Functional interactions between gyrase subunits are optimized in a species-specific manner

Received for publication, July 17, 2019, and in revised form, January 3, 2020. Published, Papers in Press, January 17, 2020, DOI 10.1074/jbc.RA119.010245

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Edited by Karin Musier-Forsyth

DNA gyrase is a bacterial DNA topoisomerase that catalyzes ATP-dependent negative DNA supercoiling and DNA decatenation. The enzyme is a heterotetramer comprising two GyrA and two GyrB subunits. Its overall architecture is conserved, but species-specific elements in the two subunits are thought to optimize subunit interaction and enzyme function. Toward understanding the roles of these different elements, we compared the activities of Bacillus subtilis, Escherichia coli, and Mycobacterium tuberculosis gyrase and of heterologous enzymes reconstituted from subunits of two different species. We show that B. subtilis and E. coli gyrase are proficient DNA-stimulated ATPases and efficiently supercoil and decatenate DNA. In contrast, M. tuberculosis gyrase hydrolyzes ATP only slowly and is a poor supercoiling enzyme and decatenase. The heterologous enzymes are generally less active than their homologous counterparts. The only exception is a gyrase reconstituted from mycobacterial GyrA and B. subtilis GyrB, which exceeds the activity of M. tuberculosis gyrase and reaches the activity of the B. subtilis gyrase, indicating that the activities of enzymes containing mycobacterial GyrB are limited by ATP hydrolysis. The activity pattern of heterologous gyrases is in agreement with structural features present: B. subtilis gyrase is a minimal enzyme, and its subunits can functionally interact with subunits from other bacteria. In contrast, the specific insertions in E. coli and mycobacterial gyrase subunits appear to prevent efficient functional interactions with heterologous subunits. Understanding the molecular details of gyrase adaptations to the specific physiological requirements of the respective organism might aid in the development of species-specific gyrase inhibitors.

DNA topology affects vital cellular processes, such as a DNA replication and recombination (reviewed in Ref. 1) and gene expression (2, 3). In vivo, DNA topology is regulated by enzymes of the topoisomerase family (reviewed in Ref. 4), which catalyze the relaxation or introduction of negative and positive supercoils or resolve catenanes (reviewed in Ref. 5). Changes in DNA topology require cleavage of one or both strands of the DNA: type I topoisomerases cleave one strand of their DNA substrate, whereas type II topoisomerases cleave both strands (6). Type I topoisomerases are subdivided into type IA and IB according to the strand that remains covalently bound to the enzyme: type IA topoisomerases form a covalent tyrosyl-ester with the 5’-end of the cleaved DNA, type IB enzymes with the 3’-end (7–10). Type II topoisomerases bind covalently to the 5’-ends of the cleaved DNA (6). They are divided into type IIA and IIB subfamilies according to structural similarities (11).

The type IA topoisomerase reverse gyrase and the type IIA topoisomerase gyrase catalyze the introduction of positive or negative supercoils, respectively. The introduction of DNA supercoils is energetically disfavored, and both enzymes couple these reactions to ATP hydrolysis (reviewed in Refs. 12–14). Relaxation of DNA, on the other hand, is an energetically favorable reaction. Type IB enzymes catalyze relaxation in an ATP-independent reaction through controlled rotation of the DNA (15–17), whereas type II topoisomerases relax DNA in an ATP-dependent reaction through a strand-passage mechanism (reviewed in Ref. 18).

Gyrase is a type IIA topoisomerase present in bacteria, some archaea, and plants that catalyzes DNA supercoiling and decatenation of DNA in an ATP-dependent reaction (19). In the absence of ATP, gyrase is able to relax negatively supercoiled DNA. Most bacteria contain two type IIA topoisomerases, gyrase and topoisomerase IV (Topo IV)2. In bacteria, gyrase removes positive supercoils that accumulate ahead of replication forks (20), whereas Topo IV decatenaes replication intermediates (21). Mycobacteria (22) and other human pathogens, including Campylobacter jejuni, Helicobacter pylori (23), and Treponema pallidum (24), contain only one type IIA topoisomerase, which has to perform both tasks in vivo (25).

The active gyrase heterotetramer is formed by the assembly of two GyrB subunits and two GyrA subunits (26, 27) (Fig. 1). GyrA consists of an N-terminal part, comprising a winged-helix domain (WHD), a tower domain, and a coiled-coil domain, as well as the C-terminal domain (CTD) (28–30). GyrA is a stable dimer in solution (28, 31). The dimer is stabilized by two protein-protein interfaces, formed by the WHDs and by the globular domains at the end of the coiled-coil domains of GyrA (28).

This work was supported by Deutsche Forschungsgemeinschaft Grant KL1153/9-1. The authors declare that they have no conflicts of interest with the contents of this article.

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2 The abbreviations used are: Topo IV, topoisomerase IV; CTD, C-terminal domain (of GyrA); G-segment, gate segment; GHKL, GyrB-Hsp90-histidine/serine protein kinases-MutL; n.s., not significant; NTD, N-terminal domain; TOPRIM, topoisomerase-primase; WHD, winged-helix domain; kDNA, kinetoplast DNA; ADPNP, 5’-adenyl-β,γ-imidodiphosphate.
These interfaces are termed the DNA-gate and the C-gate, respectively. The WHDs contain the catalytic tyrosines for DNA cleavage and religation (6). The GyrB subunit harbors an ATPase domain of the GHKL superfamily (GyrB-Hsp90-histidine/serine protein kinases-MutL), a transducer domain, and a topoisomerase-primase (TOPRIM) domain (32, 33). Binding to GyrA is established by the interaction of the GyrB TOPRIM domains with the WHDs of GyrA, which leads to formation of the DNA-gate (28, 31, 34). GyrB dimerizes on binding of ATP or the nonhydrolyzable analog ADPnP to its ATPase domain (32, 35–37). In the gyrase heterotetramer, GyrB dimerization leads to formation of a third protein-protein interface, termed the N-gate (38–40). The N-gate operates as an ATP-dependent clamp that captures a DNA segment during supercoiling and decatenation (41).

Although the overall architecture of gyrases from different organisms is similar, structural studies have revealed subtle organism- or clade-specific differences in both subunits. The presence of these structural differences suggests a fine-tuned species-specific modulation of the functional interplay between GyrA and GyrB subunits and of DNA supercoiling and decatenation activities of gyrase. GyrB from Escherichia coli (and other Gram-negatives) contains a 170-amino acid insertion in its TOPRIM domain (34) that is absent in GyrB from Gram-positives, such as Bacillus subtilis, and in GyrB from Mycobacteria (Fig. 1 and Table 1). This insert forms a globular domain with an α/β fold and provides contacts with the coiled-coil domain of GyrA; it has been implicated in DNA binding and interdomain communication (34). GyrB from Mycobacterium tuberculosis contains a short insertion within the GHKL domain of GyrA; it has been implicated in DNA binding and interdomain communication (34). GyrA contains an insertion in the tower domain of the NTD, the only exception is a DEEE-loop, which is lacking in M. tuberculosis GyrA (42) (Table 1). This interaction might play a role in stabilizing M. tuberculosis gyrase in a resting state with a wide-open N-gate, which may explain the low ATPase activity of the enzyme (42) and might be related to its role in decatenation. GyrA from E. coli contains a 34-amino acid insertion of uncharacterized function in the coiled-coil domain that is not present in B. subtilis gyrase (29) or the mycobacterial enzyme (Table 1). It forms a small globular domain that packs against the coiled coil and the globular domains at their end that form the C-gate (34) and might contribute to C-gate stabilization. The CTDs of GyrA contain C-terminal tails that are species-specific and regulate the propensity of gyrase to wrap DNA and its supercoiling activity in different ways (43–46).

To shed light on the interdomain communication and the species-specific tuning of gyrase activities, we analyzed the DNA-stimulated ATPase, DNA supercoiling, and decatenation activities of B. subtilis, E. coli, and M. tuberculosis gyrase. We compared these activities with the activities of heterologous enzymes reconstituted from subunits from two different organisms. We show that B. subtilis and E. coli gyrase are proficient DNA-stimulated ATPases and efficiently supercoil and decatenate DNA. M. tuberculosis gyrase, on the other hand, exhibits a low ATPase activity that is not DNA-stimulated and shows low supercoiling and decatenation activities. Heterologous enzymes are generally less active than their homologous counterparts, indicating species-specific optimization of the functional interaction between subunits. The only exception is a heterologous gyrase reconstituted from mycobacterial GyrA and B. subtilis GyrB, which has increased activities compared with the homologous M. tuberculosis enzyme, and comparable activities with those of the B. subtilis enzyme. The activity pattern observed for heterologous gyrase can be explained by structural considerations: B. subtilis gyrase is a minimal enzyme, and its subunits can functionally interact with subunits of other species.
from other organisms. For *E. coli* gyrase and mycobacterial gyrase subunits, on the other hand, the species-specific insertions prevent efficient functional interactions with heterologous subunits.

**Results**

**Supercoiling activities of homologous gyrase**

To investigate the functional interaction of subunits from *B. subtilis, E. coli,* and *M. tuberculosis,* we analyzed the supercoiling activity of homologous gyrase subunits, assembled from GyrA and GyrB from the same organism, and heterologous gyrase subunits from other organisms. Tetrameric gyrase was formed by incubating GyrA and GyrB for 3 min at 37 °C before starting the supercoiling reaction with ATP.

To be able to compare the activities of the different enzymes under identical conditions, we first identified buffer conditions that support supercoiling activity for all three gyrase subunits. Buffer conditions previously used to monitor supercoiling activity of gyrase from *B. subtilis, E. coli,* and *M. tuberculosis* differ: a representative buffer for measuring *E. coli* gyrase activity is composed of 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 10 mM MgCl2, 10% glycerol (compare Refs. 43, 47, and 48), and standard conditions for *B. subtilis* gyrase are 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 10 mM MgCl2 (31, 37, 38, 49). For *M. tuberculosis* gyrase, different buffers have been used by different laboratories (42, 50–53) (see Supporting Methods).

When we tested *M. tuberculosis* gyrase activity in these buffers, we observed the highest activity in a buffer composed of 20 mM Tris/HCl, pH 7.5, 100 mM KOAc, 10 mM MgCl2, 35 μg/ml BSA, 1.2 mM DTT, 10% glycerol (Fig. S1), previously used by Blower et al. (53). We cannot exclude the possibility that *M. tuberculosis* gyrase may show higher activity in other buffers. However, as *B. subtilis* and *E. coli* gyrase also showed robust activity in this buffer (Fig. S2), we performed all subsequent experiments in this buffer.

As a reference for the activities of heterologous gyrase subunits, we next compared the supercoiling activity of the three homologous enzymes (Fig. 2A). At standard concentrations (100 nM gyrase, formed from 200 nM GyrA and 800 nM GyrB), *B. subtilis* and *E. coli* gyrase supercoiled relaxed DNA completely within 30 s. Titration experiments confirmed that GyrA is saturated with GyrB under these conditions (Fig. S3, A and B). Reactions at reduced gyrase concentrations (12.5 nM gyrase; Fig. S3C) show that both enzymes are highly processive. The *E. coli* enzyme catalyzes DNA supercoiling slightly faster than *B. subtilis* gyrase. Mycobacterial gyrase (100 nM, formed from 200 nM GyrA and 400 nM GyrB) reached the end point of the reaction after 15 min (Fig. 2A). Again, we confirmed that GyrA is saturated with GyrB under these conditions in titration experiments (Fig. S3, A and B). The presence of intermediates with different levels of supercoiling at earlier time points indicates a limited processivity of this enzyme (Fig. 2A and Fig. S3, A and B).

**Supercoiling activities of heterologous gyrase**

Heterologous gyrase were formed by incubating GyrA from one organism with GyrB from another organism for 3 min at 37 °C prior to the supercoiling reaction. Reactions with gyrase subunits formed by *B. subtilis* and *E. coli* subsunits were stopped after 5 min. Reactions containing one gyrase subunit from *M. tuberculosis* were stopped after 30 min because of the low supercoiling activity observed for *M. tuberculosis* gyrase (Fig. 3A).

Heterologous gyrase containing GyrA from *B. subtilis* (GyrA<sub>Bs</sub>) and GyrB from *E. coli* (GyrB<sub>Ec</sub>) showed supercoiling
Activities of heterologous gyrase

Figure 3. Supercoiling and decatenation activity of homo- and heterologous gyrase. A, DNA supercoiling. Reactions were performed with 200 nM GyrA and 800 nM GyrB_{Esx} (400 nM for GyrB_{Esx}) in 20 mM Tris/HCl, pH 7.5, 100 mM KOAc, 10 mM MgCl₂, 3 μg/ml BSA, 1.2 mM DTT, 10% glycerol at 37 °C and stopped after 5 min (if only B. subtilis or E. coli subunits were present) or 30 min (if one or both subunits were from M. tuberculosis). GyrA_{Ec} shows the highest activity with its cognate GyrB_{Ec}, slightly reduced activity with GyrB_{Esx} and strongly reduced activity with GyrB_{Mt}. GyrA_{Mt} shows similar activity with the cognate GyrB_{Ec} and with GyrB_{Esx}, but reduced activity with GyrB_{Mt}. GyrA_{Mt} shows highest activity with GyrB_{Esx}, followed by GyrB_{Ec}. Here, the lowest activity is reached with its cognate partner GyrB_{Ec}. B, time dependence of DNA supercoiling by gyrase containing mycobacterial GyrA. Reactions were performed with 200 nM GyrA_{Mt} and 400 nM GyrB in 20 mM Tris/HCl, pH 7.5, 100 mM KOAc, 10 mM MgCl₂, 3 μg/ml BSA, 1.2 mM DTT, 10% glycerol at 37 °C and stopped at the indicated time points. C, time dependence of DNA supercoiling by gyrase containing mycobacterial GyrB. Reactions were performed with 200 nM GyrA and 2000 nM GyrB_{Mt} in 20 mM Tris/HCl, pH 7.5, 100 mM KOAc, 10 mM MgCl₂, 35 μg/ml BSA, 1.2 mM DTT, 10% glycerol at 37 °C and stopped after 5 and 30 min. Bs, B. subtilis; Ec, E. coli; Mt, M. tuberculosis; rel, relaxed DNA; sc, negatively supercoiled DNA. Bands visible as relaxed DNA that are not supercoiled represent nicked DNA.

activity. The presence of intermediates that are not (yet) fully supercoiled points to a reduced processivity of the heterologous enzyme compared with the homologous B. subtilis gyrase. Gyrase with GyrA_{Esx} and GyrB from M. tuberculosis (GyrB_{Mt}), on the other hand, showed only marginal activity. Gyrase harboring GyrA_{Ec} showed supercoiling activity with GyrB_{Esx} from B. subtilis (GyrB_{Esx}), but little activity with GyrB_{Mt}. Gyrase containing mycobacterial GyrA (GyrA_{Mt}) showed supercoiling activity with GyrB_{Esx} and GyrB_{Ec}. Thus, GyrA and GyrB subunits from E. coli and B. subtilis can be interchanged, although gyrase with B. subtilis GyrB and E. coli GyrA shows higher supercoiling activity than the reverse mixture, in agreement with previous findings (54). GyrB_{Esx} cooperates with either GyrA_{Ec} or GyrA_{Mt}, leading to supercoiling activity comparable to that of the homologous B. subtilis gyrase. In contrast, both heterologous gyrases with GyrB_{Esx} were impaired compared with the homologous E. coli gyrase. GyrB_{Mt} with GyrA_{Esx} or GyrA_{Ec} also shows severely reduced supercoiling activity compared with the homologous M. tuberculosis gyrase.

To test whether the reduced activity is due to a lower affinity of the heterologous subunits and incomplete heterotetramer formation, we performed supercoiling reactions as a function of the GyrB concentration, keeping the concentration of GyrA constant (Fig. S4). For heterologous enzymes formed by B. subtilis and E. coli subunits, the reaction was complete after 1 min at 400 nM GyrB, lower than the 800 nM GyrB used for the single-time point reactions in Fig. 3 (Fig. S4A). The same behavior was observed for heterologous gyrases containing GyrA_{Mt} and GyrB_{Esx} or GyrB_{Ec} (Fig. S4B). Thus, in these experiments, the GyrA subunit was saturated with GyrB. In contrast, an increase in the concentration of GyrB_{Esx} led to an increase in the fraction of supercoiled DNA both in combination with GyrA_{Esx} and with GyrA_{Ec}, but the reaction did not reach completion even at the highest concentration (2000 nM; Fig. S4, B and C). Thus, the reduced activity of the heterologous GyrA_{Esx}/GyrB_{Mt} and GyrA_{Ec}/GyrB_{Mt} can in part be ascribed to a reduced assembly of heterotetramers (see below).

Next, we compared the time dependence of supercoiling for heterologous gyrases containing GyrA_{Mt}, which had supercoiled DNA (almost) completely within 30 min (Fig. 3A). Whereas GyrA_{Mt}/GyrB_{Esx} supercoiled DNA completely in 30 s to 1 min, GyrA_{Mt}/GyrB_{Ec} needed 5 min for complete DNA supercoiling (Fig. 3B). Both heterologous enzymes thus catalyze DNA supercoiling faster than the homologous mycobacterial enzyme, which had completely supercoiled the DNA after 5–10 min, pointing to a gain of function. Finally, we also compared the time dependence of supercoiling for heterologous enzymes containing GyrB_{Mt} at saturating concentrations of...
GyrB (Fig. 3C). For these enzymes, GyrA was not saturated with GyrB under the standard conditions in Fig. 3A. The velocity and processivity for supercoiling was virtually identical for GyrABs, GyrBMt, and GyrAEc/GyrBSMt and comparable with the velocity and processivity of the homologous mycobacterial enzyme.

**Homologous and heterologous gyrase activity**

To determine the degree of supercoiling of the reaction products generated by homo- and heterologous gyrase, we resolved the highly supercoiled species by agarose gel electrophoresis in the presence of 10 μg/ml chloroquine (Fig. S5). In the absence of chloroquine, highly supercoiled species migrate as one band with high electrophoretic mobility. Intercalation of chloroquine into DNA leads to a decrease in twist and increase in writhe. Negatively supercoiled DNA becomes more relaxed and migrates more slowly in the presence of chloroquine, whereas relaxed DNA becomes more positively supercoiled and migrates faster than in the absence of chloroquine (see controls). In the presence of chloroquine, highly negative supercoiled DNA species can thus be separated according to the degree of supercoiling.

The reaction products of mycobacterial gyrase showed decreased mobility in the presence of chloroquine. For the B. subtilis enzyme, the mobility was moderately reduced, and for E. coli gyrase only slightly. Thus, E. coli gyrase reaches the highest supercoiling density and mycobacterial gyrase the lowest. The heterologous gyrase also showed differences with respect to the level of supercoiling reached. Gyrase containing E. coli GyrB reached a high negative supercoiling density with its cognate GyrAEc, but the supercoiling level was reduced strongly with the noncognate GyrABs and GyrAMt. Gyrase containing B. subtilis GyrB showed a slightly lower level of supercoiling with GyrAEc than with the cognate GyrABs, with GyrAMt, the degree of supercoiling was further reduced. GyrBMt showed lower levels of supercoiling with its cognate GyrAMt and showed little or no supercoiling with GyrAEc and GyrABs, respectively. Compared from the perspective of GyrA, GyrAEc reaches the highest supercoiling density with its cognate GyrB Ec. With GyrB Ec, the final supercoiling density is reduced. With GyrBMt, little supercoiling activity is observed. GyrABs also reaches the highest supercoiling density with its cognate partner, GyrB Ec; the supercoiling density reached is lower; with GyrBMt, little supercoiling activity is observed. GyrAMt reaches only a low level of negative supercoiling with the cognate GyrBEc.

Overall, both E. coli and B. subtilis gyrase supercoiling DNA rapidly, but the E. coli enzyme reaches a higher degree of supercoiling. M. tuberculosis gyrase is the least efficient enzyme and reaches the lowest level of supercoiling. Heterologous enzymes containing E. coli and B. subtilis subunits show supercoiling activity. Enzymes containing mycobacterial GyrA show supercoiling activity with GyrB from all three organisms, whereas mycobacterial GyrB does not associate efficiently with the noncognate GyrA subunits from E. coli and B. subtilis, but shows supercoiling activity with GyrA subunits from all three species when provided under saturating conditions.

**Decatenation activity of homologous and heterologous gyrase activity**

In most prokaryotes, supercoiling and decatenation reactions are performed by gyrase and Topo IV, respectively. Mycobacteria possess gyrase as the only type IIA topoisomerase (22); the enzyme thus has to perform both activities in vivo (25). To compare the decatenation activities, we also monitored the time dependence of decatenation for the homologous enzymes (Fig. 2B). Whereas B. subtilis and E. coli gyrase completely decatenated kinetoplast DNA (kDNA) within 15 min, decatenation by M. tuberculosis gyrase was slower, and the reaction was not complete after 60 min. All homologous gyrase also (rapidly) supercoiled the decatenated minicircles.

For the heterologous gyrase, the decatenation reaction was stopped after 5 and 60 min, respectively, to take into account the different decatenation velocities of the three homologous enzymes (Fig. 3D). Whereas homologous B. subtilis gyrase exhibited robust decatenation activity, gyrase reconstituted from GyrABs and GyrAEc showed no detectable decatenation after 5 min and only limited decatenation within 60 min; with GyrBMt, no decatenation was observed. Mixing of GyrBS with GyrAEc or GyrAMt resulted in decatenation within 5 min. Gyrase with GyrBS showed the same robust decatenation activity as the homologous E. coli gyrase, whereas no decatenation was observed with GyrBMt. GyrAEc showed modest decatenation (and modest subsequent supercoiling) activity with GyrABs; decatenation was equally modest with GyrAMt. Gyrase containing GyrAMt and GyrBEc showed the same low decatenation activity as the homologous mycobacterial enzyme; with GyrBMt, the decatenation activity was similar to the robust activity of the homologous B. subtilis enzyme. GyrBMt did not decatenate DNA in conjunction with either GyrAEc or GyrABs.

Taken together, GyrA and GyrB subunits from B. subtilis and E. coli are partially interchangeable for decatenation activity. Mycobacterial GyrA can decatenate DNA with GyrB from all three organisms; the highest activity is reached with GyrBS. In contrast, mycobacterial GyrB cannot cooperate functionally with GyrA from B. subtilis or E. coli, which is due to inefficient heterotetramer formation. The activity pattern of heterologous enzymes is thus similar for DNA supercoiling and decatenation.

**DNA-stimulated ATPase activity**

To dissect intersubunit communication through the coupling of DNA binding to ATP hydrolysis, the steady-state ATPase activity of homologous and heterologous gyrase was examined in the absence and presence of pUC18 (Fig. 4, Table 2, and Tables S1 and S2). These experiments were performed with an excess of GyrA over GyrB to ensure saturation of the ATPase (37). Original data and analyses with the Michaelis–Menten or Hill equation are shown in Fig. S6.

**Homologous gyrazes**

Whereas B. subtilis and E. coli gyrase showed DNA-dependent ATPase activity with increased kcat and decreased KM,ATP values in the presence of DNA, mycobacterial gyrase did not show significant changes in kcat and KM,ATP in the presence of DNA (Fig. 4, A and B, Table 2, and Tables S1 and S2). B. subtilis
gyrase hydrolyzed ATP with a $k_{cat}$ value of 0.09 s$^{-1}$ in the absence and 0.56 s$^{-1}$ in the presence of DNA, corresponding to a 5.9-fold DNA stimulation ($p < 0.01$), in agreement with previous data (37, 55). For E. coli gyrase, the unstimulated $k_{cat}$ was 0.25 s$^{-1}$. In the presence of DNA, this value increased 2.9-fold ($p < 0.001$) to 0.75 s$^{-1}$. Again, these values are in the range of values reported previously (56–58). M. tuberculosis gyrase hydrolyzed ATP with $k_{cat} = 0.05$ s$^{-1}$ in the absence and 0.06 s$^{-1}$ in the presence of DNA. The $k_{cat}$ values are similar to values reported previously (42, 59). The DNA stimulation is modest (1.2-fold; $p < 0.05$); others have observed a stronger stimulation of ATP hydrolysis in the presence of DNA (42). The $k_{cat}$ values for E. coli and B. subtilis gyrases in the presence of DNA were similar ($p > 0.05$, n.s.; Table S4). M. tuberculosis gyrase showed the lowest $k_{cat}$ values, both in the absence and presence of DNA. The $K_{M, ATP}$ value of E. coli gyrase was lower than the value for

**Table 2**

Michaelis–Menten parameters for homologous and heterologous gyrases, grouped according to the same GyrB subunit

| GyrA | GyrB | DNA | $k_{cat}$ | $K_m$ | $K_{cat}/K_m$ | $n_{Hill}$ |
|------|------|-----|---------|-------|-------------|-----------|
| Bs   | Bs   | −   | 0.09 ± 0.01 | 0.66 ± 0.09* | 0.14 ± 0.02 | 2.1 ± 0.3 |
| Bs   | Bs   | +   | 0.56 ± 0.28 | 0.33 ± 0.10* | 1.70 ± 0.84 | 2.0 ± 0.5 |
| Ec   | Bs   | −   | 0.05 ± 0.02 | 0.08 ± 0.26 | 0.06 ± 0.02 | NA        |
| Ec   | Bs   | +   | 0.21 ± 0.04 | 0.45 ± 0.06 | 0.48 ± 0.08 | NA        |
| Mt   | Bs   | −   | 0.04 ± 0.006 | 1.2 ± 0.76 | 0.03 ± 0.005 | NA        |
| Mt   | Bs   | +   | 0.58 ± 0.09 | 0.49 ± 0.13 | 1.2 ± 0.19 | NA        |
| Ec   | Ec   | −   | 0.25 ± 0.14 | 0.44 ± 0.15 | 0.57 ± 0.31 | NA        |
| Ec   | Ec   | +   | 0.75 ± 0.17 | 0.12 ± 0.11 | 6.1 ± 1.4 | NA        |
| Bs   | Ec   | −   | 0.29 ± 0.10 | 0.65 ± 0.08 | 0.64 ± 0.22 | NA        |
| Bs   | Ec   | +   | 0.47 ± 0.14 | 0.47 ± 0.08 | 1.01 ± 0.29 | NA        |
| Mt   | Ec   | −   | 0.21 ± 0.05 | 0.43 ± 0.12 | 0.49 ± 0.13 | NA        |
| Mt   | Ec   | +   | 0.46 ± 0.17 | 0.46 ± 0.19 | 1.01 ± 0.37 | NA        |
| Mt   | Mt   | −   | 0.05 ± 0.01 | 0.96 ± 0.26* | 0.05 ± 0.01 | 1.9 ± 0.2 |
| Mt   | Mt   | +   | 0.06 ± 0.01 | 0.52 ± 0.15 | 0.12 ± 0.01 | NA        |
| Bs   | Mt   | −   | 0.08 ± 0.01 | 0.76 ± 0.15* | 0.11 ± 0.02 | 2.0 ± 0.6 |
| Bs   | Mt   | +   | 0.12 ± 0.03 | 0.57 ± 0.06* | 0.21 ± 0.05 | 1.9 ± 0.3 |
| Ec   | Ec   | −   | 0.02 ± 0.018 | 0.66 ± 0.33 | 0.037 ± 0.027 | NA        |
| Ec   | Ec   | +   | 0.05 ± 0.015 | 0.80 ± 0.31 | 0.068 ± 0.019 | NA        |

Figure 4. ATPase activity of homo- and heterologous gyrases. A, C, E, and G, $k_{cat}$ values for homologous and heterologous gyrases. B, D, F, and H, $K_m$ values for homologous and heterologous gyrases. A and B, homologous gyrases; C and D, heterologous gyrases with the B. subtilis GyrB subunit; E and F, heterologous gyrases with the E. coli GyrB subunit; G and H, heterologous gyrases with the M. tuberculosis GyrB subunit. Values from 3–7 experiments are shown; error bars, S.D. Note that in C–H, the first two bars show the data for the homologous enzyme. See “Results” for more information. Experiments were performed with 0.5 μM GyrA, 0.1 μM GyrB, 0.1 μM negatively supercoiled pUC18 at 37 °C in 20 mM Tris/HCl, pH 7.5, 100 mM KOAc, 10 mM MgCl$_2$, 35 μg/ml BSA, 1.2 mM DTT, 10% (v/v) glycerol and ATP concentrations from 0 to 5 μM. See Fig. S6 and Table S1 for original data and $k_{cat}$ and $K_m$ values from individual experiments. Data shown here are summarized for enzymes with the same GyrB subunit. See Fig. S7 for a comparison of $k_{cat}$ and $K_m$ values for gyrases with the same GyrA subunit: Bs, B. subtilis; Ec, E. coli; Mt, M. tuberculosis; n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 

In the presence of DNA, this value increased 2.9-fold ($p < 0.001$) to 0.75 s$^{-1}$. Again, these values are in the range of values reported previously (56–58). M. tuberculosis gyrase hydrolyzed ATP with $k_{cat} = 0.05$ s$^{-1}$ in the absence and 0.06 s$^{-1}$ in the presence of DNA. The $k_{cat}$ values are similar to values reported previously (42, 59). The DNA stimulation is modest (1.2-fold; $p < 0.05$); others have observed a stronger stimulation of ATP hydrolysis in the presence of DNA (42). The $k_{cat}$ values for E. coli and B. subtilis gyrases in the presence of DNA were similar ($p > 0.05$, n.s.; Table S4). M. tuberculosis gyrase showed the lowest $k_{cat}$ values, both in the absence and presence of DNA. The $K_{M, ATP}$ value of E. coli gyrase was lower than the value for
the B. subtilis enzyme, both in the absence and in the presence of DNA (Table S4; p < 0.05). The decrease in $K_{M,\text{ATP}}$ with DNA was 3.6-fold for E. coli gyrase ($p < 0.001$), and 2.0-fold ($p < 0.01$) for B. subtilis gyrase (Table S2). M. tuberculosis gyrase had the highest $K_{M,\text{ATP}}$ value, both in the absence and presence of DNA, and the lowest decrease in the presence of DNA (1.8-fold, $p < 0.05$). The $K_{M,\text{ATP}}$ values are similar to values determined by others (59). Altogether, the catalytic efficiency $k_{cat}/K_M$ in the presence of DNA is highest for the E. coli enzyme (6.1 s$^{-1}$ mm$^{-1}$; Table 2), followed by B. subtilis gyrase (1.7 s$^{-1}$ mm$^{-1}$). Mycobacterial gyrase is a poor ATPase because of a lower turnover number and a higher $K_{M,\text{ATP}}$; its catalytic efficiency $k_{cat}/K_M$ is only 0.12 s$^{-1}$ mm$^{-1}$.

Heterologous gyrases

Gyrases containing the GyrB<sub>ms</sub> subunit showed a prominent increase in $k_{cat}$ in the presence of DNA with GyrA<sub>Ec</sub> and GyrA<sub>Mt</sub> (Fig. 4C, Table 2, and Tables S1, S2, and S5). The enzyme with GyrA<sub>Ec</sub> showed a lower $k_{cat}$, and the enzyme with GyrA<sub>Mt</sub> showed a similar $k_{cat}$ value compared with the homologous B. subtilis gyrase. All enzymes reached similar $K_{M,\text{ATP}}$ values in the presence of DNA (Fig. 4D, Table 2, and Tables S1, S2, and S5). Whereas gyrase with GyrB<sub>ms</sub> and GyrA<sub>Ec</sub> showed a reduced $K_{M,\text{ATP}}$ in the presence of DNA ($p < 0.05$), gyrase with GyrA<sub>Mt</sub> did not ($p > 0.05$). The catalytic efficiencies were 0.48 s$^{-1}$ mm$^{-1}$ (with GyrA<sub>ms</sub>) and 1.2 s$^{-1}$ mm$^{-1}$ (with GyrA<sub>Mt</sub>), compared with 1.7 s$^{-1}$ mm$^{-1}$ for the homologous B. subtilis enzyme.

Heterologous gyrase containing GyrB<sub>Ec</sub> showed different levels of DNA stimulation in combination with the three GyrA subunits (Fig. 4E and F, Table 2, and Tables S1, S2, and S5). The $k_{cat}$ value in the presence of DNA did not significantly increase with GyrA<sub>Ms</sub> ($p > 0.05$, n.s.) and only increased slightly with GyrA<sub>ms</sub> (1.6-fold; $p < 0.05$), compared with a 2.9-fold increase for the homologous E. coli enzyme ($p < 0.001$). The heterologous enzymes also did not show a decrease in $K_{M,\text{ATP}}$ in the presence of DNA ($p > 0.05$, n.s.), whereas the homologous E. coli gyrase exhibited a 3.6-fold decrease ($p < 0.01$). The catalytic efficiency of 6.1 s$^{-1}$ mm$^{-1}$ for E. coli gyrase decreases to 1.0 s$^{-1}$ mm$^{-1}$ for the two heterologous variants.

The turnover numbers $k_{cat}$ for heterologous gyrase containing GyrB<sub>ms</sub> were modest, as for the homologous M. tuberculosis enzyme (Fig. 4G, Table 2, and Tables S1, S2, and S5), and showed only a small increase (1.4–2.2-fold) in the presence of DNA. The enzyme with GyrA<sub>Ec</sub> showed no decrease in $K_{M,\text{ATP}}$ with DNA ($p > 0.05$, n.s.; Fig. 4H, Table 2, and Tables S1, S2, and S5); the enzyme with GyrA<sub>ms</sub> had a slightly decreased $K_{M,\text{ATP}}$ in the presence of DNA ($p < 0.05$). The catalytic efficiency of the enzyme with GyrA<sub>ms</sub> ($k_{cat}/K_M = 0.21$ s$^{-1}$ mm$^{-1}$) exceeded the efficiency of the homologous M. tuberculosis enzyme ($k_{cat}/K_M = 0.12$ s$^{-1}$ mm$^{-1}$) slightly, and the enzyme with GyrA<sub>Ec</sub> had the lowest value with $k_{cat}/K_M = 0.07$ s$^{-1}$ mm$^{-1}$.

Although the ATPase activity is provided by the GyrB subunit, it is also informative to compare heterologous enzymes containing the same GyrA subunit, combined with GyrB subunits from the different organisms (Fig. S7 and Tables S3 and S6). Gyrase containing GyrA<sub>ms</sub> shows a DNA-stimulated ATPase activity with a prominent increase in $k_{cat}$ and decrease in $K_{M,\text{ATP}}$ with its cognate GyrB<sub>ms</sub> subunit. In combination with GyrB<sub>Ec</sub> and GyrB<sub>Mt</sub>, the DNA-induced changes in $k_{cat}$ and $K_{M,\text{ATP}}$ are small or not significant. In contrast, GyrA<sub>Ec</sub> shows a DNA-dependent increase in $k_{cat}$ with all three GyrB subunits. The $K_{M,\text{ATP}}$ value is decreased in the presence of DNA when GyrB is from E. coli or B. subtilis ($p < 0.01$, $p < 0.05$); with GyrB<sub>Ms</sub>, the $K_{M,\text{ATP}}$ value is not DNA-dependent. Finally, GyrA<sub>Mt</sub> shows no DNA-dependent increase in $k_{cat}$ or decrease in $K_{M,\text{ATP}}$ with GyrB<sub>Ec</sub>. With GyrB<sub>Ms</sub>, $k_{cat}$ is slightly DNA-dependent; with GyrB<sub>Mt</sub>, both $k_{cat}$ and $K_{M,\text{ATP}}$ are slightly DNA-dependent.

In summary, GyrB<sub>ms</sub> and GyrB<sub>Ec</sub> are both robust ATPases. GyrB<sub>ms</sub> cooperates most efficiently with its cognate GyrA subunit and with mycobacterial GyrA, but also with GyrA<sub>Ec</sub>. GyrB<sub>Ec</sub> cooperates with GyrA from all three organisms, but shows little coupling of ATP hydrolysis and DNA binding when it is paired with a noncognate GyrA. GyrB<sub>ms</sub> is a poor ATPase with its cognate GyrA subunit, as well as with GyrA<sub>bs</sub> or GyrA<sub>Ec</sub>. The homologous enzyme shows moderate stimulation of its ATPase activity by DNA. Paired with GyrA<sub>ms</sub>, the catalytic efficiency is higher than for the homologous enzyme; with GyrA<sub>Ec</sub>, it is lower. Overall, the subunits from B. subtilis and M. tuberculosis appear to be more similar than E. coli and M. tuberculosis in terms of DNA-dependent ATP hydrolysis.

Subunit interaction and heterotetramer formation

The activity data of homo- and heterologous gyrases (Fig. 3) indicate that GyrB<sub>ms</sub> and GyrB<sub>Ec</sub> functionally interact with all other GyrA subunits, although to different extents. Thus, these subunits must also interact physically. Gyrase reconstituted from GyrB<sub>Ms</sub> and GyrA<sub>bs</sub> or GyrA<sub>Ec</sub> is the only case where little supercoiling or decatenation activity is detected. These enzymes also do not show DNA stimulation of ATP hydrolysis. We have shown above that heterotetramers are formed under the conditions used in the activity tests between subunits from the same organism, as well as in heterologous enzymes formed from E. coli and B. subtilis subunits and heterologous enzymes containing GyrA<sub>Ms</sub> (Figs. S3, S4, A and B). In contrast, the activity of heterologous gyrases formed by GyrA<sub>ms</sub> and GyrA<sub>bs</sub> or GyrA<sub>Ec</sub> increases when the GyrB concentration is increased (Fig. S4, B and C), pointing to a reduced interaction between these subunits and incomplete heterotetramer formation under conditions of the supercoiling and decatenation reaction.

To further test whether the mycobacterial subunits can interact with B. subtilis and E. coli subunits, we performed complementation assays of homologous B. subtilis and E. coli gyrases by an excess of GyrB<sub>Ms</sub> and of M. tuberculosis gyrase by an excess of B. subtilis and E. coli GyrB (Fig. 5, A and B). The supercoiling activities of B. subtilis and E. coli gyrases were not affected by a 4- or 10-fold excess of GyrB<sub>Ms</sub>. However, the relaxation activity of B. subtilis gyrase was inhibited by mycobacterial GyrB, indicating that GyrB<sub>Ms</sub> can compete with GyrB<sub>ms</sub> for the interaction with GyrA<sub>bs</sub> (Fig. 5A). For E. coli gyrase, no inhibition by mycobacterial GyrB was detected, indicating that GyrB<sub>ms</sub> and GyrA<sub>Ec</sub> do not interact. In the reverse experiment with mycobacterial gyrase and an excess of GyrB<sub>ms</sub> or GyrB<sub>Ec</sub> (Fig. 5B), the supercoiling activity of mycobacterial gyrase was
not altered. This is expected, as any heterologous enzymes formed would also show supercoiling activity. In contrast, the negligible relaxation activity of mycobacterial gyrase was increased in the presence of excess heterologous GyrB, demonstrating that GyrAMt can capture GyrBBs and GyrBEc to generate a heterologous enzyme with relaxation activity.

To test directly whether cognate and noncognate subunits interact physically, we performed pulldown assays with His-tagged GyrA subunits immobilized on Ni²⁺-nitrilotriacetic acid–Sepharose (Fig. S8). However, conclusions on subunit interactions were not possible due to the significant nonspecific binding of GyrA and GyrB to the chromatographic material. We therefore analyzed the formation of heterologous gyrases containing one mycobacterial subunit by size-exclusion chromatography with 4 μM GyrA and 0.8 μM GyrB (Fig. 5, C and D), reflecting the concentration ratios used in ATPase assays (Fig. 4). The retention times of the individual GyrB and GyrA subunits from the three different organisms were determined in control experiments (Fig. S9). All GyrB subunits eluted at ~12 ml; the GyrA subunits eluted between 9.5 ml (Bs and Ec) and 10.5 ml (Mt). The elution of the gyrase heterotrimer overlaps with the elution of the GyrA dimer. Therefore, we used the shift in elution volume of GyrB from 12 ml (free GyrB) to 10 ml (GyrB bound to GyrA) as a readout for complex formation and co-elution of GyrB and GyrA. To directly detect GyrB in fractions from size-exclusion chromatography, we analyzed the protein content of individual fractions by SDS-PAGE (Fig. 5, C and D). GyrBMt co-eluted with GyrAMt as well as with
GyrA<sub> Bs</sub> no co-elution was observed with GyrA<sub> Ec</sub> (Fig. 5C). Thus, GyrB<sub>Mt</sub> forms heterotetramers with GyrA<sub>Mt</sub> but not with GyrA<sub>Ec</sub> under these conditions. Both GyrB<sub>Mt</sub> and GyrB<sub> Bs</sub> co-eluted with GyrA<sub>Mt</sub> (Fig. 5D), in agreement with the observed subunit interaction in titration experiments (Fig. S4B). GyrA<sub>Mt</sub> and GyrB<sub>Ec</sub> have similar electrophoretic mobilities and cannot be separated by SDS-PAGE. In this case, we therefore had to interpret the chromatogram directly. Compared with the chromatograms of the individual subunits (Fig. S9), it is evident that a large fraction of GyrB<sub>Bs</sub> elutes as free GyrB (peak at ~12 ml), leaving only a small fraction of GyrB<sub>Ec</sub> to co-elute with GyrA<sub>Mt</sub> (at ~10 ml; Fig. 5D). From these experiments, we can therefore conclude that GyrA<sub>Mt</sub> forms heterotetramers with GyrB<sub>Bs</sub> but not (or to a lesser extent) with GyrB<sub>Ec</sub> under these conditions.

Altogether, the interaction studies show that mycobacterial GyrA can physically interact with <i>B. subtilis</i> and (to a lesser extent) with <i>E. coli</i> GyrB. Similarly, mycobacterial GyrB interacts with GyrA<sub>Bs</sub> but not to the same extent with GyrA<sub>Ec</sub>.

Discussion

Within the set of gyrases we have chosen for comparison in this study, <i>B. subtilis</i> gyrase is a “minimal” gyrase: it contains none of the insertions present in <i>E. coli</i> and mycobacterial gyrase (see Figs. 1 and 6), and its GyrA subunit has a short version of the C-tail. Differences of <i>B. subtilis</i> gyrase activities from the activity of <i>M. tuberculosis</i> gyrase must be related to the DEEE loop in GyrA<sub>Mt</sub> and the C-loop in the GHKL domain of GyrB<sub>Mt</sub>. Differences from <i>E. coli</i> gyrase, on the other hand,
Activities of heterologous gyrases

must be caused by the uncharacterized insert in the coiled-coil region of GyrAEC by the insertion in the TOPRIM domain of GyrBEC characterized previously (34), and/or by the longer C-tail of E. coli GyrA. A comparison of E. coli and M. tuberculosis gyrases is more complex, as these enzymes differ with respect to a number of elements: the DEEE- and C-loops, the long and short C-tails of the GyrA CTDs, the insert in the coiled coil, and the insert in the TOPRIM domain.

Homologous enzymes differ in catalytic activities

Both E. coli and B. subtilis gyrase show DNA-stimulated ATPase activity with a prominent decrease in $k_{cat,ATP}$ and increase in $k_{cat}$ in the presence of DNA. The DNA-stimulated $k_{cat}$ values for E. coli and B. subtilis gyrase are similar; these enzymes also catalyze DNA supercoiling and decatenation with similar velocities. In contrast, M. tuberculosis gyrase is the slowest ATPase; its ATPase activity shows little DNA stimulation. Furthermore, it is the slowest supercoiling enzyme and the slowest decatenase of the three gyrase homologs studied here. This observation is in contrast to previous studies, which reported similar supercoiling activities of M. tuberculosis and E. coli but a higher decatenase activity of mycobacterial gyrase compared with the E. coli enzyme (25). The discrepancy might be due to different reaction conditions used for these enzymes in previous work, whereas we used identical buffers for all three gyrase homologs in this work. Overall, the low supercoiling and ATPase activities of mycobacterial gyrase are consistent with the timescales reported previously (42, 60). Altogether, gyrase from B. subtilis and E. coli show similar efficiencies in DNA-stimulated ATP hydrolysis, DNA supercoiling, and decatenation, whereas M. tuberculosis gyrase catalyzes all three reactions with much lower efficiency. Whereas the E. coli and (to a lower extent) B. subtilis enzymes are evolutionarily optimized for high catalytic efficiencies supporting rapid cell division, mycobacteria as a much more slowly growing species might be able to afford a less efficient gyrase. Mycobacterial gyrase might thus constitute an evolutionary compromise to provide both functions provided by the only type IIA topoisomerase in mycobacteria.

The final level of supercoiling is higher for E. coli gyrase than for B. subtilis gyrase, whereas M. tuberculosis gyrase reaches the lowest supercoiling level. These differences may reflect different supercoiling levels of genomic DNA of these bacteria in vivo. Such a connection has been demonstrated for E. coli and Salmonella typhimurium, where the higher superhelical density of genomic DNA in E. coli compared with S. typhimurium has been linked to different levels of supercoiling reached by the respective gyrases (46). The supercoiling densities of the mycobacterial and B. subtilis genomic DNA in vivo are unknown, precluding a comparison of supercoiling levels in vivo with end points reached in vitro. The high negative supercoiling density of DNA in E. coli might explain why the mycobacterial gyrase does not relax negatively supercoiled plasmids isolated from E. coli.

It has been shown previously that the end point of the supercoiling reaction is determined by the C-tail following the CTD of GyrA (43, 45, 46). B. subtilis and M. tuberculosis GyrA both have a short C-tail, whereas the C-tails of E. coli and S. typhimurium GyrA are longer. The effect of the C-tail has been analyzed in E. coli and M. tuberculosis GyrA (43, 44), in B. subtilis GyrA (45), and in E. coli and S. typhimurium GyrA (46). The end point of the supercoiling reaction is indeed different between the short-tail enzymes from B. subtilis and M. tuberculosis gyrase on one hand and the long-tail enzyme from E. coli gyrase on the other hand. However, supercoiling end points also differ between the short-tail enzymes from B. subtilis and M. tuberculosis gyrase (this work), and between the long-tail gyrases from E. coli and S. typhimurium (46). Thus, the regulation of the supercoiling end point must be more complex and likely involves elements other than just the C-terminal tail.

Heterologous enzymes are less efficient than their homologous counterparts

In general, all heterologous gyrases studied here are less efficient than their homologous counterparts in DNA-stimulated ATP hydrolysis, DNA supercoiling, and decatenation, pointing to an evolutionarily optimized functional interaction in the cognate pairs. Functional and hence physical exchange between E. coli and B. subtilis subunits is (at least partially) possible. Both heterologous enzymes show DNA-stimulated ATPase activity as well as supercoiling and decatenation. Notably, GyrBbs and GyrBks show similar rate constants of ATP hydrolysis in these enzymes, which are coupled productively to the action of the noncognate GyrA subunit. Interestingly, the heterologous enzyme with higher supercoiling and decatenation activities, GyrAEC/GyrBbs, shows ATP hydrolysis rates similar to those of the homologous B. subtilis enzyme, whereas the inverse enzyme GyrAbS/GyrBEC with lower supercoiling and decatenation activities has a reduced ATPase activity compared with the homologous E. coli enzyme. In addition, the ATPase activity of the heterologous GyrAEC/GyrBBS enzyme with the higher supercoiling activity is reduced, both in the absence and presence of DNA, but stimulated by DNA. In contrast, the inverse GyrAEC/GyrBbs enzyme has a higher $k_{cat}$ but hydrolysis is barely stimulated by DNA, which points to reduced coupling. On a structural level, the different activities show that GyrBbs tolerates the insertion in the coiled-coil domain of GyrAEC, whereas the presence of the insertion in the TOPRIM domain of GyrBEC has adverse consequences for the functional interaction with GyrAEC. Thus, the insert in GyrAEC may play merely a structural and/or stabilizing role, whereas the insertion in the TOPRIM domain in GyrBEC exerts a functional role in interdomain communication (34), which is optimized for the cooperation with its cognate GyrA, but not with B. subtilis GyrA.

Previous studies of heterologous gyrases reconstituted from E. coli and B. subtilis subunits also report activity. Orr et al. (61) and Gubaev et al. (54) detected activity for gyrase reconstituted from GyrAEC and GyrBES, whereas the inverse combination of GyrAEC and GyrBBS leads to inactive enzyme. This is in qualitative agreement with the observations we present here, although the inverse GyrAEC/GyrBBS enzyme still shows appreciable supercoiling activity, possibly due to the different buffer conditions and subunit ratios used and due to longer reaction times. Two other studies comparing the activities of heterologous enzymes reconstituted from E. coli and S. typhimurium (St) or Xanthomonas albilineans (Xa) subunits (46, 62) also observed
supercoiling activity only for one combination: the heterolo-
gous enzymes containing the GyrB\textsubscript{Ec} subunit in combination with either GyrA\textsubscript{Mt} or GyrA\textsubscript{Xa} yielded a functional enzyme. In contrast, the heterologous enzymes reconstituted from GyrA\textsubscript{Ec} and GyrB\textsubscript{Bs} or GyrB\textsubscript{Xa} were supercoiling-deficient. \textit{E. coli} and \textit{S. typhimurium} GyrA and GyrB are highly similar, and both contain the insertions in the tower and TOPRIM domains characteristic of gyrases from Gram-negatives. The \textit{X. albilineans} enzyme is more distant in sequence from \textit{E. coli} gyrase: the GyrA and GyrB subunits also contain the insert in the tower and TOPRIM domains but additionally feature two insertions within the GyrA CTD and a short insertion in the ATPase domain of GyrB. How these structural differences are related to the differences in activities is unclear.

In contrast to the partial compatibility of \textit{B. subtilis} and \textit{E. coli} subunits, neither \textit{E. coli} nor \textit{B. subtilis} GyrA cooperate efficiently with mycobacterial GyrB, setting GyrB\textsubscript{Mt} apart. Titration experiments show that the subunits interact, but even under saturating conditions, the supercoiling activity is slow and limited by the slow ATP hydrolysis by GyrB\textsubscript{Mt}. On the other hand, \textit{M. tuberculosis} GyrA functions both in combination with \textit{E. coli} and \textit{B. subtilis} GyrB, indicating that the rapid DNA-stimulated hydrolysis of ATP by GyrB\textsubscript{Ec} and GyrB\textsubscript{Bs} can be coordinated with the action of GyrA\textsubscript{Mt}. Furthermore, gyrase reconstituted from GyrA\textsubscript{Mt} and GyrB\textsubscript{Bs} is the only example where the combination of noncognate subunits leads to a gain of function compared with the homologous counterpart: the heterologous enzyme shows a higher DNA-stimulated ATPase activity as well as higher supercoiling and decatenation activities than the homologous mycobacterial enzyme. This behavior further supports the notion that mycobacterial gyrase is not optimized for maximum activity and catalytic efficiency.

**Structural reasons for the functional interplay between heterologous gyrase subunits**

The activity pattern (Fig. 6) observed for the heterologous gyrases shows that the insertion in the TOPRIM domain of \textit{E. coli} GyrB constitutes no or only a minor hindrance for the functional interaction with GyrA\textsubscript{Mt} or GyrA\textsubscript{Bs}, respectively. Examination of the structural data available reveals that the presence of the insert in \textit{E. coli} GyrB and of the DEEE-loop in GyrA\textsubscript{Mt} is compatible without causing steric hindrance (Fig. 6). \textit{B. subtilis} GyrA does not contain any elements that could sterically interfere with the insert in GyrA\textsubscript{Ec}. On the other hand, the insertion in the coiled-coil domain in \textit{E. coli} GyrA does not interfere with a functional interaction with GyrB\textsubscript{Bx}, but prevents the cooperation with GyrB\textsubscript{Mt}. Again, the lack of insertions in \textit{B. subtilis} GyrB explains why GyrB\textsubscript{Bs} can tolerate the insertion in GyrA\textsubscript{Ec} in the heterologous enzyme. In the recently reported cryo-EM structure of mycobacterial gyrase, the ATPase domains of the two GyrB\textsubscript{Mt} subunits are folded back, such that they interact with the DEEE-loop in GyrA\textsubscript{Mt}, through the C-loop inserted into the ATPase domain (42). Although such an arrangement would bring the C-loop in GyrB\textsubscript{Mt} closer to the insert in \textit{E. coli} GyrA, no steric hindrance between these elements is evident from the structural model (Fig. 6). The conventional subunit arrangement captured in the structures of \textit{Thermus thermophilus} (39) and \textit{E. coli} gyrase (63) or \textit{Saccharomyces cerevisiae} topoisomerase II (64) also does not predict a clash between the C-loop in GyrB\textsubscript{Mt} and the insertion in GyrA\textsubscript{Ec} (Fig. 6). Although the C-loop of one GyrB\textsubscript{Mt} subunit would lead to a steric hindrance with one of the CTDs, this effect does not appear to be detrimental for activity, as the heterologous GyrA\textsubscript{Ec}/GyrB\textsubscript{Mt} enzyme and the homologous mycobacterial gyrase supercoil and decatenate DNA despite this potential clash. On the other hand, it is conceivable that the longer C-tail of \textit{E. coli} GyrA may cause a more severe interference and prevent the interaction between GyrA\textsubscript{Ec} and GyrB\textsubscript{Mt}.

The only clear exception to the generally observed loss of function is the heterologous gyrase reconstituted from GyrA\textsubscript{Mt} and GyrB\textsubscript{Bs}, which shows higher supercoiling and decatenation activities than the homologous mycobacterial enzyme and activity comparable with that of \textit{B. subtilis} gyrase. GyrB\textsubscript{Mt} also shows similar catalytic efficiencies in ATP hydrolysis with GyrA\textsubscript{Mt} and GyrA\textsubscript{Bx}, indicating that GyrA\textsubscript{Mt} and GyrA\textsubscript{Bs} are closely related and functionally interchangeable. The only difference between these subunits is the DEEE-loop present in GyrB\textsubscript{Mt}. In its absence, neither supercoiling nor decatenation activities are affected.

**Conclusions**

We show here that, in contrast to \textit{B. subtilis} and \textit{E. coli} gyrase, mycobacterial gyrase is a very inefficient enzyme that shows slow, DNA-independent hydrolysis of ATP and low supercoiling and decatenation activities. Whereas \textit{B. subtilis} gyrase is a minimal version of gyrase with high enzymatic efficiency both in supercoiling and decatenation, \textit{E. coli} gyrase is a specialized and highly efficient supercoiling enzyme, but a less efficient decatenase. Mycobacterial gyrase, on the other hand, is not evolutionarily optimized for enzymatic efficiency, neither in supercoiling nor in decatenation. Instead, it represents a compromise with a broader range of activities \textit{in vivo} to meet the physiological requirements of mycobacteria. The species-specific variations between GyrA and GyrB subunits lead to optimized functional cooperation of gyrase subunits with their cognate partners and limit the compatibility and functional cooperation with noncognate subunits. Whereas the GyrB\textsubscript{Bs} subunit is functionally promiscuous and cooperates with GyrA\textsubscript{Ec} and GyrA\textsubscript{Mt}, GyrB\textsubscript{Ec} shows a preference for GyrA\textsubscript{Mt} Gyra, presumably because of the interaction between the two insertions in the GyrB and GyrA subunits that optimize inter-subunit communication and coupling. The species-specific insertions lead to a reduced stability of the mycobacterial gyrase heterotetramer and interfere with complex formation of GyrB\textsubscript{Mt} with GyrA\textsubscript{Ec}. GyrB\textsubscript{Mt} does not support rapid supercoiling in combination with noncognate GyrA subunits. In contrast, GyrA\textsubscript{Mt} can functionally cooperate both with \textit{B. subtilis} and \textit{E. coli} GyrB. The heterologous enzymes reach higher supercoiling and decatenation activities than the homologous mycobacterial enzyme. Presumably, the slow hydrolysis of ATP by mycobacterial GyrB is a limitation for the topoisomerase activities of mycobacterial gyrase, which can be overcome by the cooperation with faster-hydrolyzing GyrB subunits in heterologous enzymes. Understanding the molecular details of individual adaptations of gyrase to the physiological require-

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**Activities of heterologous gyrases**

\textit{J. Biol. Chem.} (2020) 295(8) 2299–2312 2309
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ments of the respective organism might provide a basis for the development of species-specific gyrase inhibitors.

Experimental procedures

Protein production and purification

All proteins were produced recombinantly in E. coli BL21 (DE3). Cells were grown in Luria-Bertani medium at 37 °C to OD600 = 0.6 and gene expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside for 4 h at 30 °C. Cells were harvested by centrifugation and resuspended in lysis buffer (GyrAmt, GyrBmt, GyrAmEc, and GyrBmEc) or 50 mM Tris/HCl, pH 7.5, 1 mM NaCl, 10% glycerol, 2 mM -mercaptoethanol (buffer A) and applied to a 5-ml heparin-sepharose column equilibrated with the same buffer. Proteins were eluted with a 100-ml gradient from 200 mM NaCl to 2 M NaCl in buffer A. Fractions containing GyrA were pooled, concentrated, flushed-frozen in liquid nitrogen, and stored at -80 °C.

Crude extracts from cells overproducing GyrB were supplemented with ammonium sulfate to 50% saturation, incubated on ice for 30 min, and centrifuged. The pelleted protein was precipitated with ammonium sulfate to 50% saturation, incubated with 500 mM KCl, 1 mM EDTA, 10% glycerol, 2 mM -mercaptoethanol, and applied to an S200 column equilibrated in buffer C (GyrBmt). Fractions containing GyrA were pooled, concentrated, flushed-frozen in liquid nitrogen, and stored at -80 °C. The protein was lyophilized.

Preparation of relaxed plasmid

Negatively supercoiled pUC18 was obtained from E. coli XL1 blue transformed with pUC18 using the QiAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Relaxed pUC18 was purified from reactions containing 100 nm negatively supercoiled pUC18, 400 nm GyrA, 1600 nm GyrB in 50 mM Tris/HCl, pH 7.5, 100 mM KCl, 50 mM NaCl, 10 mM MgCl2 using the Promega Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) with subsequent ethanol precipitation.

Supercoiling and decatenation

DNA supercoiling and decatenation activity of gyrase was tested with relaxed pUC18 or kDNA (Inspiralis, Norwich, UK). For supercoiling reactions, 200 nM GyrA and 800 nM GyrBmt or 400 nM GyrBmEc (if not specified otherwise) were incubated with 20 mM pUC18 and 1.5 mM ATP in buffer containing 20 mM Tris/HCl, pH 7.5, 100 mM KOAc, 10 mM MgCl2, 35 μg/ml BSA, 1.2 mM DTT, 10% (v/v) glycerol. After preincubating the samples for 3 min at 37 °C, reactions were started with ATP and stopped with 0.5% (w/v) SDS and 12.5 mM EDTA, pH 8.0, at different time points. The decatenation activity of gyrase was tested under the same conditions, using 12.5 μg/ml kDNA as a DNA substrate. Reaction products were separated on 1.3% (w/v) agarose gels in TEP buffer (36 mM Tris, 36 mM NaH2PO4, 1 mM EDTA, pH 8.0; 2.6 V/cm, 3.5 h).

Steady-state ATPase activity

Steady-state ATPase activity was measured in a coupled enzymatic assay as described (37) with 0.5 μM GyrA, 0.1 μM GyrB, 0.1 μM negatively supercoiled pUC18 (if present) at 37 °C in 20 mM Tris/HCl, pH 7.5, 100 mM KOAc, 10 mM MgCl2, 35 μg/ml BSA, 1.2 mM DTT, 10% (v/v) glycerol, and ATP concentrations from 0 to 5 mM. Data were analyzed with the Michaelis–Menten or Hill equation to obtain $k_{\text{cat}}$, $K_M$, and $V_{\text{max}}$ values. Reaction velocities of homologous B. subtilis gyrase (in the absence and presence of DNA), of M. tuberculosis gyrase (in the absence of DNA), and of heterologous gyrase formed by GyrAmp and GyrBmp (in the absence and presence of DNA) did not follow Michaelis–Menten behavior but required description with the Hill equation, giving Hill coefficients of ~2 (Table 2 and Table S1). Errors reflect S.D. from 3–7 independent experiments.

The errors $\sigma$ for the -fold change $x$ and $y$ in $k_{\text{cat}}$ and $K_M$ in the presence of DNA and for $k_{\text{cat}}/K_M$ were propagated from the errors $\sigma_a$ and $\sigma_b$ of the individual values $a$ and $b$ according to Equation 1,

$$
\sigma \left( \frac{a}{b} \right) = \sqrt{\sigma_a^2 \cdot \left( \frac{1}{b} \right)^2 + \sigma_b^2 \cdot \left( -\frac{a}{b^2} \right)^2} \quad (\text{Eq. 1})
$$

where $x = k_{\text{cat},+DNA}/k_{\text{cat},-DNA}$, $y = K_M,+DNA/K_M,-DNA$, and $k_{\text{cat}}/K_M = k_{\text{cat},+DNA}/K_M,+DNA$ and denote the turnover number in the absence and presence of DNA, respectively, and $K_M,-DNA$ and $K_M,+DNA$ denote the $K_M$ in the absence and presence of DNA. p values were calculated from two-sample t tests using the hypothesis testing routine of OriginPro 2019.
Analytical size-exclusion chromatography

The formation of homologous and heterologous gyrase was analyzed on a calibrated S2000 10/300 GL column (GE Healthcare, Freiburg, Germany) in 20 mM Tris/HCl pH 7.5, 100 mM KCl, 10 mM MgCl₂, 10% (v/v) glycerol with 4 μM GyrA and 0.8 μM GyrB at 25 °C. Protein elution was monitored via the absorbance at 280 nm, and fractions were analyzed for GyrA and GyrB by 10% SDS-PAGE.

Author contributions—D. W. investigation; D. W. and D. K. visualization; D. W. and D. K. methodology; D. W. and D. K. writing-original draft; D. W. and D. K. writing-review & editing; D. K. conceptualization; D. K. and D. W. formal analysis; D. K. supervision; D. K. funding acquisition; D. K. project administration.

Acknowledgments—We thank Jessica Guddorf for excellent technical assistance, and Frederic Collin, Airat Gubaev, and Markus Rudolph for comments on the manuscript.

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Activities of heterologous gyrases

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