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Analysis of the Ciprofloxacin-Induced Mutations in *Salmonella typhimurium*

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The mutagenic events induced by ciprofloxacin, a potent antimicrobial agent, have been characterized. For this, a battery of His⁻ mutants of *Salmonella typhimurium* (*hisG428, hisG46, hisC9070,* and *hisG1775* targets) that detects the six possible transitions and transversions [Levin and Ames (1986): Environ Mutagen 8:9–28] and two additional His⁻ strains (*hisC3076* and *hisD3052* targets) carrying frameshift mutations have been used. Our results indicate that GC→TA transversions are the major base-pair substitution induced by ciprofloxacin and that GC→AT transitions are also produced, but to a lesser degree. However, we cannot discard the fact that AT→TA transversions are also induced. In addition, the data indicate that the mutational specificity of ciprofloxacin depends on the location of the target. Intragenic base-pair substitutions are the most frequent mutations at the *hisG428* target when it is on the chromosome, whereas 3 or 6 base-pair deletions are the major mutagenic events when this target is on the plasmid pAQ1. We have shown that ciprofloxacin also induces deletions/insertions at the *hisC3076* and *hisD3052* frameshift targets. Therefore, this inhibitor of DNA gyrase promotes a wide pattern of mutations including different kinds of base-pair substitutions, 3 or 6 base-pair deletions, and insertions/deletions resulting in frameshifts. All of these mutagenic events require the MucAB proteins involved in the error-prone repair, with the exception of base-pair insertions/deletions at the *hisD3052* target, which are independent of the presence of plasmid pKM101.

Key words: ciprofloxacin, mutational specificity, base-pair substitutions, deletions, frameshifts

INTRODUCTION

4-Quinolone antimicrobial agents (4-Qs) are known to be inhibitors of DNA gyrase, showing interesting clinical properties [for review, see Hooper and Wolfson, 1993]. These compounds exhibit a clear genotoxic activity in bacteria, in that they are strong inducers of the SOS system of *E. coli* [Gudas and Pardee, 1975; Phillips et al., 1987; Piddock and Wise, 1987; Ysern et al., 1990]. In contrast to many DNA-damaging agents whose target is the DNA itself, 4-Qs appear to exert their genotoxic action through binding to the gyrase-DNA complex, stabilizing it, and preventing the enzyme turnover [Shen, 1993; Yoshida et al., 1993]. This complex is termed a "cleavable complex," because, on the addition of a protein denaturant, it yields a double-stranded break in DNA [Reece and Maxwell, 1991; Shen, 1993]. Mutagenesis induced by 4-Qs has been shown in several prokaryotic assays, but the most clear results have been found with the Salmonella mutagenicity test. Thus, it has been reported that 4-Qs (e.g., nalidixic and oxolinic acids) induce deletions in the plasmidic *hisG428* target [Levin et al., 1984]. Also, we have shown that nalidixic acid and fluoroquinolones (ciprofloxacin, enoxacin, and ofloxacin) revert the plasmidic *hisG428* strain TA103, despite the fact that they are nonmutagenic in other Salmonella tester strains [Ysern et al., 1990]. More recently, it has been shown that fluoroquinolones produce a significant increase in reversion of both chromosomal *hisG428* and *hisG46* strains when the tester strains are Uvr⁺ and are carrying the plasmid pKM101 [Clerch et al., 1992]. Likewise, reversion of the *hisD3052* allele by the fluoroquinolone fleroxacin depends on a functional Uvr excision repair system [Gocke, 1991]. These findings have allowed us to use an appropriate battery of His⁻ mutants of *S. typhimurium* designed to study the mutagenic specificity of chemicals [Levin and Ames, 1986] and two His⁻ mutants of *S. typhimurium* that detect frameshift mutations. In this work, we present the characterization of the different mutagenic events induced by the potent fluoroquinolone ciprofloxacin in *Salmonella typhimurium.*

MATERIALS AND METHODS

Bacterial Strains and Bacteriophage

The bacterial strains that were used in this work are listed in Table I. P22 *int4*HT and ø1 bacteriophage and ø262, ø266, and ø491 nonsense

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TABLE I. Strains of *Salmonella typhimurium* Used in This Work

Strain	Genotype	Revertible target	Source or reference
DB7155	*leuA-am414 hisC-am527 supE20*		Winston et al., 1979
DB7302	*hisC527 leu414 supG50*		Winston et al., 1979
DB7303	*his*C527 leu414 supC80		Winston et al., 1979
DB7304	*hisC527 leu414 tyrU90 (supM)*		Winston et al., 1979
hisC3076	*hisC3076*	*hisC3076*	Ames et al., 1975
hisD3052	*hisD3052*	*hisD3052*	Ames et al., 1975
hisG46	*hisG64*	*hisG46*	Ames et al., 1973
hisG428	*hisG428*	*hisG428*	Hartman et al., 1971
TA92	*hisG46*/pKM101	*hisG46*	Ames et al., 1975
TA94	*hisD3052*/pKM101	*hisD3052*	Ames et al., 1975
TA103	*hisG428*/pKM101	*hisG428*	Levin et al., 1982
TA2425	*hisC3076*/pKM101	*hisC3076*	Ames et al., 1975
TA2661	*his*Δ *(G) 8476*/pAQ1	*hisG428*	Levin et al., 1982
TA2662	*his*Δ *(G) 8476*/pAQ1/pKM101	*hisG428*	Levin et al., 1982
TA2665	*his*Δ *(G) 8476*		Levin et al., 1982
TA3661	*hisG428 supB532*		Levin and Ames, 1986
TA4011	*hisG1775*	*hisG1775*	Levin and Ames, 1986
TA4011/pKM101	*hisG1775*/pKM101	*hisG1775*	This work
TA4016	*hisC9070*	*hisC9070*	Levin and Ames, 1986
TA4016/pKM101	*hisC9070*/pKM101	*hisC9070*	This work
TT2385	*zii-614*::Tn10 *hisT1529 his01242 hisG6608*		J. Roth
TT6577	*zbh-1009*::Tn10		J. Roth
TT7613	*zbf-98*::Tn10		J. Roth
TT7617	*zde-605*::Tn10		J. Roth

mutants of P22 [Levin and Ames, 1986] were kindly provided by B.N. Ames (University of California, Berkeley, CA). Lysates of all these bacteriophage were obtained in strain DB7155.

Mutagenicity Assays

Mutagenicity assays with ciprofloxacin were performed according to the standard method described by Maron and Ames [1983]. The plasmid pKM101 was introduced into strains TA4011 and TA4016 by conjugation. TA4011 and TA4016 and their conjugative derivatives were used to study the reversion of *hisG1775* and *hisC9070* mutations, respectively. In all mutagenicity assays, negative and positive controls were included. Ciprofloxacin was purchased from Bayer S.A (Barcelona, Spain).

Isolation and Characterization of *hisG46* and *hisG428* Revertants

A minimum of 100 histidine-independent spontaneous or ciprofloxacin-induced revertants of TA2662, TA103, and TA92 strains were purified on minimal glucose medium. The analysis of *hisG428* and *hisG46* revertants was performed basically as described [Levin and Ames, 1986]. First, *hisG428* revertants were characterized phenotypically as deletions, point mutations, or extragenic ochre suppressors, and *hisG46* revertants were characterized phenotypically as point mutations or extragenic suppressors by their sensitivity to both histidine analogs thiazole alanine and triazole alanine (both chemicals were from Sigma, St. Louis, MO). Also, *hisG428* and *hisG46* revertants were characterized genetically to identify extragenic suppressor mutants by their ability to transfer histidine independence to the recipient strains used in transduction experiments with P22*int4*HT [Levin and Ames, 1986]. All of these experiments were performed in duplicate. Diagnostic mutagens were used as controls when possible.

Identification of *hisG428* Extragenic Ochre Suppressors

The *hisG428* revertants of strain TA103, which were identified as extragenic suppressors, were distinguished among the glutamine (*supB*), lysine (*supG*), and tyrosine (*supC/M*) ochre suppressors by their ability to support growth of ø1, ø262, ø266, and ø491 bacteriophage. The procedure that was followed was modified by recommendation of Levin and Ames (personal communication). Overnight cultures (0.1 ml) of *hisG428* revertants were added to 2 ml of molten top agar and were poured on nutrient agar plates. Then, 20 μl of each phage (10^4 pfu/ml) were spotted onto sections of the plates. The pattern of phage growth indicated the identity of the suppressors. To confirm these results, the *hisG428* extragenic ochre suppressors were also mapped by transduction experiments by using them as recipients and by using strains carrying Tn10 linked to wild-type alleles of the affected tRNAs [*tyrT* (TT7617), *tyrU* (TT2385), *lysT* (TT6577), and *glnU* and *glnV* (TT7613)] as donors [Levin and Ames, 1986]. In these experiments, TA3661 (*supB*), DB7302 (*supG*), DB7303 (*supC*), and DB7304 (*supM*) strains were included as controls.

RESULTS

Reversion of *hisG46* Mutant Strains

Strain TA92 was chosen for analysis of revertants induced by ciprofloxacin at the *hisG46* allele, because this strain is wild type for the Uvr excision repair system and harbors the plasmid pKM101. It has been shown that strains carrying the *hisG46* missense mutation can be reverted to wild type by intragenic GC→AT transitions or GC→TA and GC→CG transversions and by extragenic

TABLE II. Characterization of Spontaneous and Ciprofloxacin-Induced Revertants of *hisG428* and *hisG46* Mutations*

TA2662 (plasmidic *hisG428*)					TA103 (chromosomal *hisG428*)					TA92 (*hisG46*)			
His^+ rev/plate	Del. (%)	P.M. (%)	Supp. (%)	UN (%)	His^+ rev/plate	Del. (%)	P.M. (%)	Supp. (%)	UN (%)	His^+ rev/plate	P.M. (%)	Supp. (%)	UN (%)
Spontaneous													
(425)	43	23	34	0	(146)	4	20	75	1	(37)	97	3	0
Ciprofloxacin (0.05 μg per plate)													
1,335	84	13	2	1	261	2	75	23	0	56	97	3	0

*Del., deletion revertants; P.M., point mutations; Supp., extragenic suppressors; UN, unidentified. The spontaneous values (in parenthesis) have been subtracted from the total number of revertants per plate (rev/plate) and from each class of revertants. Experiments were done in duplicate.

suppressors, which have been identified recently, arising by AT→CG transversions in $tRNA^{Thr}$ genes [Kupchella et al., 1994].

Table II shows the distribution of different classes of mutations found in *hisG46* spontaneous and ciprofloxacin-induced revertants that were screened by their sensitivity to the histidine analogs. Spontaneous and ciprofloxacin-induced mutations at the *hisG46* target were due mainly to intragenic point mutations (97%), whereas the frequency of extragenic suppressors was very low in both cases (3%). Similarly, Levin and Ames [1986] have reported frequencies of 98% for point mutations and of 2% for extragenic suppressors in the spontaneous revertants of strain TA100, which also carries the *hisG46* site and pKM101 plasmid. All of the chemicals reported by Levin and Ames [1986], with the exception of angelicin, preferentially induced intragenic point mutations at the *hisG46* site in strain TA100. Our results also suggest that the intragenic changes induced by ciprofloxacin at the *hisG46* mutation depend on the presence of pKM101, because we have not detected reversion of the *hisG46* strain in the absence of this plasmid [Clerch et al., 1992].

Reversion of *hisG428* Mutant Strains

The strains TA2662 (with the *hisG428* target on the multicopy plasmid pAQ1) and TA103 (with the *hisG428* target on the chromosome) were chosen to analyze the specific changes induced by ciprofloxacin at the *hisG428* allele. Both strains are wild type for the Uvr excision repair system and harbor the plasmid pKM101.

It has been reported that strains carrying the *hisG428* ochre mutation can be reverted to wild type by the following intragenic processes: an AT→GC transition, AT→TA transversions, or 3 or 6 base-pair deletions. The *hisG428* ochre mutation can also be suppressed by the following mutations in tRNA genes: a GC→AT transition (*supB*), an AT→TA transversion (*supG*), and a GC→TA transversion (*supC* or *supM*) [Levin and Ames, 1986].

Table II illustrates the frequencies for the three classes of mutations in spontaneous and ciprofloxacin-induced revertants of both strains carrying the *hisG428* mutation. It is remarkable that pKM101 produced variations in the distribution of the different kinds of *hisG428* spontaneous revertants. Thus, the comparison of data from Table II to results previously reported with isogenic strains without this plasmid, TA2661 and *hisG428*, respectively [Levin and Ames, 1986], indicated that pKM101 increased the frequency of extragenic suppressor revertants regardless of the *hisG428* plasmidic or chromosomal location. In contrast, the frequency of intragenic point mutations at *hisG428* was not affected significantly by the presence of pKM101.

Our results also showed that ciprofloxacin induced a different pattern of mutations, depending on the location of the *hisG428* site. Intragenic deletions were the most frequent event when the *hisG428* target was on the multicopy plasmid pAQ1, whereas intragenic point mutations (an AT→GC transition or an AT→TA transversion) were the main mutational events when this target was on the chromosome (84% deletions in strain TA2662 vs. 75% point mutations in strain TA103). We also found that the frequency of extragenic ochre suppressors induced by ciprofloxacin was lower in the strain carrying plasmidic *hisG428* (2%) than in the strain with the chromosomal *hisG428* site (23%).

Furthermore, we tested whether ciprofloxacin induced preferentially some kind of extragenic ochre suppressor. This study was only possible with extragenic suppressor revertants of chromosomal *hisG428*, because the number of induced suppressors of the plasmidic *hisG428* target was too low (2%). Results found with both methods (Table III) indicated that ciprofloxacin induced mainly *supC* or *supM* (GC→TA transversions) as well as *supB* (a GC→AT transition) at low frequency. Induction of *supG* (an AT→TA transversion) was not detected. All of the results presented above must depend on the plasmid pKM101, because we have not detected any increase in reversion induced by ciprofloxacin in isogenic strains TA2661 or *hisG428* lacking this plasmid [Clerch et al., 1992].

TABLE III. Identification and Mapping of Spontaneous and Ciprofloxacin-Induced Suppressor Revertants of Chromosomal *hisG428* Mutation*

Identification by growth phage mutants					Mapping by transduction				
No. supp. characterized	*supB* (CG → TA)	*SupG* (TA → AT)	*supC/M* (CG → AT)	UN	No. supp. characterized	*supB* (CG → TA)	*supG* (TA → AT)	*SupC* (CG → AT)	*supM* (CG → AT)
Spontaneous									
75	10	16	41	8	75	6	12	21	36
Ciprofloxacin (0.05 μg per plate)									
15	1	0	14	0	15	2	0	5	8

*Values of 4-quinolone-induced suppressors have been calculated after subtraction of spontaneous occurrences from each class of suppressor revertant. UN, unidentified suppressors that present an uncharacteristic pattern of phage growth.

Reversion of *hisG1775* and *hisC9070* Mutations

Two strains have been reported to detect specifically an AT→GC transition (strain TA4011 with the *hisG1775* missense mutation) and a GC→CG transversion (strain TA4016 with the *hisC9070* mutation) [Levin and Ames, 1986]. We found that ciprofloxacin did not induce these changes, because this chemical did not revert TA4011 or TA4016 or their isogenic analogs carrying plasmid pKM101 at a range of concentrations from 0.00625 to 0.2 μg/plate (data not shown).

Reversion of Frameshift Mutations

Mutagenicity assays were carried out with *hisC3076* and *hisD3052* strains and isogenic strains carrying pKM101: TA2425 and TA94, respectively. Strains carrying the *hisC3076* mutation are mainly reverted by single base-pair deletions, but base-pair insertions (+2C) and, presumably, extragenic suppressor mutations have also been reported [Cebula and Koch, 1990]. In contrast, a complex pattern of insertion and deletion frameshifts involving palindromic repeats has been found in *hisD3052* revertants, although a -2 deletion of GC or CG within the alternating CG octamer is the most frequent event [Hartman et al., 1986; Cebula and Koch, 1990; Bell et al., 1991; Kupchella and Cebula, 1991]. Our results showed that both frameshift mutations were reverted by ciprofloxacin, but *hisC3076* reversion by this chemical depended on the presence of pKM101 (Fig. 1).

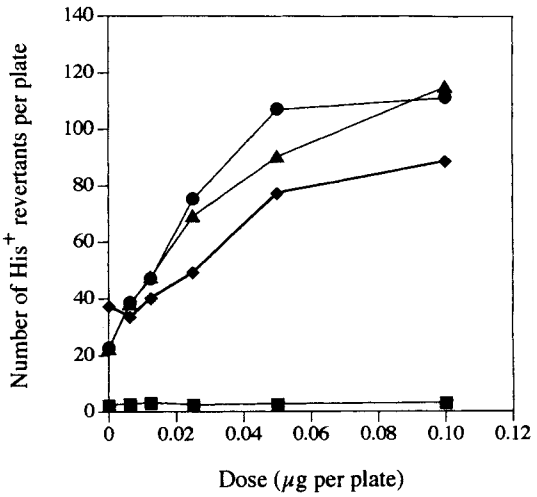

Fig. 1. The number of revertants of *hisC3076* (squares), *hisC3076*/pKM101 (diamonds), *hisD3052* (circles), and *hisD3052*/pKM101 (triangles) induced by ciprofloxacin.

DISCUSSION

A remarkable feature of the mechanisms of ciprofloxacin mutagenesis is that this chemical produces a different kind of mutation depending on the chromosomal or plasmidic location of the mutational target. Consistent with data reported for both 4-Qs, nalidixic and oxolinic acids [Levin et al., 1984], and for ciprofloxacin [Mamber et al., 1993], we have found that 3 or 6 base-pair deletions at plasmidic *hisG428* are the most frequent mutagenic events induced by this 4-Q. However, our results also show that intragenic point mutations are induced mainly when the *hisG428* site is on the chromosome. In relation to this, three pathways have been described as generating *hisG428* deletion revertants [Levin et al., 1984]. The *recA*$^-$, pKM101-dependent mechanism is the only one that is inducible by some mutagens that cause breaks in DNA, such as nalidixic acid, bleomycin, and x-rays, and its activity is limited only to the *hisG428* site located on the plasmid pAQ1 [Levin et al., 1984]. This mechanism also seems to be responsible for generating deletions by ciprofloxacin, because reversion of the plasmidic *hisG428* target by this compound depends on pKM101 [Clerch et al., 1992] and RecA (data not shown). The basis of these mutational events are unknown, but this mechanism seems to be different from frameshift mutagenesis [Levin et al., 1984].

The analysis of the data about the kinds of base-pair

substitutions induced by ciprofloxacin on the chromosome of *S. typhimurium* indicates that this compound reverts neither *hisC9070* nor *hisG1775*. It is probable, then, that GC→CG transversions and AT→GC transitions were not induced in significant numbers by ciprofloxacin. On the other hand, we have found a high level of induced *hisG46* revertants due to intragenic base-pair substitutions, which can be GC→AT transitions and GC→TA or GC→CG transversions, whereas there is a low percentage of revertants due to AT→CG transversions in $tRNA^{Thr}$ genes. The AT→CG transversion is probably a rare event induced by ciprofloxacin, whereas GC→AT transitions or GC→TA transversions are involved in reversion of the *hisG46* mutation by ciprofloxacin. According to this, the analysis of *hisG428* revertants induced by ciprofloxacin shows that 23% are mutations in tRNA genes, and the identification of these ochre suppressors indicates that a GC→TA transversion (*supC* or *supM)* is the major mutagenic event and that a GC→AT transition (*supB)* is also induced, but to a minor degree.

The characterization of *hisG428* revertants induced by ciprofloxacin also indicates that, like in *hisG46*, intragenic base-pair substitutions are the major class of mutations (75%). These can be an AT→GC transition or an AT→TA transversion. We have mentioned above that an AT→GC transition should not be a major event. Therefore, we believe that ciprofloxacin could induce AT→TA transversions at *hisG428*. This suggestion is apparently in disagreement with our results when identifying the induced ochre suppressors of *hisG428*, because we have not found any *supG* suppressor that would be due to an AT→TA transversion. However, we do not exclude the fact that this transversion also occurs, but it is possible that it was not detected due to the low number of induced ochre suppressors identified. Furthermore, other considerations should be taken into account. It must be remarked that the genotoxic activity of 4-Qs appears to be a consequence of their interaction with gyrase-DNA complexes and that the binding of this enzyme to DNA does not occur randomly. This suggests that the detection of 4-Q mutagenic effects could be greatly influenced by the location of the bacterial target studied. It seems that mutations in *hisG* are good targets for detecting 4-Q mutagenic effects, because, in our work, we have found that intragenic point mutations are the most frequent reversion events. In this sense, a prominent site of 4-Qs action has been located at a distance of about 80 base pairs from the plasmidic or chromosomal *hisG428* mutation [Gocke, 1991]. Likewise, our data suggest that *hisC* and *hisD* genes also seem to be suitable targets. Perhaps the lysine tRNA gene, *lysT*, was not a good target for the 4-Q action, and this could explain why we do not find the induction of the *supG* suppressor by ciprofloxacin. These considerations could explain the conflicting results that were found with 4-Qs by using several eukaryotic or prokaryotic assays of mutagenesis.

In addition, results presented in this work and other data reported previously by us [Ysern et al., 1990; Clerch et al., 1992] indicate that the induction by ciprofloxacin of base-pair substitutions at *hisG46*, base-pair substitutions or deletions at *hisG428*, suppressors of *hisG428,* and base-pair deletions/insertions at *hisC3076* depends on the presence of pKM101 plasmid. Therefore, it seems that the majority of mechanisms of ciprofloxacin mutagenesis require MucAB proteins involved in error-prone repair that are encoded by the pKM101 plasmid. In contrast, pKM101 is not required for ciprofloxacin-induced reversion of the *hisD3052* mutation, which can be reverted by base-pair deletions/insertions. This is not a new finding. Similar results have been found for several frameshift mutagens in strain *hisD3052* [Levine et al., 1994a,b; Shelton and DeMarini, 1995] and in *E. coli* [Watanabe et al., 1994]. In all of these cases, chemicals induce specifically -2 deletions of GC or CG in alternating CG sequences through a mechanism that is independent of MucAB proteins. Exceptionally, it has been reported that MucAB enhances -2 frameshift mutagenesis induced in alternating GC sequences by N-2-acetylaminofluorene [Janel-Bintz et al., 1994]. Therefore, we believe that -2 deletion of GC or CG might be the major event induced at the *hisD3052* allele by ciprofloxacin, but molecular studies are necessary to prove our suggestion.

In conclusion, the characterization of the mutagenic specificity of ciprofloxacin shows that this potent inhibitor of bacterial DNA gyrase exhibits a wide mutational spectrum. This could reflect the likelihood that the inhibition of DNA gyrase by ciprofloxacin probably generates different kinds of damage that could be repaired through different mechanisms. Moreover, the observation that ciprofloxacin produces all of these mutational changes at subinhibitory concentrations [see Mamber et al., 1993] strengthens the suggestion that ciprofloxacin could be responsible for the emergence of ciprofloxacin-resistant clinical strains with decreased susceptibilities to drugs and also supports the need for the appropriate use of this antimicrobial agent [Fung-Tomc et al., 1993].

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