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ORIGINAL ARTICLE

The role of *gyrA*, *gyrB*, and *dnaA* functions in bacterial conjugation

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Abstract The role of DNA gyrase in F'*lac* plasmid conjugation was studied using *Escherichia coli gyrA43* (Ts), *gyrB41*(Ts), and *dnaA46*(Ts) thermosensitive mutants as donor or recipient organisms, and a rifampicin or nalidixic acid-resistant J-53 strain in the presence or absence of nalidixic acid. Mating experiments were also performed employing Hfr derivatives of the thermosensitive strains. Conjugation was carried out in broth for 60 min using a standard method at permissive and non-permissive (32 and 43 °C) temperatures, with or without drugs. At 32 °C, nalidixic acid reduced the number of transconjugants by about 97 % in comparison to the control, while at 43 °C, the drug inhibited F'*lac* transfer by about 98 % from *dnaA46*(Ts) mutant and by about 6.5 % from *gyrA43*(Ts) and 15 % from *gyrB41*(Ts) hosts. Using the temperature-sensitive mutants as recipient strains, the transconjugants found were approximately the same under all conditions. The number of transconjugants did not change significantly when nalidixic acid-resistant strains were used as donor or recipient strains. Lastly, nalidixic acid reduced the number of transconjugants from Hfr selected in the above mutants under all experimental conditions. These findings suggest that F'*lac* transfer does not involve DNA gyrase activity.

Keywords DNA gyrase · Nalidixic acid · Conjugation

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Introduction

DNA gyrase and topoisomerase IV are essential enzymes for bacterial DNA metabolism. DNA gyrase is a tetramer composed of two A and two B subunits that introduces negative superhelical twists into bacterial chromosomes and maintains a particular level of supercoiling. This protein plays a key role in DNA replication processes such as initiation, propagation, and termination. Topoisomerase IV is similarly structured and is composed of *parC* and *parE* subunits; it acts in the terminal stages of DNA replication allowing for the decatenation of daughter chromosomes. DNA gyrase and topoisomerase IV are the targets of 4-quinolones (Khodursky et al. 1995; Blondeau 2004; Drlica et al. 2009; Aldred et al. 2014), a class of antibacterial agents largely employed in therapy since they have been found to be clinically effective (Hooper 2001). Four quinolones and temperature-sensitive mutants in both DNA gyrase and topoisomerase IV subunits have been used for a variety of physiological studies. In particular, chromosome DNA metabolism results in susceptibility to quinolone activity or gene mutation, while extrachromosomal genetic elements appeared more resistant to these drugs or thermal inactivation of gyrase enzymes. It has been reported, in fact, that the growth of phage T7 is inhibited by nalidixic acid, but does not required a functional DNA gyrase activity (Kreuzer and Cozzarelli 1979). In addition, the conjugation process is blocked by quinolones (Barbour 1967; Debbia et al. 1994), and under some experimental conditions, Hooper et al. (1989) found that the non-permissive temperature reduced the transfer of R64*drd-11* by 13-fold from *gyrB41*(Ts) mutant in comparison to that observed in the permissive condition.

The present study was undertaken to investigate the role of DNA gyrase during conjugation in the presence of nalidixic acid under normal conditions and after selective inactivation of *gyrA43*(Ts) and *gyrB41*(Ts) subunits by temperature. In

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comparison, conjugation was carried out under the same experimental conditions, employing *dnaA46*(Ts) and Hfr strain derivatives from the thermosensitive mutants.

Methods

Bacterial strains The microorganisms used in this study were originally obtained from Mary K.B. Berlyn (1998) of the *Escherichia coli* Genetic Stock Center. Strains carrying relevant characteristics for this study were: CTR4610 (*dnaA46*[Ts]) (Hirota *et al*. 1968), KNK453 (*gyrA43*[Ts]) (Kreuzer and Cozzarelli 1979) and N4177 (*gyrB41*[Ts]) (Menzel and Gellert 1983), which had been described previously (Dolcino *et al*. 2002). F'*lac* plasmid (F'128) was one of the F' kit, HfrC (*thiA32*) was used in some experiments as a control strain. All these mutants cultured at 43 °C were found to be defective for functions required for DNA metabolism, but they continued to divide and form non-nucleated cells because the remaining cellular functions were not affected. J-53 (*proB22*, *metF63*) rifampicin-resistant (J53-rif) and J-53 nalidixic acid-resistant (J53-nal) were employed as donor or recipient organisms depending on the experiments. The rich LB medium and minimal medium were those described by Miller (1972). Media were supplemented with thymine (25 mg/L) when necessary. The F'*lac* plasmid carrying transposon Tn*10* which codes for tetracycline resistance was used in mating experiments. Nalidixic acid, rifampin, and tetracycline were obtained from commercial sources (Sigma, Milan, Italy) and stock solutions were prepared following the manufacturer's instructions.

Transfer of F'*lac* from various hosts under different experimental conditions

Conjugation was carried out by standard methods employing $2x10^8$ cells/mL in Mueller-Hinton broth (Willetts 1988). The mating mixtures were incubated for 60 min at permissive and non-permissive temperatures (32 °C and 43 °C) with and without nalidixic acid.

When the experiments were carried out at the non-permissive temperature, the temperature-sensitive mutants were pre-incubated at 43 °C for 15 min before mating to inactivate the thermolabile enzymes. Immediately after mixing donors and recipients, nalidixic acid was added at a concentration of 100 mg/L.

Transconjugants were selected on plates containing rifampin or nalidixic acid (100 mg/L) with tetracycline (15 mg/L). The frequency of recombination was calculated at the end of the experiment, it was expressed as number of transconjugants per number of donors.

Transfer of F'*lac* from *dnaA46* (*Ts*) pre-incubated at 43 °C for the indicated period of time before mating

Mating experiments were carried out as described above, with the exception that donor strains were pre-incubated at 43 °C for 0, 30, and 60 min before mating.

Conjugation between Hfr derivatives of various hosts under different experimental conditions

Hfr strains were selected by F'*lac* integrative suppression in *gyrA43*(Ts), *gyrB41*(Ts), and *dnaA46*(Ts) (Miller 1972). In these Hfr strains at high temperature, chromosome replication and cell division are under the control of the F plasmid. These strains were further tested for susceptibility to rifampin and acridine orange at 43 °C, a condition that provides evidence that cell division is driven by the F mechanism (Nishimura et al. 1971; Bazzicalupo and Tocchini-Valentini 1972). These Hfr were mated with J-53 rif at 43 °C for 90 min with and without nalidixic acid, after this period of time, bacterial cells were centrifuged, suspended in saline solution, and then plated on minimal medium selecting for methionine.

Results

Transfer of F'*lac* from various hosts under different experimental conditions

When the temperature-sensitive mutants were used as donors under permissive conditions (32 °C), F'*lac* was transferred into recipients at a rate that was dependent on the bacterial host considered (Table 1). As expected, under identical experimental situations, nalidixic acid reduced the number of transconjugants from about 85 to 97 % in comparison to the respective counterpart. The same conjugations were then repeated at the non-permissive temperature for the growth of the donor strains. Under these experimental conditions (see Material and Methods) the number of transconjugants registered was not significantly influenced by temperature, irrespective of the donor strain considered. Conversely, when the mating mixtures were exposed to nalidixic acid the number of transconjugants found, in the crosses with *gyrA*(Ts) and *gyrB*(Ts), were about the same order of magnitude as those obtained under the same experimental conditions in the absence of nalidixic acid. A different situation was observed employing *dnaA*(Ts) as the donor strain. F'*lac* transfer was in fact inhibited by the quinolone with the same incidence, regardless of the temperature used.

Similar results to those reported above were found when the thermosensitive mutants were employed as recipient

Table 1 Transfer of F'*lac* from various hosts under different experimental conditions

Donor	Recipient	Nalidixic acid (mg/L)	N. of transconjugants/100 donors after incubation of the mixture at the indicated temperature		
			32 °C	43 °C	*P**
gyrA43(Ts)	J53-rif	0	16±1.2	15±1.1	0.27
		100	0.5±0.08	14±0.6	0.00049
p			0.002	0.21	
gyrB41(Ts)		0	610±45	570±52	0.101
		100	12±2.3	490±54	0.0039
p			0.00189	0.138	
dnaA46(Ts)		0	90±8	70±6.5	0.187
		100	3.0±0.1	1.4±0.4	0.0115
p			0.00281	0.00299	
J53-rif	*gyrA43*(Ts)	0	20±0.6	18±1.2	0.831
		100	11.5±0.2	17±1.1	0.0088
p			0.00184	0.347	
	gyrB41(Ts)	0	150±12.5	140±15	0.20
		100	80±6	130±11.8	0.0044
p			0.0022	0.415	
	dnaA46(Ts)	0	8.3±0.6	13±0.4	0.0060
		100	1.0±0.1	1.2±0.2	0.7
p			0.00272	0.00002	
gyrA43(Ts)	J53-nal	0	50±5.4	30±3.8	0.00212
		100	6.0±0.7	20±0.6	0.00017
p			0.00507	0.04596	
gyrB41(Ts)		0	900±60	750±45	0.033
		100	150±17	600±40	0.0087
p			0.0023	0.01249	
dnaA46(Ts)		0	160±20	130±18	0.015
		100	5.0±0.4	10±0.7	0.012
p			0.00553	0.00743	
J53-nal	*gyrA43*(Ts)	0	50±6	45±4.8	0.018
		100	30±1.2	55±4.5	0.0057
p			0.02981	0.05803	
	gyrB41(Ts)	0	450±52	500±61	0.113
		100	200±30	480±46	0.0012
p			0.00549	0.67376	
	dnaA46(Ts)	0	25±1.5	31±1.8	0.08
		100	7.0±0.6	6.0±0.5	0.03
p			0.003	0.00198	

Values are means±standard deviations of the means for at least five separate experiments

*by paired Student's test

strains (Table 1). In fact, under these experimental conditions, there was inhibition of plasmid transfer by nalidixic acid at the permissive temperature and under all experimental conditions with *dnaA*(Ts), but not at 43 °C with *gyrA*(Ts) and *gyrB*(Ts). The only difference observed in comparison with the previous experiments was in the rate of the reduction of the number of transconjugants caused by nalidixic acid at 32 °C. With gyrase mutants, in fact, there was a reduction in the number of transconjugants by about 50 % in comparison to 97 % found in the previous experiments under similar mating conditions. On the other hand, when *dnaA*Ts mutants were employed as the recipient strain, no significant difference was found in the rate of transconjugants when the mating mixture was exposed to nalidixic acid at the permissive and non-permissive temperatures as observed above.

F'*lac* transfer was also studied employing the thermosensitive mutants as donors and a nalidixic acid resistant derivative J-53 as the recipient strain (Table 1). The results obtained were similar to those registered with J-53 rif. In particular, nalidixic acid affected the efficiency of F'*lac* transfer under experimental conditions permissive for bacterial growth with all the mutant strains. When the same conjugation experiments were carried out at 43 °C there was no significant reduction of the number of transconjugants found in which gyrase mutants served as donor strains, however, the number of survivors were greater than those obtained in similar above mentioned experiments. With the *dnaA*(Ts) donor, nalidixic acid influenced the efficiency of F'*lac* transfer at both temperatures tested, but as observed with the other mutants, the number of viable cells found in the selective media were greater than those registered in the first experiment employing the nalidixic acid susceptible strain as the recipient.

Finally, conjugation experiments in which J-53 nalidixic acid-resistant strains acted as donors and temperature mutants as recipient strains, manifested a similar behavior to that observed in all the above tests with the quinolone affecting the F'*lac* transfer at 32 °C, but not at 43 °C, with the exception of a *dnaA*(Ts) mutant which confirmed its susceptibility to the drug in all the cases considered. Under these last experimental conditions all strains mated about 10-fold more efficiently, especially in the presence of nalidixic acid, than in the other assays.

Transfer of F'*lac* from *dnaA46* (Ts) pre-incubated at 43 °C for the indicated period of time before mating

The dnaA gene product is an essential protein that interacts with the *oriC* site in the *E. coli* chromosome, promoting and regulating a new synthesis of the bacterial genome. In *dnaA*(ts) mutants, the dnaA gene product is inactivated by temperature; therefore, when the strain is incubated at the non-permissive temperature, the DNA replications that are under way complete their chromosome synthesis cycle, but initiation of new genome duplications cannot start. This mutant gives the opportunity to verify the effect of nalidixic acid in strains where DNA gyrase enzymes that are required for DNA metabolism are working and thus can be inhibited, but chromosomal DNA synthesis is halted. Under these conditions there is no chromosome duplication, but conjugative plasmid DNA is synthetized; therefore, it is possible to verify if quinolones affect this duplication and DNA transfer. Table 2 reports the effects of nalidixic acid on the F'*lac* transfer from *dnaA*(Ts) pre-incubated at the non-permissive temperature for different periods of time before adding nalidixic acid. As reported in Table 2, after 60 min at 43 °C, when it is assumed that bacterial DNA metabolism is not working, nalidixic acid exhibited a modest inhibition of F'*lac* transfer in comparison with the control, providing evidence that the quinolone can only affect plasmid transfer when DNA metabolism is going on, irrespective of the presence of a functional DNA gyrase activity.

Conjugation between Hfr derivatives of various hosts under different experimental conditions

The role of DNA gyrase enzymes, as well as dnaA proteins in bacterial conjugation was evaluated in Hfr derivatives of the various thermosensitive mutants. In these Hfr strains at high temperature, chromosome replication and cell division are under control of the F plasmid (Nishimura et al. 1971; Bazzicalupo and Tocchini-Valentini 1972). Acridine orange and rifampin, respectively, inhibit cell division at 43 °C in these strains, suggesting that the F replication system is sensitive to these chemicals. These last properties were also confirmed in the Hfr derivatives selected here. The Hfr strains

Table 2 Transfer of F'*lac* from *dnaA46* (Ts) pre-incubated at 43 °C for the indicated period of time before mating

Donor	Recipient	Pre-incubation (min)	Nalidixic acid (mg/L)	N. of transconjugants/100 donors after incubation of the mixture at the indicated temperature		
				32 °C	43 °C	*p*
dnaA46(Ts)	J53-rif	0	0	12±0.8	13±1.1	0.287
			100	4.0±0.2	1.2±1.1	0.0327
p				0.00352	0.00019	
		30	0	13±1.4	11±0.7	0.38
			100	0.8±0.3	1.4±0.5	0.035
p				0.00456	0.00014	
		60	0	16.5±3.1	12.2±1.8	0.219
			100	1.0±0.4	8.0±0.6	0.00027
p				0.01329	0.05473	

See also Table 1

obtained from thermo-susceptible mutants were then exposed to nalidixic acid. No changes were found in the MIC values of the Hfr strains derivatives from *dnaA*(Ts) mutant at both 30 °C and 42 °C, while those obtained from *gyrA*(Ts) and *gyrB* (Ts) demonstrated an increase in MIC values by 1 to 2 dilutions (data not shown) in comparison to the control at the non-permissive temperature.

They were then used for mating experiments carried out to 43 °C under conditions where the enzymes were inactivated by the temperature. As reported in Table 3, under the experimental conditions employed, all Hfr strains derivatives of the above thermosensitive mutants were able to transfer a chromosomal marker, the addition of nalidixic acid; however, reduced the number of recombinants by about 95 % in all the conjugations. The number of transconjugants registered in these conjugations varied in unpredictable way depending of the donor strain.

Discussion

The present findings demonstrate that the F'*lac* plasmid can be transferred from *gyrA*(Ts), *gyrB*(Ts), and *dnaA*(Ts) at the non-permissive temperature for growth to the recipient bacterial hosts. The addition of nalidixic acid to the mating mixture did not prevent transfer of the F'*lac* plasmid from DNA gyrase mutants, but reduced the number of transconjugants in the conjugations where the *dnaA*(Ts) mutant was involved. In this last strain, plasmid transfer was restored when DNA metabolism was inhibited, in the majority of donors, by temperature for 1 h before mixing together the donor and recipient bacterial cells. The conjugation carried out with Hfr strain derivatives from thermosensitive mutants appeared susceptible to inhibition by nalidixic acid under all the experimental conditions.

Table 3 Conjugation between Hfr derivatives of various hosts under different experimental conditions

Hfr donor derivative	Recipient	Nalidixic acid (mg/L)	N. of transconjugants/100 donors after incubation of the mixture at 37 °C
gyrA43(Ts)	J53-rif	0	$(59.1\pm2.9)\times10^{-3}$
		100	$(0.78\pm0.14)\times10^{-3}$
p			0.00090
gyrB41(Ts)		0	0.8±0.16
		100	0.014±0.003
p			0.014
dnaA46(Ts)		0	2.9±0.26
		100	0.16±0.03
p			0.0023
HfrC		0	$(14.8\pm0.77)\times10^{-3}$
		100	$(0.32\pm0.04)\times10^{-3}$
			0.00085

See also Table 1

In an attempt to explain all the present results some considerations and hypotheses can be argued. Under the experimental conditions employed here, bacterial DNA replication appears to be the most important physiological situation that influences F'*lac* transfer in the presence of nalidixic acid. In DNA gyrase thermosensitive strains, incubation at the non-permissive temperature rapidly arrests DNA synthesis (Kreuzer and Cozzarelli 1979; Menzel and Gellert 1983). In these last strains, when DNA replication is not in progress, the only biological function that is working is conjugative DNA synthesis, but, as reported here, nalidixic acid had no effect on the plasmid transfer process; therefore, in this particular single strand DNA synthesis required for the transfer of genetic material there is no enzyme or other protein that can interact with the quinolone inhibiting conjugation (Willetts and Wilkins 1984).

A similar experimental condition was obtained after the *dnaA*(Ts) mutant was incubated for 1 h at 43 °C before mating. After this period of time the great majority of the bacterial population had completed the residual cycle of DNA duplication and F'*lac* transfer was no longer influenced by nalidixic acid.

Conjugation is a process that occurs in many different situations; donors may be non-viable bacteria, mini-cell-like elements, starved bacterial cells suspended in saline solution, and in any of these conditions the presence of an antibiotic, particularly quinolone, does not seem to affect the process (Debbia 1992; Debbia et al. 1994). Under all these last conditions, the only determinant factor is the absence of chromosomal DNA synthesis. Thus, quinolones that interfere with DNA gyrase enzymes block the conjugation process only if bacterial cells are viable and the chromosome is duplicating. All these observations support the hypothesis that F'*lac* transfer does not require a functional DNA gyrase activity and suggest that inhibition of plasmid transfer by quinolones may be a consequence of the interaction of these molecules with other cellular targets.

The results obtained in the conjugation experiments performed with the Hfr strain derivatives from the F'*lac* integrative suppression in *gyrA43*(Ts), *gyrB41*(Ts), and *dnaA46*(Ts) appear to be in apparent contrast with this interpretation. In these experiments nalidixic acid seems to affect the process of conjugation, reducing the number of transconjugants. While for the Hfr derivative of *dnaA*(Ts), the inhibition of DNA transfer is justified by the presence of quinolone targets, such as DNA gyrase, in the other Hfr strains obtained from the thermosensitive mutants in the topoisomerase enzymes, the target of nalidixic acid might be inactivated by temperature, and therefore, no antibacterial activity is expected. However, nalidixic acid is known to interact not only with DNA gyrase

enzymes, but also with topoisomerase IV (*parC*). Therefore, a simple explanation of these results is that when DNA gyrase is inactivated by temperature, the quinolone focuses its activity on topoisomerase IV enzymes blocking the conjugation process (Khodursky et al. 1995).

As reported and demonstrated in this report, the direct involvement of DNA gyrase in bacterial conjugation seems to be excluded, rather there is a strong indication that the physiological perturbations induced by the interaction of nalidixic acid with topoisomerases is the main factor that affects bacterial DNA transfer. In fact, this interaction of the drug with the enzymes is responsible for DNA damage that involves the activation of DNA repair mechanisms or the SOS system. During this period of time, cell division and many other bacterial functions are blocked until the damage is repaired. With this in mind, it has been hypothesized that conjugation cannot occur because the SOS mechanism, induced by nalidixic acid, blocks conjugative DNA synthesis since all DNA inside the bacterial cell requires repair.

A second more intriguing possibility is that when DNA metabolism is working, an antibiotic might induce metabolic perturbations that generate reactive oxidants including reactive oxygen species (ROS), which contribute to cell death. Thus, it is sufficient that chromosome synthesis occurs to mobilize the pool of enzymes required for this metabolism including those of the ROS components. The fact that the ROS-mediated DNA double-strand breaks is enough for SOS induction with the consequent block of any DNA synthesis (Dwyer et al. 2015). This is a condition where conjugation cannot take place.

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