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Biochemical complementation studies in vitro of gyrase subunits from different species

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Abstract To investigate the functional equivalence of DNA gyrase subunits from different bacterial sources hybrid enzymes were formed using purified A and B subunits from three species of *Streptomycetes*, *E. coli* and *B. subtilis*. The activity of gyrase hybrids composed of heterologous gyr A and gyr B proteins and of the gyrases containing homologous subunits was characterized by binding studies and a cleavage assay with two different DNA fragments. Likewise the enzyme activity was monitored by the super-coiling and relaxation assay with pBR322 DNA. We found that cleavage reactions are largely determined by the source of the gyr B subunits whereas DNA supercoiling and relaxation reactions of pBR322 catalyzed by DNA gyrase are limited to a combination of homologous A and B subunits or of heterologous A and B subunits from the taxonomically related bacteria *Streptomycetes*.

Key words: DNA gyrase; Subunit; Gyrase hybrid; DNA binding; DNA cleavage; Immunology

1. Introduction

DNA gyrases are bacterial enzymes, which possess the unique property to catalyze the supercoiling of relaxed closed circular DNA [1–3]. The holoenzyme exists as a tetramer, A_2B_2, composed of two subunits A and B encoded by the genes gyr A and gyr B. The *E. coli* gyrase activity is known to be inhibited by two classes of drugs. It is believed that quinolones act on the Gyr A-DNA complex [4–6] whereas coumarins block the gyrase mediated reaction by competing with ATP for the interaction with the B subunit [7]. The structural and biochemical functions of the *E. coli* enzyme have been extensively investigated [1–3]. Interspecies complementation studies indicated that the *M. luteus* gyr A protein can complement *E. coli* gyr B protein and vice versa to give active enzymes [8]. It was also reported that the B subunit from *B. subtilis* can complement the *E. coli* A subunit while the reciprocal complementation did not result in an active enzyme [9]. The general functional equivalence of gyrase subunits from different bacterial species with respect to the reaction steps of the enzyme is still an unsolved issue. On the other hand in view of eukaryotic DNA topoisomerase II, which contains domains with homologies to the gyrase B and A subunits [10], the interaction of heterologous gyrase subunits is of certain interest. To obtain more insight into the aspects of the exchangeability of heterologous subunits in relation to the gyrase function we have performed biochemical complementation (studies in vitro) using purified subunits from five different bacterial strains and three taxonomically different sources. Our study includes subunits A and B from *E. coli*, *B. subtilis* and from three species of *Streptomycetes*.

2. Materials and methods

2.1. Enzymes

DNA gyrases were purified from five different bacterial strains: *Escherichia coli* K12, *Bacillus subtilis* 170, *Streptomyces noursei* N 840, *Streptomyces griseus* 3933 and *Streptomyces netropsis*. The isolation of gyrases was performed according to Staudenbauer and Orr [11] using a novobiocin-sepharose column. For enzymes from *Streptomycetes* distinct modifications of the method were applied [12].

2.2. DNA

pBR322, isolated according to a known procedure [13] was relaxed with topoisomerase I from nuclei of chicken erythrocytes [14,15]. The 162 bp fragment from pBR322 was that described previously [16]. The 169 bp fragment containing a high GC content was prepared from the recombinant plasmid pUC19 carrying the nourseothricyl-acetyl-transferase (nat) gene of *S. noursei* inserted into the *Bam*HI site [17]. The fragment was derived by digestion of the plasmid with *Pst*I–*Kpn*I and subsequent treatment of the 250 bp fragment with *Sal*I.

2.3. DNA gyrase assays

Gel retardation and cleavage assays have been described previously [16].

Supercoiling reactions of gyrases were performed with relaxed pBR322 DNA in 20 μl assays containing about 500 ng DNA in 40 mM Tris-HCl (pH 7.5), 19 mM KH_2PO_4, 34 mM KCl, 1 mg/ml BSA, 2 mg/ml spermidine, 1.2 mg/ml ATP, 0.5 mg/ml t-RNA and 3 mM $MgCl_2$. Samples were incubated for 50 min at 30°C and applied to electrophoresis in 1% agarose gels (Sigma II) in Tris-acetate-EDTA (pH8) buffer at 30 V and about 200 mA overnight.

The gyrase-mediated relaxation reaction was carried out with supercoiled pBR322 DNA in the absence of ATP in a buffer system containing 67 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 25 mM KCl, 1 mg/ml BSA and 0.5 mg/ml t-RNA. Samples were incubated 24 h at 30°C and applied to electrophoresis as given above.

3. Results

3.1. Binding of various gyrases to DNA fragments

The first step of the gyrase mediated reaction is its binding to the substrate. Since it is not yet clear whether gyrases from different bacteria possess similar sequence affinity for the same substrate we compared the binding ability of gyrases from *S. noursei*, *E. coli* and *B. subtilis* to two DNA fragments differing in the sequence and cleavage sites. Formation of gyrase complexes with DNA fragments was detected by gel retardation assay and the results are shown in Fig. 1. It is evident that the binding curves of the gyrases from *E. coli* and *B. subtilis* are similar for both fragments (Fig. 1A and B) with a saturation level around 200 ng of the added enzymes. Binding to the 162 base pair (bp) fragment of *S. noursei* gyrase is comparable to that of the two other enzymes only within the initial part of the

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binding curve up to 100 ng of the enzyme added, but differs near the saturation level. For the 169 bp fragment the binding ability of *S. noursei* gyrase is strikingly lower relative to the *E. coli* and *B. subtilis* enzymes (Fig. 1B). A 50-fold molar excess of the *S. noursei* gyrase over the 162 bp DNA binds about 80% of the fragment, while 60% of the 169 bp DNA are bound by this enzyme at 200 ng under comparable conditions. These results suggest a slightly different binding to the 162 bp DNA but a markedly lower binding strength to the 169 bp DNA of *S. noursei* gyrase compared to the enzymes from *E. coli* and *B. subtilis*. Fig. 1C shows that Gyr B from *E. coli* and *B. subtilis* complemented with *S. noursei* Gyr A strongly favours the binding in a cooperative manner to the 169 bp fragment of the hybrid enzymes relative to the *S. noursei* enzyme (full circles), which is valid for the initial binding region between 100 and 250 ng protein. The A-protein of *S. noursei* alone has a clearly lower affinity to this fragment in the same protein concentration range (Fig. 1C, crosses). It should be noted that the actual protein concentrations of Gyr A are different between the curves of the hybrids and that of Gyr A alone.

3.2. Biochemical complementation studies of interspecies gyrase subunits

To test the functional equivalence of gyrase subunits from various taxonomic sources we employed the following in vitro assays: cleavage of the substrate in presence of ciprofloxacin, the supercoiling assay and the slow relaxation reaction in the absence of ATP. A representative example of the gel electrophoretic analysis for Gyr A from *S. noursei* mixed with four heterologous Gyr B proteins is shown in Fig. 2. The results of various subunit combinations are summarized in Table 1. Since it was important to exclude that subunit fractions do not contain traces of the second subunit we have controlled all subunits alone at 2–3 times higher concentrations than used in the assays.

The cleavage reaction of gyrase mediated by ciprofloxacin was examined with pBR322 and two DNA fragments differing in their sequence. Relaxed pBR322 and the 162 bp fragment from pBR322 containing a strong gyrase site at nucleotide 990 as known for *E. coli* [18,19] are cleaved by homologous and all heterologous combinations of subunits investigated (Fig. 2A and C, Table 1). It has to be mentioned that cleavage activity of gyrase hybrids with Gyr A from *E. coli* and *B. subtilis* with Gyr B from *Streptomycetes* required higher concentrations of the latter. In contrast to that the 169 bp fragment is not cleaved by all three gyrases from *Streptomycetes* and the heterologous combinations of *S. noursei* Gyr A with Gyr B from *S. griseus* and *S. netropsis* (Fig. 1B, Table 1). Different from this the heterologous combinations of Gyr A from *S. noursei* with Gyr B from *E. coli* or *B. subtilis* resulted in gyrase hybrids with strong cleavage activity as in the case of the homologous subunit mixtures of *E. coli* and *B. subtilis* as well as their reciprocal subunit combination (Table 1). Clearly, these cleavage results strikingly correlate with the distinct higher binding ability to the 169 bp fragment of gyrases from *E. coli* and *B. subtilis* (Fig. 1B) as well as of the gyrase hybrids containing *E. coli* or *B. subtilis* Gyr B and *S. noursei* Gyr A (Fig. 1C).

Fig. 2 shows that DNA supercoiling is catalyzed only by homologous gyrase hybrids or by a combination of Gyr A from *S. noursei* with Gyr B from other *Streptomycetes*, but not with Gyr B from *E. coli* or *B. subtilis* (Fig. 2C, Table 1). This could mean, that the mechanism of DNA supercoiling involving a

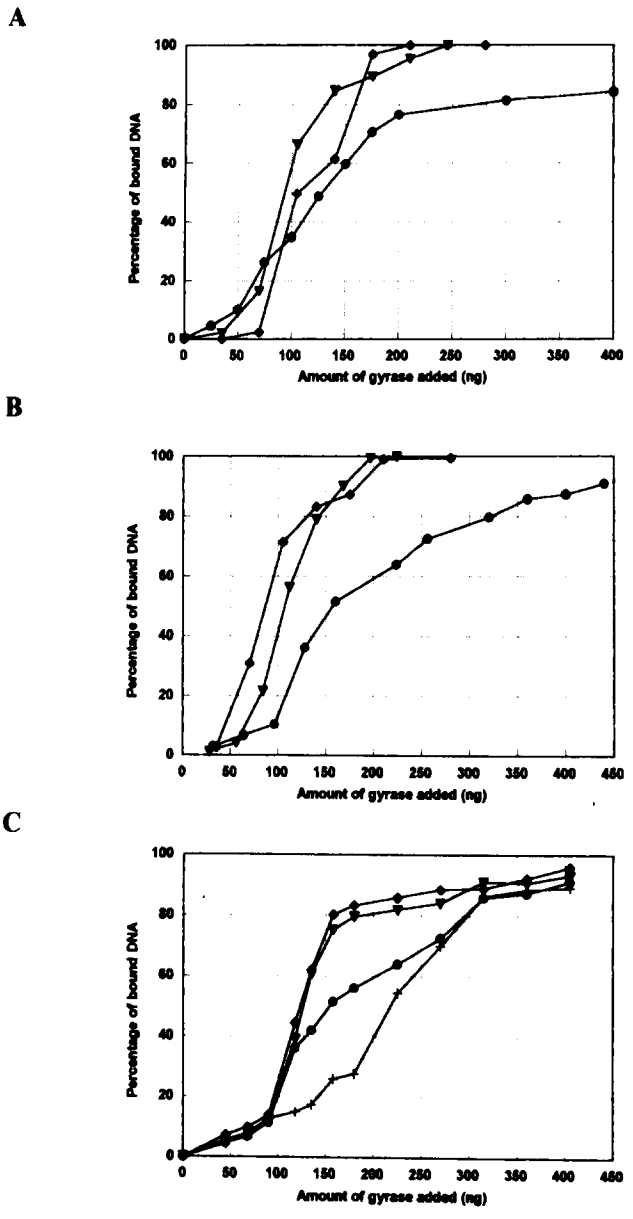

Fig. 1. Binding curves of DNA gyrases to DNA fragments. (A) 162 bp fragment; (B) 169 bp fragment: gyrases from –●–● *S. noursei*; –◆–◆ *B. subtilis*; –▼–▼ *E. coli*; (C) binding to the 169 bp fragment of hybrid enzymes: *S. noursei* Gyr A plus *E. coli* Gyr B; –◆–◆ *S. noursei* Gyr A plus *B. subtilis* Gyr B; –●–● *S. noursei* Gyr A plus *S. noursei* Gyr B; –×–× *S. noursei* Gyr A alone. The amount of bound DNA was detected densitometrically from band shift assays; 1 ng of ^{32}P-labelled DNA was retarded by gyrases in 5% PAA gels in TBM buffer (90 mM Tris-borate, pH 7.0; 3 mM $MgCl_2$).

concerted reaction cycle requires a very specific binding between the A_2 and B_2 dimers in the gyrase tetramer complex which can be formed only with homologous Gyr A and Gyr B proteins or with subunit species of the same bacterial genus as observed in case of *Streptomycetes*. This interpretation was supported by restoration of the supercoiling activity by adding homologous Gyr B to a gyrase hybrid, e.g. addition of a defined amount of *E. coli* Gyr A to the inactive hybrid of *S. noursei* Gyr

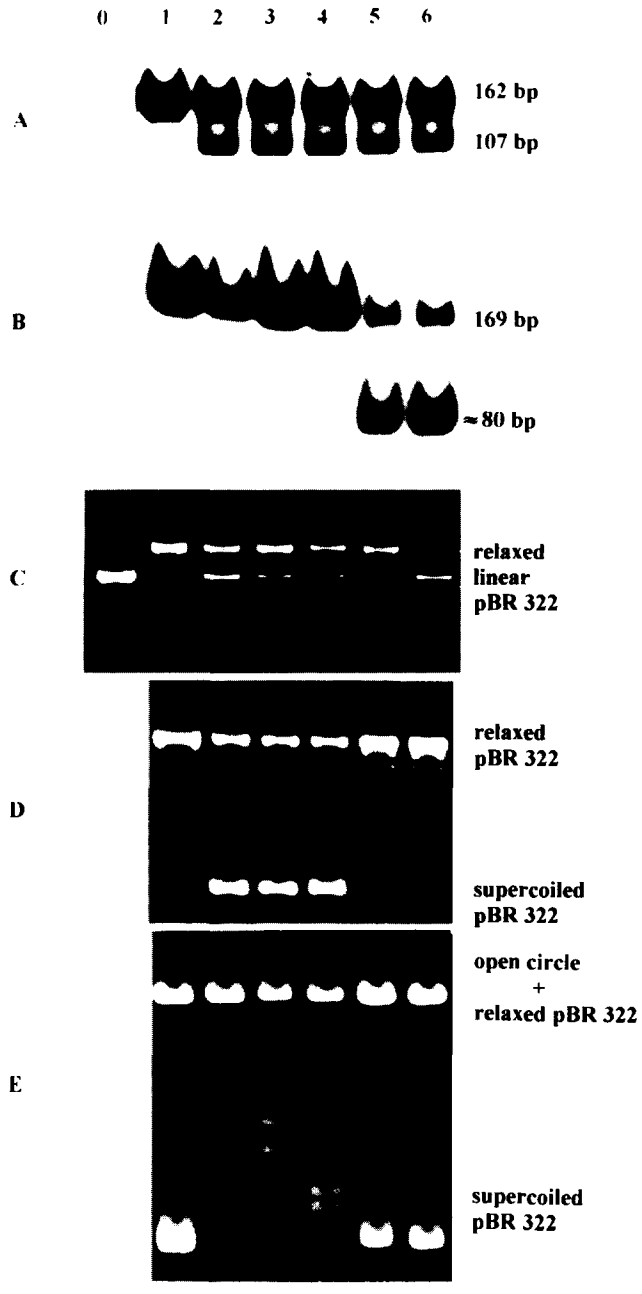

Fig. 2. Characterization of the activity of reconstituted hybrid gyrases using different assays: gyr A protein from *S. noursei* was complemented by gyr B proteins from different species. A, cleavage of the 162 bp fragment and B, of the 169 bp fragment in presence of ciprofloxacin; C, linearization of pBR322 in presence of ciprofloxacin; D, supercoiling of relaxed pBR322; E, relaxation reaction of supercoiled pBR322 DNA in the absence of ATP. Lanes: 1, Gyr A from *S. noursei* alone at 3 times higher concentration compared to the A_2B_2 complex; 2, plus Gyr B from *S. noursei*; 3, plus Gyr B from *S. griseus*; 4, plus Gyr B from *S. netropsis*; 5, plus Gyr B from *E. coli*; 6, plus Gyr B from *B. subtilis*; lane 0 (in C) pBR322 linearized by EcoRI, for details see section 2.

A plus *E. coli* Gyr B completely restored the supercoiling ability (data not shown).

We further examined the role of various subunit combinations in the gyrase mediated relaxation reaction of negatively supercoiled DNA in the absence of ATP, which is a very slow process [1]. The results in Fig. 2E and Table 1 show exactly the same behaviour as in the supercoiling assay; e.g. combinations of Gyr A from *S. noursei* with Gyr B from other *Streptomycetes* only or the reconstituted gyrase from homologous A and B subunits are able to relax negatively supercoiled DNA. Thus it seems, that as in case of DNA supercoiling the reaction cycle of the DNA relaxation requires also a more specific A_2B_2 structural complex, which can be formed only by combination of homologous or heterologous subunit species of the same bacterial genus. These results demonstrate, that the functional equivalence of gyrase subunits from taxonomically different bacteria is strongly limited with respect to the overall gyrase reaction in the in vitro system.

Immunochemical studies with polyclonal rabbit antisera against Gyr A from *S. noursei* and Gyr B from *E. coli* support structural differences and related substrate specificity of gyrase subunits. All of the tested gyrase subunits were used as inhibitors in very sensitive ELISA assays (working in a ng range of antigens and at high dilutions of antisera) with both antisera and the homologous antigen, respectively. The specificity of antibodies for the homologous Gyr A or Gyr B proteins was very high: the I_{50} concentrations of the homologous subunits were 150-fold lower in case of *E. coli* Gyr B and 100-fold lower for *S. noursei* Gyr A (data not shown). By means of Western blot analysis cross-reactivity of both antisera with heterologous gyrase subunits could not be demonstrated even at high protein concentrations. This suggests a high specificity of the antibodies to the respective gyrase subunits.

4. Discussion

The present findings provide evidence that gyrase subunits from taxonomically different bacterial species are not functionally equivalent in any hybrid enzyme composed of heterologous Gyr A plus Gyr B combinations. Our results demonstrate, that enzyme binding to a defined DNA sequence containing a gyrase cleavage site may be different for various holoenzymes. This is evidenced by the binding ability to the 162 bp fragment and 169 bp fragment of the gyrases from *E. coli* and *B. subtilis*, which is very similar (Fig. 1A and B), whereas binding of the *S. noursei* enzyme is significantly lower to the 169 bp fragment (Fig. 1B). Although both fragments contain cleavage sites for the *E. coli* and *B. subtilis* gyrase the cleavage ability is quite different for holoenzymes from *Streptomycetes* and for various gyrase hybrids (Fig. 2A to C, Table 1). *S. noursei* Gyr A complemented only with Gyr B from *E. coli* or *B. subtilis* forms a hybrid enzyme which cleaves the 169 bp fragment whereas in all combinations with Gyr B from *Streptomycetes* no cleavage occurs for this substrate (Fig. 2B, Table 1). Thus it seems that the B subunits of *E. coli* and *B. subtilis* only are able to support the Gyr A mediated cleavage of the 169 bp fragment due to an enhancement of the affinity of the A_2B_2 complex of the hybrids to this DNA sequence (Fig. 1C). In contrast the 162 bp fragment as well as pBR322 plasmid DNA are cleaved by all combinations of Gyr A and Gyr B subunits examined (Table 1). It seems that interaction of *E. coli* Gyr B with *S. noursei* Gyr A leads to a hybrid enzyme which more strongly binds and cleaves the 169 bp fragment whereas the combination of *S. noursei* Gyr A and Gyr B has a lower binding affinity associated with no cleavage capability for this fragment.

It was shown that the *E. coli* B subunit supports DNA bind-

Table 1
Comparison of the activity of DNA gyrases and hybrids reconstituted from gyr A and gyr B proteins from various sources using pBR322 and two fragments of 162 bp and 169 bp as substrates

Source of gyrase subunit		Cleavage		Supercoiling		Relaxation
Gyr A	Gyr B	162 bp	169 bp	pBR 322	pBR 322	pBR 322
S. noursei	*S. noursei*	+	−	+	+	+
	S. griseus	+	−	+	+	+
	S. netropsis	+	−	+	+	+
	E. coli	+	+	+	−	−
	B. subtilis	+	+	+	−	−
E. coli	*S. noursei*	(+)	−	(+)	−	−
	S. griseus	(+)	−	(+)	−	−
	S. netropsis	n.d.	−	(+)	−	−
	E. coli	+	+	+	+	+
	B. subtilis	+	+	+	−	−
B. subtilis	*S. noursei*	(+)	−	(+)	−	−
	S. griseus	(+)	−	(+)	−	−
	S. netropsis	n.d.	−	(+)	−	−
	E. coli	+	+	+	−	−
	B. subtilis	+	+	+	+	+

Cleavage, reaction in presence of ciprofloxacin; supercoiling and relaxation reaction as given under experimental procedures; signs denote: +, active; −, inactive; n.d., not detected.

ing of Gyr A, and strand-breakage mediated by Gyr A can only occur in presence of Gyr B [20]. Our present results show that the cleavage reaction mediated by Gyr A may depend also on the nature of the B subunit (very probably related to the structure), which influences the binding ability to the DNA sequence encompassing the cleavage site; e.g. *E. coli* Gyr B supports both, binding and cleavage of the 169 bp fragment by the hybrid enzyme composed of *S. noursei* Gyr A plus *E. coli* Gyr B, whereas in the corresponding A_2B_2 complexes Gyr B from all *Streptomycetes* is unable to promote cleavage of the 169 bp DNA. Clearly, this is in accordance with the lower binding affinity of *S. noursei* gyrase to this DNA fragment (Fig. 1B).

The results on the supercoiling activity and relaxation reaction (in the absence of ATP) of hybrids formed by various combinations of A and B subunits reveal that active holoenzymes can be obtained only for heterologous Gyr A plus Gyr B proteins within the genus *Streptomycetes* and for the reconstituted enzymes with the homologous subunits. Our more extended study only partially agrees with a previous report [9] in which no supercoiling activity was detected for *B. subtilis* Gyr A plus *E. coli* Gyr B, whereas an active enzyme was found for the reciprocal combination. This latter result of Orr and Staudenbauer [9] cannot be supported by our data (Table 1). Another finding on cross-complementation between a mutated gyr A gene in *E. coli* complemented with *B. subtilis* gyr A [21] does not contradict our results, since it was reported later, that only plasmids with both the *B. subtilis* gyr A and gyr B genes can complement an *E. coli* gyr A mutation [22]. Different from the supercoiling activity the linearization reaction of pBR322 mediated by ciprofloxacin is not limited by various combinations of different subunit species. This result could mean that different from the supercoiling cycle the cleavage reaction requires not a very specific interaction between nonhomologous A and B subunits to support the Gyr A mediated cleavage by Gyr B. Apparently, this conclusion is valid for some gyrase recognition sites only, if one compares the cleavage activity of hybrids for the 162 bp and 169 bp fragment (Table 1).

Our present findings show that the whole catalytic cycle of DNA supercoiling and relaxation (in the absence of ATP) mediated by gyrase is limited to a combination of homologous A and B subunit species or heterologous species of taxonomically related bacteria, e.g. of the genus *Streptomycetes*. Apparently the B subunit, which is responsible for DNA strand movement [1,3] is the major determinant of the catalytic cycle. In addition to the strand-passage function Gyr B can support DNA-Gyr A binding and the cleavage reaction for a certain gyrase site (Table 1), which may be the result of a tight interaction with the Gyr A protein. Our results support the view [9] that the supercoiling activity of gyrase is dependent on the specificity of the interaction between A and B subunits. It is possible that binding between A and B subunits may cause a conformational change which could differ for certain combinations of gyr A and gyr B proteins and hence changes the affinity of the enzyme to the sequence of the substrate. Immunochemical investigations also revealed structural differences for the corresponding gyrase subunits (data not presented).

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