrB* are at separate locations, with a conserved genetic environment 5' to *gyrB*. Group 2, orange: *gyrA* and *gyrB* are at separate locations, with a non-conserved genetic environment 5' to *gyrB*. Group 3, green: *gyrBA* operon, conserved genetic environment 5' to *gyrB*. Group 4, red: *gyrBA* operon, non-conserved genetic environment 5' to *gyrB*. Phyla names are indicated.
Consequences of producing DNA gyrase from a synthetic gyrBA operon in Salmonella enterica serovar Typhimurium

German Pozdeev, Aalap Mogre and Charles J Dorman*

SUPPLEMENTARY FILES
A

OD$_{600}$

WT
gyrBA

Time (h)

B

CFU/ml

WT
gyrBA

Time (h)
Fig. S1. Growth characteristics of SL1344 gyrBA.
A. Growth of the gyrBA strain as measured by absorbance at 600nm. OD$_{600}$ measurements were made every hour until 3 h, then every 30 min until 8 h and lastly at 24 h. B. Growth of the gyrBA strain as measured by viability counts. Dilutions of bacterial cultures were spread on agar plates, incubated at 37°C and colonies were counted. C. Growth of the WT and the gyrBA strains as measured by absorbance at 600 nm in minimal medium N. Pre-conditioned culture was subcultured into 25 ml of fresh minimal medium of the required Mg$^{2+}$ concentration, normalizing to an OD$_{600}$ of 0.03. OD$_{600}$ values were measured every hour from 2 h until 15 h and at 24 h. All plots are the results of at least three biological replicates, error bars represent standard deviation.
Fig. S2. Cell morphology of SL1344 gyrBA at the exponential and stationary phases of growth. Bacteria were harvested at the mid-exponential or at the late stationary phases of growth, washed with PBS, heat-fixed, stained with crystal violet and viewed under 1000x magnification with an oil immersion lens. Standard rod-shaped Salmonella cells were observed in the WT and the gyrBA strains. All images are representative of three biological replicates. A 10 µm scale bar is given for reference. Cell morphology in LB liquid cultures is shown for wild type SL1344 in exponential phase (A), stationary phase (B), SL1344 gyrBA in exponential phase (C) and stationary phase (D).
Fig. S3. Full gel data for reporter plasmid DNA supercoiling in SL1344 and SL1344 gyrBA.

Samples of pUC18 plasmid were extracted from the WT and the gyrBA at the stationary growth phase and run on a 0.8% agarose gel containing 2.5 µg/ml chloroquine to separate pUC18 topoisomers according to the degree of DNA supercoiling. Arrow shows the direction of electrophoretic flow with the more supercoiled plasmid topoisomers at the right of the gel. A) Global DNA supercoiling pattern of the WT and the gyrBA when grown in LB. The positions of relaxed and supercoiled plasmid topoisomer dimers and monomers are shown. B) Global DNA supercoiling pattern of the WT and the gyrBA when grown in minimal medium N with high (10 mM) Mg$^{2+}$ and low (10 µM) Mg$^{2+}$. Plasmid topoisomer monomers are at the righthand end of the distribution; dimers and higher order oligomers are to the left.
## Table S1 Oligonucleotides used in this study

| Name                        | 5'-3' sequence |
|-----------------------------|----------------|
| **Genetic mutation**        |                |
| confirmation primers:       |                |
| gyrA_check_Pf2              | GACTAAGGTAGCGGTAAATG |
| gyrA_check_Prev2            | GTAGATGACGAAAGAATCG |
| gyrB_check_Pf2              | CAACGAATCCATTCGATG |
| gyrB_check_Prev2            | CTGATGACGAGACTGTAAC |
| gyrA check del R            | GCATTGTCTGCTGATTC |
| gyrB check del R            | CTTTGTACGCGAATTAGC |
| gyrA_midcheck               | CGATGCTGCTACACTG |
| gyrB_midcheck               | GAACGCTGCTACACTT |
| **qPCR primers:**          |                |
| RT_gyrB_F                   | CTCGTTTACGCTCGGTATCAG |
| SL_gyrB_R                   | ATGATGTTTACGCTGATGAGCG |
| SL_gyrA_qPCR_Pf             | CAGCGTTACGCTGAAAGAA |
| SL_gyrA_qPCR_Prev           | AGCATGACTTCGCTGAGAACC |
| RT_hemX_F                   | CGCCTGAGCGGTATTTTTT |
| RT_hemX_R                   | CCCAACAGGAGCTTATTTAC |
| **Deletion mutations – kan insertions** | |
| Kan_gyrA_Pf                 | CCCCTGCCACGCAATAAACATTTAATCGTCGCTAGCGGTATCAG |
| Kan_gyrA_Prev               | CTTTTGATCACCCTGATAGTAGGATAGCGTATGATGCGGATGAGG |
| Kan_gyrB_Pf                 | GGCCTGCCAGATAGGAGATCGCGATATCAGTAGGATGAGGCTGAC |
| Kan_gyrB_Prev               | AGCGATTACACAAGATTAAATGAGCGAGAAAAAGTTGTAGGCTGAGGA |
| **gyrB::kan insertion downstream of gyrA** | |
| Kan ins gyrB F              | TCAATGTGCCGCCGCACTCGATCCGCCGGGATGATGCGGATGAGG |
| Kan ins gyrB R              | GGAAGAAGCCGCCCTGATGAGGAGATCGCGATATCGATGATGAGG |
| gyrA.int.gyrB::Kan_Pf       | GCCAAAGAAAAAGGCGCATCGCGGCTCAGCGGATGAATGAGG |
| gyrA.int.gyrB::Kan_Prev     | AAGCGATGAGCGATCTGCGGATGAGCAGATTTAATGAGGAGG |
| **gyrA::kan insertion downstream of gyrB** | |
| Kan ins gyrA F              | GGCCTGAGAAAGGCGGATGATCGCGGCTCAGCGGATGAGG |
| Kan ins gyrA R              | AAGCGATGAGCGATCTGCGGATGAGGAGATGAGGATGAGG |
| gyrB.int.gyrA::kan_Pf       | TCAATGTGCCGCCGCACTCGATCCGCCGGGATGATGCGGATGAGG |
| gyrB.int.gyrA::kan_Prev     | GGAAGAAGCCGCCCTGATGAGGAGATCGCGGATGAGGATGAGG |
For kan insertion primers, the black portion is an annealing end and the red portion is an overhanging end.