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An improved assay for the detection of alterations in bacterial DNA supercoiling *in vivo*

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Dedicated to Prof. Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.

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Due to the increasing prevalence of antibiotic resistance and the yet low output of the genomics-based drug discovery approach novel strategies are urgently needed to detect new antibiotics. One such strategy uses known ubiquitous targets like DNA topoisomerases. However, to detect inhibitors of these enzymes by an *in vitro* assay time-consuming isolation of enzymes and DNA followed by electrophoretic separation of topoisomers are required. Instead, this study aimed at developing an *in vivo* assay for the detection of alterations in DNA supercoiling indicative of topoisomerase inhibition by a reporter gene assay. A pair of plasmids was developed which carry the reporter gene *luc* for firefly luciferase under control of either promoter p*topA* (pPHB90) or p*gyrA* (pPHB91), whose activities are reciprocally affected by alterations of the supercoiling degree. Each plasmid is individually transferred into *E. coli* cells. The quotient of the luciferase activities determined using cells with either plasmid was taken as relative measure of the global supercoiling degree Qsc (quotient of supercoiling). Using isogenic reference strains with known alterations of the global DNA supercoiling degree due to mutations in either *gyrB* or *topA,* the reporter gene system was able to detect both a decrease and an increase of the negative supercoiling degree compared to the isogenic parent strain. Treating cells with known inhibitors of DNA gyrase, like fluoroquinolones, novobiocin as well as simocyclinone D8 from *Streptomyces antibioticus* which has been identified as an inhibitor of DNA gyrase *in vitro*, also caused decreases of the Qsc value *in vivo*. The suitability of this reporter gene system to screen for anti-topoisomerase I and II compounds from various natural sources like plant extracts by sensing alterations of the DNA supercoiling was demonstrated and offers a new application to identify novel compounds active against bacterial topoisomerases I and gyrase.

1. Introduction

The ability of bacteria to rapidly develop resistance to any new antibiotic requires continuous efforts to identify novel drugs. Current strategies include i) the modification of known lead structures to increase their potency and / or their stability against resistance mechanisms, ii) the identification of novel targets, e.g. by a genomics-based approach, and iii) the screening for new lead structures using known targets (Levy and Marshall 2004; Walsh 2003).

According to the latter approach in the present study an *in vivo* assay system to detect inhibitors of DNA topoisomerases was developed. Topoisomerases are ubiquitous, essential enzymes which act in concert with structural proteins, such as histones in eukaryotes and histone-like proteins in prokaryotes, to maintain a balanced degree of DNA supercoiling required for DNA replication, transcription, recombination, and repair (Champoux 2001; Wang 1996). In *Escherichia coli*, type I topoisomerases are monomeric enzymes encoded by a single gene, *topA* (topoisomerase I) and *topB* (topoisomerase III), respectively. Both enzymes relax negatively supercoiled DNA by introducing a transient single-strand break into DNA. In contrast, bacterial type II enzymes like DNA gyrase (topoisomerase II) and topoisomerase IV introduce a double-strand break. These two enzymes consist of two pairs of dimers (A_2B_2) which are encoded by two genes *gyrA* and *gyrB* (DNA gyrase) and *parC* and *parE* (topoisomerase IV), respectively. Clinically used topoisomerase inhibitors target type II enzymes: fluoroquinolones, broad-spectrum antibiotics with good Gram-positive and Gram-negative coverage, acting on the respective A subunits (GyrA and ParC) by inhibiting their strand rejoining activity and aminocoumarin-derivatives, like novobiocin and coumermycin, affecting the respective B subunits (GyrB and ParE) by inhibiting their ATPase activities required for conformational changes preceding the strand passage reaction (Maxwell 1997).

To maintain a balanced degree of DNA supercoiling in eubacterial cells three different levels of regulation are known:

i) Opposing enzymatic activities of DNA gyrase which increases negative supercoils and topisomerases I and IV reducing the number of negative supercoils (Champoux 2001; Wang 2002; Dorman 2006).

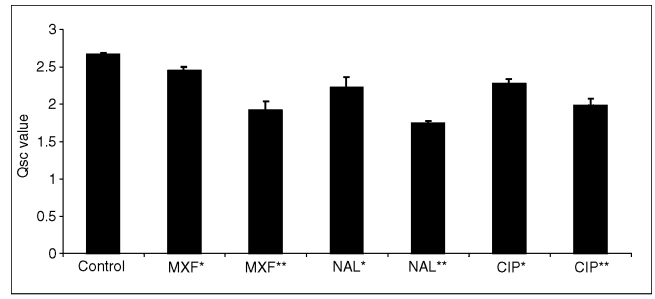
ii) Inverse affinities to DNA topoisomers of topoisomerase I which decreases with a reduction in the negative supercoiling degree and of gyrase which increases with a reduced negative supercoiling degree and *vice versa* (Masse and Drolet 1999; Snoep et al. 2002; Stone et al. 2003).
iii) Inverse regulation of the activities of promoters p*topA* as well as p*gyrA* and p*gyrB*. p*topA* activity is elevated with an increased degree of negative supercoiling while activities of promoters p*gyrA* and p*gyrB* are reduced by increased negative DNA supercoiling (homeostatic control) (Menzel and Gellert 1983, 1987; Tse-Dinh1985; Tse-Dinh and Beran 1988).

Thus, while the maintenance of a balanced global cellular supercoiling degree which is essential for bacterial survival requires the activities of at least topoisomerases I, II, and IV, the inhibition of one of these enzymes will disturb this balance.
One labour-intensive and time consuming approach to detect alterations of the degree of DNA supercoiling uses a reporter plasmid which is isolated from cells before separating different topoisomers by gel electrophoresis in the presence of DNA intercalating agents, like chloroquine (Aleixandre et al. 1991). The resulting pattern of DNA band intensities corresponds to a certain degree of supercoiling. Other systems use single reporter genes such as *lacZ* (encoding (β-galactosidase) or *bla* (encoding a (β-lactamase) placed under the control of one supercoiling-dependent promoter either on a plasmid or in the chromosome (Bagel et al. 1999; Parekh et al. 1996). However, such systems suffer from several drawbacks: A chromosomally integrated reporter gene has to be placed into the identical gene locus in each strain to compare different strains. The plasmid encoded reporter genes use a single promoter, whose activity usually indicates alterations of the supercoiling degree only in one direction. Moreover, to determine the specific activity, protein concentration has to be determined additionally in order to compare different strains.
The present study provides a novel approach by using a pair of reporter plasmids exploiting the homeostatic control, i.e. the reciprocal response of the promoters p*topA* and p*gyrA* to alterations of the supercoiling degree. Both promoters were fused individually to the reporter gene *luc* encoding luciferase from North American firefly *Photinus pyralis*. This test system was used to determine the impact on the DNA supercoiling degree in *E. coli* cells of different factors such as mutations in genes *gyrA* and *topA* associated with reduced enzymatic activity as well as different inhibitors of gyrase, like fluoroquinolones, aminocoumarins, and simocyclinone D8 (Fig. 1), a new antibiotic isolated from *S. antibioticus* Tü 6040 (Holzenkämpfer and Zeek 2002; Schimana et al. 2000; Theobald et al. 2000). Additionally, we checked the applicability of the test system to screen for topoisomerase inhibitors from various environmental sources and therefore used extracts from two medicinal plants, *Angelica archangelica* ssp. *litoralis* and *Ruta graveolens*, comprising different structural types of coumarins, to determine the presence of anti-gyrase and/or anti-topoisomerase I activity.

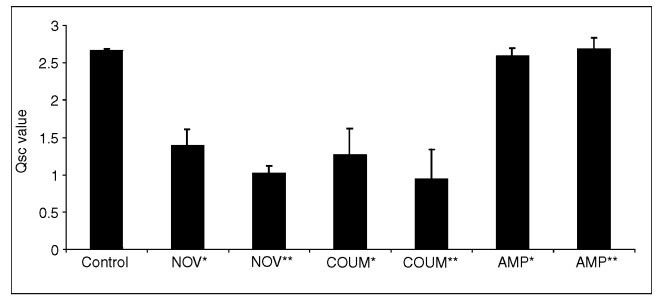
2. Investigations and results

2.1. Impact of topoisomerase mutations on Qsc (quotient of supercoiling)

To evaluate if different alterations in the DNA supercoiling level can be detected by use of the reporter gene system, isogenic reference strains derived from parent *E. coli* K12 strain JTT1 were used to determine the Qsc values. These show known alterations



(a)



(b)

Fig. 1: Impact of increasing concentrations (*one-half and **equivalent to MICs) of A) GyrA-inhibitors: moxifloxacin (MXF), nalidixic acid (NAL), ciprofloxacin (CIP); B) GyrB-inhibitors: novobiocin (NOV), coumermycin A (COUM), and ampicillin (AMP) on the Qsc value of JTT1. The data are averages of the percentage of Qsc values with respect to untreated control. The error bars denote the standard deviations. $P \leq 0.001$.

Table 1: Qsc values of reference strain JTT1 and its topoisomerase mutants RS2 (Δ*topA*), and KD112 (*gyrB*226).

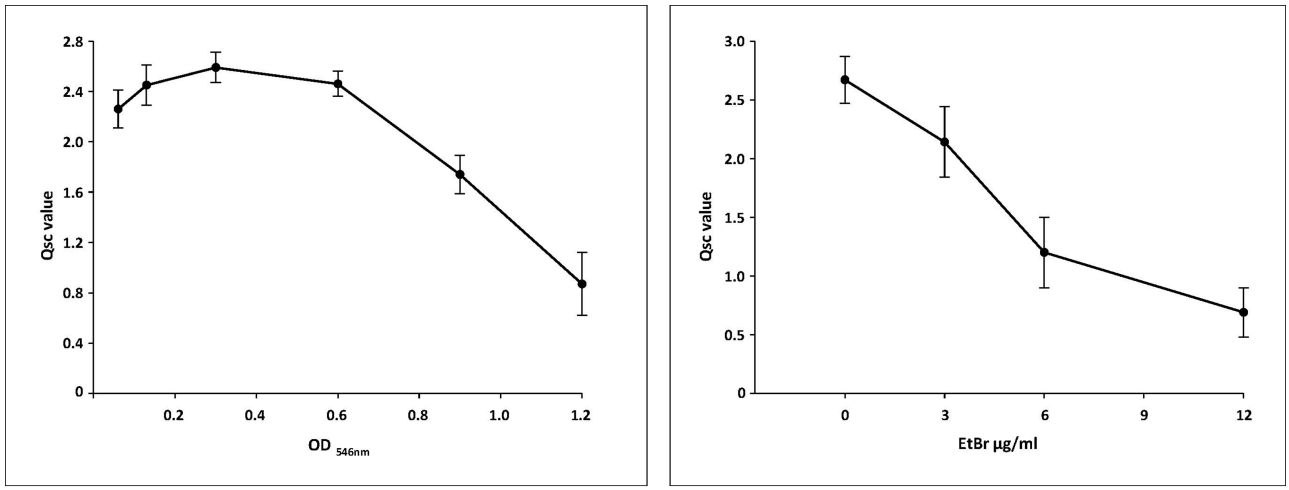
Strain	Qsc	ΔQsc (%)
JTT1	2.67	0
RS2	5.29	+ 98
KD112	1.10	− 59

The data are means of the percentage of Qsc values with respect to JTT1 = 100%. Error bars denote the standard deviations (n = 6).

in the supercoiling degree due to mutations in either topoisomerase I RS2 (Δ*topA*10) resulting in an increased negative supercoiling degree, or gyrase KD112 (*gyrB*226) resulting in a decreased negative supercoiling degree. Compared to JTT1, the Qsc value increased by more than 1.9 fold (from 2.67 to 5.29) for RS2 while it decreased by about 0.6 fold to 1.1 for *gyrB* mutant KD112 (Table 1). These alterations have already been confirmed by gel electrophoresis (Bagel et al. 1999).

2.2. Alteration of the DNA supercoiling degree by known inhibitors targeting GyrA or GyrB

In Gram-negative bacteria, like *E. coli*, DNA gyrase is the preferred, i.e. more susceptible target of known inhibitors such as quinolones and aminocoumarines, primarily interacting with the A and B subunits, respectively. To examine whether the reporter gene system is also able to detect changes in the degree of DNA supercoiling caused by inhibition of gyrase activity, the Qsc values were determined in the presence of inhibitors targeting preferentially either subunit GyrA or GyrB.
All applied inhibitors targeting GyrA (moxifloxacin, nalidixic acid, and ciprofloxacin) or GyrB (novobiocin, coumermycin A) showed a concentration-dependent decrease of the Qsc of parent strain JTT1 (Fig. 1 A,B). However, GyrB inhibitors achieved a



(A) (B)

Fig. 2: A) Alteration of Qsc in JTT1 during growth from early log phase to stationary phase. B) Effect of EtBr intercalation in DNA on the relative supercoiling degree. The data are averages of the Qsc values from four different biological experiments.

higher decrease of the Qsc (> 50%) compared to GyrA inhibitors (∼ 20%).

The β-lactam antibiotic ampicillin, which targets the cell wall biosynthesis, had no impact on the Qsc values of JTT1 even at a concentration equivalent to the MIC (Fig. 1B).

2.3. Effect of growth phases on DNA supercoiling

A possible impact of the growth phase on the supercoiling degree was investigated by comparing the Qsc values of JTT1 at OD_{546nm} 0.06 (early log phase) and OD_{546nm} 1.2 (stationary phase) (Fig. 2A). The Qsc values increased slightly after the early log phase and were almost constant during the log phase (OD_{546nm} 0.2-0.6). A drop in the Qsc values was observed when cells reached stationary phase.

2.3.1. Impact of GyrA- and GyrB-inhibitors on global supercoiling degree of WT and the fluoroquinolone resistant gyrA-mutant WT-3-1

Mutant strain *E. coli* WT3-1 carries a double mutation (S83L + D87G) in parent strain WT (Bagel et al. 1999) resulting in reduced susceptibility of DNA gyrase to the inhibition by ciprofloxacin by 2 to 3 orders of magnitude (Heisig et al. 1993). The MIC of ciprofloxacin is increased to 0.25 µg/ml compared to WT (0.03 µg/ml) whereas the susceptibility to novobiocin (MIC of 128 µg/ml for WT and WT-3-1) is not affected. Addition of ciprofloxacin at a concentration equivalent to the MIC resulted in a decrease of the Qsc value from 1.32 to 0.98 (−26%) for WT, whereas no significant alteration for WT3-1 (Fig. 3) was detectable. However, in the presence of novobiocin at 1/4 MIC (32 µg/ml) Qsc values were reduced for both, WT (from 1.32 to 0.45) and WT-3-1 (from 0.91 to 0.48). For comparison, the Qsc values determined in the absence of antibiotics for WT (1.32) and WT3-1 (0.91) indicates a reduced Qsc in WT-3-1 (−31%).

2.3.2. Impact of DNA intercalation by ethidium bromide (EtBr) on DNA supercoiling

For further validation of the assay, the effect of DNA intercalating agent EtBr on supercoiling was tested. For this purpose different concentrations of EtBr were added to JTT1 growing in the exponential growth phase. After an incubation phase of 20 min the Qsc values were determined. Deployed EtBr concentrations ranging from 3 to 12 µg/ml reduced the Qsc values in a concentration dependent manner. Concentrations higher than 12 µg/ml showed a toxic effect on bacteria (Fig. 2B).

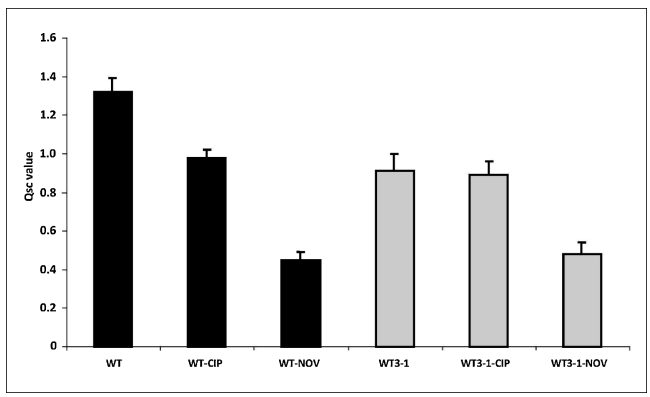


Fig. 3: Alteration of Qsc values of *E. coli* isolate WT and its *gyrA* double-mutant WT-3-1 due to treatment with different DNA gyrase inhibitors. Ciprofloxacin (CIP) targeting subunit A of DNA gyrase was used in concentrations equivalent to respective MICs of 0.03 µg/ml (WT) and 0.25 µg/ml (WT3-1) as well as equivalent to one-fourth of the MIC (32 µg/ml) of novobiocin (NOV). The error bars denote the standard deviations. $P < 0.005$.

2.3.3. Impact of simocyclinone D8 on the relative supercoiling degree

Previously published data from *in vitro* experiments have provided evidence that simocyclinone D8 (Fig. 4) is an inhibitor

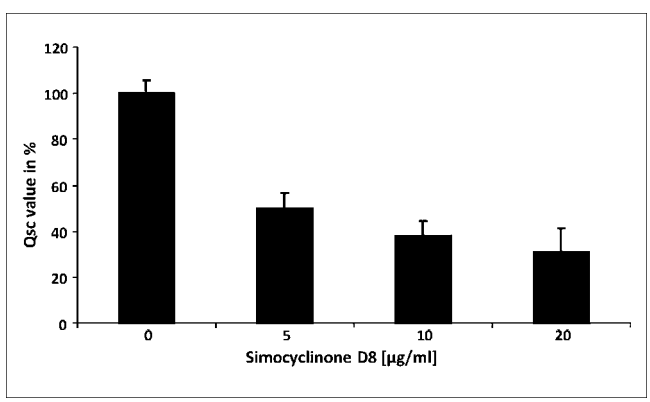
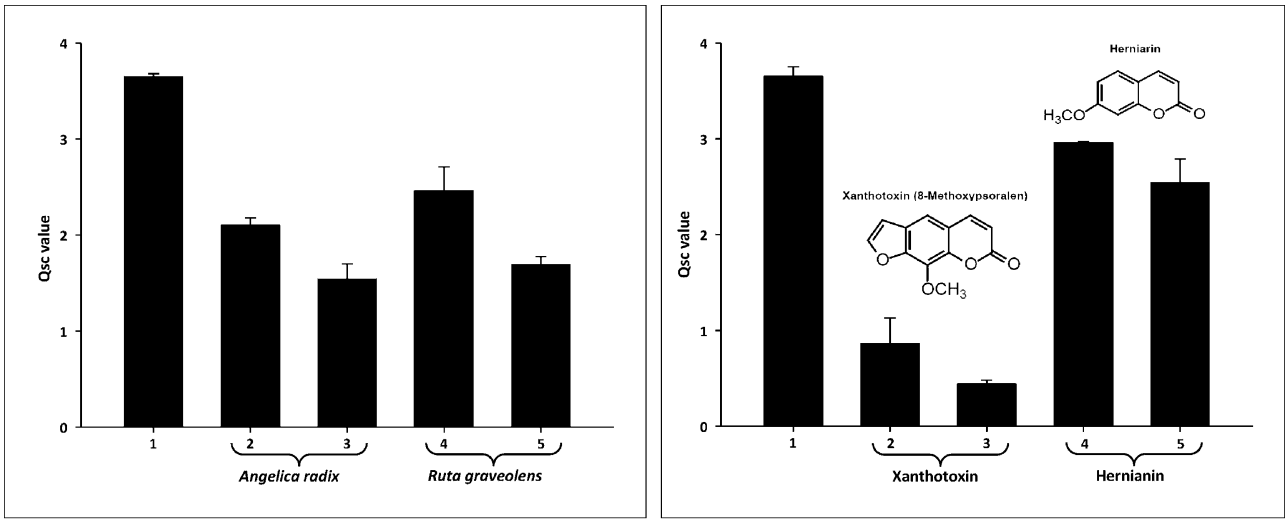


Fig. 4: Impact of different simocyclinone D8 concentrations on the degree of relative supercoiling. The data are averages of the Qsc values with respect to untreated control.



(A) (B)

Fig. 5: A) Impact of *Angelica archangelica* and *Ruta graveolens* extracts on Qsc in *E. coli* DE112. 1 = Control including 1.5% vol. ether; 2 and 3 = 0.125% and 0.25% vol. *Angelica archangelica* root extract; 4 and 5 = 0.5% and 1.0% vol. *Ruta graveolens* (ground above parts). B) Effect of xanthotoxin and Herniarin on *E. coli* DE112. 1 = control; 2 and 3 = 50 and 100 μg/ml xanthotoxin; 4 and 5 = 50 and 100 μg/ml Herniarin.

of DNA gyrase by a novel mode of action different from that of the currently used gyrase inhibitors (Flatman et al. 2005). Treatment of JTT1 with different concentrations of simocyclinone D8 resulted in a strong decrease of the supercoiling degree (Fig. 4) indicating DNA gyrase inhibition *in vivo* to be similar to that observed for GyrB-targeting inhibitors (Fig. 1).

2.4. Impact of Angelicae radix and Ruta graveolens extracts on supercoiling

2.4.1. Effect of plant extracts

Our aim was to apply the test system not only to detect inhibitory activity of pure compounds against topoisomerase I or gyrase, but also in natural extracts from various environmental sources. Extracts from two medicinal plants, *Angelica archangelica* ssp. *litoralis* and *Ruta graveolens*, comprising different structural types of coumarins, were tested for anti-gyrase and/or topoisomerase I activity. Aminocoumarin antibiotics like novobiocin and coumermycin A have well established anti-gyrase activities which were detectable by the system (Fig. 1A,B).

The roots from *Angelica archangelica* ssp. *litoralis* and the ground above parts of *Ruta graveolens* were prepared and tested as described in Experimental.

In order to elevate the sensitivity of the system we used the hypersensitive *E. coli* strain DE112 which has a defect in the proton dependent efflux systems according to a mutation in the TolC locus (Van Dyke 1994; Nishino 2003). Comparing the MIC of novobiocin for DE112 (2 μg/ml) to other *E. coli* strains like JTT1 (128 μg/ml) demonstrate the high sensitivity of DE112 according to increased accumulation of the antibiotics. Therefore the use of DE112 increases the detection ability and sensitivity of the test system.

Addition of 0.125% and 0.25% vol. *Angelica archangelica* ssp. *litoralis* roots extract to DE112 cultures reduced the Qsc value from 3.65 (100%) to 2.10 (58%) and 1.54 (42%), respectively (Fig. 5A).

Addition of 0.5% and 1.0% vol. *Ruta graveolens* extract showed a similar effect reducing the Qsc value to 2.46 (68%) and 1.69 (46%), respectively (Fig. 5 A). Both plant extracts reduced Qsc values in a concentration dependent manner. The differences in the extract concentrations used were due to the difference in the amounts of plant material used for the extraction, 50 g *Angelicae radix* and 14 g *Ruta graveolens.*

An unspecific effect of diethylether on the viability of the treated bacterial cells or on the promoter activities of p*topA* and/or p*gyrA* can be excluded since addition of up to 1.5% vol. of diethylether did not change the Qsc values in comparison to untreated samples probes (Fig. 5A).

2.4.2. Effect of xanthotoxin and herniarin

In further analyses the impact of xanthotoxin and herniarin on the bacterial supercoiling degree was investigated. Both compounds contain a core structure of coumarin. Xanthotoxin, a furanocoumarin, occurs in the aerial parts of *Ruta graveolens* and in roots of *Angelica archangelica* ssp. *litoralis* while herniarin is a hydroxycoumarin occurring in *Ruta graveolens*. In the presence of different concentrations of either xanthotoxin or herniarin (50 and 100 μg/ml) a concentration dependent decrease of the supercoiling degree of DE112 was detectable by the Qsc values. A comparison of the results showed that xanthotoxin caused a higher reduction of the Qsc values than herniarin. Addition of 50 μg/ml xanthotoxin alleviated the Qsc value from 3.65 to 0.86 (24%), whereas the same concentration of herniarin caused a reduction to 2.96 (81%). Similar tendency was determined using 100 μg/ml of both substances (Fig. 5B).

3. Discussion

The degree of supercoiling of the closed circular bacterial chromosome is maintained within a narrow range in part by histone-like, basic DNA-binding proteins and in part by the opposing enzymatic activities of DNA gyrase (negative supercoiling) and topoisomerases I and IV (relaxing) (Wang 1971; Gellert et al. 1976; Peng and Marians 1993).

The reporter gene system which was developed in the present study is based upon a pair of reporter gene fusions which are able to sense alterations of the level of DNA supercoiling *in vivo*. The system uses the relative activities of the reciprocally regulated promoters p*topA* and p*gyrA* as indicators of alterations of the global supercoiling degree. Promoter p*gyrA* was used instead of p*gyrB,* because the gene expression from p*gyrA* is more sensitive to alterations of the DNA supercoiling (Neumann and Quinones 1997). The reporter gene *luc* of the firefly luciferase has been chosen since its activity can be determined easily and with high sensitivity from crude cell extracts (Alam and Cook 1990; Bornstein et al. 1994).

The suitability of this reporter gene system for the determination of the relative degree of supercoiling has been demonstrated by several lines of evidence:

(I) Using mutants RS2 (Δ*topA*10) and KD112 (*gyrB*226) in comparison to their isogenic parent strain JTT1 (Steck et al. 1993; Sternglanz et al. 1981) alterations in the degree of DNA supercoiling were detectable both as increase and decrease of the Qsc value, respectively (Table 1). In a previous investigation using a precedent system a correlation of these data with results obtained by electrophoretic mobility shift assays using chloroquine for detection of different topoisomers of plasmid-DNA has been demonstrated (Bagel et al. 1999).

(II) Selective inhibition of DNA gyrase activity due to aminocoumarins or quinolones (Maxwell 1993, 1997; Pruss et al. 1986) can be sensed by a concentration-dependent reduction of Qsc values compared to that of untreated control (Fig. 1A,B). In accordance with the concept of homeostatic control of DNA supercoiling both drug classes cause a decrease in the expression *topA* and an increase in that of *gyrA* which by definition is detected as a reduction of the Qsc (specific activity of p*topA* / specific activity of p*gyrA*).

Moreover, the system is able to detect quantitative differences in the reduction of the Qsc due to the different modes of action of aminocoumarins and quinolones at corresponding concentrations. The strong reduction of the Qsc by aminocoumarins which inhibit the ATP-dependent strand passage activity by interacting with the N-terminal domain of gyrase subunit GyrB and subsequent stabilization of an enzyme conformation with lowered affinity for ATP contrasts with the lower reduction of the Qsc by quinolones which inhibit DNA gyrase by stabilizing a ternary cleavage complex of drug, enzyme and DNA and subsequently preventing the religation of the double strand break, result in a lower reduction in Qsc as that of aminocoumarins (Fig. 1A,B). Recently, this system has successfully been applied to determine the variations in the global supercoiling degree as Qsc values for fluoroquinolone resistant mutants in dependence of the type and number of mutations which simultaneously affect drug susceptibility and enzymatic activity of type II topoisomerases, DNA gyrase and topoisomerase IV (Marcusson et al. 2009).

The presence of the *gyrA* double mutation in WT-3-1 (S83L + D87G) is associated with increased quinolone resistance. In the presence of ciprofloxacin at a concentration equivalent to the MIC, the Qsc of WT3-1 was not affected, while that of WT was reduced (Fig. 3). This can be explained by the higher preference of quinolones for the primary target gyrase introducing negative supercoils into DNA in WT compared to topoisomerase IV relaxing negative supercoils in WT3-1. Inhibition of GyrB activity by novobiocin results in a comparable strong decrease of the Qsc for both strains, WT and WT3-1, due to the presence of the identical non-mutated target *gyrB* gene: thus, enzymatic inhibitors of DNA supercoiling, such as novobiocin reduce the supercoiling activity whereas relaxation activity is maintained probably causing a comparably higher reduction of supercoiling.

The specifity of the system has been demonstrated by the inability of ampicillin, an inhibitor of cell wall biosynthesis, to alter the Qsc value.

Another aspect regarding changes of the global supercoiling degree during different growth phases has been analyzed by the reporter system. Previous studies reported on relaxation of DNA in the stationary phase *E. coli* cells (Balke and Gralla 1987; Dorman et al. 1988; Reyes-Domínguez et al. 2003; Tse-Dinh and Beran 1988). A reduction of the Qsc determined for cells of WT in the stationary growth phase correlate with published data (Fig. 2A). In order to survive starvation, stationary-phase cells undergo morphological and physiological changes including condensation of nucleotides and a general decrease in transcription and translation (Kolter et al. 1993).

The reporter gene system has also been able to detect non enzymatic changes in the superhelicity caused by the DNA intercalating dye ethidiumbromide (EtBr). Previously published work of Steck et al. (1993) showed that EtBr is able to remove negative supercoils, which has been confirmed by decreased Qsc vales (Fig. 2B).

Simocyclinone D8, isolated from *S. antibioticus* Tü 6040, is a potential new antibiotic (Holzenkämpfer and Zeek 2002; Schimana et al. 2000; Theobald et al. 2000). Earlier data indicated high degrees of similarity between the biosynthetic gene cluster of simocyclinone and those involved in the biosynthesis of the aminocoumarin moieties of novobiocin, clorobiocin, and coumermycin (Galm et al. 2002; Trefzer et al. 2002). Like the coumarins, simocyclinone D8 contains a 3-amino-4,7-dihydroxycoumarin structure with an acyl moiety attached to the amino group (Fig. 4). In contrast to other aminocoumarin antibiotics, simocyclinone D8 contains D-olivose instead of L-noviosyl sugar at the 7-OH group of the aminocoumarin ring. This D-olivose is *O*-glycosidically linked to an angucyclic polyketide. Like clorobiocin, simocyclinone D8 contains a chlorine atom at position 8 of the coumarin ring. However, Flatman et al. (2005) provided evidence for simocyclinone D8 inhibiting the supercoiling activity of *E. coli* gyrase by interacting with the GyrA subunit and thereby preventing gyrase from binding to DNA *in vitro.* These authors suggested a novel mode of action different from that of fluoroquinolones and aminocoumarins. The concentration-dependent decrease of Qsc values determined after treatment with simocyclinone D8 supports the *in vitro* data about the effect of simocyclinone D8 on the DNA supercoiling in *E. coli* (Fig. 4). These data combined to the results showing the ability of the test system to screen for inhibitors in extracts from *Angelica archangelica* ssp. *litoralis* and *Ruta graveolens* as well as pure compounds like xanthotoxin and herniarin indicates that the system can be exploited to screen for new antibiotics.

In conclusion, alterations of global DNA supercoiling in bacteria can be due to different causes, e.g. mutations in genes encoding topoisomerases, presence of different inhibitors, and various environmental conditions. The reporter gene system developed in the present study is able to sense all these kinds of alterations of DNA supercoiling which are determined as Qsc value. The data are consistent to those published previously by using other, more time-consuming supercoiling determination methods. Thus, our two plasmid system offers a sensitive and rapid method for identifying potential novel inhibitors of bacterial topoisomerase I and gyrase.

4. Experimental

4.1. Bacterial strains

The *E. coli* strains used in this work include JTT1 and its isogenic mutants RS2 (Δ*topA*10) and KD112 (*gyrB*226) (Steck et al. 1993; Sternglanz et al. 1981) which have been generously provided by Dr. S. Suerbaum, Hannover. The quinolone-susceptible isolate WT and its double *gyrA*-mutant (S83L + D87G) have been described previously (Bagel et al. 1999; Heisig and Tschorny 1994). The hypersensitive *E. coli* strain, DE112, includes a mutation in the *tolC* locus (Van Dyke 1994). TolC is a porine component in the outer membran of Gram-negative bacteria and has a function in many proton dependent efflux systems (Nishino 2003).

4.2. Oligonucleotids and plasmids

The oligonucleotides used for amplification and sequencing are listed in Table 2. The plasmids used or constructed in this study are listed in Table 3.

Table 2: Primers used for this study

Primer	Sequence 5′→ 3′	Source
GYRA5-10	CGTCTGCAGCGATCTCTTCGTGGTC	This work
GYRA3-10	GTTTTTGGCGTCTTCCATCTAACCGCTATCCCTC*	This work
LUC5-2	GAGGGATAGCGGTTAGATGGAAGACGCCAAAAAC	This work
TOPA5-1	GAGACTGCAGCGTATCCTCATGCTCA	This work
TOPA3-1	GTTTTTGGCGTCTTCCATATTCACCTTACCTAATTT	This work
LUC5-1	AAATTAGGTAAGGTGAATATGGAAGACGCCAAAAAC	This work
LUC3-3	GTCCTGCAGTCGGAGGATTACAATAGCT	This work
LUC5-3	GAGACTGCAGATGGAAGACGCCAAAAAC	This work

Nucleotides in bold face indicate the start codon ATG of *luc* in normal or complementary sequence. CTGCAG indicates the restriction site for *Pst*I.

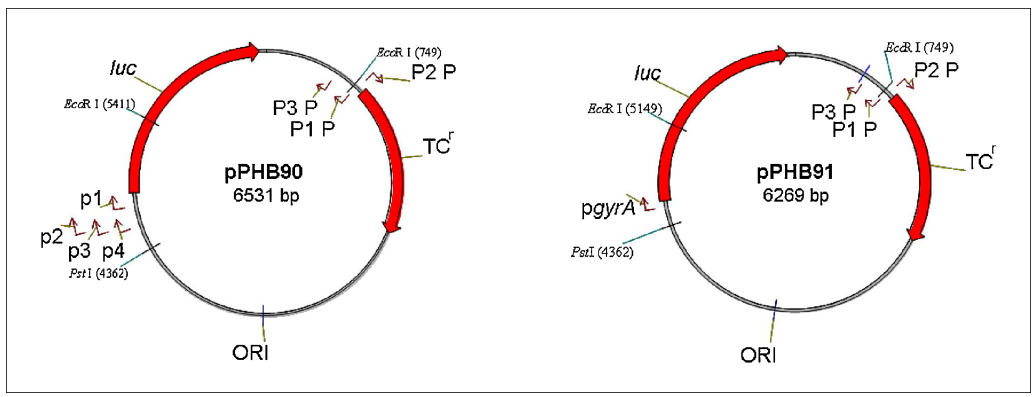


Fig. 6: Physical maps of recombinant plasmids used for the determination of Qsc values. pPHB90 = pBR322-p*topA-luc* / pPHB91 = pBR322-p*gyrA-luc*.

4.3. Antimicrobial agents and chemicals

Moxifloxacin and ciprofloxacin were obtained from Bayer HealthCare AG, Leverkusen. Novobiocin, ampicillin, coumermycin A and tetracycline were purchased from Sigma-Aldrich. Simocyclinone D8 was generously provided by Dr. Lutz Heide, Department of Pharmaceutical Biology, University of Tubingen, Germany. Expand® High Fidelity Polymerase was purchased from Roche (Basel, Switzerland), lysozyme and ethidium bromide from Sigma-Aldrich (Munich, Germany), bovine serum albumin (BSA) from Serva (Heidelberg, Germany), and the restriction enzymes from New England Biolabs (Schwalbach, Germany). The chemicals herniarin and xanthotoxin were purchased from Roth (Karlsruhe, Germany).

4.4. Plant material

Roots of *Angelica archangelica* ssp. *litoralis* (Fr.) Thell. (Apiaceae) were harvested from plants growing near Selmsdorf at the Baltic Sea (Germany) and above ground parts of *Ruta graveolens* L. (Rutaceae) had been collected from the garden of the Institute of Pharmacognosy (Hamburg). Plants were dried for 48 h by artificial heat (40 °C) under air flow. The dried roots were cut into small pieces (about 1 cm length) and subjected to extraction. The aerial parts of *Ruta graveolens* L. were dried for 24 h under the conditions described above. The dried plant material was cut into pieces of about 1 cm length and prepared for extraction.

4.5. Construction of the reporter plasmids

For fusing either promoter p*gyrA* or p*topA* to the reporter gene *luc* two subsequent PCRs were performed. The first PCR used chromosomal template DNA from wild type *E. coli* strain JTT1 with primer pairs TOPA5-1 and TOPA3-1 or GYRA5-10 and GYRA3-10, to yield DNA fragments carrying supercoiling-depended promoters p*topA* (topoisomerase I) or p*gyrA* (gyrase), respectively. The resulting PCR fragments contain the identical 3′-extension including the first six N-terminal codons of the *luc* gene.

Under comparable conditions the plasmid pGEM-*luc* carrying the reporter gene *luc* from *Photinus pyralis* was used as template for two separate PCRs with either primer pair LUC5-2 and LUC3-3 or primer pair LUC5-1 and LUC3-3, to yield DNA fragments carrying a 5′-extension containing sequences from either promoter p*gyrA* or p*topA*, respectively.

For a precise transcriptional fusion of the promoter to the reporter gene, two separate reactions were set up in the absence of primers. Each contained one PCR fragment with either promoter p*gyrA* or p*topA* and a second PCR-fragment carrying *luc* with the 5′-extension complementary to either p*gyrA* or p*topA* under identical cycling conditions. Following an initial denaturation step single strands of both fragments were annealed via the complementary overlap extensions. The 3′-ends of those hybrids were extended by Expand® High Fidelity Polymerase using the respective 5′-overhangs as templates. After five cycles one respective primer pair, either TOPA5-10 and LUC3-3 or GYRA5-10 and LUC3-3, was added for a subsequent conventional PCR to amplify full-length fusion products p*topA-luc* and p*gyrA-luc*, respectively. These fusion products which carry recognition sites for *Pst*I restriction endonuclease introduced via the 3′- and 5′-extensions of the amplification primers TOPA5-1, GYRA5-10, and LUC3-3 were digested with *Pst*I and separately cloned into the *Pst*I-site of pBR322. Resulting recombinant plasmids pPHB90 and pPHB91 (Fig. 6) were chosen since they carry the reporter gene in an orientation which precludes unspecific *luc* expression from known vector promoters. Additionally, the control plasmid pBR322-*luc* carrying a promoterless *luc* gene from the plasmid pGEM-*luc* was generated to determine the background activity of luciferase.

Table 3: Plasmids used in this study.

Plasmid	Relevant markers	Source or reference
pBR322	TET^r, AMP^r	Bolivar et al. (1977)
pPHB90	TET^r, p*topA-luc*	This study
pPHB91	TET^r, p*gyrA-luc*	This study
pBR322-luc	TET^r, *luc*	This study
pGEM-luc	AMP^r, *luc*	Promega

Relevant markers include determinants mediating resistance to Tetracyline (TET^r) and Ampicillin (AMP^r). pPHB90 = pBR322-p*topA-luc* / pPHB91 = pBR322-p*gyrA-luc*.

4.6. Luciferase activity assays

For determining the global degree of DNA supercoiling each strain was separately transformed either with a plasmid containing p*topA-luc,* p*gyrA-luc* or pGEM-*luc*. The luciferase activities of the resulting three recombinant strains were determined by using the Luciferase-Assay-System® (Promega, Germany) modified for bacteria. Briefly, cells grown overnight in standard broth no. I (Merck Darmstadt) containing tetracycline (final concentration 10 µg/ml) were diluted 200-fold into fresh medium and grown under shaking at 37 °C to mid-log phase (OD_{546} of 0.5). Test compounds were added and incubation was continued for 1 h. A sample of 90 µl of bacterial cultures was

added to 10 µl of 1 M K_2HPO_4 (pH 7.8), 20 mM EDTA, mixed and stored at –80 °C. For luciferase assays the cells were thawed at room temperature and 300 µl of cell culture lysis reagent (CCLR: 25 mM Trisphosphat, pH 7.8; 2 mM DDT; 2 mM 1,2-Diaminocyclohexan-*N,N,N,N*- tetraacetic acid; 10% glycerol; 1% Triton X-100) containing 1.25 mg/ml Lysozyme and 2.5 mg/ml BSA were added to each sample.

For determining luciferase activity, 50 µl Luciferase Assay Reagent® (Promega, Germany) containing luciferin, Mg^{2+} and ATP were added to 5 µl of the prepared sample in a microtiter plate and mixed. The intensity of light (560 nm) emitted from these samples was determined as relative light units (RLU) in a luminometer Luci2 (Anthos, Germany). Background activity of luciferase determined as RLU with cells carrying control plasmid pBR322-*luc* was subtracted from the RLU values determined with cells carrying either plasmid pPHB90 or pPHB91. The resulting RLUs correlate with the luciferase activity in the sample according to the following reaction:

$$\text{luciferin} + \text{ATP} + O_2 \xrightarrow[Mg^{2+}]{\text{luciferase}} \text{oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light}$$

4.7. Supercoiling assay

The quotient of supercoiling, Qsc as a measure for the relative supercoiling degree was calculated by dividing the specific luciferase activity of bacteria carrying plasmid pPHB90 (p*topA-luc*) by that of those carrying plasmid pPHB91 (p*gyrA-luc*). Specific activities are defined as RLU values per OD_{546nm}.

$$\text{Qsc} = \text{specific activity of p}topA\text{/specific activity of p}gyrA$$

4.8. Susceptibility testing

The MICs were determined by the broth micro-dilution method in Mueller-Hinton broth (Difco, Detroit, MI, USA) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2009). The drug susceptibilities of the strains were read after incubation for 18 h at 37 °C in 96 well microtiter plates.

4.9. Extraction of plant material

The content of 14 g cut herb of *Ruta* or 50 g cut *Angelica* roots was extracted in diethylether under shaking for one hour at room temperature. Then the extract was filtered and concentrated under low vacuum to 3 ml. The subsequent experiments were carried out using different amounts of these concentrated solution.

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