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Genetic analysis of the requirements for SOS induction by nalidixic acid in *Escherichia coli*

Kathryn G. Newmark, **Erin K. O'Reilly**[1], **Jennifer Reineke Pohlhaus**, and **Kenneth N. Kreuzer**[*]

Department of Biochemistry, Duke University Medical Center, Durham, NC 27710

Abstract

Nalidixic acid, the prototype antibacterial quinolone, induces the SOS response by a mechanism that requires the RecBCD nuclease/helicase. A key step inferred for this induction pathway is the conversion of a drug-induced gyrase cleavage complex into a DNA break that can be processed by RecBC. We tried to clarify the nature of this step by searching for additional gene products that are specifically necessary for SOS induction following nalidixic acid treatment. A transposon library of approximately 19,000 insertion mutants yielded 18 mutants that were substantially reduced for SOS induction following nalidixic acid but not UV treatment, and which were also hypersensitive to nalidixic acid. All 18 mutants turned out to have insertions in *recB* or *recC*. As expected, *recA* insertion mutants were uncovered as being uninducible by either nalidixic acid or UV treatment. Insertions in 11 other genes were found to cause partial defects in SOS induction by one or both pathways, providing possible leads in understanding the detailed mechanisms of SOS induction. Overall, these results suggest that nalidixic acid-induced DNA breaks are generated either by RecBC itself, by redundant activities, and/or by an essential protein that could not be uncovered with transposon mutagenesis.

Keywords

DNA gyrase; RecBCD; DNA damage; quinolones; transposon mutagenesis

1. Introduction

Type II DNA topoisomerases are essential enzymes involved in numerous cellular processes, including DNA replication, DNA segregation and transcription. These enzymes catalyze changes in DNA topology by creating a transient double strand break in one segment of duplex DNA and passing a second duplex segment through that break. Many clinically important antibacterial and antitumor agents target type II topoisomerases by altering the equilibrium of the reaction cycle to stabilize the normally transient intermediate called the cleavage complex (Drlica and Zhao, 1997; Burden and Osheroff, 1998). These include the antibacterial quinolones (e.g. nalidixic acid) that target DNA gyrase (and topoisomerase IV in certain bacteria), and antitumor drugs such as mitoxantrone, adriamycin and etoposide, which target mammalian type II topoisomerase. In each case, the drug-stabilized cleavage complex consists of the topoisomerase linked via phosphotyrosine bonds to both 5' ends of the staggered double-

* Corresponding author: Kenneth N. Kreuzer, Email: kenneth.kreuzer@duke.edu, Phone: 919 684 6466, Fax: 919 684 2790.
[1]Present address: bioM rieux, 100 Rodolphe St., Durham, NC 27712

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strand break. Results from numerous systems demonstrate that the cytotoxicity of these inhibitors is dependent on formation of cleavage complexes rather than inhibition of topoisomerase catalytic activity (for reviews, see Chen and Liu, 1994; Drlica and Zhao, 1997).

Although much research has been devoted to understanding how stabilized cleavage complexes lead to cell death, the detailed mechanism(s) remains unclear. Formation of cleavage complexes is necessary but not sufficient for cytotoxicity. The 5' ends of the broken DNA within the cleavage complex are safely linked to protein, and cleavage complexes are readily reversible upon drug removal both in vivo and in vitro (Hsiang and Liu, 1989; also see Drlica and Zhao, 1997). The energy poison dinitrophenol blocks cytotoxicity of mammalian type II topoisomerase inhibitors but does not prevent cleavage complex formation (Kupfer et al., 1987). Likewise, dinitrophenol (as well as chloramphenicol) protects *Escherichia coli* from the cytotoxic action of nalidixic acid (Cook et al., 1966). Evidently, a cellular processing event must occur to convert a subset of the cleavage complexes into cytotoxic lesions. What is this event, and what is the exact nature of the cytotoxic lesion?

A number of observations strongly suggest that the cytotoxic lesion is some type of double-strand break (for review, see Chen and Liu, 1994; Drlica and Zhao, 1997). For example, mutational inactivation of functionally conserved recombination proteins in phage, bacterial and eukaryotic systems leads to drug hypersensitivity. The gene products required for the repair of topoisomerase-mediated DNA damage are similar or identical to those required for the repair of endonuclease-generated double-strand breaks. Furthermore, there is evidence for overt chromosomal breaks after nalidixic acid treatment, but the location of the breaks and the possibility of covalently attached protein were not determined (Chen et al., 1996).

A variety of models could in principle explain the relationship between drug-stabilized cleavage complexes and cytotoxicity. First, a nuclease such as SbcCD might directly recognize the covalent protein-DNA complex and cleave the DNA nearby (Connelly et al, 2003). Second, the replication complex or associated helicase may be able to extract the lagging strand template from the cleavage complex upon collision (Howard et al., 1994). Third, DNA breaks might result as “collateral damage” from recombination nucleases that act after replication fork blockage by the cleavage complex, as in the phage T4 system (Hong and Kreuzer 2003). We have recently found that quinolone-stabilized gyrase cleavage complexes block *E. coli* replication forks on plasmid pBR322 *in vivo* (Pohlhaus and Kreuzer, 2005). In that study, some of the blocked forks were broken, consistent with the collateral damage model.

To approach the mechanism of cytotoxicity, we have taken advantage of an SOS reporter system and screened for *E. coli* mutants specifically deficient in SOS induction upon nalidixic acid treatment. The primary target of nalidixic acid in *E. coli* is DNA gyrase (see Maxwell and Critchlow, 1998), and this drug was one of the first inducers of the SOS regulon studied in detail. The SOS regulon consists of about 30 different genes, many of which are involved in damage repair or bypass reactions (Friedberg et al., 1995). The LexA protein normally represses SOS genes, but is cleaved to trigger SOS as a result of DNA damage. Cleavage of LexA depends on the activated form of the RecA protein, bound to single-stranded DNA.

DNA gyrase cleavage complexes stabilized by nalidixic acid are necessary but not sufficient for induction of the SOS response. There is conflicting evidence as to whether DNA replication is required for induction of SOS by nalidixic acid (Gudas and Pardee, 1976; Sassanfar and Roberts, 1990). Aside from RecA, the only known protein that is required for SOS induction by nalidixic acid is the multifunctional RecBCD enzyme (for review, see Myers and Stahl, 1994). Since RecBCD generally requires a DNA end to gain entry to DNA, its involvement in

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SOS induction suggests that a free DNA break is somehow generated from the nalidixic acid-stabilized cleavage complex.

The above results provide strong parallels between the mechanism of cytotoxicity and the mechanism of SOS induction by nalidixic acid, particularly since *recA*, *recB* and *recC* mutants are all hypersensitive to the drug (McDaniel et al., 1978). It seems highly likely that whatever mechanism creates DNA breaks to induce the SOS pathway is also a mechanism that leads to cytotoxicity. In this report, we identify and analyze *E. coli* mutants with deficiencies in the SOS response to nalidixic acid.

2. Materials and methods

2.1. Materials and media

Kanamycin and nalidixic acid were purchased from Sigma, while 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was purchased from Gold Biotechnology Inc. L-broth (LB) contained Bacto-Tryptone (10 g/L), yeast extract (5 g/L) and sodium chloride (10 g/L) while LB plates had the same composition plus Bacto-agar (14 g/L). Top agar contained Bacto-Tryptone (13 g/L), sodium chloride (8 g/L), sodium citrate dihydrate (2 g/L), glucose (5 g/L) and Bacto-agar (6.5 g/L).

2.2. Transposon mutagenesis and primary nalidixic acid screening

A transposon library was created in *E. coli* strain JH39 [F^- *sfiA11*, *thr-1*, *leu-6*, *hisG4*, *argE3*, *ilv(Ts)*, *galK2*, *srl(?) rpsL31*, *lac U169*, *dinD1*::MudI(Ap^r *lac*); Heitman and Model, 1991; O'Reilly and Kreuzer, 2004] using the EZ::TN™ <KAN-2> Tnp Transposome™ Kit from Epicentre (Madison). Electrocompetent cells were pulsed at 1.8 kV in 0.1-cm cuvettes, followed by outgrowth in TB for one hour at 37°C with aeration. The cells were then plated at appropriate dilutions on LB plates containing kanamycin (60 μg/ml). Following overnight incubation at 37°C, the colonies were lifted using 85-mm circular nitrocellulose filters (Schleicher and Schuell) and placed, colony-side up, onto LB plates containing nalidixic acid (50 μg/ml) and X-gal (60 μg/ml). The lifted colonies were incubated overnight at 37°C and the plates were checked for color development at approximately 4 and 12 hours. Colonies that turned darker blue than the wild-type were marked as "dark blue" while those that were white or lighter than wild-type were marked as "white" and "intermediate", respectively.

2.3. DNA techniques and sequencing

Genomic DNA was purified using the MasterPure™ DNA Purification Kit of Epicentre (Madison) essentially as described by the manufacturer (a phenol-chloroform extraction followed by an ethanol precipitation was added after the RNase treatment). The final purified DNA pellet was resuspended in 30 μl of T_5E_1 buffer (5 mM Tris-HCl, pH 7.8; 1 mM EDTA). The DNA samples were sequenced by the Duke University Cancer Center DNA Analysis Facility using a modification of the automated sequencing protocol (60 cycles, 60°C annealing temperature). The sequencing primer (10 pmol/μl) was KAN-2 FP-1 (5′–ACCTACAACAAAGCTCTCATCAACC–3′), the forward primer from the EZ::TN™ <KAN-2> Tnp Transposome™ Kit.

PCR-amplification of the *recB* gene was performed with primers recB-FP (5′–CGGCAGGATGTTCATCTCCCGCC–3′) and recB-RP (5′–TCTTGTGCTGCACGAGTCAGCC–3′).

2.4. Phage P1 transductions

Transductions were performed by the method of Silhavy et al. (1984), selecting for the kanamycin-resistance gene of the transposon.

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3. Results

3.1. Isolation of transposon mutants with reduced SOS induction in response to nalidixic acid

As described in the Introduction, DNA breaks are very likely generated from nalidixic acid-stabilized cleavage complexes as a requisite step in SOS induction. To search for genes that may be necessary for this step, we used the EZ::TN™ <KAN-2> Tnp Transposome™ Kit (Epicentre) to create a library of transposon mutants in the JH39 strain of *E. coli*. This strain has a *dinD1::lacZ* fusion that provides a convenient reporter for the SOS response, allowing non-inducible mutants to be identified by a simple color screen (Heitman and Model, 1991). The strain also has an *sfiA* (*sulA*) mutation, which reduces filamentation and inhibition of cell division upon SOS induction (Friedberg et al., 1995).

The transposon-protein complexes were transformed into JH39 by electroporation, and approximately 19,000 colonies with random transposon insertions were selected based on kanamycin resistance. The colonies were lifted onto nitrocellulose filters, which were then transferred to plates containing nalidixic acid and X-gal. After 4-hour and overnight incubation, a small fraction of the colonies displayed unusual color intensities, indicating different amounts of β-galactosidase induction. We collected 25 “white mutants” with no apparent induction and 105 “intermediate mutants” with reduced induction relative to JH39. We also isolated 66 “dark blue mutants” with increased induction relative to JH39; these are detailed elsewhere (O'Reilly and Kreuzer, 2004).

3.2. Secondary screens for induction with nalidixic acid or UV

We began analyzing the white and intermediate mutants, which have apparent defects in SOS induction after nalidixic acid. As a secondary screen for SOS induction we performed a disk test in which a lawn of each mutant was exposed to three levels of nalidixic acid during growth on X-gal plates (Figure 1A). The zones of inhibition were also measured to allow for a determination of drug sensitivity (Table 1). All of the 25 white mutants showed normal-sized zones of inhibition and again displayed no blue color in the presence of the drug (e.g., compare W16 to wild-type JH39 in Figure 1A and Table 1). Sixty of the intermediate mutants looked indistinguishable from the parental JH39 in this test, both for nalidixic acid sensitivity and the intensity of β-galactosidase induction. These were therefore judged to be false positives and were discarded. The remaining 45 intermediate mutants again showed reduced induction compared to JH39, with 20 displaying noticeably less color than the others. Each of these 20 mutants was also modestly hypersensitive to nalidixic acid, as exemplified by mutants I78 and I40 (Figure 1A and Table 1). Of the remaining 25 intermediate mutants, one (I28) appeared to be hypersensitive to nalidixic acid (Figure 1A and Table 1), while the other 24 were either noticeably resistant or similar to the wild-type (Tables 1 and 2; also see Figure 1A, I104).

As an additional secondary screen, all 25 white mutants and the remaining 45 intermediate mutants were tested for their SOS response to UV. Like nalidixic acid, UV treatment also induces the SOS response, but the pathway for induction requires *recFOR* rather than *recBC*, presumably because UV damage leads to single-stranded DNA rather than double-strand breaks (McPartland et al., 1980). Cells were spotted twice onto X-gal plates, and one of the spots was exposed to UV. In the wild-type control, strong induction was evident after four hours incubation (Figure 1B, JH39).

All of the 25 white mutants were also uninducible in this UV test (e.g., Figure 1B, W16). Previous analysis of the JH39 genetic background has shown a background level of β-galactosidase expression without any SOS-inducing agent, suggesting a low level of SOS-independent transcription of the *dinD1::lacZ* fusion (Heitman and Model, 1991; also see

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Kenyon and Walker, 1980 and Figure 1B, JH39 minus UV). Particularly with the discovery of *recA*, *recB* and *recC* mutants in the intermediate class (see below), we surmised that the white mutants are completely unable to express β-galactosidase, likely due to transposon insertions in the *dinD1::lacZ* fusion, and did not analyze them further.

The intermediate class of 45 mutants turned out to be more interesting. Twenty-nine of these showed apparently wild-type levels of SOS induction in response to UV (e.g., Figure 1B, I40), while the other 16 showed reduced induction (e.g., Figure 1B, I78, I104 and I28; Table 2). Mutants that are unable to process drug-stabilized cleavage complexes into an SOS signal should be among the 29 with a normal SOS response to UV. Those deficient in SOS response to both nalidixic acid and UV apparently have a more general defect in SOS induction.

The small amount of β-galactosidase expression shown by intermediate mutants in response to nalidixic acid might indicate that the mutated gene is not strictly required for the SOS response and/or that the *dinD1::lacZ* fusion is expressed at a low level without an SOS response (as mentioned above).

3.3. Analysis of insertions by DNA sequencing and P1 transduction

We were most interested in the 29 intermediate mutants that showed normal UV responses, particularly those 18 that were hypersensitive to nalidixic acid. Nonetheless, to avoid missing any potentially interesting genes, we decided to sequence all of the intermediate mutants.

We used the MasterPure™ DNA Purification Kit (Epicentre) to purify genomic DNA from each mutant and then used the forward primer from the EZ::TN™ transposon kit to sequence from one end of the transposon. Using a modified automatic sequencing protocol, we were able to obtain at least 100 bp of unambiguous sequence from each mutant. Using those sequences, the *E. coli* K12 genome was then searched using BLAST to determine which gene had been interrupted by the transposon (Table 2 and Table 3).

Two of the insertion mutants were found to have the transposon within the *trpB* gene, which is part of the *dinD1::lacZ* fusion (Casadaban and Cohen, 1980). The SOS phenotypes caused by these insertions presumably result from polar effects on *lacZ* expression, and these two mutants were therefore discarded from the collection (not included in the Tables).

Two of the transposon insertions from the intermediate class were in the *recA* gene. Both *recA* mutants were hypersensitive to nalidixic acid and were very light blue in both the nalidixic acid and UV tests. Given that JH39 produces a small amount of β-galactosidase without exposure to a DNA-damaging agent (Heitman and Model, 1991), the very weak level of β-galactosidase in the two *recA* insertion mutants is probably unrelated to the SOS pathway. We found that a previously constructed *recA56* derivative of JH39, called JH59 (Heitman and Model, 1991), also turned very light blue in a nalidixic acid disk test. Furthermore, Kenyon and Walker (1980) found that RecA was required for induced but not for basal expression from several *din* fusion constructs (the *dinD1* fusion was not tested). In summary, the two new *recA* mutants had the expected phenotype: defective in the SOS response to both nalidixic acid and UV, and hypersensitive to nalidixic acid.

We expected to find *recB* and *recC* mutants among the mutants that were specifically defective in the SOS response to nalidixic acid (see Introduction). Indeed, sequencing revealed eight insertions in *recB* and eight in *recC*, and all 16 were hypersensitive to nalidixic acid (see Tables 1 and 2). Finding *recB* and *recC* mutants in this group validated our mutant hunt, and also focused our attention on other mutants with the same phenotype in our search for additional genes necessary for the SOS response from nalidixic acid-stabilized cleavage complexes.

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Besides the 16 *recBC* mutants, the only other intermediate mutants that fit this category of interest had an insertion in *ompA*, the gene for a major outer membrane protein, or *yhcL*, the product of which has been suggested to be involved in dicarboxylate transport (Janausch and Unden, 1999). Because neither protein function suggested a role in the processing of cleavage complexes and because each gene was found only once in the screen, we were suspicious that the transposon insertions in these two strains might not be causing the observed phenotype. To resolve this uncertainty, we performed phage P1 transductions to move each transposon insertion mutation into a clean JH39 background, using the kanamycin selectable marker. The transductants behaved like wild-type JH39 in both the nalidixic acid and UV tests, indicating that the transposon mutations in *ompA* and *yhcL* were not causing the interesting phenotype. Because we had screened about 19,000 colonies for the phenotype of interest, it seemed quite possible that these two had acquired a spontaneous mutation that inactivated either *recB* or *recC*. Indeed, PCR amplification of genomic DNA from the *ompA* and *yhcL* strains revealed that their *recB* genes were approximately 1000 bp longer than the wild-type *recB* gene from JH39. It seems very likely that an endogenous insertion sequence had interrupted the *recB* genes in both strains, giving them the *recBC*-like phenotype. The *ompA* and *yhcL* insertions were therefore discarded from the collection and are not included in the Tables.

These results with the *ompA* and *yhcL* insertions raised the concern that other transposon insertions in our collection might not be causing the phenotype of interest. Insertions in 10 different genes, including *recA*, *recB* and *recC*, were isolated multiple times with the same phenotype, arguing very strongly that the transposon is causative in those cases. However, in addition to *ompA* and *yhcL*, we isolated single-hit insertions in 6 additional genes/reading frames. To test whether these insertions were causing the SOS phenotype, we again performed P1 transductions using the insertion mutants as donors and JH39 as recipient. Kanamycin-resistant transductants from the *hns*, *intQ*, *moaD* and *mobA* donors recapitulated the SOS phenotypes of their donor parents, confirming that the transposon was causative in each case. Two other insertion mutants failed this transduction test and were therefore eliminated from the collection (not included in Tables).

4. Discussion

We are interested in the pathway(s) that leads to DNA breaks after treatment with inhibitors that stabilize topoisomerase cleavage complexes. Such DNA breaks are thought to be involved in cytotoxicity and DNA rearrangements caused by inhibitors of type II topoisomerases (see Introduction). Elucidation of the detailed mechanism of DNA break formation may reveal important aspects of replication fork dynamics, and might also have implications for antibacterial and anticancer chemotherapy with topoisomerase inhibitors. We approached this issue experimentally by taking advantage of the SOS response, isolating mutants with reduced SOS induction upon nalidixic acid treatment.

Mutational inactivation of *recB* or *recC* blocks SOS induction by nalidixic acid but not by UV (McPartland et al., 1980), so we focused our attention on mutants with this phenotype. Out of 19,000 transposon insertion mutants, we found 18 that were defective for induction by nalidixic acid but normal for induction by UV. Sixteen of these had a transposon insertion in the *recB* or *recC* gene, which confirms the validity of the genetic screen. Another two had insertions elsewhere, but we found that these two strains had an approximately 1-kb insertion in the *recB* gene, which explains the induction defect. *E. coli* has numerous insertion sequences that are roughly 1-kb in size (Galas and Chandler, 1989), so we surmise that these mutants had undergone a transposition event that inactivated the *recB* gene at some time prior to our genetic screen. Since the competent cells for generating the 19,000 transposon insertion mutants were grown as a single batch, these two strains may have started out as siblings with the insertion sequence in *recB* prior to the transformation of the transposon.

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Thus, we found a total of 18 mutants with the desired phenotype, but all 18 were defective in *recB* or *recC*. Since we screened 19,000 colonies and isolated so many *recBC* mutants, the screen was quite thorough, and it is unlikely that any novel gene would be uncovered by screening additional mutants using this method and these criteria. Because this was a transposon mutagenesis strategy, one possibility is that RecBCD is the only non-essential protein involved in SOS induction by nalidixic acid but not by UV. Only the helicase activity of RecBCD is necessary for SOS induction after nalidixic acid treatment (Chaudhury and Smith, 1985). Therefore, the role of RecBCD is probably limited to generation of single-stranded DNA from the break, and the enzyme is not likely involved in break formation.

These results are consistent with the possibility that essential cellular proteins are involved in DNA break formation from the cleavage complex. For example, transcription and/or DNA replication could be necessary. One specific model is the replication fork run-off model, in which the replisome directly causes DNA breaks upon collision with the cleavage complex (see Introduction). If transcription and/or replication are necessary for break formation, then some of our mutants might have reduced SOS induction simply because of slower growth. We measured the growth rate of one insertion mutant for each of the genes uncovered here, and found that several did indeed have significantly decreased growth rates: *cyaA*, *icdA*, *mcrA*/*elbA*, *crp*, and *tufA* (data not shown; we did not test *tufB*, but it would presumably grow similarly to the *tufA* mutant). While any of these mutants could have reduced SOS induction due to poor growth, we note that the other mutants we isolated had little or no growth defect, and we would have expected to isolate many other poor-growth mutants if that was all that was necessary to reduce SOS induction.

A second possible explanation for finding only *recBC* mutants with the desired phenotype is that multiple pathways process the cleavage complex into an SOS-inducing signal. Some candidates for genes that might be involved in redundant pathways were uncovered in mutants that were modestly defective for SOS induction after nalidixic acid but seemingly normal after UV, namely *icdA*, *intQ*, *mcrA*/*elbA*, *moaD*, *mobA*, *tufA*/*B* and *trkH* (Table 2). The only mutants in this group with altered sensitivity to nalidixic acid were those in *trkH*, which were modestly resistant to the drug (Table 1). The *trkH* gene encodes an integral membrane protein involved in potassium uptake (Silver, 1996) and *trkH* mutations might somehow impact nalidixic acid uptake or efflux. Thus, the decreased SOS response of *trkH* mutants could simply reflect a lower effective level of inhibitor inside the cell. None of the other genes mentioned above has a function that clearly suggests an involvement in cleavage complex processing. However, two of the gene products have known or suspected DNA cleavage activities: McrA, which is a restriction endonuclease, and IntQ, which is encoded by a cryptic prophage and is related to the integrase family of proteins.

An inherent limitation of our genetic screen raises a third possible explanation for finding only *recBC* mutants with a strong SOS defect that is specific for nalidixic acid treatment. Perhaps the mutants of interest were missed in our screen because they were partially constitutive for the SOS response. That is, even in the absence of nalidixic acid, the desired mutants might have levels of β-galactosidase similar to or greater than the levels in wild-type cells with nalidixic acid. Insertions in a variety of important DNA metabolism genes cause an SOS constitutive phenotype (O'Reilly and Kreuzer, 2004), and we are currently analyzing these to see if any are defective for SOS induction after nalidixic acid treatment.

Our collection also included insertions in four genes that caused SOS defects after either nalidixic acid or UV treatment. Mutations in each of these genes are known to cause pleiotropic effects by altering nucleiod structure and gene expression (*hns*), protease function (*clpP*) or the cyclic AMP regulatory system (*cyaA* and *crp*) (Dorman, 2004; Saier et al., 1996; Gross, 1996). Although the SOS defects in each of these four mutants is likely to be caused by some

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indirect effect of their pleiotropy, these results may provide clues about subtleties of the SOS regulatory system. The cAMP system, involving the products of the *crp* and *cyaA* genes, has previously been linked to the SOS system in the context of cAMP-dependent SOS induction in resting cells (Taddei et al., 1995).

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Abbreviations

LB
: L-broth

UV
: ultraviolet light

X-gal
: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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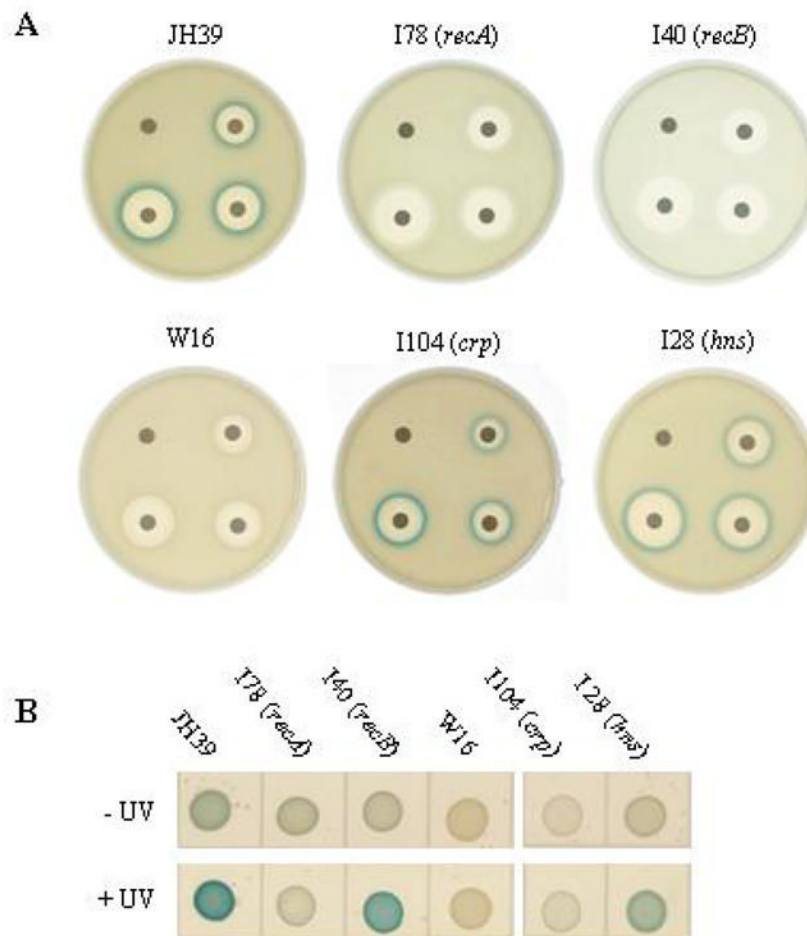

Fig. 1.

Nalidixic acid disk tests and UV tests of wild type and insertion mutants. **A.** Overnight cultures of each strain in LB were diluted to an OD_{560} of approximately 1.0 and 200 μL of the diluted cultures were added to 3.0 mL of top agar. The top agar mixture was then poured onto an LB plate containing X-gal at 60 μg/mL. Four circular glass fiber filters (GF/C; approximately 1 cm in diameter) were placed onto the cooled plate. A solution of nalidixic acid was then delivered to the filter disks (0, 2, 4 or 8 uL of a 5 mg/ml stock in 10 mM NaOH, corresponding to 0, 10, 20 or 40 μg), starting from the top left position and continuing clockwise on each plate. The plates were incubated overnight at 37°C and photographed the next morning. The strain name is given above each plate, along with the gene interrupted by the transposon in parentheses (except for parental JH39, which has no transposon, and W16, which has not been sequenced). **B.** Overnight cultures of each strain were spotted (8 μL) in duplicate onto LB plates containing X-gal at 60 μg/mL. After four hours of incubation at 37°C, half the plate was exposed to UV (52.5 J/m^2, from a General Electric G15T8 germicidal bulb) while the other half was protected with a sheet of cardboard. The plates were then wrapped in foil to protect them from light and incubated for an additional four hours at 37°C. The plates were then stored at 4°C and photographed the next day.

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Table 1

Zones of inhibition from nalidixic acid.

	Zones of Inhibition (mm)[a]			
Nalidixic Acid (μg):	10	20	40	
JH39	14.6 (±1.0)	17.2 (±1.3)	19.8 (±1.4)	
recA	17.0	20.5	24.0	HS
recB	17.8 (±0.7)	20.8 (±0.9)	23.6 (±0.7)	HS
recC	18.3 (±1.0)	21.6 (±1.3)	24.5 (±0.9)	HS
hns	17.0	20.0	23.0	HS
clpP	14.0	16.5	20.0	N
cyaA	13.3 (±2.5)	17.0 (±1.7)	20.3 (±1.5)	N
icdA	14.0	16.0	18.0	N
intQ	14.0	16.0	20.0	N
mcrA/elbA	13.0	16.0	18.5	N
moaD	15.0	17.0	21.0	N
mobA	15.0	17.0	20.0	N
tufA/B	14.5	16.5	19.5	N
crp[b]	11.7 (±0.6)	15.7 (±0.6)	19.7 (±0.6)	r
trkH	12.0	14.0	17.0	R

[a]The diameter of the zones of inhibition was measured on plates prepared identically to those depicted in Figure 1A. HS, hypersensitive, R, markedly resistant, r, slightly resistant, N, normal (similar to parental JH39). The values shown represent the average measurements of independent transposon insertion mutants. Standard error is shown when three or more transposon insertion mutants were available (see Table 3). The JH39 values represent the average and standard deviation of 18 different measurements of this parental strain.

[b]Although they did not significantly differ, the measurements of the *crp* promoter insertion mutant (I42) are not included in this calculation.

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Table 2

Summary of intermediate mutants.

Gene[a]	#	NAL Color[b]	UV Color[c]	NAL Sensitivity[d]	Putative or known function[e]
recA	2	+	+	HS	Homologous pairing and strand exchange protein
recB	8	+	+++	HS	RecB subunit of the helicase-nuclease ExoV
recB (IS)[f]	2	+	+++	HS	RecB subunit of the helicase-nuclease ExoV
recC	8	+	+++	HS	RecC subunit of the helicase-nuclease ExoV
hns	1	++	++	HS	DNA-binding protein H-NS
clpP	2	++	++	N	ATP-dependent serine protease
cyaA	3	++	+	N	Adenylate cyclase
icdA	2	++	+++	N	Isocitrate dehydrogenase (TCA)
intQ	1	++	+++	N	*Putative integrase gene within defective prophage Qin*
mcrA/elbA[g]	2	++	+++	N	Restriction endonuclease (McrA)/*isoprenoid biosynthesis (elbA)*
moaD	1	++	+++	N	Molybdopterin converting factor, small subunit
mobA	1	++	+++	N	Molybdopterin-guanine dinucleotide biosynthesis protein A
tufA/B	2	++	+++	N	Protein chain elongation factor EF-Tu
crp[h]	4	++	+	r	cAMP receptor protein
trkH	2	++	+++	R	Membrane protein, K+ transport

[a] For each gene isolated only once, P1 transductions demonstrated that the transposon insertion is causative.

[b] Intensity of blue color in response to nalidixic acid, as determined by disk test. +++ = normal, ++ = light blue, + = very light blue. These descriptions are relative to JH39 and are not directly comparable to the UV tests.

[c] Intensity of blue color in response to UV radiation, as determined by UV test. +++ = normal, ++ = light blue, + = very light blue. These descriptions are relative to JH39 and are not directly comparable to the nalidixic acid disk tests.

[d] Drug sensitivity to nalidixic acid, as determined by disk test (Table 1). N = normal (similar to parental JH39), HS = hypersensitive, r = slightly resistant, R = markedly resistant

[e] Functions as defined by the NCBI website for *E. coli* K12. Italicized function is putative.

[f] These are the two strains with a transposon in *yhcL* or *ompA* and an apparent IS element in *recB*.

[g] The transposon insertion is located between these two genes.

[h] Three of the *crp* insertions are within the coding sequence, the fourth is located 115 nucleotides upstream of the start codon.

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Table 3

Locations and directions of transposon insertions.

gene[a]	strain number	9 bp direct repeat[b]	last nucleotide transcribed
clpP	I35	ACTTTGCAC	34
	I75	ACATGGCTA	163
crp	I42	TCACATTAC	−114[c]
	I100	GTGCTTGGC	12
	I104	GGGTAAAGA	176
	I105	CGGTAAAGA	608
cyaA	I65	GTGTGGAAG	451
	I102	GCATATACT	542
	I103	CCGCAGGAT	1242
hns	I28	AACCTGGAC	329
icdA	I37	GTTTGAAGC	1010
	I107	CTCCAAGCC	418
intQ	I6	GGGTTAAGC	2
mcrA/elbA	I24	GTTCAGAGT	+361[d]
	I44	GGGTTGGAC	+248[d]
moaD	I19	ACCATCCGC	196
mobA	I22	GCCTTACAT	314
recA	I7	GTCCAACAC	563
	I78	GGTGAAGAC	99
recB	I40	GGTATGTGC	1568[e]
	I57	GTTCGATGG	1940
	I68	GGATGTGGT	1922
	I73	GATTTACAC	3470
	I79	GGTATGTGC	1568[e]
	I83	TTCTTGCAC	2868
	I93	ACCAGGCCT	491
	I94	ATGGTGAAC	1365
recC	I32	GATAGCAGT	1119
	I36	CCCGCCACC	1558
	I50	GCTGGGAAT	2039
	I52	GTCTGGACG	34
	I54	ACATGATGG	3241
	I85	GGGTAAACA	699
	I90	GCCTGGCAC	442
	I97	GTTGTTGGG	1601
trkH	I23	TAACTACGG	761
	I27	GATTATCCC	71
tufA	I29	CACGTAGAC	959
tufB	I17	GGCGTAGAG	1047

[a]Alternate gene names: *elbA* (*ycgW*), *intQ* (b1579)

[b]The 9 bp repeat was determined using a single primer from within the transposon. The last base of the inferred repeat shows the last intact nucleotide of the gene, reading in the 5′ to 3′ direction with respect to transcription.

[c]This insertion is located 115 nucleotides upstream of the start codon of the *crp* gene.

[d]These insertions lay 361 and 248 nucleotides downstream of the stop codon of the *mcrA* gene.

[e]These insertions, while identical, were isolated independently and are therefore not clones.