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Cloning and characterization of a DNA gyrase B gene from *Streptomyces sphaeroides* that confers resistance to novobiocin

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A *gyrB* gene from *Streptomyces sphaeroides*, a producer of novobiocin, has been cloned in *Streptomyces lividans*, where it conferred resistance to novobiocin. The *Streptomyces gyrB* gene was sufficiently similar to a *Bacillus subtilis gyrB* probe to be specifically recognized during Southern analysis. Partial purification of DNA gyrase by affinity chromatography revealed the presence of two such activities (differing in their responses to novobiocin) in the clone. The product of the cloned gene, a novobiocin-resistant DNA gyrase B subunit, was identified *in vitro* by coupled transcription-translation as a 79-kd protein.

Key words: novobiocin resistance/DNA gyrase/*Streptomyces sphaeroides*/antibiotic auto-immunity/*Streptomyces lividans*

Introduction

The ability of organisms to tolerate autogenous drugs is an essential prerequisite for antibiotic biosynthesis. In particular (although not exclusively so), the actinomycetes have been intensively studied in this regard, since they produce an extravagant array of antibiotics (including many that are clinically important) and are amenable to biochemical and genetic analysis. Collectively, such strains have available a range of defensive options, including (among others) modification of the target sites to which given antibiotics normally bind, drug-inactivation and/or the chanelling of biosynthesis along innocuous pathways (for a review, see Cundliffe, 1984). Much of the available data relates to inhibitors of protein synthesis that normally act against the ribosome, but can be prevented from doing so in the respective producing-organisms either by detoxification of the drugs or via ribosomal modification. In other strains, that produce inhibitors of RNA synthesis, RNA polymerase is selectively resistant to the autogenous drug (Watanabe and Tanaka, 1976; Blanco *et al.*, 1984) as is isoleucyl-tRNA synthetase, fatty acyl synthetase or protein synthesis factor EFTu in the organisms that produce pseudomonic acid, cerulenin or kirrothricin respectively (Hughes *et al.*, 1980; Kawaguchi *et al.*, 1979; Glöckner and Wolf, 1984). As yet, however, there have been no reports concerning the mechanism(s) of auto-immunity in strains that produce inhibitors of DNA gyrase.

Bacterial DNA gyrase (DNA topoisomerase II) introduces negative supercoils into relaxed closed circular duplex DNA *in vitro* (Gellert *et al.*, 1976) and also interconverts other topologically isomeric DNA structures, including knotted rings and catenanes. In doing so, gyrase utilizes the energy of ATP hydrolysis to break and reseal double-stranded DNA, with the intervening passage of another DNA duplex through the site of scission. In the intact bacterium, gyrase acts together with DNA topoisomerase I to regulate the superhelical density of the chromosome and of plasmid(s) and thereby influences DNA replication and repair, promoter activity, transposition and recombination (for reviews, see Gellert, 1981; Wang, 1985).

The structure and action of DNA gyrase have been studied in detail with enzyme from several different organisms. The tetrameric enzyme (A_2B_2) contains two different protein subunits that are encoded by *gyrA* and *gyrB* respectively. Those genes from *Escherichia coli* and *Bacillus subtilis* have recently been sequenced and the mol. wts of their products, calculated from the predicted amino acid sequences, are reasonably consistent with earlier estimates derived from the physical properties of the proteins. Thus, the DNA gyrase A and B subunits from *E.coli* (97 and 90 kd respectively; Yamagishi *et al.*, 1986; Swanberg and Wang, 1987) are significantly larger than those from *B.subtilis* (93 and 72 kd; Moriya *et al.*, 1985). The A and B subunits appear to have clearly definable roles in DNA gyrase activity and are also the targets for two separate groups of antimicrobial agents. The A protein is involved in DNA strand scission/reunion and appears to be the target for quinolone drugs, such as nalidixic acid and oxolinic acid (Gellert *et al.*, 1977; Sugino *et al.*, 1977), and for the fluoroquinolones (norfloxacin, ciprofloxacin, etc; for review, see Wolfson and Hooper, 1985). The B subunit mediates energy transduction, involving ATP binding and hydrolysis, and is the target for coumarin antibotics, such as novobiocin and coumermycin A1 (Mizuuchi *et al.*, 1978; Sugino *et al.*, 1978). Both subunits are required to reconstitute full DNA gyrase function (assayed here as supercoiling activity) which can therefore be inhibited by either class of drugs.

The quinolone drugs are not natural products. Therefore, in wishing to study possible resistance mechanisms in organisms that produce DNA gyrase inhibitors, our attention was necessarily directed to the 4-hydroxy-8-methylcoumarins. Since others (Murakami *et al.*, 1983) had already cloned DNA from *Streptomyces sphaeroides* (a novobiocin producer) in *Streptomyces lividans* and thereby obtained novobiocin-resistant strains, we began our studies likewise.

Results

Generation of novobiocin-resistant clones of S.lividans

Fragments of total genomic DNA from *S.sphaeroides* were introduced into protoplasts of *S.lividans* TK 24, using pIJ 487 as vector, and resultant thiostrepton-resistant transformants were replica plated on minimal medium containing novobiocin plus thiostrepton. A single colony grew on the plates and from that strain a plasmid 'pLST 18' (13.3 kb, comprising pIJ 487 plus ~7.2 kb of *S.sphaeroides* DNA) was isolated. This plasmid conferred resistance to novobiocin

plus thiostrepton when reintroduced into *S.lividans* TK 24 and one such transformant was designated 'AT 18'. That strain grew and sporulated readily on plates containing 750 μg novobiocin/ml, whereas the minimal inhibitory concentration for the control strain 'AT 1' (i.e. *S.lividans* TK 24 containing pIJ 487) was ~20 μg/ml.

Following restriction analysis of the *S.sphaeroides* DNA fragment contained in pLST 18 (Figure 1), a probe containing the whole of that DNA was generated (using *Eco*RI and *Hin*dIII restriction sites within the flanking polylinkers) and shown, by Southern analysis, to hybridize back to genomic DNA separately prepared from *S.sphaeroides* (data not given). That same *Eco*RI–*Hin*dIII fragment was also subcloned in pUC 18 and pUC 19 (generating pLST 1818 and pLST 1819 respectively) for subsequent ease of manipulation.

The basis of resistance in the clone

Initially, we addressed the possibility that the *S.sphaeroides* DNA present in pLST 18 might have encoded an enzyme that inactivated novobiocin, although such activity had not previously been described from any source. However, S30 extracts from strain AT 18 failed to inactivate the drug to any detectable extent (*Micrococcus luteus* was the indicator strain) even when supplemented with possible cofactors for modification such as ATP or acetyl CoA (data not given). Accordingly, the effects of novobiocin on DNA gyrase in the clone were examined directly.

In preliminary experiments, it was clear that crude S100 extracts from *S.lividans* contained demonstrable DNA gyrase activity. Thus, in a reaction that was absolutely dependent upon ATP and powerfully inhibited by novobiocin or ciprofloxacin, the relaxed, covalently closed circular form of pBR 322 was progressively converted into the fully supercoiled species via a family of topoisomeric intermediates, as revealed by agarose gel electrophoresis. Moreover, such activity in extracts from strain AT 18 was significantly more resistant to novobiocin than was that from the control strain AT 1 (data not given). This raised the obvious possibility that strain AT 18 might contain a novobiocin-resistant DNA gyrase, although other explanations were not excluded. For example, resistance could have resulted from sequestration of novobiocin due to overproduction of a normally-sensitive gyrase (or more specifically, its B subunit) encoded on the multicopy plasmid, pLST 18. In order to resolve this matter and also to address the possibility that the clone might contain more than one species of DNA gyrase, it was decided to abandon the use of crude extracts and to proceed with partially purified enzyme preparations.

DNA gyrase activity in the clone

Columns containing novobiocin, immobilized on Sepharose, can be used to purify DNA gyrase via the affinity of the drug for the B subunits of the A_2B_2 tetrameric enzyme (Staudenbauer and Orr, 1981). Here, S100 extracts from *S.lividans* AT 1 (control strain) and AT 18 (clone) were applied to such columns at relatively low ionic strength followed by stepwise elution with buffers of progressively increasing ionic strength, culminating in a combination of salt plus urea. Gyrase activity of the various eluates was assayed following dialysis (Table I). As expected, DNA gyrase from the novobiocin-sensitive strain AT 1 adhered tightly to the column and was displaced only under extreme

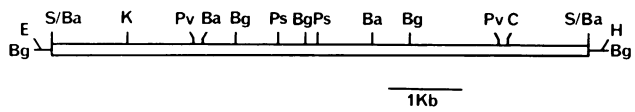

Fig. 1. Restriction analysis of cloned DNA from *S.sphaeroides* present in pLST 18. Within cloned DNA (broad band) S/Ba: *Sau*3A/*Bam*HI; K, *Kpn*I; Pv, *Pvu*II; Ba, *Bam*HI; Bg, *Bgl*II; Ps, *Pst*I; C, *Cla*I. Within flanking polylinker (not drawn to scale and bounded by Bg sites): E, *Eco*RI; H, *Hin*dIII.

Table I. Elution of DNA gyrase from novobiocin–Sepharose

Source of gyrase activity	Gyrase activity in eluates[a]			
	150 mM KCl	300 mM KCl	500 mM KCl	2 M KCl plus 5 M urea
S.lividans AT 1 (control strain)	0	0	0	+++
S.lividans AT 18	0	+++	0	+++

[a]For full composition of elution buffer, see Materials and methods.

conditions, whereas two forms of the enzyme were obtained from strain AT 18 (in roughly equivalent amounts) and one of these was very readily eluted from the column. These data implied that the clone contained two types of gyrase B subunit that differed in their affinities for novobiocin and, therefore, it was expected that the two gyrase activities in strain AT 18 would also differ in their functional responses to the drug. Such proved to be the case (Figure 2). The enzyme that adhered tightly to the column and could only be displaced by salt plus urea ('sensitive' enzyme in Figure 2) was inhibited ~50% by novobiocin concentrations around 1.6 μg/ml, whereas for the other activity (the 'resistant' enzyme that was eluted with 300 mM KCl) the corresponding novobiocin concentration was at least a 100-fold higher. These data eliminated the possibility, alluded to above, that resistance in the clone might have been due to mopping up of intracellular novobiocin by excess gyrase B protein. Rather, it now remained to be established whether a novel *gyrB* gene from *S.sphaeroides* had been cloned in strain AT 18 or whether an otherwise-sensitive B protein was being subjected to post-translational modification, resulting in resistance. This matter was addressed by Southern analysis. However, before describing those experiments, there is an additional point arising from Figure 2 that deserves comment. Reproducibly, DNA gyrase from the control strain AT 1 was more sensitive to novobiocin than the 'sensitive' gyrase activity from strain AT 18. The significance of this important observation is discussed later.

Southern analysis of the cloned S.sphaeroides DNA

Restriction fragments from pLST 18 were blotted onto membranes and probed using a 1.7 kb *Sal*GI–*Bam*HI fragment from pML 2 (Lampe and Bott, 1985) that contains exclusively *gyrB* DNA from *B.subtilis*. Despite the wide disparity in the G+C contents of DNA from *Bacillus* and *Streptomyces*, which necessitated the use of moderate stringency levels, the results were positive (Figure 3). The *Bacillus gyrB* probe (see track a) hybridized specifically with the internal *Bam*HI fragment (2.3 kb–see tracks c,e) of *S.sphaeroides* DNA but not with any other part of pLST 18. In contrast, a *Bacillus gyrA* probe, consisting of the *Bam*HI–*Eco*RI fragment (2.2 kb) of pML 2, failed to

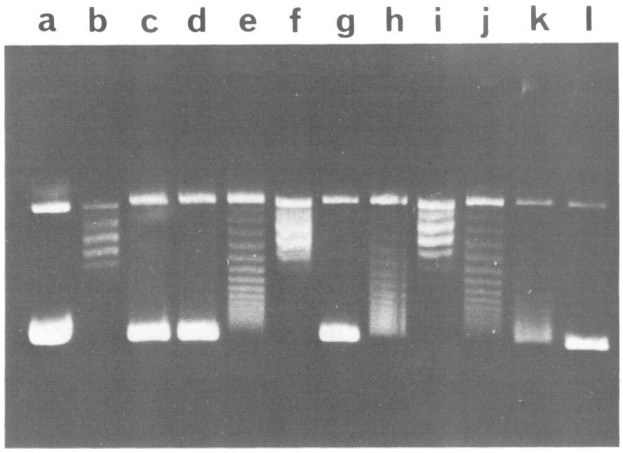

Fig. 2. Effects of novobiocin on supercoiling activity of DNA gyrase. Gyrase was incubated with novobiocin prior to addition of relaxed pBR 322 and ATP. Controls minus gyrase were: (**a**) pBR322 supercoiled *in vivo* (lower band) but still containing some open circular DNA (upper band) and (**b**) relaxed pBR322. Other tracks show the substrate after exposure to DNA gyrase and drug. (**c**–**f**) DNA gyrase from control strain AT1, novobiocin concentrations (μg/ml) were zero, 0.1, 0.4, 0.8 respectively; (**g**–**i**) 'sensitive' DNA gyrase from clone AT 18, novobiocin concentrations (μg/ml) were 0.8, 1.6, 2.4 respectively; (**j**–**l**) 'resistant' DNA gyrase from clone AT 18, novobiocin concentrations (μg/ml) were 240, 160, 80 respectively.

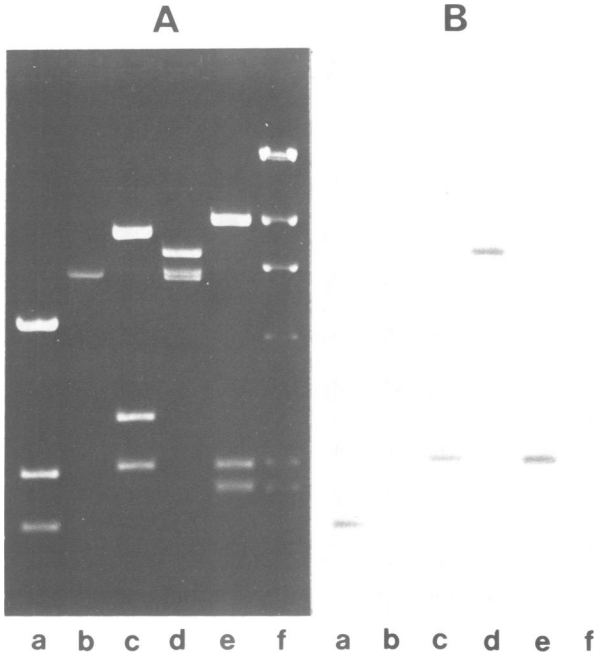

Fig. 3. Southern analysis of cloned DNA from *S.sphaeroides*. (**A**) Agarose gel electrophoresis of restriction digests of pLST 18 plus controls pIJ 487 and pML 2. (**B**) Autoradiogram of blot (from gel shown in A) probed with *B.subtilis gyrB* DNA (1.7-kb *Sal*I–*Bam*HI fragment) derived from pML 2. Plasmids, restriction digests and fragments generated (sizes given in kb) in each track were as follows: (**a**) pML 2, *Sal*I plus *Bam*HI plus *Eco*RI, 5.4 plus 2.2 plus 1.7; (**b**) pIJ 487 linearized with *Eco*RI, 6.3; (**c**) pLST 18, *Bam*HI plus *Hin*dIII, 8.3 plus 2.9 plus 2.3; (**d**) pLST 18, *Eco*RI plus *Hin*dIII, 7.2 (total cloned DNA) plus 6.3; (**e**) pLST 18, *Eco*RI plus *Bam*HI, 9.2 plus 2.3 plus 2.0; (**f**) size markers: λ DNA digested with *Hin*dIII, 23.5 plus 9.6 plus 6.8 plus 4.5 plus 2.3 plus 2.0.

hybridize with pLST 18 although it did recognize total DNA from *S.sphaeroides* under similar conditions of stringency (data not given). It was therefore concluded that pLST 18 contains a *gyrB* gene that originated in *S.sphaeroides* and encodes a novobiocin-resistant variant of the gyrase B subunit. However, *gyrA* DNA did not appear to have been cloned in strain AT 18.

Expression of the novobiocin-resistance gene in vitro

Plasmids pLST 1818 and pLST 1819 (comprising the entire cloned piece of *S.sphaeroides* DNA inserted into pUC 18 and pUC 19 respectively) were introduced into a coupled transcription–translation system prepared from *S.lividans* (Thompson *et al.*, 1984) and protein products, radiolabelled with [^{35}S]methionine, were separated on SDS–polyacrylamide gels (Figure 4). In addition to the intact 31-kb β-lactamase pre-protein and other smaller bands derived from it (typically seen in this system–see Thompson *et al.*, 1984), pLST 1818 and pLST 1819 each gave rise to a 79-kd band that was absent from controls primed by pUC 18. Presumably, this band corresponded to the novobiocin-resistant gyrase B protein from *S.sphaeroides* and the fact that it was produced with both plasmids suggested that the gene was being read *in vitro* from its own promoter. That being so, it was concluded that the authentic product, as opposed to a fusion protein, had been visualized.

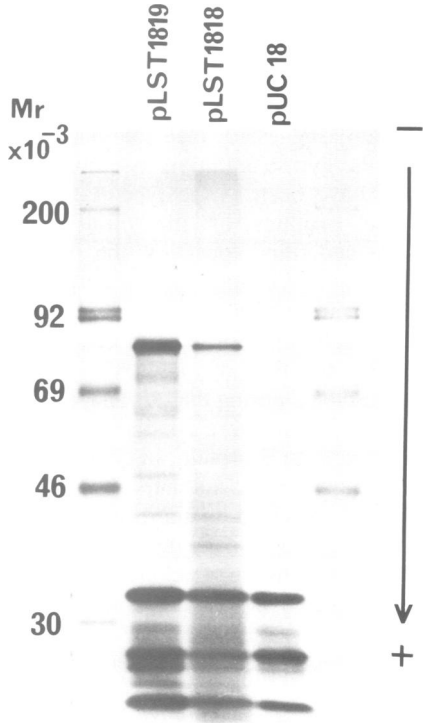

Fig. 4. Coupled transcription–translation *in vitro*. Products were analysed by electrophoresis in SDS–polyacrylamide gels together with [^{14}C]proteins (Amersham International) as mol. wt markers.

Discussion

The *gyrB* gene from *S.sphaeroides*, cloned here in *S.lividans*, represents the first resistance determinant from a novobiocin-producing organism to have been characterized. What is not clear, however, is whether this same gene was cloned previously (Murakami *et al.*, 1983), since restriction data were not included in that report and gyrase activity was not examined. Here, the cloned *gyrB* gene was not obviously

linked to *gyrA*, at least not within 2–3 kb. This calls to mind the arrangement in *E.coli*, where *gyrA* and *gyrB* are well separated in the chromosome (Bachmann, 1983) in contrast to that in *B.subtilis*, where the two genes are only ~150 bp apart (Moriya *et al.*, 1985). On the other hand, in terms of molecular size, the *Streptomyces* gyrase B protein (as produced *in vitro*) more closely resembled the *B.subtilis* homologue rather than the *E.coli* gyrase B protein, which appears to be significantly larger.

In assaying the effect of novobiocin on DNA gyrase supercoiling activity, a pronounced variation in the dose response was observed depending upon the order of addition of the various components. Thus, in the experiments described here, the drug was added to the enzyme (i.e. S100 or affinity-purified gyrase) prior to addition of plasmid plus ATP. However, in alternative protocols (data not given) when the enzyme and substrate were mixed prior to addition of novobiocin, the system was 5–10 times more resistant to inhibition. Under the latter conditions, the amount of novobiocin needed to inhibit the 'resistant' gyrase from clone AT 18 *in vitro* was close to the MIC value for the intact organism.

It has been estimated that *E.coli* contains ~500 copies of the DNA gyrase tetramer (Higgins *et al.*, 1978; see also Liu and Wang, 1987). Whether such is also the case in *S.lividans* remains to be established but, nevertheless, sufficient A protein was presumably available to provide partners for at least some of the 'additional' B subunits that are produced in strain AT 18 from the cloned *gyrB* gene. In that strain, 'sensitive' and 'resistant' gyrase activities were present in approximately equivalent amounts (an imbalance of 2:1 either way would certainly have been detected) but this does not necessarily imply that equal amounts of B protein were being produced from the two *gyrB* alleles (which, after all, were present in strain AT 18 at different copy numbers). Rather, the crucial observation (emphasized above), that the 'sensitive' gyrase from strain AT 18 was less sensitive to novobiocin than was the single gyrase from the control strain, suggests a plausible alternative model. Thus, some of the gyrase tetramers assembled in strain AT 18 might well have been of the type A_2BB^* (where B* represents the product of the cloned *gyrB* from *S.sphaeroides*) and this form of the enzyme would presumably have co-purified with A_2B_2 on novobiocin–Sepharose. Then, following elution with 5 M urea plus 2 M salt and consequent dissociation of the tetramers, $A_2B_2^*$ together with A_2BB^* and A_2B_2 would have been reconstituted during dialysis and would have been assayed as such. If that were so, the clone should have contained more B* than B protein, in qualitative agreement with the relative gene copy numbers.

This model will be tested in future work, at which time it will be interesting to determine the relative levels of DNA gyrase A and B proteins in the clone AT 18. It will also be interesting to discover whether, in *S.sphaeroides* as elsewhere (for review, see Hopwood *et al.*, 1986), antibiotic production genes are physically linked to the resistance determinant(s).

Materials and methods

Bacterial strains and media

The following strains were used: *S.sphaeroides* NCIB 11891 (producer of novobiocin) and *S.lividans* TK24. The latter strain, a streptomycin-resistant, plasmid-free derivative of *S.lividans* 66, was used as host in the generation of clones using pIJ 487 as vector (Ward *et al.*, 1986). The control strain (TK 24 containing unmodified pIJ 487) is referred to here as *S.lividans* AT 1. Strain NM522 of *E.coli* (Gough and Murray, 1983) was the host for pUC 18, pUC19 and pML 2. The latter plasmid carries the *gyrA* and *gyrB* genes from *B.subtilis* as closely adjacent DNA sequences from which they can be liberated as *Bam*HI–*Eco*RI (2.2 kb, *gyrA*) and *Sal*GI–*Bam*HI (1.7 kb, *gyrB*) restriction fragments (Lampe and Bott, 1985).

All *Streptomyces* strains were maintained at 30°C on NE agar (Skeggs *et al.*, 1985). For the preparation of S30 and S100 extracts (see below) *Streptomyces* were grown in YEME liquid medium supplemented with polyethylene glycol and $MgCl_2$ as in Thompson *et al.* (1984). Chromosomal DNA and plasmids were prepared from *Streptomyces* according to standard procedures (Hopwood *et al.*, 1985). Regeneration of protoplasts and initial selection of thiostrepton-resistant transformants on R2YE medium, replica plating on minimal medium to select novobiocin resistant strains and the determination of antibiotic MIC values, again on minimal medium, were also carried out according to Hopwood *et al.* (1985). For growth of *E.coli*, L-broth or L-agar was used (Hopwood *et al.*, 1985).

Antibiotics used were: thiostrepton (Squibb Institute, Princeton, NJ), ampicillin (Sigma), novobiocin (Upjohn, Kalamazoo, MI) and ciprofloxacin (Bayer, UK).

Cloning procedure

Total genomic DNA (40 μg) from *S.sphaeroides* was partially digested with *Sau*3A and size-fractionated by electrophoresis in 1% (w/v) low-melting-point agarose. Fragments of DNA (~4 μg total) in the size range 3–10 kb were then extracted with the aid of cetyl trimethyl ammonium bromide (Langridge *et al.*, 1980) and ligated into 2 μg of pIJ487, that had previously been digested to completion with *Bam*HI and then treated wtih calf intestinal alkaline phosphatase. Ligation was carried out at 20°C for 16 h at a final DNA concentration of 40 μg/ml. The entire ligation mixture was then used to transform ~10^9 protoplasts of *S.lividans* TK24 which were allowed to regenerate on R2YE agar medium before being flooded with 1.5 ml sterile distilled water containing thiostrepton (final concentration, 20 μg/ml in the plates). After 5–6 days at 30°C, the thiostrepton-resistant transformants were replica plated on to minimal agar plates containing thiostrepton (20 μg/ml) plus novobiocin (100 μg/ml) and incubation was continued at 30°C.

Southern analysis

Digests of pML2 (*Sal*GI plus *Bam*HI for the *gyrB* probe and *Eco*RI plus *Bam*HI for *gyrA*) were prepared and the products separated on low-melting-point agarose. The desired restriction fragments were excised, denatured, annealed to mixed sequence hexadeoxynucleotides (Pharmacia), and extended using DNA polymerase Klenow fragment (BRL) in the presence of [^{32}P]-dCTP [Amersham International: 3000–4000 Ci/mmol (111–148 TBq/mmol)] according to Feinberg and Vogelstein (1984). After 30 min at 37°C, the radiolabelled probes were each diluted to a final volume of 500 μl with 3 mM Tris–HCl (pH 7.0 at 20°C) containing 0.2 mM Na_2EDTA, heated for 10 min at 90–100°C, cooled rapidly in an ice-bath and then used directly in Southern analysis without removal of unincorporated nucleotides.

The DNA fragments from digests of pLST 18, pIJ 487 or pML 2 (see legend to Figure 3) were separated on a 0.7% (w/v) agarose gel using as running buffer 40 mM Tris-acetate (pH 7.8 at 20°C) and 2 mM Na_2EDTA (TAE buffer). The DNA in the gel was then denatured, neutralized and blotted onto Hybond-N membrane (Amersham International) and cross-linked to the membrane using UV light as recommended by the manufacturer. Prehybridization (for 30 min) and hybridization of the probe (for 16 h) were carried out at 55°C in buffer containing 270 mM NaCl, 15 mM sodium phosphate (pH 7.7), 1.5 mM Na_2EDTA, 0.5% (w/v) dried milk (Cadbury's Marvel), 1% (w/v) SDS and 6% (w/v) polyethylene glycol 6000. The hybridization washes were performed at 55°C in 75 mM NaCl, 7.5 mM sodium citrate (pH 7.6) containing 0.1% SDS. The filter was then dried at 20°C and subjected to autoradiography at 20°C using Fuji RX film.

Preparation of substrate for DNA gyrase assays

Supercoiled pBR 322, prepared by standard procedures involving centrifugation in a CsCl density gradient containing ethidium bromide, was converted to the relaxed, covalently closed circular form by treatment with calf thymus topoisomerase I in the presence of T4 DNA ligase, as recommended by the supplier (Bethesda Research Laboratories). Progress of the reaction was monitored by agarose gel electrophoresis. The reaction was stopped by addition of an equal volume of phenol (saturated with Tris–HCl, pH 8.0 adjusted at 20°C) and the relaxed DNA was precipitated from the aqueous phase using ethanol. Finally, the DNA was centrifuged

in a CsCl density gradient containing ethidium bromide and, after removal of the dye followed by precipitation with ethanol, was stored at 4°C in buffer containing 10 mM Tris−HCl (pH 8.0 at 20°C) plus 1 mM Na_2EDTA.

Purification of DNA gyrase from S.lividans

Cultures were grown for 18−24 h at 30°C in YEME-PEG medium containing thiostrepton 10 μg/ml (control strain AT1) or thiostrepton 10 μg/ml plus novobiocin 30 μg/ml (novobiocin-resistant strain AT 18, see text). Subsequent manipulations were carried out at 0−4°C. Mycelium was harvested by centrifugation and washed three times by resuspension in buffer A followed by recentrifugation. Buffer A contained 25 mM Hepes−KOH (pH 8.0 at 20°C), 10 mM magnesium acetate, 56 mM KCl (to give a total K^+ concentration of 75 mM), 2 mM dithiothreitol, 10% (w/v) ethylene glycol. Washed mycelium (5 g wet weight) was then thoroughly resuspended in 10 ml of buffer A and passed through a chilled French pressure cell at 10 000−12 000 p.s.i. (70−80 MPa). After a clearing spin at 18 000 rev/min for 30 min in the Beckman JA21 rotor, the supernatant ('S30') was recentrifuged at 45 000 rev/min for 2 h in the Beckman Ti 75 rotor. The final supernatant ('S100') was either stored as small samples at −70°C after quick-freezing in CO_2/ethanol or used directly for the bulk preparation of DNA gyrase, by affinity chromatography, as follows.

Novobiocin was coupled to epoxy-activated Sepharose (Pharmacia) as described by Staudenbauer and Orr (1981) and the resin was equilibrated with buffer A before being packed into a column (7.0 cm × 1.0 cm). The S100 supernatant, prepared in buffer A as above, was then applied to the column, which was washed overnight with buffer A. Subsequently, the column was eluted successively with 5 vols each of buffer A supplemented with KCl to give K^+ concentrations of 150, 300, 500 mM and, finally, 2 M KCl plus 5 M urea. The various eluates were then dialysed against buffer A, frozen and stored at −70°C as above.

Assay of DNA gyrase activity

Activity of DNA gyrase was assayed by following the introduction of supercoils into relaxed pBR 322 (prepared as above). As indicated in the text, the reaction was absolutely dependent upon the addition of ATP and, in preliminary experiments which led to the formulation of buffer A, the optimal requirements for Mg^{2+} and K^+ (both of which were also essential) were determined. The standard assay mixture (20 μl final volume) contained partially purified gyrase (prepared as above) in buffer A supplemented with 10 mM spermidine−HCl, *E.coli* tRNA (10 μg/ml), and bovine serum albumin (50 μg/ml). The reaction was started by the addition of ATP (1.5 mM final concentration) together with substrate (relaxed pBR322−see below) and was terminated by the addition of an aqueous mixture containing 20% (w/v) Ficol 400, 0.1% (w/v) SDS and 0.04% (w/v) bromophenol blue. Resultant plasmid DNA was analysed by electrophoresis in 0.8% (w/v) agarose gels made up in TAE buffer.

For comparison of gyrase activity in different preparations, the enzyme input was standardized as that required to convert ~50% of a fixed input of substrate (i.e. 125 ng) from the relaxed to the fully supercoiled state in 60 min at 30°C. The effects of novobiocin on gyrase activity were determined by incubating the drug with the enzyme for 5 min at 20°C in the absence of substrate prior to initiation of the supercoiling reaction as above.

Coupled transcription−translation in vitro

Expression of the cloned novobiocin-resistance gene (see text) was studied *in vitro* as described previously (Thompson *et al.*, 1984). Here, the system was primed with 1 μg of pUC 18 (control template) or 2 μg of pLST 1818 or pLST 1819. Protein products containing [^{35}S]methionine were separated by electrophoresis in 12% (w/v) polyacrylamide gels containing SDS which were then fixed, treated with Amplify (Amersham International) according to the manufacturer's instructions, dried onto Whatman no.3 paper and subjected to autoradiography using Fuji RX film at 20°C.

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