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REVIEW

In front of and behind the replication fork: bacterial type IIA topoisomerases

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Abstract Topoisomerases are vital enzymes specialized in controlling DNA topology, in particular supercoiling and decatenation, to properly handle nucleic acid packing and cell dynamics. The type IIA enzymes act by cleaving both strands of a double helix and having another strand from the same or another molecule cross the DNA gate before a re-sealing event completes the catalytic cycle. Here, we will consider the two types of IIA prokaryotic topoisomerases, DNA Gyrase and Topoisomerase IV, as crucial regulators of bacterial cell cycle progression. Their synergistic action allows control of chromosome packing and grants occurrence of functional transcription and replication processes. In addition to displaying a fascinating molecular mechanism of action, which transduces chemical energy into mechanical energy by means of large conformational changes, these enzymes represent attractive pharmacological targets for antibacterial chemotherapy.

Keywords DNA-topology · Topoisomerases · DNA Gyrase · Topoisomerase IV · Prokaryotic enzymes · Antibacterials

Introduction

When we think of DNA, our mind springs to the inspiring image of a double helix. Although duplex structure represents a hallmark in modern biology, at first glance base stacking and braided architecture lend it unusual stiffness, so it might appear as a biological device more suited to safe genetic information storage than to dynamic action in living systems. Rather, the nucleic acid behaves as a flexible molecule and shows a remarkable plasticity when performing essential functions such as replication and transcription. In fact, we should take into account that DNA is organized in large loops both in eukaryotic and prokaryotic cells, which essentially render it a closed circular system. As a consequence, it may adopt several different configurations (relaxed, positively or negatively supercoiled, knotted, catenanes) (Fig. 1). They refer to otherwise identical molecules differing in their spatial arrangement only, called topological isomers or topoisomers. These forms are dynamically generated and interconvert during DNA processing. For example, replication, which demands local opening of the double helix, produces positive supercoiling ahead of the replication fork and interwinding of daughter chromosomes (precatenanes) behind it, an event that manifestly requires topological intervention. The same is true for transcription, which leads to the production of positive supercoiling ahead of the transcription machinery and negative supercoiling behind it. More importantly, it is now clear that DNA topology plays an active role in regulating DNA processing [1–3]: basic biological processes, including control of gene transcription, faithful DNA replication and segregation, maintenance of the genome and cellular differentiation are largely directed by the energetics of DNA structure in addition to the regulation provided by the sequence itself [4, 5]. If topology were not taken care of appropriately, these fundamental processes could not occur. Hence, to dynamically modulate its activity and grant physiologic occurrence of

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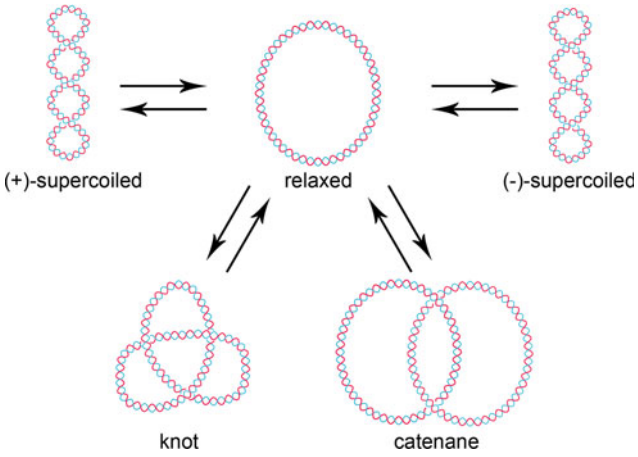

Fig. 1 Different topological states of covalently closed circular DNA

essential cellular events, (circular/constrained) DNA must be efficiently and timely converted from one topological state to another [6].

Topoisomerases

In all organisms, the DNA topology control is carried out by an essential family of enzymes, the topoisomerases. Variation in the degree of supercoiling of a circular duplex as well as separation of catenated circles require phosphodiester bond cleavage followed by DNA strand passage and, finally, resealing of the original linkage. Therefore, the mechanism underlying topoisomerase action cannot be simple. Indeed, it foresees the combination of functions characteristic of nucleases and ligases, besides the possibility of large conformational changes to cope with a strand-passing process. Moreover, in most instances, these functionalities are coupled to energy-producing ATPase activities [7]. In fact, topoisomerases consist of several domains. They can be divided into type I enzymes, which operate by introducing DNA single-strand breaks only and type II enzymes, which produce double-strand breaks. In turn, depending upon amino-acid sequence, structural, mechanistic, and evolutionary differences, type I can be subdivided into the A, B, and C subclasses, while type II consists of the A and B subclasses. All these subclasses reflect differences in the enzyme structure and mechanism of action.

Several recent reviews describe in detail the structural and biological properties of topoisomerases [8–15]. Here, we will focus on bacterial topoisomerases belonging to the type IIA subclass, with special attention to the most recent knowledge rendered available in the field.

Bacterial type IIA topoisomerases: DNA Gyrase (Gyr) and topoisomerase IV (Topo IV)

In general, every organism requires at least one type I and one type II enzyme to fully deal with maintaining appropriate levels of DNA supercoiling and removing chromosomal entanglements. In addition, most bacteria express two distinct topoisomerases of type IIA, referred to as DNA Gyrase (Gyr) and Topoisomerase IV (Topo IV). As notable exceptions, a few bacteria such as *Treponema pallidum*, *Deinococcus radiodurans*, *Helicobacter pylori*, *Campylobacter jejuni* together with the actinobacteria *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Corynebacterium glutamicum* are reported to express one topo II (Gyr) only [16–20].

Gyr activity was discovered over 30 years ago [21]. The *E. coli* functional enzyme was soon after shown to consist of a tetramer (A_2B_2, MW 400 kDa) formed by two pairs of identical subunits (GyrA and GyB) having molecular weights of about 97 and 90 kDa respectively [22]. About 14 years later, a new bacterial topoisomerase II, named topoisomerase IV, was discovered in *E. coli* and shown to be assembled in a way similar to Gyr, with subunit ParC (MW 83.7 kDa) corresponding to GyrA and ParE (MW 70.2 kDa) to GyrB [23, 24]. The above subunits are encoded by the *GyrA*, *GyrB*, *ParC* and *ParE* genes, respectively [25, 26].

Enzymatic cycle of bacterial type II topoisomerases

Gyr and Topo IV share mechanistic features common to all type II enzymes described to date, spanning from archeabacterial to eukaryotic species. In fact, a transphosphorylation process generates a transient covalent linkage between the protein and the nucleic acid, resulting in dsDNA breakage (cleavage complex) [21, 27].

Global catalytic reaction is performed by two Tyr residues conveniently located at the active site, which form phosphotyrosine linkages at the 5′ side of the attacked phosphodiester bond. Topoisomerase II-mediated breaks are not produced at the level of the two residues belonging to the same base pair, but are staggered 4 base pairs apart from each other [28, 29].

A generally accepted mechanism for the DNA topological changes mediated by type II topoisomerases, the so-called “two-gate mechanism”, was proposed long ago [30, 31]. Based upon this, the DNA segment being transported to produce strand passing (T) can exit from the interior of the enzyme through a gate (C) located on the opposite side of the entrance gate (N) (Fig. 2). The DNA double-stranded breakage forms an additional temporary gate (DNA gate) through which the T DNA double helix

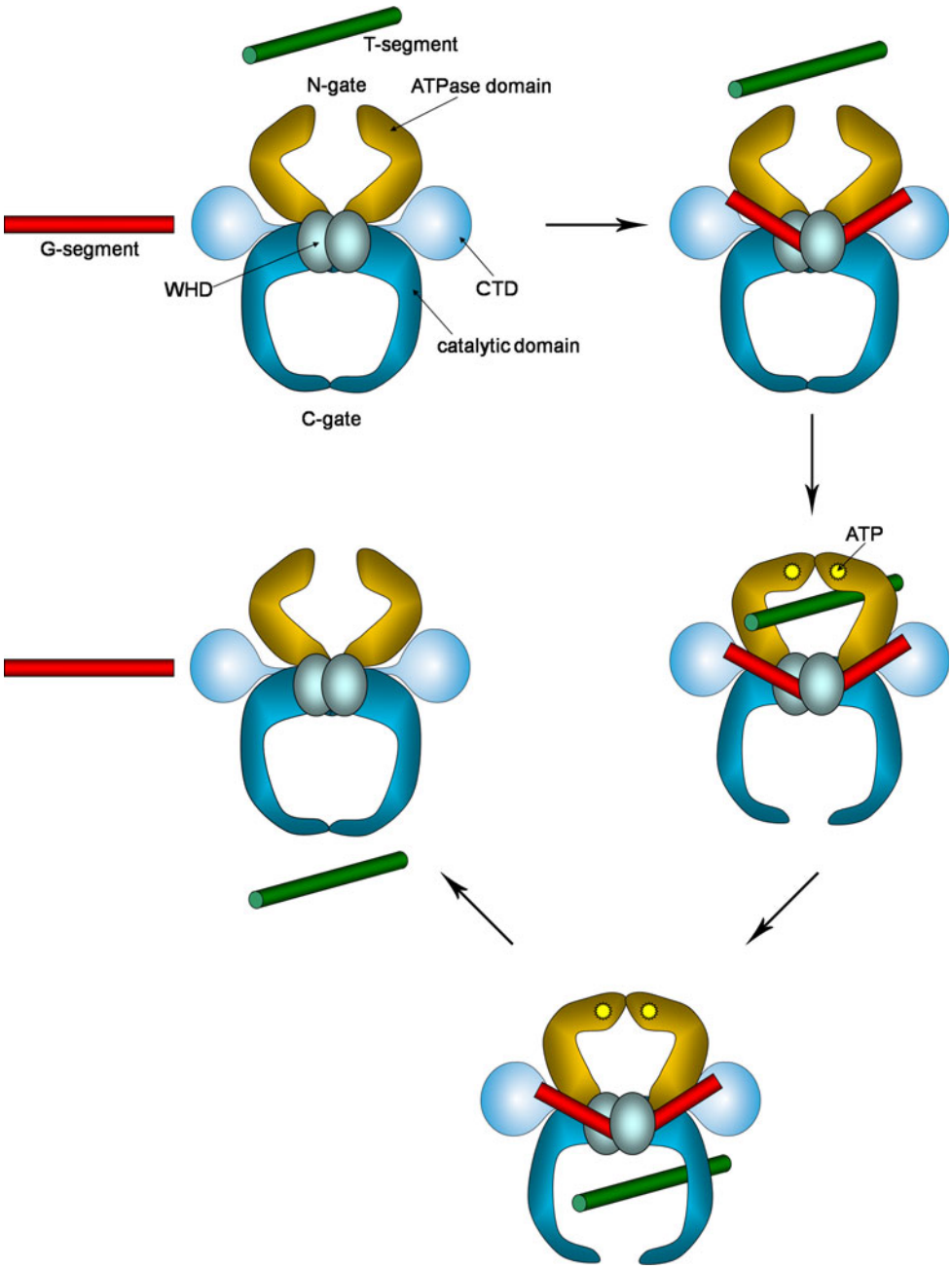

Fig. 2 Relevant steps of the two-gate mechanism for type IIA topoisomerases. *CTD* C-terminal domain, *WHD* winged-helix domain, containing the catalytic Tyr residue. The G-segment is engaged at the GyrA/GyrB or ParC/ParE domain interface, where it is sharply bent, T-segment is recruited in the ATP-clamp (N-gate), which is closed when ATP binds. Then, the G-gate opens, forming the cleavage complex and allows T-segment transport. Finally, T-segment exits through the C-gate and the enzyme is reset for a subsequent catalytic cycle

strand-passage occurs, which actually leads to a "three-gate mechanism". The concerted gates opening/closure grants one-way strand passage of the T segment: indeed each gate can be opened only when the others are closed according to a double-lock rule [32, 33]. This allows a powerful unidirectional action.

ATP hydrolysis and catalytic metal ions are the two main cofactors required by type IIA topoisomerases for successful DNA processing [21].

At the moment we do not know precisely which step makes use of the energy of ATP hydrolysis, but this might be needed (1) to securely hold the two G segments while DNA is cleaved to provide the gate for transport and/or (2) to favor the conformational changes (N gate closure) needed to push the T segment through the G segment into the enzyme central cavity. Additionally, the enzyme is functionally reset only through hydrolysis of a second ATP molecule. As a consequence, all type II enzymes require two ATP molecules to undergo subsequent rounds of catalysis [34].

Concomitantly, a divalent metal ion co-factor (usually Mg^{2+}) is required to catalyse the DNA cleavage/rejoining chemical process [21]. Mg^{2+} is physiologically present in the bacterial intracellular environment and is expected to

assist the transphosphorylation process in several ways. In particular, it can stabilize the incoming negative tyrosinate species, neutralize the penta-coordinated negatively charged transition state and/or assist in the stabilization of the negatively charged leaving group. The information presently available suggests participation of two ions per DNA strand cleavage event [35].

Clearly, the requisites of tetramer assembly, ATP hydrolysis and metal ion binding confer a remarkable specificity to the system, which can be activated/deactivated under individually modulated environmental conditions during the cell cycle and localized at selected loci as appropriate [36]. In addition, topoisomerases of different organisms exhibit different co-factor dependencies of their catalytic activity to optimally respond to individual needs [37, 38].

Domain organization

Since the DNA cleavage-resealing process performed by type IIA topoisomerases proceeds parallel on the two DNA strands, a symmetrical arrangement of the functional enzyme is expected [29]. In fact, the double-strand cutting process is handled by the formation of a protein tetramer as mentioned above [22]. Analytical ultracentrifugation and X-ray scattering studies enabled the overall shapes of Gyr subunits in solution to be defined [39, 40]. GyrA forms a dimeric heart-like core accompanied by two pear-shaped lateral densities (the carboxyl terminal domains), closely flanking the core and lying in the lower part of the protein, far from the cleavage site [39]. GyrB as such is monomeric and shows a tadpole-like structure, suggestive of a modular organization of the protein domains thus revealing potential structural flexibility of the various subdomains [40]. Dimerization of this subunit is then triggered by the presence of GyrA (and the DNA substrate). In addition, the results indicate that GyrB should be located on the top of GyrA in the active tetrameric assembly [40].

The domain organization of the various subunits is schematically reported in Fig. 3.

Interestingly, Gyr and Topo IV share a common distribution of functional domains.

The catalytic Tyr is located in the N-terminal domain of GyrA/ParC, whereas DNA binding occurs at the C-terminal domain of these proteins. In turn, the ATP-binding region is positioned at the N-terminal domain of GyrB/ParE, while their C-terminal domains contain the TOPRIM region, spanning about 100 residues, involved in catalytic Mg^{2+} coordination. Interestingly, the latter region is shared by all type IA and type II topoisomerases as well as by several other nucleases [41].

Although the various functional domains of the bacterial enzymes are situated in distinct subunits, a close similarity to the eukaryotic counterpart(s) is apparent. Additionally, Gyr and Topo IV subunits from various sources (including both Gram-negative and Gram-positive bacteria) share similar functional and structural arrangements [15], in spite of their relatively modest sequence homology (Table 1).

Since the crystal structure of the full-length Gyr/Topo IV subunits is not available as of yet, domain similarity has been used to combine crystallographic data from several topoisomerases fragments to yield very valuable structure–function relationships. Although the conformation examined in a crystal structure represents but one of the (several) possible stable conformations adopted by the enzyme (fragment) during the catalytic cycle, different conditions used to obtain the crystals may give insight into the conformational changes the fragment under investigation can undergo. In fact, studies on different topoisomerase II

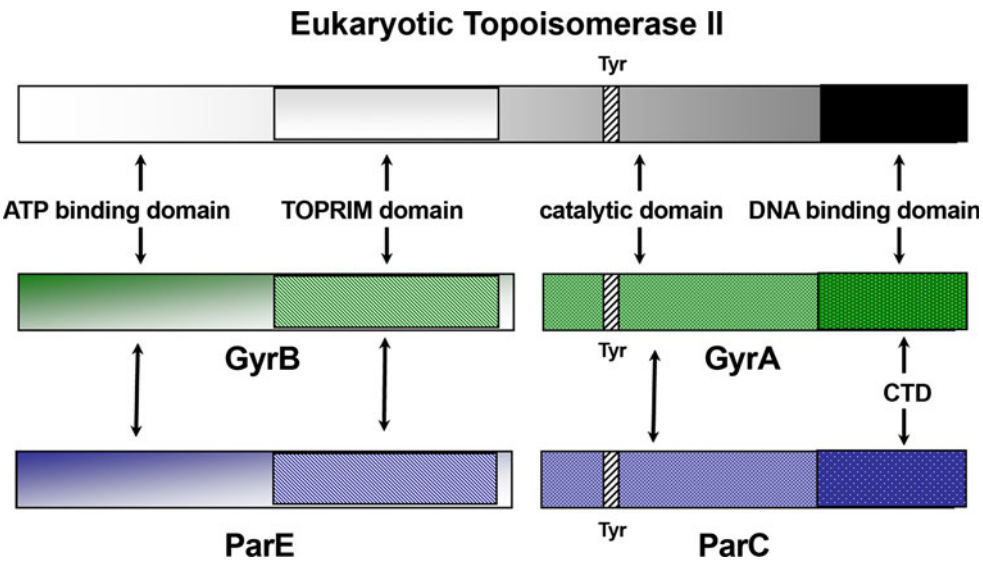

Fig. 3 Schematic organization of functional domains in type IIA topoisomerases. Location of catalytic tyrosine (Tyr) is indicated

Table 1 Optimal sequence similarity scores for selected DNA Gyrase and Topoisomerase IV subunits from Gram-negative and Gram-positive bacteria

	GyrA						ParC				
	E. coli	*B. subtilis*	*S. aureus*	*S. pneum.*	*B. burg.*	*M. tub.*	*E. coli*	*B. subtilis*	*S. aureus*	*S. pneum.*	*B. burg.*
No. of amino acids	875	821	889	822	810	838	752	806	800	823	599
GyrA											
E.coli		53	48	49	41	45	33	40	37	36	20
B. subtilis			64	59	42	49	32	42	39	35	24
S. aureus				59	41	46	34	39	38	33	24
S. pneum.					42	46	31	40	38	39	23
B. burg.						39	29	33	35	32	23
M. tub.							31	36	36	33	22
ParC											
E. coli								28	29	27	19
B. subtilis									58	53	23
S. aureus										52	24
S. pneum.											23
	GyrB						ParE				
	E. coli	*B. subtilis*	*S. aureus*	*S. pneum.*	*B. burg.*	*M. tub.*	*E. coli*	*B. subtilis*	*S. aureus*	*S. pneum.*	*B. burg.*
No. of amino acids	804	638	644	648	639	675	630	655	663	647	626
GyrB											
E.coli		44	54	53	52	48	38	49	48	49	32
B. subtilis			68	66	56	55	39	56	55	55	36
S. aureus				62	52	54	38	52	52	50	35
S. pneum.					53	51	38	51	50	49	34
B. burg.						49	38	48	48	48	35
M. tub.							39	49	46	48	32
ParE											
E. coli								37	36	39	29
B. subtilis									70	69	34
S. aureus										68	35
S. pneum.											31

DNA-binding and cleavage domains show that, in addition to substantial conformational changes in the DNA-opening region, a dramatic reorganization of accessory domains may occur during catalysis as well [42]. Also, ion and nucleotide co-factors play a remarkable role in dictating the three-dimensional architecture of the protein.

The DNA cleavage–rejoining process

The functional domains needed for bacterial topoisomerases to catalyse DNA cleavage-rejoining are located in both enzyme subunits, GyrA/ParC containing the catalytic Tyr residue and GyrB/ParE enclosing the TOPRIM region.

The GyrA 59-kDa N-terminal domain, GyrA59, entails the breakage–reunion machinery, including the winged-helix domain (WHD) with the active-site Tyr, and the 'tower' domain that stands on it and participates in DNA binding. It is catalytically functional in the presence of GyrB. Its crystal structure is dimeric, as expected by the symmetric features of enzyme action (Fig. 4) [43]. At the top of the interface, the 'recognition' helices make a head-to-head antiparallel dimer contact. Dimer formation generates a large concave surface on the top comprising the upper surface of the WHD domains and the sides of the two 'tower' domains. The active-site tyrosines are located in loops at either extreme of the dimer interface, 30 Å apart from each other, a distance very similar to that observed in yeast topoisomerase II [44]. They are positioned at the ends of strongly basic grooves created by the dimer-related monomers, ideally suited to accommodate a G segment. A 30-Å-wide central cavity generated by dimerization is

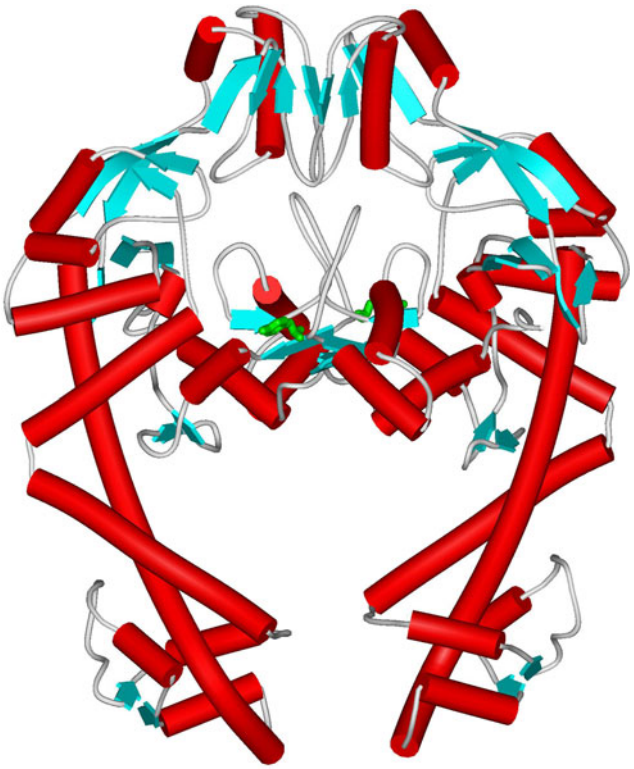

Fig. 4 Crystal structure of *E. coli* GyrA59 dimer (PDB ID 1AB4). *Red tubes* refer to α-helix regions, *blue arrows* to β-sheet. The catalytic Tyr is highlighted in *green* (ball-and-stick model)

adequate to let a T segment translocate through the cleaved G-segment. Two long helices produce the "tail" encompassing the primary dimerization interface. The latter can open to generate the C-gate during catalytic cycle progression. As it approaches its target, the G segment will dramatically change the local electrostatic potential and definitely promote further conformational changes to optimize the protein–DNA complex [43].

The structural features of ParC fragments from *E. coli* and *S. pneumoniae*, containing WHD and tower regions (the *S. pneumoniae* domain also in complex with DNA), have been characterized as well [45, 46]. The 55-kDa N-terminal breakage-reunion domain of Topo IV Par C from *S. pneumoniae* reveals a "closed" dimer folded like *E. coli* GyrA, but divergent from the "open-gate" structure of *E. coli* ParC [46, 47]. These differences probably reflect structural changes the proteins undergo to process DNA. In addition, while the G-segment binding surface in GyrA exhibits a large positive charge density, the DNA-binding site of ParC reveals a distinct pattern with alternating regions of opposite electron distribution matching the grooves and phosphate backbone of DNA [46]. Given the non-negligible distances between the catalytic tyrosine(s) and the fitted DNA phosphate backbone, a local re-shaping is required to allow their closing up in the cleavage-reunion process. Possible key steps in DNA binding and recognition involve interactions with the two active-site helices and the flexible loop between amino-acids 100 and 122. This rearrangement is indeed observed in the ParC-DNA covalent complex, although the presence of a drug molecule at the cleavage site might locally remodel the structure [48].

As far as the TOPRIM domain is concerned, the crystal structure of the 485–714 sequence of GyrB from *M. tuberculosis* has been solved very recently [49]. TOPRIM spans about 120 residues (486–604) and is characterized by the presence of a Glu residue and an Asp-x-Asp-x-Asp (DxDxD) motif in the IIA enzymes [41]. It includes a Rossmann-like fold containing a central β-sheet sandwiched by two α-helical bundles, with a tertiary scaffold nearly identical to other topoisomerases. The glutamate is located in a sharp turn at a β-α connection, a structurally analogous arrangement occurring at the level of the DxDxD element. As a consequence of TOPRIM folding, the above acidic residues are close in space and become available for concerted catalytic assistance through Mg^{2+} coordination [35].

Valuable direct information about the cleavable complex structure in bacteria stems from the successful co-crystallization of the *S. pneumoniae* ParC cleavage-reunion domain with the ParE TOPRIM domain and a DNA substrate (Fig. 5) [48]. Both the proteins and the nucleic acid are profoundly affected by mutual interaction, which is consistent with the results obtained for yeast topoisomerase II [44]. The protein provides a large positively charged surface for nucleic acid binding and the effective positioning of tyrosine and ion co-factors is guaranteed by the closing up of the Mg^{2+} coordination tetrad to the phosphodiester bond to be processed. In line with the *M. tuberculosis* and yeast structures, the TOPRIM core exhibits four parallel β-sheets surrounded by α-helices [44, 49].

The relative structural organization of the WHD, tower, and TOPRIM regions to form the Topo IV cleavage complex are shown in Fig. 6.

The DNA G-segment, similar to the yeast enzyme, is sharply bent by the protein (Fig. 5) [44]. Waiting for the experimental proof from a crystal structure, a significantly curved G segment structure can be reasonably expected to characterize the Gyr-DNA complex as well.

Biochemical role of bacterial topoisomerases II

The above mechanistic and structural similarities notwithstanding, an impressive amount of biological and biochemical evidence shows that Gyr and Topo IV are in fact specialized in performing distinct functions in bacterial cells [14, 50–52].

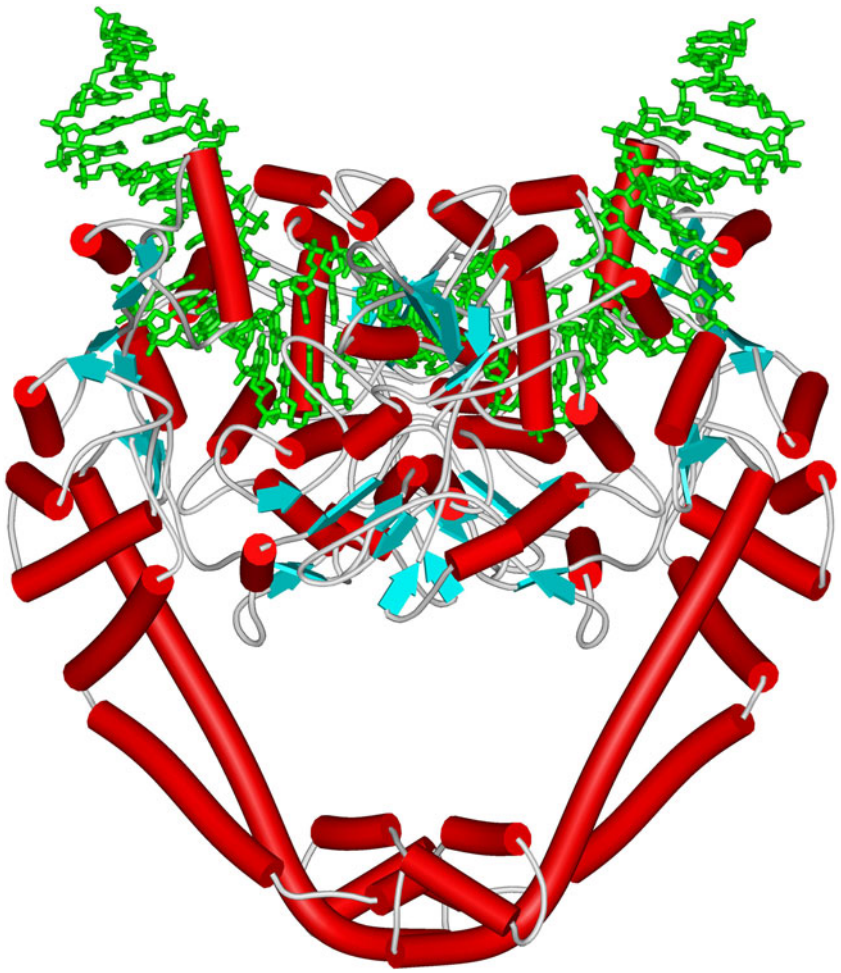

Fig. 5 Crystal structure of the *S. pneumoniae* ParC breakage-reunion domain (ParC55) and ParE TOPRIM domain (ParE30) in the cleavage complex with DNA in the presence of the quinolone moxifloxacin (PDB ID 3FOF). *Red tubes* refer to α-helix regions, *blue arrows* to β-sheet. The DNA portion is in *green sticks*

In particular, DNA Gyrase is suited for:

- introducing negative supercoils into covalently closed, positively supercoiled or relaxed, double-stranded circular DNA molecules;
- catenating and decatenating DNA rings;
- relaxing negative supercoils in the absence of ATP.

Topoisomerase IV activities include:

- catenation and decatenation of double-stranded circular DNA molecules;
- relaxation of both positive and negative supercoils.

Considering these main functions, Gyr is effective in producing intramolecular DNA strand passing processes, in particular supercoiling, whereas Topo IV is fit for intermolecular events, such as catenation/decatenation.

The above individual features allow maintaining the supercoiling level of the bacterial chromosome as appropriate and dealing with the topological constraints that arise during DNA replication and transcription. To explain how a common general mechanism can be tuned to favor different biological pathways, DNA supercoiling and catenation must be more closely examined.

DNA supercoiling/relaxation

Both Gyr and Topo IV can relax supercoiled DNA but only gyrase is able to introduce negative supercoiling. Biological and structural studies indicate that Gyr can support DNA supercoiling by allowing DNA wrapping at its exterior [53, 54]. This process produces a single positive supercoil with a left-handed superhelical crossing: after passing through the open G segment, the supercoil handedness is converted from positive to negative. Footprinting experiments and electron microscopy studies show that Gyr is able to bind a sequence of about 130–140 bp, which allows processing G and T segments close to each other [55, 56]. In turn, Topo IV covers a small region of 34 bp centered about the cleavage site and, as a result, it bends DNA to an extent that is not sufficient to generate a T segment flanking a G segment [47, 57]. Hence, Topo IV

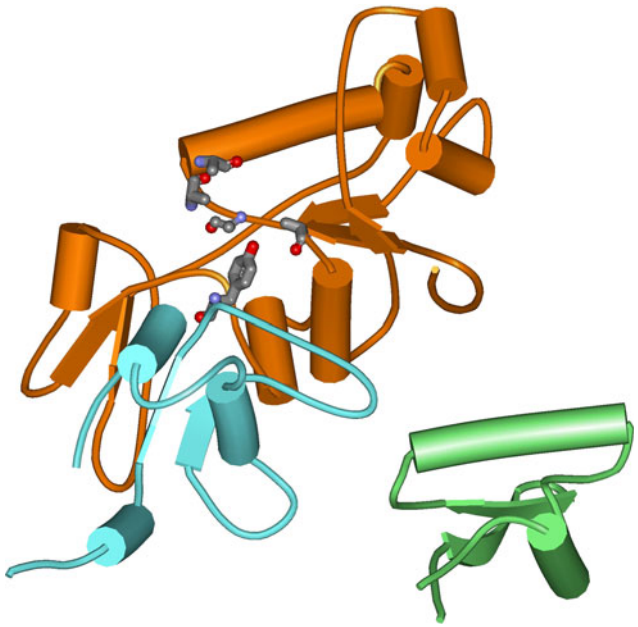

Fig. 6 Relative location of the WHD (*cyan*), tower (*green*), and TOPRIM (*brown*) domains in the catalytic DNA cleavage-resealing pocket as observed in the crystal structure of the *S. pneumoniae* ParC breakage-reunion domain (ParC55) in combination with the ParE TOPRIM domain (ParE30) in the cleavage complex with DNA(PDB ID 3FOF). The catalytic Tyr residue in WHD and the four acidic residues involved in Mg^{2+} binding in TOPRIM are highlighted (ball-and-stick model). *Tubes* represent α-helix regions, *arrows* β-sheets

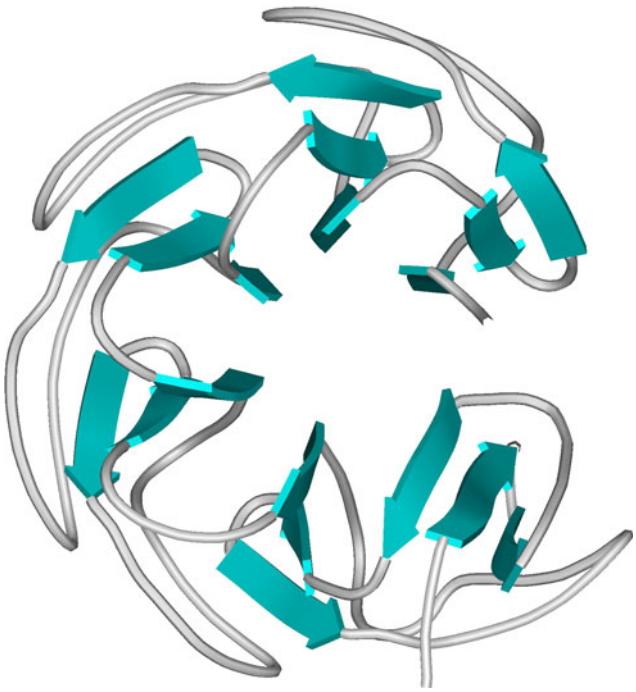

Fig. 7 The β-pinwheel structure of the *E. coli* GyrA CTD (PDB ID 1ZI0). *Blue arrows* refer to β- sheet tracts

processes DNA segments at a distance and is unable to introduce negative supercoils thus exhibiting a mechanism reminiscent of eukaryotic topoisomerases, rather than of Gyr [33].

The GyrA/ParC C-terminal domains are apparently responsible for DNA wrapping versus bending [58].

It is worth recalling that the prokaryotic CTDs, in particular GyrA CTD, are structurally distinct from the corresponding domains in eukaryotes, which strengthens the idea that they perform special functions in bacteria [47, 59]. Several three-dimensional structures are now available to assess their mechanistic significance. The GyrA CTD, a pear-shaped domain located aside the catalytic core according to X-ray scattering, adopts an unusual fold, first seen in the *B. burgdorferi* domain, namely a β-pinwheel, that is globally reminiscent of a β-propeller but is made up of six blades with a peculiar topology (Fig. 7) [39, 60]. A large, conserved basic amino acid sequence on the outer edge of this domain represents a likely site for binding and wrapping/bending DNA. FRET assays show that the GyrA CTD can sharply bend DNA over a 40-bp region to generate a T-segment in *cis* [60]. Such a combination fixes the positive crossover which, after strand passing, introduces a negative supercoil into circular DNA [53, 54].

The CTDs shape can vary from species to species. In fact, whereas the *E. coli* GyrA-CTD is spiral, the corresponding *B. burgdorferi* domain is flat [60, 61]. However, this difference is probably related to the peculiar character of CTD in *B. burgdorferi*, the only one expressed as an independent gene product [62]. Site-directed mutagenesis of *M. tuberculosis* GyrA suggests a key role played by one Tyr and two Arg residues in the third CTD blade as their mutation produced loss of enzymatic activities, without affecting ATPase activity [63].

Another distinctive feature of Gyr CTD is the so-called GyrA box characterized by the heptapeptide sequence Gln-Arg-Arg-Gly-Gly-Lys-Gly located at the first amino-terminal blade of the GyrA CTD [64]. Mutation/deletion of this box inhibits DNA wrapping around itself [65].

In addition, GyrA CTDs are characterized by a flexible connection to the catalytic core [39], which may help them interchange between at least two positional states: one located close to the N gate to grant DNA-wrapping prior to T segment engagement and the second, close to the C gate, possibly representing a resting conformation after strand passage. Sequence alignment and secondary structure analysis evidenced two notable differences between GyrA and ParC CTDs in *E. coli*. In fact, (1) there are six blades in the β-pinwheel fold for GyrA and five for ParC and (2) the GyrA box is missing in ParC [44, 65]. The superhelical spiral motif is conserved among different species as it occurs also in the *B. stearothermophilus* ParC CTD, which

is structurally similar to the corresponding region in GyrA, but still lacks the GyrA-box [66]. Apparently, the Gyr domain is better suited to wrap DNA around itself as compared to the Topo IV domain [47]. In addition, the mobility of GyrA CTD is not observed in ParC CTD, which binds more tightly to the catalytic portion of the protein and cannot assist T fragment translocation [39, 47].

To summarize, the unique supercoiling activity exhibited by Gyr is explainable by the combination of CTD features: GyrA box (perhaps synergistically with the GyrB tail domain [40]) is possibly capable of electrostatically directing the G segment from the catalytic core to the CTD, where it can be appropriately wrapped to generate the T segment. The flexible connection of the CTD to the core domain would additionally help in transferring the T segment through the DNA gate by shifting from the "upper" to the "lower" position further facilitating DNA supercoiling [39].

In turn, supercoiled and entangled regions of DNA are expected to be locally bent, which renders them ideal substrates for recognition and processing by Topo IV [67].

This picture is fully consistent with the biochemical and structural data thus far available.

DNA catenation/decatenation

The use of isogenic *E. coli* strains that allowed selective in vivo inhibition of Gyr, Topo IV, or both, unequivocally clarified that Topo IV, not Gyr, is responsible for the decatenation process [68]. In fact, lack of Topo IV essentially leaves all newly synthesized plasmid DNA in the catenated form, Gyr exhibiting at least a hundred-fold less effective decatenase activity [69, 70]. Topo IV decatenates linked cyclic DNAs 10–40 times faster than it relaxes supercoiled DNA and unlinks supercoiled catenanes at an even faster rate [71]. Thus, supercoiling seems to favor (rather than inhibit) decatenation [72]. Additionally, Topo IV binds preferentially to positively supercoiled DNA possibly in a different conformation as compared to other topoisomers, including negatively supercoiled DNA [57, 73]. Further studies on the interplay between supercoiling, catenation, and knotting in *E. coli* cells with impaired Topo IV activity indicate that catenation and negative supercoiling compete with each other in replication intermediates formed in vivo, high catenation levels being accompanied by low supercoiling and, conversely, high supercoiling levels corresponding to low catenation as segregation proceeds [74].

This confirms that effective decatenation by Topo IV relies on its ability to recognize the type and level of DNA supercoiling, thus indicating that the topological state of the genome plays a dynamic role during progressive in vivo decatenation of sister chromosomes [74].

Together with catenation, knotting should be kept at as low a level as possible (see below) since it interferes with the normal processing of DNA [75]. Like catenanes, knot crossings are preferentially removed by Topo IV [70], although it should be considered that DNA supercoiling renders elimination of intramolecular knotting energetically favored [76].

Besides helping to rationalize different individual activity profiles, the above findings foreshadow the need for concerted actions of the two bacterial topoisomerases II (and, obviously, of the type I enzyme) and the need for a finely tuned equilibrium between time and location of enzyme action/expression. However, in bacteria not expressing Topo IV, Gyr is probably engineered to perform decatenation in addition to supercoiling. Indeed, Gyr from *Mycobacterium smegmatis* behaves as an efficient decatenase [77].

A cartoon model for the DNA-processing mechanisms exhibited by Gyr and Topo IV is presented in Fig. 8.

DNA topological simplification

Unexpectedly, the steady-state fractions of relaxed and decatenated products generated by topoisomerase II action in vivo were found to be substantially higher than the corresponding equilibrium fractions [78]. Hence, the enzymes not only assist the reaching of equilibrium but also force it towards topological simplification. Moreover, the topoisomer distribution appears to be narrower than expected [78]. Though this fact is clearly rational from a biological point of view, it is not apparent how small enzymes could selectively affect the topology of very large DNA molecules sensing their complexity, as this corresponds to a global property of the assembly and cannot be determined by a spatially confined DNA–protein interaction. A three-binding-sites model was originally proposed, according to which binding of topoisomerase to a DNA crossover divides the circular DNA into two smaller cyclic domains [78]. This increases the probability of G-T segment collision in catenated, knotted, and supercoiled systems. As a corollary, sliding of the protein clamp along the DNA can trap a catenane or knot node in a small loop, thereby facilitating removal of topological links from DNA. Other models were afterwards suggested to account for the experimental topology simplification. One foresees kinetic proofreading by the enzyme which binds to entangled DNA in two successive binding events, the first of which activates the enzyme for subsequent strand passage [79]. A simpler explanation was next proposed considering a sharp bending of the G segment into a hairpin-like structure when bound to the enzyme [67]. As a result, the doorway for the T segment is placed inside the hairpin.

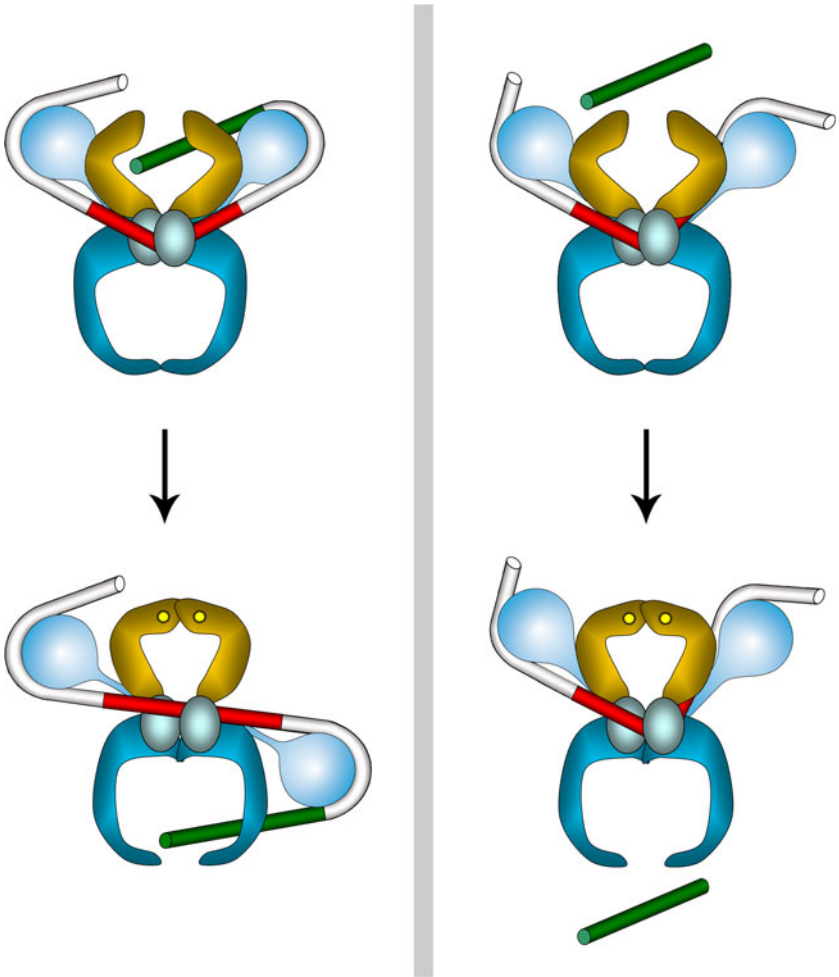

Fig. 8 Proposed model for diverse recruitment of G (*red*) and T (*green*) segments by DNA Gyrase (*left panel*) and Topoisomerase IV (*right panel*). Labeling as in Fig. 2

Thus, the T segment can move across the G segment only from the interior to the exterior of the bend. This would represent a specific tracking element, allowing the strand passing reaction to occur monodirectionally relative to the curved G segment. Further statistical mechanic simulations considered a G segment oriented in a left-handed helical conformation, characterized by a winding angle and a height parameter [80]. The results show that the winding angle plays an important role in directing the chiral preference of the topoisomerase II for relaxation of positive versus negative supercoils, favoring the former when it exceeds 180°, as in the case of Topo IV [80, 81].

The use of tightly bound mutant *EcoR*I complexes on a plasmid allows ruling out a mechanism of the sliding of a protein clamp along DNA to trap supercoiled regions. In addition, the simplification process appears to be independent of circle size, which also disfavors tracking mechanisms [34, 82]. Moreover, the amount of free energy obtainable from ATP hydrolysis does not affect the extent of topological simplification, suggesting an energy facilitated geometric (or kinetic) selection of T segments for unidirectional strand passage [34, 82]. Apparently, the "hairpin" model is more adequate to explain the experimental results of enhanced relaxation and decatenation levels. No straightforward rationale can explain how the suggested "hairpin DNA" mechanism produces simplification of DNA topology; however a computational analysis shows that this is indeed the case [67]. Topoisomerase preference for hooked juxtapositions, where the nucleic acid strands are curved toward each other, might also be invoked to explain selectivity [83]. However, simultaneous binding of the enzyme to two juxtapositions is an unlikely event that would dramatically slow down disentanglement [49, 75]. In addition, the CTD regions principally responsible for DNA bending do not appear to contribute to DNA structure simplification [82]. Indeed, CTDs would provide an ideal physical location for bent T-segment selection to effectively recognize hooked crossovers.

An important issue to be further considered is the real significance of equilibrium information given the continuous topological changes occurring in the bacterial

chromosome [84], which make disentanglement a process that is more kinetically than thermodynamically controlled.

A final concern deals with the substantially different simplification efficiency produced by enzymes of the topoisomerase II family, Topo IV being most effective in this regard [82]. This means that individual enzymes react differently to entanglement, shifting the equilibrium fraction of simplified structure to different extents. Hence, the fine details of entanglement sensing and resolution, although based on general rules, are dictated by (modest) adjustments in G and T segment recognition.

ATPase activity

The ATP requirement for topoisomerase II enzymes has been thoroughly discussed in recent reviews [7].

ATP hydrolysis by Gyrase follows a non-Michaelis–Menten kinetics and is consistent with dimerization of the protein in line with the symmetric assembly of the functional subunit [85]. Accordingly, the activity of the reconstituted enzyme prepared with varying ratios of the inactive mutant at Lys103 and wild-type GyrB suggest an interdependent action of both B subunits in ATP binding and hydrolysis to produce DNA supercoiling [86].

Available crystallographic data indicate that the 43-kDa N-terminal GyrB fragment accommodates a nonhydrolysable ATP analogue in a series of β-pleated sheets, containing both antiparallel and parallel motifs [87]. The protein undergoes dimer formation as a result of a polypeptide exchange involving 14 N-terminal amino acids (Fig. 9). The hydrophobic Ile10 residue and the nucleotide-protein contact mediated by the Tyr5 side chain appear to be particularly relevant for dimerization [88]. Moreover, according to ATPase activities of mutant proteins dimerization enhances the ATP-hydrolysis turnover [88]. In the complex, the Tyr109 residue provides a scaffold for the ATP-hydrolysis center. A channel formed at the dimer interface allows reactive water molecules to access and process the ATP γ-phosphate. Besides neutralizing the negative local charge density, a catalytically required Mg^{2+} ion provides appropriate orientation of the triphosphate group inside the reactive pocket [89]. A flexible GHKL loop, having conserved Gly residues allows accommodation of the phosphates, their negative charge being additionally counterbalanced by the conserved Lys103. In addition, a negatively charged Asp residue accounts for ATP over GTP specificity [87].

Besides the ATPase domain, a large surface containing basic residues in the hole generated by dimerization was identified as a possible DNA-binding region [87]. Such a transducer region mediates signal transduction and coordinates the subsequent cascade of catalytic events. In human

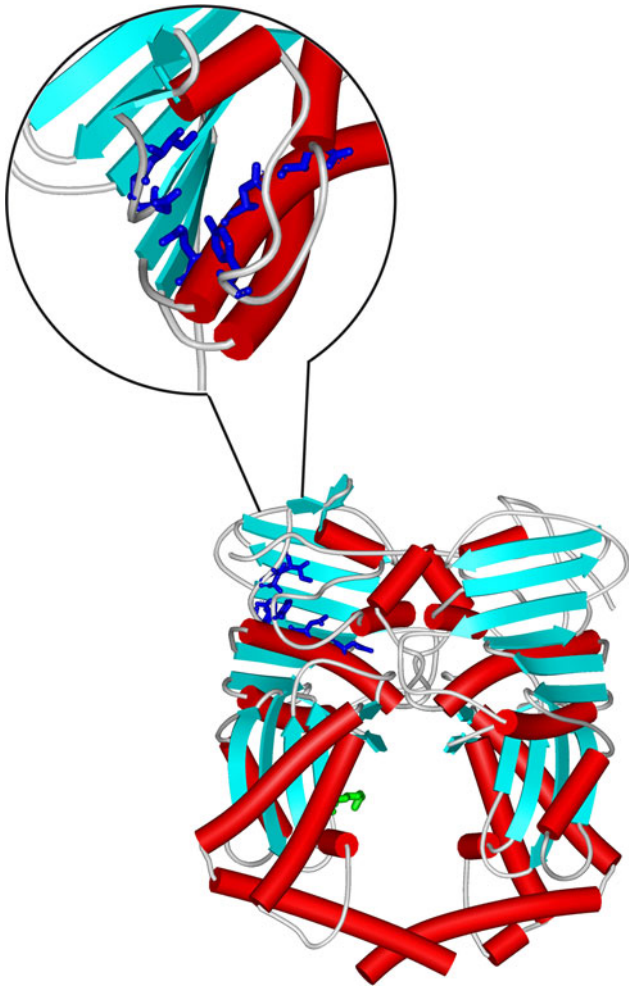

Fig. 9 Crystal structure of the 43-kDa *E. coli* GyrB dimer in complex with ADPNP, a nonhydrolyzable analogue of ATP (PDB ID 1E11). *Red tubes* refer to α-helix regions, *blue arrows* to β-sheet. Residues Glu42, Asn46, Glu50, Asp73, Arg76, Gly77, Ile78 and Tyr109 in the ATP hydrolysis region are highlighted in *dark blue* (ball-and-stick models, see also close-up), Asp286 in the DNA binding region in *green* (see text for details)

topoisomerase IIα, it is thought that a specific motif (QTK loop) mediates communication between ATPase and DNA cleavage regions [89]. Interestingly, the same motif is observed in *E. coli* and *S. aureus* GyrB as well as in *E. coli* and *S. pneumoniae* ParE transducer region at positions 335–337, 346–348, 332–334, and 333–335, respectively [90].

Computer-modeling studies using the dimeric structure of GyrB43 suggest that the putative DNA-binding cavity contains a constriction produced by Arg286 [91]. In a Gln mutant, unable to produce supercoiling, ATPase activity was not stimulated by DNA, which implies that nucleic acid binding to GyrB is a prerequisite for stimulation of the ATPase activity [91]. Similar studies on other GyrB mutants (Glu42, Asn46, Glu50, Asp73, Arg76, Gly77, and Ile78) suggest that all the above residues are directly

involved in ATP hydrolysis, while Pro79 and Lys103 mutants support ATPase function, but not supercoiling. In addition, nucleotide hydrolysis at only one GyrB subunit is sufficient to promote supercoiling [86, 92, 93].

DNA gate opening represents a crucial step to trigger DNA-stimulated ATP hydrolysis, as indicated by GyrB cross-linking studies [94]. Moreover, blocking the ATP-clamp in the closed form by means of ADPNP, a non-hydrolysable ATP analogue, is compatible with reduced DNA binding and cleaving activity, but not with supercoiling [95]. These results suggest that a T segment may reach equilibrium across the DNA gate by entering through the ATP-operated clamp or through the exit gate [95].

The mechanism emerging from the available information envisages an ATP-operated clamp, which segregates a T segment prior to DNA-gate opening. The conformational changes required for effective DNA processing catalysis can be triggered by T-segment trapping.

The ATP-binding region ParE43 from *E. coli*, examined by X-ray techniques, is structurally very similar to its Gyr counterpart in all essential amino acids [45]. Also, ADPNP binding occurs in a strictly analogous fashion. This essentially overlapping architecture, which could not be anticipated from the subunits' moderate sequence similarity, suggests a substantial conservation of the mechanism and effects of ATP binding and hydrolysis. Interestingly, a single amino-acid mutation, Met74Ile (Ile is found at the corresponding position in GyrB) produces remarkable changes in the enzyme's substrate affinity and catalytic efficiency, showing fine tuning of biochemical response produced by minor changes in the sequence of homologous enzymes [45].

The above similarities notwithstanding, a certain heterogeneity is apparent when examining the ATPase process in related species. In fact, Gyr from *B. subtilis*, exhibits an activity profile comparable to the *E. coli* enzyme [96]. However, subtle differences are evidentiated by the fact that the rate-limiting step in the nucleotide cycle of *B. subtilis* GyrB is ATP hydrolysis, not product dissociation or a related conformational change. Moreover, no cooperativity phenomena connect DNA and ATP binding in *B. subtilis* with single steps varying significantly even when comparing closely related enzymes [96].

Another interesting issue refers to the sequential hydrolysis of the two ATP molecules reported for yeast topoisomerase II, one prior to and the other after the strand passage event [97]. While ATP (but also ADP) binding grants N-gate closure, the first hydrolytic process possibly allows T-segment pushing for strand passage, the second ATPase event, responsible for enzyme resetting, is not molecularly defined. So far, it is not clear whether this mechanism could also apply to the bacterial topoisomerases, in particular to the supercoiling process mediated by Gyr though the experimental results presented above are compatible with such a view.

However, it is less simple to rationalize the linkage between energy input and the energetically favored relaxation, decatenation and unknotting processes, since they would not require ATPase activity [7]. The simplest explanation rests in the energy requirements for the observed reduction of supercoiling or catenation below equilibrium. Apparently, this process requires less energy than provided by nucleotide hydrolysis (0.2 versus 150 kJ/mol), nor is simplification sensitive to the ATP:ADP ratio [82]. Hence, in terms of energy transduction, it would represent poorly efficient machinery. Perhaps, a proofreading process could account for selective T segment testing followed by removal of those not properly oriented towards successful topological simplification [7].

Single molecule studies

Deeper insight into critical issues related to topoisomerases mechanism of action at the molecular level can be gained by monitoring progression of the catalytic cycle "on line". Recently, this was rendered feasible by applying single molecule techniques [98]. Torsional strain can be built up in the molecule when the DNA duplex ends are firmly bound one to a suitable surface and the other to a magnetic bead that could be rotated in a magnetic field. Under tension, as turns are added, the duplex extension remains essentially unchanged until a critical amount of torque accumulates and the molecule finally buckles, exchanging twist with writhe to form supercoiled structures [99]. This reduces the apparent DNA extension as the number of superhelical turns increases. Such a single molecule device can be useful to investigate enzyme action on differently stressed DNA as well as on DNA–protein assemblies like chromatin [100].

Topoisomerases are natural candidates for single-molecule studies. In fact, the activity of Gyr was monitored on a stretched DNA molecule [101]. Bursts of rotation were observed in the presence of ATP, which implies a processive introduction of negative supercoils in multiples of two. Increasing applied tension did not affect supercoiling kinetics, but reduced processivity. In view of that, processivity is explained in terms of kinetic competition between dissociation of the DNA–enzyme complex and tension-sensitive DNA wrapping [101]. Some features of this model were later criticized in view of discrepancies with biochemical data concerning hydrolysis product like the rate-limiting step and variation in the efficiency of coupling between ATP binding and strand passage [102].

Three distinct modes of Gyr activity were subsequently monitored as a function of force and torque [103, 104].

Under low mechanical stress, Gyr introduces negative supercoils by wrapping DNA. Instead, elevated tension or positive torque stimulates a mode of activity typical of the other topoisomerase II enzymes producing relaxation of left-handed braids and positive supercoils. A third mode accounts for the ATP-independent relaxation of negative supercoils [94]. It is interesting to note that applied tensile strength represents a key element to preferentially activate a specific mechanism of action.

Topo IV was shown to preferentially relax positively supercoiled DNA, probably by discriminating the supercoiling geometry of the left-handed versus right-handed arrangements [81]. DNA binding and single-molecule braiding experiments confirmed that Topo IV recognizes positive supercoils by a mechanism related to the chiral geometry of G- and T-segment crossings [105]. The enzyme is found to bind cooperatively to supercoiled DNA, suggesting that the enzyme subunits assemble efficiently upon binding to the nucleic acid [106]. Like Gyr, but unlike the eukaryotic homologues, Topo IV bends DNA changing local twist and/or writhe by 0.16 turns per complex unit. The enzyme/DNA complex changes between two states, one weakly bound and the other strongly bound, possibly reflecting different enzyme conformations upon T or G segments binding [106].

Subsequent studies on the relaxation rates of single and multiple DNA crossings by Topo IV showed that the enzyme processes DNA strands oriented almost perpendicularly [107]. Moreover, relaxation of positively supercoiled DNA is highly processive, whereas relaxation of negatively supercoiled DNA is purely distributive, which may account for the chiral substrate discrimination observed in Topo IV. These results show the importance of enzymatic processivity as compared to the direction of the crossing angle in chiral discrimination by Topo IV [107]. Interestingly, the eukaryotic type II enzyme from *Drosophila melanogaster* relaxes left-handed and right-handed braids at essentially the same rate. In addition, the prokaryotic and eukaryotic enzyme exhibit substantially different rate-limiting steps [108]. The remarkably different efficiencies exhibited by enzymes performing equivalent functions possibly reflect the different need to resolve decatenation in bacterial versus mammalian chromosomes.

Although the results from magnetic micromanipulation are quite informative and may give quantitative information on the biochemical processes at the single molecule level, the biological significance of experimental outcome remains still somewhat controversial, especially when the technique calls for application of high tensile strength onto DNA, which might represent a non-physiological state, which hence induces enzyme responses not directly transferable to real situations.

A further mechanistic issue addressed by a single-molecule smFRET approach refers to topoisomerase gate opening/closure timing during catalysis [109]. The experimental results bear evidence that DNA binding, cleavage, and gate opening are mechanistically distinct events. Additionally, DNA gate was shown to be prevalently in a closed form. This addresses concerns about the possible chromosomal damage generated by high content and long persistence of cleaved DNA intermediates produced by the action of topoisomerases [110].

At this point, a few mechanistic remarks are warranted:

- DNA bending appears to represent a key determinant for bacterial topoisomerases functions in vivo, as strongly indicated by available information on the molecular mechanisms of enzyme action. The level of bending is sufficient to address DNA cleavage intra- or inter-molecularly with quite different biological consequences; bending is invoked to account for monodirectionality of the topological event, T segment passing through the interior of the curved G segment; bending is deemed responsible for sensing catenane handedness and resolving entanglement. It would be interesting to examine DNA hot spots for topoisomerase binding to assess their intrinsic tendency to produce bent conformations, hence to act as favored enzyme substrates.
- Most mechanistic implications were obtained by combining data from crystal structures of topoisomerase fragments from different sources. Hence, relevant information for a detailed description of the interplay among domains either located in the same subunit or getting close in space as a result of functional tetramer assembly is still elusive. For example, the poorly understood functional role of the GyrB "tail" domain (residues 605–648), ubiquitous among type IIA topoisomerases, is now being clarified starting from the very recent crystal structure of the GyrB sequence 485–714, including the TOPRIM and the "tail" domains, and a C-terminal segment [49].
- Finally, the significance of selective changes occurring in catalytically and structurally crucial residues in topoisomerases from different species needs to be analyzed much more deeply to explain important modifications in substrate recognition and processing on a case-by-case basis.

Cellular localization of Gyr and Topo IV

Progression of the replicative fork generates a condition where positive supercoils accumulate in front of it while precatenanes (strongly similar to catenanes) are generated

behind [111]. This uneven topological distribution drives differential recruitment of the two enzymes along the genome being processed and, possibly, requires different timing of expression [51, 112].

This is confirmed by topoisomerases mutations that lead to a dramatic reduction in replication fork progression and production of double-stranded breaks into the nucleic acid [113]. DNA microarray experiments further suggest that in *E. coli*, both Gyr and Topo IV promote replication fork progression, which contributes to the elongation rate [114]. The same effect is present in transcription elongation as a solution to the positive supercoiling generated during this process. Hence, Gyr should be especially effective in front of the replication fork and Topo IV behind it, while they could cooperate in reducing positive superhelicity during transcription.

In addition to specific abilities of Gyr and Topo IV in processing DNA in vivo, an important issue refers to their cellular distribution as it can further differentiate the physiological role of the two enzymes [115, 116]. In living *B. subtilis* cells Topo IV and Gyr are reported to differentially localize on the nucleoids. Topo IV is apparently uniformly distributed, whereas Gyr forms discrete accumulations on the nucleoids in a large fraction of the cells, which showed highly dynamic movements. Three-dimensional time-lapse microscopy indicated that Gyr builds up and vanishes within a 1-min time scale. In addition, it is frequently co-localized with the central DNA replication machinery, confirming a major role for Gyr at the level of this process.

We should also recall that the supercoiling level changes locally due to compartmentation of DNA sequences during selected cell-cycle dependent functions, which may recruit different actions at defined genomic regions.

Altogether, the data thus far presented show how modest structural changes may produce specialized biological functions. Indeed, Topo IV might derive from Gyr by progressive loss or modification of structural characters of the CTD region, in particular deletion of the GyrA-box. This step would be sufficient to cause loss of Gyr supercoiling activity, while preserving relaxation and promoting decatenation abilities.

Bacterial IIA topoisomerases as drug targets

Since both Gyr and Topo IV are vital enzymes, any agent able to interfere with their catalytic cycle can be in principle exploited for antibacterial chemotherapy [50]. To grant effective pharmacological action and avoid toxic effects, a drug molecule should be able to inhibit the prokaryotic topoisomerases' activities without impairing the host topoisomerases' functions.

Two principal targets for effective drug intervention can be envisaged:

– impairment of the DNA cleaving-rejoining functions;

– inhibition of ATPase activity.

Representative drugs acting by the above mechanisms are reported in Fig. 10.

Bacterial topoisomerase II poisons

The first target exploits the topoisomerase action, transforming a physiological process into a lethal event. In fact, an intermediate stage of the strand-passing event corresponds to the formation of a covalent DNA–enzyme complex in which duplex DNA is cut and its 3′-ends are temporarily uncapped. If the steady-state level of this "damaged" DNA is kept low by a physiological procedure of re-sealing, damage is transient and will soon be repaired without consequences. On the contrary, if the damage level is increased by the presence of an interfering drug molecule that stacks in the cleavage site and changes its local conformation, the phosphotyrosine group will no longer be located sufficiently close to the 3′-OH to engage it in the resealing transphosphorylation [117]. In this case, the steady-state level of cleaved DNA will increase and the cut DNA will trigger at least two types of events, one dependent and the other not upon protein synthesis. In particular, (1) the replication machinery is arrested and (2) oxidative damage is activated via peroxide/hydroxyl radical production [118, 119]. As a consequence, the bacterial chromosome becomes lethally fragmented and the SOS response is activated, finally leading to bacterial cell death [120]. Hence, antimicrobial action is the result of "poisoning" the topoisomerase II enzyme, which actively participates in producing bacterial cell death [121, 122]. Indeed, this topoisomerase-mediated mechanism has successfully led to clinically effective antibacterial agents. A similar mechanism is exploited by eukaryotic topoisomerase poisons which are currently used in anticancer chemotherapy.

Fluoroquinolones

The principal family of prokaryotic topoisomerases II-targeted drugs is represented by fluoroquinolones [123–125]. They consist of a 6-fluoro-quinoline ring system, with a keto-carboxylic function at position 3 and 4, with several possible substituents at positions N-1 and C-7. Successful representatives of this family are shown in Fig. 10, in the upper panel.

At the molecular level, the interactions of quinolones were found to involve mainly subunit A of Gyrase and

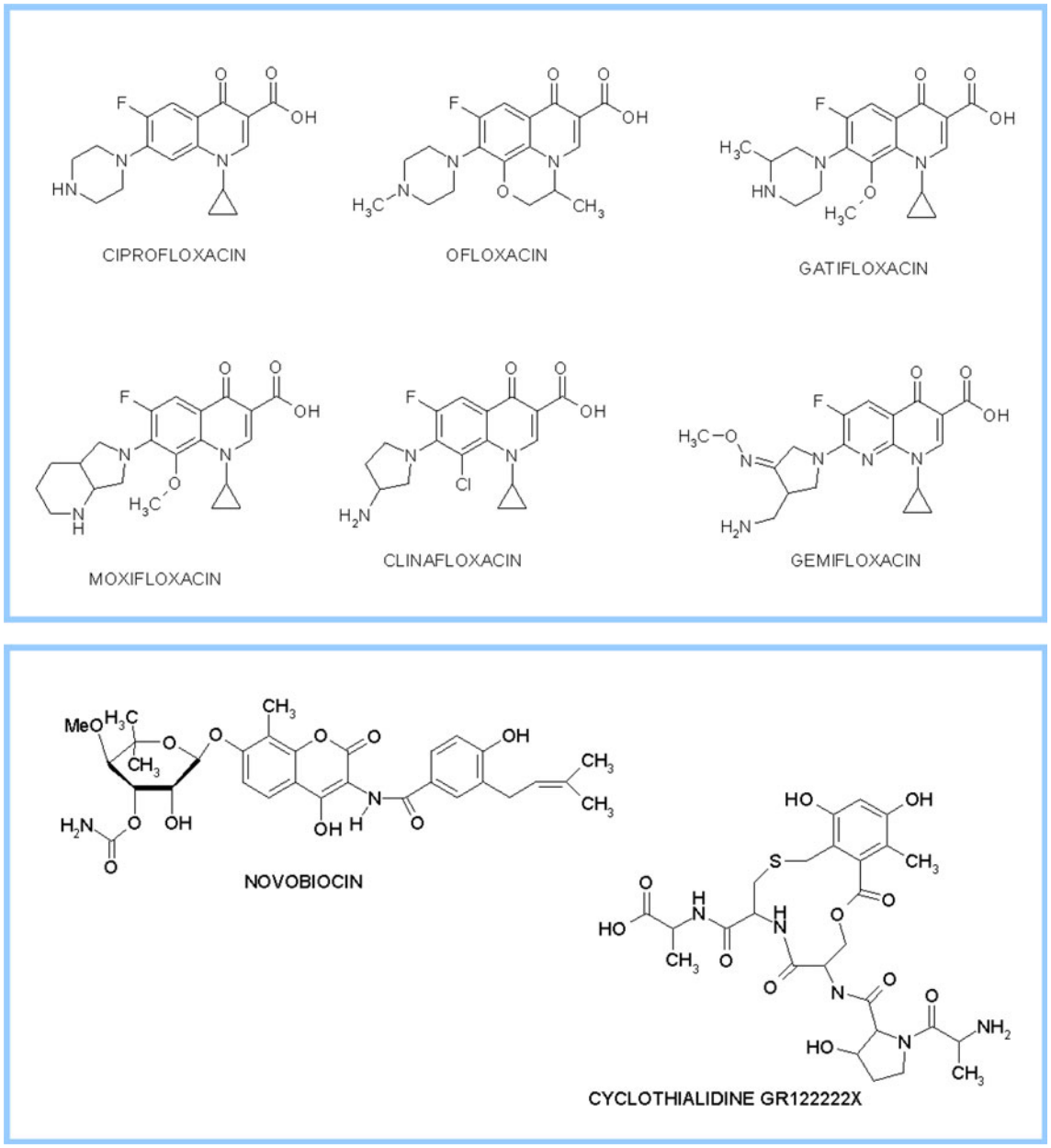

Fig. 10 Representative bacterial topoisomerase II inhibitors. Chemical structure of cleavage complex poisons (*upper panel*) and ATPase inhibitors (*lower panel*)

ParC of Topo IV. In fact, a region spatially close to the active tyrosine called Quinolone Resistance Determining Region (QRDR) has been identified. Amino acid mutations in this region greatly reduce the affinity of quinolones for the enzyme–DNA complex. The most relevant positions in *E. coli* are Ser83 and Asp87 in GyrA and the corresponding Ser79 and Asp83 in ParC [126, 127]. Mutations in GyrB and ParE, although implicated in the fluoroquinolone resistance, are less frequently observed. The current hypothesis is that they occur at the interface between the two subunits inducing subtle structural changes in the overall protein architecture.

The precise mechanism of topoisomerase II inhibition by quinolones has long been an object of debate, specific issues dealing on mode and cooperativity of interaction with DNA and participation of metal-ion co-factors [128–130]. Very recently, the structural features of a quinolone bound to a Topo IV cleavage complex (*S. pneumoniae* ParC breakage-reunion, ParE TOPRIM and G-DNA) have been elucidated by crystallographic studies (Fig. 5) [48]. The quinolones employed were moxifloxacin and clinafloxacin, two very effective antimicrobial agents.

Moxifloxacin complex is formed from a ParC55 heart-shaped homodimer flanked on either side by ParE30

monomers. Structurally, the ParC55 dimer closely resembles the unliganded protein in the absence of ParE30 as well as GyrA59, with the active site Arg117 and Tyr118 on the 100–122 loop (see above). The C-gate is organized in the closed form and TOPRIM domain is correctly located to interact with DNA along with the WHD domain (Fig. 6). The nucleic acid participates in the cleavage complex with the active site tyrosines covalently bound to the 5′-phosphate residue. In this region, DNA produces notable local changes, closing up the protein domains, so that the inner part of the 100–122 loop leans toward the DNA backbone. A moxifloxacin residue is inserted into the cleft at each side of the 4-bp staggered cleavage site. The structure of the drug molecule intercalated between the bases immediately preceding (−1) or following (+1) the cleaved bond is reported in Fig. 11. The quinolone stacks against a purine belonging to the residue immediately adjacent to the cleaved (+1) phosphodiester link. The cyclopropane ring points in the direction of Ser79 and Asp83, which are known to mutate in quinolone-resistant bacterial strains. Apparently, the reason for resistance is not correlated to a direct interaction, but it might rest in changes in local conformation when a hydrophilic amino acid is eventually replaced by an aromatic residue (Phe, Tyr) in the mutants. The C7 substituent gets close to the DNA base on the opposite DNA strand and is easily accommodated inside a large, solvent-accessible cavity generated by cleavage. No particular interaction is evidenced by the 6-fluorine atom, which possibly optimizes the interaction through its negative charge density.

Surprisingly, the essential keto-carboxylic substituents are separated from the metal ion-binding TOPRIM residues. Since the supposed function of the drug substituents at positions 3 and 4 was to chelate the active site Mg^{2+} ion, the present data lead to the conclusion that this is not the case. However, given the fact that some domains of the full-length enzyme are missing and considering that the catalytic TOPRIM region must get close to the phosphotyrosine linkage (and the cleaved 3′-OH group) to complete the catalytic cycle, there might be a further conformational change needed to reseal the gap, which could account for "later" sequestering of the metal ion by the quinolone. This is, of course, a speculative matter. In any event, inspection of Fig. 11 shows the possibility of quinolone and phosphotyrosine oxygens' engagement in coordination to a divalent ion. The DNA-base preferences observed (+1 G, +1 A) possibly account for the more efficient stacking of the planar portion of the quinolone onto a purine rather than a pyrimidine base.

The clinafloxacin analogue yielded essentially the same picture, which would suggest a common mode of quinolone recognition of the cleavage complex. Information at the molecular level will surely help in the future rational design of effective inhibitors.

New drugs are in fact badly needed, as the high efficiency and broad spectrum of activity shown by quinolones are now seriously challenged by the selection of resistant strains, even for naturally highly susceptible species like *E. coli* [126, 131–134]. Thus, great effort is presently devoted to overcoming resistance phenomena that are emerging as the most relevant complication in antibacterial therapy. Resistance to quinolones is generally acquired through mutation. In fact, as mentioned above, quinolone resistance is seen in selected GyrA and/or ParC mutants [135]. However, plasmid-mediated genes encoding quinolone resistance were recently discovered [136–138]. Some of them (QnrA1, QnrB, QnrS; QnrC) protect Gyr/Topo IV from drug attack, others (QepA) code for enzymes able to modify quinolones or for efflux pump. The promotion of resistance by these genes, their horizontally spreading

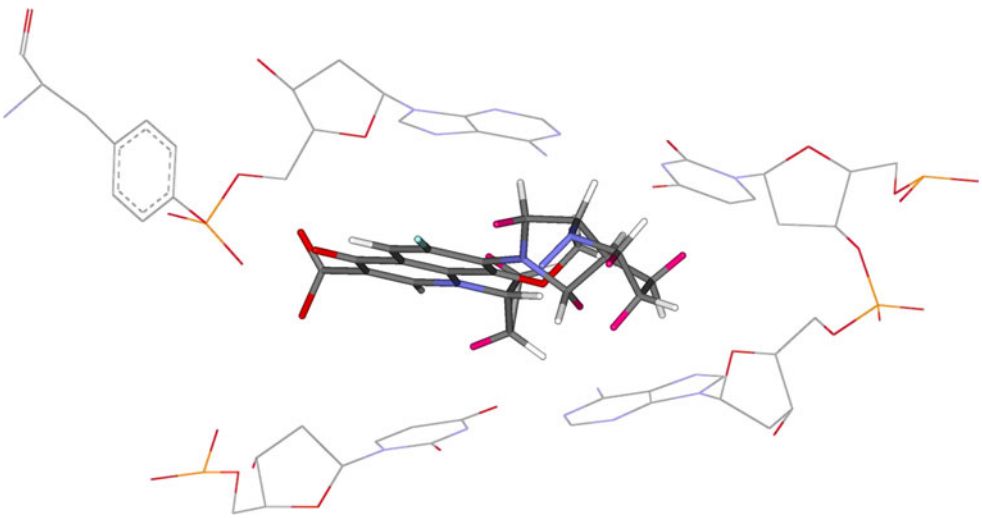

Fig. 11 Close-up view of the quinolone moxifloxacin (*stick model*) bound to the *S. pneumoniae* ParC55-ParE30-DNA cleavage complex (*line model*). Source: PDB ID 3FOF

abilities and the combined selection of resistance patterns raise serious concern on the practical use of quinolones.

New inhibitors

Antibacterials of the fluoroquinolone family were first utilized almost 40 years ago. Since then, well over 10,000 analogues were reported. Solid SAR (structure activity relationships) studies are available in the literature, which allow the exploitation of a large number of substituents in different positions of the quinolone ring [139, 140]. Besides positions 3 and 4, particularly significant in the modulation of activity are positions 1, 7, and 8. Bioisosteric substitutions as well as modifications in the ring structures were additionally performed. Recent studies suggest the potential usefulness of gatifloxacin and ofloxacin derivatives, quinolines, pyrazoles, isothiazolopyridones, and aminoquinazolinediones, some of which are especially suited to counteract the onset of resistance [141–148].

Dual inhibitors

When tested against Gyr and Topo IV, many quinolones inhibited both enzymes. This is not surprising, given the close mechanistic similarities exhibited by the latter. Nonetheless, traditional quinolones active against Gram-negative bacteria are more effective against Gyr than Topo IV, whereas the reverse is true for quinolones aimed at Gram-positive bacteria. However, this is not a general rule as preferential enzyme inhibition is species specific. Compounds exhibiting dual targeting are desirable since mutations must be introduced in both topoisomerases to produce resistant strains, hence the onset of resistance is less likely [149, 150]. Among quinolones showing dual targeting clinafloxacin, gemifloxacin, moxifloxacin, besifloxacin, and amminopyrrolidyl analogues deserve to be mentioned [151, 152]. Recently, isothiazolone derivatives and a ciprofloxacin analogue with a methyl substituent at position C-8 and a 4-hydroxy-3-methyl-1-piperidinyl moiety at C-7 have been reported to behave as effective dual inhibitors [153–155].

As an extension of dual inhibition, hybrid antibiotics targeting bacterial topoisomerases II together with DNA- or RNA-polymerase were also investigated and shown to develop resistance at quite low rates [156, 157].

Finally, a quinoline derivative linked to a piperidine carboxylic acid, exhibited potent activity against Gram-positive bacteria resistant to inhibition by fluoroquinolones [158]. Although it interferes with Gyr and Topo IV activities, its molecular mechanism of action seems to impair formation of the cleavage complex, rather than stabilizing it.

Inhibition of ATPase activity

Although until now no antibacterial drug presently in clinical use acts by inhibiting ATPase, the absolute requirement for ATP of the bacterial topoisomerases prompts significant efforts to design new derivatives that impair nucleotide hydrolysis. In fact, naturally occurring cyclic peptides of the coumarin family (novobiocin) and the cyclothialidines, have been known for a long time to represent effective Gyr inhibitors acting on the N-terminal GyrB subunit (Fig. 10, lower panel). The structural features of cyclothialidine recognition of the ATP site when complexed to a short (24-kDa) GyrB fragment in a monomeric assembly include a major contribution from the resorcinol group and several hydrogen bonds mediated by water molecules [159]. Novobiocin, in turn, shows a large network of hydrogen bonds, involving primarily the novobiose sugar. An interesting notion emerging from these studies is that the various inhibitors only partially overlap the ATP-binding site, which shows how different drugs may use different target-recognition strategies to reach the same goal. More informative is an investigation dealing with the structure of a dimeric GyrA43 fragment form *T. thermophilus* in complex with novobiocin. This antibiotic produces large conformational changes of the subdomains within the dimer. In particular, loop 98–118, closing the active site through dimeric contacts, generates an "open" conformation of the N gate as opposed to the "closed" conformation adopted by the competitor ATP [160]. More recent studies on the equivalent ParC fragment explain that novobiocin is less potent against Topo IV than against Gyr as a result of an unfavorable contact of the drug with a Met residue, replaced by Ile in Gyr [45]. Unfortunately, members of the above-mentioned natural families, although exhibiting activities in the nanomolar range, were not useful as drugs due to their inadequate pharmacological profile. However, their structures and the biochemical knowledge stemming from their use can be valuably employed to devise new potential inhibitors.

New ATPase inhibitors

Since the ATP-binding site of GyrB possesses a remarkable level of flexibility, consensus functionality maps were employed to identify functional groups able to recognize the ATPase region [161]. The calculations show that some yet unexplored binding sites could be considered for drug design and suggest the potential usefulness of several new chemical scaffolds. Moreover, since the crystal structure of a nonhydrolyzable ATP analogue bound to GyrB43 is available [87], a generally employed approach is to mimic key interactions occurring between the adenosine functional groups and protein invariant

residues, in particular Asp73, Gly77, and Thr165 (Fig. 9). Another way to select new active compounds, unrelated to the previous ones, is the use of virtual screening techniques [162]. Although these techniques give valuable initial directions for drug discovery, their indication should be considered as preliminary. High-throughput screening of existing libraries represents another reasonable strategy for lead discovery.

Novel inhibitors thus far tested include indolinones, catechins, azoles, benzopyranones, imidazolo- and triazolo-pyridines, benzimidazoles and chiral barbituric acid derivatives [163–170]. Among these compounds, members of the latter two families were endowed with the ability to inhibit both GyrB and ParE ATPases, hence behaving as especially valuable dual inhibitors.

Natural antibacterial compounds

A number of natural compounds aimed at bacterial topoisomerases II have been discovered and investigated. The interest in these compounds stems from the fact that they efficiently recognize their targets, so that they behave as remarkably specific inhibitors. In addition, starting from the natural structure, a pharmacophoric pattern can be identified and subsequently used for drug design. Among the derivatives recently reported the toxins CcdB, Microcin B17, albicidin and the small protein YacG deserve attention [171–175].

A paradigmatic, well-studied, example is CcdB. The toxin (T) CcdB is encoded by the ccd operon on plasmid F of *E. coli* together with its antitoxin (A) CcdA. Protein TA systems are utilized by plasmids to ensure that only the daughter cells that inherit the plasmid survive after cell division [176]. CcdB has two effects on Gyr. In CcdB overproducing cells, the enzyme (A subunit) is bound to CcdB and loses its ability to bind and supercoil DNA. As a second mode of action, CcdB converts Gyr into a cytotoxic agent with a mechanism similar to that of quinolones. In fact, CcdB, as a dimer, can trap Gyr in a special conformation during strand passage, with the opening of the tower and catalytic domain, when the G segment is cleaved but crossing of the T segment has not yet occurred [175, 177]. In fact, the crystal structure of CcdB bound to the dimerization domain of the A subunit of Gyr (GyrA14) reveals an "open" conformation for the catalytic core when in complex with the toxin. This represents a novel mechanism, which freezes the cleavage complex before re-sealing, hence in a toxic arrangement [178]. This mechanism could be profitably exploited to develop new effective drug molecules. As a matter of fact, peptides based on the CcdB structure showed inhibitory properties towards Gyr-mediated DNA supercoiling [179].

Recently, a new antibiotic, simocyclinone D8, was isolated from *S. antibioticus* Tű 6040 [180, 181]. It shows activity against Gram-positive bacteria, as well as cytostatic effects against human tumor cell lines, while it is apparently ineffective against Gram-negative species. The interest for this compound arises from the fact that, notwithstanding the presence of a 3-amino-4,7-dihydroxycoumarin function, reminiscent of the coumarin antibiotics, it does not interfere with ATPase functions. Instead, it is a potent inhibitor of Gyr, acting by a novel mechanism possibly related to Gyr-DNA-binding impairment [182, 183], likely occurring through effective drug interactions with the GyrA subunit [184]. Once this mechanism is fully elucidated, new drug-development strategies on the identified target(s) could be envisaged.

Finally, a biotechnological approach has been employed to down-regulate Gyr A gene expression in *E. coli* and *P. falciparum* through antisense ribozymes and it shows remarkable effects on cell viability [185, 186].

Conclusions

The more we learn, the more we see nature's amazing ability to create remarkable biological functionalities. In the case of topoisomerases, chemical energy is transduced into mechanical energy to grant effective and safe means for the cell to deal with DNA topology. Powerful methods such as X-ray crystallography and single-molecule techniques enable us to "see" such huge proteins at various stages of their action and to dissect the mechanisms by which they perform DNA strand passage to produce supercoiling or decatenation. By the additional contribution of other biochemical, biophysical, and biotechnological methods our understanding of the biological pathways of cell function is further and further enriched. Quite interestingly, evolution from one topoisomerase species to the other has allowed acquisition of very specialized functions. Gyr and Topo IV are a clear example of how modest structural rearrangements can make them solve specific topological problems generated by essential cellular actions like DNA replication and transcription. Not only do they act individually, they are synergistically operating to maintain the proper structural organization of the genome. In addition, DNA topology plays a more general role in the expression of virulence genes in pathogenic bacteria at different phases of the host–pathogen relationship. In fact, supercoiling levels control the gene regulatory network of the cell hierarchically, influencing the activities of a large number of promoters simultaneously [187].

Learning how essential enzymes work allows us to rationally devise new tools for interfering with their action not only to dissect known functions or unravel new ones

but also to develop appropriate therapeutic strategies, for example when dealing with bacterial infections. Also, different targets in the same enzyme can be exploited, improving the chances for successful therapeutic intervention. In this connection, it would be interesting to identify drug leads able to interfere with the bacterial CTD functions, not present in the human enzymes therefore maximally reducing the concern that compounds acting against bacterial topoisomerases might exhibit minor albeit non-negligible effects also on the corresponding eukaryotic homologues, thus producing undesired toxic side-effects [188].

As a final remark, a programmed manipulation of the genes involved in the biosynthetic pathways of antibiotic-producing microorganisms could represent a valuable approach to yield combinatorial libraries of re-designed antibiotics [189, 190] to be successfully exploited against the so-called life-threatening "superbugs".

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