Characterization of high frequency of recombination strains selected by integrative suppression of F'*lac* in *dnaA*, *gyrA* and *gyrB* temperature sensitive mutants

Eugenio A. Debbia,[1] Anna Marchese[1,2]

[1]"C.A. Romanzi" Section of Microbiology, School of Medical and Pharmaceutical Sciences, Department of Surgical Sciences and Integrated Diagnostics, University of Genoa; [2]Microbiological Unit, San Martino Polyclinic Hospital, Genoa, Italy

Abstract

Integration of F'*lac* plasmid into chromosome of both *gyrA*(Ts) and *gyrB*(Ts) cells phenotypically suppress the thermo-sensitive mutations of the DNA gyrase enzymes. As the comparative strains isolated from *dnaA*(Ts), these high frequency of recombination (Hfr) derivatives were able to transfer chromosomal markers to recipient strains, showed a growth rate of about 60 min, and developed filamentous forms when incubated at the temperature of 43°C. Conversely to *dnaA*(Ts) Hfr selected isolates, the great majority of Hfr derivative of gyrase mutants resulted resistant to acridine orange and rifampin. Time-kill experiments carried out at the non-permissive temperature also revealed that nalidixic acid has no antibacterial activity on these Hfr strains while derivatives of *dnaA*(Ts) mutant, as well as the control strain HfrH, were strongly inhibited by this drug. Therefore F plasmid induced duplication of chromosome in the mutants even if the DNA gyrase enzymes are not working. Of a certain interest is that these bacteria exhibit physiological perturbations that affect the main cellular functions, however, they do not appear essential for the survival of the strains.

Correspondence: Eugenio A. Debbia, "C.A. Romanzi" Section of Microbiology, School of Medical and Pharmaceutical Sciences, Department of Surgical Sciences and Integrated Diagnostics, University of Genoa, Largo Rosanna Benzi 10, 16132 Genoa, Italy.
Tel: +39-010-353 38136 - Fax: +39-010-353 7651.
E-mail: eugenio.debbia@unige.it

Key words: F integrative suppression; DNA gyrase; Thermo-resistant; Nalidixic acid-resistant.

Received for publication: 23 January 2017.
Revision received: 13 April 2017.
Accepted for publication: 5 May 2017.

Journal of Biological Research 2017; 90:6595
doi:10.4081/jbr.2017.6595

Introduction

Integration of F or other conjugative plasmids such as R1, R100 or ColV2, into the chromosome of a *dnaA*(Ts) mutant generates Hfr clones which show the concomitant ability of growing at the restrictive temperature.[1] In these Hfr derivatives, at high temperature (43°C), chromosome and cell division are under the control of the F replicative functions, because the plasmid origin replication site replaces that of the chromosome. It has been assumed that this latter genome does not require or utilizes the residual part of the DnaA protein for its duplication. Nishimura *et al.*[2] and Tocchini-Valentini[3] have found that both acridine orange and rifampin inhibit cell division at 43°C in *dnaA*(Ts) derivative Hfr strains, providing evidence that the F replicative system is sensitive to these drugs. It has been suggested that F-messenger synthesis, RNA primer formations or other unidentified plasmid functions are the preferential targets of rifampicin.[3] Some classes of rifampin-resistant mutants have also been shown to be capable of suppressing a given *dnaA*(Ts) allele of *Escherichia coli* and *Salmonella typhimurium* as well as *gyrB*(Ts) phenotypes.[4,5] It appears therefore that some specific altered RNA-polymerases or the rifampicin-susceptible F plasmid functions play a role in the growth of these strains at the non-permissive temperature. Since the products of *dnaA, gyrA* and *gyrB* genes are required in the first steps of the chromosome duplication, and the regulation of cell division in *dnaA*(Ts) is under the control of the integrated F plasmid, this phenomenon suggested of placing *gyrA*(Ts) and *gyrB*(Ts) cells under the control of F at the restrictive temperature. In a previous study,[6] already, some Hfr strains were selected from all the thermo-sensitive mutants, but only few strains were tested and chosen on the basis of their ability to grow at the restrictive temperature, and the susceptibility to rifampin and acridine orange. These criteria have been suggested by the main traits that are exhibited by Hfr derivatives of *dnaA*(Ts) mutant. Any other property of these microorganisms was not explored in detail. Whether F replication system may compensate the thermo-sensitivity of DNA gyrase mutants, in fact, has never been evaluated. Therefore the present study was aimed at the selection of thermo-resistant clones by integrative suppression in F'*lac* carrying both *gyrA*(Ts) and *gyrB*(Ts) cells. The characterization of the Hfr derivatives for their donor ability and susceptibility to acridine orange, rifampin and nalidixic acid was also performed.

Materials and Methods

Bacterial strains

Bacterial strains used in this study are listed in Table 1. The microorganisms used were originally obtained from Mary K.B. Berlyn[7] of the *E.coli* Genetic Stock Center. Strains carrying relevant characteristics for this study were: CTR4610 (*dnaA46*[Ts]),[8] KNK453 (*gyrA43*[Ts])[9] and N4177 (*gyrB41*[Ts]).[10] They were previously described.[6] All these mutants cultured at 43°C resulted defective for functions required for DNA metabolism, but they continued to divide and form non-nucleated cells because the remaining cellular functions are not affected. Plasmid F'*lac* (F'128) was one of the F' kit. F'*lac* plasmid carrying transposon Tn*10* that codes for tetracycline resistance was used for strain construction. J-53 and AB1157 were employed as recipient organisms depending on the experiments. *E. coli* ATCC 25922, was used as quality control strains, when necessary.

Media and reagents

The rich medium LB and the minimal medium were those described by Miller.[11] Agar plates for testing acridine orange were adjusted to pH 7,6 with 1 N NaOH. Log phase bacterial cultures were streaked on the agar plates with a loop. Media were supplemented with thymine (25 mg/L) when necessary. Acridine orange, nalidixic acid, rifampin, and tetracycline were obtained from commercial sources (Sigma, Milan, Italy) and stock solutions were prepared following manufacturer's instructions.

Selection of high frequency of recombination strains by integrative suppression

Hfr strains were selected by F'*lac* integrative suppression in *gyrA43(*Ts), *gyrB41*(Ts) and *dnaA46*(Ts).[11] 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 F mechanism in *dnaA46*(Ts).[2,3]

Conjugation of putative high frequency of recombination with the appropriate recipient

Conjugation was carried out by standard methods employing $2x10^8$ cells/mL in Mueller-Hinton broth.[12] The cells were incubated for 90 minutes at permissive temperature (32°C) washed in saline and plated on minimal media.

Dynamic bactericidal activity

Time-kill experiments were performed by adding the drug at concentrations indicated in the results section, to log-phase bacterial cultures diluted to 10^6-10^7 CFU/mL growing in 500 mL flasks at 43°C. Just before the compounds were added (zero time) and at 2, 6 and 24h thereafter, bacterial counts were carried out. Survivors were evaluated by determining CFU on agar plates. Colonies were counted after 48h of incubation at 35°C.

Results

Selection of high frequency of recombination strains by integrative suppression

Spontaneous temperature-resistant revertants arose with a frequency which ranged from $3.1X10^{-8}$ to $4.5X10^{-8}$ in *gyrA*(Ts) and *gyrB*(Ts) cells respectively. When the same experiments were performed with F'*lac* carrying derivatives the incidence of clones growing at the restrictive temperature increased to $1.3X10^{-7}$ for *gyrA*(Ts) and $7.1X10^{-7}$ for *gyrB*(Ts). In comparative tests carried out with a *dnaA*(Ts) mutant the frequency increased from about $3X10^{-6}$ to $9X10^{-5}$ (Table 2). Among the colonies found at 43°C, 10 random chosen strains of each different experiment were characterized for their susceptibility to acridine orange and rifampin at permissive and non permissive temperature (Table 3). As expected the *dnaA*(Ts) derivatives resulted susceptible to both drugs at the restrictive temperature. When the same experiments were performed with the Hfr derivatives from *gyrA*(Ts) and *gyrB*(Ts), the number of susceptible strains to acridine orange and rifampin varied in an unpredictable way, in particular, the experiments were repeated several times and thermo-resistant clones were selected in separate experiments but the number of susceptible strains to the compounds ranged from 3 to 38% of the isolates. Regardless this different behavior found with *dnaA*(Ts) derivatives and the thermo-resistant obtained with *gyrA*(Ts) and *gyrB*(Ts), all the isolates were considered putative Hfr and were therefore further characterized.

Table 1. List of Escherichia coli strains used in this study.

Strain	Main characteristics	Source or reference
CTR4610	*dnaA46(Ts)*, *leu*, *thy*, *thi*	Marchese and Debbia (2016)[6]
db1983	Hfr from *dnaA46*(Ts)	This work
KNK453	HF4704 *gyrA43*(Ts), *thyA*, *polA*, *uvrA*, *phx*	Marchese and Debbia (2016)[6]
db1873	Hfr from *gyrA43*(Ts), AO-s, Rif-s	This work
db1874	Hfr from *gyrA43*(Ts), AO-r, Rif-r	This work
N4177	*galK*, *gyrBII*(Ts), cou-r, Sm-r	Marchese and Debbia (2016)[6]
db1607	Hfr from *gyrB41*(Ts) AO-s, Rif-s	This work
db1654	Hfr from *gyrB41*(Ts) AO-r, Rif-r	This work
J-53	*proB22*, *metF63*	Laboratory collection
J-53 (F*lac*::Tn10)	*proB22*, *metF63*, Tn*10*, Tet-r	Laboratory collection
HfrH	prototroph	Laboratory collection
AB1157	*thr-1*, *leuB6*, *proA2*, *argE3*, *his-4*, *thi-1*, *lac Y1*, *galK2*, *rpsL31*	Marchese and Debbia (2016)[6]

AO, acridine orange; Rif, rifampicin; Cou, coumermycin; Sm, streptomycin; Tet, tetracycline; s, susceptible; r, resistant.

Conjugation of putative high frequency of recombination with the appropriate recipient

The putative Hfr isolates were then mated with an appropriated recipient and the results are reported in Table 4. In detail, Hfr strains derivative of *gyrA*(ts) were crossed with AB1157 selecting for histidine recombinants at 32°C. At least 50 colonies for each experiment were then analyzed for their ability of growing at 43°C. From 12 to 53% of the clones resulted thermo-sensitive. A further experiment was carried out using a spontaneous nalidixic acid resistant of AB1157. After selection for histidine recombinants the strain were also tested for their susceptibility to nalidixic acid, all isolates that lost the resistance to this drug resulted susceptible to 43°C. Similar results were obtained employing Hfr *gyrB*(Ts) derivative. In this case the recipient strain was J-53 and selection was for metionine. The metionine positive isolates found demonstrated a temperature sensitive trait from 33 to 50% of the cases. All the clones that inherited the temperature-sensitivity trait manifested also the coumermycin-resistance marker, in contrast, all the strains that retained the thermoresistance of the recipient exhibited susceptibility to this coumarine. These results provided evidence that donor strains retained the *gyrB*(Ts) mutation. In comparative experiments carried out with Hfr strains selected by integrative suppression in a *dnaA*(Ts) cells, the isolates found susceptibility to the temperature ranged from 23 to 56% of the cases. There was no particular difference in the number of recombinants obtained with rifampin and acridine orange resistant or susceptible Hfr (Table 4).

Growth rate and morphology of the high frequency of recombination strains

The generation times calculated for all the cultures of the Hfr strains were approximately ranged from 55 to 65 min, while the control strain showed a generation time of about 26 min. In Figure 1 are shown the shape of the Hfr derivative and the control strain incubat-

Table 2. Incidence of thermoresistant strains found in *dnaA*(Ts), *gyrA*(Ts) and *gyrB*(Ts) mutants with and without F'*lac* incubated at 43°C.

Strain	Incidence of thermo-resistant strains found
dnaA(Ts)	3×10^{-6}
dnaA(Ts)F'*lac*	9×10^{-5}
gyrA(Ts)	3.1×10^{-8}
gyrA(Ts)F'*lac*	1.3×10^{-7}
gyrB(Ts)	4.5×10^{-8}
gyrB(Ts)F'*lac*	7.1×10^{-7}

Mean of at least 3 separate experiments.

Table 3. Susceptibility to acridine orange and rifampicin.

Hfr derivative	32°C Control	32°C AO (20mg/L)	32°C Rif (1mg/L)	43°C Control	43°C AO (20mg/L)	43°C Rif (1mg/L)
dnaA(Ts)						
db1983	+	+	+	+	-	-
gyrA(Ts)						
db1873	+	+	+	+	-	-
db1874	+	+	+	+	+	+
gyrB(Ts)						
db1607	+	+	+	+	-	-
db1654	+	+	+	+	+	+
ATCC25922	+	+	+	+	+	+
HfrH	+	+	+	+	+	+

AO, acridine orange; Rif, rifampicin. Ten isolates randomly selected for at least 5 separate experiments by integrative suppression in a *dnaA46*(Ts), *gyrA*(Ts) and *gyrB*(Ts) mutant.

Table 4. Transfer of the temperature-sensitive marker from the high frequency of recombination derivatives of *dnaA*(Ts), *gyrA*(Ts) and *gyrB*(Ts) to J-53 or AB1157.

Hfr donor	Recipient strain	Selected marker	Range of incidence of recombination	Range of thermo-susceptibility (%)
dnaA(Ts)	J-53	Metionine		
db1983			2.4×10^{-5}-1.3×10^{-6}	23-54
gyrA(Ts)	AB1157	Histidine		
db1873			4.6×10^{-6}-1.6×10^{-7}	12-53
db1874			2.1×10^{-6}-4.1×10^{-7}	17-46
gyrB(Ts)	J-53	Metionine		
db1607			1.4×10^{-6}-3.2×10^{-6}	37-51
db1654			2.4×10^{-7}-1.6×10^{-7}	26-44

Hfr, high frequency of recombination. Representative of at least 5 random selected donor for each group.

ed at the non permissive temperature. The microscopic observation of the culture revealed the presence of elongated form of all strain derivative by the thermo-sensitive mutants in comparison with the control HfrH. Donor ability of chromosomal markers and in particular for the transfer of the original thermo-sensitive defect which should be present in the putative Hfr strains, produce physiological perturbations in these strains manifested by the growth rate and the morphological alterations.

Bactericidal activity of nalidixic acid against high frequency of recombination strains

The inactivation of the DNA gyrase enzyme at the restrictive temperature prompt us to evaluated in the Hfr strains selected the activity of nalidixic acid an antibiotic which interferes with the activity of these enzymes. The strains studied HfrH, and the Hfr strains derivative of *dnaA*(Ts), *gyrA*(Ts) and *gyrB*(Ts) were cultured in broth at 43°C ad exposed to different concentrations of nalidixic acid (0, 25, 50, 100 mg/L). These time-kill experiments demonstrated a strong bactericidal effect against the control strain HfrH, a similar lethal effect on Hfr *dnaA*(Ts) derivative, while both Hfr strains selected in *gyrA* and *gyrB* mutants grew in all the culture exposed to the drug also after 24 hours of exposure (Figure 2). These experiments were carried out with Hfr strains that showed susceptibility and resistance to both acridine orange and rifampin and similar results were obtained.

Discussion and Conclusions

Integration of F'*lac* plasmid into chromosome of both *gyrA*(Ts) and *gyrB*(Ts) cells was shown here to phenotypically suppress the thermo-sensitive mutations of the DNA gyrase enzymes. Similarly to the comparative strains derived from *dnaA*(Ts), these Hfr derivatives transferred chromosomal markers to recipient strains, showed a growth rate of about 60 min, and developed filamentous forms when incubated at the temperature of 43°C. On the contrary to *dnaA*(Ts) Hfr selected isolates, the great majority of Hfr derivative of DNA gyrase mutants resulted resistant to acridine orange and rifampin. Time-kill experiments carried out at the non-permissive temperature also revealed that nalidixic acid have no antibacterial activity against these Hfr strains while derivatives of *dnaA*(Ts) mutant as well as the control strain HfrH were strongly inhibited by this antibiotic.

On the basis of the present findings, it should be underlined that the Hfr strains selected from *gyrA(*Ts) and *gyrB*(Ts) mutants differ significantly from those isolated from *dnaA*(Ts). The first difference is the susceptibility to acridine orange and rifampin of these latter strains in comparison with those obtained in DNA gyrase mutants where the resistance to these compounds is exhibited by the great majority of the bacterial cells, and varies in an unpredictable way. The other difference is the resistance to nalidixic acid at the non-permis-

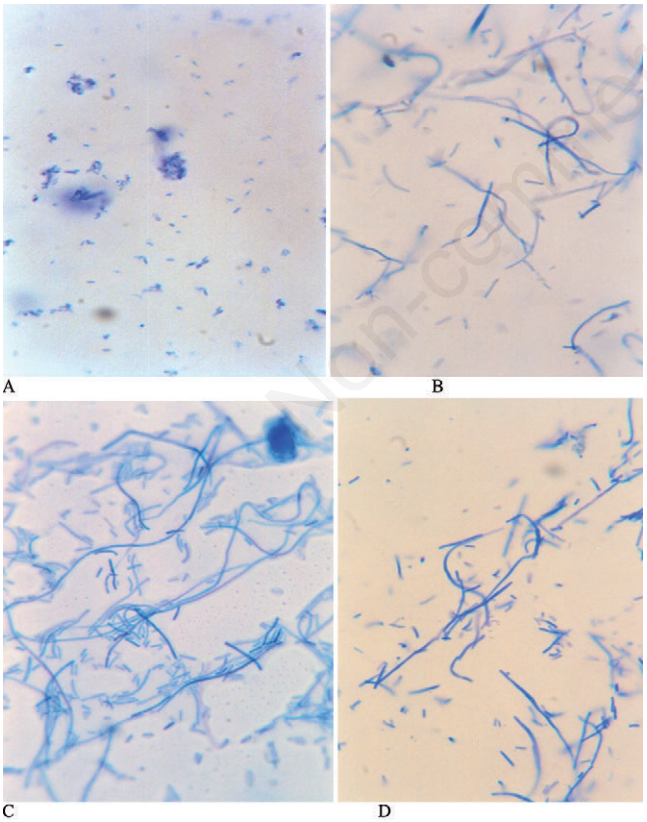

Figure 1. Shape of the high frequency of recombination derivatives and the control incubated at restrictive temperature (43°C) regardless of their susceptibility or resistance to acridine orange or rifampicin. A=HfrH; B=*dnaA*(Ts); C=*gyrA*(Ts); D=*gyrB*(Ts).

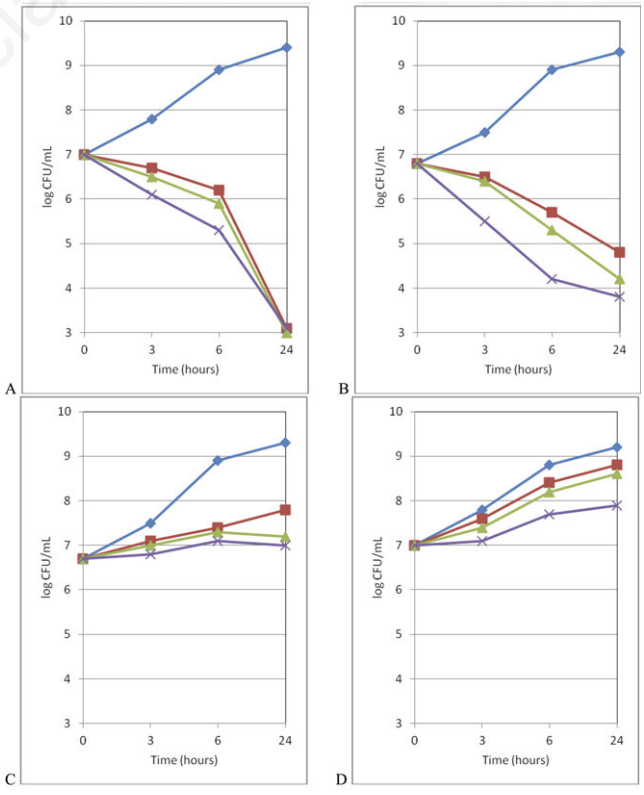

Figure 2. Dinamic bactericidal activity of nalidixic acid against high frequency of recombination derivative of A=control HfrH; B=*dnaA*(Ts); C=*gyrA*(Ts), and D=*gyrB*(Ts). ◆control; ■25 mg/L; ▲50 mg/L; ×100 mg/L. Representative of 3 different isolates regardless of their susceptibility or resistance to acridine orange and rifampicin.

sive temperature shown by the Hfr selected from the DNA gyrase mutant but not the other strains. F replicative functions are employed to duplicate chromosome in both of these cells. Although there is no evidence here in order to recognize specific F-coded proteins involved in the above phenomenon one can hypothesized an important role for RNA-polymerase because of the following reasons. Rifampin has shown to affect F replication and maintenance at a concentration too low to prevent bacterial growth suggesting a specific target among the F-messanger synthesis. On the other hand, as reviewed by Lane,[13] F plasmid possesses two independent replicative system called f5 and f7 from the restriction fragments which contained the sequence of genes responsible of the primary and secondary replication system of F respectively. The f7 functions are resistant to acridine orange as well as to rifampicin, while the primary (f5) does not. Therefore, it is reasonable to assume that at the restrictive temperature the f7 system is responsible of the growth of the *gyrA*(Ts) and *gyrB*(Ts) Hfr derivative strains. It should be considered that in *dnaA*(Ts) mutants, at the restrictive temperature, the DnaA protein is not working but all the enzymatic pool of the strains is completely operative. Therefore when the integrated F promotes its own DNA synthesis, the chromosome is involved, and all the bacterial functions result operative including DNA gyrase enzymes that can be inhibited by nalidixic acid. On the contrary, when DNA gyrase proteins are inactivated by the temperature all the cellular functions should be compromised because DNA gyrase enzymes modulate global gene expression and are of paramount importance for the physiological activity of the bacterial cell.[14,15] The alteration of DNA topology in particular, affect not only the duplication of the chromosome but transcription and gene expression. Although it has been reported that certain thermo-sensitive mutations in *gyrA* or *gyrB* genes are compensated by the enzyme produced by *topA* gene and *vice versa*,[16-19] the present findings seem to exclude any attenuation or compensatory activity that restore at least in part these functions in the Hfr studied. In particular, growth rate morphological alteration and especially the resistance to nalidixic acid suggest that DNA gyrase enzymes are not working in these bacterial cells. It is worth to underline that in spite of these severe defective lesions exhibited by these Hfr strains they were able to survive. The integration of the F factor into the chromosome supplies the power to start the duplication of the bacterial genome. Previous work from this laboratory, however, showed the *F'lac* replication is not affected by nalidixic acid or ciprofloxacin at a concentration many times greater than that required to inhibit bacterial cell growth or promoting plasmid loss.[20] These results suggest an interesting possibility that DNA gyrase enzymes should not be required for F'*lac* replication and for the Hfr derivative of DNA gyrase mutants selected here. Under a certain point of view, when the episome integrates into the genome it transforms the chromosome in a *mega-F-prime* or a mega plasmid, a replicon which expresses all its own genetic information for its autonomous replication without taking into account many enzymes or proteins coded by the bacterial DNA.

References

1. McMacken R, Silver L, Georgopoulos C. *Escherichia coli* and *Salmonella typhimurium*. Cell Mol Biol 1987;1:565-612.
2. Nishimura Y, Caro L, Berg CM, et al. Chromosome replication in *Escherichia coli*, IV. Control of chromosome replication and cell division by an integrated episome. J Mol Biol 1971;55:441-56.
3. Bazzicalupo P, Tocchini-Valentini GP. Curing of an *Escherichia coli* episome by rifampicin. P Natl Acad Sci USA 1972;69:298-300.
4. Bagdasarian MM, Izakowska M, Bagdasarian M. Suppression of the *dnaA* phenotype by mutation in the *rpoB* cistron of ribonucleic acid polymerase in *Salmonella typhimurium* and *Escherichia coli*. J Bacteriol 1977;130:577-82.
5. Filutowicz M, Jonczyk P. The *gyrB* gene product functions in both initiation and chain polymerization of *Escherichia coli* chromosome replication: suppression of the initiation deficency in *gyrB*(Ts) mutants by a class of *rpoB* mutations. Mol Gen Genet 1983;191:282-7.
6. Marchese A, Debbia EA. The role of *gyrA*, *gyrB*, and *dnaA* functions in bacterial conjugation Ann Microbiol 2016;66:223-8.
7. Berlyn MKB. Linkage map of *Escherichia coli* K12, edition 10: the traditional map. Microbiol Mol Biol Rev 1998;62:814-94.
8. Hirota Y, Jacob F, Ryter A, et al. On the process of cellular division in *Escherichia coli* I: asymmetrical cell division and production of deoxyribonucleic acid-less bacteria. J Mol Biol 1968;35:175-92.
9. Kreuzer KN, Cozzarelli NR. *Escherichia coli* mutants thermosensitive for deoxyribonucleic acid gyrase subunit A: effects on deoxyribonucleic acid replication, transcription, and bacteriophage growth. J Bacteriol 1979;140:424-35.
10. Menzel R, Gellert M. Regulation of the genes of *E. coli* DNA gyrase: homeostatic control of DNA supercoiling. Cell 1983;34:105-13.
11. Miller JH. Experiments in molecular genetics. New York, NY: Cold Spring Harbor; 1972.
12. Willetts N. Conjugation. In: Bennett PM, Grinsted J (eds.) Methods in microbiology. London: Academic; 1988. pp. 49-77.
13. Lane HED. Replication and incompatibility of F and plasmids in the IncFI group. Plasmid 1981;5:100-26.
14. Drlica K, Hiasa H, Kerns R, et al. Quinolones: Action and resistance updated. Curr Top Med Chem 2009;9:981-98.
15. Aldred KJ, Kerns RJ, Osheroff N. Mechanism of quinolone action and resistance. Biochemistry 2014;53:1565-74.
16. Usongo V, Drolet M. Role of type 1A topoisomerases in genome maintenance in *Escherichia coli*. PloS Genet 2014:10: e1004543.
17. Heddler JG, Lu T, Zhao X, et al. gyrB-225, a mutation of DNA gyrase that compensate for topoisomerase I deficiency: investigation of its low activity and quinolone hypersensitivity. J Mol Biol 2001;309:1219-31.
18. Reckinger AR, Kyeong SJ, Arkady B. Khodursky1 and Hiroshi Hiasa RecA can stimulate the relaxation activity of topoisomerase I: Molecular basis of topoisomerase-mediated genome-wide transcriptional responses in *Escherichia coli*. Nucl Acid Res 2007;35:79-86.
19. Mitscher LA. Bacterial topoisomerase inhibitors: quinolone and pyridone antibacterial agents. Chem Rev 2005;105:559-92.
20. Debbia EA. Filamentation promotes F'*lac* loss in *Escherichia coli* K12. J Gen Microbiol 1992;138:2083-91.